Heterospecific Immunity Help to Sustain an Effective BK-virus Immune Response and Prevent BK-virus-associated Nephropathy

Manon Dekeyser
Assistance Publique - Hôpitaux de Paris (AP-HP), Paris-Saclay University, Gustave Roussy Institut, INSERM - UMR 1186, Integrative Tumor Immunology and Immunotherapy https://orcid.org/0000-0002-1829-3767

Marie-Ghislaine de Goër de Herve
Paris-Saclay University, Gustave Roussy Institut, INSERM - UMR 1186, Integrative Tumor Immunology and Immunotherapy

Houria Hendel-chavez
INSERM and Université Paris-Sud

Emmanuelle Boutin
Assistance Publique - Hôpitaux de Paris (AP-HP), INSERM, IMRB

Florence Herr
Paris-Saclay University, Gustave Roussy Institut, INSERM - UMR 1186, Integrative Tumor Immunology and Immunotherapy

Romain Lhotte
Assistance Publique - Hôpitaux de Paris (AP-HP), INSERM U976 (Team 3)

Jean-Luc Taupin
Assistance Publique -Hopitaux De Paris

Yassine Taoufik
French Institute of Health and Medical Research

Antoine Durrbach (antoine.durrbach@aphp.fr)
Assistance Publique - Hôpitaux de Paris (AP-HP), Paris-Saclay University, Gustave Roussy Institut, INSERM - UMR 1186

Article

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Abstract

BK-virus-associated nephropathy (BKvAN) is a major complication in kidney transplant recipients, associated with a higher level of BK-virus (BKv) replication and leading to poor graft survival. In a large cohort of kidney transplant recipients with various BKv reactivation levels, we found that BKvAN was associated with a low BKv-specific memory CD8 T-cell functionality and an exhausted-like phenotype. This severe lymphocyte impairment was associated with a low level of class II HLA mismatches. *In vitro* experiments showed that allogeneic CD4 T cells partly restored BKv-specific memory CD8 T-cell responses. Our results suggest that in kidney transplant recipient, allogeneic CD4 T cells may provide a heterospecific help that maintain an effective BKv-specific memory CD8 T-cell response, and allows a better control of BKv replication in the kidney graft.

Introduction

With the development of potent immunosuppressive therapies, kidney transplantation has become the standard treatment for patients with end-stage renal disease, improving patient survival over dialysis[1]. Over the last decade, BK-virus (BKv) has emerged as a major cause of opportunistic infections in kidney transplant recipients (KTRs).

BKv reactivation occurs in 30 to 40% of KTRs, and may lead to BK-virus-associated nephropathy (BKvAN)[2], which affects almost 10% of KTRs, and may result in premature kidney allograft failure and irreversible graft loss[3]. In the context of kidney transplantation, the initial event is BKv replication in urine, and then in blood, which may be associated with BKvAN. There is currently no specific antiviral treatment against BKv. Decreasing the intensity of therapeutic immunosuppression to restore BKv-specific immune responses remains the only therapeutic option, but can lead to allogeneic graft rejection[2]. The management of BKv replication and BKv-specific immune restoration therefore remains a major challenge in kidney transplantation.

BKv is a widespread double-stranded DNA polyomavirus, with seroprevalence rates of up to 80% in adults worldwide[4,5]. Primary infection frequently occurs during childhood, after which, BKv establishes viral latency in the reno-urinary tract, in which the virus is controlled by the BKv-specific T-cell response[4]. The early-gene region encodes proteins involved in viral replication, such as the “large tumor antigen” (LT-Ag). These regulatory proteins facilitate replication of the viral genome. By contrast, the late-gene region encodes capsid proteins, such as viral protein-1 (VP1)[6]. LT-Ag and VP1 are immunodominant proteins widely recognized by BKv-specific T cells, which contribute to the immune response and the control of viral quiescence in immunocompetent patients[7].

The mechanisms of BKv reactivation and BKvAN are poorly understood. Immunosuppressive therapy is known to impair BKv-specific T-cell responses, which may result in a failure to control BKv replication[8]. Viral replication is thought to result from viral reactivation and a defective virus-specific immune

response[9,10]. Monitoring of the BKv-specific T-cell response and BKv viral load may therefore be useful for assessing the risk of BKv reactivation and the occurrence of BKvAN.

We prospectively studied BKv-specific T-cell responses and the impact of human leukocyte antigen (HLA) matching in a cohort of kidney transplant recipients with various levels of BKv reactivation (no reactivation, viruria, viremia, or BKvAN, 25 patients per group).

Results

We compared BKv-specific T-cell responses in 100 kidney transplant recipients with various degrees of BKv reactivation (no BKv reactivation, BKv-viruria, BKv-viremia and BKvAN, 25 patients per group). The patients were followed prospectively for a median of 36 [26–38] months, to identify risk factors for BKvAN.

Clinical characteristics of KTRs according to level of BKv reactivation

We found no significant differences between the KTR groups in terms of characteristics before or at the time of transplantation (Table 1). There were no significant differences in graft function during the first month after transplantation, the occurrence of graft rejection during follow-up or in CD4 and CD8 T-cell counts (Table 1). Due to the study design, the time from kidney transplantation to inclusion was longer for patients without BKv reactivation and for the BKv-viruria group than for the BKv-viremia and BKvAN groups (Table 1, $p = 0.0014$).

Table 1
Demographics of the study population, comparison of immunosuppressive treatments and outcome after kidney transplantation

Demographic comparison	Without	Viruria	Viremia	BKvAN	p^{i}
n	25	25	25	25	
Age of patients at inclusion, years	49 [39–63]	59 [47–67]	62 [49–70]	60 [54–64]	0.129
Male, n (%)	16 (64)	18 (72)	18 (72)	17 (68)	0.916
DIALYSIS, n (%)	20 (80)	23 (92)	23 (92)	23 (92)	0.431
Time on dialysis, months	32 [16.8–46]	28 [11–43]	40 [20.8–61.8]	47 [28–74]	0.137
Hemodialysis, n (%)	18 (90)	19 (82.6)	21 (91.3)	22 (95.7)	0.526
Peritoneal dialysis, n (%)	2 (10)	4 (17.4)	2 (8.7)	1 (4.3)	
CAUSE OF END-STAGE RENAL DISEASE					
Glomerulonephritis, n (%)	6 (24)	10 (40)	8 (32)	6 (24)	0.611
Hypertensive or diabetic nephrosclerosis, n (%)	5 (20)	7 (28)	10 (40)	9 (36)	
Polycystic kidneys, n (%)	6 (24)	3 (12)	3 (12)	2 (8)	
Tubular or interstitial diseases, n (%)	2 (8)	1 (4)	1 (4)	4 (16)	
Unknown and other causes, n (%)	6 (24)	4 (16)	3 (12)	4 (16)	
TRANSPLANTATION CHARACTERISTICS					
Donation after brain death, n (%)	16 (64)	21 (84)	17 (68)	20 (80)	0.319
Donation after cardiac death, n (%)	4 (16)	0 (0)	5 (20)	2 (8)	
Living donation, n (%)	5 (20)	4 (16)	3 (12)	3 (12)	
Highly sensitized patients[ii], n (%)	7 (28)	6 (24)	6 (24)	5 (20)	0.932

Without: patients without plasma or urinary BKv reactivation; Viruria: patients with urinary BKv reactivation; Viremia: patients with plasma BKv reactivation; BKvAN: patients with BKv-associated nephropathy; BKv: BK-virus; CMV: Cytomegalovirus; CMV R + status: recipient seropositive for CMV; CMV D+/R- status: recipient seronegative for CMV transplanted with a graft from a CMV-seropositive donor; DSA: Donor-specific alloantibodies; EBV: Epstein Barr Virus; eGFR: estimated glomerular filtration rate (MDRD4-formula); n: number of patients; NA: not applicable; p: p-values. Continuous data are expressed in medians and interquartile ranges. *(i)* p-values indicate the significance of differences between groups in Kruskal-Wallis or $\chi 2$ tests. *(ii)* defined as > 85% panel reactive antibodies. *(iii)* The comparison of immunosuppressive treatments between KTR groups was performed at BKvAN diagnosis or 12 months after transplantation.

Demographic comparison	Without	Viruria	Viremia	BKvAN	p^{i}
First transplant, n (%)	19 (76)	22 (88)	24 (96)	19 (76)	0.147
Pre-existing DSA, n (%)	10 (40)	8 (32)	10 (40)	10 (40)	0.917
De novo DSA status, n (%)	9 (36)	7 (28)	7 (28)	5 (20)	0.662
CMV R + status, n (%)	17 (68)	22 (88)	22 (88)	19 (76)	0.212
CMV D+/R- status, n (%)	4 (16)	1 (4)	2 (8)	3 (12)	0.528
BKV REACTIVATION					
Plasma BKv viral load (copies/mL)	*NA*	0	2.2×10^3 [8.9×10^2 – 1.2×10^4]	3.5×10^5 [5.0×10^4 – 7.8×10^5]	< 0.0001
Time from kidney transplantation to plasma BKv reactivation, months	*NA*	*NA*	5.6 [3.2–9.1]	3.4 [2.9–13.4]	0.859
Urinary BKv viral load (copies/mL)	*NA*	2.4×10^4 [3.9×10^3 – 2.9×10^5]	8.5×10^7 [2.2×10^6 -2.9×10^8]	1.0×10^9 [1.9×10^8 -1.6×10^9]	< 0.0001
Time from kidney transplantation to urinary BKv reactivation, months	*NA*	14.8 [2.6–51]	3.9 [1.7–11]	3.6 [1.7–13.4]	0.047
BKvAN diagnosis, months	*NA*	*NA*	*NA*	11 [5–24]	
IMMUNOSUPPRESSIVE TREATMENT COMPARISON at BKvAN diagnosis or 12 months after transplantation[iii]					
INDUCTION and MAINTENANCE TREATMENTS					
Thymoglobulin, n (%)	*NA*	6 (24)	13 (52)	15 (60)	0.027
Tacrolimus, n (%)	*NA*	19 (76)	16 (64)	21 (84)	0.516

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Demographic comparison	Without	Viruria	Viremia	BKvAN	p^i
Levels of tacrolimus, ng/mL	*NA*	9.1 [8.7–9.7]	9.1 [7.7–11.1]	8.6 [6.7–10.2]	0.670
Mycophenolate mofetil exposure, AUC h.mg/L	*NA*	35.8 [22.8–48.8]	49.3 [33.9–64.7]	60.4 [46.5–74.3]	< 0.001
CURATIVE TREATMENT FOR REJECTION					
Intravenous steroid boluses, n (%)	*NA*	5 (20)	9 (36)	10 (40)	0.130
Thymoglobulin, n (%)	*NA*	1 (4)	4 (16)	1 (4)	> 0.9999
Plasma exchanges, n (%)	*NA*	4 (16)	8 (32)	6 (24)	0.508
Rituximab, n (%)	*NA*	4 (16)	8 (32)	4 (16)	> 0.9999
Bortezomib, n (%)	*NA*	5 (20)	3 (12)	4 (16)	0.743
OUTCOME AFTER TRANSPLANTATION					
Delayed graft function, n (%)	11 (44)	6 (24)	7 (28)	8 (32)	0.462
eGFR at month 1 after transplantation, mL/min/1.73 m^2	40 [32.3–68.8]	51 [31–81]	45.5 [31.3–63.5]	41 [26.8–56.8]	0.740
eGFR at BKvAN diagnosis or 12 months after transplantation, mL/min/1.73m^2	51 [42.5–69.5]	43 [35-69.5]	48 [38–56]	29 [21-42.5]	< 0.0001
eGFR at the end of the follow-up, mL/min/1.73 m^2	49 [37-67.5]	47 [31.5–58.5]	45 [28.5–60]	13 [9.5–29]	< 0.0001
Decrease in eGFR at the end of follow-up, mL/min/1.73 m^2	0 [0–7]	3 [0-6.5]	4 [0–13]	12 [3.5–17]	0.0079
CMV reactivation, n (%)	10 (40)	8 (32)	7 (28)	9 (36)	0.828
EBV reactivation, n (%)	13 (52)	18 (72)	16 (64)	15 (60)	0.531

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Demographic comparison	Without	Viruria	Viremia	BKvAN	p^i
Graft rejection, n (%)	6 (24)	5 (20)	9 (36)	4 (16)	0.381
Graft loss, n (%)	0 (0)	0 (0)	1 (4)	9 (36)	< 0.0001
Patient death after transplantation, n (%)	1 (11.1)	3 (33.3)	2 (22.2)	3 (33.3)	0.720
Time from transplantation to inclusion, months	26 [16–34]	27 [9–79]	9 [7–18]	8 [4–28]	0.0014
CD4 T-cell count, absolute number/µL	247 [137–514]	342 [260–495]	253 [156–443]	210 [76–376]	0.472
CD8 T-cell count, absolute number/µL	210 [161–271]	311 [162–448]	172 [92–278]	159 [122–353]	0.197

Without: patients without plasma or urinary BKv reactivation; Viruria: patients with urinary BKv reactivation; Viremia: patients with plasma BKv reactivation; BKvAN: patients with BKv-associated nephropathy; BKv: BK-virus; CMV: Cytomegalovirus; CMV R + status: recipient seropositive for CMV; CMV D+/R- status: recipient seronegative for CMV transplanted with a graft from a CMV-seropositive donor; DSA: Donor-specific alloantibodies; EBV: Epstein Barr Virus; eGFR: estimated glomerular filtration rate (MDRD4-formula); n: number of patients; NA: not applicable; p: p-values. Continuous data are expressed in medians and interquartile ranges. *(i)* p-values indicate the significance of differences between groups in Kruskal-Wallis or $\chi 2$ tests. *(ii)* defined as > 85% panel reactive antibodies. *(iii)* The comparison of immunosuppressive treatments between KTR groups was performed at BKvAN diagnosis or 12 months after transplantation.

BKv reactivation in KTRs

We compared the plasma and urinary BKv viral loads between the KTR groups (Table 1).

At inclusion, the BKvAN group had higher plasma BKv viral loads than the BKv-viremia group (Fig. 1a, p = 0.0019). Similarly, patients in the BKvAN group also had a higher urinary BKv viral load than those with BKv-viruria or viremia (Fig. 1b, p < 0.0001 and $p = 0.022$ respectively).

Impact of BKv reactivation on allograft outcome and graft survival

We then analyzed the impact of BKv reactivation on the outcome of kidney transplantation (Table 1). The median time from transplantation to BKvAN diagnosis was 11 [5–24] months. Despite having a similar renal function and proportion of delayed graft function one month after renal transplantation, patients diagnosed with BKvAN had poorer renal function (eGFR of 29 [21-42.5] mL/min/1.73m^2) than those of the other groups at month 12 ($p < 0.0001$) (Fig. 1c and Table 1). Immunosuppressive treatment was modified for patients with BKvAN, with a decrease in tacrolimus dose and decreases in antimetabolite exposure or antimetabolite withdrawal (Supplementary data: Table S1). Treatment with mTor inhibitor

was initiated before BKvAN diagnosis in patients displaying sustained BKv replication in the plasma. Despite the decrease in tacrolimus level ($p < 0.0001$) (Table S1), graft function was significantly altered in the BKvAN group at the end of follow-up (eGFR: 13 [9.5–29] mL/min/1.73m^2 versus eGFR at BKvAN diagnosis: 29 [21-42.5] mL/min/1.73m^2, $p < 0.0001$). Patients with BKvAN displayed severe graft dysfunction, with a larger decrease in eGFR at the end of follow-up than was observed in the other groups (decrease in eGFR: 12 [3.5–17] mL/min/1.73m^2 versus 0 [0–7], 3 [0-6.5] and 4 [0–13] mL/min/1.73m^{2,} respectively, $p = 0.0079$) (Fig. 1c and Table 1). Graft survival was poorer in patients with BKvAN, for whom the rate of graft loss was 36%. The rate of graft loss in the other groups was < 5% ($p < 0.0001$) (Fig. 1d and Table 1). By contrast, allograft outcome was similar between the other groups (Figs. 1c and 1d, Table 1).

Risk factors for BKvAN

We analyzed factors potentially associated with the occurrence of BKvAN in cases of BKv reactivation.

Identification of risk factors associated with BKv reactivation

At BKvAN diagnosis or 12 months post-transplantation, patients with BKvAN had significantly higher levels of exposure to thymoglobulin ($p = 0.027$) and mycophenolate mofetil ($p < 0.001$) (Table 1).

We analyzed the relationship between donor/recipient [HLA-A,-B,-DR,-DQ] matching and BKv replication. BKv-viremia was associated with a larger number of total [HLA-A,-B,-DR,-DQ] and [HLA-DR] mismatches ($p = 0.011$ and $p = 0.0036$, respectively) (Figs. 2a, 2d and Table 2a). By contrast, KTRs with BKvAN had a significantly smaller number of HLA-DR mismatches than patients with BKv-viremia ($p = 0.039$) (Fig. 2d). We assessed the impact of the degree of HLA-matching as a risk factor for BKvAN, by stratifying the absolute number of HLA mismatches into subgroups. We found that cutoff values of at least five [HLA-A,-B,-DR,-DQ] mismatches and of two [HLA-DR] mismatches were associated with BKv-viremia without the occurrence of BKvAN (Figs. 2f, 2i and Table 2b). In the presence of at least five [HLA-A,-B,-DR,-DQ] mismatches, 23.8% of patients had viruria, 50% had BKv-viremia without BKvAN and 26.2% had BKvAN. In the presence of fewer than five mismatches, 45.5% of patients had viruria, 12.1% had BKv-viremia without BKvAN and 42.4% had BKvAN ($p = 0.0025$ - Fig. 2f and Table 2b). In terms of HLA-DR compatibility, in the presence of two [HLA-DR] mismatches, 20.8% of patients had viruria, 54.2% had BKv-viremia without BKvAN and 25% had BKvAN. In the presence of one [HLA-DR] mismatch, 28.6% of patients had viruria, 35.7% had BKv-viremia without BKvAN and 35.7% had BKvAN. In the absence of HLA-DR mismatches, 52.2% of patients had viruria, 8.7% had viremia without nephropathy and 39.1% had BKvAN ($p = 0.018$ - Fig. 2i and Table 2b). Compared to BKv-viremia without BKvAN, we identify HLA-DR compatibility as a risk factor associated with BKvAN ($p = 0.030$), while there are no statistical differences in terms of HLA-A,-B and DQ-compatibility (Figs. 2g, 2h and 2j).

Table 2. Comparison of HLA-A,-B,-DR,-DQ mismatches between KTR groups defined on the basis of BKv reactivation

ıte number of HLA mismatches

Comparison of HLA-A,-B,-DR,-DQ mismatches	Viruria	Viremia	BKvAN	p^i
n	25	25	25	
Total [HLA-A,-B,-DR,-DQ] mismatch number	4 [3-6]	6 [5-7]	4 [3-6]	***0.011***
HLA-A mismatch number	1 [1-1.5]	1 [1-2]	1 [0-2]	0.710
HLA-B mismatch number	2 [1-2]	2 [2-2]	2 [1-2]	0.052
HLA-DR mismatch number	1 [0-1]	2 [1-2]	1 [0-1.5]	***0.0036***
HLA-DQ mismatch number	1 [0-2]	1 [1-2]	1 [0-1]	0.310

ïication of the HLA mismatches into subgroups

Comparison of HLA-A,-B,-DR,-DQ mismatches	Total [HLA-A,-B,-DR,-DQ] < 5 mismatches	Total [HLA-A,-B,-DR,-DQ] ≥ 5 mismatches		HLA-DR no mismatches	HLA-DR one mismatch	HLA-DR two mismatches
Viruria, n (%)	15 (45.5)	10 (23.8)		12 (52.2)	8 (28.6)	5 (20.8)
Viremia, n (%)	4 (12.1)	21 (50)		2 (8.7)	10 (35.7)	13 (54.2)
BKvAN, n (%)	14 (42.4)	11 (26.2)		9 (39.1)	10 (35.7)	6 (25)
p^i	***0.0025***			***0.018***		

Viruria: patients with urinary BKv reactivation; Viremia: patients with plasma BKv reactivation; BKvAN: patients with BKv-associated nephropathy; BKv: BK-virus; n: number of patients; *p:* p-values. Continuous data are expressed in medians and interquartile ranges. *(i)* p-values indicate the significance of difference between groups in Kruskal-Wallis or χ^2 tests.

We then analyzed the BKv-specific T-cell functionality. We first evaluated the antiviral T-cell response by studying T-cell phenotype and functionality after stimulation with BKv or CEF (cytomegalovirus, Epstein Barr virus and influenza virus) in KTRs without BKv reactivation. A polyfunctional T-cell response was observed, and the antiviral T cells were able to proliferate, produce cytokines, and develop CD8 cytotoxicity in the presence of BKv or CEF peptides (Figs. 3a, 3b, 4a, 4b, Supplementary data: Figures S1, S2a, S2b and Table S2).

For the specific response to BKv in the context of BKv reactivation, we observed a gradual or total loss of BKv-specific T-cell polyfunctionality according to BKv reactivation level (Table 3). Based on the distribution of data for the BKv-specific T-cell response, we defined a cutoff value of 2.4 proliferative cells and 0.3 cytokine-producing cells for distinguishing between responder and non-responder patients. The proliferative capacity of BKv-specific CD4 and CD8 T cells was significantly lower in KTRs with BKvAN than in those with BKv-viruria or BKv-viremia (for CD4 T cells: $p = 0.0002$; for CD8 T cells: $p < 0.0001$ and $p = 0.0026$, respectively) (Figs. 3a, 3b). Using a cutoff value of 2.4 proliferative CD4 or CD8 T cells to distinguish between responders and non-responders, we observed a gradual decrease in the number of patients able to respond to BKv peptides with increasing BKv reactivation level ($p = 0.032$ and $p < 0.001$,

respectively) (Figs. 3c, 3d and Table 3). The TNFα secretion capacity of BKv-specific CD8 T cells was significantly lower in KTRs with BKvAN than in those with BKv-viremia ($p = 0.0012$) (Fig. 4a). Similarly, the co-secretion of TNFα and IFNγ (TNFα/IFNγ) was less frequently observed in patients with BKvAN than those with BKv-viruria or viremia ($p = 0.0004$ and $p = 0.025$, respectively) (Fig. 4b). Using a cutoff value of 0.3 cytokine-producing CD8 T cells to distinguish between responders and non-responders, we observed a gradual decrease in the number of patients able to respond to BKv peptides with increasing BKv reactivation level, in terms of IFNγ/TNFα secretion capacity ($p = 0.013$) (Figs. 4c, 4d and Table 3). We observed no significant differences in IFN_{γ} secretion or cytokine-secreting CD4 T-cell levels after BKv-specific stimulation (data not shown). We then assessed the specific cytotoxicity of CD8 T cells against BKv. Specific cytotoxicity tended to be lower in the BKvAN group than in the other groups, with a mortality of BKv-target cells of 0.32 [0.32–3.61] dead cells in the BKvAN group, versus 3.63 [0.32–8.6] in the BKv-viremia group and 6.04 [0.32–10.22] in the BKv-viruria group. Nevertheless, this difference was not significant ($p = 0.267$) (Table 3 and Supplementary data: Figures S3).

Table 3
Comparison of BKv-specific T-cell functionality between KTR groups defined on the basis of BKv reactivation

BKv-specific T-cell functionality	Viruria	Viremia	BKvAN	p^{i}
PROLIFERATION CAPACITIES (n^{ii})	16	13	15	
$CFSE^{low}$ BKv (LT-Ag and VP1) CD4 T cells, cell nb	4.36 [3.15–5.37]	3.39 [0.10–5.09]	2.01 [0.10–3.31]	0.0003
Response for LT-Ag, cell nb	3.90 [3.0-4.61]	1.80 [0.10–4.59]	2.12 [0.10–3.26]	0.023
Response for VP1, cell nb	4.92 [3.44–5.42]	3.60 [1.57–5.30]	1.93 [0.10–3.46]	0.008
No BKv-specific peptide response, n (%)	2 (12.5)	2 (16.6)	6 (40.0)	0.032
BKv-specific response against LT-Ag or VP1 peptides, n (%)	2 (12.5)	5 (41.7)	6 (40.0)	
BKv-specific response against LT-Ag and VP1 peptides, n (%)	12 (75.0)	5 (41.7)	3 (20.0)	
Correlation between LT-Ag CD4 T cells and BKv viral load	*NA*	-0.02 [-0.42;0.39]		0.94
Correlation between VP1 CD4 T cells and BKv viral load		-0.53 [-0.77;-0.16]		0.006
$CFSE^{low}$ BKv (LT-Ag and VP1) CD8 T cells, cell nb	4.88 [4.10–5.95]	2.33 [0.09–4.22]	0.09 [0.09–2.51]	< 0.0001
Response for LT-Ag, cell nb	4.52 [3.72–5.01]	2.29 [0.10–3.68]	0.10 [0.10–1.65]	< 0.0001
Response for VP1, cell nb	5.51 [4.75–6.05]	2.58 [0.67–4.84]	1.60 [0.10–2.57]	< 0.0001
No BKv-specific peptide response, n (%)	1 (6.25)	5 (38.5)	8 (53.3)	< 0.001
BKv-specific response against LT-Ag or VP1 peptides, n (%)	1 (6.25)	2 (15.4)	6 (40.0)	
BKv-specific response against LT-Ag and VP1 peptides, n (%)	14 (87.5)	6 (46.1)	1 (6.7)	

Viruria: patients with urinary BKv reactivation; Viremia: patients with plasma BKv reactivation; BKvAN: patients with BKv-associated nephropathy; BKv: BK-virus; n: number of patients; cell nb: normalized BKv-specific T-cell frequencies; NA: not applicable; *p: p*-values. Continuous data are expressed in medians and interquartile ranges. *(i)* p-values indicate the significance of differences between groups in Kruskal-Wallis or χ^2 tests. Correlations were evaluated with the nonparametric Spearman's rank correlation test. *(ii)* For each patient, two BKv-specific responses were analyzed, after stimulation with LT-Ag and VP1 peptides.

BKv-specific T-cell functionality	Viruria	Viremia	BKvAN	p^{i}
Correlation between LT-Ag CD8 T cells and BKv viral load	*NA*	-0.15 [-0.52;0.27]		0.48
Correlation between VP1 CD8 T cells and BKv viral load		-0.45 [-0.72;-0.05]		0.025
CYTOKINE SECRETION CAPACITIES (n^{ii})	16	14	14	
TNF + BKv (LT-Ag and VP1) CD8 T cells, cell nb	0.03 [0.03–1.33]	1.23 [0.03–2.11]	0.03 [0.03–0.03]	0.0018
Response for LT-Ag, cell nb	0.03 [0.03–1.31]	1.43 [0.03–2.23]	0.03 [0.03–0.03]	0.037
Response for VP1, cell nb	0.03 [0.03–1.30]	1.10 [0.03–1.74]	0.03 [0.03–0.37]	0.153
No BKv-specific peptide response, n (%)	7 (43.7)	3 (21.4)	9 (64.3)	0.116
BKv-specific response against LT-Ag or VP1 peptides, n (%)	4 (25)	4 (28.6)	4 (28.6)	
BKv-specific response against LT-Ag and VP1 peptides, n (%)	5 (31.3)	7 (50)	1 (7.1)	
Correlation between LT-Ag CD8 T cells and BKv viral load	*NA*	-0.56 [-0.78;-0.23]		0.002
Correlation between VP1 CD8 T cells and BKv viral load		-0.46 [-0.72;-0.10]		0.012
TNF^{+}/IFN^{+} BKv (LT-Ag and VP1) CD8 T cells, cell nb	0.2 [0.03–0.54]	0.03 [0.03–0.593]	0.03 [0.03–0.03]	0.0005
Response for LT-Ag, cell nb	0.12 [0.03–0.52]	0.03 [0.03–0.58]	0.03 [0.03–0.03]	0.197
Response for VP1, cell nb	0.31 [0.03–0.73]	0.03 [0.03–0.61]	0.03 [0.03–0.03]	0.021
No BKv-specific peptide response, n (%)	5 (31.3)	7 (50)	13 (92.9)	0.013
BKv-specific response against LT-Ag or VP1 peptides, n (%)	8 (50)	4 (28.6)	1 (7.1)	
BKv-specific response against LT-Ag and VP1 peptides, n (%)	3 (18.7)	3 (21.4)	0 (0)	

Viruria: patients with urinary BKv reactivation; Viremia: patients with plasma BKv reactivation; BKvAN: patients with BKv-associated nephropathy; BKv: BK-virus; n: number of patients; cell nb: normalized BKv-specific T-cell frequencies; NA: not applicable; *p: p*-values. Continuous data are expressed in medians and interquartile ranges. *(i)* p-values indicate the significance of differences between groups in Kruskal-Wallis or χ^2 tests. Correlations were evaluated with the nonparametric Spearman's rank correlation test. *(ii)* For each patient, two BKv-specific responses were analyzed, after stimulation with LT-Ag and VP1 peptides.

BKv-specific T-cell functionality	Viruria	Viremia	BKvAN	p^{i}
Correlation between LT-Ag CD8 T cells and BKv viral load	*NA*	-0.33 [-0.63;0.06]		0.09
Correlation between VP1 CD8 T cells and BKv viral load		-0.33 [-0.63;0.07]		0.09
CYTOTOXIC CAPACITIES (n[ii])	8	9	9	
7AAD^{+} BKv (LT-Ag and VP1) target cells (cell nb)	6.04 [0.32–10.22]	3.63 [0.32–8.6]	0.32 [0.32–3.61]	0.267
Response for LT-Ag (cell nb)	6.04 [0.32–8.60]	6.09 [1.03–11.17]	0.85 [0.32–3.54]	0.522
Response for VP1 (cell nb)	5.16 [0.32–11.57]	0.32 [0.32–7.05]	0.32 [0.32–3.01]	0.342
No BKv-specific peptide response, n (%)	5 (62.5)	6 (66.7)	7 (77.8)	0.622
BKv-specific response against LT-Ag or VP1 peptides, n (%)	2 (25)	3 (33.3)	2 (22.2)	
BKv-specific response against LT-Ag and VP1 peptides, n (%)	1 (12.5)	0 (0)	0 (0)	
PD1 EXPRESSION ON BKv CD4 T cells, % of cells	NA	1.82 [0.46–5.30]	8.11 [2.00-14.88]	0.028
PD1 EXPRESSION ON BKv CD8 T cells, % of cells	NA	1.16 [0.39–4.95]	2.70 [1.23–9.86]	0.041

Viruria: patients with urinary BKv reactivation; Viremia: patients with plasma BKv reactivation; BKvAN: patients with BKv-associated nephropathy; BKv: BK-virus; n: number of patients; cell nb: normalized BKv-specific T-cell frequencies; NA: not applicable; *p: p*-values. Continuous data are expressed in medians and interquartile ranges. *(i)* p-values indicate the significance of differences between groups in Kruskal-Wallis or χ^2 tests. Correlations were evaluated with the nonparametric Spearman's rank correlation test. *(ii)* For each patient, two BKv-specific responses were analyzed, after stimulation with LT-Ag and VP1 peptides.

We ruled out a global inhibition of antiviral T cells in patients with BKvAN, by assessing the ability of CD8 T cells to respond to viral peptides other than BKv peptides. In the presence of a CEF peptide pool, we observed a similar polyfunctional CD8 T-cell response for T-cell proliferation, cytokine secretion and CD8 cytotoxicity in all KTR groups, regardless of the level of BKv reactivation (Supplementary data: Figure S2 and Table S3).

These results indicate that a global antiviral response was maintained regardless of BKv status, and suggest a specific impairment of the BKv-specific T-cell response in patients with BKvAN.

Multivariate analysis to identify risk factors associated with BKv reactivation

For the identification of factors associated with BKv viremia and BKvAN, we performed a multivariate analysis with the factors identified as relevant in the univariate analysis (Table 4a and 4b). Three independent risk factors were found to be associated with BKv viremia and/or BKvAN: the thymoglobulin use, the total number of [HLA-A,-B,-DR,-DQ] mismatches and the loss of the proliferative BKv-specific CD8 T-cell response (Table 4b).

Table 4. Risk factors associated with BKv viremia and BKvAN in the context of BKv reactivation

a. **Univariate analysis**

Risk factors associated with BKv viremia and BKvAN in the context of BKv reactivation	Viremia (versus viruria)		BKvAN (versus viruria)		BKvAN (versus viremia)	
	OR [95% CI]	p^i	OR [95% CI]	p^i	OR [95% CI]	p^i
Immunosuppressive treatment						
Thymoglobulin	3.43 [1.03-11.48]	***0.068***	4.75 [1.41-16.05]	***0.036***	1.38 [0.45-4.25]	0.569
Antimetabolite exposure	1.12 [1.02-1.23]	***0.030***	1.17 [1.06-1.30]	***0.006***	1.05 [1.00-1.10]	0.058
HLA mismatches						
Total [HLA-A,-B,-DR,-DQ] mismatch number	1.57 [1.13-2.19]	***0.024***	1.08 [0.81-1.45]	0.605	0.69 [0.50-0.95]	***0.036***
No [HLA-DR] mismatch number	1 (ref)	***0.036***	1 (ref)	0.692	1 (ref)	0.070
One [HLA-DR] mismatch number	7.50 [1.29-43.69]		1.67 [0.47-5.93]		0.22 [0.04-1.30]	
Two [HLA-DR] mismatch number	15.60 [2.53-96.08]		1.60 [0.37-6.95]		0.10 [0.02-0.63]	
Proliferative BKv (LT-Ag and VP1) CD4 T-cell response						
Response for LT-Ag (cell nb)	0.72 [0.48-1.07]	0.159	0.62 [0.42-0.92]	0.054	0.86 [0.59-1.27]	0.453
Response for VP1 (cell nb)	0.82 [0.56-1.20]	0.298	0.57 [0.38-0.84]	***0.015***	0.69 [0.47-1.02]	0.093
No BKv-specific peptide response	2.40 [0.26-22.10]	0.278	12.00 [1.56-92.29]	***0.048***	5.00 [0.58-42.80]	0.34
BKv-specific response against LT-Ag or VP1 peptides	6.00 [0.86-41.90]		12.00 [1.56-92.29]		2.00 [0.31-12.84]	
BKv-specific response against LT-Ag and VP1 peptides	1 (ref)		1 (ref)		1 (ref)	
Proliferative BKv (LT-Ag and VP1) CD8 T-cell response						
Response for LT-Ag (cell nb)	0.58 [0.36-0.92]	***0.030***	0.39 [0.23-0.66]	***0.003***	0.67 [0.43-1.05]	0.084
Response for VP1 (cell nb)	0.54 [0.33-0.87]	***0.017***	0.37 [0.21-0.64]	***<0.001***	0.68 [0.45-1.04]	0.075
No BKv-specific peptide response	11.67 [1.11-	0.090	112.00 [6.13-	***0.006***	9.60 [0.88-	0.09

	122.38]		2,045.17]		105.17]	
BKv-specific response against LT-Ag or VP1 peptides	4.67 [0.35-61.83]		84.00 [4.48-1,576.52]		18.00 [1.27-255.74]	
BKv-specific response against LT-Ag and VP1 peptides	1 (ref)		1 (ref)		1 (ref)	

b. **Multivariate analysis**

Risk factors associated with BKv viremia and BKvAN in the context of BKv reactivation	Viremia (versus viruria)		BKvAN (versus viruria)		BKvAN (versus viremia)	
	OR [95% CI]	p^{ii}	OR [95% CI]	p^{ii}	OR [95% CI]	p^{ii}
Thymoglobulin	34.15 [2.44-477.71]	***0.027***	15.85 [0.88-284.45]	0.092	0.46 [0.06-3.44]	0.452
Total [HLA-A,-B,-DR,-DQ] mismatches	1.90 [1.06-3.38]	***0.045***	1.02 [0.56-1.87]	0.94	0.54 [0.31-0.94]	***0.045***
Proliferative BKv CD8 T-cell response						
No BKv-specific peptide response	14.34 [0.65-314.56]	0.17	214.88 [7.11-6,498.61]	***0.009***	14.99 [0.92-243.80]	0.156
BKv-specific response against LT-Ag or VP1 peptides	9.39 [0.38-229.70]		239.98 [6.99-8,237.53]		25.56 [1.11-586.42]	
BKv-specific response against LT-Ag and VP1 peptides	1 (ref)		1 (ref)		1 (ref)	

Viruria: patients with urinary BKv reactivation; Viremia: patients with plasma BKv reactivation; BKvAN: patients with BKv-associated nephropathy; BKv: BK-virus; cell nb: normalized BKv-specific T-cell frequencies, OR [95% CI]: odds ratio with 95% confidence interval; *p: p*-values. p-values indicate the significance of differences between groups in *(i)* multinomial univariate logistic regression analyses with correction for false discovery rate or *(ii)* Wald tests (multivariate multinomial logistic regression) with correction for false discovery rate.

Thymoglobulin, which was used as an induction treatment, was significantly associated with BKv-viremia compared to BKv-viruria ($p = 0.027$ - OR [95% CI] = 34.15 [2.44-477.71]), but not with BKvAN (p = ns) when compared to BKv viruria or BKv Viremia (Table 4b).

The total number of [HLA-A,-B,-DR,-DQ] mismatches was significantly associated with BKv-viremia compared to BKv-viruria ($p = 0.045$ - OR [95% CI] = 1.90 [1.06–3.38]). Interestingly, [HLA-A,-B,-DR,-DQ] mismatches were significantly associated with BKvAN compared to BKv-viremia ($p = 0.045$), but with an OR [95% CI] of 0.54 [0.31–0.94] highlighting that the presence of HLA mismatches in greater numbers reduces the risk of BKvAN (Table 4b).

A decrease in the proportion of patients displaying a BKv-proliferative CD8 T-cell response was associated with BKvAN ($p = 0.009$). In this group, when a BKv-specific CD8 T-cell response against LT-Ag and VP1 peptides was used as the reference, the OR [95% CI] was to 214.88 [7.11-6,498.61] for no BKv-specific peptide response and to 239.98 [6.99-8,237.53] for a BKv-specific CD8 T-cell response against LT-Ag or VP1 peptides. This result highlights that the decrease of the proliferative BKv-specific CD8 T-cell response increases the risk of BKvAN. Following introduction of the specific CD4 response into the model in place of the CD8 response, the occurrence of BKvAN was also found to be associated with a defect of the proliferative CD4 T-cell response to the VP1 peptide ($p = 0.024$).

Exhaustion of BKv-specific CD4 and CD8 T cells

BKv-specific T cells were not able to elaborate a functional response against BKv-peptides, but exhibited a preserved response against CEF-peptides, suggesting an exhaustion of BKv-specific T cells in patients with BKvAN.

We have evaluated the memory T-cell subpopulations of CD4 and CD8 T cells (Supplementary data: Figure S4). We detected an expansion of the TEMRA CD8 T-cell population in KTRs with BKv-viruria compared to the BKv-viremia and BKvAN groups (p = 0.0007) (Supplementary data: Figure S5h and Table S4).

To confirm the exhaustion of BKv-specific T cells in context of BKvAN, we assessed the relationship between BKv-specific lymphocyte functionality and the plasma BKv viral load. We observed a significant inverse correlation between BKv-specific CD4 and CD8 T-cell proliferation capacity and plasma BKv viral load (for VP1 peptides: $p = 0.006$ and $p = 0.025$, respectively) (Figs. 5a, 5b and Table 3). There was also a significant inverse correlation between the TNFα secretion capacities of BKv-specific CD8 T cells and plasma BKv viral load (for LT-Ag peptides: $p = 0.002$ and for VP1 peptides: $p = 0.012$) (Table 3).

We then assessed the expression of the inhibitory receptor PD1 and CTLA4 on BKv-specific CD4 and CD8 T cells (Supplementary data: Figure S6). The level of PD1 expression on BKv-specific CD4 and CD8 T cells was significantly higher in KTRs with BKvAN than in those with BKv-viremia ($p = 0.028$ and $p = 0.041$, respectively) (Figs. 5a, 5b and Table 3). We observed no significant differences in CTLA4 expression. Combined with a severe impairment of BKv T-cell functions and a highest plasma BKv loads in patients with BKvAN, these results are consistent with a BKv-specific CD4 and CD8 T-cell exhaustion in context of BKvAN.

Allogeneic CD4 T cells restore BKv-specific CD8 T-cell functionality

We then investigated the link between the severe impairment of BKv-specific T-cell functions, the exhausted-like phenotype of BKv-specific CD4 and CD8 T-cells and the smaller number of class II HLA mismatches in patients with BKvAN.

As we previously showed, heterospecific CD4 help provided by CD4 T cells generated from a distinct antigenic source could rescue memory CD8 T cell responses[11]. We hypothesize that heterospecific immunity, provided by allogeneic CD4 T cells, may contribute to sustain the effective BKv CD8 T-cell response. Therefore, PBMCs from 11 KTRs with plasma BKv reactivation were stained with a fluorescent dye, depleted from autologous CD4 T cells, and incubated for five days with BKv-specific peptide pools in the presence of autologous CD4 T cells or allogeneic third-party CD4 T cells (heterospecific allogeneic CD4-help). Unstimulated CD4-depleted PBMCs incubated in the presence of autologous CD4 T cells or allogeneic third-party CD4 T cells were used as controls. At day 5, we evaluated the proliferation capacity of BKv-specific CD8 T cells by fluorescent dye dilution (Fig. 5c). We observed a significant increase in the proliferative capacities of BKv-specific CD8 T cells in the presence of third-party allogeneic CD4 T cells (with autologous CD4 T cells = 1.03 [0.06;2.32]; with allogeneic CD4 T cells = 2.2 [1.77;4.82] – $p = 0.0137$ – Fig. 5d). Interestingly, the proportion of proliferative BKv-specific CD8 T cells in conditions of allogeneic CD4 T cells was restored near from the response range of the BKv-viruria group (p = ns – Fig. 5d). There was no statistical difference in unstimulated CD4-depleted PBMCs incubated with autologous or allogeneic CD4 T cells (p = ns – Fig. 5d).

Discussion

Our results highlight the loss of BKv-specific T-cell responses during the course of BKv replication, with an exhausted-like phenotype of BKv-specific CD4 and CD8 T-cells in KTRs with BKvAN.

BKv infection remains a major challenge in kidney transplantation, because it can lead to BKvAN, which causes graft loss in 50% of cases in this context[12–14]. There is currently no specific antiviral treatment for BKvAN, and decreasing the intensity of immunosuppression is the only treatment shown to date to be effective[15,16]. We show here that decreasing immunosuppression does not prevent high rates of graft loss in cases of established BKvAN (histologically proven), probably because of the delayed restoration of BKv T-cell responses and irreversible tissue lesions. Immunosuppressive regimens should therefore be adapted sooner after the detection of BKv in plasma, but achieving the balance between the control of BKv replication and the risk of acute allogeneic graft rejection when lowering immunosuppressive treatments remains a major challenge[17]. Indeed, after immunosuppression reduction for BKv replication, more than 20% of patients experience acute rejection, with lower graft function and survival[18].

The use of intensive immunosuppressive strategies has been identified as a key factor in BKv reactivation[19,20]. We found that thymoglobulin, a polyclonal antibody induction therapy that causes profound T-cell depletion, was a risk factor for BKv-viremia, consistent with current knowledge[21], but not for BKvAN. This result implies that thymoglobulin could be a trigger for the blood replication of BKv, but not sufficient for the occurrence of BKvAN. Other authors have also reported a higher risk of BKvAN with certain immunosuppressive regimens, including high levels of exposure to steroids or tacrolimus/mycophenolate mofetil combinations[22–24]. The choice of immunosuppressive drugs plays a role in the risk of BKvAN, as the use of tacrolimus was associated with a higher risk of developing BKvAN

than the use of cyclosporine A (CsA)[25]. The effect of tacrolimus may be explained by an activation of BKv replication, possibly in relation to its interaction with FKBP-12, whereas CsA reduces BKv replication *in vitro*[25–27]. We also found that mycophenolate mofetil exposure was associated with a higher rate of BKvAN, but this factor was not identified as an independent risk factor in our model. Nevertheless, precise AUC determinations may be useful for monitoring the inhibition of T-cell activation and proliferation in kidney transplant patients[28,29].

Despite stepwise immunosuppression reduction, some patients clear BKv, whereas others develop persistent BKv replication, highlighting the role of individual mechanisms in viral control. In solid organ transplantation, the individual risk of opportunistic infection is largely determined by the “net state of immunosuppression”. Many factors contribute to this net state, including the immunosuppressive regimen and other individual predisposing factors, such as immune-aging, concomitant viral infection, and antiviral T-cell efficacy[30].

The BKv-specific T-cell response plays a crucial role in controlling BKv replication, although the precise pathophysiological mechanisms involved remain unknown[8,31,32]. To date, there is several evidence to underline that the presence of BKv-specific CD8 T cells is predictive of the successful control of BKv reactivation after transplantation[33–35]. We provide here an exhaustive characterization of BKv-specific T cells, in terms of their phenotype and functional assessment. We describe the changes in the BKv-specific T-cell response occurring during the course of BKv reactivation, with the progressive loss of BKv-specific T-cell responses. We observed changes in T-cell functionality that appeared to be limited to BKv-specific T cells, as none of the other antiviral responses tested were affected in patients with BKvAN.

Viruses during chronic infection can make use of the negative regulatory signal mediated by the immune checkpoint pathway to escape immune system control[36]. In our work, the inverse correlation between BKv viral load and BKv-specific T-cell responses, and the higher levels of PD1 inhibitory receptor expression suggest an exhaustion of BKv-specific T cells in patients with BKvAN, as observed in other chronic viral infections[37–39].

HLA matching is a major challenge for successful transplantation, as HLA mismatches have a significant negative impact on allograft survival in kidney transplant patients[40–42]. However, the impact of HLA matching on the development and progression of BKvAN remains a matter of debate[43]. HLA is important for viral peptide presentation to both CD4 and CD8 T cells. In this point of view, BKv-specific CD8 T cells directed against the LPLMRKAYL 9-mer epitope are associated with the clearance of BKv[44,45], which binds to several HLA-B molecules[44,46,47].

In transplant recipients, antiviral T cells must operate under suboptimal (immunosuppressed) conditions, with differential individual effects. In addition, allogeneic kidney transplantation constitutes a complex immunological situation in which viral peptides may be presented by the donor or recipient HLA. In mismatch situations, the recognition, by recipient BKv-specific memory T cells, of the BKv peptides presented by donor HLA molecules on kidney epithelial cells may be altered, leading to BKv replication.

Conversely, allogeneic responses may provide a stimulatory environment to BKv-specific T cells and induce additional help to restore or elaborate a functional BKv-specific CD8 T-cell response. We showed that BKvAN was mostly observed in cases of HLA-DR compatibility and was associated with an impairment of BKv-specific T-cell functions. This is consistent with previous reports showing that BKvAN occurs even in the context of good HLA matching[13,48], with a negative impact of HLA matching in the outcome of BKvAN[49]. Conversely, in some cases, HLA mismatching may promote the development of BKvAN[21,50,51] because of the use of heavier immunosuppression[52]. In such cases, a similar inhibition of viral responses, not restricted to BKv, would be attempted.

Interestingly, we found that class II HLA-DR mismatches played an important role in BKv control, suggesting the contribution of CD4 T cells. CD4 T cells play a crucial role for the generation, maintenance and reactivation of memory CD8 T-cell responses[53–55]. We hypothesized that mismatched CD4 T cells receiving allogeneic stimuli would be able to provide the necessary help to rescue BKv-memory CD8 T-cell responses (Fig. 6). This hypothesis was confirmed by replacing *in vitro* autologous CD4 T cells with allogeneic CD4 T cells and evaluating the improvement in the BKv-specific CD8 T-cell response. We previously reported that functional CD4 T cells generated in response to a different antigen (heterospecific helpers) can provide effective help to memory CD8 T cells in experimental mouse models[11]. A higher degree of HLA mismatching can provide heterospecific CD4 help to BKv-specific memory CD8 T cells. This heterospecific help may increase the ability of the host to maintain an effective BKv-specific memory CD8 T-cell response that can protect against BKvAN. Along similar lines, Zeng et al. showed that kidney biopsy specimens from patients with BKv viremia contained more abundant alloreactive T-cell clones than virus-reactive clones, with a cell ratio of up to 7.7[56]. In addition, Sawinski et al. showed that persistent BKv-viremia does not increase graft loss but was associated with the development of class II de novo donor-specific antibodies[57]. Furthermore, we cannot rule out the possibility that, in the context of allogeneic transplantation, heterospecific immunity involves a potential cross-reactivity of virus-specific T cells with multiple HLA complexes[58].

In summary, we show that BKvAN is associated with a progressive severe loss of BKv-specific T-cell responses and an exhausted-like phenotype. These BKv-specific T-cell responses are crucial for the control of viral replication in the kidney. Our results suggest that a higher degree of HLA mismatching provides more effective control of BKv replication, possibly due to the involvement of heterospecific CD4 help processes. Thus, although a high degree of HLA matching may protect against graft rejection, it may, paradoxically, also be a significant risk factor for BKvAN. This finding should be taken into account in strategies aiming to mitigate the risk of BKvAN.

Methods

- Patients and group classification

We performed a longitudinal observational study in the Kidney Transplant Department, University Hospitals, AP-HP. We assigned KTRs with BKv reactivation to three groups on the basis of the level of BKv reactivation, and we compared these groups. KTRs without BKv reactivation were used as the control group. The groups were defined as follows:

- Patients with BKv viruria (urine BKv viral load > 200 copies/mL and plasma BKv viral load < 200 copies/mL in the last 12 months),
- Patients with BKv viremia (stable or increasing plasma BKv viral load > 200 copies/mL in the last 6 months, without BKvAN diagnosis on kidney biopsy)
- Patients with BKvAN (histologically proven BKvAN with a plasma BKv viral load > 200 copies/mL),
- Patients without BKv reactivation (plasma and urine BKv viral loads < 200 copies/mL in the last 12 months).

We prospectively included 100 KTRs over the age of 18 years in the four groups (25 patients per group). The patients were followed prospectively between April 2014 and April 2018.

Patients who had undergone combined multiple-organ transplantation were excluded, as were those with chronic viral infections, such as viral hepatitis or human immunodeficiency virus infection. KTRs with BKv reactivation not meeting the study criteria were not included.

Written informed consent was obtained from all patients. The study was performed in accordance with the rules of the local ethics committee.

We evaluated clinical characteristics before kidney transplantation, kidney graft outcome, immunosuppressive regimen, plasma and urine BKv viral load, HLA mismatches between donor and recipient, and BKv-specific T-cell functionality.

- Collection of clinical and biological data

Demographic data, for age and sex, the cause of end-stage renal disease, and dialysis and transplantation characteristics, were recorded for all patients (Table 1).

We recorded median plasma and urine BKv viral loads and the duration of BKv reactivation for each patient. Plasma and urine samples were obtained during routine visits to our center, and during acute allograft failure. BKv screening was performed in accordance with international guidelines, by assessing plasma BKv load monthly in the first six months after transplantation, and then every three months until two years after transplantation, and annually thereafter, with additional determinations in the event of an unexplained increase in serum creatinine concentration or after treatment for acute rejection[2,20]. Urinary BKv load was also determined during plasma screening for BKv. We quantified BKv DNA by BKv-specific real-time PCR (BKV R-GENE, BioMerieux®).

Glomerular filtration rate was estimated with the MDRD-4 formula (eGFR), in the first month after transplantation, 12 months post-transplantation or at BKvAN diagnosis, and at the end of follow-up for each patient. The end of follow-up was defined as the occurrence of graft loss or the time at which the last follow-up visit took place. We determined levels of tacrolimus, everolimus, and mycophenolate mofetil exposure (area under the curve – AUC in h.mg/L) before and after BKvAN diagnosis or 12 months after transplantation.

- Histological assessment

Kidney biopsy was performed, in accordance with the transplantation protocol, three and 12 months after transplantation or in cases of acute kidney injury (increase in basal serum creatinine concentration of more than 25%). BKvAN was diagnosed histologically by the Histopathology Department of Bicêtre Hospital, in accordance with international guidelines. The lesions observed included enlarged nuclei with smudgy chromatin changes, and intranuclear viral inclusions associated with a mononuclear inflammatory infiltrate and/or fibrosis. The presence of the virus was confirmed by immunohistochemistry with an antibody against large-SV40 tumor antigen[59,60].

- Assessment of HLA matching

We collected donor and recipient HLA typing data from the French organ allocation organization. For each donor–recipient combination, the degree of HLA matching was determined by counting the number of HLA-A, HLA-B, HLA-DR and HLA-DQ mismatches between donor and recipient. We then assessed the effect of HLA matching on BKv reactivation and the occurrence of BKvAN.

HLA typing was performed on recipients and living donors, with DNA molecular biology methods, using reverse sequence-specific oligonucleotides (SSOs) for the A/B/DRB1/DQB1 loci from the standard kit, and from a high-definition kit from November 2016 onwards (One Lambda, Canoga Park, CA). For deceased donors, HLA typing was performed with low-resolution sequence-specific primers (SSPs) for the A/B/DRB1/DQB1 loci (Olerup Reagents, Bionobis, France). These techniques provide at least split antigen-level resolution (most probable allele, depending on the technique, locus and antigen).

- Assessment of BKv-specific T-cell functionality

BKv-specific T-cell functionality was evaluated for all patients at inclusion. Absolute values of BKv-specific CD4 or CD8 T-cells were normalized to CD4 or CD8 T-cell frequencies within PBMCs and expressed per 10,000 CD4 or CD8 T cells.

We isolated peripheral blood mononuclear cells (PBMCs) from a whole-blood sample by centrifugation on a Ficoll gradient. PBMCs were frozen at a final concentration of 1×10^7 cells/mL. For all patients, one or two tubes of cells were cryopreserved for a mean of less than 12 months, in a room strictly maintained at the same temperature so as to preserve T-cell viability and function[61,62].

For BKv stimulation, PBMCs were incubated with two different BKv-specific antigens (LT-Ag and VP1). These overlapping BKv-specific peptides activate both CD4 and CD8 T cells (PepTivator BKV LT-Ag or VP1 used at a final concentration of 1 µg/mL/peptide, Miltenyi Biotec®). We also used CEF peptides (pool of peptides from class I-restricted T-cell epitopes from Cytomegalovirus, Epstein-Barr and Influenza viruses) as a global antiviral immune control. All stimulations were performed relative to a negative control (unstimulated cells) and a positive control (staphylococcal enterotoxin B – SEB). We assessed lymphocyte functionality[9] by measuring proliferation, cytokine production and cytotoxic capacities[63] by flow cytometry with a BD LSRFortessa™ (Supplementary data: Figure S1). Due to the lymphopenia presented by the KTRs, the low frequencies of BKv-specific T cells as previously described[7,64] and cell mortality during the freezing and thawing processes, lymphocyte function was assessed on a limited number of KTRs in each group.

BKv-specific T-cell proliferation in response to BKv antigens

We assessed lymphocyte proliferation in response to BKv peptides. PBMCs were stained with 0.5 µM CFSE and incubated for five days with BKv-specific peptide pools. The stimulated cells were then stained with antibodies against surface markers (antibodies against CD3, CD4, and CD8, BD Bioscience®) and their proliferation capacity was evaluated by CFSE dilution (Supplementary data: Figure S1).

BKv-specific T-cell cytokine secretion in response to BKv antigens

We assessed cytokine secretion in response to BKv peptides. PBMCs were incubated overnight, in the presence of brefeldin A (at a final concentration of 1 µl/mL, BD®), and BKv-specific peptide pools. After overnight culture, the stimulated cells were stained with antibodies against surface markers, then fixed and permeabilized before incubation with anti-interferon-γ (IFNγ) and anti-tumor necrosis factor-α (TNFα) antibodies (BD Bioscience®). Lymphocyte polyfunctionality was evaluated by determining the capacity of BKv-specific T cells for single ($IFN\gamma^+$ or $TNF\alpha^+$) or double ($IFN\gamma^+$ and $TNF\alpha^+$) cytokine secretion (Supplementary data: Figure S1).

BKv-specific T-cell cytotoxicity in response to BKv antigens

We measured BKv-specific CD8 T-cell cytotoxicity by incubating PBMCs overnight with BKv peptide-coated autologous target cells (ratio 3/1). Target cells were distinguished from BKv-specific CD8 T cells by CFSE staining. After stimulation, the mortality rate of target cells was evaluated by 7-amino-actinomycin D staining (Supplementary data: Figure S1).

BKv-specific T-cell proliferation in response to heterospecific immunity provided by allogeneic CD4 T-cell help

We assessed lymphocyte proliferation in response to BKv peptides in the presence or absence of allogeneic CD4 T-cells. PBMCs from 11 KTRs with plasma BKv reactivation were stained with a fluorescent dye, depleted of autologous CD4 T cells by magnetic depletion (CD4 MicroBeads human, Miltenyi®), and incubated for five days with BKv-specific peptide pools in the presence of autologous or allogeneic third-party CD4 T cells, at a ratio of 1 CD4 to 5 non-CD4 T cells. We distinguished PBMCs from allogeneic third-party cells by using two fluorescent dyes (CFSE or VPD). Unstimulated CD4-depleted PBMCs incubated in the presence of autologous CD4 T cells or allogeneic third-party CD4 T cells were used as controls. After five days of culture, the stimulated cells were stained for CD3, CD4, and CD8 and their proliferation capacity was evaluated by fluorescent dye dilution (CFSE or VPD - Fig. 5c).

- Assessment of T-cell phenotype and inhibitory receptor expression

We evaluated the *ex vivo* phenotype of CD4 and CD8 T cells. CD4 and CD8 T cells were stained for memory T-cell subpopulation identification[65–67] (stem-cell memory [TSCM], central memory [TCM], effector memory [TEM], and terminally differentiated effector T cells [TEMRA]) (Supplementary data: Figures S4).

We also evaluated the expression of lymphocyte inhibitory receptors (programmed cell death 1 - PD1, and cytotoxic T-lymphocyte-associated protein 4 - CTLA4). Cytokine-secreting BKv-specific CD4 and CD8 T cells (boolean gate from IFNγ and/or TNFα secreting cells) were assessed for the expression of PD1 and/or CTLA4 and compared to non-responding T cells (without cytokine secretion) (Supplementary data: Figures S6).

• Statistical analysis

Percentages and bar charts were used for categorical data. Medians and interquartile ranges were calculated for the analysis of continuous data. For the normalization of data distributions, the frequencies of BKv-specific T cells were subjected to natural logarithm or square root transformation. For categorical data, groups were compared in chi-squared tests. Kaplan-Meier survival curves were plotted and compared in log-rank tests. For continuous data, comparisons between two groups were performed with nonparametric Mann-Whitney tests, whereas comparisons of more than two groups were performed with nonparametric Kruskal-Wallis tests, followed by Dunn's test for multiple comparisons. All the tests used were two-sided. Correlations were evaluated with the nonparametric Spearman's rank correlation test. Odds ratios with 95% confidence intervals (OR [95% CI]) were estimated and compared by univariate multinomial logistic regression with correction for the false discovery rate. Multivariate analysis was performed with Wald tests (multivariate multinomial logistic regression). Statistical analyses were performed with GraphPad Prism or STATA v15.0 (StataCorp, College Station, TX, US) software, and differences were considered significant if $p < 0.05$.

Abbreviations

BKv

BK-virus
BKvAN
BK-virus-associated nephropathy
CEF
pool of cytomegalovirus, Epstein Barr virus and influenza virus peptides
CTLA4
cytotoxic T-lymphocyte-associated protein 4
DNA
deoxyribonucleic acid
eGFR
estimated glomerular filtration rate
HLA
human leukocyte antigen
IFNγ
interferon-γ
KTR
kidney transplant recipients
LT-Ag
large tumor antigen
mTor
mammalian target of rapamycin
OR [95% CI]
odd ratio with 95% confidence interval
PD1
programmed cell death 1
PBMC
peripheral blood mononuclear cell
PCR
polymerase chain reaction
TNFα
tumor necrosis factor-α
VP1
viral protein-1

Declarations

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DISCLOSURE

The authors have no conflicts of interest to disclose.

DATA AVAILABILITY STATEMENT

The data supporting the findings of this study are available from the corresponding

author upon reasonable request.

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Supplementary materials and methods

Thawed PBMCs were incubated at 37°C, under an atmosphere containing 5% CO_2, in Roswell Park Memorial Institute (RPMI) medium alone (unstimulated cells), with BKv-specific antigen stimulation (LT-Ag or VP1 at a final concentration of 1 µg/mL/peptide), or with CEF peptides (cytomegalovirus, Epstein-Barr virus, and influenza virus peptide pools at a final concentration of 1 µg/mL/peptide). The positive control consisted of incubation with staphylococcal enterotoxin B (SEB) at a final concentration of 0.2 µg/mL.

For T-cell proliferation, we stained 2 x 10^6 PBMCs with 0.5 µM CFSE (THERMO FISHER SCIENTIFIC®) and incubated them for five days. The stimulated cells were stained with antibodies directed against surface markers (CD3-BV605, CD4-V500, and CD8-APC H7 antibodies, BD Bioscience®) and proliferative capacity was evaluated by CFSE dilution (Figure S1).

For T-cell cytokine secretion, we incubated 4 x 10^6 PBMCs overnight in the presence of brefeldin A (at a final concentration of 1 µl for every 1 mL of cell culture, BD Bioscience®). The cells were then stained with antibodies against surface markers (CD3-BV605, CD4-VioGreen, CD8-APC Vio770 antibodies, BD Bioscience®), fixed and permeabilized for intracellular cytokine staining (IFN_γ-PE and TNF_α-Alexa 700

antibodies, BD Bioscience®). Lymphocyte polyfunctionality was evaluated by determining the capacity of BKv-specific T cells to secrete one (IFN_{γ}^{+} or TNF_{α}^{+}) or two (IFN_{γ}^{+} and TNF_{α}^{+}) cytokines (Figure S1).

For T-cell cytotoxicity, we incubated 4 x 10^6 PBMCs overnight with BKv peptide-coated autologous target cells (ratio of 3 CD8 T cells to 1 target cell). Target cells were distinguished from CD8 T cells by CFSE staining (THERMO FISHER SCIENTIFIC®). Target cell mortality after stimulation was evaluated by 7-amino-actinomycin D staining (BD Bioscience® - Figure S1).

We performed multicolor flow cytometry analysis with a BD LSRFortessa™ flow cytometer and FlowJo® software. We ensured that the analysis of BKv-specific T-cell responses was robust, by analyzing a median of 40,492 [19,962-80,339] total T cells for CD4 T cells and 24,560 [11,443-65,902] for CD8 T cells, with a median of BKv-specific responding T cells of 590.5 [142.3-2,070].

Figures

Figure 1

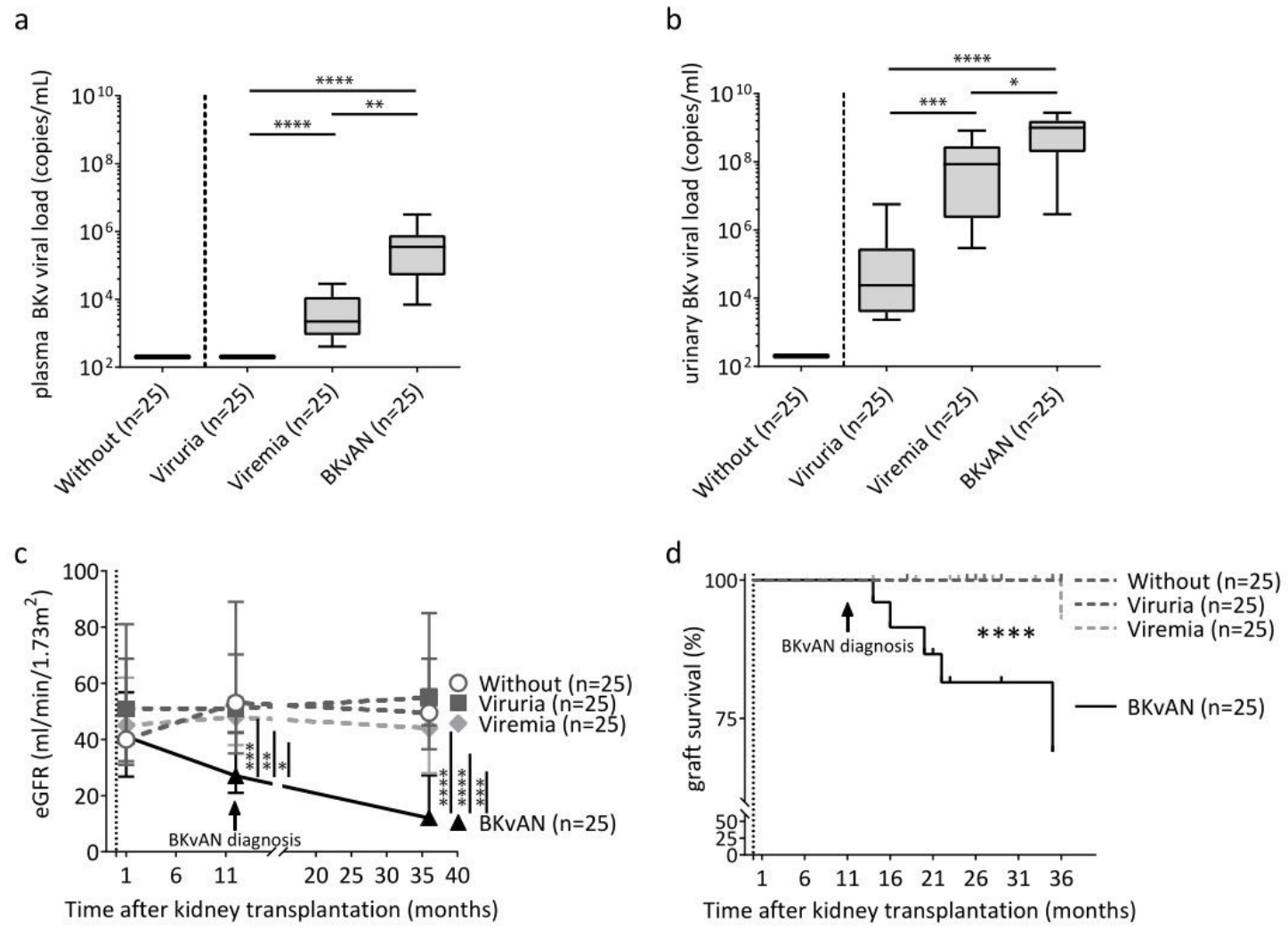

Figure 1

BKv viral load and allograft outcome in KTR groups defined on the basis of BKv reactivation

(a) Plasma and **(b)** urinary BKv viral loads in KTR groups defined according to the level of BKv reactivation. **(c)** Graft function [evaluated by eGFR at 3 time points: 1-month, BKvAN diagnosis or 12 months post-transplantation, and end of follow-up] and **(d)** Graft survival in KTR groups defined according to the level of BKv reactivation.

Without: patients with no plasma or urinary BKv reactivation; Viruria: patients with urinary BKv reactivation; Viremia: patients with plasma BKv reactivation, BKvAN: patients with BK-virus-associated nephropathy; BKv: BK-virus; eGFR: estimated glomerular filtration rate (MDRD4-formula); KTR: kidney transplant recipient; n: number of patients; p: p-values.

p-values indicate the significance of differences in Kruskal-Wallis tests followed by Dunn's multiple comparison tests between the BKv-viruria, viremia and BKvAN KTR groups **(a and b)**, nonparametric repeated-measures two-way ANOVA **(c)**, and Kaplan-Meier survival curves with log-rank **(d)** tests. Boxes represent the median and 25^{th} and 75^{th} percentiles; whiskers represent the 10^{th} and 90^{th} percentiles. eGFRs are represented as the median and interquartile range. *$p < 0.05$, **$p < 0.005$, ***$p < 0.0005$, ****$p < 0.0001$.

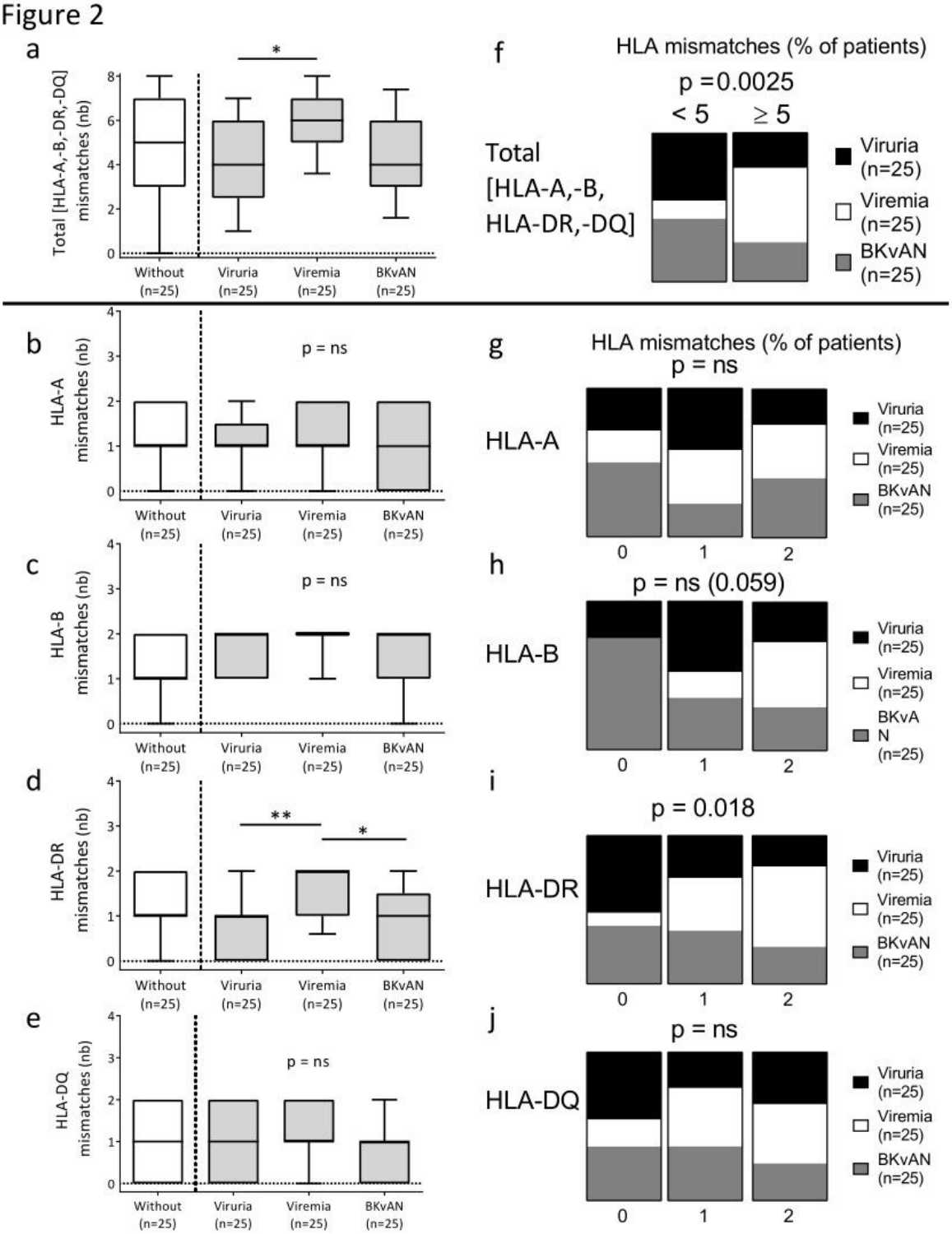

Figure 2

HLA mismatches as a risk factor for BKv-viremia and BKvAN

HLA mismatches in KTR groups defined according to the level of BKv reactivation:

Number of **(a)** total [HLA-A,-B,-DR,-DQ] mismatches, **(b)** [HLA-A] mismatches, **(c)** [HLA-B] mismatches, **(d)** [HLA-DR] mismatches, and **(e)** [HLA-DQ] mismatches.

Proportion of **(f)** total [HLA-A,-B,-DR,-DQ] mismatches expressed in classes [<5] and [≥ 5],

Proportion of **(g)** [HLA-A] mismatches, **(h)** [HLA-B] mismatches, **(i)** [HLA-DR] mismatches, and **(j)** [HLA-DQ] mismatches expressed in classes [0;1;2].

Without: patients without plasma or urinary BKv reactivation; Viruria: patients with urinary BKv reactivation; Viremia: patients with plasma BKv reactivation; BKvAN: patients with BK-virus-associated nephropathy; BKv: BK-virus; HLA: human leukocyte antigen; n: number of patients; p: p-values. p-values indicate the significance of differences between groups in Kruskal-Wallis tests followed by Dunn's multiple comparisons tests between the BKv-viruria, viremia and BKvAN KTR groups **(a, b, c, d and e)** or $\chi 2$ tests **(f, g, h, i and j)**. Boxes represent the median and 25^{th} and 75^{th} percentiles; whiskers represent the 10^{th} and 90^{th} percentiles.

*p < 0.05, **p < 0.005, ns: non-significant.

Figure 3

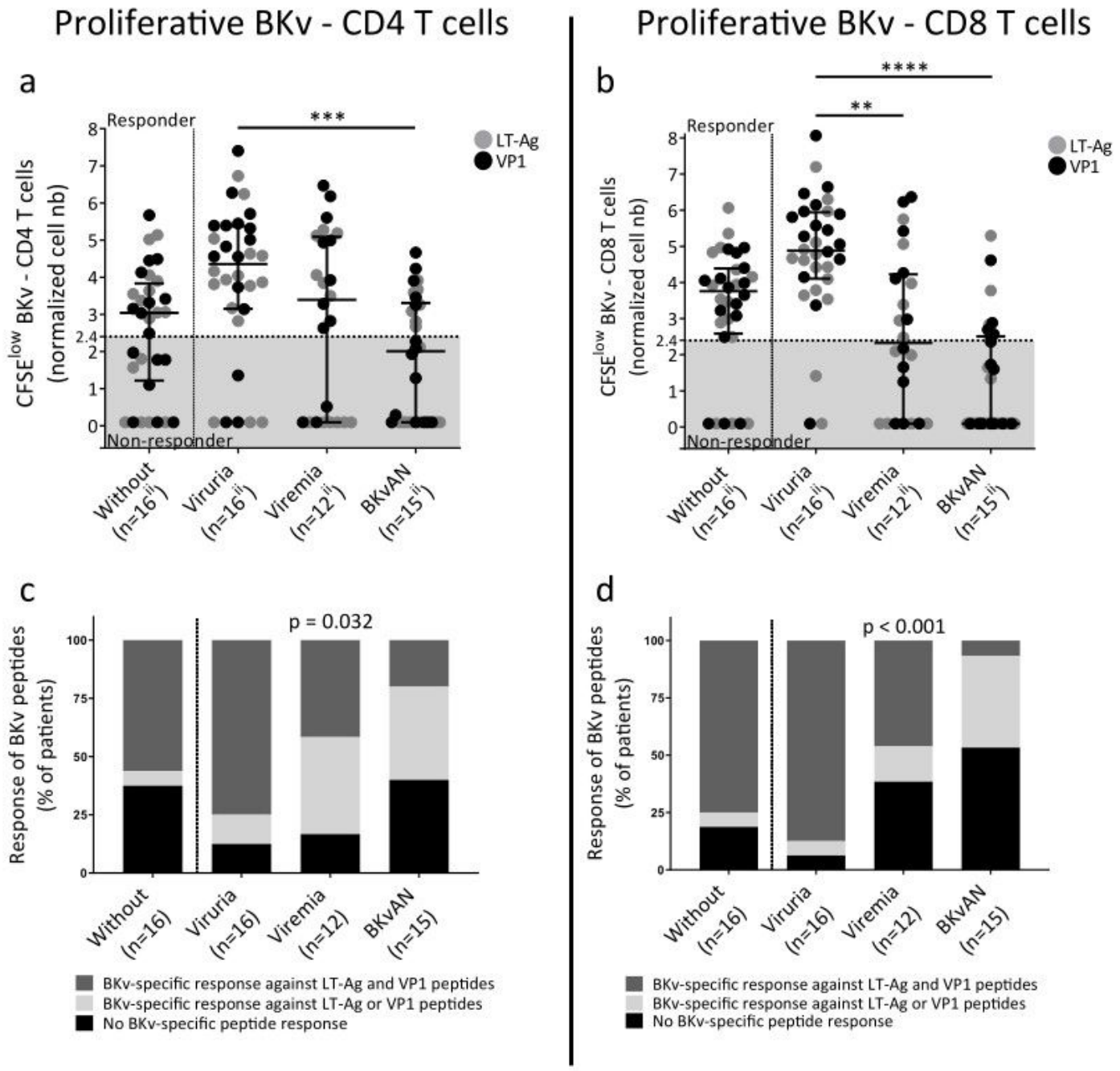

Figure 3

Impairment of the proliferative capacity of BKv-specific T cells as a risk factor for BKvAN

Proliferative capacity of BKv-specific CD4 **(left side)** and BKv-specific CD8 T cells **(right side)** for KTR groups defined according to BKv reactivation levels.

The proliferative response is expressed as **(a)** the normalized proliferative CD4 T-cell frequencies, **(b)** the normalized proliferative CD8 T-cell frequencies, **(c)** the proportion of patients displaying BKv-CD4 T-cell

proliferation according to LT-Ag and/or VP1 peptide responses, **(d)** the proportion of patients displaying BKv-CD8 T-cell proliferation according to LT-Ag and/or VP1 peptide responses.

After the culture of PBMCs in the presence of BKv-peptide pools (LT-Ag or VP1), lymphocyte proliferation was measured by carboxyfluorescein diacetate succinimidyl ester (CFSE) dilution. A cutoff of 2.4 normalized $CFSE^{low}$ T cells was used to distinguish between responders and non-responders. (n) represents the number of patients. (ii) For each patient, two BKv-specific responses were analyzed, after LT-Ag and VP1-peptide stimulations.

Without: patients without plasma or urinary BKv reactivation; Viruria: patients with urinary BKv reactivation; Viremia: patients with plasma BKv reactivation; BKvAN: patients with BK-virus-associated nephropathy; BKv: BK-virus; CFSE: carboxyfluorescein diacetate succinimidyl ester; LT-Ag: large tumor antigen; n: number of patients; normalized cell nb: normalized BKv-specific T-cell frequencies; VP1: viral protein 1.

p-values indicate the significance of differences between groups in Kruskal-Wallis tests followed by Dunn's tests for multiple comparisons **(a and b)** and $\chi 2$ **(c and d).** Scatter dot plots are shown with the median and interquartile range. **p < 0.005, ***p < 0.0005, ****p < 0.0001.

Figure 4

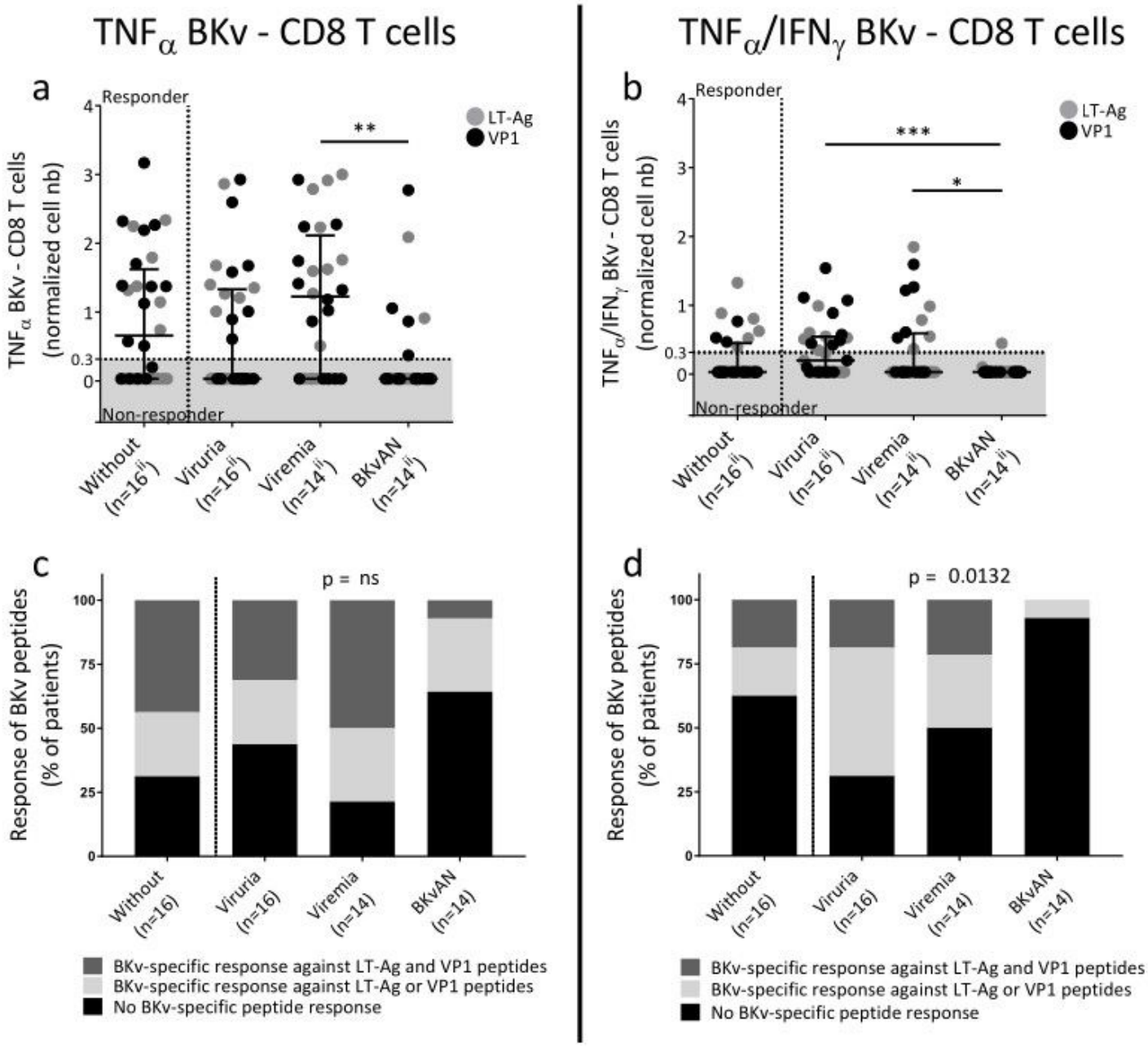

Figure 4

Impairment of the cytokine secretion capacity of BKv-specific T cells as a risk factor for BKvAN

Cytokine secretion capacity of BKv-specific CD8 T cells, **(left side)** TNFα secretion and **(right side)** TNFα/IFNγ secretion, for KTR groups defined according to BKv reactivation levels

The cytokine secretion response is expressed as **(a)** the normalized TNFα-secreting T-cell number, **(b)** the normalized TNFα/IFNγ-secreting T-cell number, **(c)** the proportion of patients displaying TNFα production

according to LT-Ag and/or VP1 peptide responses, **(d)** the proportion of patients displaying TNFα and IFNγ production according to LT-Ag and/or VP1 peptide responses.

After the culture of PBMCs in the presence of BKv-peptide pools (LT-Ag or VP1), we evaluated the secretion of IFNγ and/or TNFα by intracellular staining. A cutoff of 0.3 normalized cytokine-secreting cells was used to distinguish between responders and non-responders. (n) represents the number of patients. (ii) For each patient, two BKv-specific responses were analyzed, after LT-Ag and VP1-peptide stimulations.

Without: patients without plasma or urinary BKv reactivation; Viruria: patients with urinary BKv reactivation; Viremia: patients with plasma BKv reactivation; BKvAN: patients with BK-virus-associated nephropathy; BKv: BK-virus; IFNγ: interferon-γ; LT-Ag: large tumor antigen; n: number of patients; normalized cell nb: normalized BKv-specific T-cell frequencies; TNFα: tumor necrosis factor-α; VP1: viral protein-1.

p-values indicate the significance of differences between groups in Kruskal-Wallis tests followed by Dunn's tests for multiple comparisons **(a and b)** and χ2 **(c and d)**. Scatter dot plots are shown with the median and interquartile range. *p < 0.05, **p < 0.005, ***p < 0.0005.

Figure 5

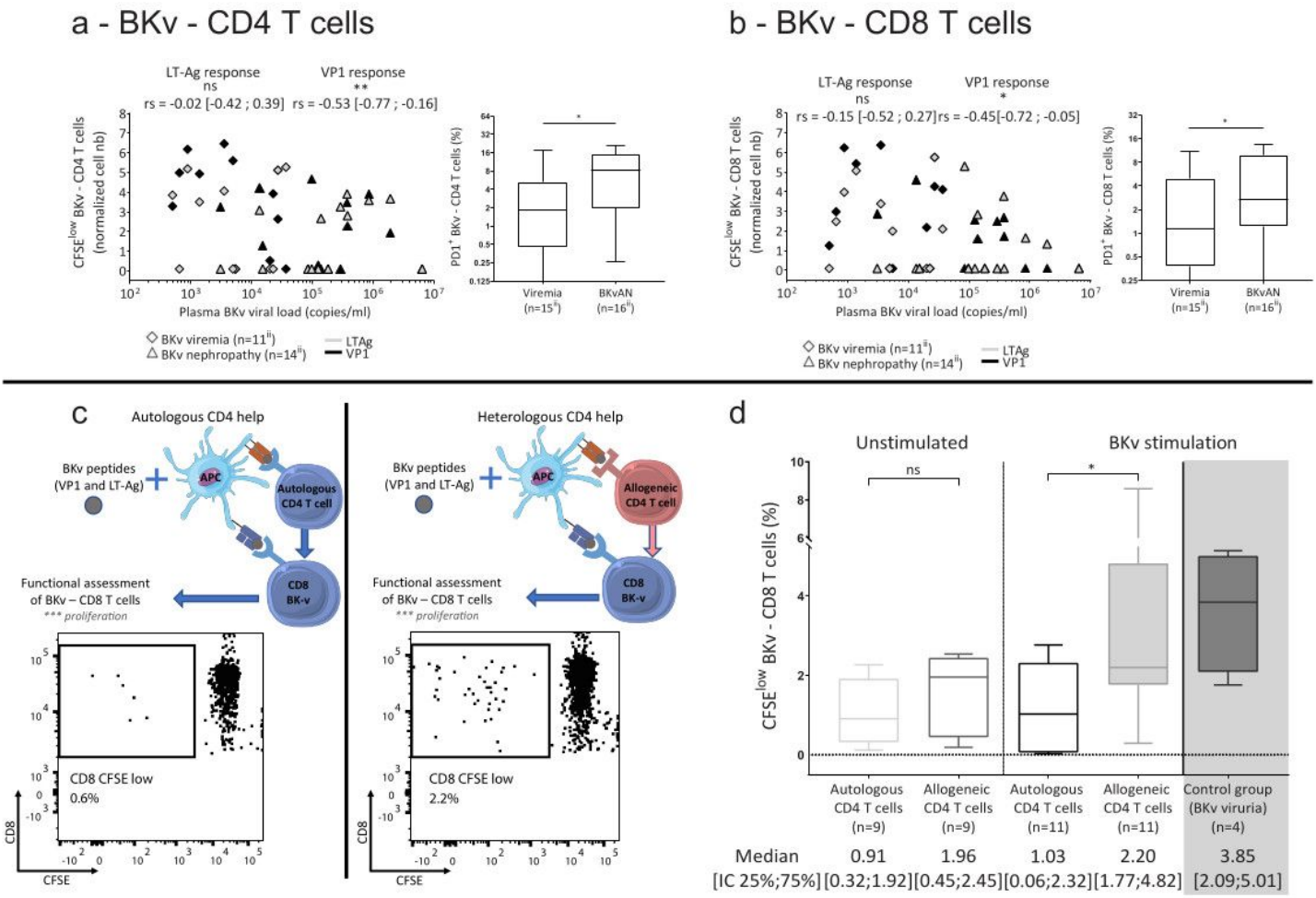

Figure 5

Restoration of the proliferative capacity of BKv-specific CD8 T cells in response to heterospecific CD4 help provided by allogeneic CD4 T cells

(**a)** Correlation between proliferative BKv-specific CD4 T cells and plasma BKv viral load. Expression of PD1 on BKv-specific CD4 T cells **(b)** Correlation between proliferative BKv-specific CD8 T cells and plasma BKv viral load. Expression of PD1 on BKv-specific CD8 T cells on BKv-viremia and BKvAN groups. (n) represents the number of patients. (ii) For each patient, two BKv-specific responses were analyzed, after LT-Ag and VP1-peptide stimulations.

(c) Schematic representation of the two experimental models. PBMCs from 11 KTRs with plasma BKv reactivation were stained with a fluorescent dye, depleted from autologous CD4 T cells by magnetic bead depletion, and incubated for five days with BKv-specific peptide pools in the presence of autologous or allogeneic third-party CD4 T cells (heterospecific CD4 help), at a ratio of 1 CD4 to 5 non-CD4 T cells. To distinguish PBMCs from allogeneic third-party cells, we used two fluorescent dyes (CFSE or VPD). After culture for five days, the stimulated cells were stained for CD3, CD4, and CD8 and their proliferation capacity was evaluated by fluorescent dye dilution. **(d)** Paired BKv-specific CD8 proliferation in the presence of autologous or allogeneic CD4 T cells. Unstimulated PBMCs incubated in the presence of autologous or allogeneic CD4 T cells were used as negative controls. Proliferative BKv-specific T cell responses from KTR with BKv viruria were used as reference.

BKv viruria: patients with urinary BKv reactivation; Viremia: patients with plasma BKv reactivation; BKvAN: patients with BK-virus-associated nephropathy; BKv: BK-virus; CFSE: carboxyfluorescein diacetate succinimidyl ester; n: number of patients; normalized cell nb: normalized BKv-specific T-cell frequencies; PD1: programmed cell death 1; rs: Spearman's r coefficient. *$p < 0.05$, **$p < 0.005$, ns: non-significant.

(a and b) Non-parametric Spearman's correlation and Mann-Whitney tests. rs values are represented with the correlation coefficient and 95% confidence interval. Box and whiskers plots showing the median and [5th-95th percentile]. **(d)** Wilcoxon matched-pairs signed-rank tests.

Figure 6

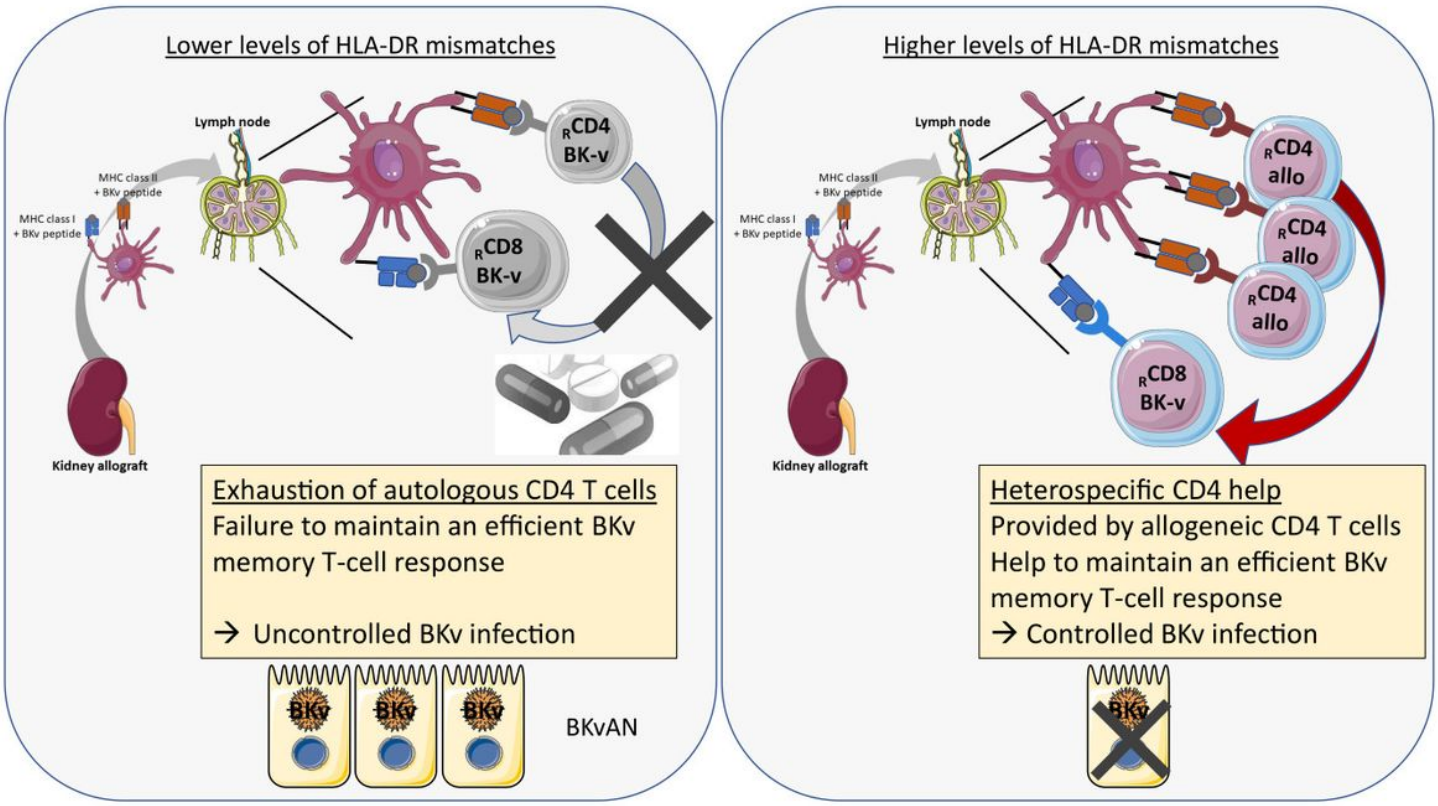

Figure 6

Schematic representation of the role of heterospecific CD4 help, provided by allogeneic CD4 T cells, to maintain an efficient BKv-specific memory CD8 T-cell response

A higher level of HLA-DR mismatches may be associated with a heterospecific CD4 help, able to sustain an efficient BKv-specific memory CD8 T-cell response.

BKv: BK-virus; BKvAN: patients with BK-virus-associated nephropathy; $_{R}$CD4 allo: allogeneic CD4 T cell; $_{R}$CD4 BK-v: BKv-specific CD4 T cell; $_{R}$CD8 BK-v: BKv-specific CD8 T cell, MHC: Major Histocompatibility Complex.

Supplementary Files

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