CD8+ Cytotoxic Lymphocyte Responses against Cytomegalovirus after Liver Transplantation: Correlation with Time from Transplant to Receipt of Tacrolimus

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The effects of the immunocompromised state after liver transplantation on the frequency of cytomegalovirus-specific cytotoxic T lymphocytes (CTL) were investigated in 93 patients by using HLA class I tetrameric complexes corresponding to HLA-A*0201, HLA-B*0702, HLA-B*0801, and HLA-B*3501 refolded with peptides from the ppUL83 matrix protein. ppUL83 CTL frequencies were suppressed during the first 6 months after transplantation. Patients with HLA-restricted response detected had high correlation among ppUL83 CD8+ CTL frequencies restricted by different HLA haplotypes (Spearman’s ρ = .67; P < .0001). There was an inverse correlation among levels of the calcineurin inhibitor, tacrolimus, and ppUL83 CD8+ CTL frequencies (r = −.31; P = .005), which is consistent with the presence of a large proportion (70%) of activated (CD38+) ppUL83 CD8+ CTL within the population of HLA class I tetramer-positive cells.

Human cytomegalovirus (CMV), a beta-herpesvirinae, infects ~60% of adults in the developed world and ~90% of third world populations [1]. In the immunocompetent host, initial infection and reactivation of latent infection are usually asymptomatic. However, in hosts with impaired cellular immune functions, such as transplant recipients and persons infected with human immunodeficiency virus (HIV), the full pathogenic potential of the virus may be realized [1, 2]. Investigations over many years indicate that CMV load is the critical factor in pathogenesis: symptomatic patients exhibit elevated levels of CMV replication, compared with those in persons who remain asymptomatic [3–10]. Similar to other viral infections, CMV replicates dynamically in the human host, with a doubling time of ~1 day [11]. We recently showed that early replication kinetics during an active infection are predictive of future outcome and can be used to identify patients destined to reach virus loads that result in pathology [12].

Despite major advances in our understanding of the virologic factors that contribute to CMV pathogenesis in the immunocompromised host, the role of CMV-specific immune function is relatively poorly understood. Nevertheless, studies of the effects of augmented immunosuppression to control organ rejection provide insight into immune effector mechanisms. For example, depletion of T cells through the administration of antithymocyte globulin increases the probability of CMV disease by increasing CMV load [9]. In contrast, methylprednisolone, which affects both T cell function and monocyte/macrophage differentiation, augments the risk of CMV disease at a given CMV load rather than by increasing CMV replication per se [4]. Taken together, these data identify an important role for specific T cell functions in the suppression of CMV replication and implicate tissue macrophages as potential contributory factors in modulating organ-specific CMV disease. Consistent with the importance of T cell function in suppressing CMV replication, data from patients undergoing bone marrow transplantation show that CD8+ cytotoxic T lymphocytes (CTL) against CMV can protect against CMV disease, whether they occur naturally or are infused as part of experimental immunotherapy [13–15].

In recent years, tacrolimus (FK506) has been increasingly used in immunosuppressive regimens [16, 17]. Tacrolimus binds to FKBP-12 and functions as a calcineurin inhibitor, to reduce interleukin-2 production and to antagonize tumor growth factor-β, which results in a reduction in T cell activation. Although the effects of tacrolimus on CD4 T cell activation and rejection are well documented [16, 17], there are few data on its effects on CD8+ T cell responses against specific pathogens such as CMV.

The CTL response against human CMV is dominated by the lower matrix protein, pp65 (ppUL83) [18–20], although responses...
against the immediate early (IE)–1 gene product (UL122) also may be important [21]. Studies that used a ppUL83 deletion mutant have shown that 70%–90% of the CMV-specific CD8+ CTL response is ppUL83 specific, with lesser contributions from glycoprotein B (gpUL55) and IE-1 (UL122) [20]. Epitope mapping has identified HLA class I-restricted peptides within ppUL83 for HLA-A2, HLA-B7, HLA-B8, and HLA-B35 [19]. By limiting dilution CTL killing assays with HLA-matched targets, the frequency of ppUL83-specific precursor CTL in healthy subjects ranges from 1:5000 to 1:12,000 [18]. However, the recent development of HLA class I tetramer complexes allowed for a reappraisal of the data obtained from classical limiting dilution assays and showed that the frequency of CD8+ CTL in many viral infections has been underestimated [22–24]. For example, during acute Epstein-Barr virus (EBV) infection, virus-specific CD8+ CTL can reach frequencies of up to 40% of the CD8+ population [22], and, in the case of CMV, CD8+ CTL against ppUL83 have been found at frequencies of 1%–10% in small numbers of immunocompetent and HIV-infected persons [25, 26].

The availability of HLA class I tetramer technology allows for the investigation of the effects of immunosuppression at various times after organ transplantation on the specific CD8+ CTL responses against CMV and their interrelationship with virologic markers [27]. In this study, we analyzed a group of liver transplant recipients with HLA tetramers specific for HLA-A*0201, HLA-B*0702, HLA-B*0801, and HLA-B*3501-restricted epitopes in ppUL83 and correlated the frequency of ppUL83 CD8+ CTL with time from transplantation, appearance of active CMV infection, and receipt of tacrolimus.

Materials and Methods

Study population. A prospective study for the detection of CMV-specific CTL responses using HLA tetramers was carried out on 93 liver allograft recipients who had undergone transplantation between November 1988 and December 1999. Patients in the study had 1 (n = 74) or more (n = 19) of the following HLA types: HLA-A*0201, HLA-B*0702 (9 samples), HLA-B*0801 (7 samples), and HLA-B*3501 (15 samples). The CMV serostatus of all persons was determined by BioELISA CMV IgG assay (Biokit).

Immunosuppressive therapy. The immunosuppressive regimen after liver allograft at the Royal Free Hospital (London) is individualized according to the underlying clinical condition. Patients who had undergone transplantation for viral hepatitis (HBV or HCV) or alcoholic liver cirrhosis received tacrolimus starting 6 h after transplantation, either orally (100–200 μg/kg/d) or by intravenous (iv) infusion over 24 h (10–50 μg/kg). Maintenance doses were adjusted according to blood concentration of tacrolimus (5–15 ng/mL) and clinical response. Patients who underwent transplantation for autoimmune hepatitis, PBC, or PSC received triple immunosuppressive therapy that began on the day of transplant (day 1), which consisted of tacrolimus (100 μg/kg/d orally), azathioprine (1 mg/kg/d iv or orally), and methylprednisolone (16 mg/d iv or 20 mg/d prednisolone orally).

Routine liver biopsies were performed on posttransplant day 5 or 6 or when clinically indicated. Histologically identified rejection was treated with 1 g/d (iv) methylprednisolone on 3 consecutive days, followed by liver biopsy to document response. Methylprednisolone-resistant rejection episodes were treated with either azathioprine (1 mg/kg/d iv or orally) or mycophenolate mofetil at 2 g/d.

Construction of HLA class I–CMV ppUL83 peptide tetrameric complexes. Four different CMV-specific tetramers were constructed for HLA-A*0201, HLA-B*0702, HLA-B*0801, and HLA-B*3501, as described elsewhere [28], by using the appropriate ppUL83-derived immunogenic peptides (NLVPVMTAV, TRPTVGGGA, DANDIYRF, and IPSINVHHY, respectively). In brief, the peptides were refolded with recombinant β2-microglobulin and the respective recombinant heavy chain bearing an engineered biotinylation site. After refolding and purification, the monomeric HLA peptide complexes were converted into tetramers by adding phycoerythrin-labeled streptavidin at a molar ratio of 1:4.

PBMC preparation and staining for FACS analysis. Fresh PBMC from HLA-A2–, HLA-B7–, HLA-B8–, and HLA-B35–positive liver transplant patients and healthy control subjects were purified from heparinized blood (20 mL) by centrifugation through Ficoll-Paque (778 g, 25 min; Amersham-Pharmacia Biotech). PBMC were washed 3 times (5 min, 437 g, 25°C) in RPMI 1640 (Sigma) supplemented with 2 mM glutamine, 50 U/mL penicillin, and 50 μg/mL streptomycin and were resuspended in 10 mL of 10% fetal bovine serum (FBS) in RPMI and then were counted. When necessary, cells were frozen in 1-mL aliquots of between 2 × 10^6 and 5 × 10^6 cells/mL in 50% FBS and 10% dimethyl sulfoxide in RPMI and were placed in a freezing box at −80°C, which allowed for the cells to cool at 1°C/min. Once the cells had frozen, the vials were transferred to liquid nitrogen for long-term storage. The influence of cryopreservation of samples was investigated: freezing samples at a controlled rate did not affect the viability of the cells, and data obtained from fresh and frozen samples were comparable. For double staining, ~2 × 10^5 PBMC in 10% FBS (in RPMI) were added to FACS tubes, were washed with PBS/0.1% sodium azide, and were pelleted (5 min, 437 g, 25°C). We added 0.3 μg (in
Table 1. Tetramer staining of healthy seropositive subjects.

<table>
<thead>
<tr>
<th>HLA class I (CMV ppUL83 peptide tetramer)</th>
<th>Frequency of ppUL83 CD8⁺ CTL using tetramers against</th>
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<tr>
<td>HLA-A*0201</td>
<td>HLA-B*0702</td>
</tr>
<tr>
<td>(CMV ppUL83 peptide tetramer) (n)</td>
<td>(CMV ppUL83 peptide tetramer) (n)</td>
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<tr>
<td>HLA-A2 (NLVPMVATV) (57)</td>
<td>2.31 (0.02-6.19)</td>
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<tr>
<td>HLA-B7 (TPRVTGGGA) (6)</td>
<td>&lt;0.001</td>
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<tr>
<td>HLA-B8 (DANDYRIF) (2)</td>
<td>0.135 (0.04-0.24)</td>
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<tr>
<td>HLA-B35 (IPSINVHYH) (13)</td>
<td>0.115 (0.11-0.12)</td>
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<td>&lt;0.001</td>
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NOTE. Data are median percentages (range). "<0.001" refers to frequencies below the detection limit of the assay CMV, cytomegalovirus; CTL, cytotoxic T lymphocytes; HLA, human leukocyte antigen; n, no. of samples analyzed.

PBS) of the HLA-A2 and HLA-B35 CMV tetramers or 1 µg (in PBS) of the HLA-B7 and HLA-B8 tetramers to each tube, and the tubes were immediately incubated for 20 min at 37°C. Then 1.5 µL of an anti-human TriColor-labeled CD8⁺ antibody (Caltag Laboratories) was added. Tubes were kept on ice and protected from light for 20 min. After incubation, the cells were washed with PBS/0.1% sodium azide (5 min, 437 g, 4°C) and were resuspended in 200 µL of fixing solution (PBS and 0.4% formalin). Cells thus stained were kept at 4°C and were protected from light until FACS analysis. For triple staining, 3 µL of a second antibody against CD38, CD45R0, HLA-DR, or CD45RA (Immunotech) labeled with fluorescein isothiocyanate was added at the same time as the anti-CD8⁺ antibody and was incubated and fixed in the same fashion. All analyses were done in quadruplicate, and mean values were used for further comparisons.

Cell sorting. PBMC were stained as described for FACS analysis, replacing PBS/0.1% sodium azide for sterile filtered 2% FBS (in PBS). After the final wash, cell pellets were resuspended in 200 µL of sterile filtered 2% FBS and were kept at 4°C. Exclusion cell sorting was done in a FACSCalibur cell sorter (Becton Dickinson). Sorted cells were resuspended in 10% FBS (in PBS) and were recovered by centrifugation (437 g, 20 min) at 4°C.

Enzyme-linked immunospot (ELISPOT) for interferon (IFN)-γ-producing cells. A multiscreeb 96-well filtration plate (Millipore) was coated with 50 µL of a 1:66 dilution of anti-human IFN-γ 1-DIK antibody (Mabtech) and was incubated overnight at 4°C. The following day, 1 x 10⁵ to 2 x 10⁵ PBMC from patients or healthy seropositive control subjects or 100–200 tetramer-positive (Tet⁺) sorted cells (see above) were added to each well. For PBMC, we added 50 µM of peptide; for Tet⁺ sorted cells, 10⁶ antigen-presenting cells (Tc cells that express HLA-A2 and SC10, an HLA-B8/B35 B cell line) were pulsed with 20 µM of relevant peptide for 1 h at 37°C, were washed once in RPMI, and were used as antigen-presenting cells at 10⁵ cells/well. Controls included an irrelevant peptide or no peptide. A second biotinylated antibody against IFN-γ (monoclonal antibody 7-B6-1-biotin; Mabtech) was added after overnight incubation, and the assay was developed by using streptavidin-ALP (Mabtech) and an AP conjugate substrate kit (50 µL/well; Bio-Rad Laboratories). The resulting spots were counted by dissecting microscope (Leica GZ6).

DNA extraction and quantitative-competitive PCR for CMV. Patients were tested for CMV viremia by qualitative PCR on each day a blood sample was collected for PBMC purification. DNA was extracted from 200 µL of whole blood by commercial kit (Quagen), according to the manufacturer’s instructions. Subsequent qualitative and quantitative competitive PCR amplifications for the detection and quantification of CMV viremia were done, as described elsewhere [8, 9], with minor modifications, to allow for the use of nonradioactive detection methods.

Statistical analyses. Between-group differences were analyzed by nonparametric Mann-Whitney U test. Correlations between groups were done by Spearman test. P ≤ .05 (2-sided) was regarded as significant.

Results

Evaluation of specificity of ppUL83-specific HLA tetramers. HLA class I heavy chains corresponding to A*0201, B*0702, B*0801, and B*3501 were synthesized and were refolded with the appropriate CMV ppUL83 peptide and β2-microglobulin, and tetramer formation effected, as described in Materials and Methods. We used a panel of PBMC (n = 96) isolated from 11 CMV-seropositive and 12 CMV-seronegative healthy volunteers to assess the performance characteristics of each tetramer.

Having shown that each ppUL83 tetramer could identify HLA class I-restricted ppUL83-specific CD8⁺ CTL in normal persons, we used the tetramers to assess the frequency of CTL directed against this protein in 93 liver transplant patients recruited over a 1-year period. CD8⁺ frequency of CTL in normal persons was summarized in table 1 for all HLA types analyzed and ranged from 0.02% to 6.2% (median, 0.84%). Of importance, the individual ppUL83 tetramers did not react with CD8⁺ CTL in any HLA-mismatched samples (table 1).

Frequency of CMV ppUL83-specific CD8⁺ CTL after liver transplantation. Having shown that each ppUL83 tetramer could identify HLA class I-restricted ppUL83-specific CD8⁺ CTL in normal persons, we used the tetramers to assess the frequency of CTL directed against this protein in 93 liver transplant patients recruited over a 1-year period. CD8⁺ CTL frequencies in samples (n = 261) from these patients were stratified into 5 groups for these analyses (before transplantation; 0–6 months, with and without CMV viremia; and >6 months after transplantation, with and without CMV viremia; figure 1).

The median frequency of ppUL83 CTL before transplantation was 0.61%, which was comparable with the frequencies observed in healthy persons (median frequency, 0.84%). ppUL83-specific CTL frequencies were significantly lower in samples from patients with and without viremia during the first 6 months after transplantation (0.19% and 0.16%, respectively), compared with frequencies in the 2 comparable groups after 6 months (0.54% and 0.36%, respectively; figure 1).
Seventeen patients possessed 2 HLA class I types amenable to analysis by the ppUL83 tetramers. Thus, the frequencies obtained for each ppUL83 HLA-restricted epitope could be correlated in the 73 PBMC samples derived from these patients. The results showed that the frequencies of ppUL83 CD8<sup>+</sup> CTL identified for each HLA-restricted tetramer within the same sample were highly correlated ($r = .67; P < .0001$). Furthermore, there were no statistically significant differences in the median frequency of ppUL83 CD8<sup>+</sup> CTL restricted by the different HLA types (HLA-A*0201, 0.22% [n = 80]; HLA-B*0702, 0.22% [n = 40]; HLA-B*0801, 0.33% [n = 36]; HLA-B*3501, 0.12% [n = 32]) for the patients who did not experience CMV viremia.

Functional analysis of ppUL83 CTL. To confirm that these ppUL83-specific CD8<sup>+</sup> CTL in the liver transplant recipients were functional, a randomly chosen subset of samples (n = 47) was subjected to a CD8<sup>+</sup>-specific ELISPOT assay, and results were compared with the CD8<sup>+</sup> CTL frequencies obtained by using the ppUL83 specific tetramers on the same samples. Despite the immunosuppressive therapy received by these patients, functional CD8<sup>+</sup> CTL could be detected in the majority of patients, and the frequencies obtained by the 2 methods were significantly correlated ($P = .004$). However, the absolute frequency of ppUL83-specific CTL by the ELISPOT assay (median, 0.12% of CD8<sup>+</sup> T cells) was significantly lower than the frequencies obtained by the HLA tetramer analysis (median, 0.82% of CD8<sup>+</sup> T cells; $P = .001$). Stratification of these samples, according to the time from transplantation, showed that the ELISPOT procedure yielded a CD8<sup>+</sup> CTL frequency that was the same regardless of the time from transplantation (median frequencies, 0.16% for <6 months vs. 0.11% for >6 months; $P$ value was not significant). In contrast, the frequencies of CD8<sup>+</sup> CTL by the ppUL83 tetramer approach were significantly higher at later time points after transplantation (median frequencies, 0.56% at <6 months vs. 2.3% at >6 months; $P = .01$; figure 2).

Further characterization of the functional capacity of the ppUL83-specific CTL was done after cell sorting of the tetramer-positive population. Analysis of 2 healthy subjects and 2 liver transplant patients (underwent transplantation many years previously) without active CMV infection (i.e., CMV PCR negative in blood) showed that an average of 6.8% of the ppUL83
tetramer staining: 0–6 m, 0.56%; ELISPOT: frequencies are indicated by horizontal line (ELISPOT: 0–6 m, 0.16%; (HLA-A*0201, HLA-B*0702, HLA-B*0801, and HLA-B*3501). Median transplantation and combine data from all 4 HLA-restricted tetramers on same samples derived from patients at 0–6 and autologous peptide-pulsed T2 cells (for HLA-A*0201) or SC10 produce IFN-γ. A population of cells in the transplant recipients were able to tetramer population in healthy persons and 7.3% of the same frequency was observed (®gure 3 B).

Figure 2. Frequency of ppUL83-specific CD8+ cytotoxic T lymphocytes identified by enzyme-linked immunospot (ELISPOT) assay for interferon-γ or ppUL83-specific HLA tetramers. Both analyses were done on same samples derived from patients at 0–6 and >6 months (m) after transplantation and combine data from all 4 HLA-restricted tetramers (HLA-A*0201, HLA-B*0702, HLA-B*0801, and HLA-B*3501). Median frequencies are indicated by horizontal line (ELISPOT: 0–6 m, 0.16%; tetramer staining: 0–6 m, 0.56%; ELISPOT: >6 m, 0.11%; tetramer staining: >6 m, 2.32%).

Longitudinal analysis of ppUL83 CTL and virologic markers. In the majority of patients, multiple blood samples were available for analysis of CTL directed against ppUL83. In addition, the results of qualitative and quantitative CMV PCR allowed for a superimposition of the virologic data on the immunologic parameters. These data then were analyzed in the context of immunosuppressive therapy. Figure 3 shows a representative group of 6 patients. In patients who remained CMV PCR-negative in blood (figure 3A), there was a marked fluctuation in the levels of ppUL83 CTL over time, despite no evidence of active CMV infection (CMV load <200 genomes/mL of blood). The median level of CD38 expression (as a marker of activated CD8 cells) in these cells was 70% (range, 0%–100%). In patients who experienced active CMV infection (CMV load >200 genomes/mL blood; n = 11), a similar variation in ppUL83 frequency was observed (figure 3B), although a temporal relationship between CMV load and ppUL83 CTL was apparent in all cases. In some patients (patients D and F), the frequency of ppUL83 CTL increased before the elevation in CMV load, whereas, in other patients, this sequence was coincident or reversed. Nevertheless, the median time between peak ppUL83 CD8+ CTL frequency and peak CMV load was 0 days (range, –28 to +30 days). When multiple HLA class I-restricted epitopes were analyzed simultaneously, there was good concordance between the frequencies of the 2 ppUL83 CTL populations (see profiles for patients A and B).

The phenotype of the ppUL83-specific CTL present during the periods of active infection was determined by FACS analysis with antibodies against CD38, CD45-RO, CD45-RA, and HLA-DR. The ppUL83 CD8+ CTL populations identified by tetramer staining showed high levels of activation (median level of CD38 cells, 82.5%; median level of HLA DR+ cells, 100%). The majority of ppUL83 CD8+ CTL also possessed a memory phenotype (median CD45RO level, 98.5%), although naive cells also were observed (median CD45-RA level, 53.5%). In contrast, ppUL83 CD8+ CTL from immunocompetent persons exhibited low levels of CD38 staining (median, 4.8%; range, 4.3%–5.3%).

Effects of tacrolimus on ppUL83 CD8+ CTL frequencies. As illustrated in figure 3, an association between tacrolimus levels and the frequency of ppUL83 CTL was apparent. Consequently, we analyzed this relationship in more detail by using correlation methods in patients who remained CMV PCR-negative during follow-up. The data shown in figure 4 illustrate an inverse relationship between tacrolimus levels and ppUL83 CD8+ CTL (r = −.31; P = .005). This effect was most apparent with frequency of ppUL83 CD8+ CTL, but there was also a correlation between absolute numbers of ppUL83-specific CD8+ CTL and quantity of tacrolimus (r = −.18; P = .05). In contrast, there was no correlation between CD8+ cell percentage (as a proportion of the total lymphocyte population) and quantity of tacrolimus.

Discussion

The direct detection of CD8+ CTL using HLA tetramers allows for a reappraisal of this component of the immune system and its role in the control of viral infections. We used HLA tetramers corresponding to HLA-A*0201, HLA-B*0702, HLA-B*0801, and HLA-B*3501 refolded with epitopes from the CTL immunodominant ppUL83 protein of human CMV to assess the impact of liver transplantation on the frequency of CD8+ CTL. Previous studies showed that liver transplant recipients are at an increased risk of CMV infection and disease as a consequence of the immunosuppression required to maintain the allograft in the new host and the administration of augmented immunosuppression to control acute rejection episodes [4, 29, 30]. We show that each ppUL83 tetramer can specifically detect a population of ppUL83-specific CD8+ CTL in healthy CMV-seropositive persons that did not react with CD8+ cells from CMV-seronegative or HLA-mismatched control subjects, and we used these reagents to study ppUL83 CTL in multiple PBMC samples derived from 93 liver transplant patients.

The results revealed that the frequency of ppUL83 CD8+ CTL during the first 6 months after transplantation was suppressed, compared with that in patients before transplantation or in patients who underwent transplantation >6 months pre-
Figure 3. Relationship of ppUL83-specific CD8+ cytotoxic T lymphocytes (CTL) with virologic and immunologic markers. A, Representative longitudinal profiles of ppUL83-specific CD8+ CTL in patients without cytomegalovirus (CMV) viremia: patient A (□, HLA-B7; ▲, HLA-B8), patient B (●, HLA-A2; ○, HLA-B35), and patient C (●, HLA-A2). B, Patients with active CMV replication >200 genomes/mL blood: patient D (●, HLA-A2), patient E (●, HLA-A2), and patient F (▲, HLA-B8). CD8+ CTL frequencies are shown with plasma levels of tacrolimus (■, ng/mL) and blood CMV load (B; △, log10 genomes/mL of blood).

Previously, indeed, even among patients with active CMV infection, ppUL83 CD8+ CTL responses were suppressed at early time points after transplantation. The frequencies of ppUL83 CD8+ CTL observed in the present study are comparable with frequencies reported in other smaller studies [25, 26, 31, 32]. However, unlike other studies that used only 1 ppUL83 HLA class I tetramer [25, 26, 31, 32], we used 4 different HLA class I tetramers and thus could correlate the frequency of CD8+ CTL among different class I alleles in patients possessing combinations of HLA-A*0201, HLA-B*0702, HLA-B*0801, and HLA-B*3501. The results showed that the frequencies determined for each allele were highly correlated (P < .0001), and the median frequencies observed were not significantly different between tetramers. It will be of interest to determine the relative frequencies of other HLA class I-restricted CD8+ CTL for which peptides within the ppUL83 have yet to be mapped and to correlate virus load with the number of HLA mismatches between donor organ and recipient.

As expected from other studies that used HLA tetramers, there was a significantly higher frequency of ppUL83 CD8+ CTL detected by using the tetramer reagent when compared with results obtained with peptide stimulation and detection of
by polymerase chain reaction for human cytomegalovirus. done on samples derived from patients consistently negative in blood liver transplantation. To minimize confounding variables, analysis was

CD8\(^{+}\) CD8\(^{+}\) CTL frequencies increased simultaneously with or after virus detection in blood. It is plausible that these fluctuations may reflect reactivation of virus with elevations in CD8\(^{+}\) CTL occurring before the appearance of virus in the blood. The investigation of such a phenomenon will require many more cases to be studied, including representatives of each donor/recipient serostatus group to separate those with past immune experience of this virus. Similar kinetics of CD8\(^{+}\) CTL and CMV load have been observed in liver transplant recipients by using an HLA-B7 CMV-specific tetramer [32].

In recent years, tacrolimus has become the agent of choice in immunosuppressive regimens after liver transplantation [14]. Although tacrolimus is used to reduce CD4 T cell activation, which is important in the rejection process, it also impacts on CD8\(^{+}\) CTL activation. Therefore, we were interested in determining the direct effects of tacrolimus on the frequency of ppUL83 CD8\(^{+}\) CTL after liver transplantation. The results showed a significant inverse correlation between tacrolimus levels and CMV-specific CD8\(^{+}\) CTL that was not accounted for by a general suppression in the relative proportion of CD8\(^{+}\) cells within the CD3 population. These data are consistent with the presence of low-grade active CMV replication that occurs in the immunocompromised state after transplantation (i.e., the presence of a significant proportion of activated T cells that would be inhibited by tacrolimus).

The level of activation of ppUL83 CD8\(^{+}\) CTL in nonviremic patients (70%) in the present study was significantly higher than in the same tetramer-positive population in healthy subjects (>5%) and in CD8\(^{+}\) cell populations of healthy subjects reported elsewhere [37]. However, data from clinical studies suggest that tacrolimus is not associated with an increased incidence of CMV infection or disease within liver transplant or renal transplant patients [38–41]. Thus, a paradox exists. If ppUL83 CD8\(^{+}\) CTL are inhibited by tacrolimus, why does CMV replication not continue in an uncontrolled fashion and cause high-level viremia and disease? A potential answer may relate to the levels of CD8\(^{+}\) CTL required to maintain suppression of CMV. We previously showed in liver, renal, and bone marrow transplant recipients that virus load thresholds exist above which the probability of disease accelerates rapidly [3, 4, 8, 9]. Extension of this concept to the T cell arm of the immune system suggests that reductions in the frequency of these effector cells could be sustained without giving rise to a substantial increase in viral replication; once the frequency falls below a critical level, the bias would be in favor of CMV replication. Of interest, augmentation of natural killer responses against CMV in mice treated with tacrolimus has also been documented [42]. Work will be required to investigate these possibilities further and to assess the utility of combining measures of viral dynamics and specific T cell responses to identify patients at risk of CMV disease [12, 43, 44].

In conclusion, we used HLA class I tetramers to investigate CMV-specific CD8\(^{+}\) T cell responses to 4 different HLA-re-
strict epitopes present within the ppUL83 protein of CMV. We found that their frequency is associated with time from transplantation, receipt of tacrolimus, and CMV load. Ongoing studies will provide further insight into the levels of CD8+ CTL required to maintain CMV replication below the levels needed for pathology.

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References


