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Overview of the cellular immunity against JC virus in progressive multifocal leukoencephalopathy

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The human polyomavirus JC (JCV) infects most healthy adults without causing any disease. In the setting of severe deficit of cell-mediated immunity, such as in acquired immunodeficiency syndrome (AIDS), malignancies or in organ transplant recipients, JCV can reactivate and cause progressive multifocal leukoencephalopathy (PML), a deadly demyelinating disease of the central nervous system. The humoral immune response, measured by the presence of virus-specific immunoglobulin G (IgG) in the blood or by intrathecal synthesis of IgG in the cerebrospinal fluid (CSF), is unable to contain the progression of PML. CD4+ T lymphocytes recognize extracellular viral proteins that have been degraded into peptides through the exogenous pathway and presented on major histocompatibility complex (MHC) class II molecules at the surface of antigen-presenting cells. Consistent with their underlying immunosuppression, the proliferative response of CD4+ T lymphocytes to mitogens or JCV antigens is reduced in PML patients. CD8+ cytotoxic T lymphocytes recognize intracellularly synthesized viral proteins that have been degraded into peptides through the endogenous pathway, and presented on MHC class I molecules at the surface of virus-infected cells. One of such JCV peptide, the $VP1_{p100}$ ILMWEAVTL, has been characterized as a cytotoxic T lymphocyte (CTL) epitope in HLA-A*0201+ PML survivors. Staining with the corresponding A*0201/JCV $VP1_{p100}$ tetrameric complex showed that $VP1_{p100}$-stimulated peripheral blood mononuclear cells (PBMCs) of 5/7 (71%) PML survivors had JCV-specific CTL, versus none of 6 PML progressors ($P = .02$). This cellular immune response may therefore be crucial in the prevention of PML disease progression and the tetramer staining assay may be used as a prognostic marker in the clinical management of these patients. *Journal of NeuroVirology* (2002) **8(suppl. 2),** 59–65.

Keywords: acquired immunodeficiency syndrome (AIDS); cytotoxic T lymphocyte (CTL); human immunodeficiency virus (HIV); JC virus (JCV); progressive multifocal leukoencephalopathy (PML)

Introduction

Recent advances in our understanding of the immune response against infectious agents have led to the development of new functional assays to assess it. These have been adapted to shed some light on the cellular immune response against JC virus (JCV).

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Immune response against infectious agents: Innate and adaptive

An effective immune response against infectious organisms requires the collaboration of both innate and adaptive immunity. Innate, or non–antigen-specific, immunity, is the first line of defense against pathogens, and is carried out mainly by macrophages, neutrophils, the complement system, and natural killer (NK) cells. However, of all of these, only activated NK cells are able to destroy virus-infected cells, whereas the others are more efficient in fending off bacterial and parasitic infections. Adaptive, or antigen-specific, immunity is carried out by B

and T lymphocytes, working in a complementary manner. Although B lymphocytes produce antibodies to neutralize free virus in fluid phase, T lymphocytes can kill infected cells before virus maturation has occurred, thereby decreasing the release of infectious virus and preventing further cell-to-cell transmission.

Humoral immune response

Antibodies recognize intact viral proteins in the extracellular space, and therefore can prevent binding of the virus to its target cells (neutralizing antibodies), and facilitate elimination of opsonized virus by mononuclear phagocytes. Antibodies can also recognize viral proteins on the surface of the infected cells, and mediate antibody-dependant cell-mediated cytotoxicity (ADCC). This has been commonly identified *in vitro*, but its contribution *in vivo* is still unknown. Because viruses are obligate intracellular organisms, they escape to the control of antibodies, and usually delay the display of cell-surface proteins until the moment when viral maturation has occurred and ADCC would simply release infectious virions. Therefore T cells are the most important component of the immune response against viruses once they have already entered the cells.

Cellular immune response

T cells are subdivided into CD4+ cells, which are mostly helper cells, and CD8+ cells, the majority of which are cytotoxic T lymphocytes (CTLs). Both harbor T-cell receptors (TCRs), which are crucial for antigen recognition. Unlike antibodies, T cells do not recognize intact viral proteins, but viral peptides processed by the cells and presented to the TCR in association with a major histocompatibility complex (MHC) molecule. The concept of MHC restriction is central to the understanding of cellular immunity. As MHC molecules are the quintessential cellular signature of the "self," it is the "self" modified by a viral antigene that triggers the cellular immune response, and not the presence of the viral antigen alone. Because different individuals have different combination of MHC molecules, they will present different parts of viral proteins, or viral "epitopes" to the T cells and therefore mount a T-cell response of different epitope specificities. Figure 1 illustrates antigen processing pathways to CD4+ and CD8+ T cells.

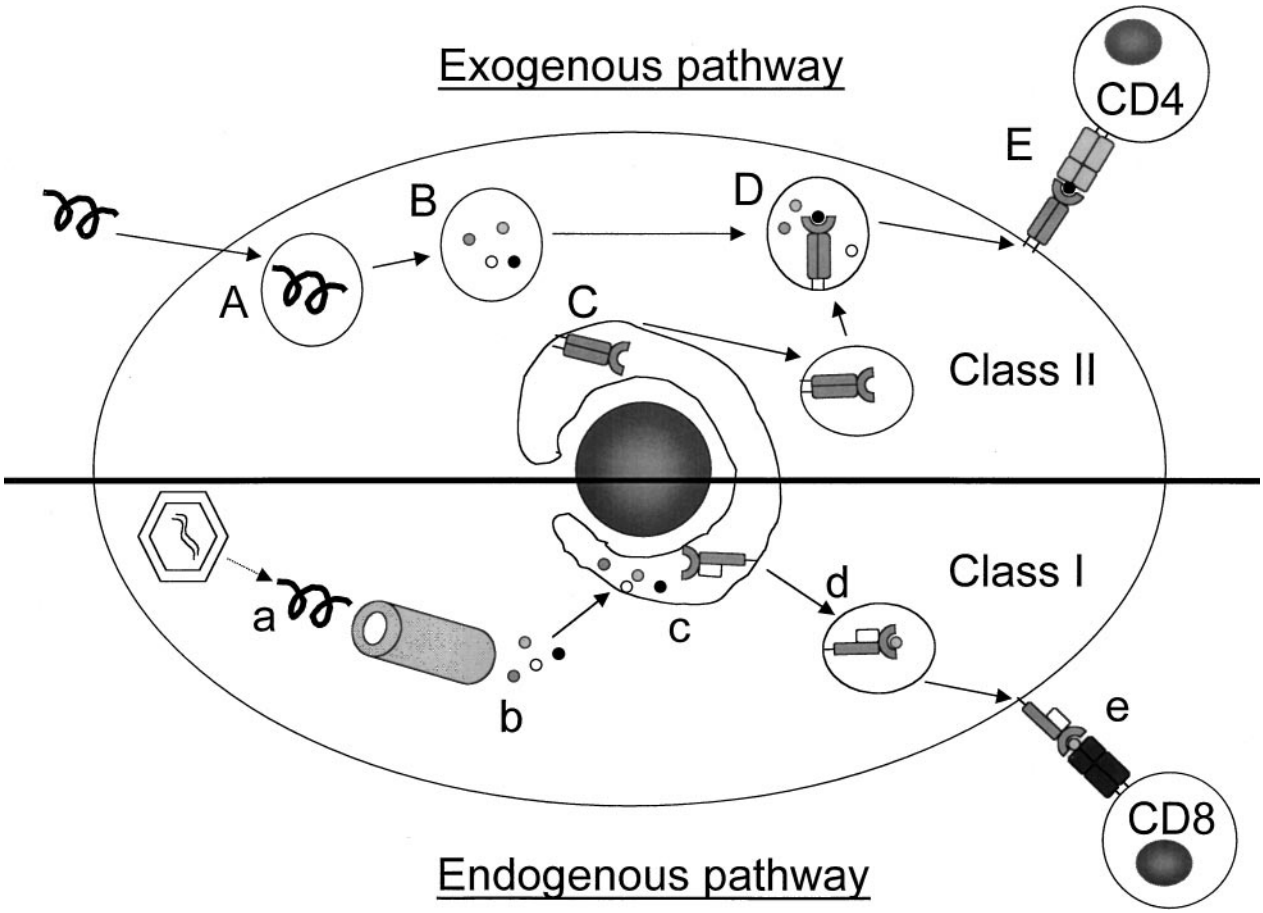

Figure 1 Exogenous and endogenous antigen-processing pathways. Antigen presentation via class II (A–E) and class I (a–e) pathways are represented (see text for details). Modified after Whitton JL *et al*, *Fields Virology*, 4th ed.

Role of CD4+ T cells

CD4+ cells, whose role is mainly to stimulate macrophages and CD8+ cells (T_H1 response) or B cells (T_H2 response) by producing specific cytokines, recognize viral epitopes presented on MHC class II molecules, which are expressed solely by specialized antigen-presenting cells (APCs) (Figure 1). APCs, which include macrophages, B cells, and dendritic cells, internalize foreign antigens by endocytosis (A). These antigens are then degraded into peptides by proteases in an acidic environment (B). MHC class II molecules synthesized in the endoplasmic reticulum (ER) (C) are modified as they migrate through the Golgi and the class II vacuoles fuse with the endosome containing the peptides. There, one viral peptide, usually 9 to 15 amino acids long, can bind to the groove of a class II molecule based on chemical affinity (D). The mature complex migrates to the cell surface and can then be recognized by the TCR of the CD4+ T cells (E). Because antigen presentation on MHC class II molecules involves the processing of extracellular proteins, it has been called the "exogenous pathway." Stimulated CD4+ cells will then proliferate and produce cytokines such as interferon (IFN)-γ, granulocyte-macrophage colony-stimulating factor (GM-CSF), and tumor necrosis factor (TNF)-α (T_H1 cells) or interleukin (IL)-4, IL-5, and IL-10 (T_H2 cells).

Therefore, CD4+ T lymphocyte function is usually tested *in vitro* by their capacity to proliferate in presence of polyclonal mitogens or specific antigens such as phytohemagglutinin (PHA) or viral proteins, respectively. Such assays are based on the incorporation of [^{3}H]-thymidine into DNA. Actively dividing cells will incorporate more [^{3}H]-thymidine than nondividing cells, and a stimulation index is calculated as the ratio between the radioactivity measured in antigen-stimulated and nonstimulated culture samples. However, these assays reveal little on the functional capacities of the responding T cells. Hence, functional assays for CD4+ cells have been developed that involve the measurement of the type and amount of cytokines produced by the cells in response to a specific or nonspecific stimulation by an extracellular antigen. The readout of these assays can be done either by enzyme-linked immunosorbent assay (ELISA) or enzyme-linked immunospot (ELISPOT) in the supernatant, or by intracellular cytokine staining followed by fluorescence-activated cell sorter (FACS) analysis.

Role of CD8+ T cells

CD8+ T lymphocytes, whose role is mainly to kill virus-infected cells, recognize viral epitopes bound to MHC class I molecules, which are produced by most nucleated cells (Figure 1). This occurs when a newly synthesized viral protein (a) gets tagged for destruction in the cytoplasm of an infected cell and is degraded into peptides by the proteasome (b). The viral peptides are then transported to the ER where they meet the heterodimeric MHC class I complex comprising the class I protein and the β2-microglobulin (c). There, one viral peptide, usually 8 to 10 amino acids long, can bind to the groove of an MHC class I molecule on the basis of chemical affinity (d). This complex will then migrate trough the Golgi apparatus towards the cell surface where it can be recognized by the TCR of the CD8+ CTL (e). Because antigen presentation on MHC class I molecules involves the processing of proteins synthesized inside the cell, it has been called the "endogenous pathway." Stimulated CTL will then produce perforin and granzymes that induce pore formation in the membrane of the infected cell and degrade its DNA, respectively, as well as INF-γ and TNF-α. This results in the destruction of the virus-infected cell, which can occur early in the viral replication cycle, before full maturation of a viral progeny.

Hence, the function of CD8+ CTLs is usually tested *in vitro* by their capacity to destroy autologous cells producing viral antigens. Such assays necessitate that the viral protein of interest be expressed by a vaccinia virus recombinant in an autologous B-lymphoblastoid cell line (B-LCL), used as target cells. Peripheral blood mononuclear cells (PBMCs) from the patient are usually stimulated *in vitro* with autologous fixed B-LCL expressing the viral protein of interest. They are then placed in contact with live autologous vaccinia-infected B-LCL cells pulsed with ^{51}Cr. The amount of radioactivity measured in the supernatant is proportional to the killing of the target cells by the effectors. The percent of specific lysis, given at various effector to target ratio (E:T), is calculated as the difference between the specific release of radioactivity from autologous target cells expressing the viral protein of interest and that of autologous control cells infected with wild-type vaccinia virus alone. This type of functional lysis assay is time-consuming and labor-intensive, and is therefore not appropriate for studying the cellular immune response in the setting of a rapidly progressive disease.

MHC class I/viral peptide tetrameric complexes

Once a viral epitope has been characterized, however, epitope-specific CTLs can be more readily detected by staining with an MHC class I/viral peptide tetramer complex. Tetramers are constructed by expressing *in vitro* the heavy chain of the MHC class I molecule of choice and the β2-microglobulin (Figure 2). These proteins are folded with the synthetic viral epitope peptide to form a momomer. Monomers are then biotinylated and mixed with avidin, a molecule with four sites that bind biotin with extremely high affinity, leading to tetramer

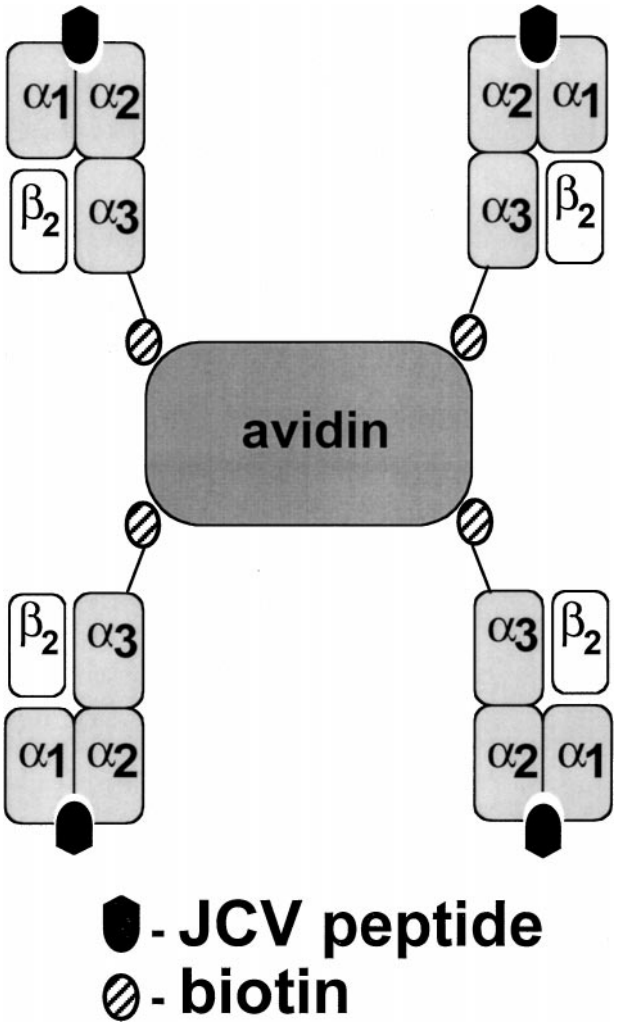

Figure 2 MHC class I/JCV peptide tetrameric complex. The JCV peptide sits in the groove of the MHC class I molecule (subunits $\alpha 1$ to $\alpha 3$), which is bound to the $\beta 2$-microglobulin ($\beta 2$), forming a monomer. Four of these monomers have been linked to a fluorochrome-tagged molecule of avidin by biotinylation, inducing the tetramer structure.

formation. Because the avidin is tagged with a fluorochrome, the tetramer can now be used as a soluble probe to stain epitope-specific CTL in fresh blood or in cultured cell samples, and quantify them easily by flow cytometry (Altman *et al*, 1996). Initially, most studies have focused on epitopes presented on the A*0201 molecules, because it is the most frequent class I allele, which is expressed approximately in 40% of people across ethnic groups in North America (Krausa *et al*, 1995).

Immune response against JCV

Which component of the immune response is most important against viruses? Antibodies alone are sufficient to prevent or modify diseases caused by hepatitis A and B viruses, poliovirus, measles virus, and varicella-zoster virus. This has led to successful vaccines that confer long lasting immunity. Antibodies-mediated neutralization of infectivity is especially advantageous in case of large amount of infectious virus being disseminated through the bloodstream. On the other hand, the presence of CTL memory response is particularly important against slow-replicating viruses that spread mainly by cell-to-cell contact without viremic phase, or those that cause persistent infection. This is indeed the case for JCV. Primary infection with JCV is asymptomatic and occurs in childhood, probably via an urine-oral route (Bofill-Mas *et al*, 2001; Bofill-Mas and Girones, 2001). Close to 90% of healthy adults have anti-JCV immunoglobulin G (IgG) (Weber *et al*, 1997). JCV then remains quiescent in the kidneys and the lymphoid organs, and approximately 30% of healthy individuals regularly excrete it in their urine. PML occurs as a reactivation of JCV in the setting of severe depression of the cellular immune response. Although an intrathecal synthesis of anti-JCV IgG has been demonstrated in 76% of patients, this was not associated with an improvement in their clinical outcome (Weber *et al*, 1997). Therefore, this humoral immune response is unable to prevent the development of PML, and modify the course of this disease.

What is then the role of the cellular immune response against JCV? Progressive multifocal leukoencephalopathy (PML) occurs in patients with depressed cellular immunity, such as in people with acquired immunodeficiency syndrome (AIDS), who have markedly decreased CD4+ T cell counts. Accordingly, these patients have been found to have a reduced CD4+ T cell response measured by cell proliferation assays after stimulation with PHA or JCV antigen, compared to normal controls (Knight *et al*, 1972; Marriott *et al*, 1975; Weber *et al*, 2001; Willoughby *et al*, 1980). Moreover, CD4+ T cells from PML patients showed a decreased production of INF-γ compared to healthy individuals after stimulation with PHA (Weber *et al*, 2001).

We have recently begun exploring the role of CD8+ CTL in patients with PML. We initially used vaccinia virus recombinant to express JCV T or VP1 proteins in autologous B-LCL, and we have shown that JCV antigen-stimulated PBMCs of human immunodeficiency virus (HIV)-infected patients who were survivors of PML had detectable JCV-specific CTL activity against these proteins. However, these findings were not definitive because the assay employed in this study was not quantitative, and the number of subjects studied was limited (Du Pasquier *et al*, 2001; Koralnik *et al*, 2001). In addition, this approach, which requires the use of radioactive isotopes, is time-consuming and labor-intensive, as it can take up to 2 months to derive a B-LCL from a patient with advanced AIDS and grow enough cells to perform a single experiment. Therefore, it is not suitable to study large groups of patients with a rapidly progressive disease such as PML.

These technical limitations can be circumvented if we have knowledge of the peptide epitope presented by the MHC class I molecules to the cytotoxic effector cells. Virus-infected cells are recognized by CTLs throught the interaction of a viral peptide presented in the groove of the HLA class I molecule on its surface and the TCR of the CTL. Class I molecules are divided into types A, B, and C, and each type has 13 to 32 different alleles, which can be further divided in as many as 20 subgroups. Because individuals receive a set of class I alleles from each parent, and because the groove of each class I molecule will only accommodate certain peptides, each individual will present viral antigens to the CTL in a different and unique fashion.

In this context, we have initially chosen to characterize the JCV epitope peptides presented to CTLs by the commonly expressed MHC class I molecule HLA-A*0201 for the following reasons. First, the A*0201 is the major subgroup of the HLA A2 allele, which is the most commonly expressed class I allele in approximately 40% of people across ethnic groups (Krausa *et al*, 1995). Second, the crystal structure of the A*0201 molecule has been established (Gao *et al*, 1997), and its interaction with peptide epitopes thoroughly studied (Garboczi *et al*, 1996). It has thus been demonstrated that the size of these epitopes is 9 amino acids, with the anchor residues at aa positions 2 and 9 being the most important. Third, based on this knowledge, computer predictive analysis have been created to estimate which 9–amino acid epitopes of any given protein is most likely to bind to the groove of the A*0201 molecule (Parker *et al*, 1994, 1995). Fourth, the usefulness of A*0201/viral peptide tetrameric complexes has recently been demonstrated in the study of the cellular immune response against HIV and simian immunodeficiency virus (SIV) (Altman *et al*, 1996; Kuroda *et al*, 1998; Ogg *et al*, 1998).

Using such an approach, we have tested 11 predicted nonamers from the T ($n = 5$), VP1 ($n = 4$), and VP2 ($n = 2$) proteins, for their ability to elicit a CTL response. The ^{51}Cr release assay employed is similar as the one described above, except that the stimulation of effector cells was performed directly with the JCV peptides in presence of IL-2, and the percent specific lysis was measured by the difference between the lysis of target cells pulsed by the JCV peptide of interest and an irrelevant control peptide. Of these 11 JCV nonamers, only 1, JCV VP1 p100–108 peptide ILMWEAVTL ($VP1_{p100}$), was recognized by CTL from three of the six HLA-A2+ HIV+/PML survivors evaluated. The cytolytic activity against target cells pulsed with the $VP1_{p100}$ peptide was MHC class I restricted (Koralnik *et al*, 2002).

We then used the $VP1_{p100}$ epitope peptide to create a tetrameric HLA-A*0201/JCV peptide complex. Fresh whole blood and cultured lymphocyte specimens from 24 HLA-A2+ study subjects were stained with the tetrameric HLA-A*0201/$VP1_{p100}$ complex, and analyzed by flow cytometry, gating on the CD8+CD3+ cells. We did not observe tetramer staining of CD8+ T cells from any of the fresh blood samples. However, the lymphocytes of four of six HIV+/PML survivors had between 2.1% and 13.5% $VP1_{p100}$-specific CD8+ T cells demonstrable after *in vitro* peptide stimulation (average 8%). Tetramer binding was also detected to 1.1% of CD8+ T cells of one HIV − /PML survivor, and to 5.7% and 6.0% of CD8+ T cells, respectively, from two HIV+ individuals with JCV-negative leukoencephalopathy of unknown etiology. No tetramer binding was detected in CD8+ cells of six HIV+ and HIV − /PML progressors, four other HIV+ patients without PML, and five healthy control subjects. A representative sample of tetramer-staining results is shown in Figure 3. There was no correlation between the percentage of tetramer-staining CD8+ cells and the duration of PML.

A good correlation was found between the functional lysis assay and the tetramer-staining assay, indicating that these were both measuring the same population of functionally active effector cells. However, the tetramer assay was found to be more sensitive, as illustrated by the fact that two patients had 1.1% and 2.1% tetramer-staining cells when the percent specific lysis at E:T 20:1 was below the cut-off of 10% generally used for this assay.

JC viremia was detected by polymerase chain reaction (PCR) in 3 of 6 PML progressors, but in none of the 7 PML survivors and 11 other study subjects. In addition, HIV+/PML survivors had a higher CD4 counts and lower HIV viral load than HIV+/PML progressors, indicating that JCV-specific immunity as well as immune reconstitution was associated with clearance of JCV from the blood.

Discussion

The characterization of the first CTL epitope of JCV in humans and the construction of the corresponding HLA A*0201/$VP1_{p100}$ tetrameric complex provide us with important insights on the cellular immune response against this virus. First, the tetramer-binding assay is quantitative. Staining of fresh blood cells was negative in all patients and controls, indicating that the frequency of $VP1_{p100}$-specific CTL was below 0.1% CD8+ T cells, which is the limit of detection of this assay. In peptide-stimulated PBMCs, PML survivors had between 1.1% and 13.5% tetramer-staining CD8+ T cells. The presence of $VP1_{p100}$-specific CTL was clearly associated with a better clinical outcome as these cells were detectable in 5 of 7 PML survivors versus 0 of 6 PML progressors (Fisher exact test, two-tailed: $P = .02$).

Are these cells truly instrumental in containing PML, or is their presence a mere consequence of longstanding stimulation by JCV? Indeed, PML survivors had improved or been stable 2 to 8.5 years after

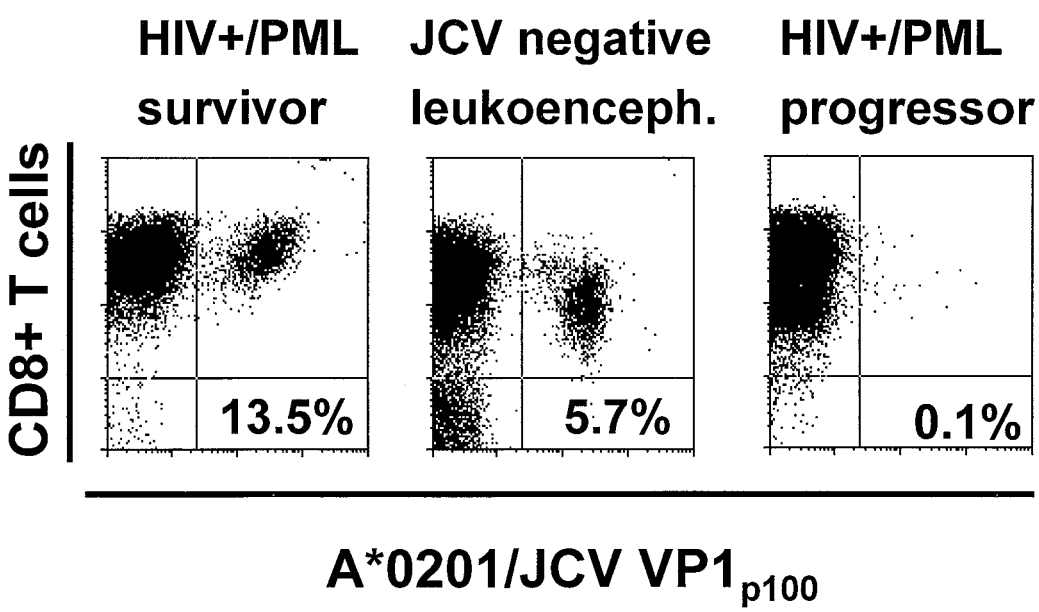

Figure 3 Dot plot of representative tetramer staining assay results in HLA-A*0201 individuals. Tetrameric HLA-A*0201/JCV $VP1_{p100}$ complex binds to a population of $VP1_{p100}$-stimulated CD8+ T lymphocytes in PBMCs of an HIV+/PML survivor and an HIV+ patient with JCV-negative leukoencephalopathy, but not of an HIV+/PML progressor. Cells analyzed by flow cytometry are represented by dots. *Y* axis: CD8+ cells. *X* axis: tetramer + cells. In the left upper quadrant of each panel are CD8+/tetramer−, whereas in the left lower quadrant are CD8−/tetramer− cells. The percentage of CD8+/tetramer+ cells located in the right upper quadrant of each panel is indicated.

disease onset. If the latter was true, one would expect to find a linear correlation between the percentage of tetramer-positive CD8+ T cells and the time elapsed since PML onset. This was not the case in our study. Indeed, the longest PML survivor had 1.1% tetramer-staining CD8+ T cells 8 years after the onset of PML. In other viral diseases, virus-specific CTLs are usually at their peak during acute infection, and tend to decrease when the disease enters a chronic or latent stage (Altfeld *et al*, 2001; Kalams *et al*, 1999). In this context, it is already remarkable to be able to find $VP1_{p100}$-specific CTLs in PML patients who have clinical and radiological evidence of an inactive, burnt-out disease, for so many years. Indeed, PML survivors usually have negative JCV PCR in their blood and cerebrospinal fluid (CSF), and their magnetic resonance imaging (MRI) show only atrophy in areas of demyelination and no new lesions overtime.

Conversely, PML progressors by definition have active brain disease, a high JC viral load in the CSF (Koralnik *et al*, 1999; Taoufik *et al*, 1998), and are most likely to have detectable JC viremia by PCR (Koralnik *et al*, 2002). Therefore, the absence of $VP1_{p100}$-specific CTLs in these individuals is significant, especially because most of them were studied early after the onset of PML, and had a fatal outcome within 5 months. We are currently following a group of PML patients whose anti-JCV cellular immune response was tested soon after PML onset and who are still alive. Preliminary results suggest that a temporal association exists between the appearance of JCV-specific CTLs and a favorable clinical evolution of PML.

Because most healthy individuals are infected with JCV, and if JCV-specific CTLs are instrumental in the prevention of disease progression, one could wonder why we did not find $VP1_{p100}$-specific CTLs in any of the five HIV-negative normal controls subjects tested. JCV VP1 is a 354–amino acid protein and only the four highest ranked nonamer CTL epitope candidates were tested in this study. The yield of the computer predictive analysis was only 9%, which indicates that the nonamers most likely to bind to the groove of the A*0201 molecule are not necessarily the most immunogenic *in vivo*. In addition, the specificity of this approach is unknown. Therefore, it is possible that other A*0201-restricted CTL epitopes exist on the VP1 protein, which may be recognized by the CTL of healthy individuals. A CTL epitope–mapping study of the VP1 protein is now ongoing in our laboratory to address this issue. Alternatively, immunodominant CTL epitopes could also be present in the five other JCV proteins, or be restricted by alleles different than the A*0201.

Thus, the discovery of the $VP1_{p100}$ epitope is only a first step in our understanding of how JCV-infected cells are recognized by CTLs. This epitope is located at nucleotide positions 1766 to 1792 on the JCV genome, which is conserved among the different JCV genotypes at the amino acid level (Agostini *et al*, 1997). This indicates that the $VP1_{p100}$ epitope should not be affected by the geographic origin of the patients. In addition, because the coding region of JCV is extremely conserved, it is also unlikely that a CTL epitope escape mutant will arise at this level. Finally, the JCV $VP1_{p100}$ epitope has two amino

acid changes, at amino acid positions 1 and 9, compared to the corresponding amino acid sequence of the other human polyomavirus BK (BKV), making a cross-recognition between JCV and BKV unlikely (Tevethia *et al*, 1998). Therefore, this tetramer-staining assay may provide a useful prognostic tool in the clinical management of A*0201+ patients with PML.

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