The individuality of (virus-specific) CD8 T cells
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CLINICALLY RELEVANT REACTIVATION OF POLYOMAVIRUS BK (BKV) IN HLA A02-POSITIVE RENAL TRANSPLANT RECIPIENTS IS ASSOCIATED WITH IMPAIRED EFFECTOR-MEMORY DIFFERENTIATION OF BKV-SPECIFIC CD8\(^+\) T CELLS

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ABSTRACT

Polyomavirus BK (BKV) frequently reactivates in immunosuppressed renal transplant recipients (RTRs) and may lead to graft loss due to BKV-induced interstitial nephritis (BKVN). Little is known on the differentiation of CD8+ T cells targeting BKV in RTRs. Here we investigated whether BKV-specific CD8+ T cell differentiation differs in RTRs with varying degrees of BKV reactivation and/or BKVN.

Using combinatorial encoding with tetramers carrying BKV major capsid protein (VP1) and large T antigen protein (LTAG) epitopes, we investigated CD8+ T cell responses to BKV in longitudinally obtained PBMC samples from 46 HLA-A02-positive RTRs and 20 healthy adults. We were also able to isolate BKV-specific CD8+ T cells from five renal allografts, two of which were affected by BKVN.

Before transplantation, BKV-specific CD8+ T cells targeting VP1 and LTAG epitopes appeared predominantly as central-memory and CD27+/CD28+ effector-memory (T EM ), and naïve-like PD-1-expressing cells, respectively. After viral reactivation, BKV-specific CD8+ T cells assumed CD28− T EM and T EM RA states in patients who were able to control BKV, whereas differentiation lagged behind in patients with severe viral reactivation or BKVN. Furthermore, VP1-specific CD69+/CD103+ tissue-resident memory (T RM ) cells accumulated in BKVN-affected allografts but lacked signs of effector differentiation. In contrast, granzyme B-expressing effector cells were detected in allografts not affected by BKVN.

In conclusion, effector-memory differentiation of BKV-specific CD8+ T cells in patients with high viral load or BKVN is impaired. Further characterization of the specific mechanisms behind this altered cellular differentiation is necessary to develop therapies that can prevent the emergence of BKVN.
AUTHOR SUMMARY

In immunosuppressed renal transplant recipients (RTRs), BKV frequently reactivates from latency and may cause severe interstitial nephritis in the allograft (BKVN). Not only is there no effective treatment, it also not understood why BKVN arises in some RTRs but not in all. In the current study we investigated populations of CD8+ T cells targeting epitopes from structural and non-structural BKV proteins in RTRs over the course of transplantation. In contrast to RTRs who suffered from self-limiting reactivation of BKV, patients who developed severe viral reactivation and BKVN were found to have BKV-specific CD8+ T cells which did not, or less often differentiate into CD28- effector-memory cells during viral reactivation. Moreover, virus-specific CD8+ T cell activation and differentiation was not only impaired in the circulation, but possibly also in BKVN-affected renal allografts. In contrast to the CD8+ T cells in kidneys from three patients who did not develop BKVN, T cells in two BKVN-affected kidneys did not display typical cytotoxic effector traits. These findings suggest that impaired BKV-specific CD8+ T cell maturation in response to viral reactivation, possibly owing to inter-individual differences in sensitivity to immunosuppressive medication or to certain viral quasispecies, underlies the emergence of severe viral reactivation and BKVN.

INTRODUCTION

Polyomavirus BK (BKV) establishes a mode of latent infection in the vast majority of the general, immunocompetent population \(^2\),\(^3\). However, in immunosuppressed renal transplant recipients (RTRs), BKV can escape the weakened immunological response leading to reactivation in up to 60% of the patients. In as much as 10% of these reactivations, the virus causes a severe interstitial nephritis (BKVN) in the allograft that is associated with graft loss \(^4\),\(^5\). Until now, the only effective treatment option for BKV reactivation following renal transplantation involves tapering of the immunosuppressive drug therapy, allowing the patient’s immune system to recover and overcome the virus. However, this also increases the chance on allograft rejection \(^4\),\(^5\).

For these reasons, effective and more specific treatment strategies are urgently needed. It is here that modern immunotherapies, such as adoptive transfer of virus-specific T cells, come into view. Recently, it was shown that BKV reactivation occurs concomitantly to a loss of T cells specifically targeting BKV epitopes, emphasizing the importance of T cells for effective immunological control of this virus \(^6\),\(^7\). T cell populations specific for BKV can be expanded in vitro and may then theoretically be used to treat BKV reactivation \(^8\). However, because each human virus triggers the formation of a specialized subset of T cells, carrying a distinct armamentarium to combat the respective virus \(^9\), it is essential to understand what type of T cells confers protection against BKV.

Previously, we used BKV virion protein 1 (VP1) peptide-loaded HLA A02-restricted tetramers to determine the phenotype and function of VP1-specific CD8+ T cells in the circulation of healthy individuals. We found that these cells largely exist in a central-memory (T_{CM}) or early-differentiated state \(^10\), a phenotype that was recently associated with stem cell-like properties \(^11\). However, in healthy individuals BKV-specific T cells may
seldom encounter their cognate antigen, whereas in RTRs BKV frequently reactivates, thus exposing the host’s T cells to substantial amounts of antigen and inflammation. Because of their specific capacity to detect and control intracellular pathology, as caused by viruses, we here investigated the phenotypic and functional differentiation of BKV VP1- and large T antigen (LTAG)-specific CD8\(^+\) T cells in the circulation of RTRs suffering from various degrees of BKV reactivation over the course of transplantation. In addition, we characterized BKV-specific T cells obtained from the allograft of some patients. Using this approach we aimed to identify whether differences in clinical outcome of BKV-infection are associated with altered differentiation pathways and/or effector functions of CD8\(^+\) T cells targeting this virus.

Using combinatorial encoding with six different HLA A02-restricted tetramers we confirmed that VP1-specific cells before transplantation mainly exist in a central-memory (T\(_{cm}\)) or early-differentiated effector-memory (T\(_{em}\)) state, whereas LTAG-specific CD8\(^+\) T cells unexpectedly exhibit a naïve-like phenotype with frequent expression of PD-1. After transplantation, both VP1 and LTAG-specific cells showed CD28\(^-\) T\(_{em}\) differentiation, sometimes with CD45RA re-expression (T\(_{em}\)RA). This mainly occurred in RTRs with low or undetectable viral load but not in patients with high viral load and/or BKVN. Within the renal allograft of two BKVN patients, we detected a high frequency of CD69/CD103-expressing tissue-resident BKV VP1-specific memory cells that, in contrast to the CD69/CD103-negative recirculating BKV-specific cells in kidneys from non-BKVN-affected patients, did not express granzyme B.

**RESULTS**

**Patients and Virology**

We included longitudinally obtained samples from 46 HLA-A02-positive RTRs: 21 in whom BKV replication had not been observed in the first year after transplantation (not-reactivating or NR patients), 11 RTRs in whom BKV had reactivated with a peak viral load below \(1 \times 10^4\) copies/ml (R\(_{low}\) patients), 6 RTRs showing BKV reactivation with a peak viral load higher than \(10^4\) copies/ml (R\(_{high}\) patients), and 8 RTRs with peak viral load higher than \(10^4\) copies/ml and biopsy-proven BKVN (BKVN patients). From five other patients who underwent a graft biopsy because of deterioration in renal allograft function during active BKV-infection, we obtained graft-eluted cells. Histological examination revealed BKVN in 2 of them, and no BKV infection in the other three patients. All grafts contained various degrees of interstitial fibrosis, tubular atrophy and cellular infiltrates. Samples from 20 HLA A02-positive healthy individuals served as a control.

Serological assessment showed the presence of anti-BKV antibodies in all patients before transplantation. Antibody titres increased significantly in the first year after transplantation in all RTRs in whom BK viremia was detected, but not in the NR patients (Figure 1a). Therefore, the rise in antibody titres is a reflection of viral reactivation as measured in the circulation but does not necessarily seems to prevent the reactivation as was shown previously. Peak viral load in RTRs were detected most often in the second
and third quarter of the first year post transplantation (Figure 1b). The viral load in the R\textsubscript{low} patients had dropped close to the quantifiable detection threshold of 1000 copies/ml already at the ≤6 months post peak viral load points. In the R\textsubscript{high} patients, this did not occur until somewhere in between the ≤1 year and ≤ 2 year post peak viral load time points. BKVN patients did not drop below this threshold during follow-up (Figure 1b). In response to detection of BKV viremia, the dosage of immunosuppressive drugs was carefully diminished, aimed at decreasing the BKV-load and preserving renal allograft function. First, the dose of mycophenolate mofetil was tapered in steps of 250 to 500 mg per 2 weeks, followed by decreasing the dose of tacrolimus by 0.5 to 2 mg per 2 weeks.

Detection of BKV-specific CD8\textsuperscript{+} T cells in healthy individuals and in RTRs before and following transplantation

Previously, BKV-specific CD8\textsuperscript{+} T cells were shown to be present in the circulation of both healthy individuals and RTRs at extremely low frequencies \textsuperscript{10,15-18}. To enhance the sensitivity and specificity of detection of BKV-specific CD8\textsuperscript{+} T cells, we here used combinatorial encoding of HLA-A02 tetramers loaded with two different immunodominant BKV VP1 peptides and one immunodominant LTAG peptide (Figure S1a) \textsuperscript{13}. Using this technique, and staining a large number of PBMCs (up to 12*10\textsuperscript{6} PBMCs per sample), we detected BKV VP1-specific CD8\textsuperscript{+} T cells in 6 out of 20 healthy individuals, and in 2 of 21 NR patients; 8 of 11 R\textsubscript{low} patients; 6 of 6 R\textsubscript{high} patients; and in 5 of 8 BKVN patients at some time point(s) during follow-up. We detected LTAG-specific cells in 12 of 20 healthy individuals, and in 4 of 21 NR patients; in 2 of 11 R\textsubscript{low} patients, in 2 of 6 R\textsubscript{high} patients and in 4 of 8 BKVN patients during follow-up (Figure S2). In RTRs, both VP1 and LTAG-specific cells were detected more frequently during viremia. Expansion of BKV-specific CD8\textsuperscript{+} T cell populations occurred in some individuals after transplantation, but not in all patients (Figure 1c). This was corroborated by a rise in Ki-67 expression after transplantation, particularly by the VP1-specific cells, indicating active cell proliferation. Ki-67\textsuperscript{+} expressing cells were detected neither in the samples from the NR patients, nor in those from the healthy individuals (Figure 1d).

The differentiation status of BKV-specific CD8\textsuperscript{+} T cells in RTRs before transplantation is similar to that in healthy individuals

Using multichannel flowcytometry, we determined the expression of various molecules characteristic for T cell differentiation and function (Figure S1b). Previously, we found that circulating BKV VP1-specific CD8\textsuperscript{+} T cells in healthy individuals were predominantly T\textsubscript{CM} cells (CD45RA\textsuperscript{−}CCR7\textsuperscript{+}CD27\textsuperscript{+}) or early-differentiated T\textsubscript{EM} (CD45RA\textsuperscript{−}CCR7\textsuperscript{−}CD27\textsuperscript{+}) cells \textsuperscript{19}. In the current study, adding the expression of CD28 to the classification, we confirmed these findings (Figure 2a).

Both LTAG and VP1-specific CD8\textsuperscript{+} T cells circulating in RTRs before transplantation showed similar phenotypes as in healthy individuals (Figure 2a). Comparison of LTAG and VP1-specific CD8\textsuperscript{+} T cells, however, revealed substantial differences in both healthy individuals and RTRs, with the LTAG-specific CD8\textsuperscript{+} T cells displaying a predominant CD45RA\textsuperscript{−}CCR7\textsuperscript{−}CD28\textsuperscript{−}CD27\textsuperscript{+} surface phenotype (Figure 2a). This phenotype may
Figure 1. A. anti-VP1 antibody levels in NR, Rlow, Rhigh and BKVN patients shortly before transplantation and one year after transplantation. B. Viral load during follow-up of Rlow, Rhigh and BKVN patients (left panel) and viral load plotted against the peak viral load (right panel). C. From left to right: Population sizes of VP1- (open symbols) and LTAG-specific (closed symbols) CD8+ T cells detected in healthy individuals, in all RTRs before transplantation, in NR patients before - and one year after transplantation and in the Rlow, Rhigh and BKVN RTRs during follow-up. D. Expression frequency of Ki-67 by VP1- (open symbols) and LTAG-specific (closed symbols) CD8+ T cells in healthy individuals, in NR patients before - and one year after transplantation and in the Rlow, Rhigh and BKVN RTRs during follow-up.
define antigen-inexperienced T cells, but also a subset of very early differentiated antigen-experienced CD8⁺ T cells with stem-cell-like traits, that, amongst others, is defined by expression of the tumour necrosis factor receptor family member CD95 (FAS receptor)²⁰,²¹. However, only about 16% of LTAG-specific CD8⁺ T cells with a “naïve” CD45RA⁺CCR7⁺CD28⁺CD27⁺ phenotype expressed CD95, which equalled the CD95 expression on the total population of CD45RA⁺CCR7⁺CD28⁺CD27⁺ CD8⁺ T cells (not shown). Thus, based on this surface marker, only a fraction of LTAG-specific cells could be assigned as typical stem-cell memory cells. Importantly, the LTAG-specific cells were significantly enriched for the expression of PD-1 when compared to the total naïve CD8⁺ T cell pool (Figure S3) suggesting that they have indeed been stimulated by antigen.

In addition, no major differences were found between the BKV-specific CD8⁺ T cells of patients just before renal transplantation and healthy control individuals regarding other immunological characteristics of BKV-specific cells like their T-bet- or Eomes expression; expression of granzyme B or granzyme K, and IL-7Rα (CD127), PD1, or CD95 (Figure 2).

Figure 2. A. Scatter plots and pie charts showing the distribution of the seven largest CD45RA/CCR7/CD28/CD27-defined human CD8⁺ T cell populations, as described previously²², amongst VP1- (first column) and LTAG-specific (second column) CD8⁺ T cell populations detected in healthy individuals (first row) and all RTRs (second row) before transplantation. B. from left to right the expression of T-bet, Eomes, granzyme B, granzyme K (first row) and IL-7Rα (CD127), PD-1 and CD95 (second row) by VP1- (open symbols) and LTAG-specific (closed symbols) CD8⁺ T cells detected in healthy individuals and in all RTRs before transplantation.
BKV-specific CD8+ T cell effector-memory differentiation is impaired in renal transplant recipients with high viral load and BKVN.

During BKV reactivation, the composition of both VP1- and LTAG-specific CD8+ T cell populations changed, as shown in Figure 3a and S4. The most profound changes were noted in the Rlow patients, in whom substantial proportions of normally cytotoxic intermediately-differentiated (CD45RA−CCR7−CD28−CD27+), CD45RA− effector-type (CD45RA−CCR7−CD28−CD27) T EM and T EM RA (CD45RA−CCR7−CD28−CD27) CD8+ T cell subsets specific for either VP1 or LTAG became detectable during and after the time point of peak viral load. In the Rhigh and BKVN group, these subsets were also formed amongst the VP1-specific CD8+ T cells but later in time and in smaller proportions. CD28− T EM subsets also emerged amongst LTAG-specific populations, but primarily at the moment of peak viral load in the Rhigh, after which their sizes diminished during the later time points. CD28− T EM differentiation was seldom observed in the BKVN patients. Differentiation had also occurred within the LTAG-specific cell-populations from NR patients at one year post-transplantation (Figure 3 and S4).

The frequency of T-bet and Eomes-expressing LTAG-specific CD8+ T cells is highest in patients with low BK viral load.

Recently, we found that the expression levels of T-bet and Eomes, master transcriptional regulators of type 1 (cytotoxic) T cell differentiation, are strong indicators of the degree of CD8+ T cell differentiation 22. We also showed that BKV VP1-specific CD8+ T cells circulating in healthy individuals mostly express low or intermediate levels of T-bet, whereas they lack expression of Eomes 10. Here, we studied whether the expression of T-bet and Eomes was influenced by the BK viremia occurring in RTRs.

Figure 4a shows that at all time points and in each patient group, VP1- and LTAG-specific cells expressed significantly more T-bet than Eomes. The frequency of T-bet- and Eomes-expressing VP1-specific cells was comparable between the different study groups. Although referring to data from only six patients, the frequency of both T-bet and Eomes-expressing LTAG-specific CD8+ T cells appeared to be higher in the Rlow patients than in the other study groups. This is also illustrated by Figure 4b, which shows two representative patients from the Rlow-, respectively BKVN group. Remarkably, despite the clear CD28− T EM differentiation detected in the LTAG-specific CD8+ T cells from NR patients around the first year after transplantation (Figure 3a), these populations did not contain increased frequencies of T-bet- and Eomes-expression at that time point (Figure 4a).

During BKV-replication, IL-7Ra expression on LTAG-specific CD8+ T cells in patients with low BK viral load is down regulated.

The cytokine IL-7 is important for T cell homeostasis in the absence of antigen and inflammation and IL-7Ra expression is rapidly lost following T cell receptor-dependent activation 23. As described previously, nearly all VP1-specific cells in healthy individuals expressed IL-7Ra, further suggesting that these cells infrequently encounter their antigen
Figure 3. A. Pie charts depicting the distribution of the seven largest CD45RA/CCR7/CD28/CD27-defined human CD8\(^+\) T cell populations, as described previously, amongst VP1- (left panel) and LTAG-specific (right panel) CD8\(^+\) T cell populations detected in NR, R\(^{low}\), R\(^{high}\) and BKVN RTRs during follow-up. B. Representative dot plot overlays showing the fluorescence intensities of CD45RA, CCR7, CD28 and CD27 with the total CD8\(^+\) T cell events shown in grey and LTAG-specific events in black from one R\(^{low}\) patient (upper row) and one BKVN patient (lower row) during follow-up.
BKV-SPECIFIC CD8+ T CELL RESPONSES IN RENAL TRANSPLANT RECIPIENTS

Figure 4. A. Scatter plots showing the expression frequencies of T-bet (upper panel) and Eomes (lower panel) by VP1- (open symbols) and LTAG-specific (closed symbols) CD8+ T cell populations detected in NR patients before - and one year after transplantation, and in the R^low, R^high and BKVN RTRs during follow-up. B. Representative dot plot overlays showing the fluorescence intensities of T-bet and Eomes with the total CD8+ T cell events shown in grey and LTAG-specific events in black from one R^low patient (upper row) and one BKVN patient (lower row) during follow-up.

(Figure 2) 10. As shown above, we found similar data for the LTAG-specific cells in healthy individuals and in patients just before renal transplantation (Figure 2b). IL-7Rα was also expressed on the majority of BKV-specific CD8+ T cells in NR-patients, R^high and BKVN patients (Figure 5a). In sharp contrast, IL-7Rα expression in the R^low patients was clearly downregulated during BKV-reactivation, especially on the LTAG-specific cell populations, as is also illustrated by two representative patients from the R^low - , respectively the BKVN group (Figure 5b).
Functional characteristics of BKV-specific CD8+ T cells

Next, we studied functional properties of BKV-specific CD8+ T cells, viz. their cytotoxic capacity as judged by both the presence of the serine proteases granzyme K and granzyme B, expression of the degranulation marker CD107a and their cytokine-producing capacity.

Previously, we found that a small number of BKV VP1-specific CD8+ T cells in healthy individuals expressed granzyme K and/or B 19, which we confirmed in the present study (Figure 2) 10. Despite the CD28− T EM differentiation occurring after BKV reactivation, particularly in the Rlow group, no clear differences in granzyme expression were observed at any time-point between these and other patients (Figure 6). As a marker for degranulation, we studied the surface expression of CD107a on BKV-specific CD8+ T cells after stimulation in vitro. Figure 7 shows in all groups at all time points a rather low frequency of CD107a+ cells, suggesting minimal degranulation of these cells, at least in the peripheral circulation.
The cytokine production capacity of the different BKV-specific CD8+ T cell populations was tested by stimulating PBMC with PMA/ionomycin, followed by visualization of the BKV-specific CD8+ T cells using combinatorial encoding with tetramers. This approach is hindered by downregulation of the T cell receptor upon T cell activation. For unknown reasons, this particularly affected the LTAG-specific cells in the R^{high} and BKVN patients. As such, we were unable to detect sufficient LTAG-specific cells in these patient groups for analysis. In the R^{low} group, where LTAG-specific cells were still detectable after stimulation, we observed that a modest proportion produced IL-2, TNFα and INFγ (Figure 7 and S5).

Previously, we found that the majority of VP1-specific CD8+ T cells in healthy individuals produced combinations of three cytokines, most commonly IL-2, INFγ and TNFα. This was confirmed in the present study, and was also observed in patients before renal transplantation and thereafter, irrespective of detectable BKV reactivation (Figure 7). No major differences in cytokine production capacity of VP1-specific cells were observed during follow-up.

Tissue-resident memory CD8+ T cells directed against the BKV VP1-epitope accumulate in the renal allograft from patients with BKVN

Because BKV nephropathy is the final consequence of uncontrolled BKV-replication in the kidney allograft, we studied the presence of BKV-specific CD8+ T cells within the graft of two patients and compared them to their peripheral blood counterparts. As a control, we studied graft-eluted cells from three RTRs without BKVN. In the two BKVN grafts, we detected only VP1-specific CD8+ T cells, whereas in the three non-BKVN-affected grafts we detected one VP1- and two LTAG-specific populations. We found that in the BKVN grafts the VP1-specific T cells were about 10^5 times enriched when compared to the peripheral blood compartment. In contrast, the frequencies of the one VP1-specific population and two LTAG-specific populations that we detected in the non-BKVN allografts, were similar to those in the paired peripheral blood samples (Figure 8a).

Tissue-resident memory T-cells (T_{RM}) are characterized by expression of CD69 and CD103, both molecules ensuring that T_{RM} populations are retained in the respective tissue and that they do not re-enter the circulation. In the two patients with BKVN, most of the graft-eluted VP1-specific CD8+ T cells expressed both CD69 and CD103, designating them as T_{RM} cells (Figure 8b). In contrast, in patients without BKVN, a minority of the BKV-specific CD8 T cells stained double-positive for these markers. The graft-eluted VP1-specific CD8+ T cells from the two BKVN patients were comparable to those in peripheral blood, showing a CD45RA^-CD27^+/^- T_{EM} phenotype (Figure 8c). Both VP1- and LTAG-specific graft-eluted cells from patients without BKVN were also quite similar to their PB counterparts but showed a more advanced differentiation state, bearing a CD27^- T_{EM} or T_{EM}RA phenotype. The accumulated VP1-specific CD8+ T cells in the two BKVN-affected kidneys contained very few granzyme B positive cells. In contrast, a considerable proportion of the BKV-specific CD8+ T cells in the non-BKVN-kidneys expressed this serine protease, although the percentage was lower than in the PB compartment (Figure 8d).
**DISCUSSION**

Here, we document that in renal transplant patients with high viral load and/or BKVN, the effector-memory differentiation of circulatory BKV VP1- and LTAG-specific CD8⁺ T cells is distinct from that in patients with low viral load. In line with this, the frequency of circulating T-bet and Eomes-expressing LTAG-specific cells was highest in patients with low viral replication. Furthermore, the BKV-specific CD8⁺ T cells in R<sub>low</sub> patients...
Figure 7. Scatter plots showing the production of CD107a (first row), IL-2 (second row), IFNγ (third row) and TNFα (last row) by VP1- (open symbols) and LTAG-specific (closed symbols) CD8+ T cell populations detected after stimulation in vitro in healthy individuals, NR patients before - and one year after transplantation, and in the R^low, R^high and BKVN RTRs during follow-up.

downregulated their expression of IL-7Rα, emphasizing the activation of these cells. Despite these dissimilarities in differentiation patterns, the BKV-specific cells in the distinct patient groups expressed similar but low levels of granzyme K and B. Also, we did not find any difference between the groups in cytokine production by the BKV-specific CD8+ T cells, which were polyfunctional as we showed before [8].
Figure 8. A. Line graphs showing the paired percentages of BKV VP1- and LTAG-specific CD8+ T cells amongst the total CD8+ T cell pool in the peripheral blood (PB) and in the kidney for 2 BKVN patients (first column) and three other RTRs (middle and right columns) B. Dot plot overlays showing the fluorescence intensities of CD013 and CD69, and of C. CD45RA and CD27, and D. CD103 and granzyme B in the PB and in the kidney.
Because we found no differences in properties of BKV specific CD8+ T cells between healthy individuals and patients shortly before transplantation, possible effects exerted by the uremic state or by any drug medication at present or in the past seem not to be involved. Recently, Schaenman et al. also reported that there is no apparent association between any type of immunosuppressive medication and the emergence of severe BKV reactivation in RTRs.

VP1-specific CD8+ T cells collected before transplantation started off with a T_CM or early-differentiated T_EM phenotype, whereas the LTAG-specific cells curiously primarily displayed a naïve-like phenotype. Nevertheless, following transplantation and viral reactivation in the R^low patients, both VP1- and LTAG-specific populations differentiated into CD28¯ T_EM cells, with LTAG-specific cells even acquiring the T_EM RA state. In the R^high and BKVN patients, VP1- and LTAG-specific CD8+ T cells instead generally persisted in their T_CM and CD28^CD27^ T_EM differentiation state. When compared to human cytomegalovirus (hCMV) or Epstein-Barr virus (EBV)-specific CD8+ T cells, the frequencies of BKV-specific cells in the circulation are very low, making them difficult to detect [8, 13-16, 34]. Therefore, Schachtner et al. used in vitro stimulation with overlapping BKV peptide pools in an Interferon-γ Elispot assay, and showed that the overall BKV-specific CD4+ and CD8+ T cell response was significantly delayed in patients who developed BKVN [5]. The same group recently demonstrated that this delay concerns mainly the T cell response targeting LTAG epitopes [4], which is in line with the data presented here.

The naïve-like LTAG cells detected prior to transplantation expressed PD-1 significantly more often when compared to the total CD45RA/CCR7/CD28/CD27 CD8+ T cell population. Apart from being a marker of functional exhaustion, PD-1 is also recruited into the immunological synapse upon T cell activation [30, 31]. Therefore, this naïve-like state may represent a subset of antigen-experienced T cells in a very early differentiation state, close to the CD95-expressing naïve-like population of stem-cell memory cells that was described recently [32, 33]. Despite the CD28¯ T_EM differentiation by T cells in the R^low group, a differentiation state that is normally associated with increasing expression levels of granzyme B [28,29], CD28¯ cells specific for BKV epitopes detected after viral reactivation had not significantly upregulated their granzyme B expression.

In view of the low percentage of granzyme-expressing cells, it may therefore well be that the normal immunological control of BKV by CD8+ T cells is not exerted by granzyme K or B. For example, human CMV-specific CD8+ T cells highly express granzyme B and T-bet. Instead, CD8+ T cells targeting EBV epitopes, primarily express granzyme K and Eomes, suggesting that each virus is controlled by a distinct type of CD8+ T cell equipped with a specific armamentarium [8, 21, 35, 36]. Therefore, CD8+ T cells may also have adopted a distinct strategy to control BKV, especially considering the long relationship between man and this virus [37]. Given the polyfunctionality with regard to cytokine production, BKV-specific CD8+ T cells may rely much more on production of typical cytokines to control BKV proliferation than on exerting cytotoxicity against infected cells.

It is important to mention that we only investigated the immunodominant HLA-A02-restricted T cell response in this study. Whilst this was done because this is the most abundant HLA class I molecule expressed by the general Western population, immunodominant
BKV T cell responses indeed also occur via other HLA class I molecules as shown recently by Cioni et al. 30.

One should also consider that the mechanism by which viral control is executed, may not be reflected by T cells located in the peripheral blood compartment. Indeed, the epicentre of BKV infection and inflammation is located within the renal allograft and not in the circulation. In the two patients with BKVN, from whom we obtained graft-eluted cells, the frequency of VP1-specific CD8+ T cells in the graft was indeed much higher than in their paired peripheral blood samples, suggesting sequestration of virus specific cells within the allograft. The majority of these graft-eluted cells consisted of CD69/CD103 double-positive T_{RM} cells [27]. Surprisingly, also here only very few of these cells expressed granzyme B. Considering the immunopathology in BKVN grafts, as evidenced by histological damage and deteriorated graft function, this large T_{RM} population appeared not capable to control the viral infection. In contrast, in patients without BKVN, only few BKV specific T_{RM} cells were detected in the graft, that apparently possibly contributed to local control of the virus.

Although more granzyme B-expressing cells were present than in the BKVN patients, they were mainly CD103-negative and their frequency was still lower than in the peripheral blood compartment. Probably, the intragraft BKV-specific CD8+ T-cells with the CD103-negative effector phenotype, are recirculating cells. In fact, in paired peripheral blood samples, similar phenotypes were found. Whether the T_{RM} cells originate from in situ differentiation of these recirculating effector cells, or vice versa, is unknown. Neither do we understand why so few effector cells were detected in the BKVN-allografts, and why the large population of T_{RM} cells in the BKVN-allografts failed to contain the infection. This situation is reminiscent of so-called tumour-infiltrating lymphocytes (TILs), which are in general dysfunctional 31. By analogy with that, we suppose to name these cells as Virus-specific Tissue-Infiltrating Lymphocytes (V-TILs). Given the small sample size in the current study, further research into (BKV-specific) kidney-resident T cell memory populations is required.

Specific reasons for the impaired effector-memory differentiation of circulating BKV-specific CD8 T cells in the patients with high viral loads / BKVN require further research. One possibility is that differentiation did occur, but was not measurable in the peripheral blood compartment due to retention of these cells in the tissue. Considering truly impaired differentiation, this may be the consequence of defective CD4+ helper cell function, insufficient costimulation, individual differences in susceptibility to immunosuppressive medication, or differences in the virulence of various BKV sub- or quasispecies. More knowledge on these possibilities, also on BKV-specific CD4+ T cell differentiation in these patients, is needed to better understand the disease process in order to develop effective BKV-directed immunotherapy in the future.

In conclusion, our findings show an impaired effector-memory differentiation program of BKV-specific CD8+ T cells in patients with severe BKV reactivation and/or BKVN. This offers an explanation for the pathogenesis of this clinical entity in RTRs, as well as a rationale for the potential effectiveness of immunotherapies to treat BKV reactivation in the future.
MATERIALS AND METHODS

Subjects and study groups
From the cohort of renal transplant recipients (RTRs) who were transplanted at the Academic Medical Center (AMC, Amsterdam, The Netherlands) between 2008 and 2013, we selected 25 HLA A02-positive patients, who experienced a reactivation of BKV-infection as demonstrated by a positive DNA real-time quantitative PCR (qPCR) in plasma within the first two years after a first transplantation. We included only HLA-02-positive individuals in this study because this is the most ubiquitously expressed HLA subclass (~50%) by the Western population. BKV DNA was quantified before and at regular intervals of 3 months after transplantation, and more frequently when qPCR had become positive, or earlier when BKV reactivation was clinically suspected. Peripheral blood samples were collected at the same time points; mononuclear cells (PBMC) and sera or plasma samples were frozen.

Time points chosen for analyses comprise: pre-transplantation (pre Tx); the period prior to detection of the peak viral load (pre-peak); the moment of peak viral load; the period of the first 6 months after detection of the peak viral load (≤6 months post peak); the period from month 6 to month 12 after detection of the peak viral load (≤ 1 year post peak); and the period between the first year and the second year after detection of the peak viral load (≤2 years post peak). Data points of individual patients shown and analysed were the ones collected closest to t = 6 months post peak, t = 12 months post peak and t = 24 months post peak. The pre-peak time point was defined as the number of months from transplantation to peak viral load divided by two. For obvious reasons, these restrictions did not apply to the pre-transplantation samples and the peak viral load samples as these concerned single sampling moments. Each time frame holds no more than one data point from an individual patient. All other data points collected and measured during follow-up were excluded from the analyses and the graphs shown in this manuscript.

Immunosuppressive treatment included induction with CD25mAb (Basiliximab), and maintenance therapy, consisting of corticosteroids 10 mg/day orally, mycophenolate mofetil 2 gram/day and tacrolimus aimed at serum trough levels of 6 – 10 ng/ml. Exclusion criteria comprised previous transplantation, PRA > 5%, inadequate viral load monitoring frequency, inadequate sampling frequency and/or treatment with immunosuppressive medication other than the agents described above. From the same cohort of RTRs, 21 HLA-A02-positive patients were included in whom no BKV reactivation occurred. These patients were treated, monitored and sampled according to the same protocol. In addition, we isolated mononuclear cells from renal allograft tissue and paired peripheral blood of 5 RTRs. Two patients who underwent a graft biopsy because of deterioration in renal allograft function during active BKV-infection were diagnosed to have BKVN based on histological analysis and a positive SV40 staining. BKV was not actively replicating in the three other RTRs and histological signs of BKV infection were lacking. All grafts contained various degrees of interstitial fibrosis, tubular atrophy and cellular infiltrates. As a control, we also included PBMC isolated from 20 HLA A02-positive buffy coats from
healthy blood donors ranging between 18 and 64 years of age (Sanquin, Blood Supply, Amsterdam, the Netherlands, Table I). For these latter subjects we could not obtain serum samples. We chose a viral load of $1 \times 10^4$ copies/ml as cut-off value between $R_{\text{low}}$ and $R_{\text{high}}$ patients, because it was previously proposed as a critical threshold for developing BKVN. However, as opposed to the BKVN patients, we were unable to detect BKVN in the $R_{\text{high}}$ patients by immunohistochemistry of their allograft biopsies.

**ETHICS STATEMENT**

The study was approved by the Medical Ethical Committee of the AMC, and written-informed consent was obtained from all patients in accordance with the Declaration of Helsinki.

**Isolation of mononuclear cells from peripheral blood and renal allograft tissue**

PBMC were obtained using standard density gradient centrifugation and subsequently cryopreserved until the day of analysis. Samples of human renal cortex were obtained from transplantectomies and renal allograft biopsies. Kidney mononuclear cells were isolated using mechanical disruption and enzymatic digestion. Renal cortex tissue was cut into small pieces, washed thoroughly with PBS to remove blood and incubated with collagenase type IV (150 U/ml, Worthington, Lakewood, NJ, USA) and DNase I type IV (50 U/ml) in HBSS + 2% fetal calf serum (FCS) + 0.6% bovine serum albumin (BSA) for 20’ at 37°C. The tissue pieces were washed and processed through a single-cell strainer. Renal biopsy eluates were analyzed directly. Isolates of larger kidney samples underwent density gradient centrifugation and were cryopreserved.

**Virological analyses**

Viral DNA was isolated from 200 ul plasma sample by Magnapure96 isolation (Roche applied Science) using the total nucleic acid isolation kit according to the instructions of the manufacturer. Subsequently, isolated DNA was amplified by an internally controlled quantitative realtime TaqMan PCR targeting the Large T-antigen Gene. Quantification was based on standard curves using quantified plasmid DNA containing the target sequence. Values over 1000 copies/ml were considered to be positive.

**Serological analyses**

Serum samples were analysed by Luminex for IgG reactivity against the BKV-genotype Ib1 major capsid protein 1 (VP1) according to a published protocol. Glutathione–casein (GC) coupled Bio-Plex polystyrene beads (Bio-Rad Laboratories, Hercules, CA, USA) containing a combination of fluorescent dyes were coupled to either GST-BKV VP1.tag or GST.tag. For each antigen, 3,000 GC-coupled beads per sample were loaded with crude bacterial lysates containing relevant GST-fusion protein. Samples were preincubated with GST.tag containing bacterial crude lysates (2 mg/mL) in blocking buffer to reduce nonspecific GST binding. The antigen-coated bead mixtures were incubated with serum diluted 1:100.
<table>
<thead>
<tr>
<th>Table I. Patient characteristics</th>
<th>Low peak viral load [VL&lt;10e4 c/ml]</th>
<th>High peak viral load [VL&gt;10e4 c/ml]</th>
<th>BKVAN</th>
<th>Non-reactiv. [NR]</th>
<th>Healthy individuals</th>
<th>P-value</th>
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<tbody>
<tr>
<td>Recipient</td>
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<td>Number</td>
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<td>6</td>
<td>8</td>
<td>21</td>
<td>20</td>
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</tr>
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<td>Age (median (yr), quartiles)</td>
<td>63 (53-66)</td>
<td>62 (54-65)</td>
<td>58 (52-62)</td>
<td>56 (47-65)</td>
<td>-</td>
<td>0.79</td>
</tr>
<tr>
<td>Gender (% male)</td>
<td>45.5%</td>
<td>66.7%</td>
<td>50%</td>
<td>61.9%</td>
<td>57.1%</td>
<td></td>
</tr>
<tr>
<td>Pre-transplant CMV status (% positive)</td>
<td>45.5%</td>
<td>50%</td>
<td>87.5%</td>
<td>85.7%</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Pre-transplant EBV status (% positive)</td>
<td>90.9%</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
<td>-</td>
<td></td>
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<td>Donor</td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Age (median (yr), quartiles)</td>
<td>65 (63-70)</td>
<td>55 (46-61)</td>
<td>47 (39-58)</td>
<td>53 (46-61)</td>
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<tr>
<td>Gender (% male)</td>
<td>72.7%</td>
<td>50%</td>
<td>75%</td>
<td>52.4%</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Deceased donor (%)</td>
<td>45.5%</td>
<td>50%</td>
<td>75%</td>
<td>71.4%</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>HLA mismatches (median, quartiles)</td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>HLA A</td>
<td>1 (1-1)</td>
<td>1 (0.3-1)</td>
<td>1 (0-1)</td>
<td>1 (0-1)</td>
<td>-</td>
<td>0.67</td>
</tr>
<tr>
<td>HLA B</td>
<td>1 (1-2)</td>
<td>1 (1-1.8)</td>
<td>1 (1-1.3)</td>
<td>1 (1-2)</td>
<td>-</td>
<td>0.59</td>
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<tr>
<td>HLA DR</td>
<td>1 (0.5-1.5)</td>
<td>0.5 (0-1)</td>
<td>1 (0-1)</td>
<td>1 (0-2)</td>
<td>-</td>
<td>0.35</td>
</tr>
<tr>
<td>HLA A/B/DR</td>
<td>4 (2.5-4.0)</td>
<td>2.5 (2-3.8)</td>
<td>2 (1.8-3)</td>
<td>3 (2-5)</td>
<td>-</td>
<td>0.29</td>
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<tr>
<td>BKV infection</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Time point of reactivation (weeks post Tx) median [IQR]</td>
<td>28 (27-33)</td>
<td>20 (15-22)</td>
<td>20 (13-25)</td>
<td>-</td>
<td>-</td>
<td>0.003</td>
</tr>
<tr>
<td>Duration to peak viral load (weeks post Tx) median [IQR]</td>
<td>28 (28-43)</td>
<td>26 (22-26)</td>
<td>38 (29-49)</td>
<td>-</td>
<td>-</td>
<td>0.29</td>
</tr>
<tr>
<td>BKV DNA load at peak (median (*10^3 counts/ml blood), quartiles)</td>
<td>2 (0.5-2.9)</td>
<td>52 (16-206)</td>
<td>422 (144-985)</td>
<td>-</td>
<td>-</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Graft outcome</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Delayed Graft Function (% present)</td>
<td>27.3%</td>
<td>50%</td>
<td>50%</td>
<td>47.6%</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Cold ischemia (median (h), quartiles)</td>
<td>3 (2-14)</td>
<td>7 (2-14)</td>
<td>12 (5-18)</td>
<td>13 (3-18)</td>
<td>-</td>
<td>0.26</td>
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<tr>
<td>Rejection episode &lt; 1yr (% present)</td>
<td>9.1%</td>
<td>16.7%</td>
<td>12.5%</td>
<td>4.8%</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Rejection episode &gt; 1yr (% present)</td>
<td>10%</td>
<td>0%</td>
<td>12.5%</td>
<td>10%</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Low peak viral load [VL&lt;10e4 c/ml]</td>
<td>High peak viral load [VL&gt;10e4 c/ml]</td>
<td>BKVAN</td>
<td>Non-reactiv. [NR]</td>
<td>Healthy individuals</td>
<td>P-value</td>
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<tr>
<td></td>
<td>35.0 (29.0-43.5)</td>
<td>44.0 (36.8-50.0)</td>
<td>31.0 (29.0-43.0)</td>
<td>45.0 (38.5-46.0)</td>
<td>-</td>
<td>0.60</td>
</tr>
<tr>
<td>1 year post TX</td>
<td>35.0 (29.0-43.0)</td>
<td>44.0 (36.8-50.0)</td>
<td>31.0 (29.0-43.0)</td>
<td>45.0 (38.5-46.0)</td>
<td>-</td>
<td>0.052</td>
</tr>
<tr>
<td>2 years post TX</td>
<td>35.0 (29.0-43.0)</td>
<td>44.0 (36.8-50.0)</td>
<td>31.0 (29.0-43.0)</td>
<td>45.0 (38.5-46.0)</td>
<td>-</td>
<td>0.052</td>
</tr>
</tbody>
</table>

1 T-cell-mediated rejection and/or Antibody-mediated rejection
2 Estimated GFR (eGFR) was calculated using the abbreviated MDRD formula published by Levey et al. (1): eGFR = 175 x (P_cr ÷ 88.4)^-1.154 x age^-0.203 x 0.742 [if female] x 1.210 [if black]
For detection of bound serum antibodies, beads were incubated with goat anti-human total immunoglobulin G–biotin (1:1,000 dilution; Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA), streptavidin R–phycoerythrin (1:1,000 dilution; Invitrogen), and washed. Beads were analyzed in a Bio-Plex 100 analyzer (Bio-Rad Laboratories). Results are presented as median fluorescent intensity (MFI) units. For each sample, antigen-specific binding was obtained by subtracting the MFI for beads coated with GST alone from those of beads coated with GST VP1. The cut-off value to determine BKV-seropositivity was based on sera of healthy children aged 10-15 months old, as described 35.

**Immunofluorescence staining, flowcytometry**

For the detection of BKV-specific CD8+ T cells we utilized combinatorial encoding with tetramers loaded with different immunodominant BKV peptides. This technique significantly increases the sensitivity in comparison to single multimer staining and allows for a detection limit as low as 0.002% of total CD8+ T cells in large sample sizes (Figure S1b) 13. To achieve a large enough sample size, we stained up to twelve million PBMC with the tetramers per experiment and determined the presence of BKV VP1 and LTag-specific CD8+ T cell populations as well as their expression of various surface and intracellular markers by multichannel flowcytometry (Figure S1c). As advised previously, we used a pre-defined inclusion cut-off value of at least 10 double-positive tetramer events (Figure S1b) 13.

Tetrameric complexes were obtained from Sanquin (Amsterdam, Netherlands) and from the NIH Tetramer Core Facility. Three different and previously tested immunodominant epitopes, shared by the majority of BKV strains were selected 10,15,16,36. This concerned two BKV capsid protein VP1 epitopes: BKV VP1-derived AITEVECFL (VP1 p44) and BKV VP1 LLMWEAVTV (VP1 p108); and one large T antigen protein (LTag) epitope: BKV LTag LLLIWFRPV (LTag p579). These were incorporated in phycoerythrin (PE, Sanquin), allophycocyanin (APC) and Brilliant Violet™ 421-labeled HLA-A02 tetrameric complexes (NIH).

PBMC were washed in phosphate-buffered saline containing 0.01% (wt/vol) NaN3 and 0.5% (wt/vol) bovine serum albumin. Samples were split into aliquots of two million cells. Each aliquot was incubated with a mix of PE-, APC-, and BV421-labeled tetrameric-complexes for two different BKV VP1 epitopes and one BKV LTag epitope (Sanquin, Amsterdam, Netherlands), followed by incubation with a combination of the following antibodies: CD27 APC-eFluor780 (eBioscience Inc, San Diego, CA, USA), CD8 BrilliantViolet (BV)785, IL-7Rα BV711, CXCR6 PE-Cy7 (BioLegend, San Diego, CA, USA), CD3 V500, CD45RA BV650, CCR7 BrilliantUltraViolet (BUV)395, PD-1 BrilliantBlue515, CD14 PE-CF594, CD19 PE-CF594, CD21 PE-CF594, CD95 BV711 (BD Biosciences, San Jose, CA, USA), CD28 FITC (Sanquin). Dead cells and duplets were excluded from analysis by using Live/Dead fixable staining (Life Technologies Europe BV, Bleiswijk, Netherlands) and height- and width event characteristics, respectively (Figure S1c).

The FOX-P3 staining kit (eBioscience) was used for intracellular stainings with the following antibodies: Eomesodermin PerCP-eFluor710, granzyme K PerCP-
eFluor710, T-Bet PE-Cy7 (eBioscience), Ki-67 BUV395 and granzyme B AlexaFluor700 (BD Biosciences). Cells were washed twice, all aliquots of a sample were pooled and up to ten million PBMC per sample were measured on an LSRFortessa flow cytometer and analyzed with FlowJo Version 9.3.3 software. Only live CD19⁻CD4⁻CD20⁻CD8⁺CD3⁺ lymphocytes positive for both differently labelled but otherwise identical tetramers were considered specific for the BKV epitope presented in the HLA-A2 tetramer (Figure S1b). CD8⁺ T cell differentiation was determined by surface expression patterns of CD45RA, CCR7, CD28 and CD27. We used a classification that defines the seven largest functionally distinct subsets, involving naïve and stem-cell memory cells (sharing a similar phenotype), central-memory cells (T_CM), four different effector-memory (T_EM) subsets and the T_EM_RA subset as described previously 22,29,37-39.

Please note that due to limited numbers of available PBMCs per patient we were not always able to do stainings with all the different antibody panels. This affects the data presented on granzyme K, granzyme B and Ki-67 expression (which were stained in a separate panel), where we did not have sufficient samples to determine the expression of these markers by BKV VP1-specific CD8⁺ T cells in one NR patient, one R_C low patient and one R_C high patient at t=pre-peak; three R_C low patients and three R_C high patients at t=<6 months post-peak; and two R_C low patients at t=2 years post-peak. Expression of these markers could also not be measured in BKV LTag-specific CD8⁺ T cells for one R_C high patient and one BKVN patient at t=peak; nor in one R_C low patient at t=2 years post-peak.

Cytokine production by BK virus-specific T cells
Cytokine release after phorbol 12-myristate 13-acetate (PMA)/ionomycin stimulation was performed as described by Lamoreaux et al.24. In short, PBMC were thawed and rested overnight in suspension flasks (Greiner) in RPMI supplemented with 10% FCS, penicillin, and streptomycin (culture medium). Samples were split into aliquots of two million cells. Each aliquot was stimulated with PMA (10 ng/ml) and ionomycin (1 μg/ml) in culture medium in the presence of CD107a FITC (eBioscience); αCD28 (15E8; 2 μg/mL), αCD29 (TS 2/16; 1 μg/mL), brefeldin A (Invitrogen; 10 μg/mL); and GolgiStop (BD Biosciences) in a final volume of 200 μL for 4 hours (PMA at 10 ng/mL/ionomycin at 1 μg/mL) at 37°C and 5% CO2 in untreated, round-bottom, 96-well plates (Corning). Subsequently, cells were incubated with a mix of PE-, APC-, and BV421-labeled tetrameric-complexes for two BKV VP1 epitopes and one BKV LTag epitope (table II), followed by incubation with CD14 PE-CF594, CD19 PE-CF594, CD21 PE-CF594, CD3 V500, CD8 BV785, and Live/Dead fixable red cell stain. Cells were then washed twice, fixed, and permeabilized (Cytofix/ Cytoperm reagent; BD Biosciences) and subsequently incubated with the following intracellular mAbs: anti-IFNγ BUV 395, anti-TNFα BV650 (BD Biosciences), and anti–IL-2 PerCP-eFluor 710 (eBioscience). Cells were washed twice; all aliquots of a sample were pooled and up to ten million PBMC per sample were measured on an LSRFortessa flow cytometer and analysed with FlowJo Version 9.3.3 software.
Statistical analysis

The two-tailed Mann-Whitney test or the Kruskal-Wallis test was used to analyse differences between different patient groups in IBM SPSS v22.0. A p-value less than 0.05 was considered statistically significant.

ACKNOWLEDGEMENTS

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We are also grateful to all physicians working at the Renal Transplant Unit for their help in collecting patient’s samples and clinical data and for their stimulating discussions. We are also deeply indebted to the renal transplant recipients who participated in this study.

We acknowledge the NIH Tetramer Core Facility (contract HHSN272201300006C) for provision of MHC class I tetramers. This study was funded by the Dutch Kidney Foundation (14OKG05, Kolff Physician Researcher Grant (M. van Aalderen) and CP09.04, Consortium Grant “ALLOVIR” (K. Heutink)). We also thank Roche Nederland for financial support.
REFERENCES


Figure S1. A. Schematic overview of the detection of BKV virion protein 1 (VP1)- and large T antigen protein (LTAG)-specific CD8+ T cells using combinatorial encoding with six different fluorescently-labelled major histocompatibility complex (MHC) class I tetramers loaded with VP1 and LTAG peptides. B. Representative dot plots showing the gating strategy used to define lymphocytes, single cells (exclusion of duplets), CD8-positive and CD3-positive events, four different CD45RA and CD27-defined events, CCR7-negative and positive events, CD28-negative and positive events, T-bet and/or eomesdermin (Eomes)-positive events, IL-7Rα (CD127)-negative and positive events, CD95-positive events, PD-1-positive events, Ki-67-positive events and granzyme K and/or granzyme B-positive events, respectively. These data were obtained from one representative healthy individual.
Figure S2. Bar graphs showing the detection frequencies of VP1- (open bars) and LTAG-specific (closed bars) CD8⁺ T cells in healthy individuals, in not-reactivating (NR) patients before - and one year after transplantation, and in respectively, the reactivating patients with low (Rlow), high (Rhigh) peak viral loads and in patients with BKV-induced interstitial nephritis (BKVN) during follow-up.

Figure S3. Scatter plot showing the expression frequency of PD-1 by the total CD45⁺CCR7⁺CD28⁺CD27⁺ ‘naïve’ CD8⁺ T cell population and by all the LTAG-specific CD8⁺ T cells with a CD45⁺CCR7⁺CD28⁺CD27⁺ phenotype.
Figure S4. Line graphs showing the statistical dispersion of the CD45RA/CCR7/CD28/CD27-defined subset distribution of VP1- and LTAG-specific CD8+ T cell populations over time in NR patients, Rlow patients, Rhig patients and BKVN patients (mean and standard deviation shown).
Figure S5. Pie charts showing the distribution of cytokine combinations produced by VP1-specific CD8+ T cells detected after stimulation in vitro in healthy individuals, in NR patients before - and one year after transplantation, and in the Rlow, Rhigh and BKVN RTRs during follow-up (left panel), as well as those produced by LTAG-specific CD8+ T cells in the Rlow patients (right panel).