Patterns of Cytomegalovirus Reactivation Are Associated with Distinct Evolutive Profiles of Immune Reconstitution after Allogeneic Hematopoietic Stem Cell Transplantation

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T cell–mediated immunity is essential for the control of cytomegalovirus (CMV) infections in patients undergoing allogeneic hematopoietic stem cell transplantation (allo-HSCT). Our aims were to identify patterns of CMV-specific immune responses associated with multiple or prolonged reactivations. We analyzed findings in 116 recipients during the course of infection or reactivation and latency. CD8+ T cell responses were determined weekly, using HLA class I tetramers together with extended phenotypic analyses. Our results confirmed that recipients of allo-HSCT from unrelated donors were more susceptible to multiple reactivations and that the donor’s CMV serological status influenced the occurrence of prolonged reactivations. We found that a lack of CMV-specific T cells after the first episode of reactivation was associated with multiple subsequent reactivations. In patients with uncontrolled reactivations, CMV-specific T cells of the late differentiation phenotype CD45RA+CD27−CD28− did not develop. Longitudinal evaluation of CD27 and CD45RA expression within the tetramer-positive subset could help identify patients in whom a protective immune response is developing. Evaluation of CMV-specific immune responses during the first episode of reactivation, together with extended phenotypes, could thus improve immune monitoring, especially in recipients at risk of uncontrolled viral reactivation.
pectedly, such reactivations occur despite the presence of CMV-specific T cells and may be due to qualitative disorders rather than quantitative defects of the immune response.

Recognition of viral antigens by the immune system induces a coordinate number of changes in lymphocyte subsets, including changes in cell surface molecules, lymphocyte migratory properties, the ability to proliferate, and T cell–mediated cytotoxicity [16, 17]. Persistent viruses elicit highly diverse functional responses. A linear differentiation model based on the cell surface expression of CD8 T cells was developed, from early CD27⁻CD28⁺ to intermediate CD27⁺CD28⁻ stages to late CD27⁻CD28⁻ stages, characterized by their high perforin content and direct ex vivo cytotoxicity [19]. The expression of the lymph node homing receptor CCR7 added to the complexity of the definitions of central memory and effector memory T cell subsets [20]. On stimulation, naïve CD3⁺CD8⁻CCR7⁺CD45RA⁺ T cells give rise to distinct populations of central memory (CCR7⁺CD45RO⁻), effector memory (CCR7⁻CD45RO⁺), and differentiated memory (CCR7⁻CD45RA⁺) cells with distinct effector functions [21, 22]. During chronic infection, CMV-specific memory CD8⁺ T cells display a predominantly late phenotype [23]. We considered the possibility that the phenotype of CMV-specific T cells might differ between patients with controlled and those with uncontrolled reactivation. We benefited from the frequent monitoring of CMV reactivation after allo-HSCT and weekly determination of the viral load. We analyzed findings in 116 patients after allo-HSCT during the course of infection or reactivation and focused on the response against the tegument protein pp65, using HLA-class I tetramers and extended phenotypic analyses. Our aims were to identify biological markers that could be associated with multiple and/or prolonged reactivations.

METHODS

**Patient characteristics and blood samples.** We studied 116 HLA-A*0201 and/or HLA-B*0702 patients who underwent allo-HSCT from an HLA-identical sibling (n = 58) or a matched unrelated donor (n = 58). All recipients received non–T cell–depleted allo-HSCT (table 1) at the Hematology and Bone Marrow Transplantation Unit of Saint Louis Hospital, Paris. All donor–recipient pairs were typed at the allelic level for HLA class I and class II loci. Before transplantation, serum samples from recipients and donors were analyzed by ELISA for CMV-specific IgG antibodies. Heparin-treated blood (15 mL) was collected from recipients before and at intervals of up to 381 days after allo-HSCT. Samples were collected weekly from day 15 to day 90 and at subsequent visits, when CMV antigenemia was determined. Written informed consent was obtained from patients, and the Committee on Medical Ethics of Saint Louis Hospital approved the investigation.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>All recipients (n = 116)</th>
<th>Seropositive recipients (n = 66)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Donor’s relation to patient</td>
<td>Sibling</td>
<td>58 (50)</td>
</tr>
<tr>
<td></td>
<td>Unrelated</td>
<td>58 (50)</td>
</tr>
<tr>
<td>Source of stem cells</td>
<td>Peripheral blood</td>
<td>44 (38)</td>
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<td></td>
<td>Bone marrow</td>
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<td>Cord blood</td>
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<td>Donor sex/recipient sex</td>
<td>Female/male</td>
<td>25 (22)</td>
</tr>
<tr>
<td></td>
<td>Female/female</td>
<td>27 (23)</td>
</tr>
<tr>
<td></td>
<td>Male/female</td>
<td>20 (17)</td>
</tr>
<tr>
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<td>Male/male</td>
<td>44 (38)</td>
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<td>Means age, years</td>
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<tr>
<td></td>
<td>Donors</td>
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<tr>
<td>Diagnosis</td>
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<tr>
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<td>Non-Hodgkin lymphoma</td>
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</tr>
<tr>
<td></td>
<td>D⁺/R⁻</td>
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<tr>
<td></td>
<td>D⁻/R⁺</td>
<td>14 (12)</td>
</tr>
<tr>
<td></td>
<td>D⁻/R⁻</td>
<td>36 (31)</td>
</tr>
</tbody>
</table>

**NOTE.** Data are no. (%) of subjects, unless otherwise specified. CML, chronic myeloid leukemia; CMV, cytomegalovirus; D⁺/R⁺, seropositive donor/seropositive recipient; D⁺/R⁻, seropositive donor/seronegative recipient; D⁻/R⁺, seronegative donor/seropositive recipient; D⁻/R⁻, seronegative donor/seronegative recipient.

**Monitoring for CMV infection and preemptive therapy.** Weekly monitoring for CMV infection started from engraftment and continued until day 100 after transplantation or longer. CMV infection was defined as pp65 antigenemia of ≥1/200,000 leukocytes [24] in 2 consecutive tests. CMV infection was treated with ganciclovir or foscarnet for 2–3 weeks or until a patient had 2 consecutive tests with negative results.

**Tetramers, monoclonal antibodies, and flow cytometry.** HLA-A*0201/NLVPMVATV– and HLA-B*0702/TPRVTGG-GAM–soluble molecules were biotinylated (BirA enzyme; Avidity) and tetramerized with either streptavidin–phycoerythrin (PE) or streptavidin–allophycocyanin (APC) (Molecular Probes), as
described elsewhere [25]. For individuals assessed with both A2 and B7 tetramers, the dominant response was used for analysis. One hundred microliters of whole blood was stained with a saturating amount of tetramer, CD3 antibody conjugated to peridinin-chlorophyll–binding protein (PerCP), and CD8 antibody conjugated to fluorescein isothiocyanate (FITC). Absolute numbers were calculated using the TrueCount system (Becton Dickinson) with CD3-APC, CD45-PerCP-Cy5.5, CD8-FITC, and CD4 PE antibodies. Staining performed in healthy CMV-seronegative donors confirmed the preestablished detection limit of specificity, which was >0.1% of CD3+CD8+ T cells [15]. The following antibodies were used for phenotypic analyses: CD27-FITC, CD28-APC, CD45RO-PE, CD45RA-FITC, and CCR7-FITC/PE (all antibodies from Becton Dickinson). Flow cytometry analysis was performed immediately using a FACS-Calibur flow cytometer (Becton Dickinson).

Donor/recipient chimerism of tetramer-positive T cells. Peripheral blood mononuclear cells were cultured 5 days in RPMI 1640 medium supplemented with 10% human serum, 50 U/mL interleukin-2, and 10% RPMI 1640 medium supplemented with 10% human serum, 50 U/mL interleukin-2, and 10% RPMI 1640 medium supplemented with 10% human serum, 50 U/mL interleukin-2, and 10% RPMI 1640 medium supplemented with 10% human serum, 50 U/mL interleukin-2, and 10% RPMI 1640 medium supplemented with 10% human serum, 50 U/mL interleukin-2, and 10% RPMI 1640 medium supplemented with 10% human serum, 50 U/mL interleukin-2, and 10% RPMI 1640 medium supplemented with 10% human serum, 50 U/mL interleukin-2, and 10% RPMI 1640 medium supplemented with 10% human serum, 50 U/mL interleukin-2, and 10% RPMI 1640 medium supplemented with 10% human serum, 50 U/mL interleukin-2, and 10%

RESULTS

CMV reactivation and evaluation of specific CD8+ T cell responses. None of the patients from the seronegative donor/seronegative recipient (D-/R−) group (n = 36) had a primary CMV infection. Samples from seronegative recipients who received a graft from seropositive donors (n = 14) always remained free of CMV. Conversely, reactivation was frequent among the seropositive recipients, occurring in 45 of 66 patients. Of them, 30 patients had multiple reactivations, defined by ≥2 distinct episodes of viremia separated by >14 days.

We therefore focused the study on these 66 seropositive patients. Acute GVHD occurred at similar frequencies whether the donor was a sibling or was unrelated (46% and 57%, respectively; P = .37) or whether the donor was CMV seronegative or seropositive (53% and 63%, respectively; P = .4). The same findings were observed for chronic GVHD (57% vs. 46% [P = .37] and 53% vs. 48% [P = .71], respectively). We evaluated the effect of the donor serological status and donor type on reactivation. None of these parameters had an impact on the occurrence of a reactivation during the first 90 days after transplantation. These results were confirmed in a multivariate study; none of the factors included in the Cox analysis (acute GVHD as time-dependent covariate, chronic GVHD, donor serology, and donor type) was significantly associated with the first CMV reactivation (data not shown).

Similarly, when CMV was detected, the onset of reactivation did not differ whether the donor was seronegative (mean day of reactivation, 42) or seropositive (day 41) or whether the donor was a sibling (day 44) or unrelated (day 39). Sixteen patients had prolonged reactivations (lasting >6 weeks). This status of long-lasting viremia predominated in the group with seronegative donors (P = .03) (figure 1A). Similarly, prolonged reactivations occurred more frequently in the unrelated donor group (P = .044). Donor type was also analyzed in the context of multiple reactivations. Grafts from unrelated donors significantly increased the risk of >1 episode of reactivation (P = .02) (figure 1B), whereas donor serological status had no observed effect. The intensity of the first CMV reactivation, as indicated by the maximal viral load, did not influence the occurrence of a subsequent reactivation.

The absolute numbers of CMV-specific T cells strongly correlated with the total absolute counts of CD3+CD8+ T cells (R = .67; P < .0001), regardless of reactivation status. A correlation between CMV-specific and CD3+CD4+ T cell counts (R = .41; P = .0001) was observed only in patients with reactivation.

Relationships between T cell reconstitution and CMV reactivation. Patients were divided into 2 groups, according to CMV reactivation status. Absolute counts of CD4+ and CD8+ T cell subsets were plotted each month (figure 1C and 1D, respectively). The longitudinal evolution of these parameters was compared between the group of patients with no reactivation and the group with ≥1 reactivation. The results showed that CD4+ T cell counts during the first 3 months after transplantation provide a useful predictive marker, with a threshold value of 100 × 10^6 cells/L delineating the risk of reactivation. This was also the true for the CD8+ T cell subset, but to a lesser extent.
CMV-specific immune responses were similarly detected in seropositive recipients with or without reactivation (59.5% and 41.7%, respectively). The onset of this response after transplantation was similar in both groups (days 42.5 and 52, respectively). However, recovery of tetramer-positive T cell counts was associated with the absence of reactivation in 77% of cases ($P = .012$). Twenty-nine of the 45 patients with reactivations had a CD8$^+$ T cell response against CMV. A specific response was usually delayed after the onset of reactivation (mean interval, 22.4 days), but this interval was extremely variable between recipients and did not correlate with either serological status or donor type. Among the 21 patients in whom reactivation did not occur, 5 recipients from seronegative donor/seropositive recipient (D/R) pairs had a CMV-specific response. The lack of viral reactivation was confirmed in samples by PCR. The recipient-donor type of tetramer-positive cells was analyzed in 3 patients, and CMV-specific T cells were of recipient origin in all cases, despite a global donor-type chimerism (data not shown).

Reconstitution of distinct CMV-specific T lymphocyte subtypes in D/R recipients. The level of CMV-specific CD8$^+$ T cells might not be the only marker of protective immune response, and the differentiation profile of these cells could provide an indirect insight into their function. We conducted longitudinal multicolor stainings for 19 recipients (8 seropositive donor/seropositive recipient [D+/R+] subjects and 11 D−/R− subjects). Additional phenotypes of CMV-specific CD8$^+$ T cell subsets were determined in 6 healthy CMV-seropositive patients.

Figure 1. Effect of donor type, serological status, and CD4$^+$ and CD8$^+$ T cell counts on cytomegalovirus (CMV) reactivation. Outcomes were plotted in 66 seropositive patients. A, CMV serological status for reactivations lasting <6 or >6 weeks. R+/D+, seropositive recipient and seropositive donor; R−/D−, seropositive recipient and seronegative donor. B, Donor types for patients with single or multiple reactivations. Values above bars are nos. of patients. MUD, matched unrelated donor. C and D, T cell counts. Levels of CD4$^+$ (C) and CD8$^+$ (D) T cells at months 1, 2, and 3 are shown for recipients with ($n = 25$) (white triangles) or without ($n = 15$) (black circles) detectable reactivation. allo-HSCT, allogeneic hematopoietic stem cell transplantation.
donors. These cells were mainly of the memory and late differentiation phenotype (data not shown). In 17 cases, CMV-specific T cells were already present during R1. In $D^+/R^+$ recipients, tetramer-positive cells were equally distributed within the CD45RO$^+$CCR7$^-$ and CD45RO-CCR7$^-$ subsets (figure 3A), and the expression profile of CD27 and CD28 molecules was skewed to a predominantly late phenotype, as in healthy individuals (figure 3B). Conversely, during the first reactivation of $D^-/R^+$ recipients, tetramer-positive cells were mostly of the CD45RO$^+$CCR7$^-$ phenotype (figure 3C), with expression of early and intermediate markers of differentiation (figure 3D). $D^+/R^+$ recipients with CMV-specific CD8$^+$ T cells were followed up for 2 months after an isolated episode of reactivation. They showed sustained expression of CD45RO (figure 4A) but a shift from the early to the intermediate and then to the late CD27$^-$CD28$^-$ phenotype (figure 4B).

**Analysis of CMV-specific immune responses in the context of multiple and prolonged reactivations.** In $D^+/R^+$ recipients with multiple but limited reactivations (lasting <2 weeks), progressive enrichment of the differentiated memory CD45RA$^+$CCR7$^-$ and the late differentiation CD27$^-$CD28$^-$ subset in the CMV-specific CD8$^+$ T cell pool was observed (figure 4C and 4D, respectively). Sixteen patients with prolonged reactivations were defined as noncontrollers. Most of these patients (12/16) received transplants from a seronegative donor. CMV-specific CD8$^+$ T cells were detected in 12 patients at the time of R1, and the onset of the immune response was not delayed. This was not due to a weak expansion of these T cells, because similar levels of tetramer-positive T cells were found in single, multiple, and prolonged reactivations. Remarkably, CMV-specific CD8$^+$ T cells remained CD45RO$^+$CD27$^+$ (figure 4E), and the evolution to the CD27$^-$CD28$^-$ late phenotype was either absent or extremely delayed, compared with that during a controlled reactivation (figure 4F). CD4$^+$ T cell counts for patients with prolonged reactivations were lower than those for patients with controlled reactivations, but the difference did not reach significance. Because of the limited availability of samples, interferon (IFN)–γ enzyme-linked immunospot assays with pp65-derived peptides were performed in 5 $D^+/R^+$ patients only. In 3 patients, the end of a prolonged CMV reactivation coincided with the development of CD45RA$^+$CD27$^-$ CMV-specific T cells and the detection of IFN-γ–producing T cells (>1 cell/μL). This threshold was never reached in the other 2 patients with a single episode of reactivation and persistent CMV-specific CD45RO$^+$CD8$^+$ T cells (data not shown). CD4$^+$ T cell counts <100 × 10$^6$ cells/L did not allow the analysis of CMV-specific CD4$^+$ T cell reconstitution in our patients with reactivations.

**DISCUSSION**

Our main goal was to determine the extent to which the immune-response profile would predict protective immunity against CMV. We performed extensive longitudinal monitoring of a large cohort of recipients of allo-HSCT without T cell depletion. Our analyses focused on the seropositive recipients, who

<table>
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<th>Risk factor</th>
<th>$P$</th>
<th>OR (95% CI)</th>
</tr>
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<tbody>
<tr>
<td>Tetramer-positive cells$^a$</td>
<td>.017</td>
<td>6.401 (1.393–29.409)</td>
</tr>
<tr>
<td>Donor type$^b$</td>
<td>.036</td>
<td>6.698 (1.138–39.439)</td>
</tr>
<tr>
<td>Donor serological status for CMV$^c$</td>
<td>.856</td>
<td>1.170 (0.214–6.380)</td>
</tr>
</tbody>
</table>

**NOTE.** Logistic regression analysis was performed to determine the odds of a second cytomegalovirus (CMV) reactivation in 45 patients with >1 reactivation. CI, confidence interval; OR, odds ratio.

$^a$ Tetramer-positive cells present vs. tetramer-positive cells absent during the first reactivation.

$^b$ Matched unrelated donors vs. tetramer-positive donors.

$^c$ Seropositive vs. seronegative donors.
positive recipients (D/H11002 and 124; for D patients with different patterns of reactivation (data not shown). d, day.

CMV disease [26]. However, recurrent and prolonged reactivations could be impaired in the setting of immunodepression [5, 10]. According to this hypothesis, the cytotoxic activity of CMV tetramer–positive T cells could be related to changes in subset repartition of CMV-immune responses in 5 D+/R+ patients without viral reactivation. In 3 cases, analyses of global and virus-specific T cell chimerisms showed that all CMV-specific T cells were of recipient origin, whereas the global chimerism was of the donor type. This extends, into a setting without T cell depletion, findings already reported for cord blood transplantation [39] and T cell–depleted allo-HSCT [29].

Recurrent reactivations were diagnosed in 30 patients and correlated with the lack of CMV-specific T cells during R1. Using a different approach, one based on the simultaneous quantification of CMV-specific CD4+ and CD8+ T cells [40], Lilleri et al. suggested that repeated episodes of reactivation could occur in patients without prompt recovery of specific immunity on day 60 [6]. Moreover, multivariate analysis revealed that allo-HSCT with an unrelated donor and the absence of CMV-specific T cells during R1 were independent risk factors for recurrent reactivations.

Despite the development of CMV-specific T cells, prolonged reactivations were observed in 16 patients. As pointed out elsewhere, the cytotoxic activity of CMV tetramer–positive T cells could be impaired in the setting of immunodepression [5, 10] and could be related to changes in subset repartition of CMV-specific CD8+ T cells, as recently reported in HIV-infected individuals [41]. In the absence of T cell depletion, mature T cells are transferred and their population expands after transplantation. Accordingly, CMV-specific T cells detected in D+/R+ recipients displayed phenotypic features similar to those observed in immunocompetent individuals [23, 42]. Because >85% of prolonged reactivations occurred in D+/R+ recipients, we focused on this group. In such cases, the specific T cell responses observed during reactivation might mimic responses during pri-
mary infection, taking into account the specific restrictions conferred by the setting of allo-HSCT. When sufficient amounts of CMV-specific CD8+ T cells were detected, CD27+/CD28−/28+/CD27− early/intermediate phenotypes predominated within the tetramer-positive fraction during the first wave of reactivation. This was rapidly followed by a progression through to the CD27−/CD28− late phenotype. CD45RA and CD45RO expression did not correlate directly with the stages defined by CD27 and CD28, but enrichment of the CD45RA+CD27− subset could be associated with higher levels of IFN-γ–producing T cells. The development of CD45RA+CD8+ T cells over a period of 70 days was observed only in patients with ≥2 reactivations.

Because primary CMV infection is usually clinically silent, little is known about the longitudinal evolution of CMV-specific T cells in “healthy” individuals. In a cohort of 200 children, progressive expansion of the population of CMV-specific CD8+CD45RA+CD27− T cells was observed during postprimary infection [43]. Analyses in infected individuals enrolled in clinical protocols enabled a more detailed longitudinal follow-up of CMV-specific responses, as observed in allo-HSCT [44] or kidney transplantation [45]. In the latter study, the number of CMV-specific CD27− T cells was linearly related to the total number of CMV-specific T cells. In

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Figure 4. Evolution of cytomegalovirus (CMV)–specific CD8+ T cell subsets according to the pattern of reactivation in recipients with seropositive donors. In a single episode of reactivation, CMV-specific CD8+ T cells remained CD45RO+ with evolution to the CD27−/CD28− late phenotype (A and B). In multiple controlled reactivations, there was enrichment of CMV-specific CD45RA+ cells with less marked evolution to the late phenotype (C and D). In prolonged reactivations, CMV-specific CD8+ T cells remained CD45RO+ with early/intermediate phenotypes (E and F). Results are expressed as percentages within the tetramer (Tet)–positive CD3+CD8+ subset. Data from 4 representative patients are shown in each graph.

Figure 5. Schematic representation of distinct profiles of cytomegalovirus (CMV)–specific CD8+ immune responses, according to diverse aspects of CMV reactivation. Uncontrolled reactivation was related to a defect of T cell differentiation more than to a quantitative defect of tetramer T cell subsets, because tetramer-positive cell counts did not differ according to different patterns of reactivation. During the first 3 months after allogeneic hematopoietic stem cell transplantation (allo-HSCT), patients with multiple but controlled reactivations acquired late and differentiated phenotypes similar to the CMV-specific memory phenotypes found in healthy donors. In prolonged reactivations, CD4+ T cell counts were lower, and the process of differentiation was impaired. D+/R−, seronegative donor-seropositive recipient.
such settings, the distribution of the differentiation subsets might reflect the profiles of preexisting memory T cells. The repeated phenotypic follow-up provided by our study clearly showed that, in D−/R+ patients with uncontrolled reactivations, CMV-specific T cells of the late phenotype were unable to develop. The source of CMV-specific T cells was mainly T cells generated de novo, and the number of CMV-specific T cells of the late phenotype was not linearly related to the total number of CMV-specific CD8+ T cells.

These data confirmed the general consensus that most CMV-specific CD8+ T cells in infected individuals controlling CMV infection were IFN-γ-secreting CD27−CD28− T cells [22]. In a quite different setting of immunodeficiency, higher numbers of early/intermediate CMV-specific T cells were associated with long-term recovery. These data in HIV-positive patients with marked CD4 T cell defects highlight the importance of CD4 in supporting the differentiation of CD8+ T cells from early to late effectors [41]. In allo-HSCT, the respective reconstitution of CD4+ and CD8+ T cell subsets is not synchronized, and the emergence of CD4+ T cells is delayed compared with CD8+ T cells [46]. In prolonged CMV reactivation in D−/R+ recipients, differentiation of virus-specific CD8+ T cells may be impaired, probably because of the latency of the development of the CD4+ T cell subset. Figure 5 is a schematic representation summarizing the diverse aspects of CMV reactivation in allo-HSCT, according to distinct profiles of immune reconstitution.

Several studies have analyzed the relative contribution of immune responses against immediate early 1 protein [47] and pp65, with controversial conclusions [44, 48]. We are now investigating this question in the light of our present results.

Our study reveals that immune monitoring in allo-HSCT provides a valuable extension of virologic monitoring, notably in the era of anti-CMV preemptive therapy. We suggest some guidelines for the management of CMV-related complications and recommend the determination of CD4+ T cell counts during the first 3 months after allo-HSCT as a predictive marker of reactivation, together with monitoring of tetramer-positive CD8+ T cells (risk of multiple reactivations) and the longitudinal evaluation of CD27 and CD45RA expression within the tetramer-positive subset (risk of prolonged reactivations).

References


