Cross-reactive CTL recognizing two HLA-A*02-restricted epitopes within the BK virus and JC virus VP1 polypeptides are frequent in immunocompetent individuals

Madeva C. Sharma, Wendy Zhou, Joy Martinez, Ludmila Krymskaya1, Tumul Srivastava, Wahajul Haq, Don J. Diamond, Simon F. Lacey*

Laboratory of Vaccine Research, Beckman Research Institute of the City of Hope, 1450 East Duarte Road, Duarte, CA 91010-3000, USA

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Abstract

Two HLA-A*02-restricted epitopes have been identified within the VP1 polypeptide of a human polyomavirus, BK virus, which is associated with polyomavirus-associated nephropathy in kidney transplant patients. Immunization of transgenic mice with recombinant modified vaccinia Ankara expressing BKV VP1 (rMVA-BKV VP1) elicited functional CTL populations recognizing the sequences LLMWEA VTV (amino acids residues 108–116, BKV VP1p108) and AITEVECFL (residues 44–52, BKV VP1p44) and cross-reactive to the previously described JC virus VP1 homologs. Flow-based analyses of PBMC from a panel of thirty healthy HLA-A*02 human volunteers indicated that the majority of these subjects harbored functional CTL populations recognizing the BKV epitopes and cross-reactive with the JCV homologs. CTL recognizing the JCV VP1p100 and JCV VP1p36 epitopes have previously been associated with prolonged survival in progressive multifocal leukoencephalopathy patients. These findings suggest that infection with BKV or JCV could potentially induce cross-protective T-cell immunity against diseases associated with these viruses.

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Introduction

The polyomaviruses BK virus (BKV) and JC virus (JCV) infect the majority of humans early in life and establish asymptomatic persistent infection in the kidney as well as other tissues (Chesters et al., 1983). The two viruses are closely related (~70% sequence homology), and both reactivate in the context of immune suppression. BKV reactivation in recipients of hematopoietic stem cell transplantation (HSCT) or kidney transplantation (KTx) is evidenced by viruria and/or viremia. In a minority of individuals, BKV can cause clinical disease including hemorrhagic cystitis, ureteric stenosis and BK-virus-associated nephropathy (BKVN), which is associated with significant morbidity and mortality in KTx patients. JCV reactivation is associated with progressive multifocal leukoencephalopathy (PML), a fatal demyelinating disease of the CNS seen in immunocompromised patients with AIDS, cancer or recipients of organ transplantation. Two T-cell epitopes have been identified within the JCV major capsid protein VP1, and CTL recognizing these epitopes have been associated with control of the virus (Du Pasquier et al., 2004a, 2004b). Recently, PML has been identified as a complication of natalizumab treatment of patients with multiple sclerosis (Kleinschmidt-DeMasters and Tyler, 2005) and Crohn's disease (Langer-Gould et al., 2005), with a temporal association shown between serum JCV load and natalizumab therapy. Natalizumab is a humanized monoclonal antibody against α4 integrins, and it is probable that, by blocking normal trafficking of lymphocytes, this drug

⁎ Corresponding author. Room 1001C, Fox South Bldg., Laboratory of Vaccine Research, Beckman Research Institute of the City of Hope, 1500 East Duarte Road, Duarte, CA 91010-3000, USA. Fax: +1 626 301 8981.
E-mail address: slacey@coh.org (S.F. Lacey).
1 Present address: Mannkind Corporation, 28903 North Avenue Paine, Valencia, CA 91355.

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interfered with CTL responses previously been shown to be important in control of PML in HIV+ patients (Du Pasquier et al., 2004a, 2004b).

Since BKV and JCV are so closely related, it seems probable that T-cell responses are also important in control of BKV. The incidence of BKVN in KTx recipients has increased in recent years due to the use of newer and more potent immunosuppressive agents, and the primary treatment options for BKVN are judicious reduction of immunosuppression and experimental use of cidofovir. Early studies of T-cell immunity to BKV used BKV-infected cell lysates as antigens and did not define specific antigens or epitopes recognized by the immune system (Comoli et al., 2003, 2004; Drummond et al., 1985, 1987). The first T-cell epitope to be described for BK virus, an HLA-A*02-restricted epitope, LLMWEAVTV at position 108–116 within the BKV VP1 major capsid protein was identified as a candidate epitope by using computer predictions of HLA binding and shown to be generated by in vivo processing of the full-length BKV VP1 polypeptide in a transgenic mouse model (Krymskaya et al., 2005) (Table 1). CTL populations recognizing this epitope and cross-reacting with the JCV homolog epitope ILMWEAVTL (position 100–108 in the JCV VP1 ORF) were shown to be present in a small group of normal human subjects and in a kidney transplant (Ktx) recipient expressing the HLA-A*02 phenotype (Krymskaya et al., 2005). In the present study, we identify a second HLA-A*02-restricted epitope within the BKV VP1 ORF and show that CTL recognizing this epitope also frequently cross-react with the JCV homolog. We have surveyed a panel of thirty healthy individuals expressing HLA-A*02 for the presence of CTL recognizing these four polyomavirus epitope sequences and characterized these CTL in terms of their function and phenotype after expansion in culture.

Results

*Identification of a HLA-A*02-restricted T-cell epitope within the BKV VP1 polypeptide by use of a transgenic mouse model*

An HLA-A*02-restricted T-cell epitope SITEVECFNL has previously been reported at position 36–44 within the JCV VP1 (Du Pasquier et al., 2003, 2004a, 2004b). The BKV sequence at position 44–52, AIITEVECFNL differs from the JCV homolog only at the N-terminal amino acid residue (Table 1), leading us to speculate that the BKV VP1p44–52 sequence might represent a functional CTL epitope. We immunized HHDII transgenic mice intraperitoneally with a recombinant modified vaccinia Ankara (MVA) expressing BKV VP1. Two weeks following immunization, the mice were sacrificed, the spleens harvested and the splenocytes stimulated for 2 weeks in culture with the BKV VP1p44–52 peptide. Following this stimulation, the splenocytes were tested for their ability to lyse Jurkat A2 cells pulsed with the BKV VP1p44–52 peptide, or the JCV VP1p36–44 peptide, or with an irrelevant HIV peptide. The results of these experiments (Fig. 1) indicated that the murine effector cells recognized target cells presenting the BKV and the JCV homologs of this epitope with similar affinity. BKV VP1 polypeptide is therefore processed in vivo in these transgenic mice to generate the p44–52 epitope and elicit a T-cell response restricted by HLA-A2.

Cytotoxic T-cells that cross-recognize the BKV VP1p44–52 and JCV VP1p36–44 epitopes are present in a majority of healthy humans expressing HLA-A2

We then wished to investigate the prevalence in humans of T-cell populations recognizing the BKV VP1p44–52 and JCV VP1p36–44 epitopes and to determine whether cross-reactivity similar to that found in the mouse model would be seen in the context of human lymphocytes. Aliquots of archival cryopreserved PBMC samples from healthy human volunteers expressing HLA-A*02 were stimulated for 2 weeks in culture in the presence of IL-2 with one of the following four peptides: BKV VP1p44–52 or JCV VP1p36–44 (11 subjects), BKV VP1p108–116 or JCV VP1p100–108 (25 subjects) before analysis. VP1-specific CD8+ T-cells were detected by labeling with a panel of four HLA-A*02 tetramers refolded with the peptides and conjugated to either phycoerythrin (PE) in the case of the JCV tetramers or allophycocyanin (APC) in the case of the BKV tetramers. A cut-off value for positivity was set at 0.2% of CD8+ T-lymphocytes. This threshold was based on control stainings of these expanded cultures with irrelevant HIV tetramers (data not shown). Fig. 2A shows representative flow plots of analyses of PBMC from healthy human volunteers expressing HLA-A*02 peptide, or the JCV VP1p36–44 epitope. This cross-recognition was seen in most of the eleven subjects investigated for responses to the JCV VP1p36 and the BKV VP1p44 epitopes and was seen for T-cell populations expanded by stimulation with either peptide (Figs. 3A and B). Eight of eleven (73%) individuals expanded T-cells recognizing BKV/JCV in response to stimulation with the BKV VP1p44 peptide. Six of eleven (55%) responded to stimulation with the JCV VP1p36 peptide, and ten of eleven (91%) responded to at least one of the two forms of the epitope, while four (36%) responded to both peptides.

### Table 1

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<thead>
<tr>
<th>Alignment of BKV and JCV VP1 T-cell epitope homologs</th>
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<tr>
<td>BKV VP1p44-52</td>
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<td>BKV VP1p108-116</td>
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<td>JCV VP1p100-108</td>
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Non-conserved amino acid residues are highlighted.
We have surveyed 25 normal subjects expressing the HLA-A*02 allele for T-cell responses to the BKV VP1p108–116 and JCV VP1p100–108 epitopes. The frequency of responses to the BKV VP1p108–116 (5/25 individuals, 20%) and JCV VP1p100–108 (9/25 individuals, 36%) was lower than that seen to the p36/p44 epitopes (Figs. 3C and D). Because of limitations on patient sample availability, we were unable to investigate all 31 subjects for T-cell responses to each of the four epitopes. Among the subjects was an individual (ND#26) with an unusual pattern of responses to the BKV VP1p108 epitopes. Among the subjects in this study with T-cells recognizing polyomaviruses, this individual, unlike the majority of subjects in this study with T-cells recognizing polyomaviruses, was bound by the JCV VP1p100 tetramer but did not stain with BKV VP1p108–52 peptide (squares), or JCV VP1p36–44 peptide (diamonds), or an irrelevant HIV peptide (triangles). E:T ratio, effector to target ratio. The error bars represent standard deviations. This figure is representative of three separate experiments.

Functionality of T-cells recognizing the BKV VP1p44–52 and JCV VP1p36–44 epitopes

We investigated the functionality of these CTL populations by using a combined tetramer, intracellular cytokine and cytotoxic granule mobilization assay which we have described previously (Krymskaya et al., 2005). A representative result of such an experiment on a culture containing a population of CTL recognizing the BKV VP1p44–52 peptide, approximately 65% of these cells both degranulated and produced IFN-γ, and another 14% of the cells degranulated without producing cytokine (bottom right plot in Fig. 2B). Upon stimulation with the BKV VP1p44–52 peptide, approximately 65% of these cells both degranulated and produced IFN-γ, and another 14% of the cells degranulated without producing cytokine (bottom right plot in Fig. 2B). These results suggest that these BKV/JCV VP1-specific cells expanded by peptide stimulation represent functional CTLs.

BKV/JCV-specific CD8+ T-cells are less differentiated than CMV-specific T-cells

A putative model of T-cell differentiation has been proposed (Fig. 6) in which early differentiated cells expressing the costimulatory receptors CD27 and CD28 progress from a CD27−CD28+ phenotype through to fully differentiated CD28+CD27− cells. Evidence for this model rests on the correlation of these markers and telomere length (Effros et al., 1996; Hamann et al., 1999; Roos et al., 2000). Appay et al. (2002) have reported patterns of CD27 and CD28 expression associated with CD8+ T-cells recognizing the human viruses Epstein–Barr virus (EBV, mostly early CD27+, CD28+), human immunodeficiency virus (HIV, mostly intermediate CD27+, CD28+), human cytomegalovirus (CMV, mostly late CD27−CD28+) and hepatitis C virus (HCV, mostly early CD28+, CD27+). The frequency of BKV/JCV-specific CD8+ T-cells in normal volunteers was too low to permit direct analysis without expansion by stimulation. However, at least one report indicates that, despite extensive in vitro proliferation, the CD27 phenotypes of CD8+ T-cell clones specific for HIV, CMV and EBV were typical of freshly isolated CD8+ T-cells reactive with these viruses (Ochsenbein et al., 2004). We therefore analyzed in-vitro-expanded BKV/JCV-specific CD8+ T-cells by tetramer labeling in combination with panels of antibodies to the surface markers CD8, CD45RA, CD45RO, CCR7, CD27, CD28 and the cytotoxic molecules granzyme A, granzyme B and perforin. As a control, we also characterized the surface marker phenotype of CMV-specific cells expanded from PBMC from several of the subjects by stimulation with peptides corresponding to HLA-A2-restricted epitopes within the CMV pp65 (NLVPVMATV495–503) and IE-1 (VLEETSVML316–324) polypeptides. The CMV-specific CD8+ T-cells were predominantly CD27-negative and CD28-negative (Fig. 5). This is in agreement with our previous studies on CMV-specific T-cells and with the report of Appay et al. (2002). By contrast, the BKV/JCV-specific CD8+ T-cells mostly expressed CD28 as well as CD27. Their CD27/CD28 phenotype was thus similar to the EBV and HCV-specific T-cells described by Appay et al. (2002) as being early-differentiated cells (Fig. 6).

Discussion

In this study, we have documented T-cell responses in a majority of a panel of healthy immunocompetent humans expressing HLA-A*02 to two T-cell epitopes within the BKV VP1 polypeptide that are restricted by this MHC-I allele. One of these epitopes (BKV VP1p44) has not, to our knowledge, been described before. In most cases, T-cells recognizing these epitopes cross-recognized cells presenting the JCV homolog peptide.
The majority of adults in the USA are infected with BKV; the overall seroprevalence in a large number of studies measuring neutralizing antibody titer or hemagglutination ranged between 50% and 90% in populations of healthy individuals or unselected patient groups (for a review, see Knowles, 2001). One recent study of 2435 sera in the UK reported an age-related BKV seroprevalence of 91% by the age of 5–9 but a JCV seroprevalence rate of only 50% by the age of 60–69 (Knowles et al., 2003). In addition, some molecular studies on leukocytes have suggested that almost all immunocompetent adults are infected with both BKV and JCV (Dorries et al., 1994). Given the probability that the majority of adult volunteers in our study cohort had been exposed to BKV and/or JCV, and not anticipating the high degree of T-cell immunity cross-reactivity that we observed, we did not screen sera from our subjects for BKV serostatus.

Fig. 2. Cross-reactivity and functionality of CD8+ T-cells in human samples recognizing the BKV VP1p44–52 and JCV VP1p36–44 epitopes. (A) PBMC from a normal subject were stimulated for 11 days in culture with BKV VP1p44–52 peptide in the presence of rIL2 before tetramer staining and flow analysis. In the upper row of plots, the cultures were labeled with FITC-conjugated antibody to CD8, and with BKV VP1p44 tetramer conjugated to APC, or with JCV VP1p36 tetramer conjugated to PE, or with an irrelevant HIV-PE tetramer. In the lower row of plots, the cells were labeled simultaneously with two tetramers. All plots were gated on lymphocytes as assessed by forward and side scatter. (B) Interferon-γ production and CD107a/b mobilization by BKV VP1p44 tetramer-binding CD8+ T-cells. Aliquots of the culture shown in panel A were mock-stimulated (upper row) or stimulated with BKV VP1p44–52 peptide (lower row) for 6 h before labeling and flow analysis. The right-hand column of plots is gated on CD8+ and tetramer+ lymphocytes (boxes within left plots). The numbers within the quadrants indicate events as a...
Fig. 3. CD8⁺ T-cell responses in normal humans to the BKV and JCV homologs of two HLA-A*02-restricted epitopes within the VP1 capsid proteins. Archival cryopreserved PBMC from thirty normal human subjects expressing the HLA-A*02 allele were examined by peptide IVS followed by tetramer assay of the cultures for CTL responses to four polyomavirus epitopes. Panels A and B: BKV VP1p44–52 and JCV VP1p36–44 (11 subjects). Panels C and D: BKV VP1p108–116 and JCV VP1p100–108 (25 subjects). Light bars on all charts indicate percent of CD8⁺ T-cells binding BKV tetramers and dark bars the percent binding JCV tetramers.

Fig. 4. Specificity of CTL in subject ND#26 for the JCV VP1p100–108 epitope. IVS with the BKV VP1p108–116 peptide did not expand tetramer-binding CD8⁺ cells from subject ND#26 PBMC (left column of plots). However, stimulation of ND#26 PBMC with the JCV VP1p100–108 peptide expanded a population of cells that were bound by the JCV VP1p100–108 tetramer but not by the BKV VP1p108–116 tetramer (center and right plots).
We described an individual (ND#26) who had a T-cell response to the JCV VP1p100 epitope that was unusual in this cohort for its specificity; attempts to expand T-cells from this individual by stimulation with the BKV VP1p108 peptide were unsuccessful, and the cells expanded by stimulation with the JCV VP1p100 peptide did not bind the BKV VP1p108 tetramer. It is therefore probable that these JCV-specific memory T-cells were in fact elicited in response to JCV VP1 antigenic stimulation, although the reason for lack of cross-reactivity in this case is unclear. This individual could have been infected with a JCV strain with one or more sequence alterations within the region of the VP1 sequence corresponding to the p100 epitope, such that it diverged further from the BKV sequence, thus preventing cross-recognition of the BKV p108 peptide while retaining enough homology with the archetypical JCV sequence ILMWEAVTL to allow recognition. However, a BLAST search of VP1 sequences indicated that all JCV VP1 sequences described to date are completely conserved within the p100 epitope. An alternative hypothesis is that the structure of the T-cell receptor (TCR) of the JCV VP1p100-specific CD8+ T-cell population within subject ND#26 had some character that decreased the promiscuity of cross-recognition of close homologs of the JCV VP1p100 epitope relative to the TCRs on the corresponding T-cells in subjects ND#02, ND#03, ND#21, ND#27, ND#30, and ND#31 that exhibited p100/p108 cross-reactivity.

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This concept has been explored by others in the context of murine responses to the related lymphocytic choriomeningitis and Pichinde viruses. A complex pattern of T-cell cross-reactivity was found that depended in part on the specificity of private T-cell repertoires of individual mice (Lin and Welsh, 1998; Kim et al., 2005). An example of observed differences in T-cell cross-reactivity between human subjects has been reported in the context of cells recognizing related influenza and Epstein–Barr virus epitopes and attributed to the private specificity of individual TCR repertoires (Clute et al., 2005). In short, there is ample precedent for the concept that individuals’ T-cells may differ in their degree of cross-reactivity to two closely related epitopes.

Our finding of an early-differentiated (CD27+, CD28−) phenotype of BKV/JCV-specific as compared to CMV-specific (CD27−, CD28+) CD8+ T-cells based on expression of these
two cell surface antigens may reflect the biology of the two viruses and the functional properties of the T-cell phenotype needed during chronic infection. The subjects in this study were healthy immunocompetent individuals chronically infected with JCV or BKV or both. It may be speculated that, in subjects with healthy immunocompetent individuals chronically infected with viruses and the functional properties of the T-cell phenotype needed during chronic infection. The subjects in this study were compared to the herpesviruses, but it is clear that the large T antigen and the agnoprotein, at least, modulate the host cell processes by affecting transcription and cell cycle. The net result of these viral and host cell interactions may be to cause accumulation of T-cells at distinct steps on the differentiation pathway that reflect the nature and properties of the virus in question (Fig. 6).

In this report, we have shown that low-level T-cell responses recognizing two HLA-A*02-restricted BKV epitopes within VP1 are present in a majority of healthy immunocompetent individuals expressing this HLA type. The knowledge of these epitopes allows the design and use of immunologic tools such as peptides and MHC-I tetramers for tracking of immune responses to these epitopes in populations such as kidney transplant recipients at risk for BKV reactivation and development of PVAN. Given the high homology between BKV and JCV, the cross-reactivity of these epitopes demonstrated in this report and the previous finding that CTL recognizing the JCV homologs are protective from the JCV-associated syndrome PML (Du Pasquier et al., 2001, 2004a, 2004b; Koralnik et al., 2002), it is highly likely that CTL recognizing the BKV epitopes will be associated with protection against PVAN. It will be of great interest to determine the immunological circumstances in which approximately 5% of KTx recipients develop PVAN. Presumably, this is due to a failure of T-cell-mediated immune control, and this is the subject of our ongoing investigations.

**Materials and methods**

**Study subjects**

PBMC were collected from thirty healthy HLA-A*02 donors at City of Hope and cryopreserved by standard methods (Maecker et al., 2005). These cryopreservation procedures do not normally significantly impact immunological assays (Maecker et al., 2005). The study protocol was approved by the COH Institutional Review Boards, and specimens and data were obtained prospectively after obtaining informed consent from the enrollees. HLA typing was performed by PCR, as described elsewhere (Krausa and Browning, 1996) for all blood donors.

**Peptides**

Peptides were synthesized in our laboratory with a Symphony Quartet peptide synthesizer (Protein Technologies Inc., Tucson, Arizona) using standard Fmoc protocols and purified to >95% purity by HPLC. The identity of the peptides was confirmed by MALDI TOF mass spectrometric analysis using a Kompact Probe mass spectrometer (Kratos Analytical, Shimadzu, Kyoto, Japan).

**Tetramer construction**

The HLA-A*02 BKV VP1p108, HLA-A*02 JCV VP1p100, HLA-A*02 BKV VP1p44, HLA-A*02 JCV VP1p36 and HLA-A*02 CMV IE-1 316–324 tetramers were refolded, purified and conjugated to fluorochromes in our laboratory using previously described methods (La Rosa et al., 2002).

**rMVAs expressing BKV VP1**

The preparation of this rMVA (rMVA-BKV VP1) has been described previously (Krymskaya et al., 2005).

**Immunization of Tg mice and in vitro expansion of CTLs**

The transgenic mouse strain HHDII was obtained from Dr. F. Lemonnier (Pasteur Institute) (Firat et al., 1999). This mouse expresses a transgenic monochain histocompatibility class I molecule in which the C-terminus of the human β2m is covalently linked to the N-terminus of a chimeric heavy chain (HLA-A*0201-α1, -α2, H-2Dβ-α3-transmembrane and intracytoplasmic domains). The colony of HHDII mice used in this study was seronegative for murine polyomavirus as shown by regular testing by MFI (multiplex fluorescent immunoassay) at the University of Missouri Research Animal Diagnostic Laboratory. Eight-to-twelve-week-old mice were immunized intraperitoneally with 3 to 5 × 10⁷ pfu of rMVA-BKV VP1. The animals were sacrificed after 2 weeks and the spleens retrieved. Single-cell splenocyte suspensions were prepared by passing the cells through a 70-μm Falcon cell strainer (Becton Dickinson Labware, Franklin Lakes, NJ) using the plunger from a sterile 1-ml syringe. Splenocytes were subjected to 1 round of in vitro expansion (IVS) as previously described (La Rosa et al., 2002). Briefly, splenocytes from immunized animals were cocultured with peptide-loaded LPS blasts in complete IVS medium at a ratio of 3:1 for 7 days, with the addition of 10% rat T-stim (Collaborative Biomedical Products, Bedford, MA).

**Cytotoxicity assay**

Cytolytic activity of effector cell populations was determined using a 4 h chromium release assay (CRA) following 1 IVS, as previously described (La Rosa et al., 2001; Daftarian et al., 2003). Jurkat A2.1 cells pulsed with 10 μM of the relevant or control HIV peptides were used as targets for CRA. Jurkat A2.1 cells were loaded with 200 μCi of Na⁵¹ CrO₄ (ICN, Costa Mesa, CA) for 1 h in a 37 °C water bath and further processed as described (La Rosa et al., 2001). Experimental evaluations were performed in triplicate.
**Intracellular cytokine (ICC) assays**

Splenocytes after 1 week IVS stimulation were tested for intracellular IFN-γ production by stimulation overnight with 5 μM BKV or JCV peptides. The following day, brefeldin A was added to all the cultures and incubation continued for 4 h. The cells were then washed with 3 ml PBS/0.5% BSA before labeling for 20 min at 4 °C with an FITC-conjugated antibody to murine CD8 (Pharminagen). The cells were then washed again with PBS/0.5% BSA before permeabilization (Cytofix/Cytoperm, Pharmingen) and labeling with APC- or PE-conjugated antibody to IFN-γ for 30 min at 4 °C. The cells were washed and analyzed on a FACScanto flow cytometer (Becton Dickinson).

**In vitro stimulation of PBMC**

Cryopreserved human PBMC were cultured in 24-well culture plates at a density of 3.5 million/ml in RPMI 10 containing 1 μg/ml of a single BKV or JCV VP1 peptide at 37 °C in a CO2-gassed incubator. After 3 days, recombinant human IL-2 (NIH AIDS Research and Reference Reagent Program) was added to 30 units/ml. Each 2 days thereafter, 50% of the culture medium was removed and replaced by fresh medium containing rIL2. Incubation was continued for 11–14 days before flow analysis.

**Combined ICC and CD107 mobilization/degranulation assay**

This assay was performed essentially as previously described (Bettis et al., 2003). Aliquots of 1 million cells from in vitro stimulation cultures were resuspended in 1 ml of RPMI 10 medium and FITC-conjugated antibodies to CD107a and CD107b (Pharmingen) added followed by costimulatory antibodies to CD28 and CD49d (Pharmingen) from in vitro stimulation cultures were resuspended in 1 ml of GolgiStop (Pharmingen) for 30 min at 4 °C. The cells were then labeled for 20 min at 4 °C with APC-conjugated tetramers followed by 20 min with PerCP-conjugated antibody to CD8 (Pharminagen) before permeabilization (Cytofix/Cytoperm, Pharmingen) and labeling with PE-conjugated antibody to IFN-γ (Pharminagen) for 30 min at 4 °C before flow analysis. A primary gate was set on lymphocytes using forward and side scatter and a secondary gate set on CD8 tetramer-binding cells. At least 100,000 events were collected per sample. The percentage of CD8 tetramer-binding lymphocytes expressing elevated surface CD107a/b and secreting IFN-γ was determined by reference to controls incubated with costimulatory antibodies to CD28 and CD49d but no peptides.

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**References**


