Persistent Cytomegalovirus-Specific Memory Responses in the Lung Allograft and Blood following Primary Infection in Lung Transplant Recipients

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Persistent Cytomegalovirus-Specific Memory Responses in the Lung Allograft and Blood following Primary Infection in Lung Transplant Recipients

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Primary CMV infection in lung transplant recipients (LTRs) is associated with increased mortality. We studied 22 donor CMV-positive, recipient-negative (D⁺R⁻) LTRs for the development of posttransplant CMV-specific immunity. We found that 13 of 22 D⁺R⁻ LTRs (59.1%) seroconverted (CMV IgG Ab⁺). Using pooled peptides of the immunodominant CMV Ags pp65 and IE1, we detected CMV-specific CD8⁺IFN-γ⁺ T cells in the PBMC of 90% of seroconverted individuals following primary infection by intracellular cytokine staining. In contrast, few seroconverters had detectable CMV-specific CD4⁺IFN-γ⁺ T cells during viral latency. However, the majority of IgG⁺ LTRs demonstrated CMV-specific CD4⁺ and CD8⁺ T cell proliferative responses from PBMC, with CD4⁺IFN-γ⁺ T cells detectable upon re-expansion. Examination of lung allograft mononuclear cells obtained by bronchoalveolar lavage revealed both CMV-specific CD4⁺ and CD8⁺IFN-γ⁺ T cells, including patients from whom CD4⁺IFN-γ⁺ T cells were simultaneously undetectable in the PBMC, suggesting differential effector memory populations between these compartments. Moreover, both responses in the PBMC and lung allograft were found to persist, despite substantial immunosuppression, long after primary infection. Clinical correlation in this cohort demonstrated that the acquisition of CMV immunity was associated with freedom from CMV disease (p ≤ 0.009) and preservation of allograft function (p ≤ 0.02) compared with those who failed to develop CMV immunity. Together, our data reveal immunologic heterogeneity in D⁺R⁻ LTRs, with the development and persistence of primary CMV responses that may provide clinical benefit.

Cytomegalovirus, a member of the β-herpesvirus family, remains a significant cause of morbidity and mortality in solid organ and hematopoietic cell transplant recipients (1–4). CMV infection is the most common opportunistic pathogen in lung transplant recipients (LTRs), with primary infection in high-risk D⁺R⁻ individuals recognized as a risk factor for increased mortality in several studies (5–7). In addition, CMV has been implicated as a risk factor for bronchiolitis obliterans syndrome or chronic allograft rejection (8). Enhanced susceptibility to CMV in LTRs may, in part, be due to the lung being a major reservoir for latent virus (9). Thus, because of the major impact of CMV in lung transplantation, the majority of patients undergo prophylactic treatment in the immediate posttransplant period, although the duration of therapy varies among institutions.

Several previous reports in renal transplant recipients have demonstrated that some immunosuppressed individuals are capable of developing CMV-specific humoral and cellular immune responses during primary and following infection (10–12). Earlier studies that examined CMV immune responses in LTRs following primary infection demonstrated CMV-specific Ab and total T cell proliferative responses (using [³H]thymidine incorporation) in the majority of D⁺R⁻ patients tested (13, 14). However, these studies in LTRs did not assess the frequency of CMV-specific cytokine-producing cells or the proliferative capacities within the CD4⁺ and CD8⁺ T cell subsets. Moreover, little is known regarding the presence or absence of CMV-specific responses at the level of the allograft following primary infection, or whether intragraft CMV-specific cellular memory responses are enduring in the allograft in patients after primary infection. Additionally, the prevalence of high-risk D⁺R⁻ LTRs developing CMV-specific immune responses remains unclear, and if so, whether these responses may impact allograft function and CMV disease. To address these questions, we performed a cross-sectional analysis of a cohort of 22 D⁺R⁻ LTRs and assessed CMV-specific immune responses (IFN-γ positivity and T cell proliferation) in response to overlapping pooled peptides for the immunodominant Ags pp65 and IE1, as well as CMV lysate. As a positive control in these in vitro studies, we used the superantigen staphylococcal enterotoxin B (SEB) produced by the human pathogen Staphylococcus aureus that activates T cells bearing specific Vβ regions (15). We find that a majority of D⁺R⁻ seroconverted LTRs have detectable CMV-specific cellular responses in the PBMC following primary infection during viral latency. Additionally, we show that memory response from distinct T cell subsets persist in the lung allograft and PBMC often long after primary infection. These CMV-specific immune responses were further examined with respect to CMV disease and lung allograft function in our cohort, and the results of these studies are discussed in relation to CMV surveillance and...
CMV-SPECIFIC MEMORY RESPONSES IN THE LUNG ALLOGRAFT AND BLOOD

**Materials and Methods**

**Subjects and samples**

LTRs in the Johns Hopkins Lung Transplantation clinic and clinical database were identified based on pretransplant CMV serology mismatch status (D’R”), and all available living candidates were consented for study participation using an Institutional Review Board-approved consent form and enrolled in a cross-sectional analysis study over a 2.5-year period. Therefore, D’R+ patients, at various times posttransplant, were initially tested for the presence or absence of CMV-specific IgG. We then performed tests that patients developed CMV-specific IgG that detectable CMV-specific cellular responses, compared with those who failed to convert. All patients were treated with standard two to three drug immunosuppression at the time of analyses (see Tables I, II, and III), and although calcineurin inhibitor doses were variable among patients, all patients had maintenance therapeutic levels at the time of study. Brefeldin A (10 μg/ml) (Sigma-Aldrich) was added for the final 4 h of culture as described previously (16). We used CFSE (Molecular Probes) labeling at a concentration of 625 nm to assess Ag-specific proliferation, with CFSE-labeled cultures harvested on day 6 and fluorescence intensity immediately determined. The following fluorochrome-labeled Abs were purchased from BD Biosciences: FITC-conjugated anti-CD3, PE-conjugated anti-CD4, PerCP-cyanin 5.5-conjugated (Cy5.5) anti-CD8, allophycocyanin-conjugated anti-IFN-γ, isotype control Ab, and anti-CD3. Cell fluorescence was analyzed using a FACS Calibur (BD Biosciences)cytometer. Data were analyzed using FlowJo software (Tree Star).

**CMV ELISA**

Patients were treated for CMV IgG using a qualitative ELISA (Wampole Laboratories) specific for inactivated CMV strain AD169, limit of assay 0.9 OD ratio. When multiple CMV IgG assays were obtained, the first positive determination and the most recent negative determination were used for the analysis.

**Statistical analysis**

Actuarial freedom from allograft dysfunction and CMV disease in relation to CMV serologic status were assessed using the Kaplan-Meier method, with the log-rank test used for the analysis.

### Table I. Patient characteristics and CMV serology

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age (years)</th>
<th>Gendera</th>
<th>Survival</th>
<th>Primary Diagnosis</th>
<th>Immunosuppressionb</th>
<th>CMV IgG</th>
<th>CMV IgG (time)c</th>
<th>CMV Episodes</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>55</td>
<td>F</td>
<td>No (EBV lymphoma)</td>
<td>PPHb</td>
<td>CSA 1752, MMF 0.52, pred 152</td>
<td>(-)</td>
<td>1521</td>
<td>0</td>
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<tr>
<td>2</td>
<td>52</td>
<td>M</td>
<td>Yes</td>
<td>COPD/empysema</td>
<td>TAC 21/5, MMF 12, pred 51</td>
<td>(+)</td>
<td>664</td>
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<tr>
<td>3</td>
<td>36</td>
<td>F</td>
<td>No (sepsis)</td>
<td>Scleroderma</td>
<td>CSA 302, AZA 100, pred 52</td>
<td>(+)</td>
<td>1164</td>
<td>2</td>
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<tr>
<td>4</td>
<td>39</td>
<td>M</td>
<td>Yes</td>
<td>Cystic fibrosis</td>
<td>CSA 1002, AZA 125, pred 51</td>
<td>(-)</td>
<td>1809</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>63</td>
<td>F</td>
<td>No (CMV)</td>
<td>COPD/empysema</td>
<td>TAC 652, MMF 0.52, pred 61</td>
<td>(+)</td>
<td>262</td>
<td>3</td>
</tr>
<tr>
<td>6</td>
<td>17</td>
<td>M</td>
<td>Yes</td>
<td>Bronchopulmonary dysplasia</td>
<td>TAC 2, RAPA1, pred 51</td>
<td>(+)</td>
<td>914</td>
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<tr>
<td>7</td>
<td>43</td>
<td>F</td>
<td>No (BOS)</td>
<td>COPD/empysema</td>
<td>TAC 0.52, MMF 0.54, pred 101</td>
<td>(-)</td>
<td>1323</td>
<td>6</td>
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<td>8</td>
<td>24</td>
<td>M</td>
<td>Yes</td>
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<td>TAC 21/5, MMF 12, pred 51</td>
<td>(+)</td>
<td>1432</td>
<td>0</td>
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<tr>
<td>9</td>
<td>59</td>
<td>M</td>
<td>No (respiratory failure)</td>
<td>Alpha-1-antitrypsin deficiency</td>
<td>TAC 1, pred 201</td>
<td>(-)</td>
<td>1558</td>
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<td>10</td>
<td>11</td>
<td>F</td>
<td>Yes</td>
<td>Interstitial lung disease</td>
<td>TAC 252, MMF 0.75, pred 201</td>
<td>(-)</td>
<td>785</td>
<td>0</td>
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<td>11</td>
<td>29</td>
<td>F</td>
<td>No (Sepsis)</td>
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<td>(-)</td>
<td>432</td>
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<td>12</td>
<td>25</td>
<td>M</td>
<td>Yes</td>
<td>PPH</td>
<td>TAC 100/75, AZA 100, pred 51</td>
<td>(+)</td>
<td>1333</td>
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<tr>
<td>13</td>
<td>58</td>
<td>M</td>
<td>No (CMV)</td>
<td>Idiopathic pulmonary fibrosis</td>
<td>CSA 100/75, MMF 12, pred 101</td>
<td>(-)</td>
<td>195</td>
<td>4</td>
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<tr>
<td>14</td>
<td>45</td>
<td>F</td>
<td>Yes</td>
<td>Scleroderma/ILD</td>
<td>CSA 752, AZA 753, pred 51</td>
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<td>15</td>
<td>56</td>
<td>F</td>
<td>Yes</td>
<td>Scleroderma/PH</td>
<td>CSA 1752, MMF 0.52, pred 1</td>
<td>(+)</td>
<td>1054</td>
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<td>16</td>
<td>35</td>
<td>M</td>
<td>Yes</td>
<td>COPD/empysema</td>
<td>TAC 2, MMF 12, pred 51</td>
<td>(+)</td>
<td>2190</td>
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<td>F</td>
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<td>(+)</td>
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<tr>
<td>18</td>
<td>43</td>
<td>F</td>
<td>Yes</td>
<td>Sarcoïdosis</td>
<td>TAC 1.5, MMF 12, pred 51</td>
<td>(+)</td>
<td>1731</td>
<td>2</td>
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<tr>
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<td>52</td>
<td>F</td>
<td>Yes</td>
<td>COPD/empysema</td>
<td>TAC 3, AZA 150, pred 51</td>
<td>(-)</td>
<td>2018</td>
<td>0</td>
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<tr>
<td>20</td>
<td>49</td>
<td>F</td>
<td>No (thromboembolism)</td>
<td>Sarcoïdosis</td>
<td>TAC 3, MMF 0.75, pred 51</td>
<td>(+)</td>
<td>2569</td>
<td>0</td>
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<tr>
<td>21</td>
<td>27</td>
<td>F</td>
<td>Yes</td>
<td>Veno-occlusive disease/PPH</td>
<td>TAC 4, pred 7.5</td>
<td>(+)</td>
<td>232</td>
<td>1</td>
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<td>22</td>
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<td>Yes</td>
<td>Idiopathic pulmonary fibrosis</td>
<td>TAC 21, pred 5</td>
<td>(-)</td>
<td>120</td>
<td>0</td>
</tr>
</tbody>
</table>

*a* F, female; M, male; PPH, primary pulmonary hypertension.

*b* CSA, cyclosporine (dose in milligrams with superscript times per day); TAC, tacrolimus (dose in milligrams); MMF, mycophenolate mofetil (dose in grams); AZA, azathioprine (dose in milligrams); Pred, prednisone (dose in milligrams); RAPA, rapamycin (dose in milligrams).

*c* Time in posttransplant days.
and statistical significance was calculated using the Cox-Mantel log-rank test. A \( p \) value of <0.05 was considered statistically significant.

**Results**

**D\(^+\)R\(^-\) LTRs are heterogeneous in their development of posttransplant CMV seroconversion and CMV clinical disease**

We identified 22 LTRs at our institution with CMV D\(^+\)R\(^-\) serology status at the time of transplant (Table I). Qualitative anti-CMV IgG was assessed cross-sectionally in all patients over a 2.5-year period using standard ELISA, with seroconversion, or lack thereof, indicated in posttransplant days. Heterogeneity among this cohort was revealed in regard to seroconversion, with posttransplant CMV IgG detectable in 13 of 22 individuals tested (59.1%). To ensure that CMV IgG titers were true endogenous responses and not due to exogenous administration, individuals were first assayed at least 60 days after CMV Ig infusion therapy in those cases where it was administered after \( \approx \)2–3 half-lives (17), and then rechecked 2–3 mo later to ensure the response was still detectable. Interestingly, of those individuals with evidence of seroconversion, only 8 of 13 (57.1%) had clinical episodes of CMV disease, suggesting that subclinical primary CMV infection occurs in D\(^+\)R\(^-\) LTRs and is sufficient to induce CMV-specific immune responses in some individuals.

**CMV-specific CD8\(^+\) IFN-\(\gamma\) T cells are detectable in the majority of seroconverted D\(^+\)R\(^-\) LTRs during latent infection**

Functional effector memory T cell populations in response to CMV are heterogeneous; however, the presence of CMV-specific CD8\(^+\) IFN-\(\gamma\) T cells have been demonstrated to be a dominant effector response (15, 18). Therefore, to determine whether D\(^+\)R\(^-\) LTRs acquire functional CMV-specific cellular immunity following primary infection, we performed in vitro stimulation of PBMC using pooled peptides of the immunodominant CMV Ags pp65 or IE1 and determined Ag-specific IFN-\(\gamma\) production using ICS (Fig. 1). In these experiments, PBMC were cultured for 6 h in medium alone, or in the presence of either pp65 peptides, IE1 peptides,
CMV-specific IFN-γ responses are present in the majority of seroconverted D^R^ LTRs studied with posttransplant CMV IgG seroconversion (Table II). In the same experiments, we assessed in vitro T cell proliferative responses to CMV Ags pp65 peptides, IE1 peptides, and CMV lysate. Using CFSE labeling, we assessed proliferation on day 6 following Ag stimulation. In Fig. 2, we show seroconverted patient no. 21 following recovery from acute systemic CMV infection, with similar CD4^- (Fig. 2a) and CD8^- (Fig. 2b) proliferative responses in response to CMV lysate as those seen in a typical seropositive normal donor. In contrast, the nonseroconverted D^R^- LTRs we studied did not generate substantial CMV-specific T cell proliferative responses as in patient no. 19, although weak responses could occasionally be detected as in patient no. 4 (Table III). Similar to our cytokine results, T cells from seronegative individuals are quite capable of proliferating in response to SEB or OKT3/CD28 (data not shown), suggesting there is not a major defect in the T cell proliferative capacity in patients that did not proliferate in response to CMV Ags. Thus, whereas the magnitude of CMV-specific T cell proliferation was variable in this cohort of D^R^ LTRs, the majority (9 of 10 D^R^- seroconverters or 90%) had detectable CD4^+ and/or CD8^+ proliferation of 1% or more in response to CMV lysate or pp65 peptides above medium background (Table III).

A subset of CMV-specific CD4^+ T cells produce IFN-γ upon re-expansion during latent infection

Because we did not detect CMV-specific CD4^+ IFN-γ^- T cells in the majority of seroconverted D^R^- LTRs, but did detect CMVSpecific CD4^+ proliferative responses, in most of these patients, we asked whether proliferating CD4^+ T cells demonstrated effector function(s). We examined proliferating CMV-specific CD4^+ T cells on day 6 following CMV lysate stimulation for IFN-γ positivity by gating on the CD4^+ CFSElow population as shown in Fig. 3. We found a subset of CFSElow CD4^+ IFN-γ^- cells (8.33%) in the PBMC of patient no. 21 in whom CD4^+ IFN-γ^- cells were not detectable following 6-h stimulation with CMV lysate (Fig. 1b). Similar results were seen in patient no. 17 (data not shown). We further assessed proliferating CMV-specific CD4^+ CFSElow T cells and found that a subset of these cells also secreted TNF-α, and, surprisingly, virtually all of these cells expressed the cytolytic effector molecule granzyme B. The latter is consistent with a recent report demonstrating the emergence of CMV-specific CD4^+ CD28^+ granzyme B^+ T cell subsets following primary CMV infection in renal transplant recipients (19). In contrast, nonproliferating (CD4^+ CFSElow) T cells did not exhibit effector functions (data not shown). Thus, whereas CMV-specific CD4^+ IFN-γ^- effector memory cells were not readily detectable in a short 6-h in vitro assay in the majority of our D^R^- patients during viral latency, we found that longer term 6-day cultures were sufficient to amplify the responses to detect subsets of CMV-specific proliferating CD4^+ T cells producing IFN-γ or TNF-α and the cytolytic effector molecule granzyme B.

### Table II. CMV-specific IFN-γ

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>CD4^+</th>
<th>CD8^+</th>
<th>CD4^+</th>
<th>CD8^+</th>
<th>CD4^+</th>
<th>CD8^+</th>
<th>Timea</th>
<th>Immunosuppressionb</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (−)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.44</td>
<td>1.04</td>
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<tr>
<td>4 (−)</td>
<td>0</td>
<td>0.13</td>
<td>0.11</td>
<td>0.01</td>
<td>Nd</td>
<td>Nd</td>
<td>0.41</td>
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<td>5 (+)</td>
<td>0.78</td>
<td>1.36</td>
<td>0.11</td>
<td>0.06</td>
<td>0.01</td>
<td>0.04</td>
<td>0.14</td>
<td>0.01</td>
</tr>
<tr>
<td>6 (+)</td>
<td>0.03</td>
<td>0.06</td>
<td>0.06</td>
<td>0.04</td>
<td>0.08</td>
<td>0.04</td>
<td>0.14</td>
<td>0.01</td>
</tr>
<tr>
<td>8 (+)</td>
<td>0.38</td>
<td>0.36</td>
<td>0.37</td>
<td>0.42</td>
<td>0.35</td>
<td>0.41</td>
<td>0.57</td>
<td>4.01</td>
</tr>
<tr>
<td>12 (+)</td>
<td>0.1</td>
<td>1.01</td>
<td>0.03</td>
<td>0.46</td>
<td>0.01</td>
<td>0.06</td>
<td>1.85</td>
<td>2.91</td>
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<tr>
<td>14 (+)</td>
<td>1.55</td>
<td>0.07</td>
<td>0.22</td>
<td>0.95</td>
<td>0.96</td>
<td>0.08</td>
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<tr>
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<td>0.01</td>
<td>0</td>
<td>0.24</td>
<td>0.04</td>
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<td>0</td>
<td>2.64</td>
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<td>16 (+)</td>
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<td>17 (+)</td>
<td>0</td>
<td>0.21</td>
<td>0.15</td>
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<td>0.06</td>
<td>0</td>
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<tr>
<td>18 (+)</td>
<td>1.2</td>
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<td>2.11</td>
<td>0.58</td>
<td>0.18</td>
<td>0.53</td>
<td>11.02</td>
<td>2285</td>
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<tr>
<td>19 (−)</td>
<td>0.04</td>
<td>0.07</td>
<td>0.01</td>
<td>0.01</td>
<td>0.02</td>
<td>0.03</td>
<td>0.77</td>
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<td>21 (+)</td>
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<td>0.05</td>
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<td>0.03</td>
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<td>22 (−)</td>
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<td>0</td>
<td>0</td>
<td>2.69</td>
<td>4.58</td>
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### Notes

- CD8^- T cells specific for pp65 and/or IE1 in 9 of 10 (90%) D^R^- LTRs during latent infection in those patients who did not seroconvert, as demonstrated by patient no. 2. This was not due to an inability of their CD8^- T cells to produce IFN-γ, because they had similar frequencies of IFN-γ^-CD8^- T cells in response to SEB stimulation as in those patients with detectable CMV-specific responses (Fig. 1a and Table II). Overall, we found CD8^-IFN-γ^- T cells specific for pp65 and/or IE1 in 9 of 10 (90%) D^R^- LTRs studied with posttransplant CMV IgG seroconversion (Table II). In contrast, we detected CMV-specific CD4^-IFN-γ^- T cells in response to pp65 peptides and/or CMV lysate in only 3 of 9 patients (33%); however, one of these patients (patient no. 5) was persistently viremic with ganciclovir-resistant CMV (Fig. 1b and Table II). Together, these data indicate a higher prevalence of CMV-specific CD8^-IFN-γ^- compared with CD4^-IFN-γ^- T cell responses in the PBMC of LTRs during latent infection after primary infection. Moreover, despite substantial immunosuppressive therapy, these CMV-specific responses frequently persist in patients years after transplantation and/or clinical CMV disease.

Both CMV-specific CD4^- and CD8^- T cell proliferative responses are present in the majority of seroconverted D^R^- LTRs during latent infection

In the same experiments, we assessed in vitro T cell proliferative responses to the CMV Ags pp65 peptides, IE1 peptides, and CMV lysate. Using CFSE labeling, we assessed proliferation on day 6 following Ag stimulation. In Fig. 2, we show seroconverted patient no. 21 following recovery from acute systemic CMV infection, with similar CD4^- (Fig. 2a) and CD8^- (Fig. 2b) proliferative responses in response to CMV lysate as those seen in a typical seropositive normal donor. In contrast, the nonseroconverted
It is increasingly evident that nonlymphoid tissue such as the lung is an important site for effector memory T cells (20, 21). We sought to determine whether CMV-specific T cells were present in the lung allograft after primary infection, detectable in some cases years following clinical disease.

Acquisition of CMV immunity is associated with freedom from CMV disease and preservation of allograft function

We wished to assess whether the development of CMV-specific immune responses in D\(^{+}\)R\(^{-}\) LTRs provided functional protection from clinical CMV disease (pneumonitis and/or viremia). To address this question, we performed a time-weighted analysis to assess freedom from CMV disease. Our data show that D\(^{+}\)R\(^{-}\) individuals who developed posttransplant CMV immunity (using seroconversion as a surrogate for immunity) had significantly less CMV disease following documented seroconversion compared with those D\(^{-}\)R\(^{+}\) patients (\(p \leq 0.009\)) who failed to acquire CMV immunity (CMV naive) as shown (Fig. 5a). These data are consistent with the idea that primary CMV immune responses are functional, and appear critical for effective viral control in D\(^{-}\)R\(^{+}\) high-risk patients. Although the role of CMV infection in the long-term outcome of LTRs remains controversial, the majority of studies implicate CMV infection as a risk factor for bronchiolitis obliterans, the clinical manifestation of chronic rejection. Therefore, we evaluated our D\(^{+}\)R\(^{-}\) patient cohort to assess whether the acquisition of CMV immunity during primary infection affected long-term graft function. Using a log-rank Kaplan-Meier analysis, we found that D\(^{+}\)R\(^{-}\) CMV immune individuals had relative preservation of lung allograft function over time, as determined by serial measurements of the FEV\(_{1}\), as illustrated in Fig. 5b (\(p \leq 0.02\)), compared with D\(^{-}\)R\(^{+}\) patients who failed to develop immunity. Together, these data suggest that the acquisition of CMV immunity in D\(^{-}\)R\(^{+}\) LTRs correlates with freedom from CMV disease and is associated with preservation of allograft function over time.

Discussion

In this study, we show that despite significant immunosuppression, ~60% of D\(^{+}\)R\(^{-}\) LTRs acquire posttransplant CMV-specific humoral and cellular immune responses. In those patients who develop CMV-specific immunity, we detect CD4\(^{+}\) and CD8\(^{+}\) CMV-specific cellular responses in the PBMC, as well as in the lung allograft, with persistence of these responses in some cases long

**FIGURE 2.** Representative CMV-specific and SEB-responsive proliferative responses in seroconverted and nonseroconverted D\(^{-}\)R\(^{+}\) lung transplant patients. PBMC from D\(^{-}\)R\(^{+}\) LTRs were CFSE-labeled and cultured for 6 days in the presence or absence of either CMV lysate (2.5 \(\mu\)g/ml) or SEB (1 \(\mu\)g/ml), and cultures were assessed for decreased fluorescence intensity of CFSE by flow cytometry. Gated CD4\(^{+}\) (a) and CD8\(^{+}\) (b) T cells from a seroconverted patient, a normal donor, and a seronegative patient.

CMV-specific CD4\(^{+}\) and CD8\(^{+}\) T cells are present in the lung allograft during latent infection

It is increasingly evident that nonlymphoid tissue such as the lung is an important site for effector memory T cells (20, 21). We sought to determine whether CMV-specific T cells were present in LMNC obtained by BAL (as part of routine clinical surveillance) during latent infection and, if so, how these responses compared with PBMC responses. We examined BAL LMNC for CMV-specific responses in four seroconverted D\(^{-}\)R\(^{+}\) patients simultaneously with PBMC responses, as well as two nonseroconverted D\(^{-}\)R\(^{+}\) patients without CMV-specific PBMC responses. Representative CD8\(^{+}\) responses from these patients are shown in Fig. 4a and reveal similar (patient no. 18) to slightly higher frequencies (patients nos. 21 and 17) of pp65- and IE1-specific CD8\(^{+}\) IFN-\(\gamma\) T cells in the BAL LMNC compared with the PBMC. As shown above, CMV-specific CD4\(^{+}\) IFN-\(\gamma\) were found in only a minority of patients in the PBMC; however, we detected this effector memory cell population in the BAL LMNC of all four seroconverted patients studied during latent infection with three representative patients shown (Fig. 4b). Two of these patients (nos. 21 and 17), in fact, did not have detectable CMV-specific CD4\(^{+}\) IFN-\(\gamma\) effector memory T cells in their PBMC from the same day, and all patients examined had no evidence of CMV viral replication in the BAL or blood at the time of study. These patients were examined at various times following primary CMV infection, with patient no. 21 having recently recovered from disseminated CMV 1 mo prior (8 mo posttransplant); patient no. 17 had recovered from viremia/pneumonitis 1 year prior (2 years posttransplant); and patient no. 18 had a brief episode of viremia 1 year prior, and CMV colitis 5 years prior, but, interestingly, no clinical episodes of CMV pneumonitis (6 years posttransplant). As expected, two D\(^{-}\)R\(^{+}\) patients studied, who did not have detectable PBMC responses, similarly lacked detectable CMV-specific BAL LMNC responses (data not shown). Furthermore, the frequencies CD8\(^{+}\) IFN-\(\gamma\) cells in response to SEB and the CMV peptides were often higher (2- to 12-fold) in the BAL LMNC compared with the PBMC (as in patients nos. 17 and 21), and the CD8\(^{+}\) IFN-\(\gamma\) cells in response to SEB were strikingly higher (~15-fold) in the lung, further supporting the notion that cytokine-producing effector memory T cells may preferentially localize in the lung. Together, these data show that CMV-specific CD4\(^{+}\) and CD8\(^{+}\) IFN-\(\gamma\) T cells persist in the lung allograft after primary infection, detectable in some cases years following clinical disease.

**TABLE 2.** Representative CMV-specific and SEB-responsive proliferative responses in seroconverted and nonseroconverted D\(^{-}\)R\(^{+}\) lung transplant patients. PBMC from D\(^{-}\)R\(^{+}\) LTRs were CFSE-labeled and cultured for 6 days in the presence or absence of either CMV lysate (2.5 \(\mu\)g/ml) or SEB (1 \(\mu\)g/ml), and cultures were assessed for decreased fluorescence intensity of CFSE by flow cytometry. Gated CD4\(^{+}\) (a) and CD8\(^{+}\) (b) T cells from a seroconverted patient, a normal donor, and a seronegative patient.
after primary CMV infection, suggesting that these are durable, protective immune responses. Furthermore, we found differential CD4⁺ T cell responses in the lung allograft compared with the PBMC in some patients during latent infection, suggesting distinct memory pools within these respective compartments. As far as we are aware, this is the first report that has demonstrated functional CMV-specific CD4⁺ and CD8⁺ effector memory T cell responses in an organ allograft such as the lung, and has also shown differences between these lung allograft responses and those in the peripheral blood. The detection of CMV-specific effector T cell responses in BAL cells also suggests that LTRs could serve as unique models for studying the dynamics between an immunosuppressed transplant host, an organ allograft, and CMV infection. Although we examined CMV-specific effector responses in BAL cells from only a few subjects in our study, the differences observed in overall CMV-specific frequency, particularly in the CD4⁺ T effector compartment, raise the question of whether the monitoring of CMV-specific responses at the allograft level may have clinical utility in these high-risk patients. Therefore, more studies are needed to further characterize the CMV-specific effector pools in the lung allograft and peripheral blood, and to further delineate the implications of immune responses in these compartments. To that end, we show that the acquisition of CMV-specific immunity in D⁺R⁻ LTRs might be associated with improved clinical outcomes compared with those who fail to develop CMV immunity, underscoring the potential importance of these responses, and that the factors contributing to their development warrant further investigation.

In our cohort, we detected CMV-specific CD8⁺ IFN-γ⁺ T cells in the majority of seroconverted D⁺R⁻ LTRs in response to the pooled peptides pp65 and/or IE1. These results are consistent with previous reports showing pp65 and IE1 to be the immunodominant target Ags for peripheral blood CTL in normal CMV-seropositive individuals and seropositive renal transplant recipients (22–25), although we acknowledge that these CMV-specific T cell responses may underrepresent the total cellular response, because recent evidence indicates a broader recognition of other CMV Ags (26–28). Interestingly, 4 of 8 D⁺R⁻ patients with detectable CMV-specific CD8⁺ cytokine responses had both pp65- and IE1-specific CD8⁺ IFN-γ⁺ memory populations in the PBMC, further demonstrating the importance of these immunodominant viral Ags in transplant recipients. Differences in our detection of CMV-specific CD8⁺ IFN-γ⁺ memory cells compared with CD4⁺ IFN-γ⁺ cells in these patients may reflect a greater stability of the circulating CD8⁺ effector memory pool compared with the CD4⁺ memory pool (29, 30). Conversely, several studies have shown that

![Figure 3](http://www.jimmunol.org/Downloadedfrom)
viral-specific CD8+ memory responses are depleted following infections with heterologous viral infection (31, 32). This latter mechanism may account for the lack of CMV-specific CD8+ IFN-γ+ responses from patient no. 6 who had evidence of seroconversion 2 years before and a history of multiple viral infections only demonstrating CD4+ and CD8+ T cell proliferative responses to pp65 peptides. However, we suspect that this patient has adequate immunity based on the lack of clinical CMV disease observed posttransplant. In this regard, previous investigators have demonstrated that proliferating CMV-specific CD4+ and CD8+ T cell effector functions, such as IFN-γ secretion and granzyme B/perforin positivity (19, 33). Moreover, these investigators have demonstrated that other factors, such as viral Ag stimulation and environmental cytokines, may play a significant role in the pool size and phenotype of CMV-specific CD8+ T cells (34). Recently, Ag concentration has been shown to be an important determinant in viral-specific CD8+ effector function, with cytolytic function occurring at lower Ag concentrations compared with cytokine effector function (35). Finally, other factors such as long-term immunosuppression in these patients may contribute to the more consistent finding of CMV-specific CD8+ IFN-γ+ memory cells compared with CD4+ IFN-γ+ cells in the majority of seroconverted D+R− LTRs. Thus, the detection of long-lasting pp65- and/or IE1-specific CD8+ IFN-γ+ memory responses in the PBMC of seroconverted D+R− LTRs by ICS is a sensitive measure of posttransplant CMV immunity.

In contrast to the CMV-specific CD8+ IFN-γ+ memory responses detected in our seroconverted D+R− LTRs, we only detected CMV-specific CD4+ IFN-γ+ T cells in two patients during viral latency. These findings are consistent with previous studies in renal transplant recipients, which demonstrated that the emergence of circulating CMV-specific CD4+ IFN-γ+ T cells is critical for the resolution of primary infection, yet decline rapidly following resolution of viremia, perhaps homing to secondary lymphoid tissue (10, 11, 36). However, our findings in D+R− LTRs further show that a CMV-specific CD4+ T memory pool capable of proliferating upon in vitro stimulation with CMV lysate and/or pp65 peptide stimulation is maintained in the PBMC of the majority of patients during
latently infected cells. Moreover, upon expansion, subsets of proliferating CMV-specific CD4\(^+\) T cells produce IFN-\(\gamma\) and TNF-\(\alpha\), with coexpression of granzyme B, consistent with a capacity for cytolytic function. These observations are most likely due to an initial low precursor frequency of CMV-specific CD4\(^+\) IFN-\(\gamma\) effector memory cells below the level of detection before expansion. Alternatively, these findings may suggest distinct lineage(s) of circulating T\(_{H1}\) viral-specific memory cells, which are initially IFN-\(\gamma\)\(^-\) (37). It is plausibly possible that in the absence of viremia, a circulating CMV-specific CD4\(^+\) T cell precursor memory pool (T\(_{p31}\)) may persist after primary infection, which requires Ag-induced proliferation before exhibiting effector function (38). This may result in part from incomplete CMV-specific CD4\(^+\) T cell differentiation due to the use of antiviral therapy during primary infection, resulting in rapid clearance of viral Ag from the blood in contrast to the typical untreated primary CMV infection course in normal individuals. An example of a D\(^+\)R\(^-\) patient with perhaps a “typical” primary CMV disease is patient no. 14, who never developed clinically significant primary CMV infection and, therefore, did not receive additional antiviral therapy (beyond the standard 3 mo of ganciclovir prophylaxis posttransplant), and who demonstrates persistent CMV-specific CD4\(^+\) IFN-\(\gamma\) effector memory cells in the PBMC over 4 years following transplantation. In contrast, patient no. 18 has had intermittent episodes of viremia without clinical pneumonitis (although not at the time of these studies), which may account for the persistence of detectable CMV-specific CD4\(^+\) IFN-\(\gamma\) effector memory cells in the PBMC. Thus, factors such as exposure to intermittent viremia or antiviral therapy, in addition to maintenance immunosuppression, may play a role in whether D\(^+\)R\(^-\) patients have detectable CMV-specific CD4\(^+\) IFN-\(\gamma\) responses in their PBMC during latent infection. However, we also find that circulating CMV-specific CD4\(^+\) T cells do persist in most seroconverted LTRs, and are capable of proliferation and effector functions following in vitro Ag stimulation.

Although circulating CMV-specific CD4\(^+\) IFN-\(\gamma\) effector memory T cells are not always readily detectable in seroconverted D\(^+\)R\(^-\) patients following 6-h in vitro antigenic stimulation, these cells can be detected in the lung allograft (BAL LMNC). This is perhaps due to greater accessibility to viral Ag, because the lung is a significant reservoir for CMV during latency (9). An earlier study in LTRs suggested that CMV-specific lymphocytes obtained by BAL were capable of proliferation several months following acute CMV pneumonia, although these investigators did not distinguish which T cell subsets nor evaluate viral-specific cytokine production (39). Our data suggest a potential important role for CMV-specific CD4\(^+\) IFN-\(\gamma\) effector memory cells in viral surveillance in the lung allograft, particularly because these responses are detectable during clinical quiescence, at times long after primary infection. Indeed, a recent report in HIV-infected individuals demonstrated the disappearance of CMV-specific CD4\(^+\) IFN-\(\gamma\) T cells before onset of CMV clinical disease (40). Furthermore, these data are consistent with recent reports in mice, showing that the presence of activated, virus-specific CD4\(^+\) effector memory T cells in the lung confer protection against secondary viral challenge (41, 42). Finally, the increased frequencies of CD4\(^+\) IFN-\(\gamma\) T cells detected in the lung in response to SEB compared with the PBMC in several patients further supports the notion that the lung is an important site for cytokine-producing effector T cells. Indeed, Harris et al. (43) have shown that Ag-specific nonlymphoid effector memory cells, including those in the lung, have differential function compared with their counterparts in the draining lymph node. Thus, persistent CMV-specific CD4\(^+\) IFN-\(\gamma\) memory T cells can be differentially detected in the lung allograft in contrast to the PBMC in some LTRs. Whether these differences are due to higher levels of viral Ag exposure, preferential localization of distinct memory populations, or other factors remain in question.

The development of posttransplant CMV immunity has previously been studied in LTRs. Earlier reports in LTRs that included D\(^+\)R\(^-\) patients noted seroconversion (CMV IgG Abs) in ~50–100% of the individuals tested (14, 44). A previous study by Zeevi et al. (13) reported that PBMC from 31 to 64% of D\(^+\)R\(^-\) LTRs had detectable Th proliferative responses to CMV protein Ags or whole CMV, respectively, compared with 63–65% in D\(^+\)R\(^+\) patients. However, these investigators used [\(^3\)H]thymidine incorporation to assess total T cell proliferation and did not discriminate between CD4\(^+\) or CD8\(^+\) subsets, or effector functions. Our findings indicate that CMV-specific proliferative responses from PBMC have variable contributions from both CD4\(^+\) and CD8\(^+\) subsets when assessed using CFSE labeling and flow cytometric analysis. We detected frequent CD8\(^+\) proliferative responses to CMV lysate stimulation, and suspect that these responses are most likely due to cross-priming mechanisms described in other infectious models (45, 46). Thus, CMV-specific proliferative responses in the PBMC are due to the contributions of both CD4\(^+\) and/or CD8\(^+\) memory populations that can persist long after primary infection in certain transplant recipients.

As far as we are aware, this is the first report that has formally demonstrated that the development of CMV immunity in response to primary infection can potentially impact posttransplant clinical outcomes. We report a freedom from CMV disease in those patients who develop primary CMV immunity compared with those that did not. These findings are consistent with the notion that viral-specific T cell responses confer protection in immunocompromised transplant patients, most likely by limiting viral load (47). Furthermore, these data are the first to correlate the presence of CMV responses following primary CMV infection to allograft function. We find that the FEV\(_1\), a physiologic measure known to decline with progressive allograft dysfunction, is relatively preserved in D\(^+\)R\(^-\) LTRs who develop posttransplant responses to CMV compared with those that do not, supporting the idea that these are important protective responses in this patient population. Although the role of CMV infection as a factor in the development of bronchiolitis obliterans syndrome (chronic rejection) remains controversial (44), the majority of studies suggest it is an important risk factor (8). However, we acknowledge these data do not demonstrate causality, i.e., that development of CMV-specific immunity alone leads to preservation of allograft function. Other factors such as acute rejection episodes and/or intensity of immunosuppression may also play important roles that were unable to be determined given the small cohort size. Furthermore, we point out the limitation that this was a cross-sectional study, evaluating these high-risk patients at various time points following transplant and primary CMV infection and, therefore, likely detected CMV-specific responses that were long present in some of these patients. The rapid divergence of the Kaplan-Meier curves in Fig. 5a might suggest that the acquisition of CMV immunity in the first posttransplant year is most critical in patients with primary CMV infection and is currently under prospective investigation. Nevertheless, these data suggest that the ability to acquire CMV-specific immunity might be a critical factor in determining whether CMV infection is an important cofactor for chronic rejection and/or allograft dysfunction. However, we also acknowledge that these studies were performed in a small cohort, and that although the development of CMV-specific immune responses may provide clinical benefit, other factors may play a
role in these observations. Thus, further studies are needed to determine whether monitoring patients posttransplant for the development of humoral and cellular responses over time may assist clinicians in risk stratifying patients for recurrent CMV disease and allograft dysfunction. Furthermore, these data raise the issue of whether strategies for the adoptive transfer of CMV-specific cellular immunity in individuals may benefit LTRs who fail to develop functional immunity, as previously shown in bone marrow transplant recipients (48, 49). Finally, these results provide evidence that chronic immunosuppression therapy should not be a uniform deterrent for exploring CMV vaccine strategies (50) in transplant recipients or transplant candidates in the pretransplant period, because the induction of vaccine strategies (50) in transplant recipients or transplant can-

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