Determinants of acute and chronic renal allograft injury
Kers, J.

Citation for published version (APA):
Kers, J. (2016). Determinants of acute and chronic renal allograft injury

General rights
It is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), other than for strictly personal, individual use, unless the work is under an open content license (like Creative Commons).

Disclaimer/Complaints regulations
If you believe that digital publication of certain material infringes any of your rights or (privacy) interests, please let the Library know, stating your reasons. In case of a legitimate complaint, the Library will make the material inaccessible and/or remove it from the website. Please Ask the Library: http://uba.uva.nl/en/contact, or a letter to: Library of the University of Amsterdam, Secretariat, Singel 425, 1012 WP Amsterdam, The Netherlands. You will be contacted as soon as possible.
Chapter 10

Intragraft Blood Dendritic Cell Antigen-1–Positive Myeloid Dendritic Cells Increase during BK Polyomavirus–Associated Nephropathy

Ünsal Yapıcı¹,*
Jesper Kers¹,*
Ivana Slavujevic-Letic¹
Geurt Stokman¹
Joris J.T.H. Roelofs¹
Michiel C. van Aalderen²
Jaap W. Groothoff³
Onno J. de Boer¹
Karlijn A.M.I. van der Pant⁴
Nike Claessen¹
Luuk B. Hilbrands⁴
Frédérique J. Bemelman⁵
Ineke J.M. ten Berge⁵
Sandrine Florquin¹

*Authors contributed equally

¹Department of Pathology, Academic Medical Center, Amsterdam (NL)
²Department of Internal Medicine, Renal Transplant Unit, Academic Medical Center, Amsterdam (NL)
³Emma Children’s Hospital, Academic Medical Center, Amsterdam (NL)
⁴Department of Nephrology, Radboud University Nijmegen Medical Center, Nijmegen (NL)

Journal of the American Society of Nephrology, 2015, PMID: 26701980
(Supplementary information is available at http://jasn.asnjournals.org)
CHAPTER 10

Abstract

Although both polyomavirus infection and T cell–mediated rejection (TCMR) are characterized by tubulointerstitial inflammation in the renal allograft, these conditions are treated with opposing therapeutic regimens. To gain more insight into the differences between antiviral and alloimmune responses, we performed a case-control study, in which we immunophenotyped the inflammatory infiltrates in renal biopsy specimens with BK polyomavirus-associated nephropathy (BKPyVAN) and specimens with TCMR. Compared with TCMR, BKPyVAN was diagnosed later after transplantation; therefore, BKPyVAN specimens showed more chronic damage than TCMR specimens. However, TCMR and BKPyVAN specimens had comparable levels of tubulointerstitial inflammation. Adjustment for confounders in various multivariable models revealed more blood dendritic cell antigen-1+ (BDCA-1+) myeloid dendritic cells (mDCs) present during BKPyVAN (odds ratio, 2.31; 95% confidence interval, 1.03 to 5.16; P=0.04) than during TCMR. Double immunostaining for SV40 and BDCA-1 showed that, during BKPyVAN, BDCA-1+ mDCs localized in proximity to the polyomavirus–infected tubular epithelial cells. We ensured that time of biopsy after transplantation was not a confounding factor by including additional specimens with late TCMR and protocol biopsy specimens matched for biopsy time. These additional specimens showed amounts of BDCA-1+ mDCs comparable with amounts in the early TCMR specimens. These results suggest that BDCA-1+ mDCs, known to be involved in the antiviral immune response during various viral infections, might have a pivotal role during BKPyVAN infection in the grafted kidney.
**Introduction**

Current immunosuppressive treatment has improved renal allograft survival, albeit at the expense of reduced immune surveillance to pathogens. Consequently, the incidence of viral infections in renal transplant recipients has increased (Marcen, 2009). In the last decade, BK polyomavirus–associated nephropathy (BKPyVAN) has become an important cause of renal transplant morbidity, leading to graft loss in 40%–60% of infected patients (Ramos et al., 2009). The diagnosis is generally made by renal allograft biopsy and can sometimes be difficult because of the light microscopic similarities with T cell–mediated rejection (TCMR). Both BKPyVAN and TCMR are characterized by tubulointerstitial inflammation in the renal allograft (Ahuja et al., 2001). The role of viral infections and their associated cellular infiltrates in the development of (chronic) allograft lesions is still incompletely understood and controversial. Previous studies have determined the phenotype of the cellular infiltrates in BKPyVAN compared with TCMR. Some studies reported the presence of more B lymphocytes in BKPyVAN (Ahuja et al., 2001; Latif et al., 2007; Li et al., 2013; Mannon et al., 2005), whereas others found no differences in the amount of T and B cells (Rogers et al., 2009). Buettner et al. showed a higher number of plasma cells in BKPyVAN compared with TCMR (Buettner et al., 2012). The demonstration of lymphocytic infiltrates, marked tubulitis, and tubular HLA-DR expression in areas lacking polyomavirus replication may support the diagnosis of TCMR as proposed by Nickeleit et al. (Nickeleit et al., 2000); however, the former two criteria are often observed in BKPyVAN as well and therefore, cannot be considered pathognomic (Masutani et al., 2012; Menter et al., 2013). A newly appreciated feature of BKPyVAN is that it may cause immunecomplex deposition along the tubular basement membrane. Granular staining of IgG, C3, and C4d is focally present on immunofluorescence accompanied by amorphous electron–dense deposits on electron microscopy (Batal et al., 2009; Bracamonte et al., 2007). Of special interest is also the dendritic cell (DC) dynamics in the kidney (Rogers et al., 2014). DC subsets have been shown to increase during renal damage (rejection and IgA nephropathy) (Woltman et al., 2007), whereas during BKPyVAN, peripheral blood DCs were shown to decrease (Womer et al., 2010); however, back-to-back comparison of biopsy material of BKPyVAN and TCMR is currently lacking.
The aim of this study was to investigate the demographic, clinical, histologic, and in particular, immunophenotypical differences between BKPyVAN and TCMR in a case-control setting.

**Materials & Methods**

**Renal allograft recipients**

All patients with a biopsy-proven BKPyVAN diagnosed between the years 2002 and 2010 and meeting the threshold for a minimal adequate specimen according to the Banff classification (at least seven glomeruli and one artery) were included (n=28). All biopsies were taken on clinical indication. The diagnosis of BKPyVAN was made on renal biopsies with patchy interstitial mononuclear inflammatory cell infiltrates in areas with necrotic tubular epithelium, homogenous intranuclear inclusion bodies (Ramawami et al., 2011) and positive immunohistochemistry with an anti-SV40 antibody (EMD Millipore, Billerica, MA). After revision, biopsies from six patients were considered to have both BKPyVAN and TCMR (inflammation in areas without viral inclusions), resulting in 22 patients with pure BKPyVAN. For all patients, the biopsy described in this study was the first episode of graft dysfunction that needed a renal transplant biopsy. These patients had also not experienced an episode of rejection before BKPyVAN. All follow-up biopsies and the reason of graft failure in this group were investigated. For comparison, we used a cohort of 37 patients from the same time period of transplantation with biopsy-proven TCMR. We performed SV40 staining on these biopsies to exclude concomitant BKPyVAN. For all patients, data on DSAs at time of biopsy was available: 56 of 59 had measurements by complement-dependent cytotoxicity assay (CDC), three of 59 had only DSAs measured by Luminex single-bead assay, and 11 of 59 had both CDC and Luminex data available. There were no CDC/Luminex+ DSA measurements in our cohort. On follow-up, 58 of 59 patients had DSAs measured by Luminex. All patients who were additionally classified as C4d-positive antibody-mediated rejection were excluded from analysis. Immunosuppressive medication consisted of induction therapy with basiliximab (Simulect; Novartis Pharma B.V., Arnhem, The Netherlands) and the combination of prednisolone, a calcineurin inhibitor (cyclosporin A; Neoral; Novartis, Basel, Switzerland) or tacrolimus (Prograf; Astellas Pharma), and mycophenolate mofetil (Cellcept; Roche, Basel, Switzerland), which did not change until time
of biopsy. For a secondary comparison, we included six biopsies from patients who underwent a first episode of TCMR and six protocol biopsies without signs of pathology (both groups were matched with the BKPyVAN biopsies for the time interval between biopsy and transplantation). Additionally, six reperfusion biopsies were included as negative controls. The experiments described in this study were conducted in adherence to the Declarations of Helsinki and Istanbul.

**Histology and immunohistochemical staining**

Biopsy material was formalin fixed, paraffin embedded, and stained with hematoxylin and eosin, Jones silver stain, and periodic acid–Schiff after diastase treatment for routine diagnostic workup. Electron microscopy is not part of the routine workup for renal transplant biopsies at our hospital. All biopsies were stained for T lymphocytes (CD3; Thermo Fisher Scientific, Vernon Hills, IL), B lymphocytes (CD20; Thermo Fisher Scientific), plasma cells (CD138; Dako), macrophages (CD68/PGM1; Dako), NK cells (CD56; Thermo Fisher Scientific), mDCs (BDCA-1; Miltenyi Biotec and DC-SIGN; R&D Systems, Minneapolis, MN), plasmacytoid DCs (CD123; BioLegend, San Diego, CA), mature DCs (DC-LAMP; Beckman Coulter, Inc., Brea CA ) and HLA-DR (CR3/43; Dako) in a sequential manner. For M2-type macrophages (CD163/CD68; Dako) and transitional B lymphocytes (CD23/CD20; Thermo Fisher Scientific), immunohistochemical sequential double–alkaline phosphatase staining was performed essentially as previously described (van der Loos, 2008; Yapici et al., 2011). Paraffin sections of inflamed tonsil tissue were used as positive controls for the staining and renal tissue labeled only with secondary antibodies was used as negative control. Next to immunostaining on paraffin sections, immunofluorescence staining for FITC-labeled C3c (Dako) and C4d (Bioconnect) were performed on cryosections. The specificity of the BDCA-1 antibody to stain DCs was tested with double–alkaline phosphatase staining with various markers of inflammatory cells in tonsillar tissue. We validated the specificity of the BDCA-1 staining for DCs on tonsillar tissue by double staining with the leukocyte markers CD3, CD20, CD15, CD68, CD123, CD163, c-KIT, MPO, tryptase, DC-LAMP and DC-SIGN (Supplemental Figure 2). A fraction of the BDCA-1+ cells coexpressed DC-SIGN, indicating that none of the tested cell types (at least in tonsillar tissue) coexpressed BDCA-1, except for mDCs, indicating its specific nature.
CHAPTER 10

Histopathologic examination and immunophenotypical quantification

All biopsies were scored according to the last revised version of the Banff classification by two observers in a simultaneous manner (Haas et al., 2014). The biopsies of the BKPyVAN cohort were also scored according to the BKPyVAN grading system as proposed by Hirsch et al. (Hirsch and Randhawa, 2013) and the Banff Polyomavirus Working Group [discussed in (Adam et al., 2014)]. BKPyVAN staging was classified as follows: stage A corresponds to PyV replication with no or minimal tubular injury, stages B1–B3 correspond to severe PyV-induced tubular injury with increasing amounts of inflammation, and stage C corresponds to PyV replication with severe (>50%) IF/TA. For PyV viral load (pvl), scoring is on the basis of the percentage of tubules showing SV40 immunoreactivity: pvl1, <1% of tubules with viral replication; pvl2, between 1% and 10% of tubules; pvl3, >10% of tubules. The whole cortex was examined, except for the immediate subcapsular cortex with a width of 0.5 mm. Cells that stained positive for CD3, CD20, CD23/CD20, CD138, CD56, BDCA-1, DC-SIGN, CD123, and DC-LAMP were quantified per HPF, and the cell counts per HPF were averaged. The biopsy surface that was stained for CD68+ cells and CD163+/CD68+ cells was quantified digitally with Image Pro Plus Software, version 5.0 (Mediacybernetics, Rockville, MD). HLA-DR expression was scored semiquantitatively as the percentage of tubules showing a dot–like cytoplasmic staining pattern corresponding to the endosomal localization of the HLA complexes. HLA-DR expression on epithelial cells was not considered a sign of rejection, because it was also observed in patients with BKPyVAN (Masutani et al., 2012; Menter et al., 2013). The percentages of missing staining because of unavailability of material per diagnosis are shown in Supplemental Table 1.

Statistical analyses

Differences in baseline demographic, clinical, and (immuno)histochemical parameters were evaluated with Fisher exact tests (binary and ordinal data) and Mann–Whitney rank tests (continuous data) where applicable. A Holm–Bonferroni correction of the P value was applied where indicated. We considered the missing staining as missing completely at random, because diagnostic processing was considered the sole reason for unavailability of biopsy material. We next performed multiple imputations of the data (M=10 datasets; maximum of 5 iterations). Numeric data were generated by predictive mean matching, binary data by logistic
regression, and ordered data by proportional odds models. Univariable and multivariable logistic regression models were generated for the complete patients as well as on the M=10 imputed datasets that include all patients after imputation. In case of logistic regression on the imputed datasets, the covariance matrices were used to pool the individual dataset estimates and obtain adjusted 95% CIs. Data extracted from the M=10 datasets were pooled according to the rules by Rubin (Rubin, 1987). Statistical analyses were performed using the R Computing Environment (version 2.15.2 for Macintosh). Graphs were created with use of the package ggplot2, version 1.0.0 for Macintosh. Two-tailed P values of <0.05 were considered significant.

Results

Demographic and clinical comparison between BKPyVAN and TCMR

We compared demographic and clinical parameters between patients with the diagnosis of BKPyVAN and patients with the diagnosis of TCMR. Patient characteristics are described in Table 1. Patients with BKPyVAN had significantly more HLA mismatches between donor and recipient (P=0.03) and were biopsied later after transplantation (median of 288 versus 20 days; P<0.001), and at time of biopsy, the eGFR according to the Modification of Diet in Renal Disease was significantly higher compared with that in patients with TCMR (P=0.05). In the group of patients with TCMR, 9 of 37 had donor-specific antibodies (DSAs) detectable at time of biopsy (24%) compared with 6 of 22 (27%) in the BKPyVAN group (P>0.99). On follow-up, 12 of 36 (33%) patients with TCMR compared with 9 of 22 (41%) patients with BKPyVAN developed de novo DSAs (TCMR versus BKPyVAN odds ratio [OR], 0.70; 95% confidence interval [95% CI], 0.21 to 2.20; P=0.54 adjusted for biopsy DSA+). No differences were observed for class 1 (P=0.37) or class 2 DSAs (P=0.30).

Histologic comparison between BKPyVAN and TCMR

We analyzed the acute and chronic Banff scores (Figure 1) in patients with BKPyVAN and TCMR. Tubulitis was a prominent feature in both TCMR and BKPyVAN to a similar degree (P=0.23). However, interstitial inflammation in non-sclerotic areas (Banff i score) was significantly higher in TCMR (P=0.01), whereas interstitial fibrosis and
tubular atrophy (IF/TA) scores were higher in BKPyVAN (P<0.001). Not surprisingly, there was a strong correlation between time of biopsy after transplantation and IF/TA score (Spearman Rho=0.61; P<0.001). To investigate the total burden of inflammation in the biopsies, which includes areas of fibrosis, the total inflammation score (ti score) as proposed by Mengel (Mengel et al., 2009) was determined as well. There was no difference in ti score between biopsies diagnosed with TCMR and BKPyVAN (P=0.54) (Figure 1). As expected, significant glomerulitis and per definition intima arteritis were only seen in the TCMR group. Signs of transplant glomerulopathy were not found in the TCMR group but were present in the BKPyVAN group (P=0.02). Of the BKPyVAN biopsies, four (18%) were classified as stage A, one (5%) was classified as stage B1, three (14%) were classified as stage B2, six (27%) were classified as stage B3, and eight (36%) were classified as stage C according to the classification by Hirsch et al. (Hirsch and Randhawa, 2013).

Table 1 | Demographic and clinical characteristics of the included patients

<table>
<thead>
<tr>
<th>Donor</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (median years, IQR)</td>
<td>45 (38-54)</td>
<td>51 (41-52)</td>
<td>0.31</td>
</tr>
<tr>
<td>Donation type (N, %)</td>
<td></td>
<td></td>
<td>0.15</td>
</tr>
<tr>
<td>Living donation</td>
<td>7 (19%)</td>
<td>8 (36%)</td>
<td></td>
</tr>
<tr>
<td>Donation after cardiac death</td>
<td>10 (27%)</td>
<td>2 (9%)</td>
<td></td>
</tr>
<tr>
<td>Organ perfusion support</td>
<td>20 (54%)</td>
<td>12 (55%)</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Recipient</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (median years, IQR)</td>
<td>49 (34-57)</td>
<td>47 (28-54)</td>
<td>0.53</td>
</tr>
<tr>
<td>Gender (N of males, %)</td>
<td>14 (38%)</td>
<td>10 (45%)</td>
<td>0.59</td>
</tr>
<tr>
<td>Paediatric patients (N, %)</td>
<td>3 (8%)</td>
<td>5 (23%)</td>
<td>0.14</td>
</tr>
<tr>
<td>Prior transplantations (N, %)</td>
<td>7 (19%)</td>
<td>4 (18%)</td>
<td>&gt;0.99</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Transplantation</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>HLA A/B/DR mismatch (median, IQR)</td>
<td>2 (0-3)</td>
<td>3 (2-5)</td>
<td>0.03</td>
</tr>
<tr>
<td>Cold ischaemia time (median hours, IQR)</td>
<td>23 (17-25)</td>
<td>20 (17-25)</td>
<td>0.82</td>
</tr>
<tr>
<td>Delayed graft function (N, %)</td>
<td>13 (35%)</td>
<td>7 (32%)</td>
<td>&gt;0.99</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>At time of biopsy</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Time of Bx after Tx (median days, IQR)</td>
<td>20 (10-67)</td>
<td>288 (141-776)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>eGFR (median mL/min/1.73m², IQR)</td>
<td>17 (7-31)</td>
<td>28 (13-49)</td>
<td>0.05</td>
</tr>
<tr>
<td>Proteinuria (median gr/24 hours, IQR)</td>
<td>0.33 (0.16-0.69)</td>
<td>0.24 (0.16-0.69)</td>
<td>0.54</td>
</tr>
<tr>
<td>ATG treatment (N, %)</td>
<td>6 (16%)</td>
<td>2 (9%)</td>
<td>0.70</td>
</tr>
</tbody>
</table>

1 Deceased donors only. TCMR = T cell-mediated rejection, BKPyVAN = BK polyomavirus-associated nephropathy, IQR = interquartile range, HLA = human leukocyte antigen, Bx = biopsy, Tx = transplantation, eGFR = estimated glomerular filtration rate according to the Modification of Diet in Renal Disease (MDRD) formula, ATG = anti-thymocyte globulin.
Blood DC Antigen-1+ Myeloid DCs correlate to BKPyVAN

We phenotyped the inflammatory infiltrates in both BKPyVAN and TCMR with immunohistochemical staining. Representative immunostaining in BKPyVAN and TCMR and the distribution of the different cell types in both groups are shown in Figures 2 and 3 and Supplemental Figure 1. T cells were present in tubules in both BKPyVAN and TCMR. Some glomeruli showed the presence of mostly T cells and some macrophages and B cells. Other than plasma cells, CD138 also stained the basolateral border of some tubules. In all DC staining (CD123, blood dendritic cell antigen-1 [BDCA-1], DC-SIGN, and DC-LAMP), dendrites were visible on positive cells. BDCA-1+ and DC-SIGN+ myeloid dendritic cells (mDCs) were observed around tubules with SV40+ tubular epithelial cells (Figure 2G). Hardly any NK cells or transitional B cells were present and we, therefore, excluded this staining from additional analyses. All biopsies were negative for peritubular capillary C4d per exclusionem, and we did not observe tubular basement staining with C3c. No significantly different HLA-DR staining was observed between BKPyVAN and TCMR. Univariable analyses on both complete patients and the M=10 imputed datasets showed more BDCA-1+ mDCs and fewer T cells in BKPyVAN (Table 2). DC-SIGN+ mDC counts were also higher in BKPyVAN, but the OR was lower than for BDCA-1+ mDCs (OR, 1.10; 95% CI, 0.99 to 1.22; P=0.08). Figure 3 is the quantitative representation of Figure 2 and Table 2. Plasma cells were observed to a similar extent between BKPyVAN and TCMR (OR, 1.01; 95% CI, 0.98 to 1.04; P=0.56). In multivariable analysis with CD3+ T cells, BDCA-1+ mDCs were significantly more
enriched in BKPyVAN compared with TCMR (Table 3, model 1) (OR, 1.38; 95% CI, 1.03 to 1.86; P=0.03). In the multivariable model that also included demographic parameters, similar results for BDCA-1⁺ mDCs were obtained (OR, 1.40; 95% CI, 1.03 to 1.90; P=0.03). When we constructed multivariable models with clinical (OR, 1.62; 95% CI, 1.02 to 2.55; P=0.04) and histologic parameters (OR, 2.31; 95% CI, 1.03 to 5.16; P=0.04), BDCA-1⁺ mDCs were significantly associated with BKPyVAN, and the OR per increase in cell count increased (Table 3). When we excluded the patients in both groups with any feature of ABMR (any glomerulitis, intima arteritis, peritubular capillaritis, or transplant glomerulopathy), still, a higher BDCA-1 cell count in patients with BKPyVAN was observed (mean difference in BDCA-1⁺ cells per high power field [HPF] =2.6; 95% CI, 0.1 to 5.1; P=0.04). The timing of the biopsy differed between the BKPyVAN and TCMR groups, which might have biased our observation of more BDCA-1⁺ mDCs in BKPyVAN. We compared the 22 patients with BKPyVAN with a novel group of 6 patients with a first episode of TCMR at a time point that was matched to the occurrence of BKPyVAN after transplantation to eliminate the influence of time after transplantation. To provide the reader with more insight into the possible role of BDCA-1⁺ mDCs during an episode of BKPyVAN, we determined the amount of BDCA-1⁺ cells in healthy renal transplant tissue of 6 patients with a protocol biopsy free of rejection at the same time after transplantation and a group of 6 reperfusion biopsies (n=6) as a negative control. Figure 4 shows the BDCA-1⁺ cell counts in these sets of biopsies. In the protocol biopsies at reperfusion and after transplantation, there was hardly any BDCA-1⁺ cells visible. The initial group of TCMR (n=37) and the second group of late TCMR (n=6) did not differ in their amounts of BDCA-1⁺ mDCs (P=0.74), whereas both groups had significantly lower BDCA-1⁺ mDC counts than the biopsies displaying BKPyVAN (initial cohort, P=0.01; second group of late TCMR, P=0.03). This suggests that, after transplantation, BDCA-1⁺ mDCs preferably infiltrate the grafts and migrate toward BK polyomavirus–infected tubuli during BKPyVAN.
209

**BDCA-1+ myeloid dendritic cells in BKPyVAN**

**Figure 2 | Representative photographs of different immunohistochemical staining**

Arrows indicate hard to observe stained cells. (A and B) CD3+ T cells. (C and D) CD20+ B cells. (E and F) CD138+ plasma cells. (G and H) Double staining of BDCA-1+ mDCs in blue, with SV40 antigen in red. (I and J) DC-SIGN+ mDCs. (K and L) CD123+ plasmacytoid DCs. (M and N) DC-LAMP+ mature DCs. Original magnification, X20 in A–F and I–N; X40 in G and H.

**Figure 3 | The distribution of the inflammatory cell types in BKPyVAN and TCMR**

Data are depicted as medians and interquartile ranges. Univariable and multivariable comparisons between BKPyVAN and TCMR are in Table 2.
CHAPTER 10

Table 2 | Univariable comparison of inflammatory infiltrates in BKPyVAN versus TCMR

<table>
<thead>
<tr>
<th></th>
<th>Complete-case analysis</th>
<th>Multiple imputations pooled analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>OR (95% CI)</td>
<td>P</td>
</tr>
<tr>
<td>T cells</td>
<td>0.99 (0.98 - 1.00)</td>
<td>0.02</td>
</tr>
<tr>
<td>B cells</td>
<td>1.01 (1.00 - 1.04)</td>
<td>0.18</td>
</tr>
<tr>
<td>Macrophages</td>
<td>0.81 (0.40 - 1.54)</td>
<td>0.53</td>
</tr>
<tr>
<td>M2-type</td>
<td>0.94 (0.79 - 1.10)</td>
<td>0.45</td>
</tr>
<tr>
<td>Plasma cells</td>
<td>1.01 (0.98 - 1.04)</td>
<td>0.55</td>
</tr>
<tr>
<td>Dendritic cells</td>
<td>0.80 (0.52 - 1.17)</td>
<td>0.27</td>
</tr>
<tr>
<td>Mature</td>
<td>1.07 (0.76 - 1.53)</td>
<td>0.70</td>
</tr>
<tr>
<td>Myeloid (BDCA-1*)</td>
<td>1.43 (1.13 - 1.93)</td>
<td>0.007</td>
</tr>
<tr>
<td>Mature (DC-SIGN+)</td>
<td>1.10 (1.00 - 1.23)</td>
<td>0.08</td>
</tr>
</tbody>
</table>

OR = odds ratio, CI = confidence interval. Here, the OR means that per increase in cell/HPF, the relative odds of having BKPyVAN is higher than having TCMR.

Table 3 | Multivariable models including T cells and BDCA-1+ mDCs in BKPyVAN versus TCMR

<table>
<thead>
<tr>
<th>Multivariable models</th>
<th>Multiple imputations pooled analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>OR (95% CI)</td>
</tr>
<tr>
<td>Multivariable model 1 (crude cellular model)</td>
<td></td>
</tr>
<tr>
<td>T cells</td>
<td>0.99 (0.98 – 1.00)</td>
</tr>
<tr>
<td>BDCA-1+ myeloid dendritic cells</td>
<td>1.36 (1.03 – 1.86)</td>
</tr>
<tr>
<td>Multivariable model 2 (demographic)</td>
<td></td>
</tr>
<tr>
<td>T cells</td>
<td>0.99 (0.98 – 1.00)</td>
</tr>
<tr>
<td>BDCA-1+ myeloid dendritic cells</td>
<td>1.40 (1.03 – 1.90)</td>
</tr>
<tr>
<td>Donor age</td>
<td>1.02 (0.95 – 1.09)</td>
</tr>
<tr>
<td>Recipient age</td>
<td>0.98 (0.94 – 1.03)</td>
</tr>
<tr>
<td>recipient gender (male)</td>
<td>0.78 (0.17 – 3.54)</td>
</tr>
<tr>
<td>Multivariable model 3 (clinical)</td>
<td></td>
</tr>
<tr>
<td>T cells</td>
<td>0.98 (0.97 – 1.00)</td>
</tr>
<tr>
<td>BDCA-1+ myeloid dendritic cells</td>
<td>1.62 (1.02 – 2.55)</td>
</tr>
<tr>
<td>Proteinuria</td>
<td>0.36 (0.02 – 5.26)</td>
</tr>
<tr>
<td>Estimated glomerular filtration rate (eGFR)</td>
<td>1.06 (1.00 – 1.12)</td>
</tr>
<tr>
<td>Living donation</td>
<td>1.02 (0.09 – 11.18)</td>
</tr>
<tr>
<td>Human leukocyte antigen (HLA) mismatches</td>
<td>2.05 (1.01 – 4.16)</td>
</tr>
<tr>
<td>Multivariable model 4 (histopathological)</td>
<td></td>
</tr>
<tr>
<td>T cells</td>
<td>1.00 (0.97 – 1.02)</td>
</tr>
<tr>
<td>BDCA-1+ myeloid dendritic cells</td>
<td>2.31 (1.03 – 5.16)</td>
</tr>
<tr>
<td>Timing of biopsy after transplantation</td>
<td>1.01 (1.00 – 1.01)</td>
</tr>
<tr>
<td>Total percentage of inflamed biopsy (ti score)</td>
<td>0.91 (0.82 – 1.00)</td>
</tr>
<tr>
<td>Banff tubulitis score (t score)</td>
<td>4.60 (0.84 – 25.10)</td>
</tr>
<tr>
<td>Banff interstitial fibrosis/tubular atrophy score (IF/TA score)</td>
<td>11.01 (1.86 – 65.21)</td>
</tr>
</tbody>
</table>

OR = odds ratio, CI = confidence interval. OR are presented as per increase in unit (e.g. average cell count per high power field). ORs >1.00 indicate a positive association with BKPyVAN (as compared to TCMR).
Patients with features of both BKPyVAN and TCMR have similar BDCA-1+ cell counts as patients with BKPyVAN

We encountered 6 patients who had signs of BKPyVAN as well as TCMR in the same biopsy (tubulitis in areas without viral inclusions). When we analyzed 6 patients, these patients had comparable absolute (P=0.11) and relative (P=0.43 corrected for total inflammation) BDCA-1+ mDC counts as 22 patients with only BKPyVAN but had significantly higher absolute and relative BDCA-1+ mDC counts than patients with only TCMR (both P=0.03). These analyses suggest that these 6 patients might have BKPyVAN rather than TCMR. On the basis of BDCA-1 cell counts, we cannot exclude that additional alloimmune responses take place in these six patients.

The burden of tubulointerstitial inflammation correlates to BDCA-1+ mDCs in BKPyVAN but not in TCMR

In the BKPyVAN patients, the amount of BDCA-1+ mDCs was associated with the extent of tubulointerstitial inflammation (P<0.001 with ti score and P=0.04 with t score) but not with the percentage of infected tubuli (P=0.31). The amount of BDCA-1+ mDCs was not related to the burden of fibrosis (P=0.24 with IF/TA score) or the time interval between biopsy and transplantation (P=0.86 with time of diagnosis.

Figure 4 | BDCA-1+ cell counts in BKPyVAN, TCMR, and protocol biopsies
There was a significantly higher amount of BDCA-1+ mDCs in BKPyVAN compared with the full group of TCMR biopsies (n=37) and the group of late TCMR biopsies that were matched for the time of biopsy after transplantation with the BKPyVAN group (n=6). We hardly observed BDCA-1+ mDCs in protocol and reperfusion (T=0) biopsies.
The absolute and relative BDCA-1+ mDC counts (corrected for total of biopsy inflammation) broken down per tubular viral load (P=0.20 and P=0.60 for trend, respectively) and histologic BKPyVAN stage (P=0.70 and P=0.20 for trend, respectively) did not show significant differences among the stages. In patients with TCMR, no association between the number of BDCA-1+ mDCs and the degree of tubulointerstitial inflammation was observed (P=0.84 with ti score and P=0.59 with t score).

**Discussion**

In this study, we show that the inflammatory infiltrates during an episode of BKPyVAN were enriched with BDCA-1+ mDCs compared with the inflammatory infiltrates during TCMR. These cells were found in close proximity of tubules that expressed the SV40 large T antigen.

It is generally thought that the inflammation in BKPyVAN is because of an appropriate antiviral–specific immune response and therefore, reduction of the immunosuppression is the gold standard strategy to date (Schaub et al., 2010). However, the distinction between TCMR and BKPyVAN is difficult, and this is particularly important, because treatments are opposite. The major aim of this study was to compare the inflammatory infiltrates in renal biopsies during TCMR and BKPyVAN to get more insight in the specificity of the antiviral and alloimmune responses. We showed that the composition of the inflammatory infiltrates is different between BKPyVAN and TCMR, although the total burden of tubulointerstitial inflammation in the biopsies was similar. We observed an enrichment of BDCA-1+ mDCs in BKPyVAN in proportion to the total burden of tubulointerstitial inflammation, which was not the case during TCMR. BDCA-1+ mDCs were mainly but not exclusively localized in BKPyVAN in proportion to the total burden of tubulointerstitial inflammation, which was not the case during TCMR. BDCA-1+ areas of polyomavirus–infected tubular epithelial cells, which provides additional evidence of a role for these cells in the pathogenesis of BKPyVAN and provides a possible novel marker to differentiate between the two diseases. In protocol biopsies (either reperfusion biopsies or protocol biopsies matched with the BKPyVAN biopsies for time after transplantation), we hardly observed any BDCA-1+ mDCs.
BDCA-1+ myeloid dendritic cells in BKPyVAN

mDCs, which indicates that the increase during BKPyVAN most probably represents an influx of these cells during polyomavirus infection. One can hypothesize that mDCs orchestrate the immune response during BKPyVAN or that the influx of mDCs is a bystander effect of tubulointerstitial inflammation in these patients; however, we did not observe BDCA-1+ mDCs in areas of tubulitis during TCMR. Thus far, most studies reported deficiencies of all DC subtypes in the peripheral blood during BKPyVAN (Chen et al., 2006; Comoli et al., 2004; Womer et al., 2010), whereas increased amounts of all DC subtypes have been reported in renal biopsies during acute rejection (Zuidwijk et al., 2012). However, the number of intragraft DCs in BKPyVAN has not been studied before. On the basis of our results, we postulate that the deficiency of peripheral blood DCs in BKPyVAN might be explained by the migration of these cell types to the injured or infected renal tissue. DCs are the prime antigen-presenting cells of the immune system, and both plasmacytoid cells and mDCs are known to respond to various pathogen-associated molecular patterns/danger-associated molecular patterns, including viral RNA/DNA by innate pattern recognition receptors. In this study, we did not perform pathway analysis, and we, therefore, acknowledge that the finding of an increased influx of BDCA-1+ mDCs during BKPyVAN is mainly hypothesis generating and that our observations need replication in a prospective cohort.

Studies on the role of T lymphocytes, B lymphocytes, and plasma cells in BKPyVAN have shown conflicting results. Some studies have reported a significant increase in B cells and plasma cells in BKPyVAN (Ahuja et al., 2001; Buettner et al., 2012; Kemény et al., 2010; Mannon et al., 2005), whereas other studies did not find any differences in cell counts (Meehan et al., 2005; Rogers et al., 2009). In our study, we show that the amount of T cells (in multivariable analyses), B cells, and plasma cells is comparable during BKPyVAN and TCMR as well as after we corrected the cell counts for the degree of total inflammation in the biopsy.

Tubular HLA-DR expression has been shown to be present during acute rejection but absent during BKPyVAN and was suggested as a discriminative factor (Nickeleit et al., 2000). However, recent studies showed that, in some of the patients with BKPyVAN, HLA-DR expression is also present (Li et al., 2013; Menter et al., 2013). Our study is in line with these recent publications. It is known that HLA-DR expression
can stimulate an allogeneic lymphocytic reaction and enhance T cell–mediated lysis (Rosenberg and Singer, 1992). Thus, polyomavirus infection could possibly trigger an alloimmune response by inducing HLA-DR upregulation as previously proposed for CMV (von Willebrand et al., 1986). Likewise, the polyomavirus BK large T antigen–derived peptide can elicit an HLA-DR promiscuous and polyfunctional CD4+ T cell response (Ramaswami et al., 2011).

This study has some limitations. First, during our study period, only since 2008, regular peripheral blood and urine checks for BK polyomavirus replication were introduced. This means that we could have missed patients who have active viremia, which would increase the yearly incidence of BKPyVAN in this period. We are, therefore, also unable to investigate the association between peripheral blood DC subsets, BK polyomavirus replication, and our biopsy results. Second, because we did not include all episodes of TCMR during the study period, the predictive value for the BDCA-1+ mDCs could not be calculated. Calculation of the predictive value for novel markers to distinguish between antiviral and alloimmune response and therefore, aid in the decision to either decrease or increase immunosuppressive treatment is problematic because of the lack of a true gold standard diagnostic criterion. Third, we were unable to fully address the influence of time after transplantation on our results. A prospective approach to associate peripheral blood viral dynamics, biopsy results, and outcomes after standardized therapy should resolve this possible inclusion bias.

In conclusion, in these cohorts of biopsies, BKPyVAN and TCMR showed a similar degree of tubulointerstitial inflammation, and the composition of the infiltrates was overall very comparable between the 2 entities. We observed an increased amount of BDCA-1+ mDCs during BKPyVAN that was associated with the total burden of tubulointerstitial inflammation in patients with BKPyVAN but not in patients with TCMR, and these cells were mostly found in close proximity of BK polyomavirus–infected tubules. Our data suggest that particular differences in immune cell infiltration are present between BKPyVAN and TCMR and that these differences may underlie the immune responses characteristic for both entities.