Preformed Frequencies of Cytomegalovirus (CMV)–Specific Memory T and B Cells Identify Protected CMV-Sensitized Individuals Among Seronegative Kidney Transplant Recipients

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Background. Cytomegalovirus (CMV) infection remains a major complication after kidney transplantation. Baseline CMV risk is typically determined by the serological presence of preformed CMV-specific immunoglobulin (Ig) G antibodies, even though T-cell responses to major viral antigens are crucial when controlling viral replication. Some IgG-seronegative patients who receive an IgG-seropositive allograft do not develop CMV infection despite not receiving prophylaxis. We hypothesized that a more precise evaluation of pretransplant CMV-specific immunosensitization using the B and T-cell enzyme-linked immunospot assays may identify CMV-sensitized individuals more accurately, regardless of serological evidence of CMV-specific IgG titers.

Methods. We compared the presence of preformed CMV-specific memory B and T cells in kidney transplant recipients between 43 CMV IgG-seronegative (sR⁻) and 86 CMV IgG-seropositive (sR⁺) patients. Clinical outcome was evaluated in both groups.

Results. All sR⁺ patients showed a wide range of CMV-specific memory T- and B-cell responses. High memory T- and B-cell frequencies were also clearly detected in 30% of sR⁻ patients, and those with high CMV-specific T-cell frequencies had a significantly lower incidence of late CMV infection after prophylactic therapy. Receiver operating characteristic curve analysis for predicting CMV viremia and disease showed a high area under the receiver operating characteristic curve (>0.8), which translated into a high sensitivity and negative predictive value of the test.

Conclusions. Assessment of CMV-specific memory T- and B-cell responses before kidney transplantation among sR⁻ recipients may help identify immunized individuals more precisely, being ultimately at lower risk for CMV infection.

Keywords. kidney transplantation; CMV infection; T- and B-cell ELISPOT assay; adaptive immunity.

Despite the outstanding progress made with the advent of preventive antiviral strategies, cytomegalovirus (CMV) infection remains the most common opportunistic infection in kidney transplant recipients. Although primary infection in immunocompetent hosts is usually asymptomatic, transplant recipients are at increased risk of developing CMV infection in the period immediately after transplantation. This poses a critical challenge to both graft and patient survival [1, 2].

The T-cell immune response to CMV is known to be of primary importance in controlling viral infection [3–5]. However, the humoral adaptive immune response, evaluated by serological CMV-specific immunoglobulin (Ig) G titers, is the only marker currently available for immune-risk stratification in clinical practice. Unfortunately, this surrogate approach does not entirely help identify all truly immune-sensitized transplant recipients.
at lower risk of CMV infection after transplantation. Indeed, there is clear clinical evidence for this position; although most CMV IgG–seropositive (sR+) transplant recipients receiving a seropositive allograft are unlikely to develop CMV infection after transplantation, up to 20%–30% may experience CMV disease without antiviral prophylaxis [6, 7]. Furthermore, even though most seronegative recipients of seropositive allografts will develop CMV infection if not treated with antiviral prophylaxis, a considerable proportion (30%–40%) will never experience CMV infection [8].

An important body of evidence suggests that monitoring CMV-specific T-cell responses, at different times before and after transplantation, may allow a more accurate characterization of the immune risk profile against CMV infection [9–13]. Measuring circulating CMV-specific IgG antibodies is the most common method of assessing the CMV-specific B-cell sensitization status. However, this approach may underestimate the true magnitude of the humoral immune response, because it excludes the whole memory B-cell pool. In fact, memory B cells can exist in the absence of detectable serum antibody levels [14, 15], but are able to rapidly differentiate into antibody-secreting cells (ASCs), which may be highly relevant for an effective humoral response. Therefore, a direct assessment of the CMV-specific memory T and B cells in transplant recipients could provide a more complete picture of their adaptive memory immune response against CMV.

This study aimed to investigate the baseline CMV-specific memory T- and B-cell compartments using highly sensitive enzyme-linked immunospot (ELISPOT) assays in a cohort of seronegative and seropositive kidney transplant recipients. We measured the frequency of CMV-specific interferon (IFN) γ– and IgG–producing memory T and B cells and determined whether it could illustrate the immune sensitization status against CMV more accurately than circulating CMV IgG titers. These observations could be relevant to clinical CMV risk stratification, and they provide new insights into the mechanisms of the adaptive immune response against CMV infection in kidney transplantation.

Table 1. Clinical and Demographic Characteristics of Kidney Transplant Recipients by CMV IgG Serostatus

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>sR− Patients (n = 43)</th>
<th>sR+ Patients (n = 86)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex, male/female, No.</td>
<td>30/13</td>
<td>56/30</td>
</tr>
<tr>
<td>Age, mean ± SD, y</td>
<td>47.9 ± 17.3</td>
<td>51.7 ± 11.4</td>
</tr>
<tr>
<td>Type of kidney transplant, living/deceased donor, No. (%)</td>
<td>34 (79)/9 (21)</td>
<td>25 (29)/61 (71)</td>
</tr>
<tr>
<td>Donor CMV IgG serostatus</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Seronegative, No. (%)</td>
<td>6 (14)</td>
<td>15 (17.5)</td>
</tr>
<tr>
<td>Seropositive, No. (%)</td>
<td>37 (86)</td>
<td>71 (82.5)</td>
</tr>
<tr>
<td>Preventive therapy, prophylaxis/preemptive, No. (%)</td>
<td>37 (86)/8 (14)</td>
<td>11 (12.8)/75 (87.2)</td>
</tr>
<tr>
<td>Maintenance IS, No. (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CNI-based (TAC/CsA/other)</td>
<td>39 (90.7)/4 (9.3)/0 (0)</td>
<td>79 (92)/8 (71)/1 (1)</td>
</tr>
<tr>
<td>MMF/mTor-i</td>
<td>41 (95.3)/2 (4.7)</td>
<td>78 (90.7)/8 (9.3)</td>
</tr>
<tr>
<td>Induction IS, No. (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No induction/rATG/basiliximab</td>
<td>3 (7)/20 (46.5)/20 (46.5)</td>
<td>5 (6)/35 (40.5)/46 (53.5)</td>
</tr>
<tr>
<td>DGF, yes/no, No. (%)</td>
<td>11 (25.6)/32 (74.4)</td>
<td>22 (25.6)/64 (74.4)</td>
</tr>
<tr>
<td>BPAR, yes/no, No. (%)</td>
<td>7 (16.3)/36 (83.7)</td>
<td>12 (14)/74 (86)</td>
</tr>
<tr>
<td>Allograft function (eGFR), mean ± SD, mL/min</td>
<td>40.6 ± 28</td>
<td>45.8 ± 21</td>
</tr>
<tr>
<td>Month 6</td>
<td>40.8 ± 23</td>
<td>52.4 ± 16</td>
</tr>
<tr>
<td>Month 12</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CMV infection, yes/no, No. (%)</td>
<td>11 (25.6)/32 (74.4)</td>
<td>25 (29)/61 (71)</td>
</tr>
<tr>
<td>Disease</td>
<td>8 (18.6)/35 (81.4)</td>
<td>12 (14)/74 (86)</td>
</tr>
<tr>
<td>Pretransplant anti-CMV T-cell ELISPOT count, mean ± SD (range), IFN-γ spots/3 × 10⁶ PBMCs</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CMV lysate</td>
<td>33.61 ± 97.7 (0–448)</td>
<td>150.2 ± 190 (0–856)</td>
</tr>
<tr>
<td>pp65 Antigen</td>
<td>20.5 ± 42.8 (0–259)</td>
<td>120.24 ± 181 (0–765)</td>
</tr>
<tr>
<td>IE-1 antigen</td>
<td>26.78 ± 92.5 (0–604)</td>
<td>45.1 ± 95 (0–539)</td>
</tr>
</tbody>
</table>

Abbreviations: BPAR, biopsy-proved acute rejection; CMV, cytomegalovirus; CNI, calcineurin-inhibitors; CsA, cyclosporin A; DGF, delayed graft function; eGFR, estimated glomerular filtration rate; ELISPOT, enzyme-linked immunospot assay; IE-1, immediate-early protein 1; IFN, interferon; Ig, immunoglobulin; IS, immunosuppression; MMF, mycophenolate mofetil; mTor-i, mTor-inhibitors; PBMCs, peripheral blood mononuclear cells; pp65, 65-kDa phosphoprotein; rATG, rabbit anti-thymocyte globulin; SD, standard deviation; sR−, CMV IgG–seronegative transplant recipient; sR+, CMV IgG–seropositive transplant recipient; TAC, tacrolimus.
METHODS

Study Patients
This was a retrospective study case-control study. Between February 2010 and January 2013, a total of 50 consecutive CMV IgG–seronegative (sR−) kidney transplant recipients from our renal transplant unit were eligible to participate in the study. To confirm a stable pretransplant CMV IgG serostatus, 2 serial serology tests were performed during the year before transplantation, followed by another at the time of transplantation. We excluded 7 patients from the study: 5 patients without pretransplant blood samples, 1 with low but detectable CMV IgG titers 6 months before transplantation that were not detectable at transplantation, and 1 who received numerous blood transfusions during the 6 months before transplantation. Therefore, we evaluated 43 kidney transplant recipients. We included 86 contemporary and consecutively paired sR+ transplant recipients in a 2:1 ratio with the sR− group.

CMV Preventive Strategies
A preemptive strategy was used, with all sR− patients receiving a seronegative allograft (sR−/sD+) and all sR+ patients not receiving T-cell depleting agents (eg, rabbit anti-thymocyte globulin [rATG]). Prophylaxis with valgancyclovir over 100 days was restricted to sR− patients receiving a seropositive allograft (sR+/sD+) and or rATG induction therapy.

Clinical Data and Definitions
The definition of CMV infection was based on the criteria recommended by the American Society of Transplantation for use in clinical trials [16]. Briefly, CMV viremia was defined as the detection, by either quantitative nucleic acid testing or the 65-kDa phosphoprotein (pp65) antigenemia assay, of replicating CMV in blood without symptoms, and CMV disease was defined as evidence of CMV replication/antigenemia with compatible symptoms, including both viral syndrome and invasive tissue disease.

Microbiological Studies
Surveillance of CMV antigenemia was determined in polymorphonuclear leukocytes obtained by dextran sedimentation and formaldehyde fixed, stained, and read under a fluorescence microscope (rapid antigenemia anti-human CMV ppUL83; Argene; reference 14-002). Surveillance quantitative CMV DNA detection was evaluated in plasma using a real-time CMV kit (Abbott). The cutoff value for CMV DNA detection was 1000 copies/mL assessed in plasma.

Enzyme-Linked Immunosorbent Assay for CMV IgG Detection
CMV serostatus was determined using a commercial CMV IgG enzyme-linked immunosorbent assay Kit (BioCheck) according to the manufacturer’s instructions. Two serial serological tests were performed during the year before transplantation, followed

Figure 1. Representative images of cytomegalovirus (CMV)–specific immunoglobulin (Ig) G B-cell enzyme-linked immunospot (ELISPOT) assay results from CMV IgG seronegative (sR−) and seropositive (sR+) kidney transplant recipients with positive or negative CMV-specific interferon (IFN) γ T-cell ELISPOT results; CMV-specific IgG-producing memory B cells were detected in sR+ (A) and sR− (B) patients with positive CMV-specific IFN-γ T-cell ELISPOT results but not in sR− patients with negative results (C).
by another evaluation just at the time of the transplant surgery. The CMV IgG cutoff value for seronegativity was <1.1 IU/mL.

CMV Peptides
As stimuli for the IFN-γ ELISPOT assay, we used pools derived from a peptide scan (15mer overlapping by 11 amino acids) covering the whole antigen length through the immediate-early protein 1 (IE-1) and the pp65 (Jerini Peptide Technologies; Swiss-Prot ID P13202 and P06725, respectively) of human CMV (Human Herpes Virus-5), plus a CMV lysate (Autoimmune Diagnostik). This allowed us to avoid human leukocyte antigen restrictions. We used Human CMV (AD169 strain) viral lysate (Advanced Biotech; 10-144-000) to detect CMV-specific IgG-secreting B cells.

CMV-Specific Memory/Effector B-Cell Assessment

CMV-Specific Memory/Effector T-Cell Assessment

CMV-Specific T-Cell ELISPOT
CMV-specific T-cell ELISPOT assays were performed as described elsewhere [13]. Briefly, 3 x 10^5 peripheral blood mononuclear cells (100 µL) were stimulated in triplicate with a CMV antigen peptide pool (1 µg/mL) for 18 hours, which exclusively assessed memory immune responses. We detected IFN-γ spots using a biotinylated anti-human IFN-γ antibody developed by the addition of alkaline phosphatase conjugate substrate (AID). The resulting spots were counted semiautomatically with an ELISPOT reader (AID ELISPOT Reader HR, fourth generation).

CMV-Specific IgG B-Cell ELISPOT Assay
A detailed description for the IgG B-cell ELISPOT assay can be found in the supplementary data. Briefly, we seeded 5 x 10^5 cells from the memory B-cell stimulation assay in 100 µL triplicates on a CMV purified viral lysate (Advanced Biotech; 10-144-000) coated 96-well ELISPOT plate (MAIPSWU10 MultiScreen, Millipore) after 18 hours of incubation at 37°C. The IgG spots were detected using a biotinylated human anti-IgG antibody and developed by the addition of streptavidin-conjugated alkaline phosphatase substrate (Mabtech). The resulting spots were counted semiautomatically with an ELISPOT reader (AID ELISPOT Reader HR, seventh generation).

Statistical Analysis
All data are presented as means and SDs. Groups were compared using the χ² test for categorical variables, the 1-way analysis of variance or t test for normally distributed data, and the nonparametric Kruskal–Wallis or Mann–Whitney U test for nonnormally distributed variables. Both CMV antigenemia and disease were considered outcome variables of the study. Bivariate correlation analyses were done using Pearson or Spearman tests for nonparametric variables. A sensitivity/specificity receiver operating characteristic analysis was done to investigate the value of the ELISPOT test for predicting posttransplant CMV infection. The 2-tailed statistical significance level was P < .05.

RESULTS

Baseline Patient Demographic Characteristics
Table 1 summarizes the main clinical and demographic characteristics of the 43 sR− patients and the 86 sR+ patients. Most
patients (86%) received a kidney allograft from a CMV IgG–seropositive donor (sD+). Most sR− patients received anti-CMV prophylaxis, whereas sR+ patients were followed up with the preemptive strategy. All but 1 patient in the sR+ group who re-
ceived belatacept were treated with a calcineurin inhibitor–based immunosuppressive regimen. Induction therapy was used in most patients with either anti-CD25 monoclonal antibodies or T-cell depletion (rATG). We observed CMV viremia and disease in 11 (25.6%) and 8 (18.6%) of the 43 sR− patients, respectively; the corresponding rates in the 86 sR+ patients were 25 (29%) and 12 (14%). All late-onset CMV infections in the sR− group were observed within the sR−/sD+ combination and appeared a median of 33 days after prophylactic treatment; most patients were asymptomatic or had viral syndromes diagnosed (5 of 8). The 3 cases of invasive tissue disease were located in the gastrointestinal tract. Two patients experienced CMV re-
currence after valganciclovir treatment.

Preformed T- and B-Cell CMV Sensitization Among sR− Kidney Transplant Recipients

First, we evaluated the frequency of CMV-specific IFN-γ-producing T cells against 2 specific CMV antigens (pp65 and IE-1) and a CMV lysate. As shown in Table 1 and Supplementary Figure 2, 13 (30%) and 15 (34%) of the 43 sR− patients, respective-
ly, displayed different detectable IE-1 (26.78 ± 92.5) and pp65 (20.5 ± 42.8) CMV-specific IFN-γ spots / 3×10⁵ stimulated peripheral blood mononuclear cells; pp65, 65-kDa phosphoprotein; rATG, rabbit anti-thymocyte globulin; SD, standard deviation; sD−, seronegative allograft; sD+, seropositive allograft; sR−, CMV immunoglobulin G-seronegative transplant recipient.

Abbreviations: BPAR, biopsy-proved acute rejection; CMV, cytomegalovirus; DGF, delayed graft function; eGFR, estimated glomerular filtration rate; ELISPOT, enzyme-linked immunospot; IE-1, immediate-early protein 1; IFN, interferon; IS, immunosuppression; MPA, micophenolyl acid; PBMCs, peripheral blood mononuclear cells; pp65, 65-kDa phosphoprotein; rATG, rabbit anti-thymocyte globulin; SD, standard deviation; sD−, seronegative allograft; sD+, seropositive allograft; sR−, CMV immunoglobulin G-seronegative transplant recipient.

a P < .05.
detected CMV-specific IFN-γ–producing T-cell frequencies also showed circulating CMV-specific IgG-secreting memory B cells (Figure 1B).

Preformed CMV-Specific IFN-γ T-Cell Frequencies in sR+ and sR− Patients

Next, we compared the strength of preformed IFN-γ–producing T cells against different CMV-specific antigens between sR− and sR+ patients. As shown in Figure 2, the mean preformed CMV-specific T-cell responses were significantly weaker among sR− than among sR+ patients, although a number of sR− patients displayed high IFN-γ T-cell frequencies, similar to those observed in some sR+ kidney transplant recipients.

Preformed CMV-Specific T-Cell Responses and CMV Infection in Both sR− and sR+ Patients

The main demographic and clinical variables were evaluated with regard to the advent of CMV infection after kidney transplantation (Table 2). No statistically significant associations were found between such variables as the type of CMV preventive therapy, the type of induction immunosuppression, the donor IgG serostatus (sD+ vs sD−), micopenlocy acid trough levels, the incidence of acute rejection, and the development of either CMV viremia or disease. Conversely, patients who experienced delayed graft function showed higher CMV disease incidences after transplantation. Of note, those with detectable preformed CMV-specific T-cell responses (against both IE-1 and pp65 CMV antigens) displayed significantly lower rates of CMV infection (both viremia and disease) than those with no evidence of CMV-specific T-cell sensitization before transplantation (Figure 3). Likewise, preformed CMV-specific IFN-γ–producing T-cell frequencies (both pp65 and IE-1 specific) were significantly lower among sR+ patients who developed CMV disease or viremia than among those who did not (Supplementary Figure 3).

Pretransplant Anti-CMV T-Cell Responses and Prediction of CMV Infection Risk

Receiver operating characteristic curve analysis of CMV-specific IFN-γ ELISPOT assay results for IE-1 and pp65 CMV antigens showed high sensitivity and specificity for the prediction of both CMV viremia and disease (Figure 4). The most sensitive and specific IFN-γ ELISPOT threshold against IE-1 and pp65 antigens were evaluated to establish the optimal threshold to define the CMV ELISPOT result as a binary variable (positive or negative) capable of predicting posttransplant CMV infection (both viremia and disease). As shown in Table 3, low specificities and positive predictive values were obtained for both CMV viremia and disease, but consistently high negative predictive values and sensitivities were observed for both tests, particularly for IE-1 T-cell responses. When these cutoff values were applied to the sR+ transplant group, similar low pretransplant CMV-specific T-cell frequencies predicted the development of CMV infection with high sensitivity and specificity (Supplementary Figure 4).

Discussion

The precise identification of a kidney transplant recipient’s immune susceptibility to CMV infection is a crucial goal for establishing guided preventive therapeutic strategies. Currently, the only criterion available to determine the patient’s immune status is the presence of preformed IgG antibodies against the virus. However, this is merely a surrogate of the complete humoral adaptive immunity expected to confer protection. Here, we report that evaluating the frequency of both CMV-specific IFN-γ and IgG-producing memory T and B cells allows a more precise assessment of immune-sensitized individuals without serological evidence of CMV-specific humoral immunity. Furthermore, we showed that transplant recipients with high frequencies of preformed CMV-specific IFN-γ–producing
memory T cells demonstrate clinical protection. They were unlikely to develop CMV infection after kidney transplantation compared with patients without cellular immune responses, which is consistent with our recent report on sR+ kidney transplant recipients [13].

Kidney transplantation waiting lists may contain to 20%–30% sR− individuals, and it is well known that sR−/sD+ recipients are at considerably higher risk of CMV infection. However, a significant proportion will never develop clinical infection, despite not receiving preventive treatment [8, 18], suggesting either that primary CMV-specific effector T-cell responses recover quickly and effectively immediately after transplantation, thereby providing sufficient protection and control of CMV replication [19, 20], or that these individuals may be appropriately sensitized before transplantation, despite no evidence of CMV IgG titers in their serum samples.

It is important to note that, although B-cell responses are commonly evaluated by the serological measurement of specific antibodies [21], analysis limited to this level may not provide a sufficient assessment of the absolute memory repertoire, because it excludes the memory B-cell subset [22, 23]. Indeed, memory B cells may exist in the absence of detectable serum antibody levels in different biological settings [14, 15], and their rapid differentiation into ASCs with antibody production may have high relevance for a protective humoral response [24]. To this end, the highly sensitive B-cell ELISPOT assay allows accurate detection of antigen-specific IgG ASCs at a single-cell level [17]. Using this, we observed that sR+ patients have concomitantly high frequencies of CMV-specific IgG ASCs, and some sR− patients may also have detectable frequencies of CMV-specific IgG ASCs. This suggests that these patients were already sensitized against CMV despite not showing circulating antibodies.

The T-cell compartment is thought to play a key role in viral replication and control [25–28]. Therefore, we aimed to investigate the CMV-specific memory T-cell response against the 2 dominant immunogenic CMV antigens (IE-1 and pp65) in the sR− patients. First, we observed that the sR− patients with detectable CMV-specific IgG-ASC also showed high CMV-specific IFN-γ–producing memory T-cell frequencies, reinforcing the fact that these individuals had had previous contact with CMV despite no serological evidence of IgG antibodies. Second,
although significantly lower frequencies were observed in sR− than in sR+ patients, robust T-cell responses occurred in both groups, suggesting some degree of immune sensitization variability between individuals that may appropriately be detected using a sensitive tool such as the IFN-γ ELISPOT assay.

Together with other investigators, we have recently reported that the presence of CMV-specific T-cell frequencies among sR+ patients seems to provide protection against CMV infection after kidney transplantation [11–13]. Interestingly, in our current study, sR+ patients not developing CMV viremia or disease showed significantly higher detectable IFN-γ–producing memory T-cell frequencies, especially against the IE-1 CMV antigen, than patients developing CMV infection after prophylactic treatment. Likewise, the same protective effect was observed among our control sR− patients. Moreover, when the most sensitive and specific T-cell ELISPOT cutoff value was used as a binary variable (positive or negative) to define the risk for late posttransplant CMV infection, very high sensitivity and negative predictive values for both CMV disease and viremia were obtained. This illustrates the usefulness of the assay for identifying immunized kidney transplant recipients without detectable serum CMV IgG titers.

A limitation of our study is the relatively small sample size, which precludes multivariate analysis of whether preformed T- and B-cell sensitization are independent protective variables against CMV infection after transplantation. Nonetheless, our data should be used to guide future prospective randomized interventional trials of these immune assays in the context of kidney transplantation and other solid organ transplant settings.

In summary, our observations may have relevant clinical implications, because 25%–30% of adult sR− transplant recipients may have robust undetected preformed CMV-specific memory B and T-cell memory clones, ultimately conferring sufficient immune protection to avoid CMV infection after kidney transplantation.

**Supplementary Data**

Supplementary materials are available at Clinical Infectious Diseases online (http://cid.oxfordjournals.org). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

**Notes**

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**Potential conflicts of interest.** All authors: No reported conflicts. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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