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Dendritic Cells Pulsed with Polyomavirus BK Antigen Induce *Ex Vivo* Polyoma BK Virus–Specific Cytotoxic T-Cell Lines in Seropositive Healthy Individuals and Renal Transplant Recipients

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Abstract. Polyoma BK virus (BKV)-associated interstitial nephritis has emerged as a relevant complication of immunocompromise after kidney transplantation, leading to reduced survival of the renal allograft. The limitations of current antiviral treatment and the high probability of rejection in kidney graft recipients when control of viral replication is attempted by reduction of immunosuppression warrant further efforts to develop alternative therapeutic tools. Cellular immunotherapy has proved to be a successful approach for prevention and/or treatment of other viral complications in the immunocompromised host. For assessing the feasibility of translating this strategy to the prevention of BKV-associated disease, a procedure for *ex vivo* reactivation of BKV-specific cytotoxic T cells (CTL) was developed from BKV-seropositive healthy donors and allograft recipients through stimulation with dendritic cells pulsed with inactivated BKV. The CTL lines thus obtained showed BKV specificity, as an efficient lysis of BKV-infected targets was accompanied by little or no reactivity against mock-infected autologous or allogeneic targets. *In vitro* killing of allogeneic BKV-infected targets, likely as a result of populations of TCR$\gamma\delta$+/CD3+ displaying MHC class I unrestricted cytotoxicity, was also displayed. Application of this culture system may allow a preemptive therapy approach to BKV-related complications in transplant recipients, based on CTL treatment guided by BKV DNA levels.

The human polyomaviruses are double-stranded DNA viruses that have recently received increasing attention as pathogens in immunocompromised patients. After primary infection, the polyomaviruses remain latent in the urogenital tract and in B lymphocytes, as well as in the brain (1). Asymptomatic reactivation occurs spontaneously in immunocompetent individuals, whereas overt clinical disease attributable to polyomaviruses is described to occur in the background of immunodeficiency, most often as a result of reactivation of the latent virus (2,3). Persistent active replication of polyomavirus BK (BKV) has been recently identified as an important cause of progressive graft dysfunction and graft loss in recipients of renal allograft (4–8).

Current therapeutic options are limited, and control of viral replication in kidney allograft recipients is tentatively obtained by means of reduction of immunosuppression (4–8). However, most patients with nephropathy caused by BKV slip into a disheartening cycle, alternating between viral interstitial nephritis and rejection, precipitated by the lowered dose of immunosuppressive drugs (8). Preliminary data on the use of the antiviral agent cidofovir have been reported (9); however, the drug used at the doses recommended for cytomegalovirus (CMV) infection is nephrotoxic, and its efficacy at the lower dosage proposed for BKV-related nephropathy remains to be confirmed in randomized, controlled trials. Thus, there is a need to develop alternative therapeutic tools that are able to control the infection before establishment of nephropathy.

Host immune response is of central importance in limiting primary viral infection and in controlling the virus carrier state (10). On the basis of the evidence that herpesvirus-related pathology in immunocompromised hosts originates from a deficiency of virus-specific cytotoxic T cells (11,12), clinical

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studies demonstrated how infusion of lymphocytes (13) or, better, virus-specific cytotoxic T lymphocytes (CTL) expanded *in vitro* could safely and effectively prevent or treat Epstein-Barr virus (EBV)-related lymphoproliferative disease or CMV-associated interstitial pneumonia occurring in hematopoietic stem cell transplantation or solid-organ transplant recipients (14–17). Application of this approach to the prevention and treatment of other virus-related pathologies affecting allograft recipients, such as BKV-related interstitial nephropathy, although appealing, rests on the possibility of demonstrating that cellular immunity has a central role in the control of BKV infection and that immunogenic viral antigens that are capable of eliciting CD8+ responses can be presented correctly to potentially reactive T cells.

The crucial role of T-cell immunity in the control of BKV infection is suggested by the increased incidence of reactivation and clinical disease linked to the degree of immunocompromise (5). Furthermore, it has been demonstrated that CTL are critical for the clearance of acute polyomavirus infection in the mouse model (18–20), and a recent work has suggested a role for polyomavirus JC-specific CTL in the containment of progressive multifocal leukoencephalopathy in affected humans (21). However, at the present time, there are no available data in the literature on *ex vivo* induction of human cellular immune response to BKV.

In the past few years, dendritic cells (DC) generated from peripheral blood monocytes and cultured *in vitro* in the presence of antigenic proteins or peptides have been increasingly used to induce enhanced tumor-specific or virus-specific cellular responses *in vitro* and *in vivo*, because of their optimal capacity to process and present the antigen (22–24). Recently, it has been shown that polyomavirus-infected DC induce antiviral CD8+ T-cell responses in the mouse (25).

The aim of this study was to assess the feasibility of activating autologous BKV-specific cellular immune response from the peripheral blood of seropositive healthy individuals and kidney allograft recipients by delivering inactivated virus to DC *in vitro* and using DC thus pulsed as stimulators for generation of CTL. We found that virus-pulsed DC are good stimulators of BKV-specific cytotoxic T-cell responses and are also able to reactivate BKV-specific immunity in immunocompromised subjects, such as renal allograft recipients.

Materials and Methods

Harvest and Isolation of Peripheral Blood Mononuclear Cells

Peripheral blood mononuclear cells (PBMC) from BKV-seropositive healthy individuals ($n = 6$) or renal transplant recipients ($n = 6$) were isolated by Ficoll-Hypaque density gradient centrifugation, resuspended in X-VIVO 20 medium (BioWhittaker, Walkersville, MD), and used fresh or cryopreserved. The six kidney allograft recipients were pediatric patients with BKV infection/reactivation, as demonstrated by BKV-PCR positivity in serum and/or urine. At the time of blood sampling, all patients were receiving immunosuppressive therapy for prevention of graft rejection. Approval for this study was obtained from the Institutional Review Board, and patients or guardians gave informed consent for the acquisition of additional heparinized blood samples to be used in this study during routinely performed phlebotomies.

BKV Antigen

BKV antigen was obtained from human fibroblast cell cultures infected with a wild-type BKV strain isolated at the Institute of Microbiology, University of Florence, from a bone marrow transplantation patient. For virus stock production, the viral suspension was diluted 1:100 and inoculated on a confluent monolayer of MRC-5 cells; infected cells were incubated in the presence of MEM, plus 2% FCS. After 2 wk of incubation at 37°C, the cells were subcultured and incubated again in the same conditions, until a cytopathic effect appeared and the hemagglutination assay resulted positive. The virus in the supernatant was inactivated by treatment with 0.1% β-propiolactone (26). Residual BKV infectivity was assessed by inoculation of the treated supernatant in MRC-5 cells, as described above; after 10 passages without development of cytopathic effect, the cultures were discarded. Supernatant from MRC-5 cell culture was used as mock antigen.

DC

DC were generated from peripheral blood monocytes as described previously (24). PBMC were suspended at the concentration of 1×10^6/ml in X-VIVO 20 medium, and 1-ml aliquots were plated in 24-well plates. After 90 min at 37°C, nonadherent cells were discarded, and human recombinant IL-4 (rIL-4; R&D Systems, Minneapolis, MN) at a final concentration of 500 U/ml and human recombinant granulocyte-monocyte colony-stimulating factor at a final concentration of 800 U/ml (Sandoz Pharmaceuticals, Basel, Switzerland) were added. After 6 to 7 d of incubation, cells were recovered, phenotyped to assess the degree of maturity, and cryopreserved for later use or pulsed with inactivated BKV (equivalent of final MOI 0.01).

ELISPOT Assay

ELISPOT assays to determine the frequency of IFN-γ–secreting PBMC were performed following a method modified from one previously described (27). In detail, 96-well multiscreen filter plates (MAIPS 4510; Millipore, Bedford, MA) were coated with 100 μl of primary antibody (IFN-γ; Mabtech, Nacka, Sweden) at 2.5 μg/ml and incubated overnight at 4°C. Responder cells were then seeded at 1×10^5/well in the absence or in the presence of irradiated DC pulsed with BKV antigen (DC-BKV; effector:stimulator ratio 40:1). Controls included wells plated with DC-BKV alone. After incubation for 24 h at 37°C, 100 μl of biotinylated secondary antibody (Mabtech; 0.5 μg/ml) was added, and plates were then processed according to standard procedure. IFN-γ–producing spots were counted using an ELISPOT reader (Bioline, Torino, Italy). The number of spots per well was calculated after subtraction of assay background, quantified as the average of 24 wells containing only sterile complete medium, and specific background, quantified as the sum of cytokine spots associated with responders alone, DC-BKV alone, and responders plated with unpulsed DC.

Preparation of BKV-Specific Cytotoxic T-Cell Lines

BKV-specific CTL were prepared from fresh or frozen PBMC, according to the following method: 2×10^6 PBMC were co-cultured with 5×10^4 BKV antigen-pulsed DC in 2-ml volumes of X-VIVO 20 medium, supplemented or not with 10 ng/ml IL-7 and 10 pg/ml IL-12 (both R&D Systems). After 8 d, cultures were restimulated with 5×10^4 BKV-pulsed autologous DC. On day +11, IL-2 (20U/ml) was

added to the cultures. CTL were then expanded with weekly rounds of restimulation with autologous stimulators in the presence of rIL-2. CTL were examined for immunophenotype, and for specificity in a standard ^{51}Cr-release assay against a panel of targets including autologous mock-pulsed or BKV-pulsed DC.

T-Lymphocyte Subset Depletion

CD3+/TCR$\gamma\delta$+ cells were removed from cell suspension by negative selection with anti-TRC$\gamma\delta$ mAb (Becton Dickinson, Mountain View, CA) and magnetic microspheres coated with goat-anti-mouse IgG (Dynabeads M-450; Dynal, Lake Success, NY), according to a previously described method (28).

Flow Cytometry

mAb used to characterize cultured cells were CD3 FITC and PE, anti HLA-DR PE, CD8 FITC and PE, CD56 PE, anti-TRC$\gamma\delta$ FITC, CD4 PE, CD19 FITC, CD20 PE, CD45 FITC (Becton Dickinson). Appropriate isotype-matched controls were included. Cytofluorimetric analysis was performed by means of direct immunofluorescence on a FACScan flow cytometer (Becton Dickinson).

Target Cell Lines and Cytotoxicity Assay

EBV-transformed B-cell lines (EBV-LCL) were generated and maintained following a protocol previously described (17). Tubular epithelial cells (TBE) were established from cortical portions of normal kidneys using a previously published technique (29). HSV-1–infected phytohemagglutinin blasts were obtained according to a previously described method (28). Cytotoxic activity was measured as described previously (17). Spontaneous release from the target cells was consistently <25%. Results were expressed as percentage of specific lysis. For HLA class I blocking experiments, target cells for cytotoxicity assays were incubated with 30 μl of the murine anti-human HLA class I mAb W6/32 (Dako, Glostrup, Denmark) for 30 min at room temperature. Cytotoxicity was then performed as described above.

T-Cell Receptor CDR3 Spectratyping

CDR3 size length analysis on preculture PBMC and on BKV-specific CTL was performed according to a previously described method (30). In detail, PCR amplification was performed under non-saturating condition, after titration of cDNA obtained from the different samples. In each PCR tube, we used a panel of TCR-BV family-specific primers and a constant TCRβ-chain primer labeled with a fluorochrome at the 5' end with 5'-6 carboxyfluorescein. Each reaction also contained a β-actin specific primer pair amplifying a product as internal control to check the efficiency of the single PCR (31). Spectratyping was generated by loading, after denaturation, an equivalent volume of PCR product and loading dye on a 5% acrylamide denaturing gel and run into a 377 Fluorescence based DNA sequencer (Applied Biosystems, Foster City, CA) in the presence of Rox-labeled size markers. The data were analyzed using Applied Biosystems Genescan software that allows assignment of size and peak areas to different PCR products. The data for each TCR-BV family were visualized as chromatograms. By this technique, a normal repertoire is visualized as a series of bands, separated by 3 bp, having gaussian distribution; alterations, either in intensity or in distribution of the bands, reflect a turbative of repertoire.

Statistical Analyses

Data were expressed as mean ± SD. Immunologic parameter correlation was evaluated by *t* test. $P < 0.05$ was considered statistically significant. Statistical analysis was performed using the SAS System (SAS, Cary, NC).

Results

DC Pulsed with BKV Preparation Stimulate Antigen-Specific IFN-γ Production

It is known from data reported in the literature that viral infection is an optimal mean to activate DC and that polyomavirus-infected DC are able to stimulate polyomavirus-specific CTL reactivation in the mouse (25). Nevertheless, the use of live BKV virions is not compatible with good laboratory practice (GLP) protocols of CTL generation for *in vivo* use. We therefore proceeded to investigate whether DC pulsed with inactivated BKV constituted a stimulus apt to induce specific immune responses in humans.

DC were incubated with inactivated BKV (equivalent of MOI 0.01) overnight, and cell surface phenotypic analysis was then performed. We found that upon pulsing with BKV antigen, immature DC did indeed display an activated phenotype, with upregulation of MHC and co-stimulatory molecules (data not shown). The PBMC from healthy BKV-seropositive donors and kidney transplant recipients with BKV reactivation were tested for their reactivity to autologous DC pulsed with BKV antigen by measuring specific proliferation in a standard ^{3}H-thymidine incorporation assay. IFN-γ secretion was measured on 10-d CTL culture cells by means of an ELISPOT assay. In all subjects tested, the 6-d proliferation observed after stimulation with BKV-DC was always higher than that observed with mock-pulsed DC ($10.8 \pm 3.7 \times 10^3$ cpm *versus* $5.4 \pm 2.6 \times 10^3$ cpm; $P < 0.05$; Figure 1A). Moreover, T cells from both control subjects and patients showed a measurable IFN-γ production in response to the virus (patients, 11 mean spots/10^5 PBMC; healthy donors, 17 mean spots/10^5 PBMC; Figure 1B).

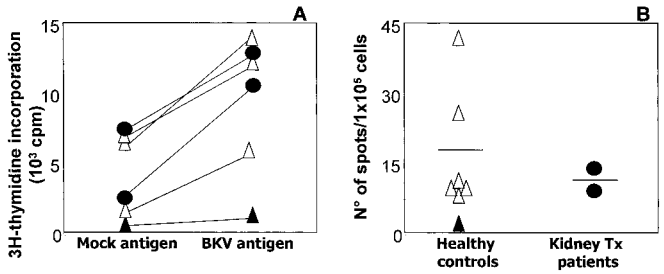
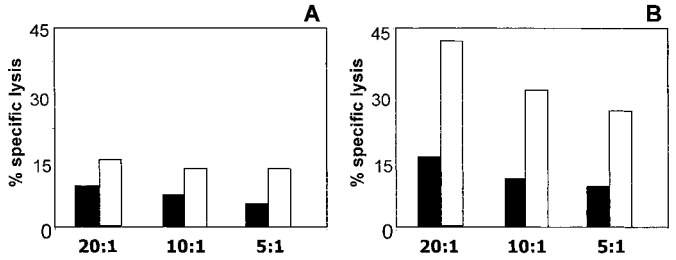


Figure 1. Proliferation as measured by ^{3}H-thymidine incorporation assay, and IFN-γ production as measured in an ELISPOT assay, in response to polyoma BK virus (BKV)-pulsed dendritic cells (DC). (A) Proliferation results for samples from three BKV-positive healthy controls (△), one BKV-negative healthy individual (▲), and two BKV-positive kidney transplant recipients (●); the results for each subject are represented linked. (B) IFN-γ secretion for 10-d BKV–cytotoxic T cell (CTL) culture samples from six BKV-positive healthy individuals (△), one BKV-negative healthy individual (▲), and two BKV-positive kidney transplant recipients (●).

BKV Antigen-Pulsed DC Induce BKV-Specific CTL In Vitro

We then proceeded to evaluate the ability of DC-BKV to reactivate *in vitro* BKV-specific cytotoxic T lymphocytes from healthy seropositive individuals. PBMC and autologous DC pulsed with BKV antigen were co-cultured for 3 to 4 wk. Once a week, cultures were restimulated with DC-BKV, and starting on day +9, rIL-2 (20U/ml) was added to stimulate growth of BKV-activated lymphocytes. Clusters of proliferation were observed around DC, and at the end of the culture, T-cell recovery was a median of 1.5-fold the initial T-cell number (data not shown). Surface marker analysis indicated that these lines were 94 (±3)% CD3+, 53 (±15)% HLA-DR+, with 71 (±7)% CD4+ lymphocytes, 19 (±5)% CD8+ cells, and contained 10 (±1)% cells with a γδ TCR and 3 (±2)% showing a CD3+/CD8+/CD56+ phenotype. Unmanipulated cells recovered from the cultures showed a low although sizable cytotoxicity against autologous BKV-pulsed DC (11 ± 4%), with absent or low activity against unpulsed DC (3.5 ± 5%).

In an attempt to increase virus-specific cell-mediated lysis, we implemented the reactivation protocol by adding rIL-12 and rIL-7 to the cultures at day 0, based on previous observations from our group in the setting of leukemia-directed CTL activation (25). With this new protocol, we obtained a mean fivefold expansion of T cells in the cultures and a significant increase of CD8+ lymphocytes and activated T cells in CTL lines (44 ± 5% CD8+ cells, with 33 ± 12% CD4+ lymphocytes, and 73 ± 6% HLA-DR+ cells). The specific lysis mediated by cytokine-treated CTL was consistently higher than cytotoxicity observed in untreated cultures (treated, 33 ± 17%; untreated, 10 ± 4%; $P < 0.05$). Data on the phenotype and cytotoxicity of CTL reactivated in the absence or in the presence of IL7/IL-12 are reported in Figure 2. The CTL lines obtained with the latter reactivation protocol, in addition to a strong lysis of the autologous BKV-pulsed DC, showed little or no reactivity against mock-pulsed autologous DC, unpulsed HLA-mismatched EBV-LCL, and HSV-1–infected autologous phytohemagglutinin blasts (Figure 3). Moreover, antibody inhibition studies showed that specific cytotoxicity was mostly HLA class I–dependent (Figure 3). Because depletion of CD3+/γδ+ T cells in the effector population caused a moderate decrease of cytotoxicity against autologous BKV-pulsed targets (≤20% of total cytotoxicity), it is likely that specific activity was mediated by both CD3+/CD8+/γδ− and CD3+/γδ+ subsets.



CD8	20%	HLA-DR	36%
CD4	79%	CD8/CD56	4%
TCRγδ	8%	CD56	5%

CD8	45%	HLA-DR	71%
CD4	25%	CD8/CD56	15%
TCRγδ	66%	CD56	3%

Figure 2. Phenotype and cytotoxicity of CTL reactivated by stimulation with BKV-pulsed DC in the absence or in the presence of IL-7/IL-12. Lysis against BKV-pulsed DC (□) is stronger in treated cultures (B), at all effector-to-target (E:T) ratios, in comparison with untreated cultures (A). Moreover, the percentage of CD8+, HLA-DR+, and TCRγδ+ cells is higher in treated cultures. Lysis against mock-pulsed DC is also reported (■). Data refer to a representative experiment and are the mean of triplicate wells.

BKV-Specific CTL Reactivation in the Presence of IL-7 and IL-12 Allows Expansion of HLA-Unrestricted T-Cell Populations in Kidney Transplant Recipients

The relevance of BKV-related pathology in renal transplantation prompted us to verify whether BKV-specific CTL lines could be reactivated from kidney transplant recipients with active BKV infection. CTL specific for the virus were successfully expanded with our protocol in all patients tested, and data on phenotype and cytotoxicity of the six CTL lines are reported in Table 1. In detail, the CTL lines contained a high percentage of CD8+ and HLA-DR+ cells and mediated BKV-specific cytotoxicity, in the absence of lysis against allogeneic EBV-LCL. For excluding a possible cytopathic effect of patients' CTL on donor graft tissue in case of *in vivo* use, cytotoxicity against allogeneic tubular epithelial cells was performed and found to be absent (see Table 1).

The success of a CTL-based preemptive treatment of BKV-related disease in renal transplant patients could also rely on the demonstration of *in vitro* killing of allogeneic BKV-infected targets, as the renal epithelial cells bearing the latent virus are of donor origin and therefore would not be a suitable

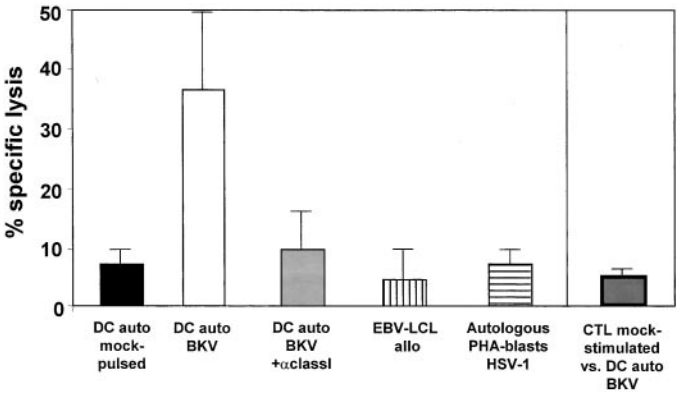


Figure 3. Cytotoxicity of CTL reactivated by stimulation with BKV-pulsed DC in the presence of IL-7/IL-12. Antigen specificity of the cultured T cells is demonstrated by low lysis of autologous mock-infected DC (■) and autologous HSV-1 infected phytohemagglutinin blasts (▤) in the presence of strong lysis of autologous BKV-pulsed DC (□). HLA-restriction is proved by prevention of lysis against autologous BKV-pulsed DC in the presence of anti-HLA class I mAb (■). Absence of alloreactivity is testified by absent cytotoxicity against allogeneic EBV-transformed B-cell lines (EBV-LCL; ▥). Lytic activity of cultures from Peripheral blood mononuclear cells (PBMC) stimulated with mock-pulsed DC, against autologous BKV-pulsed DC (▨), is also included. Data represent cytotoxicity at a 10:1 E:T ratio.

Table 1. Surface phenotype and cytotoxic activity of BKV-specific CTL lines obtained from the six kidney transplant patients with active BKV infection[a]

Patients	CD4+ Cells (%)	CD8+ Cells (%)	HLA-DR+ Cells (%)	TCRγδ+ Cells (%)	CD8/56+ Cells (%)	E:T Ratio	Auto Unpulsed Targets (% Lysis)	Auto BKV-Pulsed Targets (% Lysis)	Allo LCL (% Lysis)	Allo TBE (% Lysis)
1	29	54	58	36	7	20:1	5	15	ND	0
						10:1	0	16	ND	0
2	16	36	65	69	25	20:1	3	13	4	ND
						10:1	2	9	1	ND
3	1	40	75	43	22	20:1	31	71	16	3
						10:1	16	64	6	0
4	64	24	66	11	5	20:1	1	10	0	ND
						10:1	2	12	0	ND
5	27	51	74	54	21	20:1	15	51	8	0
						10:1	8	40	6	0
6	24	45	71	66	15	20:1	16	43	7	ND
						10:1	9	31	2	ND

[a] E:T, effector to target; ND, not done.

target for a patient's HLA-restricted CTL. The CTL lines reactivated from kidney allograft recipients showed a sizable cell-mediated lysis of a panel of allogeneic BKV-bearing targets, with little or no activity against allogeneic unpulsed DC (Figure 4). This phenomenon could be due to populations of CD3+/γδ+ and CD3+/CD56+ cells endowed with MHC class I unrestricted cytotoxic activity. In particular, the percentage of CD3+/γδ+ T cells was found increased in T-cell lines reactivated in the presence of IL-7/IL-12 compared with untreated cultures (untreated, 9 ± 2%; treated, 51 ± 17%; $P < 0.005$). Depletion experiments, performed on BKV-specific CTL lines reactivated from kidney allograft recipients with anti-TCRγδ monoclonal antibody and goat–anti-mouse IgG-coated magnetic beads, confirmed that the majority of cytotoxic activity against allogeneic BKV-infected targets was mediated by the CD3+/γδ+ T-cell subset (Figure 5).

T-Cell Repertoire Analysis by CDR3 Spectratyping

Comparative analysis of TCR CDR3 spectratypes on kidney transplant recipients' PBMC and the derived CTL was performed at +30 d from reactivation. This analysis showed that, although the TCR repertoire of patients' PBMC is polyclonal

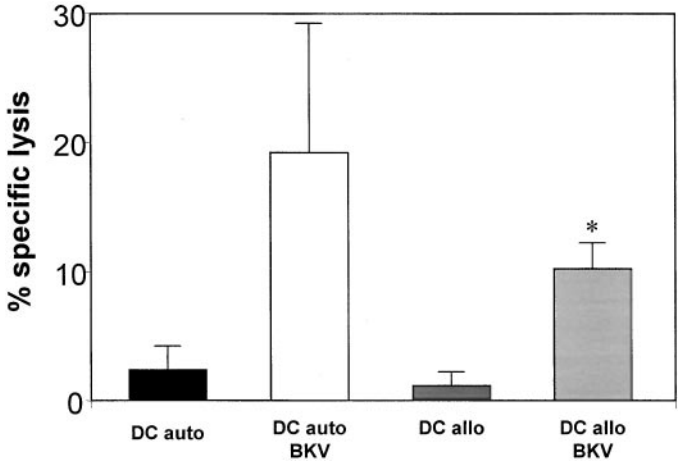


Figure 4. Cytolytic assays of cytokine-treated BKV-specific CTL reactivated from kidney transplant recipients. MHC class I unrestricted cytotoxic activity is demonstrated by the sizable lysis against allogeneic BKV-pulsed DC (■) in the absence of lysis against allogeneic unpulsed DC (▨; % lysis, 10 ± 2 *versus* 1 ± 1; $P < 0.05$). Cytotoxicity against autologous BKV-pulsed (□) and autologous unpulsed DC (■) is also reported. Data represent mean ± SD cytotoxicity at a 10:1 E:T ratio of four separate experiments.

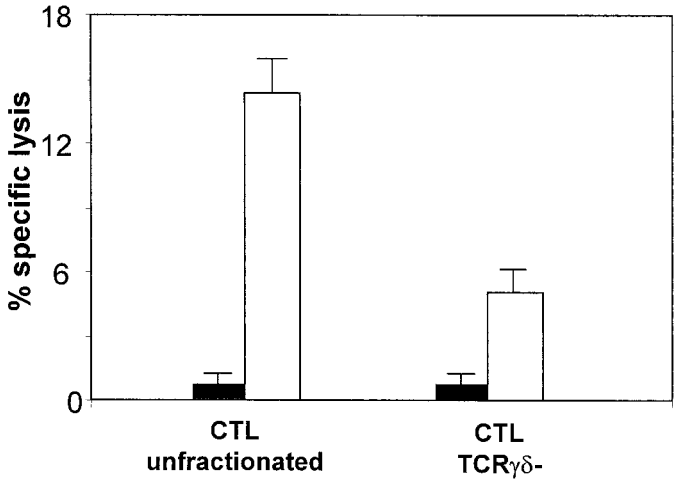


Figure 5. Effect of the depletion of TCRγδ+ T-cell population on cytolytic activity of cytokine-treated BKV-specific CTL. Cytotoxic activity against allogeneic BKV-pulsed DC (□) is markedly reduced after depletion of TCRγδ+ T cells. Cytotoxicity against autologous unpulsed DC (■) is also reported. Data represent mean ± SD cytotoxicity of triplicate wells at a 10:1 E:T ratio and are representative of two separate experiments.

with the TCR-BV families having a gaussian-like profile, a wide skewing was present in many of the BV families of CTL, suggesting that a restriction in the TCR usage induced by the antigen is present in the T-cell line (Figure 6).

Discussion

T-cell immunity is crucial for protection against BKV disease. After primary infection, the virus becomes latent and may reactivate symptomatically upon immunodeficiency (2,5). Moreover, seronegativity for BKV before kidney transplantation predisposes the patients to active viral infection and subsequent development of BKV-related nephropathy in the pediatric setting (32), likely as a result of a defective development of protective immunity to BKV secondary to immunosuppressive treatment and the allogeneic environment of the graft. Because active BKV infection seems to be a consequence of impaired specific immune function, evaluation of BKV-specific T-cell immunity and restoration of specific immunity in high-risk patients through cellular immunotherapy may represent valuable tools in the management of the emerging BKV-related pathology.

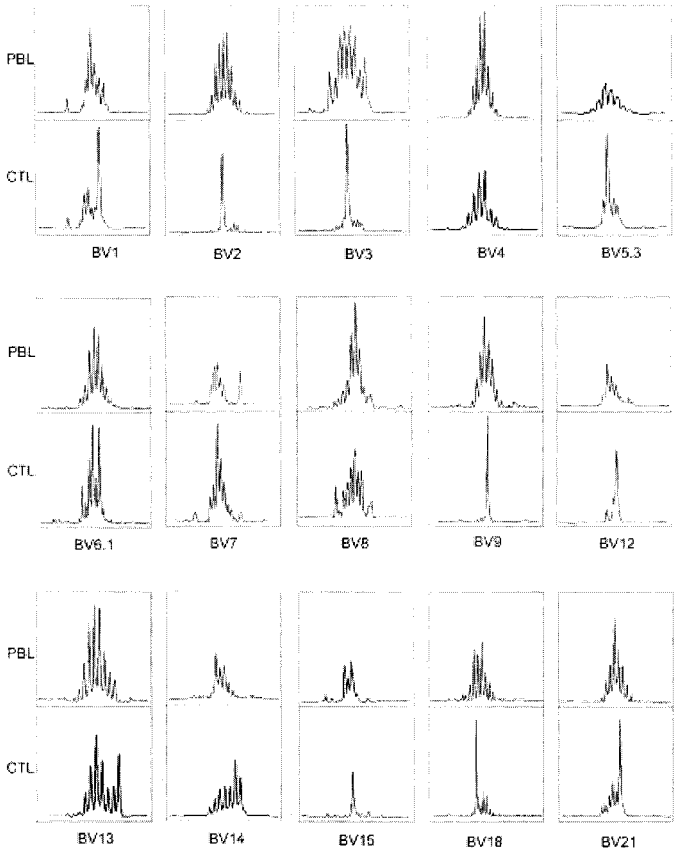


Figure 6. TCR BV spectratyping results of preculture PBMC and the 4-wk culture BKV CTL line from a single kidney transplant recipient. The spectratype representation of 15 BV gene family members in PBMC and the derived BKV-specific CTL line of a kidney transplant recipient are shown. Whereas PBMC show the normal, gaussian, distribution, a wide skewing is found in the majority of the BV families, suggesting that a restriction in the TCR usage induced by the antigen is present in the T-cell line. BV 19 and 10 are not shown because they are pseudogenes, BV 5.1, BV 6.2, BV 11, BV 16, BV 17, BV 20, BV 22, BV 23, BV 24, and BV25 were found to have a similar, gaussian, pattern both in the peripheral blood lymphocyte (PBL) and in the T-cell line.

Our results demonstrate that it is feasible to reactivate BKV-specific CTL in humans by stimulation with BKV-pulsed autologous DC. The method described allows CTL expansion under GLP conditions, as no animal serum is required in the culture medium and the use of inactivated virions, grown on human cells, minimizes the risk of virus transfer from BKV-specific T cells, as demonstrated by the failure to obtain evidence of a cytopathic effect in BKV infection–permissive cell cultures after incubation with BKV-specific CTL. The use of whole virus to pulse DC is at present mandatory, as the T-cell response to BKV is poorly characterized and immunogenic T-cell epitopes have not been defined. However, this seeming limitation may indeed represent an advantage in the context of BKV disease, as not only can this technique be used for patients of any HLA type, but it also will reactivate CTL with multiple specificities and allow expansion of HLA-unrestricted T-cell subsets.

Data obtained in the setting of human CMV (HCMV)–specific CTL reactivation indicated that DC pulsed with inactivated virus were able to induce T-cell lines that included a variable, although generally low, number of CD8+ lymphocytes (23). The percentage of CD3+/CD8+/TCR$\gamma\delta$− and CD3+/CD8+/TCR$\gamma\delta$+ T cells in our cytokine-treated cultures was decidedly augmented in comparison with what was observed in the absence of cytokines. The CD8+ T-cell increase could have been due to an IL-12–dependent activation of DC (24), which in turn boosted antigen-specific CD8 CTL induction, coupled with CD8+ T-cell proliferation and survival promotion mediated by IL-7 (33), whereas the high number of TCR$\gamma\delta$+ T lymphocytes observed might be ascribable to IL-7, which is known to be a factor involved in early development and in prolonging the life-span of mature TCR$\gamma\delta$+ T cells (34). This finding, reproducible in the individuals tested, may be relevant to a cellular immunotherapy approach for BKV-related disease, because, as already described for polyomavirus JC (21), CD8+ T-cell population may be necessary to contain infection. Moreover, most important in view of a possible cell transfer program, the protocol used to reactivate BKV-specific T cells and, in particular, the use of IL-12 and IL-7 in the early culture phase allows the expansion of sufficient CTL for infusion requirements.

The technique reported can also be exploited to evaluate BKV-specific cellular immunity. Indeed, the same stimulators used to reactivate BKV-specific CTL can be used in an ELISPOT assay to measure the frequency of IFN-γ–producing T cells. This method could highlight high-risk situations, such as absent or decreased ability to mount a cellular immune response upon development of active BKV infection, and consequently help in identifying subsets of patients who are prone to progression from active infection to BKV-related disease. Thus, serial evaluation of BKV-specific, IFN-γ–secreting T-cell populations, used in combination with BKV DNA determination, may allow application of a preemptive approach to treatment and represent a useful tool to monitor treatment

efficacy, as recently proposed for EBV-related posttransplantation lymphoproliferative disease (35).

The demonstration of the feasibility of reactivating BKV-specific CTL from samples of patients receiving immunosuppression after kidney transplantation could have immediate therapeutic implications. Although in patients with late-phase BKV nephropathy interstitial inflammation may be present, it has been suggested that renal dysfunction can be primarily a consequence of virus-mediated necrosis of tubular cells (36). In addition, a recent report showed that renal allograft with BKV infection is characterized by a decrease in cytotoxic T cells (37). Thus, induction or restoration of protective immunity to BKV through a cellular immunotherapy approach could reduce the incidence of active BKV infection and related interstitial nephropathy in kidney transplant recipients.

BKV-related nephropathy in kidney allograft recipients mostly originates from viral reactivation in the graft. Hence, a major drawback in devising a cell therapy strategy in this heterologous setting is that viral antigens on HLA-mismatched donor renal cells could escape recognition by HLA-mismatched recipient CTL. However, because treatment of established BKV nephropathy does not seem to have substantially changed the prognosis (38), in view of a preemptive immunotherapeutic strategy, lysis of donor tubular epithelial cells harboring BKV might not be essential to avoid progression from active viral infection to nephropathy. Indeed, it has been suggested that invasive renal disease may depend on the virus's gaining access to the blood via tubular fluid leaking into the interstitium and, hence, gaining access to peritubular capillaries (5). Consequently, MHC-restricted CTL of patient origin could limit viral spread to renal parenchyma and the consequent graft damage, through lysis of autologous circulating B lymphocytes carrying BKV.

In addition, the CTL lines reactivated from our patients included in most cases a high percentage of CD3+/TCR$\gamma\delta$+ cells, which could also contribute to the killing of autologous BKV-infected cells. TCR$\gamma\delta$+ T-lymphocyte subsets have been shown to proliferate in response to virus-infected cells; mediate lysis through a TCR-dependent, MHC-unrestricted antiviral effector function (39,40,28); and play a protective role against viral infections (41,42). Indeed, our CTL lines reactivated from kidney graft recipients mediated an MHC-unrestricted, BKV-directed killing, which was abrogated by depletion of CD3+/TCR$\gamma\delta$+ T cells. Therefore, it is reasonable to hypothesize that these MHC-unrestricted cytotoxic populations could control BKV reactivation from the allograft urothelium. Moreover, it cannot be excluded that in some patients, subsets of cytotoxic CD8+/TCR$\alpha\beta$+ CTL could also recognize and lyse infected cells in a BKV-specific, MHC-unrestricted manner (43), thus contributing to control BKV reactivation from the graft.

Our data support the feasibility of an adoptive immunotherapy approach to BKV-associated nephropathy occurring in kidney transplant recipients. In particular, a preemptive strategy may be valuable even when immunosuppression discontinuation is prevented by a high risk of rejection. A phase I/II clinical trial will have to be undertaken to evaluate whether early infusion of autologous BKV-specific CTL, following a strategy of preemptive therapy guided by BKV DNA levels (44), could induce control of BKV infection *in vivo* without increasing the probability of graft rejection or graft damage.

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