Surveillance of $\gamma\delta$ T Cells Predicts Cytomegalovirus Infection Resolution in Kidney Transplants

Hannah Kaminski,* Isabelle Garrigue,†‡ Lionel Couzi,*§ Benjamin Taton,* Thomas Bachelet,*§ Jean-François Moreau,§ Julie Déchanet-Merville,§ Rodolphe Thiébaut,** and Pierre Merville*§

*Department of Nephrology, Transplantation, and Dialysis and †Virology and ‡Immunology laboratories, Bordeaux University Hospital, Bordeaux, France; §National Center of Scientific Research (CNRS), Research Unit 5234, Bordeaux, France; ¶National Center of Scientific Research, Mix Unit of Research 5164, Bordeaux, France; ¶French Institute of Health and Medical Research (INSERM), Institute of Public Health and Epidemiology and Development (ISPED), Center U897–Epidemiology-Biostatistics, Bordeaux, France; and **National Institute for Research in Computer Science and Control (INRIA), Statistics in Systems biology and Translational Medicine (SISTM) Team, Bordeaux, France

ABSTRACT

Cytomegalovirus (CMV) infection in solid-organ transplantation is associated with increased morbidity and mortality, particularly if a CMV mutant strain with antiviral resistance emerges. Monitoring CMV–specific T cell response could provide relevant information for patient care. We and others have shown the involvement of $V_{\delta}^{2} neg$ $\gamma\delta$ T cells in controlling CMV infection. Here, we assessed if $V_{\delta}^{2} neg$ $\gamma\delta$ T cell kinetics in peripheral blood predict CMV infection resolution and emergence of a mutant strain in high–risk recipients of kidney transplants, including 168 seronegative recipients receiving organs from seropositive donors (D+R$^{2}$) and 104 seropositive recipients receiving antithymocyte globulins (R+/ATG). $V_{\delta}^{2} neg$ $\gamma\delta$ T cell percentages were serially determined in patients grafted between 2003 and 2011. The growing phase of $V_{\delta}^{2} neg$ $\gamma\delta$ T cells was monitored in each infected patient, and the expansion rate during this phase was estimated individually by a linear mixed model. A $V_{\delta}^{2} neg$ $\gamma\delta$ T cell expansion rate of $>0.06$% per day predicted the growing phase. The time after infection at which an expansion rate of 0.06% per day occurred was correlated with the resolution of CMV DNAemia ($r=0.91$; $P<0.001$). At 49 days of antiviral treatment, $V_{\delta}^{2} neg$ $\gamma\delta$ T cell expansion onset was associated with recovery, whereas absence of expansion was associated with recurrent disease and DNAemia. The appearance of antiviral–resistant mutant CMV strains was associated with delayed $V_{\delta}^{2} neg$ $\gamma\delta$ T cell expansion ($P<0.001$). In conclusion, longitudinal surveillance of $V_{\delta}^{2} neg$ $\gamma\delta$ T cells in recipients of kidney transplants may predict CMV infection resolution and antiviral drug resistance.

Received October 10, 2014. Accepted April 26, 2015.
Published online ahead of print. Publication date available at www.jasn.org.

Present address: Thomas Bachelet, Center for the treatment of Kidney Diseases, St. Augustin, Bordeaux, France.

Correspondence: Prof. Pierre Merville, Department of Nephrology, Transplantation and Dialysis, Bordeaux University Hospital, Place Amélie Raba Léon, 33076 Bordeaux, France. Email: pierre.merville@chu-bordeaux.fr

Clinical management of cytomegalovirus (CMV) infection remains a challenge in solid-organ transplantation (SOT), because its direct and indirect consequences increase patient morbidity and mortality occurrences. CMV infection resolution involves the synergistic action of an antiviral drug and an efficient CMV immune response. Consequently, a simple measurement of the latter would be of help in the management of infection. In consensus recommendations, the regimen of antiviral drugs includes a minimum course of 2 weeks, with a continuation of treatment until one or two consecutive virologically negative samples are obtained (often 49 days on the basis of the randomized Valcyte [valganciclovir po] compared to ganciclovir iv in patients with cytomegalovirus [CMV] disease who are solid organ transplant recipients [VICTOR] Trial).2 Notwithstanding, 30%
patients have virologic and 15% of patients have clinical recurrence of CMV infection, meaning that the management of anti-CMV therapy regimens could be improved. Patients with recurrent disease are at risk of developing an antiviral resistance (AVR), which is a serious complication observed in 6%–17% of patients with SOT and CMV disease. Risks factors associated with AVR are an extended exposure time to antiviral treatment and the absence of preexisting CMV–specific immunity (donor [D]–recipient [R]–patients). Consequently, the evaluation of cell-mediated immunity (CMI) during the course of infection may help improve the prediction of CMV infection resolution and therefore, the time of therapy discontinuation. Theoretically, CMI could also help predict the risk of emergence of a mutant strain, but it has never been shown until now. During the last decade, a variety of assays has been developed to measure CMV–specific cellular responses, but they have failed to identify a threshold of an efficient immune response that can predict CMV infection resolution. Moreover, there is a lack of data pointing to an immune marker that can help diagnose early the onset of a resistant strain.

Several years ago, we showed how a subset of γδ T cells is involved in the response toward the control of CMV infection. This subset, which is typically located within the epithelia, is characterized by the use of Vδ1, Vδ3, or Vδ5 segments (collectively called Vδ2γδ γδ T cells), whereas the most common subset in the peripheral blood uses the association of Vδ2 and Vγ9 variable regions (Vγ9/Vδ2 T cells). After CMV infection and not after other viral infections (such as HSV, VZV, EBV, and influenza), Vδ2γδ γδ T cells undergo massive expansion in the blood of recipients of kidney transplants, which is associated with the control of CMV infection. This observation has been extended to other SOTs as well as in healthy donors with positive serology, in bone marrow transplants, and during fetal life. We have shown that these cells displayed a restricted repertoire, which can be regarded as the signature of a CMV-driven selection and amplification of specific T cells in vivo. In vitro, data confirm Vδ2γδ γδ T cells as an efficient anti-CMV component of the immune response. Consequenly, their monitoring may help guide clinical decision making. Because measurement of Vδ2γδ γδ T cells in peripheral blood is easy to perform, inexpensive, and highly reproducible, we seriously measured the size of this subset by flow cytometry in recipients’ blood during the pre- and post-transplant periods both before and after the onset of CMV infection along with the monitoring of viral load. Our goal was to determine whether longitudinal monitoring of Vδ2γδ γδ T cells could predict CMV infection resolution or the occurrence of AVR in high-risk D+R− patients and R+/-antithymocyte globulin (ATG) recipients of kidney transplants.

RESULTS

Baseline Characteristics of Patients
As shown in Table 1, no significant differences were observed in D+R− patients between infected and uninfected patients. In infected R+/ATG patients compared with uninfected patients, donors and recipients were significantly older, and recipients were less HLA sensitized. No other differences were noted.

Median CMV DNAemia duration was longer in D+/R− patients: 134 (1st and 3rd quartiles: 14 and 373 days) compared with 24 days (6 and 194 days) in R+/ATG patients (P<0.001). In D+R− infected patients, median CMV DNAemia duration was 21 days (1st and 3rd quartiles: 12 and 30 days) in a 3-month prophylaxis group, 85 days (25 and 167 days) in a 6-month prophylaxis group, and 179 days (123 and 296 days) in the case of preemptive strategy (P<0.001). In R+/ATG infected patients, median CMV duration was not influenced by CMV prevention strategy: 24 (17 and 34) versus 26 (21 and 35) days for preemptive and prophylactic strategies, respectively (P=0.54).

Kinetics of Vδ2γδ γδ T Cells in Recipients of Kidney Transplants
During the 10 years of the study, on average, 10 phenotypic determinations of Vδ2γδ γδ T cells were available for each patient. For infected patients, the frequency of sampling averaged 29.2 days, with an SEM of 10.5 days, between day 0 of CMV infection and the end of the growing phase of Vδ2γδ γδ T cells. Results of the kinetics of Vδ2γδ γδ T cells are expressed in percentages of total lymphocytes (Figure 1), but similar profiles were observed with absolute counts (data not shown). D+R− infected patients (Figure 1A) had a slow and progressive growing phase of Vδ2γδ γδ T cells. The median (1st and 3rd quartiles) percentage of Vδ2γδ γδ T cells increased from 0.8% (0.4% and 2.9%) before CMV infection to 4.4% (1.4% and 10.7%) thereafter (P=0.001 from day 180). During the same period of time, the median (1st and 3rd quartiles) percentage of Vδ2γδ γδ T cells in D+R− uninfected patients (Figure 1A) remained stable at 0.7% (0.5% and 1.06%).

In infected R+/ATG patients (Figure 1B), the median percentage of Vδ2γδ γδ T cells increased from 1.6% (0.8% and 3%) before CMV infection to 6.1% (2.8% and 10.8%) thereafter (P=0.03 from day 60). Interestingly, the growing phase of Vδ2γδ γδ T cells occurred before CMV DNAemias became detectable. Moreover, Vδ2γδ γδ T cell percentages at the beginning of follow-up were significantly higher in patients who will not develop post–transplant CMV DNAemia (4% [2.1% and 9%]) than in those who will (1.6% [0.8% and 3%]; P=0.01).

Estimation of Vδ2γδ γδ T Cell Expansion Rate
A unique percentage of Vδ2γδ γδ T cells could not predict the resolution of DNAemia (Supplemental Figure 1). Instead, we considered, patient by patient, the time at which the growing phase in the whole Vδ2γδ γδ T cells kinetics occurred, and then, we estimated the Vδ2γδ γδ T cell expansion rate (Figure 2). Estimated expansion rates in infected D+R− (Figure 2A) and R+/ATG patients (Figure 2B) were not significantly different (P=0.35). We were able to determine a threshold of 0.06% Vδ2γδ γδ T cells increase per day that maximizes the
validity (according to AUC) of the definition of the growing phase of Vδ2neg γδ T cells kinetics (Figure 3, A and B). Thus, for a given patient, an increase of Vδ2neg γδ T cells >0.06% per day between two successive determinations predicted the growing phase, with sensitivity (Se) of 77%, specificity (Spe) of 90%, positive predictive value (PPV) of 88%, and negative predictive value (NPV) of 97% in prediction of CMV resolution at the end of the curative antiviral treatment. Consequently, Vδ2neg γδ T cell expansion onset at the end of the curative antiviral treatment was present in 95% of patients without recurrence, whereas absence of Vδ2neg γδ T cell expansion was found in 86% of patients with recurrent DNAemia and 93% of patients with recurrent disease. Consequently, Vδ2neg γδ T cell expansion onset had an Se of 93%, an Spe of 94%, a PPV of 87%, and an NPV of 97% in prediction of CMV resolution at the end of the curative antiviral treatment.

Correlation between the Time of Vδ2neg γδ T Cell Expansion and the CMV DNAemia Resolution

As shown in Figure 4A, the time of Vδ2neg γδ T cell expansion in infected D+R− patients was closely correlated with the CMV DNAemia resolution (r=0.91; P<0.001). Indeed, 88% of infected D+R− patients had CMV DNAemia resolution within 30 days after they reached 0.06% per day Vδ2neg γδ T cell expansion rate. In infected R+/ATG patients as well, the time of Vδ2neg γδ T cell expansion was correlated with the CMV DNAemia resolution (r=0.52; P<0.001) (Figure 4B). Of note, 92% of infected R+/ATG patients had CMV DNAemia resolution within 30 days after they reached 0.06% per day Vδ2neg γδ T cell expansion rate. Comparing R+ATG and D+/R− infected patients, the time of Vδ2neg γδ T cell expansion occurred earlier in R+/ATG than D+/R− patients (P<0.001; data not shown). To understand the worst correlation in R+/ATG infected patients compared with D+/R− infected patients, we analyzed separately the correlation in D+R− infected patients who received either ATG (n=14) or anti-ILR antibody (n=37) and found that the correlation in D+R− infected patients with ATG was the same as in R+/ATG patients (r=0.55; P<0.001) (Supplemental Figure 3).

To apply these data in a concrete clinical setting, we analyzed the Vδ2neg γδ T cell expansion onset at the end of the curative antiviral treatment according to the VICTOR Trial (49 days).2 We observed (Figure 5) that Vδ2neg γδ T cell expansion onset was present in 95% of patients without recurrence, whereas absence of Vδ2neg γδ T cell expansion was found in 86% of patients with recurrent DNAemia and 93% of patients with recurrent disease. Consequently, Vδ2neg γδ T cell expansion onset had an Se of 93%, an Spe of 94%, a PPV of 87%, and an NPV of 97% in prediction of CMV resolution at the end of the curative antiviral treatment.

### Table 1. Baseline characteristics of patients

<table>
<thead>
<tr>
<th>Criterion</th>
<th>D+R− (n=93)</th>
<th>D−R+ (n=75)</th>
<th>P Valuea</th>
<th>R+/ATG (n=74)</th>
<th>R−/ATG (n=30)</th>
<th>P Valuea</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Recipients</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age, yr (mean±SD)</td>
<td>50±12.3</td>
<td>46.8±14.7</td>
<td>0.50</td>
<td>56±11.6</td>
<td>49±9.6</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Sex (men/women)</td>
<td>65/28</td>
<td>55/20</td>
<td>0.70</td>
<td>48/26</td>
<td>17/13</td>
<td>0.50</td>
</tr>
<tr>
<td>HLA sensitization, N (%)</td>
<td>26 (28)</td>
<td>19 (25)</td>
<td>0.70</td>
<td>36 (49)</td>
<td>25 (83)</td>
<td>0.001</td>
</tr>
<tr>
<td><strong>Nephropathy, N</strong></td>
<td></td>
<td></td>
<td>0.70</td>
<td></td>
<td></td>
<td>0.40</td>
</tr>
<tr>
<td>Glomerular</td>
<td>36</td>
<td>28</td>
<td></td>
<td>21</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>Tubulointerstitial</td>
<td>12</td>
<td>8</td>
<td></td>
<td>9</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Vascular</td>
<td>7</td>
<td>11</td>
<td></td>
<td>10</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Binephrectomy for cancer</td>
<td>1</td>
<td>0</td>
<td></td>
<td>3</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Diabetes</td>
<td>4</td>
<td>2</td>
<td></td>
<td>4</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Congenital</td>
<td>24</td>
<td>18</td>
<td></td>
<td>13</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>Unknown</td>
<td>9</td>
<td>8</td>
<td></td>
<td>11</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Hemodialysis/peritoneal dialysis, N</td>
<td>89/4</td>
<td>73/2</td>
<td>0.60</td>
<td>67/5</td>
<td>26/2</td>
<td>&gt;0.99</td>
</tr>
<tr>
<td><strong>Donors</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age, yr (mean±SD)</td>
<td>47.9±13.8</td>
<td>44.9±16.6</td>
<td>0.20</td>
<td>52.6±15.3</td>
<td>45.1±15.1</td>
<td>0.01</td>
</tr>
<tr>
<td>Expanded criteria donors, N (%)</td>
<td>28 (30)</td>
<td>17 (23)</td>
<td>0.30</td>
<td>38 (51)</td>
<td>12 (40)</td>
<td>0.20</td>
</tr>
<tr>
<td>Living donors</td>
<td>3</td>
<td>3</td>
<td></td>
<td>0</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>HLA A/B/DQ/DR mismatches, N</td>
<td>3.2±1.6</td>
<td>3.4±1.3</td>
<td>0.60</td>
<td>3.4±1.4</td>
<td>3.6±1.5</td>
<td>0.10</td>
</tr>
<tr>
<td>Total ischemia time, h (mean±SD)</td>
<td>15.6±4.8</td>
<td>17.5±6.7</td>
<td>0.09</td>
<td>18.5±7.3</td>
<td>18.4±7.6</td>
<td>0.20</td>
</tr>
<tr>
<td>Delayed graft function, N (%)</td>
<td>24 (26)</td>
<td>20 (27)</td>
<td>&gt;0.99</td>
<td>52 (70)</td>
<td>19 (63)</td>
<td>0.50</td>
</tr>
<tr>
<td><strong>Immunosuppressive treatment, N</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cyclosporin A/tacrolimus/mTORi</td>
<td>27/62/4</td>
<td>17/57/1</td>
<td>0.30</td>
<td>6/62/5</td>
<td>3/26/1</td>
<td>0.50</td>
</tr>
<tr>
<td>Mycophenolate mofetil</td>
<td>92</td>
<td>74</td>
<td>0.60</td>
<td>73</td>
<td>30</td>
<td>&gt;0.99</td>
</tr>
<tr>
<td>Corticosteroids</td>
<td>93</td>
<td>75</td>
<td>0.90</td>
<td>73</td>
<td>28</td>
<td>0.90</td>
</tr>
<tr>
<td>Anti–IL2 receptor antibody/ATG</td>
<td>61/32</td>
<td>53/22</td>
<td>0.50</td>
<td>0/74</td>
<td>30</td>
<td>—</td>
</tr>
<tr>
<td>Corticosteroid duration, d (mean±SD)</td>
<td>66±1110</td>
<td>756±1173</td>
<td>0.66</td>
<td>1322±1247</td>
<td>1929±1364</td>
<td>0.24</td>
</tr>
<tr>
<td>Preemptive therapy/universal prophylaxis 3/6 mo</td>
<td>48/27/18</td>
<td>32/21/22</td>
<td>0.30</td>
<td>56/18</td>
<td>16/14</td>
<td>0.03</td>
</tr>
</tbody>
</table>

mTORi, mammalian target of rapamycin inhibitor.

aP values were obtained using Mann–Whitney or χ² tests for quantitative or discrete variables, respectively.
The Time of Vδ2neg γδ T Cell Expansion Is Delayed in Infected D+R− Patients with AVR

In infected D+R− patients, the median time between Vδ2neg γδ T cell expansion and CMV DNAemia negativation was 14 days in patients without AVR but much longer (79 days) in patients with AVR (P<0.001) (Figure 6A).

In all 15 infected D+R− patients with AVR, the median (1st and 3rd quartiles) time of Vδ2neg γδ T cell expansion was significantly delayed compared with patients without AVR: 140 (128 and 243) versus 100 (18.7 and 163) days, respectively (P<0.001) (Figure 6B). A threshold of 76 days had good predictive values of AVR occurrence (Se=100%; Spe=77%; PPV=60%; NPV=100%; AUC=0.75; data not shown).

Factors Influencing the Delay in Vδ2neg γδ T Cell Expansion in Infected D+R− Patients

To better examine the factors controlling the delay of the time of Vδ2neg γδ T cells expansion in infected D+R− patients (all infected R+/ATG patients had an early expansion), a univariate analysis (Table 2) was undertaken. Interestingly, the type of anti-CMV prevention strategy in fluenced the time of Vδ2neg γδ T cell expansion, which occurred earlier in patients treated with universal prophylaxis for either 6 or 3 months compared with preemptively treated patients. Moreover, a late-onset infection, occurring in 52.7% of D+R2 patients in our study, was also significantly associated with an earlier time of expansion (P<0.001). Universal prophylaxis (3 or 6 months) was highly correlated with late-onset infection, which means that 96% of infected patients treated with universal prophylaxis had a late-onset infection, whereas 87.5% of preemptively treated infected patients had an early infection (relative risk, 7.6; 95% confidence interval, 3.6 to 16.20; P<0.001). A multivariate analysis showed that late-onset infection was the only factor associated with the time of expansion (Supplemental Table 1).

DISCUSSION

Our results show that a longitudinal monitoring of Vδ2neg γδ T cells in D+R− and R
Figure 3. Estimation of the threshold of Vδ2neg γδ T cell expansion rate. A represents a histogram of maximal Vδ2neg γδ T cell increase (in percentage of Vδ2neg γδ T cells per day) between two determinations before the growing phase of Vδ2neg γδ T cells kinetics. B represents a histogram of estimated Vδ2neg γδ T cell expansion rates (in percentages of Vδ2neg γδ T cells per day) with the linear mixed model during the growing phase of Vδ2neg γδ T cells kinetics. A rate of expansion of 0.06% per day was the best threshold to maximize the validity of the definition of the growing phase of Vδ2neg γδ T cells kinetics. (C) Receiver operating characteristics curve of estimated Vδ2neg γδ T cell expansion rates during the growing phase of Vδ2neg γδ T cells kinetics versus maximal Vδ2neg γδ T cell increases between two determinations before this growing phase. Thus, for a given infected patient, an increase of Vδ2neg γδ T cells >0.06% per day between two successive determinations predicted the growing phase, with Se of 77%, Spe of 90%, PPV of 88%, and NPV of 75% (AUC=0.91).

Figure 4. Time of Vδ2neg γδ T cell expansion is correlated with the CMV DNAemia resolution. In (A) D+R− infected and (B) R+/ATG infected patients, linear regressions are represented between the CMV DNAemia resolution (days) and the time of Vδ2neg γδ T cell expansion (days). Scales are different between A and B with regards to the longer CMV DNAemia duration and the Vδ2neg γδ T cell expansion in D+R− compared with R+/ATG patients. P values were obtained using the Fisher test.

+ATG recipients of kidney transplants can predict the fate of CMV infection: CMV DNAemia resolution or CMV DNAemia/disease recurrence and emergence of a mutant strain with AVR; 90% of D+R− and R+/ATG patients had long-term resolution of CMV DNAemia within 30 days after they reached an expansion rate of 0.06% per day. At the end of the antiviral treatment, Vδ2neg γδ T cell expansion onset was closely associated with recovery and absence of expansion with recurrent DNAemia or disease. In addition, we observed that Vδ2neg γδ T cell expansion and CMV resolution were less correlated in D+R− and R+/ATG patients with ATG. This rare phenomenon could be explained by two hypotheses. First, after ATG, Vδ2neg γδ T cells expansion could be driven by another homeostatic process than CMV infection. Second, the persistence of CMV DNAemia in these patients with an early Vδ2neg γδ T cells expansion could be explained by a profound and persistent depletion of CMV−specific αβ T cells. Additional studies are needed to analyze more precisely the effect of ATG on both γδ and CMV−specific αβ T cells.

To our knowledge, this study has the highest number of patients (n=272) ever included in a longitudinal immunomonitoring study in organ transplantation. This study also differs from previous CMI studies in several respects. First, we analyzed R+ and R− patients separately, because CMI assays gave conclusive results mainly in R+ patients. Second, we used longitudinal monitoring, because (1) this approach is more likely to capture the dynamics of the immune response and (2) previous studies aimed at defining a threshold of CMI at a given time point as a predictive marker of CMV events rarely succeeded, especially in D+R− patients. Indeed, we used this approach to predict CMV recurrence after the first episode of CMV infection, whereas the majority of the studies focused on the prediction of CMV infection on the day of transplantation or at the end of prophylaxis therapy. In keeping with the risk of recurrence, it has been shown in recipients of lung transplants that relapsing viremia was associated with poor induction of T-bet on CMV−specific CD8+ T cells and low frequencies of pp65−specific CD8 effector T cells, although a threshold was not identified. Others found a correlation between CMI assay and recurrence but not with a threshold. Finally, most CMI assays require an in vitro activation step, whereas Vδ2neg γδ T cell monitoring relies on a simple (without stimulation) and standardized whole−blood immunophenotyping.

Uninfected R+/ATG patients had a significantly higher percentage of Vδ2neg γδ T cells at the beginning of follow−up compared with that of infected R+/ATG patients. Moreover, R+/ATG infected patients had an earlier expansion and...
In naïve D+/R− patients, universal prophylaxis is considered to hamper the development of specific immune response, causing more late-onset CMV infections than the preemptive approach,30,31 with increased morbidity and mortality.32 Conversely to this hypothesis, we found that the time of Vδ2neg γδ T cell expansion occurred earlier in patients with a late-onset infection for most under universal prophylaxis, with a faster CMV DNAemia resolution. We hypothesized that the time from transplantation could allow (1) a local priming of Vδ2neg γδ T cells in tissues without systemic dissemination of the virus and (2) a decrease of the immunosuppressive burden. Thus, when a late-onset infection occurs after the end of the universal prophylaxis, Vδ2neg γδ T cells are more prone to undergo an efficient expansion.

AVR is a major concern in recipients of SOTs,19,33 and we have shown a link between a delay in the Vδ2neg γδ T cell expansion and the emergence of mutant strain. Such a relationship between AVR and low CMI had been previously suggested in a single study in a few patients.26 With the caution of a limited size of patient sample, the time of Vδ2neg γδ T cell expansion above 76 days after the beginning of CMV infection could be a useful immune marker to suspect AVR caused by a mutant strain. Indeed, in our cohort, the virologic diagnosis of a mutant strain was performed, on average, 40 days later (namely, 116 days after the beginning of CMV infection) and also, previously reported at about 130 days.4 On one hand, a delayed immune response requiring a longer treatment can favor the emergence of a mutant strain. On the other hand, the immune response can be delayed because of the mutant strain, which has a different fitness as well as a different ability to stimulate late immune response compared with a wild-type strain.

Finally, the emergence of a mutant CMV strain was associated with a persistent CMV DNAemia after Vδ2neg γδ T cell expansion. This phenomenon may reflect some degree of clonal exhaustion of Vδ2neg γδ T cells, because it is often observed in chronic viral infections, and it deserves additional investigations.34,35 This observation emphasizes also that infection resolution is the result of a subtle balance between viral replication, specific immune response, antiviral treatment, and immunosuppressive burden, especially in the case of a viral-resistant strain.

In conclusion, Vδ2neg γδ T cell expansion is related to the resolution of CMV infection in high-risk patients, and a delayed expansion is predictive of the occurrence of a mutant strain. A prospective trial could confirm the usefulness of Vδ2neg γδ T cell longitudinal monitoring in patients with SOTs to tailor the optimal duration of treatment.
Table 2. Variables associated with the time of Vβ2<sup>neg</sup> γδ T cell expansion in patients infected with D+R

<table>
<thead>
<tr>
<th>Variable</th>
<th>Coefficient (SEM)</th>
<th>P Value&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Recipient age at the time of the graft,&lt;sup&gt;a&lt;/sup&gt;</td>
<td>+0.45 (1.1)</td>
<td>0.70</td>
</tr>
<tr>
<td>Recipient’s sex (men versus women)</td>
<td>+8 (32)</td>
<td>0.80</td>
</tr>
<tr>
<td>Induction (anti-ATG versus anti–IL-2 receptor antibody)</td>
<td>+22 (32)</td>
<td>0.50</td>
</tr>
<tr>
<td>Tacrolimus versus cyclosporin A</td>
<td>+14 (4)</td>
<td>0.70</td>
</tr>
<tr>
<td>CMV infection versus CMV disease</td>
<td>+54 (32)</td>
<td>0.09</td>
</tr>
<tr>
<td>Peak viral load&lt;sup&gt;b&lt;/sup&gt;</td>
<td>−8.6 (4.2)</td>
<td>0.80</td>
</tr>
<tr>
<td>Valganciclovir 3 mo (versus preemptive treatment)</td>
<td>−93 (47)</td>
<td>0.05</td>
</tr>
<tr>
<td>Valganciclovir 6 mo (versus preemptive treatment)</td>
<td>−109 (33)</td>
<td>0.002</td>
</tr>
<tr>
<td>Late-onset infection/disease (yes versus no)</td>
<td>−129 (25)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

<sup>a</sup>Increase in the delay of expansion (days) for each additional 1 year of age.

<sup>b</sup>Decrease in the delay of expansion (days) for each additional 10,000,000 IU CMV copies.

<sup>c</sup>P values were obtained using Fisher test.

CONCISE METHODS

Study Design and Patients
As shown in Figure 7, 168 D+R—patients with either anti–IL-2 receptor antibody (n=114) or ATG (n=54) and 104 R+/ATG patients were included between January 1, 2003 and December 31, 2011, and followed at least 2 years post-transplantation with viral (CMV PCR) and immunologic (peripheral blood immunophenotyping) determinations.

From January 1, 2003 to November 30, 2006, patients received universal prophylaxis for 3 months. From December 1, 2006 to June 30, 2010, patients were preemptively followed and treated when the PCR CMV result reached 2000 copies per 1 ml. Finally, from July 1, 2010 to December 31, 2011, patients received universal prophylaxis: 6 months for D+R—patients and 3 months for R+/ATG patients.

CMV manifestations were defined as CMV infection, CMV disease, late-onset infection, CMV tissue-invasive disease, recurrent DNAemia, and recurrent disease on the basis of standardized criteria. Intravenous ganciclovir or oral valganciclovir was given for curative treatment and always followed by oral valganciclovir at a prophylactic dose as previously described. The anti-CMV treatment...

Figure 7. Distribution of the patients. 168 D+R—patients with either anti–IL-2 receptor antibody (n=114) or ATG (n=54) and 104 R+/ATG patients were included between January 1, 2003 and December 31, 2011, who experienced or not experienced CMV infection. Peripheral blood immunophenotyping determinations were longitudinally determined for each patient.
was discontinued after two consecutive negative CMV PCR tests. The dose was adjusted according to the manufacturer’s recommendations using the Cockcroft–Gault formula. Expanded criteria for the donor and delayed graft function were defined as previously described.36,37 This study was approved by the Institutional Review Board of the Bordeaux Hospital.

**CMV Monitoring**

CMV IgG serology was performed following the manufacturer’s recommendations (Enzygnost Anti-CMV/IgM and IgG; Dade Behring, Marburg, Germany and Access CMV IgG and IgM; Beckman Coulter). The CMV PCR assay was used as previously described.38 CMV DNAemia was considered positive when detectable (namely, >500 copies per 1 ml). The assay was performed one time per week for the first 3 months, one time per month between months 3 and 6, and finally, every 2 months up to 1 year. AVR was suspected when persistent viral replication was observed after >2 weeks of appropriate antiviral therapy. AVR was confirmed by full-length sequencing of U97 and UL54 genes,19 which was performed at the French National Cytomegalovirus Reference Center (Limoges, France). Sequences were compared with the AD169 reference sequence using the Gene Librarian 3.2TM software (Visible Genetics Inc., Siemens, France).6,39

**Flow Cytometry Analyses and Monitoring of Vδ2neg γδ T Cells**

Vδ2neg γδ T cells count was obtained by immunophenotypic determination after flow cytometry was carried out on 100 µl anticoagulated whole blood taking into account at least 5000 total lymphocytes stained with anti-CD45, antipan-T (clone IMMU 510; Beckman Coulter, Krefeld, Germany), and anti-TCR Vδ2 (clone 15D; Thermo Fisher Scientific, Rockford, IL). Percentages of cell populations were obtained using CELLQUEST software (BD Bioscience), and absolute counts of lymphocytes were obtained using the Single-Platform Lyse/No–Wash Trucount (BD Bioscience). In our center, the surveillance of Vδ2neg γδ T cells was on the basis of a measurement at day 0 of the graft; months 3, 6, and 12; and then, every year. In case of CMV infection, additional Vδ2neg γδ T cells determinations were performed. A maximal interval of 100 days between two determinations of Vδ2neg γδ T cells phenotype was required; otherwise, these patients were not included in the analysis of the kinetics of Vδ2neg γδ T cells (Figure 7).

**Definitions of Time Points of Infection and Vδ2neg γδ T Cells Kinetics**

Supplemental Figure 2 illustrates the evolution of CMV DNAemias and Vδ2neg γδ T cells in a representative infected patient. Every time point is considered from the beginning of CMV infection.

Day 0 of infection was the first positive CMV DNAemia.

CMV DNAemia resolution was defined as the first negative CMV DNAemia with successive negative CMV DNAemias (at least two) without subsequent relapse until the end of the follow-up period, which means at least 1 year from day 0 of infection.

The growing phase of Vδ2neg γδ T cells was identified by careful individual examination of the whole kinetics of Vδ2neg γδ T cells.

Day 0 of Vδ2neg γδ T cell expansion was the first Vδ2neg γδ T cells determination from day 0 of infection, after which the Vδ2neg γδ T cell growing phase began.

**Statistical Analyses**

Analyses were performed with conventional statistical methods using the R statistical software (version 3.10.1) and specifically, the lme4 and ROCR packages.40 Mann–Whitney and Fisher tests were used when appropriate. P<0.05 was considered statistically significant. When required, threshold values and performances were evaluated using receiver operating characteristics. The expansion rate was determined during the growing phase of Vδ2neg γδ T cells kinetics (encompassing from 2 to 12 determinations) and estimated for each patient using a linear mixed model, which is well suited to analyze repeated, correlated, and unbalanced longitudinal data. The individual expansion rate was calculated using empirical Bayes estimates.

**ACKNOWLEDGMENTS**

We thank Catherine Río (nurse coordinator). We also thank the technicians from the Laboratories of Virology and Immunology at Bordeaux University Hospital and the Centre National de Référence des Cytomégalovirus for their significant contribution to this study.

**DISCLOSURES**

None.

**REFERENCES**


9. Egli A, Humar A, Kumar D: State-of-the-art monitoring of cytomegalo-
ivirus-specific cell-mediated immunity after organ transplant: A primer

J, Ricafort R, Madan RP, Herold BC: Dynamics of cell-mediated immune
responses to cytomegalovirus in pediatric transplantation recipients.
PEDIATR TRANSPLANT 16: 18–28, 2012

11. Pipeling MR, John ER, Orens JB, Lechtzin N, McDyer JF: Primary cyto-
megalovirus phosphoprotein 65-specific CD8+ T-cell responses and
T-bet levels predict immune control during early chronic infection in

Dazzi A, Cescon M, Morelli MC, Pinna AD, Landini MP, Lazzarotto T:
Monitoring cytomegalovirus T-cell immunity in small bowel/multivisceral

Joly P, Bonneville M, Potaux L, Moreau JF: Major expansion of
gammadelta T lymphocytes following cytomegalovirus infection in

S, Travers PJ, Lowdell MW: The role of Vdelta2-negative y T cells during
cytomegalovirus reactivation in recipients of allogeneic stem cell

15. Vermijlen D, Brouwer M, Donner C, Liesnard C, Tackoen M, Van
cytomegalovirus elicits fetal gammadelta T cell responses in utero.

P, Moreau JF, Déchanet-Merville J: Long-term expansion of effector/
memory Vdelta2-gammadelta T cells is a specific blood signature of

17. Couzi I, Pitard V, Sicard X, Garrigue I, Hawchar O, Merville P, Moreau JF,
Déchanet-Merville J: Antibody-dependent anti-cytomegalovirus activity of

Emilie D, Moreau JF, Déchanet-Merville J: Shared reactivity of Vdelta2
regulated gammadelta T cells against cytomegalovirus-infected cells and tu-

19. Couzi I, Helou S, Bachelet T, Moreau K, Martin S, Morel D, Laffon ME,
Boyer B, Alain S, Garrigue I, Merville P: High incidence of anti-
cytomegalovirus drug resistance among D+R-kidney transplant recipients

20. Cantisani S, Lara R, Montejo M, Redel J, Rodríguez-Benzot A, Gutiérrez-
Aroca J, González-Padilla M, Bueno L, Rivero A, Solana R, Torre-
Cisneros J: Pretransplant interferon-γ secretion by CMV-specific CD8+
T cells informs the risk of CMV replication after transplantation. Am J

Venkatakanam R, Humar A: Cell-mediated immunity to predict cyto-
megalovirus disease in high-risk solid organ transplant recipients. Am J
Transplant 9: 1214–1222, 2009

22. Lisboa LF, Kumar D, Wilson LE, Humar A: Clinical utility of cytomegalo-
virus cell-mediated immunity in transplant recipients with cytomeg-

23. Martin-Gandul C, Pérez-Romero P, Blanco-Lobo P, Benmarzouk-
Hidalgo OJ, Sánchez M, Gentil MA, Bemal C, Sobrino JM, Rodríguez-
Hernández MJ, Cordero E: Spanish Network for Research in Infectious
Diseases (REIPI): Viral load, CMV-specific T-cell responses and
cytomegalovirus disease in solid organ transplant recipients at higher
risk for cytomegalovirus infection during preemptive therapy. Transpl
Int 27: 1060–1068, 2014

Meyerhans A, Köhler H: Levels of virus-specific CD4 T cells correlate
with cytomegalovirus control and predict virus-induced disease after
Supplemental Figure 1

A: D+R- infected patients

B: R+/ATG infected patients

$V\delta2^{\text{neg}} \gamma\delta T$ cells (%)
Supplemental Figure 2

Growing phase of Vδ^{neg}γδT cell expansion

Time of Vδ^{neg}γδT cells expansion

CMV DNAemia cp/mL

Days

Duration of CMV infection

Vδ^{neg}γδ T cells (%)
Supplemental Figure 3

A: D+R- infected patients
(induction with anti IL2-R antibody)

B: D+R- infected patients
(induction with ATG)

CMV DNAemia resolution (days)

Time of $V\delta 2^{\text{neg}}$ $\gamma\delta$ T cell expansion (days)

$r=0.97$
$p<0.00001$

$r=0.55$
$p<0.0001$
Supplement Section

Supplemental Figure 1: Distribution of \( V\delta 2^{\text{neg}} \gamma\delta \) T cell percentages at the time of CMV DNAemia resolution. Histograms of \( V\delta 2^{\text{neg}} \gamma\delta \) T cell percentages at the time of CMV DNAemia resolution for both D+R- infected patients (Supplementary Figure 1A) and R+/ATG infected patients (Supplementary Figure 1B).

Abbreviations: D, donor; R, recipient; ATG, anti-thymocyte globulins.

Supplemental Figure 2: Concomitant evolution of \( V\delta 2^{\text{neg}} \gamma\delta \) T cells and CMV DNAemia in a representative infected patient.

Abbreviations: CMV, cytomegalovirus

Supplemental Figure 3: Correlation between the time of \( V\delta 2^{\text{neg}} \gamma\delta \) T cell expansion and the CMV DNAemia resolution in D+R- infected patients with or without ATG. In D+R- infected patients without ATG (3 A) and D+R- infected patients with ATG (3 B), linear regression are represented between the CMV DNAemia resolution (days) and the time of \( V\delta 2^{\text{neg}} \gamma\delta \) T cell expansion (days).

P-values were obtained using Fisher test.

Abbreviations: D, donor; R, recipient; ATG, anti-thymocyte globulins, CMV, cytomegalovirus.
Supplemental Table

Supplemental Table: Variables associated with the time of V\(\delta\)2\(\text{neg}\) γ\(\delta\) T cell expansion in D+R- infected patients

<table>
<thead>
<tr>
<th>Multivariate analysis</th>
<th>Coefficient (SE) (days)</th>
<th>p-value(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CMV infection vs. CMV disease</td>
<td>-6 (30)</td>
<td>0.84</td>
</tr>
<tr>
<td>Universal prophylaxis (vs. preemptive treatment)</td>
<td>30 (50)</td>
<td>0.5</td>
</tr>
<tr>
<td>Late-onset infection/disease (yes vs. no)</td>
<td>-155 (51)</td>
<td>0.004</td>
</tr>
</tbody>
</table>

Abbreviations: SE, standard error

\(^a\) p-values were obtained using Fisher test.