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ORIGINAL ARTICLE

Inflammatory activation and recovering BKV-specific immunity correlate with self-limited BKV replication after renal transplantation

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Summary

As BKV-associated nephropathy has emerged as an important cause of allograft failure, it has been of major importance to find immune mechanisms suitable to identify kidney transplant recipients (KTRs) at increased risk of BKV replication. We monitored 29 KTRs with seven measurements during the first year post-transplantation. BKV-specific T cells directed to 5 BKV proteins were analyzed in an interferon-γ ELISPOT assay. BKV-specific antibodies were measured using an ELISA. The extent of immunosuppression and inflammatory activation were quantified by measures of immune function including lymphocyte subpopulations, IP-10, and adhesion molecule serum levels. All 5 BKV-specific T cells increased significantly from diagnosis to resolution of BKV replication ($P < 0.001$). While antistructural T cells were significantly higher in KTRs with BKV replication ($P < 0.05$), no differences were observed for antismall t- and large T-antigen-directed T cells ($P > 0.05$). Interestingly, 65% of KTRs without BKV replication showed transient appearance of antismall t- and large T-antigen-directed T cells. Although no significant differences were observed for T-cell subpopulations and adhesion molecules, IP-10 levels increased significantly during BKV replication ($P < 0.05$). Assessment of BKV-specific T cells identifies recovering BKV-specific immunity in KTRs with BKV replication and suggests their protective ability in KTRs without BKV replication. Increases in IP-10 levels stress the importance of infiltrating inflammatory leukocytes in the regulation of BKV replication and point to inflammatory activation in the pathogenesis of BKV replication.

Introduction

Reactivation of latent polyomavirus BK (BKV) infection in kidney transplant recipients (KTRs) can cause so-called BKV-associated nephropathy (BKVN)—a serious complication with graft failure in up to 30% of cases [1–7]. Therefore, quantification of BKV load has been evaluated to monitor BKV replication [6,8–12]. Different previous studies demonstrated that the control of BKV replication is related to recovering BKV-specific immunity [13–21]. Recently, our own data showed that development of BKV-specific T cells differentiates KTRs with self-limited BKV replication from KTRs with progression to BKVN [13].

Although approximately 75% of cases occur within the first year after renal transplantation, knowledge of BKV-specific immunity remained incomplete during this early period of high-dose immunosuppression. It is of major importance to understand the balance between BKV replication, immune control, and immunosuppressive therapy to protect KTRs with BKV replication from viral injury. Thus, it would be most helpful to characterize immune mechanisms that can be used to identify KTRs with an increased risk of BKV replication.

We attempted to address these questions by prospectively monitoring BKV-specific immunity during the first year post-transplantation. Frequencies of BKV-specific interferon-γ-(IFNg)-secreting lymphocytes were analyzed using an ELISPOT assay after stimulation of peripheral blood mononuclear cells (PBMC) with 5 BKV proteins. BKV-specific IgM and IgG levels were measured using an enzyme-linked immunosorbent assay (ELISA). We attempted to quantify the extent of immunosuppression and inflammatory activity by enumeration of lymphocyte subpopulations, circulating interferon-g-inducible protein 10 (IP-10), soluble intercellular adhesion molecule-1 (sICAM-1), and soluble vascular cell adhesion molecule-1 (sVCAM-1).

Patients and methods

Patients

This study was approved by our local ethical review committee in compliance with the declaration of Helsinki (Ethic Committee Charité University Medicine Berlin, Germany, 126/2001, 07/30/2001). Informed consent was obtained from all patients. 30 patients who received renal transplants between September 2008 and January 2009 were consecutively enrolled. One patient was excluded for development of acute rejection and graft loss. KTRs were divided into two groups according to BKV replication. The prevalence of BK viruria and viremia was 41% and 14%, respectively.

Group 1 included 12 KTRs with BKV replication in urine and/or serum. Renal biopsies were not performed as these subjects had stable renal function. Self-limited BKV reactivation is defined as BKV load in urine or serum for less than 6 months without any therapeutic interventions. Group 2 included 17 KTRs without BKV replication during the first post-transplant year. 28 KTRs were treated with anti-interleukin-2R-antibody as immunosuppressive induction. 1 KTR received antithymocyte globulin (ATG). No differences were observed concerning calcineurin-inhibitor trough levels and mycophenolate dosages ($P > 0.05$). Treatment of acute rejection consisted of pulse steroids. All 29 study subjects are alive with a functioning graft. Detailed characteristics are shown in Tables 1 and 2.

Collection of samples

Serum and urine samples for BKV load screening and blood samples for immune monitoring were collected at +1 week, +1, +2, +3, +6, +9, and +12 months after transplantation. Samples were successfully collected in 90.1% of cases.

PBMC were isolated from 10 to 20 ml of heparinized blood using standard Ficoll-Hypaque density gradient technique. Serum was used to detect BKV-DNA by TaqMan real-time PCR, BKV-specific antibodies, IP-10, sICAM, and sVCAM by ELISA.

Design of BKV-specific overlapping peptide pools

BKV strain AS, genotype III, was used to design BKV-specific overlapping peptide pools as described previously [13, 14]. Overlapping peptide pools were synthesized by JPT (Berlin, Germany) to span the entire sequences of non-structural small t-antigen and large T-antigen, and structural VP1-3. Overlapping peptide pools are built of 15 amino acids with each peptide sharing an overlap of 11 amino acids with the previous one. Overlapping peptide pools were diluted in dimethyl sulfoxide and used at concentrations of 1 μg/ml.

Quantitative PCR for BKV-DNA detection

sBKV loads were measured by TaqMan real-time PCR as described previously (11,13,14). Briefly, DNA was isolated from serum using a QIAamp DNA Mini Kit (Qiagen Corp., Hilden, Germany) according to manufacturer's instructions. PCR was based on the TaqMan platform (ABI). PCR amplifications were set up in a reaction volume of 25 u/μl using primer and probe at final concentrations of 900 nM and 5 μM, respectively. Primers and probe were designed to amplify the VP1 region of BKV, respectively. A plasmid standard containing the VP1 coding region of respective virus was used to determine the copy number per ml. Thermal cycling was begun with an initial denaturation step at 95 °C for 10 min that was followed by 40 cycles at 95 °C for 15 s (denaturation) and 60 °C for 1 min (reannealing and extension). The detection level is the lowest viral load measured within the range of linearity, 4500 copies/ml serum.

ELISPOT Assay for IFNg detection

BKV-specific cellular immunity was determined by measuring IFNg upon stimulation of PBMC as described previously [13]. PBMC were isolated from 10 to 20 ml of heparinized blood using the standard Ficoll-Hypaque density gradient centrifugation technique. For ELISPOT assay, 96-well multiscreen filter plates (Millipore, Billerica, MA, USA, MAIPS 4510) were coated with 100 μl of primary IFNG monoclonal antibody (mAb) at a concentration of 3 μg/ml (ahu-IFNG-Endogen M700A) and incubated overnight at 4 °C. A standardized responder T-cell number of 2.5×10^5 PBMC per well was added in quadruple but at least triplicate wells with or without peptides (1 μg/ml) and with Staphylococcus enterotoxin B (SEB; Sigma, St. Louis, MO, USA, 1 μg/ml) as positive control and incubated for 24 h at 37 °C. Negative controls were always run in parallel using responder cells plus medium alone. Plates

Table 1. Clinical characteristics of KTRs.

	Recipient age/sex	Donor type	Donor age/sex	HLA-mismatch	Acute rejection (Banff grading)/ time point (months)	Maintenance immunosuppression
Group 1 (n = 12)						
1	59/f	Deceased	59/m	2	–	MP, MPA, Tac
2	66/m	Deceased	66/m	5	–	MP, MMF, Tac
3	28/f	Living	47/f	3	–	MP, MMF, CsA
4	51/m	Living	46/f	5	–	MP, MMF, Tac
5	58/m	Deceased	51/m	0	–	P, MMF, Tac
6	65/f	Deceased	50/f	4	–	P, MMF, Tac
7	44/f	Deceased	56/m	2	–	MP, MPA, Tac
8	68/m	Living	52/f	3	–	MP, MMF, CsA
9	44/m	Deceased	59/f	2	–	MP, MMF, CsA
10	71/f	Deceased	70/m	5	Borderline/3	MP, MPA, Tac
11	53/m	Living	50/f	5	–	MP, MMF, CsA
12	47/m	Deceased	50/f	0	–	MP, MMF, Tac
Mean	55		55	3		
SD	13		8	2		
Median	56		52	3		
Range	44–71		46–70	0–5		
IQR	46–65		50–59	2–5		
Group 2 (n = 17)						
13	76/f	Deceased	66/m	4	–	MP, MMF, Tac
14	49/m	Deceased	55/f	6	–	MP, MPA, Tac
15	60/f	Deceased	51/m	0	–	MP, MMF, Tac
16	30/m	Living	48/f	5	–	MP, MMF, CsA
17	59/m	Living	60/f	4	Ia/1	MP, MMF, Tac
18	68/m	Deceased	65/m	3	–	MP, MMF, Tac
19	56/f	Deceased	11/m	2	–	MP, MMF, Tac
20	30/f	Living	50/f	3	Ib/3	MP, MMF, CsA
21	66/f	Deceased	70/m	6	–	MP, MMF, Tac
22	53/f	Deceased	53/m	0	–	MP, MMF, Tac
23	48/m	Deceased	64/m	4	–	MP, MMF, Tac
24	47/f	Deceased	47/m	4	–	MP, MMF, Tac
25	71/m	Living	55/f	6	–	MP, MMF, Tac
26	30/f	Deceased	13/m	0	–	MP, MMF, Tac
27	29/f	Deceased	28/f	0	–	MP, MMF, Tac
28	42/m	Living	45/m	4	Ia/0	P, MMF, Tac
29	32/f	Deceased	55/m	0	–	MP, MPA, CsA
Mean	50		49	3		
SD	16		17	2		
Median	49		53	4		
Range	29–76		11–70	0–6		
IQR	34–60		47–60	0–4		
Mean	52		51	3		
SD	14		14	2		
Median	53		52	3		
Range	29–76		11–70	0–6		
IQR	44–65		48–59	2–5		

IQR, interquartile range; f, female; m, male; MP, methylprednisolone; P, prednisolone; MMF, mycophenolate mofetil; MPA, mycophenolic acid; CsA, cyclosporine A; Tac, tacrolimus.

were incubated overnight at 4 °C with 100 µl (1 µl/ml) biotinylated detection IFNG antibody (ahu-IFNG biotin-Endogen M701). After adding streptavidine (1 µg/ml) for 2 h at room temperature, spots were developed by adding 200 µl visualization solution, AEC (3-amino-9-ethylcarbazole, SIGMA) in acetate buffer supplemented with H_2O_2 30% for 3–5 min. Resulting spots were counted using a computer-assisted ELISPOT reader (Immunospot, Cellular Technologies, Ltd., Cleveland, OH, USA). The number of SFU/10^6 PBMC per well was calculated by

Table 2. Time-course of BKV replication and BKV loads

	Onset of first BKV replication (months)	No. of samples with positive BKV load*	Duration of BKV replication (months)†	Peak uBKV load (copies/mL)/ time point (months)	Peak sBKV load (copies/mL)/ time point (months)
Group 1					
1	2	2	4	1.65E+4/3	–
2	1	2	2	2.65E+5/2	–
3	1	4	8	4.14E+5/3	–
4	6	1	1	6.84E+4/6	–
5	3	2	6	2.89E+4/3	–
6	2	1	1	2.55E+4/2	–
7	3	1	3	2.12E+4/3	–
8	1	3	5	1.38E+8/3	–
9‡	1	3	11	7.20E+3/3	1.02E+4/12
10‡	1	6	11	1.06E+8/12	1.42E+4/12
11	1	2	2	2.35E+5/2	2.50E+4/2
12	2	2	4	7.18E+7/2	1.97E+5/2
Mean	2	2	5	2.62E+7	6.16E+4
SD	1	1	4	4.75E+7	9.04E+5
Median	2	2	4	2.35E+5	1.96E+4
Range	1–6	1–6	1–11	7.20E+3-1.38E+8	1.02E+3-1.97E+5

uBKV load, urine-BKV load; sBKV load, serum BKV load; SD, standard deviation.
*Samples were obtained at +1 week, +1, +2, +3, +6, +9, +12 months after transplantation.
†Duration from first positive BKV load to clearance of BKV reactivation or end of study period.
‡Patient did not recover from BKV replication by the end of the first year post-transplantation.

adding spot counts from quadruple. Positive ELISPOT signals were predefined as containing at least 10 SFU per well after subtraction of negative control.

ELISA for BKV-specific antibodies

BKV-specific IgM and IgG were measured in serum samples using virus-like particles (VLP) made by expressing BKV-VP1. BK polyomavirus, strain AS and major capsid VP1 protein (ab74567) expressed in *S. cerevisiae* were purchased from Abcam plc, 330 Cambridge Science Park Cambridge CB4 0FL UK for use in Sigma. Standard 96-well plates with high-level coating properties were coated by diluting BKV-VP1 to 1.0 μg/ml and adding 100 μl to each well. After an overnight incubation at 4°C, the plates were washed three times with wash solution (50 mM Tris, 0.14 M NaCl, 0.05% Tween-20, pH 8.0), treated with 200 μl of blocking solution (50 mM Tris, 0.14 M NaCl, 1% BSA, pH 8.0) for 30 min. After the plates were then washed three times with wash solution, 100 μl of the diluted serum samples (1:200 for IgM, 1:500 for IgG) was transferred to assigned wells and incubated for 60 min and washed again five times. Horseradish peroxidase conjugated secondary antibody was diluted (1:100 000 for goat anti-human IgG, 1:50 000 for goat anti-human IgM) and then 100 μl was added to each well. After the incubation of 60 min with the secondary antibody, the plates were washed five times with wash solution. The o-phenylenediamine hydrochloride color reaction was stopped after 15 min by adding 1N sulfuric acid. Optical density was measured at 405 nm with an automated microtiter plate reader. BKV-specific antibodies were measured and read as optical density (OD) units. For a cutoff, an optical density at 405 nm of <0.16 (IgM) <0.18 (IgG) was defined as nonreactive for BKV-VLP. Positive and negative control sera, sensitivity controls, and reproducibility controls were included in each run. Runs where replicate serum values fell outside the expected coefficient of variation were repeated.

ELISA for IP-10, sICAM, and sVCAM

IP-10, sICAM-1, and sVCAM-1 serum levels were determined by a sandwich enzyme immunoassay using a Human IP-10 Quantikine, a human sICAM Quantikine, and a human sVCAM Quantikine by R&D Systems, respectively. Serum dilutions for IP-10, sICAM-1, and sVCAM-1 were 30-, 20-, and 20-fold, respectively.

Flow cytometry

Cell phenotype was analyzed by staining with fluorochrome-conjugated monoclonal antibodies for the surface markers CD3, CD8, CD4 (BD Biosciences, San Jose, CA, USA) to determine T-cell subpopulations. Four-color flow cytometry was performed using FACSCalibur and CELLQuest Software (BD Biosciences, Franklin Lakes, NJ, USA).

Statistical methods

Statistical tests were performed using SPSS Version 19 (SPSS, Chicago, IL, USA). For comparisons of study groups, two-sided Mann–Whitney *U*-test for nonparametric independent samples was used. For comparisons between paired samples, Friedman and two-sided Wilcoxon signed-rank test for nonparametric dependent samples were used. Two-sided *P*-values <0.05 were considered statistical significant with Bonferroni adjustment for pairwise comparisons.

Results

Characteristics of the study population

Twenty-nine KTRs were consecutively included in this study. Patient characteristics are shown in Tables 1 and 2. According to the study design, the main difference between both groups was the presence of BKV replication. Group 1 included 12 KTRs with BKV replication. Eight of 12 KTRs (67%) showed a quick recovery without therapeutic interventions or progression to BKVN. 4 KTRs (33%) showed prolonged BKV replication of more than 6 months with 2 KTRs (16%) having ongoing BKV replication. One patient developed biopsy-proven BKVN in further follow-up. No therapeutic reduction in maintenance immunosuppression was performed in any patient. Group 2 included 17 KTRs without BKV replication during the first post-transplant year. No differences in age, sex, HLA-mismatch, C-reactive protein, serum creatinine levels, delayed graft function, cold ischemia time, time on dialysis, CMV reactivation, immunosuppression, or acute rejection episodes were observed between groups 1 and 2 ($P > 0.05$). No differences were observed concerning donor type, age, or sex, donor C-reactive protein ($P = 0.672$), and donor leukocytes ($P = 0.320$) between groups 1 and 2.

Kinetics of BKV-specific immunity

Kinetics of BKV-specific T cells in KTRs with BKV replication

All 12 KTRs (100%) with BKV replication (group 1) showed low or undetectable T-cell responses at +1 week (Figs 1a–e and 2a,b). Peak levels of BKV-specific T cells were observed at +3, +6, and +9 months and were significantly higher than at +1 week ($P < 0.05$) for all BKV proteins (Fig. 2). Overall, antistructural and antismall t- and large T-antigen immune responses were detected simultaneously in 8 of 12 KTRs (75%). In 4 of 12 KTRs (25%), however, antistructural responses preceded detection of antismall t- and large T-antigen responses. Interestingly, immune responses directed to major structural VP1 were significantly higher than to VP2/VP3 and small t-antigen ($P < 0.05$), whereas no differences were observed between VP1 and large T-antigen ($P = 0.203$).

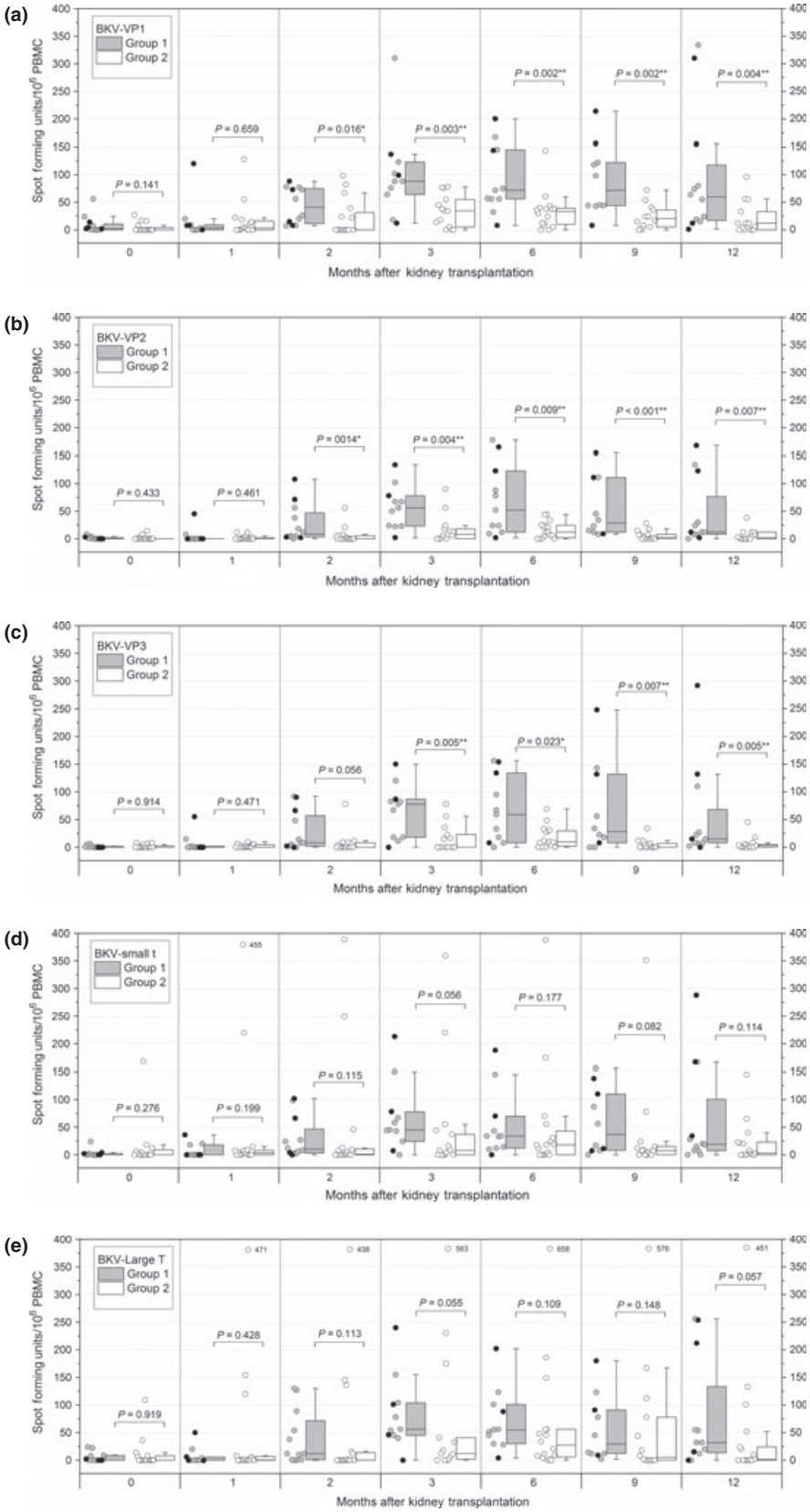
Overall, 10 of 12 KTRs (83%) showed immune responses directed to all 5 BKV proteins at least at one time. 2 KTRs did not develop BKV-specific T cells and showed ongoing BK viremia after +12 months. First detection of BKV-specific T cells in KTRs of group 1 was accompanied by recovery from BKV replication. At the time of recovery from BKV replication, BKV-specific T-cell responses directed to all BKV-specific proteins were significantly elevated. At this point in time, stimulation with BKV-VP1-3-, -small t-antigen-, and -large T-antigen elicited 132 SFU [median, range (73–334)], 105 SFU [median, range (45–165)], 108 SFU [median, range (56–150)], 95 SFU [median, range (44–288)], and 114 SFU [median, range (40–254)], respectively. No differences were observed between KTRs with BK viruria only and KTRs with BK viremia for primary onset of BKV-specific T cells, or pattern of BKV-specific immune responses to different BKV antigens. Frequencies of BKV-specific T cells in patient 11 and 12 with a quick recovery from BK viremia tend to be higher compared to KTRs with BK viruria only for all BKV antigens (Fig. 1).

Kinetics of BKV-specific T cells in KTRs without BKV replication

All 17 KTRs (100%) without BKV replication (group 2) showed low or undetectable T-cell responses at the time of kidney transplantation (Fig. 1a–e). BKV-specific T-cell responses increased continuously with peak at +3 and +6 months, which were significantly higher than at +1 week ($P < 0.01$) for all investigated BKV proteins. Overall, 6 of 17 KTRs (35%) showed immune responses directed to structural BKV proteins at least once, whereas only 3 of 17 KTRs (18%) showed immune responses directed to all structural proteins. Interestingly, 11 of 17 KTRs (65%) showed immune responses to nonstructural small t- and/or large T-antigen at least once. Three of 17 KTRs (18%) showed immune responses to all 5 BKV proteins, whereas 6 of 17 KTRs (35%) showed no BKV-specific T cells at any time.

No significant differences in frequencies of BKV-specific T cells were observed between both groups at + 1 week and

Figure 1 Significantly elevated BKV-specific T-cell responses to structural VP1-3 in KTRs with BKV replication ($P < 0.01$). Differences between KTRs with BKV replication (group 1) and KTRs without BKV replication (group 2) reach significance at +3, +6, +9, and +12 months for structural BKV antigens only (a-e; $P < 0.05$). BKV-specific T-cell responses directed to nonstructural st and LT do not reach significance at any time in the first post-transplant year. Shown are the median values with interquartile range (box borders) and individual values. Black dots mark KTRs developing BK viremia, gray dots mark KTRs developing BK viruria only.


(a)
BKV-VP1
Group 1
Group 2
P = 0.141
P = 0.659
P = 0.016*
P = 0.003**
P = 0.002**
P = 0.002**
P = 0.004**
(b)
BKV-VP2
Group 1
Group 2
P = 0.433
P = 0.461
P = 0014*
P = 0.004**
P = 0.009**
P < 0.001**
P = 0.007**
(c)
BKV-VP3
Group 1
Group 2
P = 0.914
P = 0.471
P = 0.056
P = 0.005**
P = 0.023*
P = 0.007**
P = 0.005**
(d)
BKV-small t
Group 1
Group 2
P = 0.276
P = 0.199
P = 0.115
P = 0.056
P = 0.177
P = 0.082
P = 0.114
(e)
BKV-Large T
Group 1
Group 2
P = 0.919
P = 0.428
P = 0.113
P = 0.055
P = 0.109
P = 0.148
P = 0.057
Spot forming units/10^6 PBMC
Months after kidney transplantation

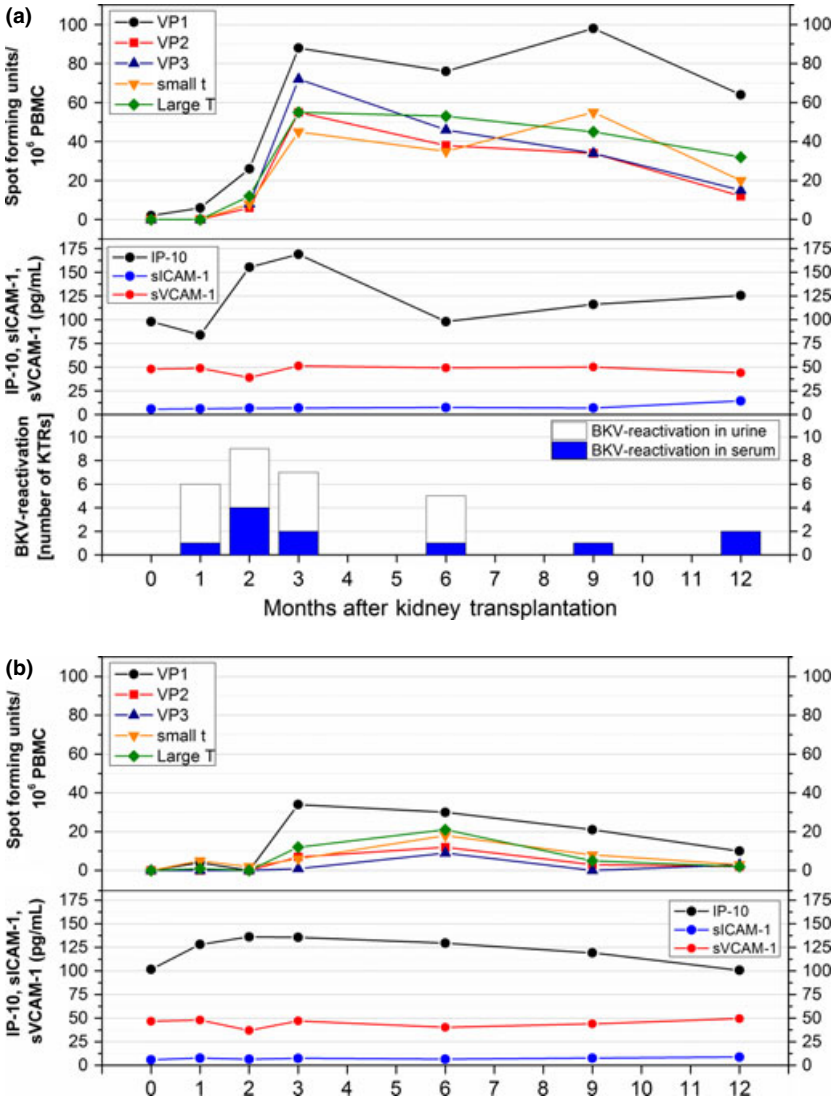


Figure 2 Increase in BKV-specific cellular immunity and IP-10 levels associated with quick recovery from BKV replication (a). KTRs with self-limited BKV replication are characterized by an increase in BKV-specific T cells. BKV-specific T cells directed to structural VP1-3 and nonstructural st and LT at +2, +3, +6, +9, and +12 months were higher than at +1 week, +1 month, and +2 months ($P < 0.05$). A tendency for higher IP-10 levels was observed at +3 months compared to +1 month ($P = 0.084$). KTRs without BKV replication show low frequencies of BKV-specific T cells (b). Upper frame: median BKV-specific IFNg-secreting T cells. Middle frame: median IP-10, sICAM-1, and sVCAM-1 serum levels. Lower frame: Numbers of KTRs with BKV replication in urine and/or serum.

+1 month ($P > 0.05$; Fig. 1a–e). At +2, +3, +6, +9, and +12 months, however, BKV-specific T cells to structural BKV proteins were significantly higher in group 1 ($P < 0.05$). Differences in BKV-specific T-cell responses directed to small t- and large T-antigen did not reach significance at any time ($P > 0.05$; Figs 1a–e, 2a,b).

Kinetics of BKV-specific antibodies in KTRs with BKV replication

All 12 KTRs of group 1 (100%) showed detectable BKV-specific IgG levels at +1 week and were classified as BKV seropositive. BKV-specific IgG levels increased as early as at diagnosis of BKV reactivation with peak BKV-specific IgG levels at +6 and +9 months. BKV-specific IgG levels at +6 and +9 months were significantly higher compared to +1 week, +1, +2, and +3 months ($P < 0.05$; Fig. 3a). As shown in Figs 1 and 3, the increase in BKV-specific antibodies was detectable as early as at diagnosis of BKV replication and earlier compared to BKV-specific cellular immune responses ($P < 0.05$).

Most KTRs of group 1 showed low or undetectable BKV-specific IgM levels at +1 week. IgM levels increased

significantly during active BKV replication phases and subsided afterward. Four of 12 KTRs (33%) showed no increase in BKV-specific IgM levels. Peak BKV-specific IgM levels were observed at +3 months and were significantly higher compared to +1 week and +1 month ($P < 0.05$; Fig. 3b). No differences for BKV-specific IgM and IgG were observed between KTRs with BK viruria only and BK viremia ($P > 0.05$).

Kinetics of BKV-specific antibodies in KTRs without BKV replication

Fifteen of 17 KTRs of group 2 (88%) showed detectable BKV-specific IgG levels at +1 week and were classified as BKV seropositive. 2 of 17 KTRs (12%) were classified as BKV seronegative. Only 3 of 17 KTRs (18%) showed a substantial increase in BKV-specific IgG levels within the first months after transplantation. Most KTRs showed low or undetectable BKV-specific IgM levels at +1 week. Three of 17 KTRs (18%) showed transient appearance of BKV-specific IgM (Fig. 3a,b).

No significant differences were observed concerning the magnitude of BKV-specific IgM and IgG levels between both groups at +1 week ($P > 0.05$). At +1, +2, +3, +6, +9, and +12 months, however, BKV-specific IgG levels increased significantly in KTRs of group 1 ($P < 0.05$). Additionally, significantly more KTRs of group 1 showed transient appearance of BKV-specific IgM, which were higher at +3, +6, +9, and +12 months (Fig. 3a,b; $P < 0.05$).

Measures of immune function and inflammatory activity

At diagnosis of BKV replication, IP-10 levels were significantly higher than before BKV replication ($P < 0.01$), whereas no difference was observed after recovery

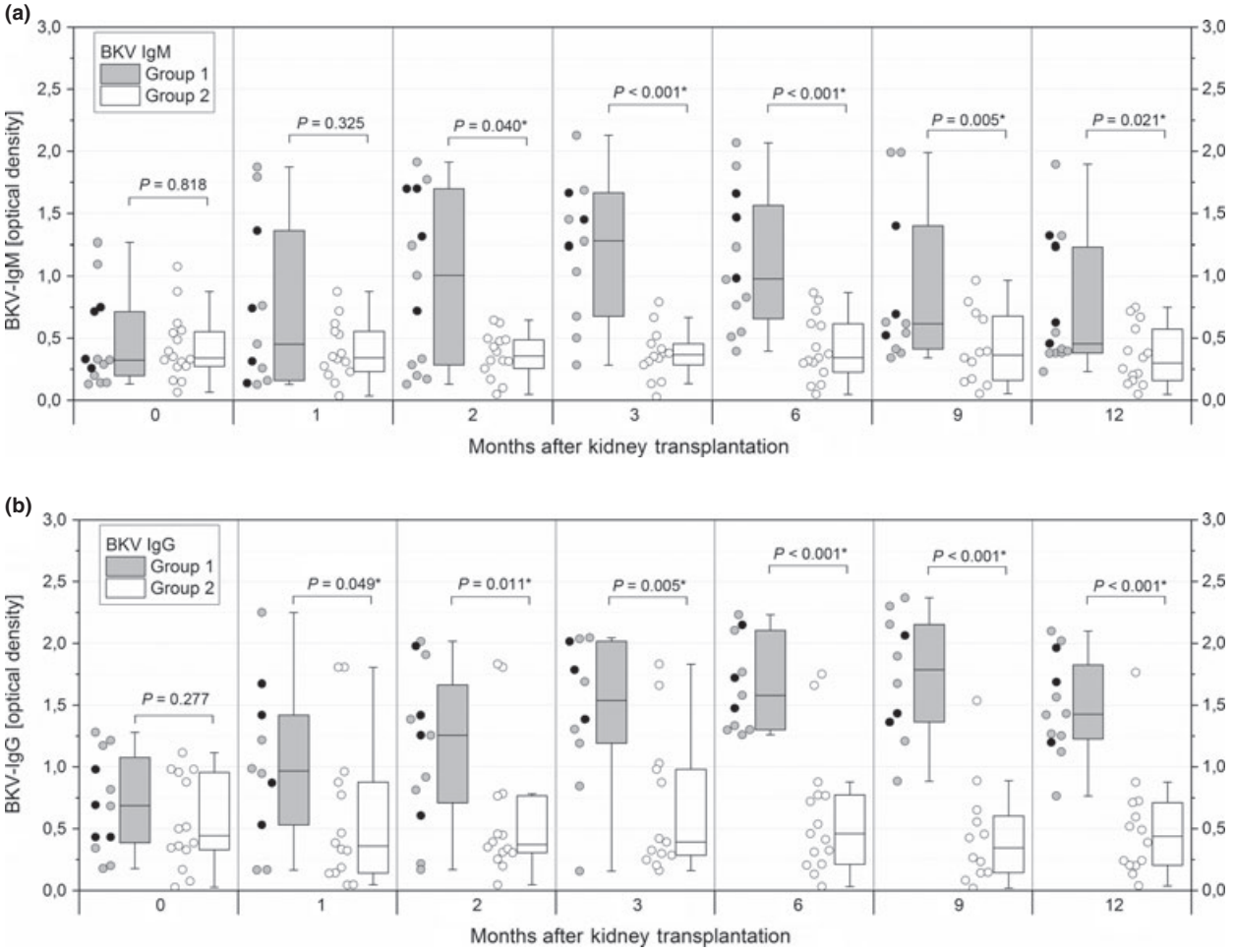


Figure 3 Increase in BKV-specific IgM and IgG levels in KTRs with BKV replication. Differences in BKV-specific IgM levels reach significance at +2, +3, +6, +9, and +12 months after renal transplantation (a; $P < 0.05$). Differences in BKV-specific IgG levels reach significance at +1, +2, +3, +6, +9, and +12 months after renal transplantation (b; $P < 0.05$). Shown are the median values with interquartile range (box borders) and individual values. Black dots mark KTRs developing BK viremia, gray dots mark KTRs developing BK viruria only.

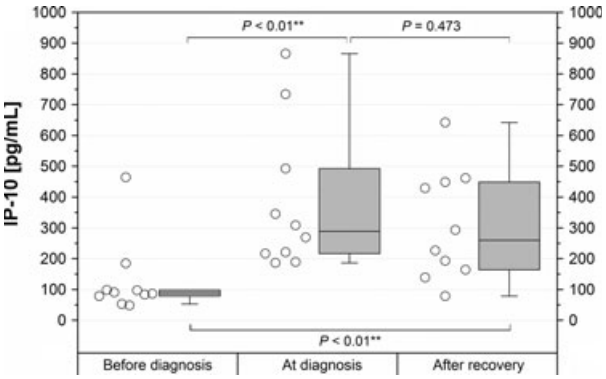


Figure 4 Significantly higher IP-10 serum levels at diagnosis of BKV replication. IP-10 levels are significantly higher in KTRs with self-limited BKV replication (group 1) at diagnosis of BKV replication than before diagnosis of BKV replication ($P < 0.01$). After recovery from BKV replication, IP-10 levels are significantly higher than before diagnosis of BKV replication ($P < 0.01$), whereas no difference is observed in comparison with the time of diagnosis ($P = 0.473$).

($P = 0.473$; Fig. 4). In follow-up after recovery, IP-10 serum levels further declined and were significantly lower than at diagnosis of BKV replication ($P = 0.021$). Patients with ongoing BKV replication showed increased IP-levels during replication. KTRs with self-limited BKV replication showed significantly higher IP-10 levels at the time of diagnosis ($P < 0.05$) and after recovery from BKV replication ($P < 0.05$) compared to KTRs without BKV replication at any time after transplantation. KTRs without BKV replication did not show a significant increase in IP-10 levels in follow-up ($P > 0.05$).

No differences were observed for sICAM and sVCAM from diagnosis to recovery from BKV replication ($P > 0.05$). No statistical significant differences were observed between both groups at +1 week, +1, +2, +3, +6, +9, and +12 months for IP-10, sICAM, and sVCAM ($P > 0.05$; Fig. 2a,b).

We found higher leukocyte counts in KTRs with BKV replication compared to KTRs without BKV replication at +1, +2, and +3 months, which were 10.26/nl vs. 7.65/nl [median; $P = 0.298$], 9.53/nl vs. 7.54/nl [median; $P = 0.034$], 9.15 vs. 6.96 [median; $P = 0.388$], respectively. Interestingly, 42% of KTRs with BKV replication showed increased leukocyte counts of >11/nl at diagnosis of BKV replication compared to 6%, 12%, and 6% in KTRs without BKV replication at +2, +3, and +6 months. The analysis of lymphocyte subpopulations included CD3 + , CD3 + CD4+, CD3+ CD8+ T-cell counts, but showed no significant differences between both groups ($P > 0.05$).

Discussion

The development of BKV-specific immunity is of central importance in limiting primary BKV infection and in controlling the BKV carrier state. Previous observations suggest that BKV-specific antibodies may contribute to protection from BKV replication and accelerate BKV clearance [16,22,23]. The crucial role of BKV-specific T cells in the containment of BKV replication, however, is suggested in recent studies showing that recovering BKV-specific cellular immunity is correlated with BKV clearance [13–19,24–27]. Previous studies showed strong inflammatory cytokine responses in KTRs with BKV replication in urine [28]. Aims of our analysis were to monitor BKV-specific immunity kinetics, markers of T-cell function, and inflammatory activity to point out differences in clinical settings.

We had originally hypothesized that development of BKV-specific T cells might protect from BKV replication during the first months after renal transplantation. Low or undetectable BKV-specific cellular immunity as shown for KTRs with/without BKV replication in the early phase post-transplantation, however, indicates a state of overimmunosuppression and increased risk of BKV replication. In this context, evaluation of pretransplant BKV-specific immunity might be of major importance to identify patients at increased risk. Our findings suggest the importance of structural VP1-3 as sensitive markers of BKV replication over nonstructural small t- and large T-antigen. Even low-level BK viruria only represents a sufficient trigger for reconstitution of BKV-specific cellular immunity.

Our data show a relevant number of KTRs without BKV replication who develop antismall t- and large T-antigen responses (65%). These findings are in line with previous observations, suggesting that antismall t- and large T-antigen responses are associated with recovery and protection from BKV replication [13,18]. The increase in BKV-specific T cells in KTRs without BKV replication may be either related to a self-limited BKV replication not detected with our sampling density or linked to the ability to mount a sufficient BKV-specific T-cell response to protect from BKV replication.

If we compare these findings of KTRs with BK viruria only with our previous observations of KTRs developing BK viremia [13] (data not shown), we observe a tendency for higher frequencies of BKV-specific T cells at recovery from BK replication in KTRs with BK viremia. Even the 2 KTRs with quick recovery from BK viremia in this study tend to show higher frequencies of BKV-specific T cells compared to KTRs with BK viruria only. Interestingly, substantially elevated BKV-specific T cells in KTRs with BK viruria only decreased within a few weeks after recovery from BKV replication, which suggests that prolonged detection of BKV-specific T cells, as shown for KTRs recovered from BK viremia and BKVN [13,14], represents a marker of severity and duration of BKV replication.

Monitoring of BKV-specific immunity and measures of immune function do not provide a characteristic profile to identify KTRs with BKV replication early after transplantation. However, increased frequencies of antismall t- and large T-antigen-directed T cells in a relevant number of KTRs without BKV replication and our previous observations in KTRs with BKVN [13] suggest their involvement in recovery and protection from BKV replication.

Initial inspections of our data suggest that KTRs with the ability to develop BKV-specific cellular immune responses show a quick and self-limited recovery from BKV replication. As almost all KTRs show well detectable BKV-specific IgG levels at the time of BKV reactivation, our data strongly suggest that BKV seropositivity and high titers of BKV-specific antibodies do not protect from BKV replication. Our results indicate that quantification of BKV-specific T cells is a better parameter for response to BKV replication than BKV-specific antibodies [13,18,25,29].

Stable allograft function with stable serum creatinine concentrations supports the hypothesis that suggested cytotoxicity and tissue damage due to BKV-specific T cells might be related to prolonged viral persistence and BKVN [19,20]. Return of BKV-specific T cells into the circulation could be rather a sign of recovery in KTRs with a short course of self-limited BKV replication. As shown previously [13], KTRs with BKVN are characterized by the failure to develop a sufficient BKV-specific T-cell response with a temporal association of antismall t- and large T-antigen responses and recovery from BKVN [13,17,18]. Our results also stress that minor structural VP2/VP3 and nonstructural small t-antigen are more antigenic in a relevant number of KTRs and should be included in stepwise manner next to VP1 and large T-antigen [13,14].

Another aim of this study was to evaluate inflammation and endothelial activation, measured as levels of IP-10, sICAM-1, and sVCAM-1. A significant finding of this investigation was the observation that BKV infection increases IP-10 levels, which precipitously declined after BKV clearance to baseline levels. However, another outcome of our research was a less pronounced response of adhesion molecules sICAM-1 and sVCAM-1, which suggests a more prominent role for immune-mediated effector mechanisms of inflammatory activation. The activation of the chemokine IP-10 provides a mechanism by which BKV infection may enhance the effector immune response to the infected tubular epithelial cells. IP-10 is predominantly chemotactic for activated T cells, natural killer cells, and monocytes and has been shown to be essential in the development of a protective TH1-response against viral infections [30–34]. In BKV replication, there is evidence of a TH1 response, characterized by elevated levels of IFNg [14,15].

These observations can be used to suggest a possible pathogenic pathway of BKV replication. Infection with BKV leads to the generation of antigen-specific activated T cells in lymphoid organs. During this period, infection of tubular epithelial cells results in expression of IP-10, attracting activated T cells which home to BKV-infected tissues. BKV-activated infiltrating T cells further express IFNg, thus enhancing T-cell differentiation.

We hypothesize that the induction of IP-10 by processes involving infiltrating inflammatory leukocytes is important in the regulation of acute BKV replication and may provide a clue for the involvement of specific immune responses in KTRs with progression to BKVN. In addition, data on increased leukocyte counts at diagnosis of BKV replication suggest that systemic inflammatory processes might trigger BKV replication.

Similar to other studies, the number of KTRs with BKV replication is limited, which leads to relatively small sample sizes. Furthermore, it cannot be excluded that KTRs without BKV replication actually had self-limited BKV replication which was not detectable due to limitations of the sampling density. Limitations of the sampling density also may have an impact on frequencies of BKV-specific T cells and measures of immune function, which already might be decreasing. Unfortunately, we were unable to collect samples pretransplantation. Therefore, the prognostic value of pretransplant BKV-specific immune responses needs to be addressed in further studies.

In summary, this is the first study prospectively monitoring BKV-specific immunity kinetics with a simultaneous assessment of T-cell function in KTRs with and without BKV replication. Our data indicate BKV-specific T cells as useful prognostic markers in the setting of KTRs with BKV replication. Unfortunately, our results do not suggest a sensible approach for risk stratification of BKV replication among all KTRs. Further studies are necessary to evaluate the prognostic value of BKV-specific immune monitoring prior to transplantation.

Authorship

TS, NB: and PR: participated in research design. TS and PR: participated in the writing of the paper. TS, MS and AS: participated in the performance of the research. TS and PR: participated in data analysis.

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