Recurrence of cytomegalovirus reactivation remains a major cause of morbidity and mortality following allogeneic hematopoietic stem cell transplantation. Monitoring cytomegalovirus-specific cellular immunity using a standardized assay might improve the risk stratification of patients. A prospective multicenter study was conducted in 175 intermediate- and high-risk allogeneic hematopoietic stem cell transplant recipients under preemptive antiviral therapy. Cytomegalovirus-specific cellular immunity was measured using a standardized interferon-γ enzyme-linked immunospot assay (T-Track® CMV). The primary aim was to evaluate the suitability of measuring cytomegalovirus-specific immunity after the end of treatment for a first cytomegalovirus reactivation to predict recurrent reactivation. Forty of 101 (39.6%) patients with a first cytomegalovirus reactivation experienced recurrent reactivations, mainly in the high-risk group (cytomegalovirus-seronegative donor/cytomegalovirus-seropositive recipient). The positive predictive value of T-Track® CMV (patients with a negative test after the first reactivation who experienced at least one recurrent reactivation) was 84.2% in high-risk patients. Kaplan-Meier analysis revealed a higher probability of recurrent cytomegalovirus reactivation in high-risk patients with a
negative test after the first reactivation (hazard ratio 2.73; P=0.007). Interestingly, a post-hoc analysis considering T-Track® CMV measurements at day 100 after transplantation, a time point highly relevant for outpatient care, showed a positive predictive value of 90.0% in high-risk patients. Our results indicate that standardized cytomegalovirus-specific cellular immunity monitoring may allow improved risk stratification and management of recurrent cytomegalovirus reactivation after hematopoietic stem cell transplantation. This study was registered at www.clinicaltrials.gov as #NCT02156479.

Introduction

Cytomegalovirus (CMV) infection and disease remain a serious cause of morbidity and mortality after allogeneic hematopoietic stem cell transplantation (HSCT). Adequate risk stratification is essential to identify and properly manage patients at highest risk for CMV reactivation. The main risk factors include donor (D) and recipient (R) CMV serostatus (D-/R+ defining high-risk patients), the use of mismatched or unrelated donors, graft-versus-host disease (GvHD), and intense immunosuppression. Close monitoring during the first 100 days after transplantation, in accordance with current guidelines, has greatly reduced the incidence of CMV-related complications. However, recurrent and late-occurring CMV reactivation remain major life-threatening issues, and effective strategies for the prevention of late CMV disease prevail as an unmet medical need. This is particularly critical in outpatient care more than 100 days after HSCT when patients are less frequently monitored. Studies clearly identified a delay in global and CMV-specific immune reconstitution as a major risk factor for recurrent and late-onset CMV reactivation. Several CMV-specific immune monitoring assays have been described. They are based on the quantification of the number and/or functionality of immune cells targeted against CMV, using flow cytometry detection, an enzyme-linked immunosorbent assay (ELISA) or enzyme-linked immunospot (ELISpot). Multiple studies demonstrated the suitability of these methods for predicting recurrent and/or late CMV reactivation, resulting in the emergence of new risk stratification models based on the monitoring of CMV-specific cell-mediated immunity (CMV-CMI) together with CMV viral load. The lack of standardized assays does, however, render the comparison of most reported results difficult. Two standardized CMV-specific interferon (IFN)-γ ELISpot assays, based on the in vitro stimulation of peripheral blood mononuclear cells (PBMC) with IE-1 and pp65 peptides (T-SOT®, CMV) or proteins (T-Track® CMV), have been described. T-Track® CMV is highly sensitive due to the use of urea-formulated T-activated® IE-1 and pp65 proteins, resulting in the activation of a broad spectrum of CMV-specific effector cells (including CD4+, CD8+ and NK cells). One study reported the utility of T-SOT®-CMV to predict the risk of a first treatment-requiring CMV reactivation after HSCT. Here we describe – to the best of our knowledge for the first time – the utility of the T-Track® CMV assay to predict recurrent and late-onset CMV reactivation after HSCT.

Methods

Study design and participants

A prospective multicenter study was conducted in 175 interme-
Results

Patients’ characteristics

One hundred and seventy-five allogeneic HSCT recipients were enrolled. Twenty-one patients were excluded from the analysis, due to either protocol violation (n=8) or the absence of valid T-Track® CMV test results (n=13). The study flow diagram is shown in Online Supplementary Figure S2. Of the 154 HSCT recipients included in the final analysis, 101 (65.5%) experienced at least one treatment-requiring CMV reactivation (hereafter designated as “CMV reactivation”) up to day 225 after transplantation (Table 1). Eight patients (4.6%) were diagnosed with CMV disease and 69 (44.8%) with GvHD (Table 1 and Online Supplementary Table S2). The majority of patients with GvHD (50/69 [72.5%] of all patients and 33/40 [82.5%] of D-/R+ patients) experienced at least one episode of CMV reactivation. This is in line with the strong immunosuppressive effect of steroids used for the treatment of GvHD.21,22 Of the 101 patients who had a CMV reactivation, 65 (64.4%) belonged to the D-/R+ high-risk group (Table 2). Sixty-one (60.4%) patients experienced only one CMV reactivation while 40 patients developed either two (n=24) or three (n=16) CMV reactivations (hereafter referred to as “recurrent CMV reactivation”) (Table 2). Most of the HSCT recipients who had recurrent CMV reactivation were high-risk patients (57/40 [92.5%]) (Table 2). Therefore, we focused on high-risk patients as the clinically relevant population with regards to the risk of CMV recurrence after HSCT. The median (range) time to CMV reactivation in D-/R+ patients for the first, second and third CMV reactivation was 37 (19-58), 109 (63-188) and 174 (119-219) days after transplantation, respectively (Online Supplementary Figure S3B). The respective median (range) CMV viral load is presented in Figure 1.

Table 1. Patients’ characteristics.

| Study population, N (%) | 154 (100%) |
|-------------------------|------------|
| Gender, n (%)           |            |
| Male                    | 88 (57.1)  |
| Female                  | 66 (42.9)  |
| Age in years, median (range) | 58 (20-75) |
| Underlying disease, n (%) |             |
| Acute myeloid leukemia  | 79 (51.3)  |
| Myelodysplastic syndrome | 24 (15.6)  |
| Acute lymphoid leukemia | 17 (11.0)  |
| Non-Hodgkin lymphoma    | 12 (7.8)   |
| Multiple myeloma        | 10 (6.5)   |
| Osteomyelofibrosis      | 4 (2.6)    |
| Chronic myeloid leukemia| 3 (1.95)   |
| Chronic lymphoid leukemia| 3 (1.95)  |
| Severe aplastic anemia  | 2 (1.3)    |
| Donor (D) / recipient (R) CMV serostatus, n (%) | |
| D+/R+                   | 53 (34.4)  |
| D+/R-                   | 19 (12.3)  |
| D-/R+                   | 82 (53.3)  |
| Stem cell source, n (%) |            |
| Bone marrow             | 9 (5.8)    |
| Peripheral blood        | 145 (94.2) |
| Donor source, n (%)     |            |
| Matched sibling         | 31 (20.1)  |
| Matched unrelated donor  | 92 (59.8)  |
| Mismatched unrelated donor | 30 (19.1) |
| Conditioning regimen, n (%) |         |
| Non-myeloablative       | 41 (26.6)  |
| Myeloablative, standard | 75 (48.7)  |
| Myeloablative, toxicity-reduced | 38 (24.7) |
| At least one treatment-requiring CMV reactivation, n (%) | 101 (65.5) |
| CMV disease, n (%)      | 8 (4.6)    |
| Graft-versus-host disease, n (%) | 69 (44.8) |
| Infections other than CMV (after day 45), n (%) | 52 (33.8) |
| Death, n (%)            | 21 (13.6)  |

CMV: cytomegalovirus.

Measurement of CMV-specific cell-mediated immunity over time after hematopoietic stem cell transplantation

CMV-CMI was evaluated using a standardized IFN-γ ELISpot-based assay (T-Track® CMV).26,27,30 A total of 647 valid test results were included in the analysis. The distribution of spot-forming cells (SFC) was analyzed over time after transplantation in response to the CMV proteins IE-1 and pp65 (Online Supplementary Figure S4A). Overall, the response to IE-1 antigen was lower than that to pp65 throughout the study, especially in D-/R+ patients. A significant increase in the response to pp65 was apparent in D-/R+ patients over time (Online Supplementary Figure S4A). Accordingly, while the proportion of IE-1-positive tests remained low (up to 33.3% around day 145), that of pp65-positive tests increased to 64.5% in D-/R+ patients (Online Supplementary Figure S4B). Interestingly, the percentage of T-Track® CMV-positive tests (considering both IE-1 and pp65 markers) was consistently higher than that of pp65 alone, reaching 77.4% of positive tests around day 145 in D-/R+ patients (Online Supplementary Figure S4B). Thus, although generating lower spot counts, IE-1 antigen contributes significantly to T-Track® CMV test positivity.

The patterns of SFC distribution relative to the start of the first CMV reactivation were comparable (Online Supplementary Figure S4C).

CMV-specific cell-mediated immunity measured after the end of treatment of a first CMV reactivation can predict recurrence of CMV reactivation

The primary aim of the study was to evaluate the suitability of measuring CMV-CMI after the end of treatment of a first-occurring CMV reactivation to predict freedom from or occurrence of a subsequent CMV reactivation (Figure 2A). CMV-CMI was measured on the day of discontinuation of antiviral treatment (day 0), and on days 7

Table 2. Cytomegalovirus reactivation according to the patients’ cytomegalovirus serostatus.

| All | D-/R+ | D+/R- | D+/R+ |
|-----|-------|-------|-------|
| Study population, n | 154   | 82    | 53    |
| No documented CMV reactivation, n | 53    | 17    | 20    |
| At least one CMV reactivation, n | 101   | 65    | 33    | 3 |
| One CMV reactivation only, n | 61    | 28    | 30    |
| Recurrent CMV reactivation, n | 40    | 37    | 3     |
| Two CMV reactivations, n | 24    | 21    | 3     |
| Three CMV reactivations, n | 16    | 16    | 0     |

CMV: cytomegalovirus; D-: CMVnegative donor; R+: CMVpositive recipient; D+: CMV-positive donor; R-: CMVnegative recipient.
and 14 thereafter. The first available measurement was considered for the analysis. In fact, measurements on days 0, 7 and 14 contributed 36/76 (47.4%), 29/76 (38.1%) and 11/76 (14.5%) test results, respectively, to this analysis.

SFC levels after a first CMV reactivation were compared between patients who experienced no further CMV reactivation and those with one or two subsequent (i.e., recurrent) CMV reactivations. A significant difference in SFC distribution was observed between the two groups, for both IE-1- and pp65-specific SFC levels, when considering all patients (Mann-Whitney U test, \( P < 0.001 \)) (Figure 2B). In the high-risk population, a significant difference was observed for pp65-induced response (Mann-Whitney U test, \( P = 0.001 \)), but not for IE-1-mediated response (Mann-Whitney U test, \( P = 0.724 \)) which was very low in both groups (Figure 2B). In accordance with these results, a ROC analysis in D-/R+ patients revealed AUC estimates significant for pp65-specific tests (AUC 0.780, 95% confidence interval [95% CI]: 0.642-0.917; \( P < 0.001 \)) but not for IE-1-specific tests (Figure 2C).

Interestingly, a post-hoc analysis of these data upon normalization of SFC values to absolute lymphocyte counts derived from peripheral blood counts (expressed as SFC/μL blood) showed a stronger discrimination of SFC distributions between patients without and with recurrent CMV reactivation, including in D-/R+ patients (Mann-Whitney U test, \( P = 0.010 \) [IE-1] and \( P < 0.001 \) [pp65]), and an improved predictive value in ROC analysis (AUC 0.760 [95% CI: 0.599-0.921], \( P = 0.002 \) for IE-1 and AUC 0.863 [95% CI: 0.741-0.986], \( P < 0.001 \) for pp65) (Online Supplementary Figure S5), compared to the normalization to 200,000 lymphocytes. SFC distributions according to conditioning regimen and GvHD occurrence are presented in Online Supplementary Figures S6 and S7, respectively.

The diagnostic accuracy of the ELISpot assay was determined in terms of sensitivity (patients with recurrent CMV reactivation had a negative test result after a first CMV reactivation) and specificity (patients free from recurrent CMV reactivation had a positive test result after a first CMV reactivation) (Table 3). Likely due to the low SFC levels and high proportion of negative test results induced by IE-1, the IE-1-specific test alone showed limited performance in the D-/R+ population (Table 3). By contrast, the performance of pp65-specific positive tests, alone or in combination with IE-1, to correctly identify patients free of future recurrent CMV reactivation was high, with a specificity of 77.8% (pp65) and 83.3% (pp65 and IE-1 combined) in D-/R+ patients (Table 3). The sensitivity of pp65-specific tests, alone or in combination with IE-1, in D-/R+ patients was 58.6% and 55.2%, respectively (Table 3). The positive predictive value (PPV; patients with a negative test after the first CMV reactivation had a subsequent recurrent CMV reactivation) of pp65-specific tests in D-/R+ patients reached 80.9% (pp65 test alone) and 84.2% (pp65 and IE-1 tests combined) while the respective negative predictive values (NPV; patients with a positive test after the first CMV reactivation did not have a subsequent CMV reactivation) were low (53.8% and 53.6%, respectively) (Table 3).

The probability of recurrent CMV reactivation in patients with positive and negative ELISpot tests was estimated using Kaplan-Meier curves (Figure 2D). In line with the previous observations, the difference in probability of recurrent CMV reactivation between patients with a positive or negative pp65-specific test result after the first reactivation was highly significant, both in the total population (HR 4.91; log-rank test, \( P < 0.001 \)) and in the D-/R+ group (HR 2.52; log-rank test, \( P = 0.013 \))(Figure 2D).
Figure 2. Legend on following page.
Figure 2 (previous page). Performance of cytomegalovirus (CMV)-specific cell-mediated immunity measured after the end of a first CMV reactivation to predict freedom from and/or occurrence of recurrent CMV reactivation. (A) Interferon-γ enzyme-linked immunospot (ELISpot) was performed after the end of antiviral therapy for a first CMV reactivation, at up to three time points relative to the end of treatment, namely day 0 (d0), day 7 (d7) and day 14 (d14). The first available measurement was considered for the analysis. (B) Quantitative ELISpot results in response to CMV proteins IE-1 and pp65 were evaluated on the basis of the mean of square root-transformed (SRM) spot-forming cells (SFC), as described in the Methods section. Differences in SFC distribution between patients with only one CMV reactivation and those with recurrent CMV reactivation were evaluated using a Mann-Whitney U test. Respective P-values are shown under each graph. For the sake of simplicity, scatter plots are depicted as squared SRM values (SRM^2). The median and interquartile range of the SRM^2 SFC are shown above each graph. Additional information (minimum, maximum, 10th and 90th percentiles) are shown in Online Supplementary Table S3. Due to the log scale representation, values of zero SRM^2 were replaced by 0.01 (y-axis), meaning that baseline values shown at y=0.01 are actually equal to zero. Red triangles and blue dots represent negative and positive tests, respectively, defined according to the rules described in the Methods section. Of note, of the three CMV-negative donor/CMV-positive recipients (D-/R+) with a documented recurrent CMV reactivation and with high pp65-SFC after the first CMV reactivation (251 to 386 SFC/200,000 lymphocytes) one was treated for recurrent CMV although the viral load was below the center-specific threshold, after which high pp65-specific SFC dropped dramatically over time before the start of treatment of a CMV reactivation with a viral load above the threshold; the third patient had a lengthy (>3 months) first CMV reactivation with a high sustained viral load (up to 310,000 copies/ml) likely reflecting refractory CMV.

Table 3. Diagnostic accuracy in identifying patients with and without recurrent cytomegalovirus (CMV) reactivation based on CMV-specific negative and positive enzyme-linked immunospot test results after the first CMV reactivation.

| Population          | Marker   | Sensitivity | Specificity | Chi-square | PPV       | NPV       |
|---------------------|----------|-------------|-------------|------------|-----------|-----------|
| All patients        | IE-1     | 73.3% (22/30) | 56.8% (25/44) | P=0.010    | -         | -         |
|                     | pp65     | 58.1% (18/31) | 88.4% (38/43) | P<0.001    | -         | -         |
|                     | IE-1, pp65 | 53.3% (16/30) | 93.2% (41/44) | P<0.001    | -         | -         |
| D/R+ patients       | IE-1     | 75.9% (22/29) | 17.7% (3/17)  | P=0.606    | 61.1%     | 30.0%     |
|                     | pp65     | 58.6% (17/29) | 77.8% (14/18) | P=0.015    | 80.9%     | 53.8%     |
|                     | IE-1, pp65 | 55.2% (16/29) | 83.3% (15/18) | P=0.009    | 84.2%     | 53.6%     |

D/R+ patients: CMV-infected patients, D/R+ patients with CMV-specific response at d0, d7 or d14; IE-1: CMV IE-1 enzyme-linked immunospot test; pp65: CMV pp65 enzyme-linked immunospot test; IE-1, pp65: CMV IE-1 and pp65 enzyme-linked immunospot test; PPV: positive predictive value; NPV: negative predictive value.

2D). Interestingly, the performance of the pp65 test was improved by the combination with IE-1 (T-Track® CMV test), in all patients (HR 5.68; log-rank test, P<0.001) and in D/R+ patients (HR 2.73; log-rank test, P=0.007) (Figure 2D).

To better understand the usability of the assay in terms of clinical cutoff, we evaluated the PPV (patients with SFC ≤ threshold after the first CMV reactivation had a recurrent CMV reactivation) and NPV (patients with SFC > threshold after the first CMV reactivation did not have a recurrent CMV reactivation) of pp65-specific response in D/R+ patients at low, intermediate and high SFC counts (Table 4). A NPV of 100% (3/3) was observed at lower (9 and less) SFC counts (Table 4), in line with the results described above (Table 3).

Benefit of monitoring CMV-specific cell-mediated immunity over absolute T-cell counts.

We next compared the performance of CMV-CMI to that of absolute lymphocyte (T and NK cells) counts measured after end of treatment of a first CMV reactivation to predict subsequent CMV reactivation episodes. Multicolor flow cytometry was performed using peripheral blood mononuclear cells. NK and T (total, naive and memory) cell levels were expressed as absolute cell counts. The first visit with existing absolute cell counts following the end of antiviral therapy (of day 0, 7 and 14) was considered for the analysis. Absolute cell counts were significantly higher in patients with no recurrent CMV reactivation in all cases, except for total and memory CD8+ T cells in D/R+ patients (Online Supplementary Figure S8A). In ROC analyses, total lymphocytes as well as total and naive CD8+ cell populations showed a good predictive value for recurrent CMV reactivation, with AUC estimates between 0.815 and 0.885 in D/R+ patients (Online Supplementary Figure S8B), thus in a
Table 4. Positive and negative predictive values of low, intermediate and high spot-forming-cell counts of a pp65-specific enzyme-linked immunospot assay after the end of treatment for a first cytomegalovirus (CMV) reactivation to predict the occurrence of future recurrent CMV reactivation in high-risk hematopoietic stem cell transplant patients.

| Patient population | Marker | Thresholda | PPV | NPV | % patients above threshold |
|--------------------|--------|------------|-----|-----|---------------------------|
| D-/R+              | pp65   | 386        | 65.9% (29/44) | 100% (3/3) | 6.4% (3/47) |
|                    |        | 40         | 74.3% (26/35) | 75.0% (9/12) | 25.5% (12/47) |
|                    |        | 9          | 87.5% (21/24) | 65.2% (15/23) | 48.9% (23/47) |
|                    |        | 0          | 100% (8/8) | 46.2% (18/39) | 83.0% (39/47) |

9 SFC (SRM2)/200,000 lymphocytes (stimulated minus unstimulated condition) rounded to closest spot count; thresholds were derived from Receiver operating characteristic curve data; the threshold of 9 SFC (SRM2)/200,000 lymphocytes showed the highest sensitivity+specificity value; SFC: spot-forming cells; SRM2: squared mean of square-root-transformed CMV, cytomegalovirus; D/R: donor/recipient CMV serostatus; NPV: negative predictive value; PPV: positive predictive value.

CMV-specific cell-mediated immunity at day 100 post-transplantation can predict late recurrent CMV reactivation (post-hoc analysis)

Accurate prediction of future recurrent CMV reactivation is particularly critical when patients are released from close monitoring in an outpatient setting around 3 months after HSCT.4–6 We conducted two post-hoc analyses to determine whether CMV-CMI monitoring at the fixed time of day 100 could identify patients at risk of future (i.e. late) CMV reactivation. The first analysis considered all patients, regardless of a possible existence of CMV reactivation prior to day 100, thus assessing occurrence of late CMV reactivation generally (Online Supplementary Figure S9). The second analysis focused on patients who experienced CMV reactivation prior to day 100, thus investigating the usefulness of the IFN-γ ELISpot measured around day 100 to predict late recurrent CMV reactivation (Figure 5). T-Track® CMV test results acquired between day 80 and day 100 after transplantation were considered in patients with ongoing CMV reactivation (Online Supplementary Methods).

Interestingly, patients (including those in the high-risk group) who did not experience CMV reactivation up to day 80-100 did not experience CMV reactivation thereafter (Online Supplementary Figure S9B, orange-labeled dots). A majority of these patients presented low IE-1 and pp65-specific test results throughout the study (Online Supplementary Figure S9B and data not shown). Such sustained low responsiveness in patients with no CMV reactivation was previously reported.29 Its cause remains to be investigated. Consequently, performance of the ELISpot test at day 100 in this global analysis of late CMV reactivation was low (Online Supplementary Figure S9B-D; Online Supplementary Table S5). On the other hand, the analysis of ELISpot test results at day 100 in patients with an earlier CMV reactivation (Figure 3A) revealed a significant difference in SFC counts between patients without and with late recurrent CMV reactivation (Mann-Whitney U test, P < 0.01 and <0.001), with the exception of IE-1-specific response in D-/R+ patients (Mann-Whitney U test, P = 0.277) (Figure 3B). All patients with late recurrent CMV reactivation belonged to the high-risk group. In ROC analyses, AUC estimates for pp65 test results were 0.811 (P < 0.003) in the D-/R+ population and 0.911 (P < 0.001) in the total population (Figure 5C). pp65-specific response showed the best diagnostic accuracy, with a sensitivity and specificity of 75.0% and 90.9%, and a PPV and NPV of 90.0% and 76.9% in the D-/R+ population, respectively (Table 5). The probability of late recurrent CMV reactivation was significantly higher in D-/R+ patients with a pp65-negative test at day 100 (HR 6.34; log-rank test, P = 0.002). In this setting, the response to IE-1 did not improve the performance of pp65 (Figure 3D).

Diagnostic accuracy and time-to-event analyses indicated that negative IFN-γ ELISpot test results could predict recurrence of CMV reactivation after HSCT, with a particular focus on clinically relevant high-risk D-/R+ patients.

Overall, the response to pp65 antigen in the ELISpot assay was higher than that of IE-1, as previously reported.29,27,30 Although the IE-1-specific response had low or no predictive value alone, it improved the assay performance in combination with pp65-specific tests after the first CMV reactivation.

Discussion

This study demonstrates for the first time the suitability of a standardized CMV-specific IFN-γ ELISpot assay to predict recurrence of CMV reactivation after HSCT, with a particular focus on clinically relevant high-risk D-/R+ patients. The diagnostic accuracy and time-to-event analyses indicated that negative IFN-γ ELISpot test results could predict recurrence of CMV reactivation after HSCT. Positivity of the T-Track® CMV test is based on several rules, the main one being that CMV antigen-stimulated conditions must yield ≥ 10 SFC (or mean of sqrt SFC ≥ 3.16) (Online Supplementary Methods). This raises the possibility that the technical cutoff of the assay might be a relevant clinical cutoff for the prediction of recurrent CMV reactivation (PPV > 80%). Whether the positivity cutoff of T-Track® CMV is indeed a valid clinical cutoff to predict recurrent CMV reactivation in high-risk D-/R+ patients could be addressed in a randomized interventional study. In fact, such an approach is currently being appraised in high-risk solid-organ transplant recipients in a study aim-
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Figure 3. Legend on following page.
The good predictive value of absolute lymphocytes, which might be a relevant normalizer of the ELISpot assay observed upon normalization to the currently implemented absolute T-cell count measurement, provides an additional possibility of introducing T-Track® CMV in complement with the described protective role of CD4+, CD8+ T cells, especially in patients with recurrent CMV reactivation.

CMV-CMI monitoring using a standardized assay, while complementing the current approach of CMV-specific immune monitoring using a standardized ELISpot measurement, in favor of the ELISpot assay measured after in vivo infection, which ranged from day 45 to 186 after transplantation, which might be of greater clinical relevance to predict recurrent CMV reactivation episodes. Moreover, dysfunctional CD8+ T cells specific for CMV accumulate in patients with recurrent CMV reactivation. Thus, functionality (i.e., the capacity of cells to respond to antigen stimulation) rather than the number of T cells is more likely to accurately reflect protection against recurrent CMV reactivation. Accordingly, we observed several cases of discordant absolute CD8+ T cell counts and IFN-γ ELISpot measurements in favor of the ELISpot assay, in particular cases of high CD8+ T-cell count together with low T-Track® CMV results prior to or during CMV reactivation episodes. Therefore, CMV-specific immune monitoring using a standardized assay, while complementing the current approach of absolute T-cell count monitoring, provides an additional value for the risk stratification of recurrent CMV reactivation in HSCT patients.

Negative ELISpot test results after the first CMV reactivation and at day 100 predicted subsequent recurrent CMV reactivation with a PPV of 80% and 90%, respectively. Measuring pp65-specific response at day 100 rather than directly after the end of a first CMV reactivation also improved the PPV (77% vs. 54%, respectively). This suggests that measuring CMV-CMI at the fixed time point of day 100 might be of greater clinical relevance to predict recurrence of CMV. The apparent improvement in PPV (and PPV) of the day-100 measurement might be in part due to the overall later measurement time (median range of time of 93 [80-100] days), compared to the time of measurement at the end of treatment for a first CMV reactivation, which ranged from day 45 to 186 after transplant-
tion (median 82 days). Moreover, most (31/44 [70.4%]) of the day-100 measurements took place 14 days or later relative to the end of a first antiviral treatment, while most (36/76 [47.4%]) of the measurements at the end of a first CMV reactivation were from the day of discontinuation of antiviral treatment (day 0). This raises the possibility that measuring CMV-CMI 14 days after the end of a treatment-requiring CMV reactivation – rather than on the day of antiviral treatment discontinuation – might improve the predictive value for protection against recurrent reactivation. This might be explained by the ongoing immune reconstitution that takes place after HSCT. Indeed, a paired comparison of existing day 0, day 7 and day 14 measurements revealed a statistically significant increase in SFC counts between day 7 (or day 0) and day 14 in patients with no future recurrent CMV reactivation (data not shown). A future study should determine whether measuring CMV-CMI 14 days after the end of antiviral treatment and/or whether monitoring the dynamics of response might improve prediction of recurrent CMV reactivation.

While the current focus on high-risk (D+/R+) HSCT patients for the prediction of recurrent CMV reactivation is of major clinical relevance, it constitutes the main limitation of this study. A recent study in intermediate-risk (D+/R−) HSCT patients partly addressed this question by measuring the dynamic changes in CMV-CMI using T-Track® CMV,52 underscoring the usefulness of multiple measurements for risk stratification of HSCT recipients. It should also be emphasized that despite the use of non-standardized conditioning regimens, viral load testing and treatment protocols in this multicenter study, the IFN-γ ELISpot assay performed very well at predicting recurrent CMV reactivation. This further highlights the applicability of using a sensitive and standardized CMV-CMI monitoring assay in a real-world setting, as well as its ease of implementation in clinical routine, for the risk stratification of HSCT patients.

In conclusion, our results suggest that using a standardized CMV-CMI monitoring assay can support clinicians in the identification and management of patients with increased risk of recurrent CMV reactivation following HSCT. Beside its applicability in the preemptive setting, the door is also open to its implementation in novel clinical settings with unmet medical needs, such as steering the duration of antiviral prophylaxis (e.g., using lertamivir)34 and monitoring immune reconstitution following adoptive T-cell transfer.34

Disclosures
The participating clinical and measurement centers (D.W., EW, DT, CW, DJ, KS-E, JG, SM, MS, GK, MK, IH, ML-T, SK, DH, SK, MV, SG, MD, TG, MK, TH, and ML) received research funding from Lophius Biosciences for this study. LD is an employee, co-founder, Chief Scientific Officer and shareholder of Lophius Biosciences. SB is an employee and shareholder of Lophius Biosciences. TS, H.G. and A.R. are employees of Lophius Biosciences. RW is Chairman of the Board and a shareholder of Lophius Biosciences. Issued patents relevant to the T-Track® CMV assay used in this study: WO/2010/115984 “METHOD FOR POLYPEPTIDE TRANSFER INTO CELLS”, WO/2003/080792 “USE OF UREA-ADJUVATED POLYPEPTIDES FOR DIAGNOSIS, PROPHYLAXIS AND TREATMENT”. WO/2003/046212 “METHOD FOR IDENTIFYING TARGET EPITOPES OF THE T CELL MEDIATED IMMUNE RESPONSE AND FOR ASSAYING EPITOPE-SPECIFIC T CELLS”.

Contributions
LD, DW, SB and RW contributed to the study conception and design; TS, LD, SB and RW organized, coordinated and supervised the study and its logistics; DW, EW, DT, CW, DJ, KS-E, JG, SM, MS, GK, MK, IH, ML-T, SK, DH, SK, MV, SG, MD and TG acquired patient samples and contributed to data collection; EW, TH, MK., M.L. and S.B. performed the ELISpot assays; SB and HG analyzed the ELISpot results; AR, TS, LD and RW conducted and supervised the interpretation of results; AR drafted the manuscript and figures; All authors contributed to, reviewed and approved the manuscript.

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