Clinical-Scale Rapid Autologous BK Virus-Specific T Cell Line Generation From Kidney Transplant Recipients With Active Viremia for Adoptive Immunotherapy

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Background. Polyomavirus-associated nephropathy (PVAN) after BK virus reactivation in kidney transplant recipients (KTR) can compromise graft survival. Lowering immunosuppression is the only established approach to prevent or treat PVAN but nonspecifically increasing host immune competence also augments rejection risk. Ex vivo T cell stimulation/expansion offers the possibility to generate BK-specific T cell lines for adoptive immunotherapy. The objective of this study was to develop and characterize a clinical-scale protocol to generate BK-specific T cell lines from viremic KTR. **Methods.** Peripheral blood mononuclear cells from healthy controls and viremic KTR were stimulated using BK virus peptide libraries loaded or not on monocytes-derived dendritic cells. Cell counts, flow cytometry, and next-generation sequencing were used to assess T cell expansion, differentiation, and clonal diversity. Enzyme-linked immunospots, cytotoxicity assays as well as adoptive transfer in NOD/SCID/IL2Rγ^{null} mice were used to assess for pathogen-specificity and evidence of nonspecific alloreactivity. **Results.** T cell lines from KTR and healthy control showed similar characteristics, implying that ongoing immunosuppression and chronic virus exposure do not compromise the differentiation, specificity, or clonal diversity of T cell lines after ex vivo production. Using antigen-loaded dendritic cells improved T cell expansion and favored central memory T cell differentiation. The T cell lines were antigen-specific and showed no nonspecific alloreactivity in vitro and in vivo. **Conclusions.** Using a rapid, clinically compliant culture system, we show that autologous BK virus-specific T cell lines can be reliably generated from viremic KTR. Our results pave the way for the treatment or prevention of PVAN with adoptive immunotherapy.

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Latent BK polyomavirus infection in the urothelium is highly prevalent.[1] Reactivations are frequent in immunocompromised patients and pose a particular problem to kidney transplantation recipients (KTR). Polyomavirus-associated nephropathy (PVAN) can occur in up to 10% of KTR[2] and lead to graft loss in up to 50% of the cases.[3,4] Many risk factors have been described, such as younger than 18 or older than 60 years,[5,6] male sex,[5,6] depleting antibody, number of HLA mismatches,[6,7] intensity of immunosuppressive regimens,[8] ABO incompatibility,[9] cold ischemia time,[7] delayed graft function,[7] ureteral stent placement,[7] and donor urinary BK virus replication.[10] Reduction of immunosuppression in response to BK virus replication (assessed by BK viremia, viruria, decoy cell,[4] or urinary polyomavirus-Haufen test[11]) is the most accepted strategy to treat BK-related complications[12] but has the disadvantage of increasing rejection risk by nonspecifically increasing immune competence. In addition, patients who are unable to control BK viremia in 3 months have been shown to have lower BK-specific T cells.[13] Hence, KTR antiviral

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immunity remains the cornerstone of PVAN prevention and treatment. The selective augmentation of host anti-BK immunity without a concomitant increase in the risk of graft rejection could solve the clinical conundrum facing transplant nephrologists treating PVAN. Adoptive immunotherapy using ex vivo expanded autologous BK-specific T cells from KTR may represent such ideal strategy.

It was recently shown in allogeneic hematopoietic cell transplantation that donor-derived T cell lines generated ex vivo and targeting several viruses, including BK virus, could control viremia and BK-related complications.[14] In solid organ recipients, adoptively transferred autologous or "third party" (neither derived from the donor nor the recipient) Epstein-Barr virus (EBV) or cytomegalovirus (CMV)-specific T cell lines appear efficacious and safe despite ongoing immunosuppression.[15-18] Whether similar promising results could be obtained in refractory BK viremia or PVAN remain to be established as no study using BK-specific T cell lines in the KTR population have been reported. An essential stepping stone for the design of such trials is the development of reliable T cell manufacturing protocols that can be readily implemented in cell processing facilities.

In this work, a clinical-compliant system to rapidly generate BK virus-specific T cell lines was adapted and validated using both healthy control (HC) and KTR suffering from active or presumptive PVAN. Using as model a culture protocol capable of expanding virus-specific T cell lines from peripheral blood mononuclear cells (PBMC) in 9 to 14 days from healthy donors,[14,19,20] we show that the reliable clinical-scale expansion of BK-specific T cell lines requires the addition of autologous BK virus antigen-pulsed monocyte-derived dendritic cells (DCs). The use of DC not only improved T cell expansion but also favored the generation of central memory T cells (Tcm) and conferred increased antigenic specificity. T cell lines derived from both HC and KTR were polyclonal, further expanded and persisted upon transfer into immunodeficient mice but did not show off-target allo/xenoreactivity in vitro and in vivo. Collectively, this work describes a readily translatable approach to manufacture autologous BK-specific T cell lines from KTR for adoptive immunotherapy.

MATERIALS AND METHODS

Donors and T Cell Line Generation

A total of 8 KTR from a single center and 5 volunteer HCs were recruited on internal review board–approved protocols (CÉR 13030 and 13125) for the various experiments conducted in this study. The KTR were all suffering from active viremia and 6 carried a diagnosis of definitive PVAN (**Table S1, SDC,** http://links.lww.com/TP/B414). PBMCs were isolated from up to 200 mL of blood by gradient density separation (Ficoll-Paque, GE Healthcare, Baie d'Urfe, Canada). When indicated, cells were cryopreserved (20 to 50 × 10^6 cells per vial) in 10% dimethyl sulphoxide (DMSO, Sigma-Aldrich, Oakville, Canada), 20% human serum (Sigma-Aldrich), and 70% Roswell Park Memorial Institute medium (RPMI).

A total of 10 to 15 × 10^6 PBMC were directly pulsed with overlapping peptide libraries from virus-encoded protein 1 (VP1) and large T antigen (LTA) as in,[19,20] at a concentration of 1 μg/mL for 30 or 120 minutes (JPT, Berlin, Germany). Alternatively, PBMC were cocultured in a 1:10 ratio (stimulator: effector) with irradiated autologous DCs (40 Gy) pulsed for 2 hours with the 2 peptide libraries (1 μg/mL). T cell lines were generated in 14 days, using either peptide-pulsed PBMC or DC as previously described.[20,21] Specifically, all T cell cultures were performed using 30 mL of T cell media (45% Advanced RPMI 1640, 45% Click's medium, 10% human serum, 1× L-glutamine, IL-4 (1666 U/mL Feldan, Québec, Canada) and IL-7 (10 ng/mL Miltenyi, Auburn, CA)) in a G-Rex10 vessel (Wilson Wolf Manufacturing, New Brighton, MN) and incubated at 37°C and 5% CO_2. Media and cytokines were changed at days 5, 8, and 12. Cells were counted by trypan blue exclusion with an automated cell counter (Countness, Invitrogen). At days 8 and 12, cultures were split if the cell concentration exceeded 45 × 10^6 cells/mL.

DC Generation

DC were prepared from circulating monocytes isolated by plastic adherence and differentiated into DC in DC medium (X-vivo 15, 5% human serum, 1× Penicillin-streptomycin-glutamine, 1 mM sodium pyruvate) supplemented with 800 IU/mL GM-CSF and 1000 IU/mL IL-4 (Feldan) in 7 days. They were then matured for 2 days with GM-CSF, IL-4, TNFα (10 ng/mL), IL-1β (10 ng/mL), IL-6 (100 ng/mL) (Feldan), and prostaglandin E2 (1 μg/mL) (Sigma-Aldrich, Oakville, Canada). IFN-γ (1000 U/mL (Peprotech, Quebec, Canada) was added on the 8th day of the culture for the final 24 hours of the maturation step.

IFNγ Enzyme-Linked Immunospot Assay, Cytotoxicity Assays, and Flow Cytometry

IFNγ enzyme-linked immunospot assay (ELISpot) assays and analysis were performed by exposing 5 × 10^4 cells to the VPI or LTA antigenic peptide libraries, a nontargeted peptide library (negative control), or stimulated with an anti-CD3 antibody (positive control) overnight, according to the manufacturer's instructions (Mabtech Inc., Cincinnati, OH and vSpot Reader Spectrum, AID, Strassberg, Germany). Cytotoxicity was performed using a standard 4-hour chromium release assay using autologous or allogenic phytohemagglutinin (Sigma-Aldrich) blasts pulsed with antigenic peptide libraries.[20] Cells were surface stained with monoclonal antibodies to: CD3, CD4, CD8, CD45RO, CD62L (BD Biosciences, Mississauga, ON), washed and fixed in phosphate buffered saline 2% fetal bovine serum 1% paraformaldehyde before acquisition on a LSRII instrument (BD Biosciences). Data were subsequently analyzed using Flowlogic software (Inivai Technologies, Mentone, Australia) or Kaluza (Beckman Coulter, Indianapolis, IN).

Adoptive Transfer in NOD/SCID/IL2Rγ^{null} Mice

Seven- to 12-week-old NOD/SCID/IL2Rγ^{null} (NSG) mice were subjected to total body irradiation (2.5 Gy). The following day, the mice were injected intravenously with 0.5 × 10^6 human T cells from either a 14-day culture (as described above) or a CD3+ selection of unstimulated T cells (Stemcell Technologies, Vancouver, BC). Intraperitoneal injections of rhIL-15 (1 μg, 2000 U; Miltenyi) were administered twice a week for 3 weeks. Venipunctures were obtained weekly from week 2 or week 3 until death or sacrifice (100-200 μL). Skin, liver, colon, and short bowel samples were fixed in 10% formalin, embedded in paraffin and stained with hematoxylin-eosin phloxine. Spleens were collected and processed into single cell suspensions for flow cytometry. When indicated, mice received 2 mg of 5-bromo-2′-deoxyuridine (BrdU) (Sigma-Aldrich) intraperitoneally every second day for 14 days

starting on day 7 posttransfer. The protocol was approved by the institutional authorities according to Canadian Council on Animal Care regulations. BrdU incorporation was assessed with flow cytometry using anti-BrdU monoclonal antibody (BD Biosciences) after a staining using BD Perm/Wash Buffer, BD Cytoperm Permeabilization Buffer Plus and Deoxyribonuclease I (Sigma-Aldrich), according to the manufacturer's instructions.

Clonality Analysis

Next-Generation Sequencing (NGS) DNA-based TCR-γ chain analysis was performed for clonality determination. DNA was extracted from T cell line or using DNAzol (Invitrogen) and quantified with the Qubit 2.0 system (ThermoFisher Scientific, Waltham, MA). TCR-γ chain was amplified from 50 ng of gDNA with LymphoTrack TRG assay (InvivoScribe, San Diego, CA) and AmpliTaq Gold DNA polymerase (ThermoFisher Scientific) in the SimpliAmp Thermal Cycler (ThermoFisher Scientific). Amplicons with sizes greater than 100 base pairs were positively selected with Agencourt AMpure XP beads (Beckman Coulter, Brea, CA). The libraries obtained were quantified on the ViiA 7 Real-Time PCR System with the Ion Library Taqman Quantitation kit (ThermoFisher Scientific). Next generation sequencing was completed on the Ion Proton semiconductor platform (ThermoFisher Scientific) using an Ion P1 v3 chip (ThermoFisher Scientific), prepared with the Ion Chef System (ThermoFisher Scientific). Fastq data were the analyzed with the LymphoTrack Bioinformatics Software (InvivoScribe). Up to 200 unique reads or 10% or total reads were obtained after the merging of duplicates using multiple sequence alignment (Multiple Alignment using Fast Fourier Transform - MAFFT software, version 7)

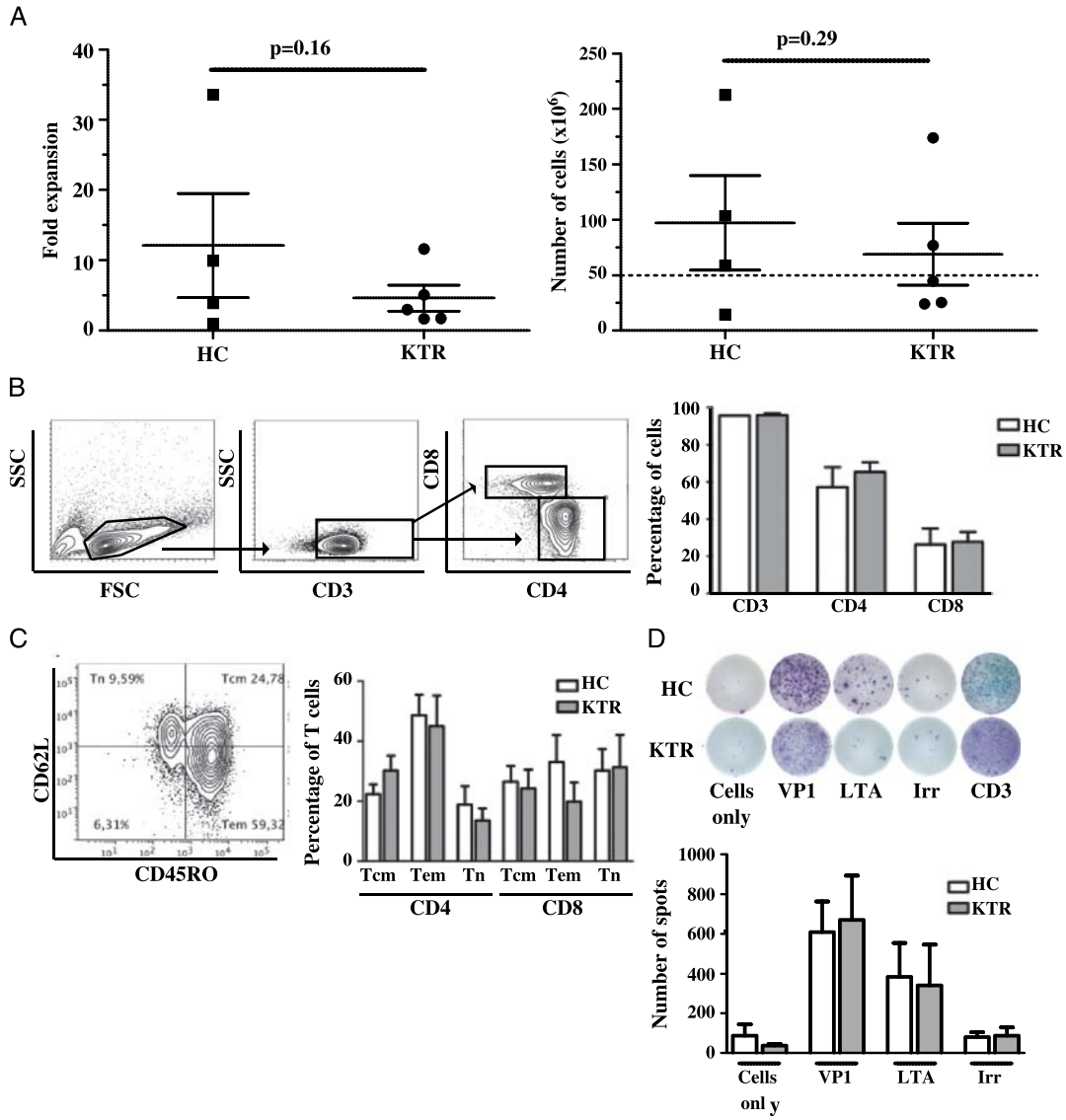

FIGURE 1. BK-specific T cell lines obtained from viremic KTRs are similar to those from HC. Comparison of T cell line from HC (n = 4) to KTR (n = 5) after 14-days in culture. A, Fold expansion (left), (horizontal bar in the middle represents the mean, top and bottom horizontal bars represent SEM), p = 0.155 and absolute cell number (right) p = 0.291. B, Representative gating and percentage of CD3 + T cells and their distribution as CD4+ or CD8+ T cells. C, CD4+ and CD8+ T cell differentiation profile, as defined by CD45RO and CD62L, 1 representative dot plot (left) and compiled results (right, mean and SEM). D, One representative ELISpot result (for 5×10^4 cells plated) (top) and compiled results (bottom, mean and SEM). Hexon (adenovirus) peptide library was used as an irrelevant control peptide library (Irr). Tn, CD45RO–CD62L+; Tcm, CD45RO+CD62L+; Tem, CD45RO+CD62L–.

software version 7 and determining the CDR3 region by IgBlast (www.ncbi.nlm.nih.gov/igblast/). Single nucleotide differences between 2 sequences outside the CDR3 region were considered sequencing errors. Clonality index was calculated using Shannon's Entropy formula, as described by Harden and colleagues.[22]

Statistical Analysis

All statistical analyses were performed using unpaired Student *t* test using GraphPad Prism (version 5.0c; GraphPad Software) unless otherwise specified. *P* values less than 0.05 were considered significant. Kaplan-Meier survival analysis was used for NSG mice.

RESULTS

BK Virus-reactive Autologous T Cell Lines Can Be Generated From Viremic KTR

We initially used a protocol that has been shown to generate clinical-scale virus-specific T cell lines in just 9 to 14 days after the stimulation of PBMC with synthetic overlapping peptide libraries that cover the entire length of immunogenic viral proteins (overlapping 15-mers).[19,20] Peptide libraries from 2 immunogenic BK virus proteins, VP1 and LTA, were used to stimulate T cells. This choice was based on previous studies highlighting the importance of these proteins in the generation of protective immune responses in the case of BK virus reactivation in KTR[4,23,24] and previous experience in allogeneic hematopoietic cell transplantation using T cell lines generated with these peptide libraries.[14] Direct stimulation of PBMC from HC (n = 4) and KTR (n = 5) revealed that VP1 and LTA-specific T cell lines are generated in both cases (Figure 1). However, cellular expansion was highly variable (fold expansion mean of 12.1 ± 7.4 for HC and 4.6 ± 1.9 for KTR, p = 0.155) (Figure 1A). Less than 50 million cells were obtained for 3/5 T cell lines generated from KTR PBMC, which may not be sufficient for treatment and ancillary testing at cell doses previously used in antiviral adoptive immunotherapy trials.[14,17,25] Irrespective of donor type, the T cell lines contained more than 90% of CD4+ or

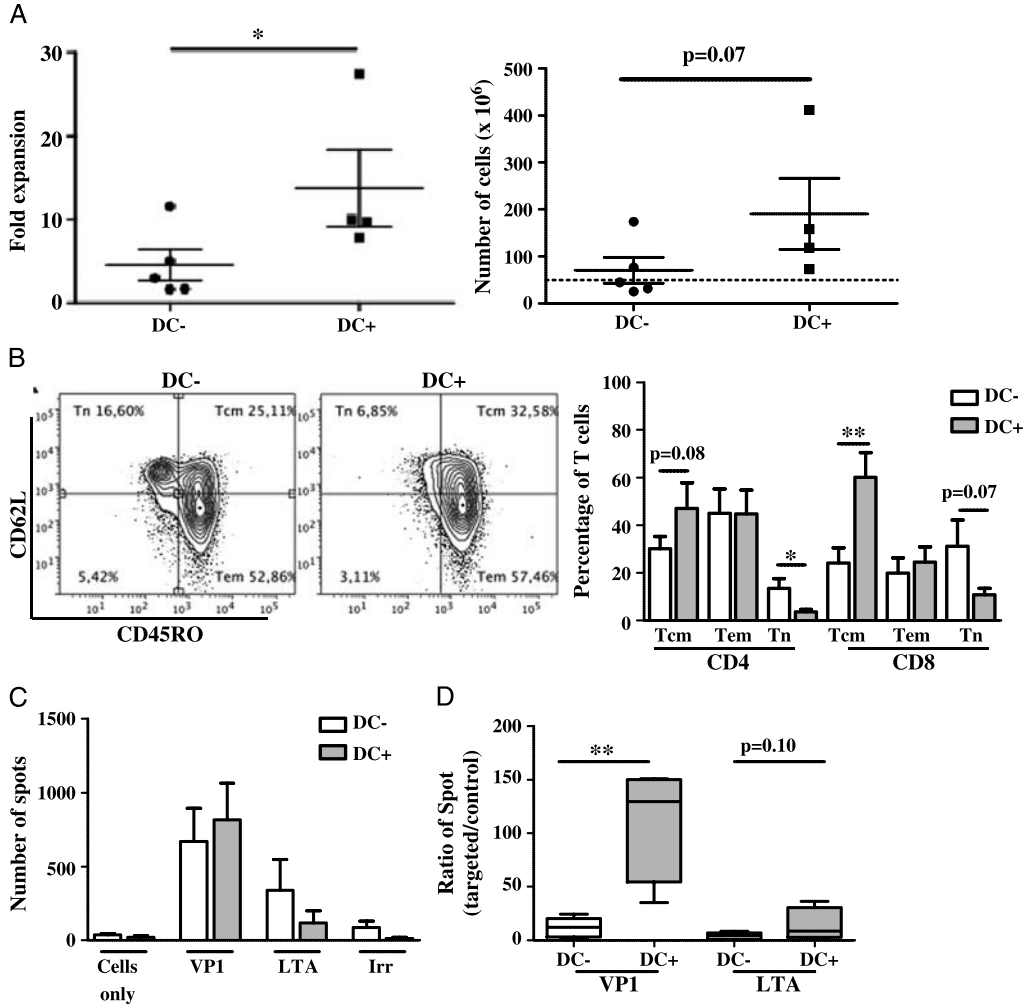

FIGURE 2. DC stimulation improves T cell expansion, specificity and differentiation. Comparison of T cell lines from KTRs without (DC–, n = 5) and with DC stimulation (DC+, n = 4) at the end of the 14-day culture. A, Fold expansion (horizontal bar in the middle represents the mean, top and bottom horizontal bars represent SEM) *P* = 0.042 (left) and absolute cell number at the end of the 14-days culture, *P* = 0.073 (right). B, CD4+ and CD8+ T cell differentiation profile, as defined by CD45RO and CD62L expression (representative dot plots on the left) and compiled results (right) represented as mean with SEM, *P* = 0.038 for Tn CD4+ T cells and *P* = 0.008 for Tcm CD8+ T cells. C, Comparison of the number of spots on ELISpot assay with and without DC stimulation and D, ratio of spots produced in response to targeted (VP1 or LTA) or control (Hexon) peptide libraries. Boxes are to represent the distribution of results with horizontal bars indicating the mean and whiskers representing SEM. *P* = 0.002 for VP1/Hexon.

CD8+ CD3+ T cells (Figure 1B). Naive T cell (Tn), Tcm, and effector memory T cell (Tem) subsets were further defined based on CD45RO and CD62L expression. No differences were noted between T cell lines from HC and KTR, which both contained a mixture of Tn, Tcm, and Tem phenotype T cells (Figure 1C). Finally, T cell lines were assessed for reactivity against VP1 and LTA peptide libraries using the IFN-γ ELISpot assay (Figure 1D). Despite clear-cut reactivity to VP1 and LTA, the T cell lines from both HC and KTR displayed low-grade reactivity against control adenoviral peptides derived from the highly immunogenic Hexon protein.

Taken together, these results show that BK-reactive T cell lines can be rapidly generated from HC and KTR, but at levels that may not support clinical use. The use of PBMC previously exposed to BK-virus did not otherwise impact T cell differentiation or reactivity relative to PBMC from HC.

Peptide-Loaded DC Stimulation Improves T Cell Expansion, Specificity, and Differentiation

We next sought to determine whether improved expansion, differentiation, and antigen specificity of anti-BK virus T cell lines from viremic KTR could be achieved using DCs instead of PBMCs as antigen-presenting cells.[21] To this end, VP1 and LTA peptide library pulsed-autologous monocytes-derived DC[21] were used at the beginning of the culture in 4 patients. PBMC containing responder T cells (cryopreserved at the time of blood collection) were added to mature DC pulsed with the antigenic libraries. This significantly increased KTR T cell lines expansion to clinical-scale levels in all donors tested (Figure 2A).[14] Although the proportion of CD4+ and CD8+ T cells were similar with both protocols (not shown), the addition of DC stimulation altered the differentiation profile (Figure 2B). At the end of the 14-day culture, we noted an increase in the percentage of Tcm and a decrease in the proportion of Tn in the cultures stimulated with DC relative to the original condition. In HC, the addition of DC similarly increased expansion, but the variation in Tn or Tcm did not reach statistical significance (**Figure S1, SDC,** http://links.lww.com/TP/B414). The use of peptide-pulsed DC also led to more specific antigen reactivity as evaluated by ELISpot. Compared with the reference condition, T cell lines generated from DC-stimulated PBMC showed little to no reactivity towards adenoviral peptides resulting in a high ratio of VP1 to Hexon spot count (Figures 2C-D). The LTA/Hexon spot count ratio also improved but the difference was not statistically significant. Importantly, the use of DC could not be substituted by extending the peptide pulse to 2 hours with PBMC alone, resulting in unpredictable expansion and reactivity toward untargeted peptides (**Figure S2, SDC,** http://links.lww.com/TP/B414). These results show that the use of a single round of peptide-pulsed autologous DC improves BK-specific T cell generation in KTR in terms of expansion, differentiation, and antigen specificity.

T Cell Lines Obtained From Viremic KTR and HC Are Similarly Polyclonal

Chronic infections, such as HIV,[26] EBV,[27] CMV,[28] and end-stage renal disease[29] are proposed to narrow TCR repertoires. In vitro expansion of antigen-specific T cells can also restrict the TCR repertoire.[30,31] Rapidly generated T cell lines from both HC and actively BK viremic KTR were polyclonal (Figure 3) as assessed by a robust TCRγ chain next-generation sequencing approach to identify clonal populations from all mature T cell subtype populations.[32] The absolute number of unique reads was not statistically different between both groups, with a mean of 724 794 ± 113 824 (HC) and 547 837 ± 44 741 (KTR) (P = 0.099) (Figure 3A). Polyclonality was further estimated using the clonality index as proposed by Harden et al[22] and applied to our results. Both groups had similar indices, with means of 0.240 ± 0.036 (HC) and 0.246 ± 0.044 (KTR) (P = 0.448) (Figure 3B). An index toward 0 is indicative of a polyclonal repertoire, whereas an index closer to 1 infers oligoclonality. No difference was found between the 2 groups in terms of number of clones

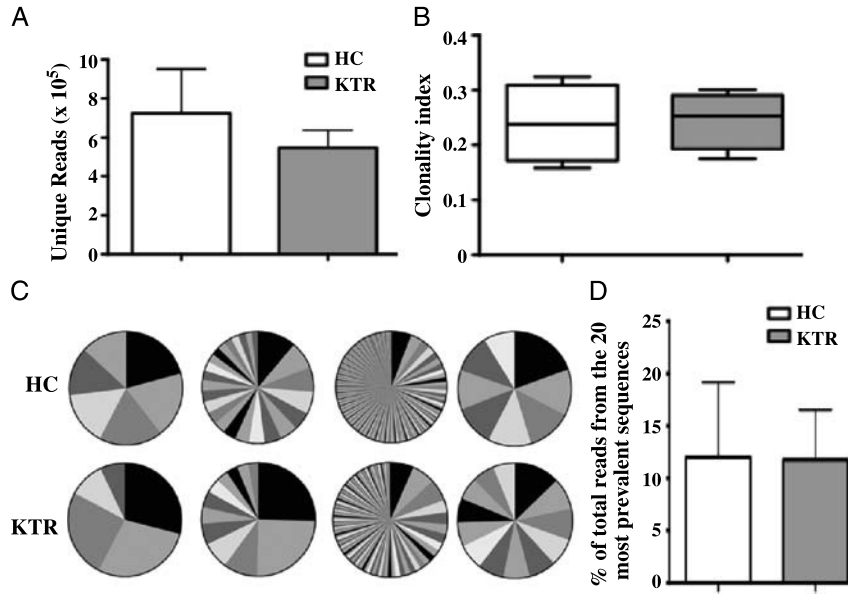

FIGURE 3. T cell line clonality from viremic KTRs and HCs are similar. A, Absolute number of unique TCRγ reads in the 2 groups (n = 4 per group) and (B) Clonality index between the 2 groups (mean with SEM). C, Schematic representation of the most abundant clones that represent 10% of total reads in all samples tested. D, Percentage of total reads represented by the 20 most prevalent sequences for both groups (mean with SEM).

required to reach 10% of the total reads (Figure 3C) or the percentage of total reads represented by the 20 most prevalent clones in each T cell line (Figure 3D). Thus, rapidly generated T cell lines from both HC and KTR remained polyclonal despite ongoing BK-virus antigen exposure in KTR, suggesting that viremic patients maintain a large repertoire that can be mobilized for adoptive immunotherapy.

BK-Specific T Cell Lines Are Not Alloreactive In Vitro and In Vivo

The prospect of infusing ex vivo activated polyclonal autologous T cells to solid organ recipients raises the concern of inducing cellular rejection, despite previous evidence that T cell lines targeting other viruses are safe in this population.[15,17,25] Hence, we tested whether VP1/LTA-specific T cell lines displayed evidence of alloreactivity in vitro and in vivo. The BK-specific T cell lines generated after peptide-loaded DC exposure did not lyse allogeneic (obtained from volunteer donors) or autologous targets (loaded or not with a control peptide library) and, as expected from the ELISpot data, we noted a dose-related specific cell cytotoxicity of autologous targets loaded with the VP1 and LTA peptide libraries (repeated-measures analysis of variance: $P = 0.057$ for 10:1, $P = 0.048$ for 20:1, and $P = 0.027$ for 40:1) (Figure 4A). To further ascertain that the T cell lines lacked nonspecific alloreactivity, we performed adoptive transfer into NSG immunodeficient mice. Mice were given hIL-15 to support engraftment in the first 3 weeks posttransfer.[33] Although NSG mice cannot reject the xenogeneic human cells, they express murine histocompatibility antigens that are recognized with high avidity by human T cells resulting in severe xenogeneic graft-versus-host disease (GVHD). As such, it represents a stringent system to test for nonspecific alloreactivity. As anticipated, T cells directly isolated from PBMC caused lethal GVHD (decreased activity, skin changes, prostration, and weight loss) 36 to 63 days postadoptive

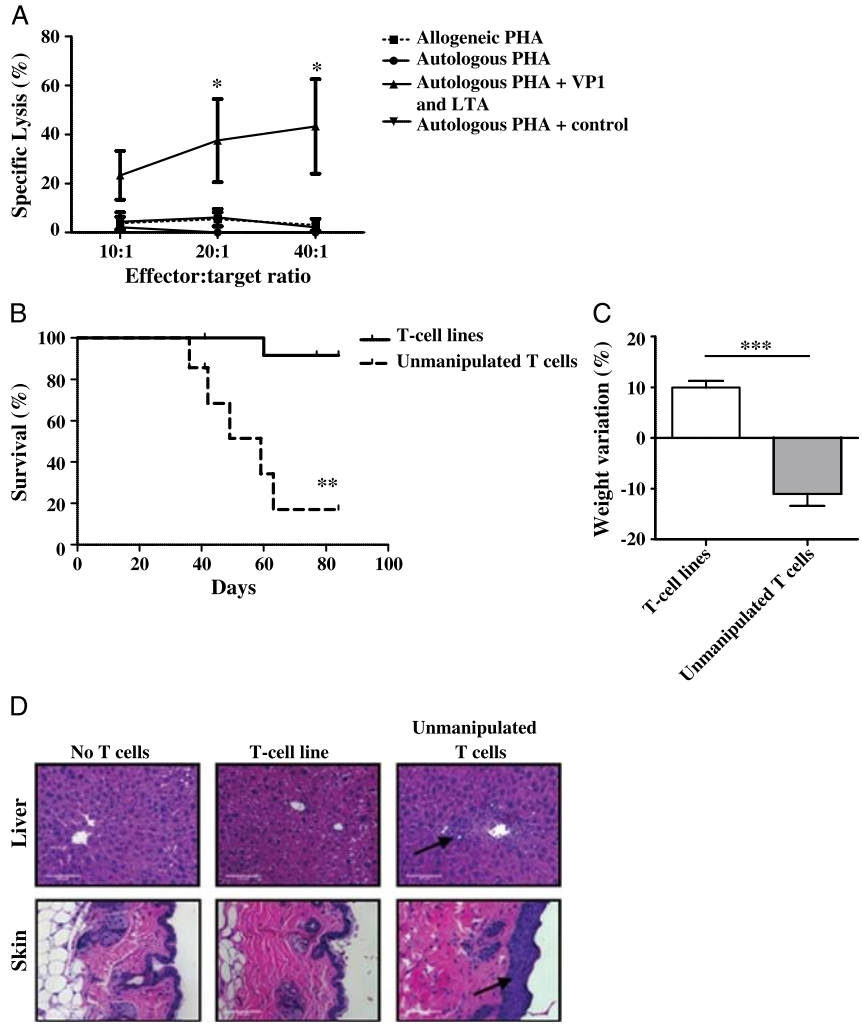

FIGURE 4. BK-specific T cell lines do not induce alloreactivity in vitro and in vivo. A, Specific lysis (%) of 4 targets at 3 different effector:target ratios in a 4-hour chromium-51 release assay (n = 4 T cell lines from HC and KTR, each value was obtained in triplicates, mean with SEM), repeated measures ANOVA for effector:target ratio of 10:1 ($P = 0.057$), 20:1 ($P = 0.048$), and 40:1 ($P = 0.027$). Allogenic or autologous PHA blasts were pulsed or not with peptide libraries, VP1/LTA or control pp65 (CMV) peptide libraries. B, Kaplan-Meier survival analysis after T cell lines (n = 14) or freshly isolated T cells from PBMC (n = 7) injections, from 4 different T cell lines, log-rank test (**$P = 0.001$). C, Percent weight variation between the weight on the day of death (or sacrifice) and the day of infusion. Mean (SEM) (***$P < 0.001$). D, One representative hematoxylin-eosin phloxine staining of liver and skin of a noninfused NSG and recipients of a BK-specific T cell line or freshly isolated unmanipulated T cells (10×). Arrows point out GVHD manifestations (portal space infiltration and epidermal thickening), white bar in images = 100μm. ANOVA, analysis of variance; PHA, phytohemagglutinin.

transfer. In contrast, the infusion of an equal number of cells from the ex vivo generated T cell lines was very well tolerated (Figures 4B-C). Histological evidence of GVHD was present in the skin and liver[34] of unmanipulated T cell recipients, but not in mice injected with T cell lines (Figure 4D). Neither group had significant evidence of intestinal GVHD (not shown). Collectively, these data support the notion that rapidly generated anti-BK T cell lines do not retain nonspecific alloreactivity.

T Cell Lines Can Expand and Persist In Vivo

To assess whether the absence of xenogeneic GVHD was due to a failure of cultured T cells to expand and persist in vivo, we conducted further experiments with NSG mice. Labeling experiments with BrdU showed that a fraction of adoptively transferred T cells proliferated in the first 3 weeks, while human IL-15 was supplemented. When compared with T cells from mice not exposed to BrdU, spleen CD4+ (mean of 11.48% ± 2.57 versus 4.20% ± 1.03) and CD8+ (mean of 13.44 % ± 1.45) from BrdU-exposed mice showed evidence of BrdU incorporation (Figure 5A). Weekly peripheral blood sampling further revealed that the percentage of human T cells continued to increase after IL-15 supplementation, indicating that the transferred T cells were not dependent on human homeostatic signals to expand and persist in a murine environment. We noted that compared with week 3, both CD4+ and CD8+ had expanded at week 6 (paired *t* test: $P = 0.028$ for CD4+ and $P = 0.034$ for CD8+) (Figure 5B). In all animals followed up until 11 to 12 weeks posttransfer (n = 11), human T cells persisted without clinical evidence of GVHD (Figure 5C). Collectively, these data indicate that ex vivo-generated BK-reactive T cell lines maintain their capacity to proliferate and persist in vivo without triggering xenogeneic GVHD.

DISCUSSION

We report herein a rapid, clinically compliant protocol to generate autologous BK-specific T cell lines from PVAN or actively BK viremic KTR from a single venipuncture. This process required stimulation with antigen-pulsed DC to achieve clinical-scale expansion in contrast to a similar culture system already used in clinical trials, which has been shown to generate EBV-specific or multivirus (including BK) specific T cell lines from PBMC alone.[14,19,20] The reasons why stimulation with BK antigenic peptides alone

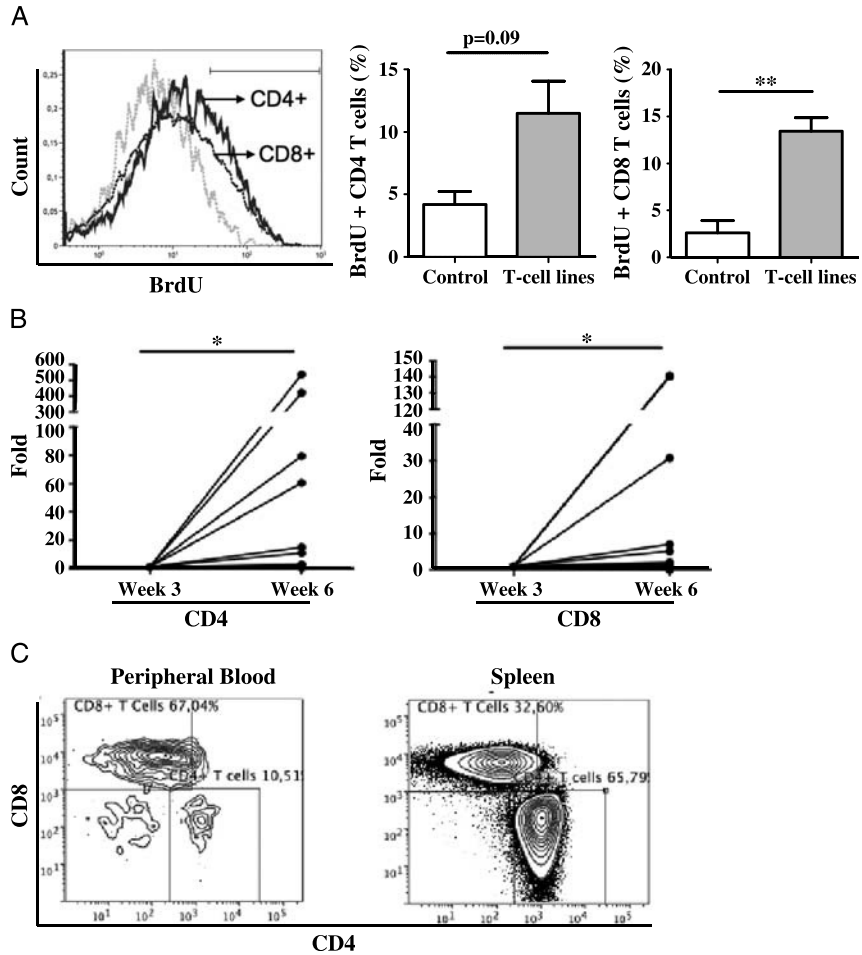

FIGURE 5. BK-specific T cell lines can persist and proliferate in vivo. A, One representative histogram showing BrdU staining of CD4+ and CD8+ from mouse spleens expressed as the % of BrdU-positive cells by flow cytometry and compiled results from 6 mice compared to mice not receiving BrdU (control) from 2 independent experiments for both CD4+ ($P = 0.087$) CD8+ ($P = 0.004$) T cells. Means are represented with SEM. B, CD4+ and CD8+ T cells expansion in the peripheral blood at week 6 compared to week 3, as measured by flow cytometry (number of events counted per standard volume of blood, time and speed of acquisition), n = 14 from 4 independent experiments. C, One representative dot plot of CD4+ and CD8+ T cell in mouse's blood and spleen, 12 weeks after T cell line injection.

resulted in low T cell expansion might be related to the immunogenicity of the VP1 and LTA peptide pools or the nature of the BK-specific repertoire in HC and immunocompromised KTR. Two other studies have similarly reported limited growth of BK-specific T cells.[30,35] Blyth et al, generated T cell lines from 12 hemodialysis patients, 1 HC and 1 KTR. The T cell line from the KTR expanded 0.9 fold and contained 75% CD3+ and 21% CD56 + CD3- cells, suggesting the presence of NK cells. Likewise, Comoli et al, generated T cell lines using inactivated virus pulsed on DC and IL-2 from 6 KTR, which expanded poorly (1.5-fold). Expansion was improved using IL-12 and IL-7 early in the culture process but this led to the presence of a large proportion of γδ T cells (up to 69%), whose potential relevance to adoptive immunotherapy of refractory viral infections is unclear. This contrasts with our results showing the reliable expansion of CD4+ and CD8+ T cells after the use of DCs for antigen presentation. Recently, Dasari and colleagues[36] proposed and elegant method to obtain multivirus-specific T cell lines from solid-organ recipients using an adenoviral vector coding for several HLA-restricted antigens as a platform for antigen presentation. Two cell lines were obtained from PVAN patients, but it is unclear whether this system expands BK virus-specific T cells to clinical-scale levels.

The use of peptide-pulsed autologous DC decreased the proportion of Tn in the T cell lines and reciprocally increased Tcm but not Tem. This was to be expected using a system where T cell stimulation is increased.[37] The improved antigen specificity of the T cell lines may be linked to the decrease in Tn representation. Moreover, the generation of a Tcm-rich T cell product may be associated with greater clinical efficacy owing to the intrinsic features of this subset (long-term persistence, capacity to self-renew, in vivo proliferative potential) relative to Tem or more differentiated effectors.[38] Therefore, the use of a single DC-based stimulation along with IL-4 and IL-7 generates T cell lines with favorable features for immunotherapy after 2 weeks of culture. Our study was limited by the number of patients included and great differences in culture yields. Despite these limitations, we could obtain clinical-scale expansions and BK-specific T cell lines from all donor tested despite the inherent variability associated with the use of human samples. We did not have the statistical power to show that T cell lines from KTR did not expand as well as those derived from HC. We nonetheless noted a trend suggesting that T cells from viremic KTR may not grow as well in culture. This might be related to the immunosuppressive therapy received by the KTR or the chronic exposure to the virus.

The T cell lines generated with our approach did not show allo/xenoreactivity in vitro and in vivo despite their polyclonality and capacity to expand and persist in NSG mice. Previous experiments with autologous anti-EBV T cell line generation from solid organ transplant recipients did not show donor-specific alloreactivity.[15,17,25] Unfortunately, donor cells were unavailable to us to confirm these findings (6 KTR out of 8 received a transplant from a deceased donor and live donor were unreachable—**Table S1, SDC,** http://links.lww.com/TP/B414). Although, it is possible that target cells from volunteer donors in the cytotoxicity assays shared common HLA allotypes with the kidney donors, the clinical translation of our approach would benefit from using donor cells to validate whether the T cell line displays evidence of donor-specific alloreactivity before infusion. Our in vivo studies were primarily aimed at evaluating whether the T cell lines would show xenoreactivity, as a surrogate indication of nonspecific alloreactive potential. Unlike unmanipulated T cells, the T cell lines did not cause xenogeneic GVHD. This occurred despite the capacity of the adoptively transferred T cells to expand and persist in NSG mice. Although this is reassuring (along with our in vitro cytotoxicity assessments and previous adoptive immunotherapy experience in both hematopoietic cell and solid organ transplantation patients[15,17,25]), it does not rule out the possibility that these ex vivo generated autologous T cell lines may not react against the transplanted organ. In addition, we could not evaluate the in vivo therapeutic efficacy of our T cell lines given that no mouse model of human BK-virus infection exists. Our in vivo experiments were aimed at evaluating the nonspecific allo/xenoreactivity of the T cell lines as the optimal evaluation of human antiviral responses in mice would require a humanized system.[39,40]

The success of adoptive immunotherapy hinges on the capacity of the infused T cells to expand in vivo after transfer and persist. Based on previous experience in humans, we would speculate that BK-virus responses following adoptive transfer in patients would increase and persist based in part on the predominant Tcm population found in the T cell lines. The degree of T cell expansion and persistence would also be determined by the intensity of the concomitant immunosuppression, which remains a central variable especially in solid organ transplant patients. Hence, several crucial issues pertaining to safety and efficacy after BK-virus adoptive immunotherapy for PVAN can only be addressed in clinical trials. Our work paves the way for such trials by establishing a clinical-compliant protocol to generate autologous BK-specific T cell lines from KTR. In addition, our system is amenable to modifications to refine the preparation of BK-specific T cell lines. Among these, genetic engineering of virus specific T cell lines appears promising.[41] For instance, the expression of resistance genes allowing the virus-specific T cells to elude pharmacological immunosuppression may further improve T cell function and persistence.[41] Moreover, our system could be used to generate third party BK-specific T cell lines given our results with HCs (Figure 1 and **Figure S1, SDC,** http://links.lww.com/TP/B414). These third-party T cell lines would require more extensive characterization to define antiviral responses restricted to specific HLA alleles before they could be used and would be unlikely to persist long term after transfer.[42-44] However, once characterized, they could be cryopreserved and banked for rapid distribution and use.

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