Polyomavirus BK after Kidney Transplantation – Role of Molecular and Immunologic Markers

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Abstract

Polyomavirus-associated nephropathy in kidney transplantation is viewed as a complication of prolonged, intense immunosuppression, which disrupts the balance between antiviral immune control and polyomavirus replication. The prevalence rate ranges from 1-10% in kidney transplant programs around the world, with graft failure and return to dialysis in > 50%. Most cases are caused by the human polyomavirus BK, which asymptomatically infects more than 80% of the general population. Reactivation of BK virus replication and high urine viral loads precede increasing plasma BK viral loads and histologically defined as well as clinically manifest nephropathy. Quantitative real-time polymerase chain reaction protocols have proven valuable as surrogate markers to follow the course of polyomavirus-associated nephropathy and to guide preemptive reduction of immunosuppression. As these assays enter clinical routine diagnostic laboratories, quality control becomes important. Testing of BK virus-specific antibodies and T-cells is currently being explored for a better characterization of the virus/host balance. The BK virus-like particles IgG in enzyme-linked immunosorbent essays are recognized as sensitive indicators of recent BK virus exposure. However, no humoral immune responses have been identified to correlate with protection from BKV viremia or disease. BK virus-specific T-cells are generally of low frequencies in the peripheral blood of both healthy donors and kidney transplant patients alike, but significantly increase at the time when plasma BK virus loads decrease, hence representing relatively late indicators of regaining control. We discuss the currently available data on molecular and immune markers regarding promises and caveats. **(Trends in Transplant. 2009;3:85-102)**

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Key words

Transplantation. Viral infections. Polyomavirus. JC virus. BK virus. Polyomavirus associated nephrology. T-cells. Epitope mapping. Vaccine. Adaptive T-cell transfer. Quality control.

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Introduction

The concept of co-evolutionary adaptation entails that both hosts and viruses are selected for genetic variants that permit coexistence at mutually acceptable costs[1]. In this equilibrium, severe viral diseases must be considered individual or epidemiologic accidents either of the virus changing towards more pathogenic characteristics and/or entering a new population, or, of the host not being able to respond adequately[2]. One kind of accident is immunosuppression, administered for transplantation, which disrupts the balance between virus replication and immune response abruptly and introduces foreign HLA-type tissues, which together increase virus-associated morbidity and mortality[3]. Adequately administered antiviral drugs, if available, can counteract this failing balance by reducing the impact of viral replication. Longer-term stability, however, requires eventual restoration of virus-specific immune control. The role of the adequate mounting of an immune response is obvious when primary viral infections are compared in immunocompetent and immunosuppressed individuals. The role of established specific immune control is unveiled by the reactivation of replication of persistent viruses following exposure to immunosuppressive regimens. The most important examples in the setting of transplantation are the human herpes viruses, especially cytomegalovirus, the hepatitis B and C virus, and the human polyomaviruses BK and JC[4].

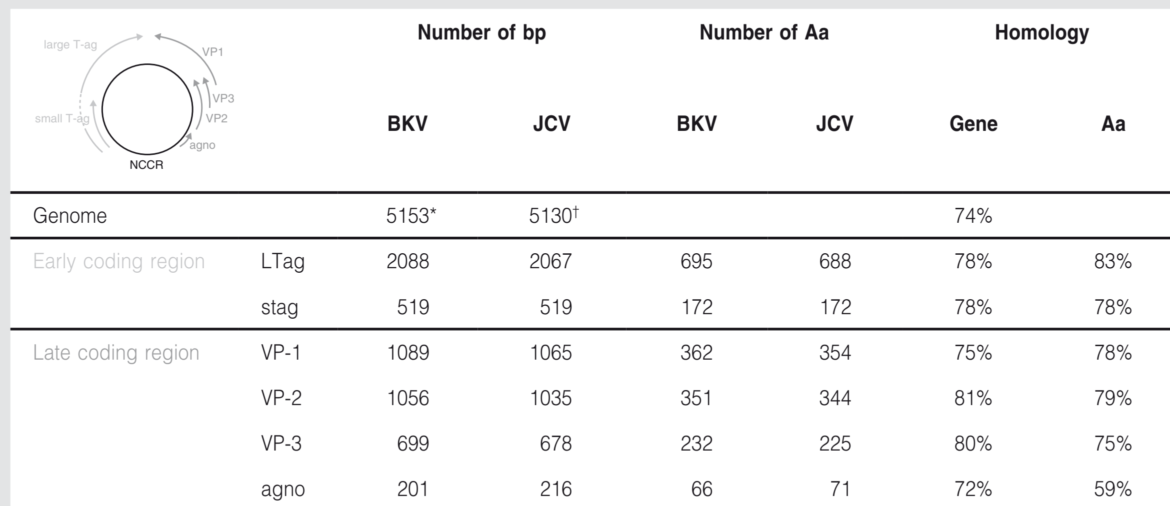
The human polyomaviruses are small, non-enveloped, icosahedral double-stranded DNA viruses of 40-45 nm and fairly resistant to environmental inactivation. To date, six polyomavirus have been detected in human specimens: BK virus (BKV)[5] and JC virus (JCV)[6], the related KI[7] and WU virus[8] in respiratory secretions, the MC virus in Merkel cell carcinoma[9], and the simian virus 40 (SV40) in contaminated polio vaccines[10]. So far, compelling evidence for circulation in human populations is only available for BKV and JCV. Both viruses are closely related (Table 1) and their epidemiology seems to suggest prototypic co-evolution: a high infection rate in the general human population without pronounced clinical symptoms, persistence in the renourinary tract[11,12], with asymptomatic reactivation and urinary shedding[10,13,14]. Polyomavirus diseases are rare in immunocompetent persons[14-16]. In addition to altered immune responses, specific tissue injuries are frequently noted. Thus, BKV can cause polyomavirus-associated nephropathy (PVAN) after kidney transplantation[17] and hemorrhagic cystitis after hematopoietic stem cell transplantation[18], whereas JCV causes progressive multifocal leukoencephalopathy in patients with severe immunodeficiency through HIV/AIDS, transplantation, or longstanding therapies for autoimmune diseases[19-22].

In immunocompetent individuals, reactivation of urinary BKV and JCV replication is found in 7 and 19% of cases, respectively, with low median urine viral loads of 3.5 and 5 log genome equivalents (geq)/ml, respectively[14,15,23-26]. When extrapolated to a metropolitan area like Zurich, with one million inhabitants, the daily polyomavirus shedding would amount to 3.5×10^{11} geq/ml of BKV and 3×10^{13} geq/ml of JCV shed into the environment. Indeed, polyomaviruses were detected in urban sewage and investigated as indicators of water quality[27-29]. Earlier studies indicated that, genomic subtypes were correlated with the migration history of human populations[30,31]. Viremia however has not been confirmed in healthy individuals[14-16].

Molecular aspects of BK virus replication in kidney transplantation

In immunosuppressed individuals, BKV shedding first can be found in about 30% of urine samples, with about 3-4 Log higher BKV loads than found in healthy individuals[32-37]. Following kidney transplantation, high-level BKV replication is detectable in urine of 20-60%

Table 1. The genome structure of the human polyomaviruses BK virus and JC virus



		Number of bp		Number of Aa		Homology	
		BKV	JCV	BKV	JCV	Gene	Aa
Genome		5153*	5130†			74%	
Early coding region	LTag	2088	2067	695	688	78%	83%
	stag	519	519	172	172	78%	78%
Late coding region	VP-1	1089	1065	362	354	75%	78%
	VP-2	1056	1035	351	344	81%	79%
	VP-3	699	678	232	225	80%	75%
	agno	201	216	66	71	72%	59%

The early genes are in red (large T antigen LTag, small T antigen stag), late genes in green (agnoprotein, VP1, VP2 and VP3). The genome length and coding sequences are given in nucleotides and amino acids (percent identity) Amount of similarity between both viruses is indicated at the genome and protein level in %. bp: base pairs; Aa: amino acids; BKV: polyomavirus BK; JCV: polyomavirus JC; NCCR: non-coding control region.
*Dunlop sequence.
†Mad-1 sequence.

of patients, with urine viral loads of 10^7 to 10^{10} geq/ml (Fig. 1)[32,38]. About 30% of kidney transplant patients with BKV-positive urine samples eventually develop BKV-positive plasma samples. Progression of histologically documented PVAN is observed in about 60% of kidney transplant patients with plasma viremia. Hence, BKV detection and quantification serve as important markers to assess the risk of PVAN[32,39]. Patients with definitive PVAN have higher viral loads than viremic patients without PVAN (28,000 vs. 2,000 geq/ml)[32]. According to expert recommendations, persistently high BKV in plasma > 4 $\log_{10}$ geq/ml for more than four weeks in kidney transplant patients defines “presumptive PVAN”[33,35,40].

Following surgical removal of PVAN-containing allografts, plasma BKV loads show a rapid drop, suggesting that the majority of plasma BKV loads is derived from replication in the graft. The calculated plasma viral half-life of 1-2 hours implies that during steady-state more than 99% of the plasma BKV loads are turned over per day, and allow estimating the tubular epithelial cell loss as in the order of 10^6 to 10^7 cells per day[41]. Moreover, persistent BKV replication in kidney transplant patients leads to emergence of BKV variants with rearrangements (rr) of the non-coding control region (NCCR) containing viral promoter and enhancer sequences. These rr-NCCR BKV variants are first detected in plasma and later in urine, which supports the notion that intrarenal tubular epithelial cells and urothelial cells of ureter and bladder are independent but partially linked replication compartments. The occurrence of rr-NCCR BKV is linked to 20-fold higher plasma viral loads (median 20,000 c/ml vs. median 440,000 c/ml), and more tissue pathology both of which can be recapitulated in tissue culture[42]. Of note, the sequences encoding the viral protein-1 (VP1) capsid and the large T-antigen were not altered, suggesting that significant immunologic pressure was not be present in patients with emerging rr-NCCR BKV.

JCV can be detected in 20-50% of urine samples and may present with shedding of “decoy cells” bearing polyomavirus particles in nuclear inclusions[32,34,41]. The JCV is rarely detected in plasma of patients with JCV-associated

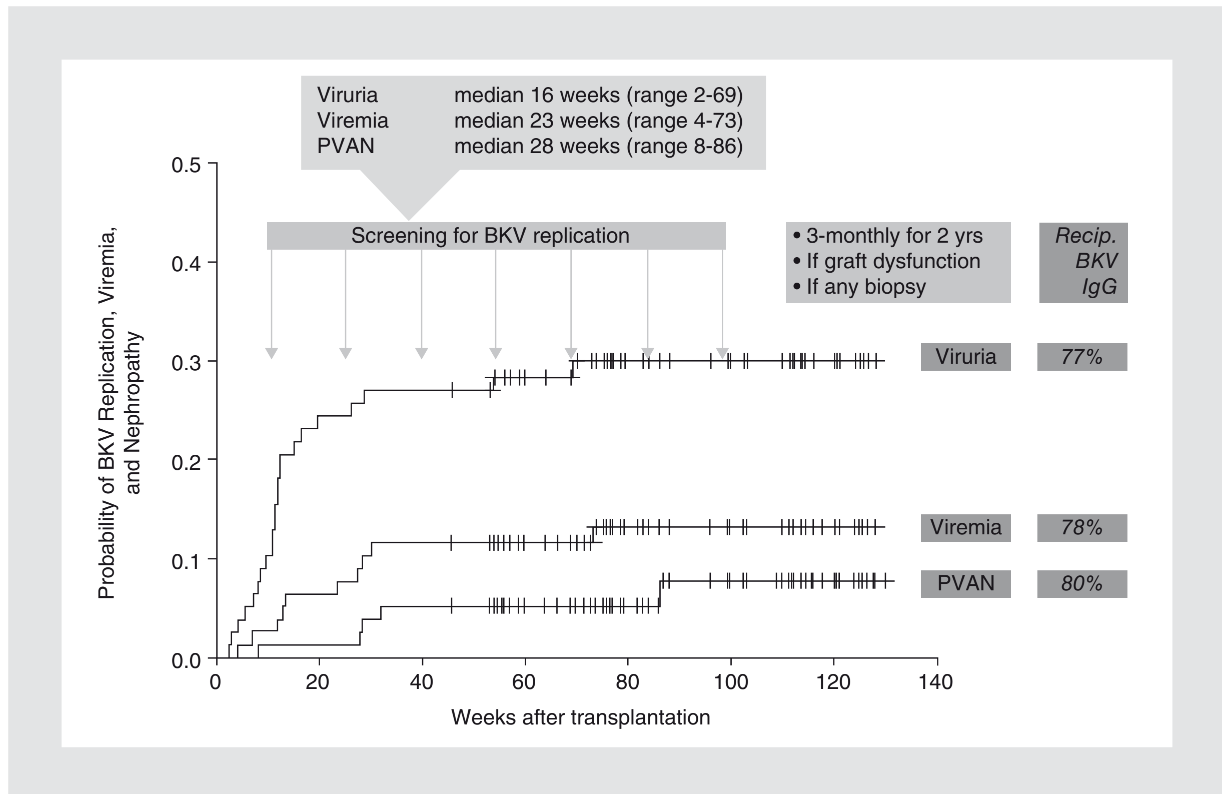


Figure 1. *Probability of BK virus viruria, viremia and nephropathy (modified after Ref. 32). BKV: BK virus; PVAN: polyomavirus-associated nephropathy; IgG: immunoglobulin G seroprevalence.*

nephropathy (14%) and then of low viral loads (mean 2,000 c/ml)[34]. Overall, JCV-associated nephropathy seems to run a more benign course than BKV-associated nephropathy, although some cases with graft failure have been reported. Rearrangement of the JCV NCCR in PVAN has not been detected, which is, however, a hallmark of JCV variants in progressive multifocal leukoencephalopathy.

Although the paradigm of viruria preceding viremia and PVAN by about 4-8 weeks has been known for almost a decade in kidney transplants (Fig. 2), it is still not defined which patients progress to plasma replication and to disease. In analogy to cytomegalovirus (CMV), molecular and immunologic markers have been investigated for the potential to better assess the risk of BKV infection to progress to replication and PVAN and thereby guiding intervention.

BK virus genome detection

One of the first BKV-specific polymerase chain reaction (PCR) assays was published by Arthur, et al. in 1989[43] to study the role of BKV in hemorrhagic cystitis after bone marrow transplantation. Since then, various qualitative and quantitative PCR assays have been developed (Table 2), with various targets in the 5.1 kb double-stranded DNA genome (Table 1). Most of these assays were designed to specifically detect BKV, but some cover both BKV and JCV, and include the possibility of distinguishing between the two. Published target sequences are located in the large T gene (LT, 15 assays), in the VP1 gene (seven assays), in the VP2 gene (three assays) or in the NCCR (three assays). Our routine in-house TaqMan® BKV PCR amplifies a conserved region of the LT gene, which was chosen to match BKV sequences without amplifying the homologous

Acc. number	pos. 1st nt	Alignment	Nr of identical sequences
Forward primer			
		ATACACAGCAAAGCAGGCAAGGGTTCTATTACTAAATACAGCTTGACT	
EU681739	107	--	137
EU681749	107	---------------------A--------------------------	60
EU681746	47	------------A--------T--------------------------	12
AB301092	4198	------------------G-----------------------------	11
Probe			
		GGTAGAAGACCCTAAAGACTTTCCCTCTGATCTACACCAGTTTCTTAG	
EU681746	140	--	33
EU681775	183	------------------------T-----------------------	181
AB263924	4291	------------------------T-G---------------------	4
EU681743	108	-------------------------------G----------------	1
AB263926	4291	--------------------------G---------------------	1
AB269864	4294	------------------------T--------------A--------	1
Reverse primer			
		CAGTGATGAAGAAGCAACAGCAGATTCTCAACACTCAACACCACCCAA	
V01109	2630	--	138
EU681749	260	---------G--G--------------G--------------------	48
EU681746	200	---------------------------G--------------------	12
AB269839	4343	---------G--G-------G------G--------------------	6
AB269825	4354	-----------G---------------G--------------------	1
		A	

Figure 2. *Primer and probe evaluation for real-time polymerase chain reaction. Alignment of published sequences of the large T-antigen targeted by the primers and probe published by Hirsch, et al.[49]. Identical nucleotide positions are indicated by a dot. Variable nucleotide positions are boxed. The sequence of the primers and probe are underlined and shaded grey. The alignment was performed with the BLAST algorithm in June 2008.*

JCV sequences[32,34,49]. In addition, the detection BKV of VP1 mRNA in urine samples and in biopsies has been described[63,64]. Next to the many published BKV PCR assays, some commercial PCR kits have now become available for qualitative and quantitative detection of BKV (e.g. Argene, Affigene, TIB Molbiol, Nanogen).

In general, the choice of a target region for a diagnostic real-time PCR assay implies that the chosen sequence is specific for the particular microorganism and that the sequence is conserved throughout all isolates of this microorganism. To identify the best target sequence with respect to sensitivity and specificity, genomic sequences should be compared from as many different isolates from various sources as possible. This is initially not achieved in the case of newly discovered pathogens when only one or few sequences are available. It should be noted that sequences coding for proteins that are recognized by the host's immune system are under significant selective pressure and may change rapidly. Typical examples are surface proteins of RNA viruses. Data from PVAN allografts suggest that this may also be the case for BKV VP1, although to a lesser extent[65]. The increasing number of sequences available in the public databases calls for a periodical routine control of the primer and probe sequences by homology search (BLAST). According to the sequences, primers and

Table 2. Polymerase chain reaction assays for the detection of BK virus

First author	Year	Method	Target region	Specificity
Arthur[43]	1989	PCR with RFLP or hybridization	LT	BK and JC (discriminatory)
Nickeleit[44]	1999	Semi-nested PCR Dilution series	LT	BK, JC (specific inner PCR)
Biel[45]	2000	Semi-nested PCR	LT	BK, JC (specific inner PCR)
		Taqman	LT	BK and JC
Limaye[46]	2001	Taqman	LT	BK
Leung[47]	2001	Taqman	VP1	BK
Whiley[48]	2001	LightCycler	VP2	BK and JC (specific Tm)
		PCR-ELAHA	VP1	BK and JC (specific probes)
Hirsch[49]	2001	Taqman	LT	BK
Merlino[50]	2003	Semi-quantitative PCR	LT	BK
Holman[51]	2003	Semi-quantitative PCR	LT	BK
Beck[52]	2004	LightCycler	VP2	BK
Whiley[53]	2004	PCR-ELAHA	VP1	BK and JC (specific probes)
Randhawa[54]	2004	LightCycler, Sybr	VP1	BK
Herman[55]	2004	Taqman	LT	BK and JC
McNees[56]	2005	Taqman MGB	LT	BK
Sehbani[57]	2006	LightCycler SybrGreen	LT	BK
Moret[58]	2006	PCR, hybridization,	n. s.	BK and JC (specific probes)
Kaigala[59]	2006	PCR, Microchip	VP1	BK
Pal[60]	2006	Taqman (3 Assays)	VP1, NCCR	BK
Elfaitouri[61]	2006	Taqman	VP2	BK, JC and SV40
Si-Mohamed[62]	2006	Taqman	LT	BK

PCR: polymerase chain reaction: RFLP: restriction fragment length polymorphism; LT: large T-antigen; VP: viral protein; NCCR: non-coding control region.

probes should be adapted when critical bases are altered. This can be achieved by including degenerated positions matching two or more sequence variants. Mismatches at the 3'-end of the primers have more impact than at the 5'-end, and may reduce the PCR efficiency with under-quantification by several logs. In the last decade, more sequences of naturally occurring BKV isolates have become available. Figure 2 shows the sequences of primers and probes used in our assay targeting the BKV LT. Although the majority of published sequences perfectly match with both primers, about one-third of sequences show one, two and three nucleotide mismatches including some at the critical 3'-end (Fig. 2). For the probe, approximately 80% of sequences bear a single nucleotide mismatch, which are, however, located in the center and are not likely to affect detection. Nevertheless, this demonstrates the requirement for periodical reevaluation and adaptation of diagnostic BKV assays.

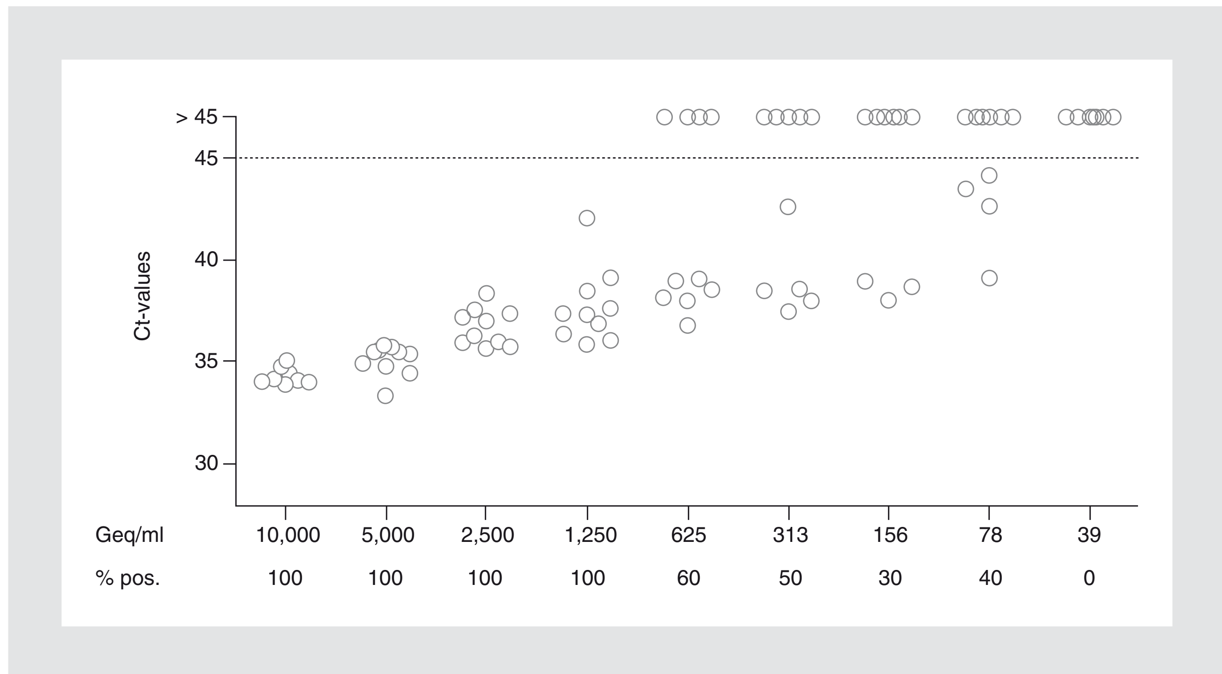


Figure 3. *Detection limit of polymerase chain reaction assay and standard curve validation. Serial 2-fold dilution of 10^2 geq per PCR reaction obtained from the standard curve. Two independent runs of five replicates were performed. Replicates that gave no detectable results are plotted at Ct > 45. In this case, the detection limit is 50% at the 3.13 geq (in 5 µL in 25 µL total PCR assay volume). Because the DNA is 2-fold concentrated relative to the extracted sample volume, the detection threshold corresponds to 313 geq/ml. This statistical approach also allows to evaluate whether or not the standard curve is too high or too low, since the dilution series goes as low as 0.78 and 0.39 geq per assay, which should yield no more than 80 and 40% positive results per 10 assays, respectively.*

Diagnostic performance of BK virus real-time polymerase chain reaction assays

The amplification efficiency and linear range of a PCR assay is typically established using reference plasmids containing the target sequence. Most assays today report a linear dynamic range from 10^2 to 10^8 per reaction. To determine the limit of detection of a PCR assay in a statistically meaningful way, we run independent replicas of five or 10 reactions of twofold dilutions of the BKV reference plasmid, starting from 100 copies per reaction down to at least two dilutions below one copy per assay. The results can be scored categorically as “yes-detectable” or “no-undetectable”, or by scoring the threshold cycles (Fig. 3). Since routine samples are analyzed in triplicates, we defined the limit of detection of our assays as being the concentration of target detected in at least 50% of the replicates.

The sample materials analyzed for BKV include urine, plasma, and, rarely, cerebrospinal fluid (CSF) for suspected cases of BKV encephalitis. Tissues such as renal biopsies can also be tested for BKV DNA, but have not entered the diagnostic routine. An optimal extraction of these samples is a prerequisite for a correct quantification of the viral loads. We routinely use the MagNA Pure LC System with the Total Nucleic Acid Kit (both from Roche Diagnostics) for urine and plasma samples. A sample volume of 200 µl is extracted and eluted in 100 µl. Manual extraction methods are used for CSF samples (Qiagen QIAamp Blood Kit). Fluid extractions using the Roche Kit and the Qiagen Kit yielded comparable results, but we observed that samples with low viral loads are occasionally under quantified with spin columns compared to beads. Biopsies are also extracted with the Qiagen Kit, with an additional lysis step by proteinase K. For the quantification of BKV

DNA from biopsies, quantitative PCR for a chromosomal host call target is used such as beta-actin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), or the aspartoacylase gene (ACY)[66]. These genes are present in two copies per cell. The quantification of host cell genes allows to normalize the BKV load per 150,000 cells (i.e. equivalent to 1 µg DNA) to correct for the cellular content of the sample.

Quality controls

Internal quality controls are part of every PCR run and allow monitoring for possible contaminations Moreover, quantitation controls i.e. specimens with known copy number of e.g. 5000 or 50000 geq/ml are run to test for inhibition, or technical problems (positive controls). Besides these, external quality controls should be tested on a regular basis. External quality controls consist of samples whose outcome is not known to the person who analyses it. These samples should be handled in the same way as patient samples, from the extraction to the interpretation and validation of the results. For many infectious agents, such controls are commercially available and are distributed in Europe for quality assurance (e.g. NEQUAS, INSTAND, QCMD). The results are collected and analyzed, and the intended results and the performance of the laboratory relative to other participants is communicated. External controls allow the laboratories to evaluate the quality of their workflow and of their assays and to compare them to those of other laboratories. A pilot study evaluating BKV and JCV external quality controls was successfully conducted recently and such controls are now available (QCMD, Glasgow, Scotland[67]). Together, these methodological and quality issues are of utmost importance when BKV load testing moves from the research laboratory into the clinical diagnostic laboratory for decision making and clinical study evaluation.

Clinical value of BK virus load screening and monitoring

The sequence of BKV viruria, viremia and histologically defined PVAN (Fig. 1)[32] provides a rationale for screening, and scrutinizing histologic diagnostics and even preemptive intervention[33,35,40]. In cases of established diagnosis, monitoring the course of plasma and urine BKV loads assists in deciding on the duration and escalation of intervention. However, as more studies are being conducted, there is considerable uncertainty about the frequency of screening and about whether or not the sample material should be urine, plasma, or both. The significance of urine BKV loads (or “decoy cells” in experienced institutions) resides in the high negative-predictive value which allows to exclude PVAN with > 95%. If urine BKV screening is negative, BKV viremia and PVAN are unlikely to occur within the next 4-8 weeks (Fig. 1). Conversely, the significance of plasma BKV loads resides in the high positive-predictive value. However, several, mostly prospective studies have reported the absence of “definitive PVAN” in biopsies, even though the plasma BKV load was > 10^4 geq/ml. Potential explanations for these failures are: i) the focality of PVAN, which is estimated to result in false negative biopsy results in 10-30% of cases, particularly in the early phase[68]; ii) less than four weeks of duration for increasing plasma BKV loads above 10^4 geq/ml according to the consensus recommendations[40]; and iii) overquantification of plasma BKV loads because of shifted standard curves. Indeed, in a first multicenter study, which included Basel, differences in the quantification of patient samples were noted to be in the order of plus or minus 1 $\log_{10}$ geq/ml[69]. Since these differences of over- and under-quantification were center-specifically maintained for most of the plasma and urine samples, it had to be concluded that the reference standards used to generate the BKV copy number were shifted. The results of this study[69] emphasized the need for international standards that allow normalization of BKV loads

Table 3. Polyomavirus-associated nephropathy histology and plasma BK viral load

Year	Center	Reference	Cases (n)	PVAN	Median BK viral load (geq/ml)	Cutoff
2002	Basel	(32)	5	definitive	28,000	7,700
2004	Atlanta	(70)	8	definitive	73,000	2,800
2004	Pittsburgh	(54)	10	definitive	443,000	5,000
2005	Leuven	(72)	21	definitive	70,800	n/a
2006	Paris	(62)	5	definitive	398,000	n/a
2006	Basel	(73)	16	definitive	57,000	10,000
2007	Houston	(74)	8	definitive	1,427,000	63,000
2007	Rochester	(71)	4	definitive	n/a	16,000
2007	Baltimore / Basel	(34)	75	definitive	2,900,000	10,000
2008	Milwaukee	(75)	8	definitive	186,000	9,100

PVAN: polyomavirus-associated nephropathy.

by transformation into International Units. Until such normalization standards become available, not only assessments of the linear dynamic range should be made, but also validation of the titer by twofold dilution series down to copy numbers of statistically less than one copy per reaction should be made (Fig. 3). In prospective studies with presumed duration of four weeks of increased risk were included, the positive predictive value was approximately 50-70%[34,70,71]. In table 3 we summarize recent studies reporting histologically confirmed (i.e. "definitive") PVAN and the respective median plasma BKV loads, as well as the lowest plasma BKV load of a confirmed case (cutoff).

Humoral immune responses to BK virus

The BKV-specific antibody responses have been measured by four different assays: plaque-based assays, hemagglutination inhibition assay (HIA), indirect immunofluorescence of BKV-infected cells, and enzyme-linked immunosorbent essay (ELISA). Neutralization assays are considered the most specific as they measure antibodies that bind to infectious BKV virions and inhibit the infection of host cells. The HIA measures antibodies that inhibit the agglutination of red blood cells mediated by the three-dimensional virion capsid by interfering with their binding to sugar residues on type 0 erythrocytes. Indirect immunofluorescence detects intracellular viral proteins including the early and late gene products. Enzyme-linked immunosorbent essays have been described using the recombinant capsid protein VP1 in the linear form, or as three-dimensional, self-assembled, virus-like particles (VLP), the BKV-early protein large-T antigen (LT) and the BKV agnoprotein. The VP1-derived VLP are most frequently now used because of their high specificity, sensitivity, and ease of handling in the ELISA format[35,76-79].

Neutralizing antibodies are usually examined by plaque assays[80]. However, this technique is too cumbersome for larger sample numbers. Neutralizing antibodies are closely correlated with antibody titers measured by HIA or by BKV VLP ELISA[10,13,81,82]. The VLP ELISA tests have a higher sensitivity, but recognize a wider range of epitopes than the HIA[82]. Cross-reactivity between polyomaviruses was examined in pre-absorption assays (pre-incubation of sera with other virus antigens) and revealed limited IgG cross-reactivity between SV40 and BKV, but not between BKV and JCV. When denaturing BKV VLP, the IgG activity significantly dropped, indicating the

importance of the three-dimensional antigen structure for the majority of BKV-specific antibodies[81]. When analyzing BK-VLP and JC VLP-specific IgG activities in healthy blood donors, no statistical evidence for IgG cross-reactivity was apparent (linear regression IgG: R^2 = 0.04), but some cross-reactivity for the respective IgM activity (linear regression R^2 = 0.32; A. Egli and H.H. Hirsch, unpublished results).

Humoral responses to BK virus in healthy individuals

Knowles, et al. used HIA to measure BKV and JCV titers[10]. In that study, BKV seroprevalence of 81% was observed, with a slow decrease of seroprevalence in older age groups, most probably due to a decrease in geometric mean titers with increasing age (8.7% reduction per 10 years). For JCV, the overall seroprevalence was only 35%, but strongly increased with increasing age. Using BKV and JCV VLP, we found a significant decrease of BKV IgG seroprevalence and IgG activity with increasing age of healthy blood donors, while JCV seroprevalence increased[14]. Given the fact that urinary BKV shedding was significantly less frequent than JCV shedding (7 vs. 19%), it was interesting to note that the activity of JCV IgG VLP antibody correlated with urine JCV load[14]. This suggests that higher VLP antibody levels are indicative of recent exposure to viral antigen and included asymptomatic virus shedding[83]. Such antibodies probably have a role in clearing and protecting from viremia, but obviously don't protect from localized polyomavirus replication in mucosal tissues. In a first study comparing antibody responses in healthy donors to various other recombinant BKV antigens (anti-LT, anti-VP1 and anti-agnoprotein), Leuenberger, et al.[84] found similar IgG activities for all three BKV proteins as in kidney transplant patients without recent BKV replication[84]. Overall, IgG activities were higher against VP1 than against LT, and only rarely were antibodies against agnoprotein detectable[84].

Humoral responses to BK and JC viruses after kidney transplantation

The role of BKV serology in kidney transplantation to assess potential risk of infection is controversial. The classical donor seropositive/recipient seronegative (D^+R^-) risk constellation is not as common. Bohl, et al. screened 198 transplant recipients, and for 66 recipients, donor serostatus was available. According to BKV serology, 48% were D^+R^+, 19.6% were D^+R^-, 18% were D^-R^+, and 13.6% were D^-R^-. Only donor BKV seropositivity was significantly associated with the risk for BKV replication posttransplantation. The likelihood of recipients' viruria in the first year posttransplantation correlated with increasing donor BKV-VLP antibody titers[78]. Thus, the BKV antibody activity may reflect recent antigen exposure and possibly higher BKV loads in the graft, which is then not met by a qualitatively and quantitatively corresponding immune response in the recipient[14].

Since none of the patients studied by Bohl, et al.[78] had "definitive PVAN", the study by Hariharan, et al. is of interest. Patients with first diagnosis of PVAN had high plasma BKV loads and showed moderate to low BKV-VLP IgM and IgG levels. Cases with stabilizing PVAN with intermediate plasma BKV loads had a strong increase in IgM and intermediate IgG levels, whereas past PVAN cases had low or undetectable BKV loads and a significant increase in IgG levels. This suggest that with increasing IgG levels, plasma BKV load decreased in part due to neutralizing antibodies[79]. However, work by Comoli, et al.[83,85], Leuenberger, et al.[84], and more recently by Chen, et al.[77] showed that BKV-VLP IgG levels already increased during rising plasma BKV loads, indicating that neutralizing antibodies were not sufficient to prevent BKV viremia and PVAN. These observations were more recently confirmed by Ginevri, et al. in a larger cohort of 62 patients and suggest rather that BKV-VLP IgG titers reflect the patient's current or recent history of antigen exposure[35].

The role BKV VLP-specific IgA was assessed by Randhawa, et al.[86]. For both donors and recipients, the rate of seropositivity was 80% for BKV-VLP IgG, 20% for IgA, and for 0% for IgM. Interestingly, some patients without evidence for BKV detection during follow-up showed an increase of BKV-VLP IgG seropositivity, which may therefore reflect BKV replication below detection levels in plasma or urine, or outside the sampling times. A significant increase in BKV IgA was observed (pretransplant 20% vs. posttransplant 75%), in line with the notion of mucosal exposure[86]. Comparison of IgG activities specific against different BKV proteins in kidney transplant patients revealed that BKV LT-specific IgG activities were high in patients after clearing prolonged episodes of BKV viremia. Unlike antibody responses to linear VP1, VLP, or LT, the vast majority of kidney transplant patients, even those clearing histologically confirmed PVAN, did not mount agnoprotein-specific immune responses, despite abundant late-phase expression of this viral protein[84].

Humoral immune responses to JCV replication in kidney transplants are currently not available. Our own observations in a large international cohort of *de novo* kidney transplant patients indicates, however, that patients with high-level JCV replication in urine, with or without detectable plasma JCV loads, have a strong increase in the JCV IgG VLP activity (H.H. Hirsch, unpublished observation)[38]. In no cases, however, was JCV-associated nephropathy documented, suggesting that, akin to BKV, the JCV-VLP IgG are sensitive enough to pick up antigen exposure in the urothelial replication without significant graft damage.

BK virus-specific cellular immunity in healthy donors and kidney transplanted patients

In kidney transplant patients, a diverse set of risk factors associated with BKV replication and progression to disease has been reported, not all of which were confirmed in independent studies. These factors include older age, male gender[87], seropositive donor[78,88], seronegative recipient[88,89], low number of BKV-specific interferon-γ (IFNγ) production of peripheral blood mononuclear cells (PBMC)[85], use of potent immunosuppressive regimens[32,33,38,90,91], HLA mismatches[32,38,92], and antirejection treatment[32,38,92]. Overall, the common denominator of these risk factors points to an impaired BKV-specific cellular immunity akin to CMV posttransplantation[4,93].

Immunologic assays for BK virus-specific cellular immune responses

In general, three immunologic assay types to quantify and qualify markers of BKV-specific immune response are commonly used: i) EliSpot assay to measure secretion of IFNγ from PBMC by detecting spot-forming units per million PBMC; ii) intracellular cytokine staining and flow cytometry to detect accumulated IFNγ in T-cells with addition of CD8 and CD4 phenotyping; and iii) tetramer assays to detect T-cells recognizing single BKV epitopes depending on a specific HLA context (also flow-cytometry based) (Fig. 4). For the first two assays, overlapping peptide libraries have been used that consist of 15 amino acid peptides overlapping by 11 amino acids that cover the entire amino acid sequence of the BKV or JCV LT, VP1, and agnoprotein. These peptide pools have been added to PBMC in order to pulse antigen-presenting cells present in the PBMC. Any peptides contained in the pool that can bind to major histocompatibility complex MHC-II, or to MHC-I after external or internal trimming, can then be recognized by the respective T-cell receptor and elicit a response such as IFNγ expression. Alternatively, antigen-presenting cells can be pulsed with BKV preparations[94] or lysates from infected cell cultures[95]. In the latter assays, slightly more spot-forming units were observed compared to stimulation by

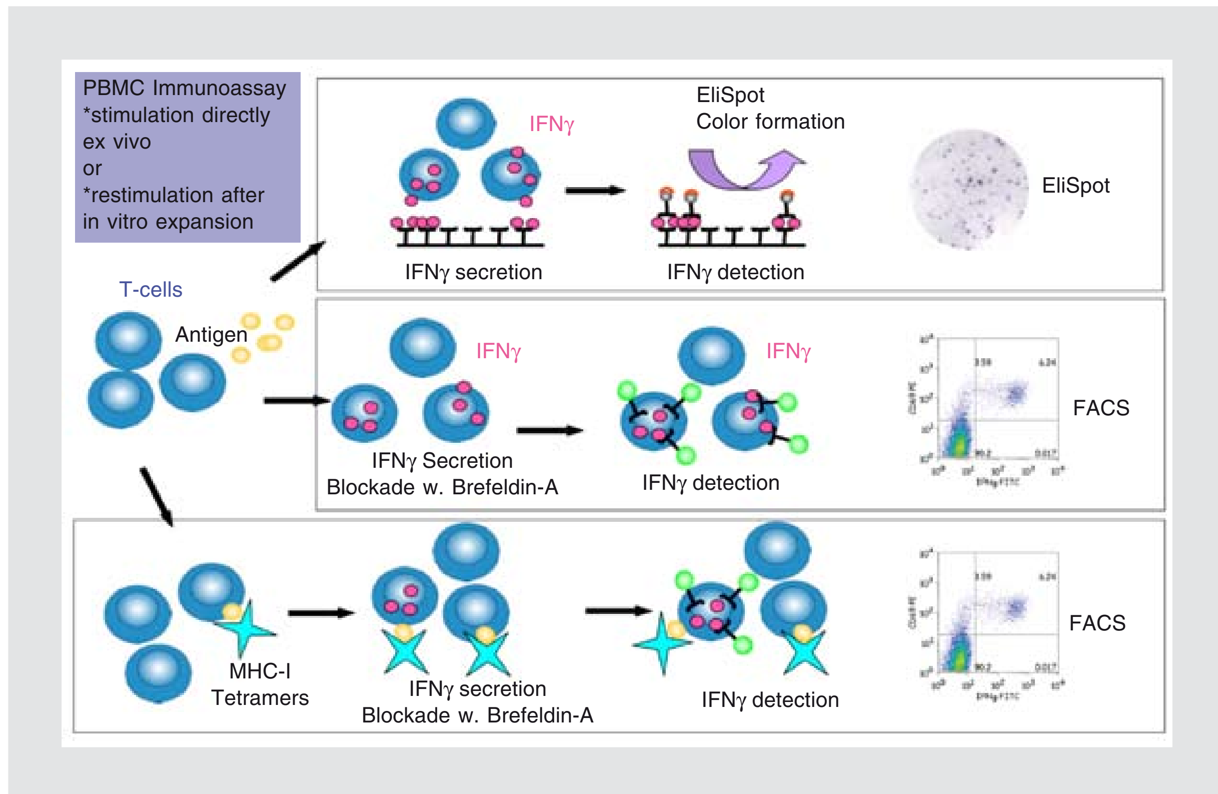


Figure 4. *Immunoassays for detecting BK virus-specific cellular immune responses. Peripheral blood mononuclear cells (PBMC) are either directly stimulated* ex vivo *or re-stimulated after* in vitro *pre-stimulation and expansion using viral antigens e.g. overlapping peptide pools of 15 amino acids covering the entire amino acid sequence of large T-antigen or VP1. EliSpot assay detects secreted interferon-γ (IFNγ) after captured by specific antibodies coated in well and second antibody for color reaction. Intracellular cytokine staining detects intracellular accumulation of IFNγ after secretory blockade with brefeldin-A and fixation/permeablization with fluorescent antibody. Tetramer staining detects T-cells binding specifically to fluorescence-labeled single peptide epitopes major histocompatibility complex MHC-I tetramer complexes which can be combined with intracellular IFNγ detection.*

peptide pools, probably a reflection of the more abundant antigens. By contrast, tetramer assays using streptavidin-fluorophore labeled MHC-I with a single 9mer bound that can be recognized by the respective T-cell. In this case, the precise epitope for the HLA-type must be known. Provided with the co-stimulatory signals, cytokine expression can also be assayed in this way. Otherwise, only the T-cell receptor frequency is enumerated.

Comparing the frequencies in direct *ex vivo* assays, it should be noted that BKV- (and JCV)-specific T-cell responses in PBMC responding to BKV VP1 and LT peptides pools are 10- to 100-fold lower than the frequencies described for CMV pp65 or CMV lysate responses[94] (Fig. 5). Because of the low precursor frequency in addition to the presence of immunosuppression, *in vitro* T-cell expansion for 7-9 days after a single BKV-specific stimulation has been used[35,96], or longer T-cell expansion for 3-6 weeks using multiple rounds of stimulation with activated monocytes or dendritic cells[77,94,97]. Every expansion protocol introduces bias, by selecting responding lymphocyte populations but a common problem is that the use of 15mer peptide pools seems to favors CD4 over CD8 T-cell phenotypes. However, BKV-specific CD8 T-cells can be fostered by initial magnetic bead separation[97], or by adding interleukins IL-12 and IL-7 to the expansion culture[94].

Using a “direct” *ex vivo* IFNγ EliSpot assay, we found low median frequencies of 24 and 25 spot-forming units of IFN-producing T-cells per 10^6 PBMC after stimulation with BKV LT and VP1 in healthy donors[96]. In a cross-sectional

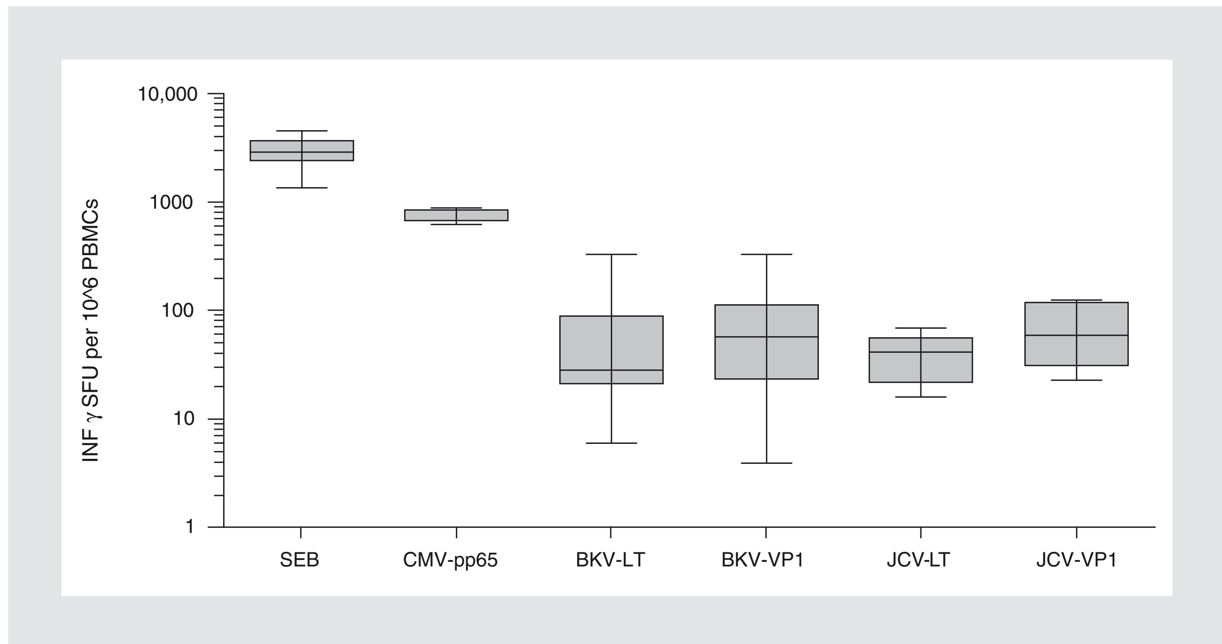


Figure 5. *Direct EliSpot assay comparing BK virus- and cytomegalovirus-specific cellular immune responses in healthy blood donors. Peripheral blood mononuclear cells (PBMC) from five healthy blood donors are directly stimulated* ex vivo *with the indicated antigens and interferon-γ release quantified by EliSpot assay (A. Egli, S. Köhli, H.H. Hirsch, unpublished): Staphylococcus superantigen (SEB) as positive control, overlapping peptide pools covering the cytomegalovirus pp65 (CMV-pp65); the BK virus large T-antigen (BKV-LT), capsid viral protein VP1 (BKV-VP1); JC virus large T-antigen (JCV-LT) and capsid protein VP1 (JCV-VP1). SFU: spot-forming units.*

cohort of adult kidney transplant patients, we detected slightly higher median values despite immunosuppression[96]. This is in contrast to what has been observed for CMV-specific cellular immune responses, which were significantly lower in kidney transplant patients than in non-immunosuppressed healthy donors[93,98]. However, in kidney transplant patients with increasing or persistently high plasma BKV loads above 10^4 geq/ml, significantly lower median frequencies of BKV-specific IFNγ responses were found than in patients with a decrease of plasma BKV loads of more than 2 logs (BKV LT: 22 vs. 78 SFU per 10^6 PBMC; BKV VP1: 53 vs. 285 SFU per 10^6 PBMC; $p < 0.05$). The BKV VP1-specific responses were generally higher than LT-specific responses, but the responses to LT over the cutoff of 69 spot-forming units correlated better with declining plasma BKV loads as a measure of emerging BKV immune control[96]. The study underlines the potential of BKV-specific cellular immunity as a monitoring assay to predict future BKV replication. It also demonstrates that risk stratification of kidney transplant patients prior to BKV replication cannot be readily achieved by direct EliSpot assays, but may require short-term expansion and/or other more sensitive asseys.

Using *in vitro* expansion, BKV-specific cellular immune responses were 10-fold to 100-fold increased after single-round stimulation with BKV LT or VP1 overlapping peptide libraries[99] for patients with decreasing rather than increasing plasma BKV loads[96]. However, expansion could also be observed in patients with increasing or high plasma BKV loads, indicating that BKV-specific T-cells may be already present in PBMC of such patients, but paralyzed by immunosuppression and hence unable to control BKV replication until *in vitro* wash out and re-stimulation. This observation is important as it suggests the possibility for using such T-cell activities could be expanded for cellular immunotherapy by adoptive transfer.

Ginevri, et al.[35] stimulated PBMC in culture for nine days with peptide pools encompassing BKV LT or VP1 and retested the

activities using an EliSpot assay or killing assay. Again, the IFNγ responses were higher for BKV VP1 than the LT response, but the LT-mediated killing activity was higher than the respective VP1 activity (median % lysis at a 5:1 ratio, BKV LT: 26% vs BKV VP1: 8%)[35]. A study by Zhou, et al.[100] suggested that a subset of BKV LT-specific CD154 positive CD4 T-cells with TNFα and IFNγ production may carry a high potential for killing[100]. Thus, the currently emerging data suggest that BKV VP1-specific cellular responses may be a first sign of the recovering BKV-specific cellular immune response, whereas the BKV LT-specific activity better correlates with the specific killing activity directed against BKV infected cells[35,96,101].

Intracellular cytokine staining and flow cytometry have been used directly on stimulated PBMC samples, but were limited by the low frequency of the BKV responses[102,103]. Similarly, only low responses could be detected by intracellular cytokine staining and flow cytometry in our cross-sectional study on kidney transplant patients[96]. However, the LT responses contained a higher proportion of CD8 T-cells compared to the VP1 responses.

Tetramer staining requires prior knowledge of the HLA class I and the corresponding peptide epitope. Despite the increased sensitivity, even tetramer staining required prior expansion. Studies measuring the frequency of CD8 T-cells binding to a tetramer complex loaded with VP1 epitope p44 and p106 showed frequencies > 1% in patients with declining plasma BKV loads[77]. Moreover, this technique has been used to examine the cross-reactive potential of identical BKV and JCV epitopes predicted by computer algorithms[77,97,99,104].

Epitope mapping of BK virus

The clearance of BKV replication is associated with increasing frequencies of BKV-specific T-cells. Spectra typing of *in vitro* expanding T-cell receptor β-chains indicated that these BKV-specific T-cell responses were oligoclonal[94]. These experiments suggest that particular immunodominant epitopes may play a major role in the control of BKV infection. Knowledge of such epitopes might have the potential to increase the sensitivity and specificity of BKV-specific immune monitoring in kidney transplant patients. In addition, vaccines could be potentially designed involving only a small parts of the BKV proteins. However, immunodominance is not simple to define and may vary considerable for different BKV strains, BKV proteins, the HLA-type of the host, and possibly also the type of virus-mediated pathology. Moreover, the cellular immunity consists of different synergizing and antagonizing arms with helper, killer, and regulatory functions. Interestingly, agnoprotein, an abundantly expressed late viral protein in BKV-infected cells *in vitro* and *in vivo* is immunologically ignored by both humoral and cellular immune response[84]. Table 3 gives an overview of the so-far identified BKV epitopes, most of which bind to HLA-A0201.

Homology between BK virus and JC virus – impact on cellular immune assays

The high degree of homology between BKV and JCV on the protein level is potentially of importance for providing some degree of cellular and humoral cross-protection. The VP1 homology is 75% for the genome and 78% for amino acid sequence, which is lower compared to the LT homology of 78% for the genome and 83% for amino acid sequence (Table 1)[17,99,106,107]. Nevertheless, it is clear that primary infection of either virus occurs independent of the other virus. According to the seroprevalence data, BKV infection occurs more frequently than JCV, and mostly prior to JCV. This is in contrast to the higher prevalence rate of JCV shedding in 19% of healthy blood donors compared to 7% of BKV shedding (A. Egli and H.H. Hirsch, unpublished). This points to differences in the epidemiology, reactivation biology, and transmission between

Table 4. T-cell epitopes in the BK virus large T-antigen and major capsid protein

BK virus large T-antigen epitopes				
Position	**Amino acid sequence**	**HLA type**	**Comment**	**Reference**
362	MLTERFNHIL	A0201		(95)
406	VIFDFLHCI	A0201		(95, 97)
410	FLHCIVFNV	A0201		(95, 97)
579	LLLIQFRPV	A0201	Cross-reaction to JCV	(95, 97)
25	GNLPLMRKAYLRKCK	B0708		(102)
613	TFSRMKYNICMGKCI	DRB1 0901	Cross-reaction to JCV	(102)
57	TLYKKMEQDVKVAHQ	DRB1 0301		(102)
553	IYLRKSLQNSEFLLE	B08	Cross-reaction to JCV	(102)
557	KSLQNSEFLLEKRIL	B08	Cross-reaction to JCV	(102)
157	TLACFAVYT	A0201		(97)
BK virus viral protein 1 epitopes				
Position	**Amino acid sequence**	**HLA type**	**Comment**	**Reference**
p44	AITEVECFL	A0201	Cross-reaction to JCV	(77, 102, 104, 105)
p108	LLMWEAVTL	A0201	Cross-reaction to JCV	(104, 105)

Position indicating the amino acid position from start of the protein (amino acid single letter code).

both polyomaviruses, akin to herpes simplex virus type 1 and type 2. Due to significant differences between the BKV and JCV VP1, the major capsid protein, IgG cross-reactivity does not seem to be a major issue. By contrast, the degree of homology is significantly higher for the LT. This is also supported by EliSpot assays performed with PBMC expanded after stimulation with BKV LT and VP1 peptide pools, followed by re-stimulation with either BKV or JCV LT and VP1, respectively[96], which indicated more cross-reactivity between cellular responses for LT, but less so for VP1[96]. Several other groups focused on certain previously identified immunodominant epitopes, which were shared between BKV and JCV. For VP1, the HLA-A0201 epitopes (AITEVECFL and LLMWEAVTL) were described to have cross-reactive potential[77,102,104,105]. For the LT protein, even more immunodominant epitopes with cross-reactive potential have been identified (LLLIWFRPV, TFSRMKYNICMGKCI, IYLRKSLQNSEFLLE, KSLQNSEFLLEKRIL)[95,97,102] (Table 4).

Conclusions

Considerable progress has been made in defining the presentation of polyomavirus BK-associated nephropathy in kidney transplantation. Viral screening and monitoring by quantitative molecular/genetic assays such as real-time PCR have been identified as valuable surrogate markers of the risk of PVAN in kidney transplant patients. Several studies have shown the feasibility of preemptive intervention by reducing immunosuppression in adult and pediatric patients. Provided there is adequate design and quality control, these BKV load assays will prove indispensable in clinical practice. For a more advanced level of characterizing the virus/host balance in kidney transplant patients, BKV-specific immunoassays are currently being tested. Whereas BKV VLP IgG ELISA tests are recognized as sensitive indicators of recent BKV exposure, no humoral markers of protection from BKV viremia or disease have emerged. Cellular immunoassays

indicate generally low frequencies in PBMC of healthy donors and kidney transplant patients alike, and only emerge in PBMC when plasma BKV loads are falling as a sign of regaining control. Further work is needed to better understand BKV-specific cellular immunity in order to contribute to risk stratification and to dosing of immunosuppressive drugs.

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