T-Cell Immunity to Subclinical Cytomegalovirus Infection Reduces Cardiac Allograft Disease

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**Background**—Asymptomatic cytomegalovirus (CMV) replication is frequent after cardiac transplantation in recipients with pretransplantation CMV infection. How subclinical viral replication influences cardiac allograft disease remains poorly understood, as does the importance of T-cell immunity in controlling such replication.

**Methods and Results**—Thirty-nine cardiac recipients who were pretransplantation CMV antibody positive were longitudinally studied for circulating CMV-specific CD4 and CD8 T-cell responses, CMV viral load in blood neutrophils, and allograft rejection during the first posttransplantation year. Nineteen of these recipients were also analyzed for changes of coronary artery intimal, lumen, and whole-vessel area. All recipients received early prophylactic therapy with ganciclovir. No recipients developed overt CMV disease. Those with detectable levels of CMV-specific CD4 T cells in the first month after transplantation were significantly protected from high mean and peak posttransplantation viral load (P<0.05), acute rejection (P<0.005), and loss of allograft coronary artery lumen (P<0.05) and of whole-vessel area (P<0.05) compared with those who lacked this immune response. The losses of lumen and vessel area were both significantly correlated with the time after transplantation at which a CD4 T-cell response was first detected (P<0.05) and with the cumulative graft rejection score (P<0.05).

**Conclusions**—The early control of subclinical CMV replication after transplantation by T-cell immunity may limit cardiac allograft rejection and vascular disease. Interventions to increase T-cell immunity might be clinically useful in limiting these adverse viral effects. (Circulation. 2006;114:1608-1615.)

**Key Words:** atherosclerosis ■ immune system ■ lymphocytes ■ rejection ■ transplantation ■ viruses

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**Editorial p 1561**

**Clinical Perspective p 1615**

Overt CMV infection is implicated in long-term adverse outcomes of the cardiac allograft, including the frequency and severity of acute rejection episodes and transplant arteriopathy. These adverse influences on the allograft have been termed indirect effects of CMV because CMV replication within the allograft has not been detected in most studies. The pathogenesis of these effects of CMV remains poorly understood and controversial. Because early acute rejection appears to be a major risk factor for subsequent transplant arteriopathy in heart transplant recipients, CMV may promote arteriopathy, at least in part, by its enhancement of rejection.

The association of CMV infection with adverse effects on the cardiac allograft was first identified in transplant recipients who did not receive antiviral therapy, were frequently treated with T-cell cytoablative immunosuppression, and had a high frequency of frank CMV disease, and, presumably, also had a high viral load. Although recent work suggests that antiviral therapy may reduce the adverse indirect effects of CMV in transplant recipients receiving less cytoablative immunosuppression, the importance of subclinical levels of CMV infection on the cardiac allograft is unclear.
In this study we examined the role of CMV-specific T-cell immunity in controlling posttransplantation subclinical levels of CMV replication and whether variation among recipients in such immunity was associated with protection from cardiac allograft sequelae during the first year after transplantation. Intriguingly, CMV-specific T-cell immunity, particularly by CD4 T cells, was associated with decreased CMV load, a reduction in episodes of acute rejection, and reduced transplant arteriopathy.

Methods

Study Population and Design

Thirty-nine patients who were CMV antibody positive (Ab⁺) and receiving their first heart transplant were enrolled at Stanford University Medical Center between January 2002 and September 2004. This included 30 consecutive patients who were CMV Ab⁺ enrolled up until October 2003, of whom 10 underwent a baseline intravascular ultrasound (IVUS), and an additional 9 CMV Ab⁺ patients enrolled between February 2004 and September 2004 for whom baseline IVUS analysis was available. Exclusion criteria were CMV antibody negative (Ab⁻) pretransplantation status, age younger than 12 years, and renal insufficiency requiring prolonged dialysis. Thirty-two patients received a graft from a CMV Ab⁺ donor, and 7 received a graft from a CMV Ab⁻ donor. All received prophylaxis with intravenous ganciclovir 5 mg/kg per dose twice a day for 2 weeks, followed by 6 mg/kg once daily for 2 weeks, with dosage adjustment based on serum creatinine levels. As part of standard care of pediatric cardiac transplant recipients at our institution, 6 patients aged 13 to 16 years also received intravenous CMV immune globulin in the first 72 hours after transplantation, and at 2, 4, 6, 8, 12, and 16 weeks afterward. All patients received the same immunosuppressive regimen, which consisted of induction therapy with daclizumab and standard-dose therapy with oral cyclosporin A, mycophenolate mofetil, and prednisone. As part of standard clinical care, patients were monitored for the occurrence of acute allograft rejection by endomyocardial biopsies performed weekly during the first month after transplantation, every 2 weeks during the second month, then monthly up to 6 months after transplantation, and at 9 and 12 months after transplantation. Peripheral blood was drawn at the time of biopsies to monitor CMV viral load and T-cell immunity.

Flow Cytometric Detection of CMV-Specific T-Cell Responses

Peripheral blood mononuclear cells were analyzed for CMV-specific CD4 and CD8 T-cell responses after stimulation with whole CMV antigen derived from a lysate of CMV-infected fibroblasts or a CMV pp65 (UL83) 15-mer peptide pool, as reported earlier. Stimulation with *Staphylococcus aureus* enterotoxin B (SEB) served as a positive control, and appropriate negative controls were those reported earlier. Permeabilized and fixed cells were stained with fluorochrome-conjugated monoclonal antibodies for interferon-γ (IFN-γ), the CD69 activation antigen, CD4, and CD8α. CD4 or CD8 T cells expressing both IFN-γ and CD69 were considered positive events if they occurred at frequencies >0.1% on the basis of data obtained from incubation with negative controls. The circulating concentration of CMV-specific or pp65-specific CD4 or CD8 T cells was determined by multiplying the frequency of positive cells by the concentration of CD4 or CD8 T cells per milliliter, respectively. Recipients with ≥400 CD4 T cells per milliliter or ≥200 CD8 T cells per milliliter that were CMV-specific or pp65-specific during the first month after transplantation were defined as belonging to the early CD4 or CD8 T-cell immune response groups, respectively. These thresholds were based on observations from our laboratory that the concentration of circulating CD4 and CD8 T cells had normal lower limits in healthy adults of 400 cells per microliter and 200 cells per microliter, respectively, which are similar to published values. We used circulating levels of CMV-specific T cells in our analyses because these levels appear to be a better predictor of viral control than the percentage of CMV-specific positive T cells. However, our assignment of recipients into early and late responders was not changed when ≥0.1% of CMV-specific positive T cells was used as the sole criterion.

Quantitative Analysis of CMV DNA

Total DNA was isolated from 5 × 10⁵ blood polymorphonuclear leukocytes (PMNs), and 1.0 × 10⁵ cell equivalents of DNA was added to a 45-µL reaction mixture containing 5 µL of SYBR Green PCR Master Mix (Applied Biosystems, Foster City, Calif) and 1 µmolar/L each of the IE-1 oligonucleotide primers 5'-TCTTGGAATTCCTGGTCA-3' and 5'-GGCCGAAATCCCTCAAAA-3'. The reaction was incubated at 50°C for 2 minutes, 94°C for 10 minutes, followed by 40 cycles of 15 seconds at 94°C, 1 minute at 60°C with the use of a GeneAmp 5700 Sequence Detection System (Applied Biosystems). On the basis of spiking of known amounts of plasmid IE-1 gene DNA into 1.0 × 10⁵ cell equivalents of DNA isolated from PMNs of CMV Ab⁺ individuals, the lower limit of detection of this assay was 3 CMV genome copies per 1.0 × 10⁵ PMNs. This approach was also used to establish a standard curve to determine sample copy number, with the results expressed as DNA copies per 1.0 × 10⁵ PMNs. In all assays, the specificity of the final amplified products was verified by heat dissociation curve analysis with the use of software supplied by the manufacturer.

Definition of Cardiac Allograft Rejection

Endomyocardial biopsies were graded for rejection according to the criteria of the International Society for Heart and Lung Transplantation.

Heart Catheterization and Quantitative IVUS

IVUS was used to assess coronary artery intimal, lumen, and whole-vessel area at baseline (within 6 weeks after transplantation) and at 12 months after transplantation in 19 of the 39 patients. After diagnostic coronary angiography was performed, a 2.6F, 40-MHz mechanical ultrasound catheter, connected to a Galaxy IVUS system (Boston Scientific Corporation, Natick, Mass), was advanced over a guidewire to position the IVUS transducer in the mid to distal left anterior descending coronary artery. An automated pullback at 0.5 mm/s was performed, and the IVUS images were recorded on videotape. The images were digitized, and 2-dimensional analysis of the first 100 equally spaced cross-sectional images (50-mm length) was performed. All measurements were performed blinded to clinical and angiographic information.

Statistical Analysis

Data were analyzed with JMP 5.0 and SAS 9.1 software (SAS Institute, Inc, Cary, NC). Categorical data are presented as frequencies and percentages and were compared between groups with the use of the Fisher exact test. Continuous variables are summarized as mean ± 1 SD or mean ± 1 SEM, as indicated. Two-group comparisons of means were performed with the use of unpaired Student *t* tests. Longitudinal data were analyzed with the use of mixed models. These models were formulated as a quadratic spline, with a knot at 4 months, a group main effect, 2-way interactions between each time point and group, and a random intercept. Denominator degrees of freedom for *F* tests for mixed models were adjusted because of the modest sample size. Type I error was controlled at 5% for between-group comparisons made across multiple time points with the use of sequential Bonferroni adjustment. Curves for time to allograft rejection were estimated by the Kaplan-Meier method and compared between groups with the use of the log-rank test. Spearman correlation coefficients were calculated to estimate any monotonous association between uncensored continuous variables. All group comparisons were 2-tailed.

The authors had full access to the data and take responsibility for its integrity. All authors have read and agree to the manuscript as written.
Results

CMV-Specific T-Cell Immunity and Clinical Characteristics

The baseline characteristics of the 39 transplant recipients are summarized in the Table. Previously published large-scale, multisite studies of first cardiac transplants\textsuperscript{24,25} were summarized in the Table. Previously published large-scale, multisite studies of first cardiac transplants\textsuperscript{24,25} were identical in average donor age (32 years), recipient age, donor age, and cold ischemic time, respectively. Compared with these studies, the sample for the present study was identical in average donor age (32 years), similar in its sex ratio (77\% male), younger on average in recipient age (43 years), and had a longer average cold ischemic time (208 minutes).

These 39 recipients were classified as having either an early (ie, within 1 month after transplantation) or late CMV-specific CD4 or CD8 T-cell response on the basis of flow cytometric analysis (Figure 1A). Twenty-six (67\%) of these recipients had an early CMV-specific CD4 T-cell response as detected by whole CMV antigen, and 12 also had a CMV pp65 peptide pool CD4 T-cell response (Table). None of the recipients had an early CD4 T-cell response to the pp65 peptide pool without a corresponding response to whole antigen. Among the 26 recipients with an early CD4 T-cell response, 12 (46\%) also had an early CD8 T-cell response obtained with either whole CMV antigen and/or the pp65 peptide pool stimulation. However, all recipients with an early CMV-specific CD8 T-cell response had a concurrent early CD4 T-cell response. The early versus late response groups for CD4 or CD8 T cells had similar baseline demographic characteristics of known risk factors\textsuperscript{25} for graft rejection and transplant arteriopathy (Table for CD4 T-cell responses and data not shown for CD8 T-cell responses). None of the enrolled patients had symptomatic CMV disease during the first year after transplantation, and only 1 recipient, a member of the early CD4 T-cell response group, had a rejection episode in the first month after transplantation that required treatment with intravenous glucocorticoids.

CMV-Specific Immunity and Other Immune Parameters

Compared with those with a late CMV-specific CD4 T-cell response, recipients with an early CD4 T-cell response had significantly greater levels of CMV-specific CD4 T cells at the time of transplantation and at the first, second, and third months after transplantation but not subsequently (Figure 1B). The differences in CMV-specific CD4 T-cell immunity between the early and late response groups were not due to differences in either the circulating number of CD4 T cells (Figure 1C) or in the overall capacity of CD4 T cells to produce IFN-\(\gamma\) in response to polyclonal stimulation, as assessed with the use of SEB stimulation (Figure 1D). We also found no significant difference between the early and late response groups in the circulating number of CD8 T cells.

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<th>Characteristics of Patients and Follow-Up Immune Function</th>
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<td>Age, y</td>
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<td>Male sex, n (%)</td>
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<td>Pretransplantation coronary artery disease, n (%)</td>
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<td>Donor age, y</td>
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<td>Positive donor CMV serology, n (%)</td>
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<td>First year after transplantation</td>
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<td>Clinical CMV-related disease</td>
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<td>CMV immune globulin and prolonged antiviral prophylaxis, n (%)</td>
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<td>Follow-up immune function</td>
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<td>Early CMV-specific CD4 T-cell response to whole antigen, n (%)</td>
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<td>Early CD4 T-cell response to CMV pp65 peptide pool, n (%)</td>
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<td>Early CMV-specific CD8 T-cell response (whole antigen and/or pp65 peptide pool), n (%)</td>
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<td>Absolute CD4 T-cell count at 1 month after transplantation, cells/(\mu)L</td>
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<tr>
<td>Absolute No. of SEB-induced IFN-(\gamma) CD4 T cells at 1 month after transplantation, cells/mL</td>
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Values are mean±1 SD or frequency (percentage). NA indicates not applicable. Early and late responses are defined in Methods.
Thus, the delayed CMV-specific CD4 T-cell immunity of the late response group was not due to a generalized quantitative or qualitative T-cell immune deficiency.

CMV-Specific T-Cell Immunity and Systemic Viral Load

Viral load was low to undetectable in the early and late response groups during the first month when prophylactic ganciclovir was administered (Figure 2A). The time after transplantation until CMV DNA was first detected also did not differ between the groups (mean ± 1 SEM of 83.1 ± 17.5 versus 94.5 ± 25.6 days, respectively; \(P = 0.64\)). In contrast, the mean viral load was estimated to be higher at 2 months \(P < 0.05\), 3 months \(P = 0.004\), and 4 months \(P = 0.0019\) for the late CMV-specific CD4 T-cell response group compared with the early response group (Figure 2A). Mean and peak levels of CMV DNA in the first year after transplantation were significantly higher for late response patients compared with early response patients (Figure 2B). There were no significant differences in these parameters of viral load for early versus late CMV-specific CD8 T-cell response groups (data not shown).
An early CMV-specific CD4 T-cell response was associated with a significant delay in the time to any allograft rejection (Figure 3A) and to moderate to severe rejection (Figure 3B) during the first year after transplantation. There was a significant positive correlation between the mean rejection score during the first year after transplantation and the number of days after transplantation at which a CMV-specific CD4 T-cell response was first detected (Figure 3C). In contrast, there were no significant differences between early versus late CMV-specific CD8 T-cell response groups for freedom from either all rejection or moderate or more severe rejection episodes ($P=0.35$ and $P=0.26$, respectively).

**CMV-Specific T-Cell Immunity and Negative Coronary Artery Remodeling**

Among the 39 patients analyzed, 19 agreed to a basal IVUS study performed within 6 weeks after transplantation. Of these 19 patients, 11 were from the early CMV-specific CD4 T-cell response group, and 8 were from the late response group. The changes in the intimal, lumen, and vessel areas from baseline to the first year after transplantation were evaluated. The losses of lumen area and vessel area in relation to baseline were significantly greater for the late response group than for the early response group (Figure 4A). The magnitude of the loss of coronary artery vessel area was significantly correlated with both the time after transplantation at which CD4 T-cell responses were first detected (Figure 4B) and with the mean rejection score of the allograft during the first year after transplantation (Figure 4C). Similarly, the magnitude of the loss of lumen area was significantly associated with the time at which CD4 T-cell responses were first detected (Figure 4D) and with the mean allograft rejection score (Figure 4E).

We reanalyzed our results after excluding 6 recipients, all children between 13 and 16 years of age, who received additional antiviral treatment in the form of CMV immune globulin. Five of these recipients were in the early response group, and 1 was in the late response group (Table). All analyses of viral load, freedom from any or moderate to severe rejection, loss of vessel and lumen area, and correlation between loss of vessel and lumen area and time after transplantation at which CMV-specific CD4 T-cell responses were first detected or rejection score remained statistically significant (results not shown).

**Discussion**

Key results of this study are as follows. (1) A CMV-specific CD4 T-cell immune response in the first month after transplantation was associated with a reduction in mean and peak CMV viral load. (2) Early immune response was associated with reduced episodes of acute rejection and less transplant arteriopathy. These studies suggest for the first time that an early CMV-specific immune response is beneficial rather than deleterious in terms of cardiac allograft rejection and early transplant arteriopathy. Our work also provides evidence that even subclinical CMV infection may contribute to the pathophysiology of cardiac allograft disease. It also suggests that methods to enhance CMV-specific T-cell immunity may represent a new therapeutic strategy in these patients. Finally, our findings could have relevance to the function of other types of solid organ allografts in recipients who develop subclinical CMV infection after transplant.

We observed an early CMV-specific CD4 T-cell response in 26 of the 39 transplant recipients studied. This early response was associated with significant protection from the postransplantation CMV load, suggesting its relevance to immune control of viral replication. In contrast, no significant differences were observed between the early and late CMV-specific CD4 T-cell response groups for the circulating levels...
of CD4 T cells or the capacity of these cells to produce IFN-γ after stimulation with SEB bacterial superantigen. Thus, our inability to detect early CMV-specific CD4 T-cell responses in 13 of the recipients was unlikely to be due to a nonspecific T-cell immunosuppressive effect, eg, either because of increased levels of immunosuppressive drugs or an enhanced sensitivity of T cells to the effects of these drugs or other posttransplantation metabolic stresses.

CMV-specific CD4 T cells in the late response group, although initially low to undetectable, increased after the first month (Figure 1B), indicating that this was not a persistent immunodeficiency. None of these late response recipients or those of the early response group had symptomatic viral disease. This delayed T-cell immunity, aided by prophylactic antiviral therapy, may have prevented symptomatic CMV disease by delaying the onset and peak of viral load in the late response group. However, in the absence of an early posttransplantation CMV-specific T-cell response, this antiviral prophylaxis did not appear to be effective in limiting the adverse effects of CMV on allograft rejection and vascular disease.

Intimal hyperplasia, with resultant loss of luminal cross-sectional area, is a central pathogenic mechanism in transplant arteriopathy as well as native atherosclerosis.26–28 In both forms of arteriopathy, intimal hyperplasia can be compensated by vessel enlargement, also known as positive remodeling, or, alternatively, exacerbated by vessel area shrinkage, ie, negative remodeling.29,30 For the 19 transplant recipients who underwent basal IVUS analysis shortly after transplantation, we found a significant association of delayed CMV-specific CD4 T-cell immunity (and, perhaps, posttransplantation viral load) with negative coronary artery remodeling, as demonstrated by overall vessel shrinkage and reduced lumen area by the first year after transplantation. These results extend an earlier study9 in which recipients who had relatively high posttransplantation levels of CMV replication, based on pp65 antigenemia, were also at a significantly greater risk for negative remodeling compared with those with low or absent levels of CMV infection. These results suggest that CMV replication early after transplantation may promote allograft vascular disease by enhancing negative remodeling rather than increasing intimal hyperplasia. The finding of a significant association between the posttransplantation level of allograft rejection and both the loss of vessel area and lumen area during the first year suggests that allograft rejection is a pathogenic mechanism for negative remodeling, as it is for transplant arteriopathy overall. Additional studies to determine the predictive value of negative remodeling versus intimal hyperplasia on long-term severity of transplant arteriopathy will be of interest.

Possible mechanisms by which CMV may promote acute rejection and its potential downstream effects, such as transplant arteriopathy, include direct infection of the allograft and cross-reaction of viral-specific T cells with graft-derived alloantigens. Most studies have not found substantial levels of CMV in the allograft vasculature, suggesting that a systemic effect of CMV is likely. Although data from experimental rodent models31 and some studies of CMV-specific CD4 and CD8 T cells from healthy human donors32,33 support cross-
reactivity between antiviral and alloantigen-specific T-cell responses, the importance of such cross-reactivity in human solid organ transplantation remains unclear. Importantly, our results found that a robust CMV-specific CD4 T-cell immune response was associated with a protective rather than deleterious impact on cardiac allograft rejection. This suggests that augmentation of early posttransplantation CMV-specific T-cell immunity in those with low basal levels might decrease rather than increase allograft rejection and its deleterious impact on the coronary artery vasculature.

The relationship between the pretransplantation and posttransplantation levels of CMV CD4 T-cell immunity was not assessed in this study. However, it is plausible that the early response group recipients had substantially higher levels of such immunity than the late response group for months to years before transplantation because, on epidemiological grounds, most CMV Ab+ transplant recipients are likely to have been infected for years to decades.34 Indeed, our findings are reminiscent of the substantial variability of the frequency and blood concentration of CMV-specific CD4 T cells among healthy immunocompetent CMV Ab+ adults.35 Although the reasons for this variation among healthy donors in CMV-specific T-cell immunity remain unclear, the particular T-cell levels for an individual were found to be relatively stable over a period of at least 6 months.35

Only 46% of transplant recipients with an early CMV-specific CD4 T-cell response, as assessed with the use of whole CMV antigen stimulation, also had a detectable CD4 T-cell response to the CMV pp65 peptide pool. However, this subgroup of recipients with an early pp65-specific CD4 T-cell response was not protected to a greater degree from high posttransplantation viral load, allograft rejection, or negative vascular remodeling (data not shown). This indirectly suggests that CMV-specific CD4 T-cell responses directed at antigens other than pp65 may be important in early control of viral replication after transplantation. This notion is also supported by a recent study36 of heart or lung transplant recipients that also used the peptide pool stimulation assay and found that an early pp65-specific CD4 T-cell or CD8 T-cell response was not associated with protection from frank CMV disease or, most likely, with high levels of viral replication. In contrast, this study found that such protection was associated with an early IE-1–specific CD8 T-cell response after transplantation,36 although it is unknown whether this applies to subclinical CMV infection.

We did not observe any significant protection in recipients with a detectable early CMV-specific CD8 T-cell response from posttransplantation CMV load or CMV-related allograft disease. However, the stimulation of peripheral blood mononuclear cells with whole CMV antigen, as we used here, may favor the detection of CD4 over CD8 T-cell responses because extracellular antigen presentation to CD8 T cells requires the cross-presentation of viral peptides on MHC class I molecules by rare dendritic cells,37 whereas presentation to CD4 T cells can also be performed by more abundant B cells and monocytes. Future studies with the use of assay systems that more readily detect CMV-specific CD8 T cells, such as monocye-derived dendritic cells infected with CMV,38 or stimulation with peptide pools for additional antigenic CMV proteins39 may help to clarify the role of CD8 T cells in this context.

In summary, our study suggests that early and relatively high levels of CMV-specific CD4 T-cell immune responses are associated with limited CMV replication that is clinically asymptomatic and that this CD4 T-cell immune response is associated with protection from acute graft rejection and negative remodeling of the cardiac allograft vasculature. These findings suggest that there may be benefits for allograft function of limiting viral replication by enhancing T-cell immunity to CMV in the posttransplantation setting, eg, by vaccination40 or the adoptive transfer of autologous CMV-specific T cells.51,42

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Disclosures
None.

References
Cytomegalovirus (CMV) in heart transplant recipients was first identified by Shumway and colleagues in the late 1980s as an accelerator of allograft rejection and transplant arteriopathy. These recipients frequently had symptomatic CMV. CMV replication by T-cell immunity in this context is also uncertain. Tu et al used flow cytometry to measure posttransplantation CMV-specific T-cell responses to cytomegalovirus after transplantation is correlated with immediate early 1-specific CD8 T cells. Curr Opin Infect Dis. 2005;18:1031–1036.


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