Programmed Death–1 Expression in Liver Transplant Recipients as a Prognostic Indicator of Cytomegalovirus Disease

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Immunological parameters that distinguish solid-organ transplant (SOT) recipients at risk for life-threatening cytomegalovirus (CMV) disease are being actively pursued to aid posttransplant management. A candidate marker is programmed death (PD)–1 receptor, whose overexpression has been associated with disease progression during persistent viral infections. To determine whether levels of this negative regulator of T cell activity are altered in SOT recipients with symptoms of CMV disease, a comparative PD-1 expression analysis was done in healthy, CMV-positive individuals and in liver transplant recipients. PD-1 levels were measured among the total population of CD8+ and CD8+ T cells binding to CMV-specific major histocompatibility complex class I tetramers. Minimal PD-1 expression was found in the healthy, CMV-positive cohort, and symptomatic SOT recipients had significantly higher PD-1 levels. PD-1 up-regulation was significantly associated with incipient and overt CMV disease and with viremia. Our findings suggest that PD-1 could be developed as a prognostic tool to predict CMV disease and guide therapeutic interventions.

In transplant recipients, therapy administered to limit T cell–mediated graft rejection impairs the ability of the immune system to control opportunistic infections such as with cytomegalovirus (CMV). Thus, antiviral therapy is often required to avoid serious clinical complications [1]. In solid-organ transplant (SOT) recipients, late CMV disease frequently arises after the suspension of the scheduled 3-month antiviral prophylaxis [2]. This complication is most severe in CMV-seronegative recipients of a graft from a seropositive donor (D+/R− patients), with approximately one-third of these patients developing late CMV disease [3], often in association with ganciclovir (GCV) resistance, rejection of the transplanted organ, and infection-related mortality [2].

There has been interest in developing immunological assays that could identify patients at highest risk for late CMV disease, because of the limitations of current monitoring approaches [4]. Studies have demonstrated a pivotal role for adaptive T cell immunity in limiting the development of CMV disease in the immune-suppressed patient [5]. In a recent longitudinal study of viremia and T cell function in D+/R− patients, the acquisition of T cell lytic function and the ability to produce interferon (IFN)–γ in response to rising CMV viremia was an unreliable indicator of a protective T cell response [6]. Additional assays of effector function, homing capabilities, or other parameters will be necessary to define protective T cell immunity to CMV in this clinical setting, in which a significant proportion of patients have a measurable outcome such as disease [4, 7, 8].

Recent studies have identified a role for the up-regulation of inhibitory molecules, such as programmed death (PD)–1 receptor, in impairing T cell immunity to
persistent viruses [9–12]. PD-1 overexpression on HIV-, simian immunodeficiency virus–, hepatitis B virus–, and hepatitis C virus–specific CD8+ T cells has been shown to diminish their ability to proliferate, produce cytokines, kill infected cells, and promote apoptosis [9–14]. CMV-specific T cells express PD-1, although in CMV- and HIV-positive individuals receiving highly active antiretroviral therapy, PD-1 levels are higher on HIV-specific CD8+ T cells than on CMV-specific CD8+ T cells (from individuals either with or without viremia) than on CMV-specific CD8+ T cells, which maintain a functional phenotype [9]. However, it is unknown whether abnormal expression of PD-1 or other immunomodulatory signals contributes to functional immune impairment in D+/R− SOT patients who develop primary CMV infection and/or disease while being immune suppressed.

To address the role played by PD-1 in late CMV disease, we have characterized PD-1 expression in a D+/R− orthotopic liver transplant (OLT) cohort comprising both asymptomatic and symptomatic patients at high risk for CMV disease [6] and compared it with that in healthy, CMV-positive volunteers. Flow cytometry methods were used to measure PD-1 levels on total CD8+ T cells and on CMV-specific CD8+ T cells, by means of a panel of 8 major histocompatibility complex (MHC) class I tetramers specific for different CMV epitopes.

**MATERIALS AND METHODS**

**Study cohorts.** Seventeen CMV-seronegative patients receiving a first OLT from a CMV-seropositive donor were enrolled at the University of Washington Medical Center (UWMC), after signing an informed-consent document. The prospective longitudinal study was approved by local review boards. Patients received 3 months of prophylaxis with valganciclovir (Valcyte; Roche) and were treated intravenously (iv) with GCV (Cytovene Roche) or valganciclovir for CMV disease [3]. Details for this cohort, including information on demographics, primary disease, induction/maintenance, and immune-suppressive treatment, have been published elsewhere [6]. Diagnostic assays included human leukocyte antigen (HLA) typing (table 1), CMV serological analysis, and plasma DNA viremia. Blood was collected 3–12 months after OLT [6]. Forty-seven consenting, healthy, CMV-positive volunteers (mean age, 49.4 years; age range, 18–60 years) were included in the study as a control group. Peripheral blood mononuclear cells (PBMCs) from all blood samples were stored at the City of Hope Laboratory of Vaccine Research [6].

**MHC class I tetramer assembly and conjugation.** On the basis of HLA-A and HLA-B types represented in the patient population (table 1), a panel of 8 tetramers folded with HLA class I–restricted CMV epitopes [15–17] was prepared as de-
scribed elsewhere [18]. Specifically, the HLA-A*02 pp65 495–503 (pp65N9V), the HLA-B*0702 pp65 417–426 (pp65T10M), the HLA-B*0702 pp65 265–275 (pp65R11L), the HLA-A*1101 pp65 13–24 (pp65S12K), the HLA-A*02 IE-1316–324 (IE-1V9L), the HLA-B*08 IE-1 199–207 (IE-1E9M), the HLA-A*01 pp50 245–253 (pp50V9Y), and the HLA-A*0301 pp150 945–955 (T11K) tetramers were conjugated to allophycocyanin (APC; Sigma) fluorochrome [8].

**PD-1 expression.** Staining of PBMCs was performed as described elsewhere [6]. Briefly, 0.3 μg of the relevant APC-tetramer was added to PBMCs for 20 min. Subsequently, PD-1 (CD279)–phycoerythrin (PE) or mouse IgG1 isotype control–PE and CD8–fluorescein isothiocyanate were added together for an additional 30 min. Intracellular cytokine staining (ICS) for IFN-γ production was performed as described elsewhere [6]. PD-1 or isotype control and CD8 antibodies were added together after stimulation. All antibodies were purchased from BD Biosciences.

**Flow cytometry analysis.** Flow cytometry analysis was performed on a FACSCanto (BD Immunocytometry Systems). Between 0.4 × 10^6 and 1 × 10^6 events were acquired for each sample. Biexponential analysis and 98% quantile contour plots for data display [19] were done using BD FACSDiva (version 5.0.1) and FlowJo (version 7.1.3; Tree Star) software. The number of tetramer-binding cells is expressed as a percentage of the CD8 + T cell population [18].

**Statistical analysis.** Welch’s t tests were used to compare patients and healthy donors (HDs) after reducing longitudinal observations to 1 mean value per person, with attention restricted to the relevant time periods. Comparisons of symptomatic and asymptomatic time periods used paired t tests. Normality was examined using Shapiro-Wilk tests and normal quantile plots and was found to be satisfactory. The association between PD-1 expression and risk of disease was tested using the Wald test from a Poisson regression model. This allows for variation in PD-1 expression through time and variation in time at risk but assumes a flat hazard function. This approach tests the association between PD-1 expression at the beginning of an interval and the risk of disease during the interval, so it directly addresses the prediction of disease, as opposed to association at the time of disease.

**RESULTS**

**CMV-specific tetramer binding and PD-1 expression in healthy volunteers.** To assess the range of PD-1 expression on CD8 + T cells among healthy individuals, T cells from 47 CMV-positive volunteers were evaluated for PD-1 expression by flow cytometry. PD-1 expression on the total population of CD8 + T cells was measured in all donors; however, PD-1 levels on CMV-specific tetramer CD8 + T cells were measured only for individuals (18/47 tested) showing >0.2% of tetramer-specific CD8 + T cells. This cutoff was chosen to allow flow acquisition of sufficient antigen-specific cells for quantitative analysis [8]. Although CMV-specific tetramer binding varied considerably (range, 0.03%–21%), the mean fluorescence intensity (MFI) of PD-1 expression on CMV-specific tetramer CD8 + T cells was consistently <100 (mean, 43.1; range, 4–82) (figure 1). In particular, the individuals with the highest (HD 11) and the lowest (HD 5) tetramer binding (105-fold difference) showed a comparably low PD-1 MFI. These findings indicate that, in the immunocompetent adults examined, PD-1 expression was low and independent of the frequency of circulating peripheral CMV-specific tetramer CD8 + T cells.

**CMV-specific tetramer binding in OLT recipients.** CMV-specific tetramer binding was longitudinally measured in D+/R− OLT patients from discontinuation of antiviral prophylaxis until 1 year after transplant, using CMV-specific class I tetramers (table 1). In the upper panel of figure 2A, the assessment of CMV-specific tetramer binding for 2 representative symptomatic pa-

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Figure 1. Tetramer binding and programmed death (PD)-1 expression in healthy donors. Bars show the percentages of CD8 + T cells binding to cytomegalovirus (CMV)–specific tetramer (CMV-TET, left Y-axis) for each CMV-positive healthy donor, and black circles identify the corresponding PD-1 mean fluorescence intensity (MFI) for CMV-specific tetramer CD8 + T cells (right Y-axis) (MFI values from PD-1 isotype controls were subtracted for each donor).
tients is shown. Rehospitalization and iv GCV treatment were required for CMV tissue invasive disease (duodenal and esophageal) in the case of unique patient number (UPN) 3 and for acute rejection with concomitant high CMV viremia in the case of UPN 13. For both patients, considerable levels of CMV-specific tetramer binding (respective to HLA-A*02 pp65N9V tetramer for UPN 3 and to HLA-A*01 pp50V9Y for UPN 13) (table 1) were measured during periods of viremia, no viremia,
and even during antiviral treatment. The magnitude of CMV-specific tetramer binding was not predictive of CMV disease and was not associated with viremia [11, 20–22].

**PD-1 temporal profiles.** Because CMV-specific IFN-γ production, degranulation, and viremia levels are insufficient indicators of a protective T cell response in D+/R− OLT patients [6, 8], the expression of an alternative marker, PD-1 receptor, was evaluated in OLT recipients (table 1) at the highest risk for life-threatening CMV disease. PD-1 up-regulation has been associated with immune dysfunction [9–12], so it was a plausible hypothesis that levels of this inhibitory molecule might be increased in D+/R− patients who develop a primary T cell response during immune-suppressive therapy [4]. As illustrated for UPN 3 and UPN 13 (figure 2A, lower panel), PD-1 expression on CMV-specific tetramer+CD8+ T cells rose just before disease, at the time of CMV diagnosis and GCV administration, and progressively declined afterward to MFI levels <100, comparable to those in healthy, CMV-positive adults (figure 1). It is interesting to note that, in the case of UPN 3, GCV was stopped at 5.5 months after OLT, on the basis of the decline in viremia (figure 2, left plots). However, PD-1 levels were still elevated, and CMV symptoms recurred, which required additional antiviral treatment. When post-OLT GCV therapy was suspended at 7 months, PD-1 levels were below an MFI of 100, and the patient did not show further CMV disease symptoms. Because of strong acute rejection, UPN 13 (figure 2, right plots) was kept under GCV antiviral therapy until the disappearance of viremia. Levels of PD-1 on CMV-specific tetramer+CD8+ T cells declined to an MFI characteristic of healthy individuals after antiviral suspension, without recurrence of disease.

Use of tetrabytes may pose some limits for clinical applications, because there are HLA alleles for which no minimal cytotoxic epitope has been described [8, 15]. Therefore, PD-1 levels were measured at symptomatic time points on IFN-γ+CD8+ T cells, a potentially broader population because of the use of full-length peptide libraries versus single 9–12mer epitopes. After short-term stimulation with CMV peptide libraries in ICS assays [6], PD-1 up-regulation was at least 3 times lower than when measured on CMV-specific tetramer+CD8+ T cells (data not shown), indicating that PD-1 levels on functional T cells are reduced [9]. In agreement with others, we pursued the hypothesis that measuring PD-1 levels independent of function is more closely correlated to disease status than measuring levels of PD-1 on functional antigen-specific T cells [9–13].

**PD-1 up-regulation in D+/R− OLT patients.** PD-1 expression was monitored in the total population of CD8+ and CMV-specific tetramer+CD8+ T cells (for a total of 72 individual observations). PD-1 levels were elevated preceding and during the symptomatic phase of CMV disease and early after antiviral administration. In sharp contrast, declining or minimal PD-1 levels were found after transplant when patients were asymptomatic (table 1 and figures 2 and 3). An MFI of >100 (mean, 174; range, 114–346) was found immediately before CMV disease symptoms appeared, during overt CMV disease, and early after iv GCV treatment (figure 3, upper panel; symptomatic time points). When patients were asymptomatic, PD-1 MFI values ranged between 1 and 113 (MFI, 62.1). In the patients screened (table 1), elevated PD-1 expression on CMV-specific tetramer+CD8+ T cells was moderately associated with viremia (figure 4A) [11] and trended higher during GCV treatment (P = .045). In contrast, percentages or absolute numbers per cubic millimeter of CMV-specific tetramer+CD8+ T cells (figure 4B and 4C), CD8+ T cells (figure 4D), and total lymphocytes per cubic millimeter (R2 = 0.01, data not shown) were not associated with CMV viremia. In all tested cases, PD-1 MFI values were not associated with total lymphocyte (R2 = 4.7 × 10−5) or with either CD8+ T cell (R2 = 0.06) or CMV-specific tetramer+CD8+ T cells (R2 = 0.02). Of importance, PD-1 expression on CMV-specific tetramer+CD8+ T cells were not significantly associated with risk of disease (P = .04), by a Poisson regression model in which the hazard of disease depended on PD-1 expression levels at the previous patient visit and blood sampling, suggesting a promising test of predictive utility.

**Comparative PD-1 expression analysis and threshold identification.** Levels of PD-1 measured on CMV-specific tetramer binding or the total population of CD8+ T cells in healthy individuals were compared with levels detected in the D+/R− OLT cohort when the patients were either symptomatic or asymptomatic for CMV disease (table 1 and figure 3). Levels of PD-1 measured on CMV-specific tetramer+CD8+ T cells at symptomatic time points (including the measurement preceding CMV disease) were higher than those measured in healthy volunteers and at asymptomatic time points in patients. The differences were strongly significant (figure 3). A similar but less marked difference [11] was measured for levels of PD-1 on the total population of CD8+ T cells; however, there was some overlap of distributions across groups that may limit the prognostic specificity of measurement of PD-1 on the total CD8+ T cell population. (figure 3, lower panel). In contrast, the clear-cut separation between PD-1 MFI values for CMV-specific tetramer+CD8+ T cells in symptomatic patients versus healthy volunteers (figure 3; upper panel) enabled us to establish a PD-1 threshold of 105.5 MFI, on the basis of an estimated upper 99th percentile for the PD-1 levels in healthy volunteers (figure 3, upper panel; dotted line). This value also separates the symptomatic and asymptomatic time points, with very little overlap (figure 3, upper panel). Thus, levels of PD-1 on CMV-specific tetramer+CD8+ T cells seems to be a promising indicator to identify when D+/R− OLT patients may be at the highest risk for late CMV disease.
DISCUSSION

The PD-1 pathway regulates the balance between the stimulatory and inhibitory signals needed for effective immune responses [23]. Pathogens that cause chronic infection can exploit this inhibitory pathway to evade host defense, down-regulating T cell responses and facilitating pathogen persistence [11]. Blockade of the PD-1/PD-L1 inhibitory pathway increases T cell survival and restores T cell proliferation and cytokine production [9–12, 24]. In the present study, we measured expression of PD-1 on CD8+ T cells in a cohort of D+/R− OLT patients in which a proportion developed symptomatic CMV disease, a favorable population to establish the significance of a molecular marker associated with T cell function [6]. Blockade of the PD-1/PD-L1 inhibitory pathway increases T cell survival and restores T cell proliferation and cytokine production [9–12, 24]. In the present study, we measured expression of PD-1 on CD8+ T cells in a cohort of D+/R− OLT patients in which a proportion developed symptomatic CMV disease, a favorable population to establish the significance of a molecular marker associated with T cell function [6].

Among healthy, CMV-positive individuals, levels of PD-1 on total and CMV-specific CD8+ T cells were uniformly low (figures 1 and 3), in agreement with previous findings [11]. The consistency of the low PD-1 expression detected using tetramers folded with different CMV epitopes contrasted with a marked variability of the levels of CD8+ T cells binding to the CMV-specific tetramers [25]. Because CMV has been speculated to play a role in immunosenescence, causing dysfunctional CD8+ T cells and cytokine imbalance in the elderly, an upper age limit of 60 years was imposed for HD enrollment [26]. Further analysis is required to assess whether PD-1 up-regulation is found in elderly individuals and is associated with impaired CMV-specific T cell responsiveness.

We detected significantly higher expression of PD-1 on total and CMV-specific CD8+ T cells in transplant recipients who developed CMV disease, compared with that in healthy, CMV-positive individuals (figures 2 and 3). Although PD-1 expression on CMV-specific T cells has been previously shown [9–11], this is the first report of longitudinal PD-1 measurements in immunosuppressed transplant patients, in whom a proportion will develop symptomatic CMV infection. The small size of our D+/R− OLT population, although appropriate for performing statistical evaluation, is a limitation of this study. Our primary
focus has been to select an appropriate study population, comprising patients who will develop symptomatic disease, to accurately assess whether measured parameters are involved in protection from disease [4]. However, the suitability of the high-risk D/H11/R/SOT recipients for conducting these studies contrasts with the difficulty of recruiting such patients, because they constitute only 20%–30% of all SOT recipients [2, 3]. Hence, investigations exclusively focusing on the highest risk D/H11/R/SOT recipients are in the minority [6, 27–29], compared with studies enrolling patients at lower risk for CMV disease [30–37].

In agreement with previous studies [11, 20–22], CMV-specific tetramer binding was not associated either with CMV disease or with viremia (figure 4B and 4C). In our SOT cohort, in which lymphopenia was mild and not associated with disease [6], viremia was not correlated with overall and absolute CMV-specific T cell numbers (figure 4). In addition, PD-1 upregulation was linked to neither antirejection treatments nor to the number of lymphocytes and specific T cells. In contrast, there was a positive correlation between PD-1 expression on CMV-specific tetramer+CD8+ T cells and plasma DNAemia (figure 4A), remarkably similar to a recent study conducted in HIV-infected individuals [10, 11]. These data provide the first evidence that, in D+/R− OLT patients, increasing amounts of viral antigen are associated with increased expression of PD-1 on CMV-specific T cells. Moreover, elevated levels of PD-1 expression on CMV-specific tetramer+CD8+ T cells appeared to be associated with the risk of disease (P = .04), suggesting a promising predictive tool for this immune parameter, temporarily and quantitatively associated with CMV disease. In particular, the MFI difference found between PD-1 levels measured on CMV-specific T cells at symptomatic and asymptomatic time points (figure 3, upper panel) may be used as a prognostic tool to identify SOT recipients developing CMV disease [2]. The goal of future studies is to expand the general applicability of the current approach to other D+/R− SOT recipients, hematopoietic stem-cell transplant recipients with impaired CMV T cell responses [38–40], or neonates with congenital CMV infection [41, 42], given that PD-1 levels may mirror our observations in the D+/R− OLT patients. A unified theme for estimating the risk for CMV disease could be derived from measurements of T cell immunomodulatory molecules such as PD-1.

PD-1 up-regulation was detected not only on CD8+ T cells specific for pp65 from different HLA types [15] but also on CD8+ T cells specific for other CMV antigens, such as IE1 and pp50 [25]. These data imply that elevated PD-1 expression related to CMV disease in D+/R− OLT patients is generalized to T

Figure 4. Association with plasma cytomegalovirus (CMV) DNAemia. Programmed death (PD)-1 mean fluorescence intensity (MFI) levels for CMV-specific tetramer+CD8+ T cells (A), percentages of CMV-specific tetramer+CD8+ T cells (B), absolute nos. of CMV-specific tetramer+CD8+ T cells per cubic millimeter of blood (C), and absolute nos. of CD8+ T cells per cubic millimeter of blood (D) among the whole solid-organ-transplant cohort are shown on the Y-axes; plasma DNAemia is shown on the X-axes. On the upper right side of each plot is indicated the strength (R², coefficient of determination) and significance (P) of the association with plasma DNAemia.
cells recognizing multiple CMV antigen targets. T cells with high PD-1 levels detected using 1 or few tetramers specific for cytotoxic epitopes is likely to be an underestimate of the magnitude [11].

A possible disadvantage of using PD-1 measurements as a clinical disease marker may be the cost associated with frequent monitoring, given that the median of detection of PD-1 upregulation is 15 days (mean, 28 days) (table 1) before the onset of disease. However, the economic impact of rehospitalization and the risk associated with late CMV disease and iv GCV administration are significantly higher [43] than the costs associated with testing PD-1 levels by use of blood samples obtained at the patient’s primary-care provider. Importantly, monitoring patients with symptomatic CMV disease by means of this marker might be clinically useful for assessing the timing of antiviral administration and its suspension, avoiding either the risks associated with overtreatment or recurrence due to early antiviral withdrawal (figure 2) [3].

In summary, our study provides novel insight into the criteria for CMV protective immunity and identifies for the first time that PD-1 levels are associated with late CMV disease after prophylaxis suspension in D+/R− OLT patients receiving immune-suppressive treatments [3]. The consistency of PD-1 elevation either before or during disease and early after antiviral treatment makes the assessment of this parameter particularly attractive as a potential prognostic indicator of CMV disease in the complex management of high-risk D+/R− OLT patients.

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References