Impact of letermovir on cytomegalovirus-specific T-cell reconstitution after allogeneic hematopoietic stem cell transplantation in the post-transplant cyclophosphamide era

The approval of letermovir (LTV) in prophylaxis after allogeneic-hematopoietic stem cell transplantation (allo-HSCT) has reduced the incidence of cytomegalovirus (CMV) clinically relevant reactivations (CRE) in the early post-HSCT period, postponing these events after LTV cessation in high-risk patients.[1-3] T-cell reconstitution is impaired in LTV recipients, and levels of both polyclonal and CMV-specific T cells are restored only some months after LTV treatment.[4-8] Since the presence of CMV-specific T cells correlates with protection against CRE,[9-13] this delay potentially exposes patients to viral reactivations after LTV discontinuation.

To identify the factors stimulating the emergence of protective CMV-specific immunity during or immediately after LTV treatment, we analyzed the dynamics of CMV-specific $CD8^+$ T-cell reconstitution in patients receiving allo-HSCT with post-transplant cyclophosphamide (PT-Cy) and calcineurin inhibitor (CNI)-free graft-*versus*-host disease (GvHD) prophylaxis. Additionally, we explored the correlation between reconstitution of CMV-specific T cells and key clinical outcomes, such as the incidence and severity of CMV reactivation and of acute and chronic GvHD (aGvHD and cGvHD). Understanding whether CMV-specific T cells can serve as a predictive biomarker may have significant implications for risk stratification and personalized management of post-HSCT patients.

In a single-center retrospective observational study, we analyzed 42 CMV-seropositive adult patients with hematological malignancies undergoing allo-HSCT with sirolimus. All patients were treated according to institutional guidelines.[14] GvHD prophylaxis was based on PT-Cy, except in patients receiving graft from cord blood. Anti-thymocite globulin was not used. The conditioning regimen was treosulfan-based; an intensified conditioning with the addition of a second alkylating agent was classified as myeloablative.[14]

Fifteen patients (36%) were treated with allo-HSCT from 2019 to 2022 and received 480 mg of LTV orally once daily from day (D)0 until D100 after transplant as CMV prophylaxis. The control group (no-LTV) was composed of 27 (64%) patients transplanted from 2016 to 2018, before LTV approval. Only patients with peripheral blood mononuclear cells (PBMC) available at 90 (D90) and 180 (D180) days after HSCT and whose donors expressed at least one HLA evaluable with Dextramer® CMV-Kit were enrolled. All patients signed written informed consent according to the principles of the Declaration of Helsinki and the non-interventional ALMON study approved by the Milan San Raffaele Hospital Institutional Ethical Committee in 2007. Samples were processed and stored by the institutional Biological Resource Center (Num_ID in BBMRI-ERIC: bbmri-eric:ID:IT_1383758011993577).

Cytomegalovirus DNAemia was quantified weekly by real-time polymerase chain reaction (PCR) on whole blood samples until D100, twice weekly in case of positivity. Subsequently, patients were routinely monitored for CMV viremia and/or presence of CMV-related symptoms: in detail, patients with no CMV reactivation or CMV-related symptoms were monitored for CMV viremia every 2-3 weeks until D180, as per institutional guidelines.[5] CRE was defined as onset of CMV disease or initiation of pre-emptive therapy, based on CMV DNAemia above 10,000 copies/mL and the CMV-infection risk of the patient, or repeated positive samples showing increased viral load during LTV prophylaxis.[13] CMV blips were defined as the detection of a single positivity for CMV DNA with subsequent spontaneous clearance.[15] Patients', transplant and immune characteristics are detailed in Table 1. CMV-specific $CD8^+$ T cells were quantified by flow cytometry using the In Vitro Diagnostic Regulation (IVDR) Dextramer® CMV-Kit (Immudex), as previously described,[10] in PBMC frozen at D90 and at D180. Dextramer reagents are composed of multiple MHC-peptide complexes and fluorophores covalently bound to a dextran backbone. This tool allows the evaluation of CMV-specific immunity with a good correlation with functional assays and higher sensitivity.[10] Data were considered evaluable if at least 15,000 events in the $CD3^+CD8^+$ region were acquired. Absolute counts of CMV-specific $CD8^+$ T cells were calculated based on the percentage of $CD3^+CD8^+Dextramer^+$ lymphocytes in frozen samples and on the absolute counts of $CD3^+$ T cells in corresponding whole fresh blood. Protective anti-viral immunity was defined based on the threshold of 0.5 CMV-specific cells/μL, which we had previously identified as an independent protective factor from subsequent CRE.[10] Absolute $CD3^+$ counts and percentages of $CD8^+$ T cells were similar in the two groups at both timepoints, with a trend for a slower $CD3^+$ reconstitution in LTV patients, in agreement with previous observations.[4,5,8] Only a minority of patients (N=2 at D90 and N=4 at D180) were treated with a dose of steroids with a potential impact on T-cell amount and functionality. As expected, the number and incidence of both CRE and overall CMV reactivations within D180 was lower in LTV compared with no-LTV patients (CRE, P=0.007; CMV reactivations, P=0.008) (Table 1, *Online Supplementary Figure 1A, B*). In agreement with our previous data in a wider cohort,[5] in patients receiving LTV prophylaxis, we

Table 1. Patients and transplant characteristics (total number of patients = 42).

Characteristic	No-LTV N=27	LTV N=15	*P*
Mean age, years (range)	53 (20-70)	53 (22-75)	0.633
Gender, N (%)			0.927
F	13 (48.1)	7 (46.7)	
M	14 (51.9)	8 (53.3)	
Diagnosis, N (%)			0.599
AML	15 (55.6)	10 (66.7)	
MDS/MPN	7 (25.9)	4 (26.6)	
ALL	3 (11.1)	0	
Other	2 (7.4)	1 (6.7)	
Graft source, N (%)			0.909
PBSC	24 (88.9)	13 (86.7)	
BM	1 (3.7)	1 (6.7)	
CBU	2 (7.4)	1 (6.7)	
Donor type, N (%)			0.035*
MUD	14 (51.9)	14 (93.3)	
MRD	4 (14.8)	0	
MMRD	7 (25.9)	0	
CBU	2 (7.4)	1 (6.7)	
GvHD prophylaxis, N (%)			0.400
PTCy-sirolimus-MMF	22 (81.5)	14 (93.3)	
PTCy-sirolimus	3 (11.1)	0	
Sirolimus-MMF	2 (7.4)	1 (6.7)	
CMV serostatus host/donor, N (%)			0.307
Pos/pos	17 (63.0)	7 (46.7)	
Pos/neg	10 (37.0)	8 (53.3)	
Conditioning intensity, N (%)			<0.001*
MAC	22 (81.5)	4 (26.7)	
RIC	5 (18.5)	11 (73.3)	
HSCT, N (%)			0.435
1	23 (85.2)	14 (93.3)	
2	4 (14.8)	1 (6.7)	
D30 mean % donor chimerism (range)	98.4 (90.0-99.9)	98.7 (96.2-99.9)	0.447
D90 mean % donor chimerism (range)	98.6 (87.0-100)	99.4 (96.7-100)	0.246
D90 mean absolute $CD3^+$ counts (range)	731 (108-2,226)	474 (82-890)	0.202
D180 mean absolute $CD3^+$ counts (range)	1,157 (89-3,068)	948 (231-2,571)	0.561
D90 mean % $CD8^+/CD3^+$ (range)	39.1 (11.5-67.5)	32.9 (3.1-56.0)	0.306
D180 mean % $CD8^+/CD3^+$ (range)	42.1 (13.3-77.8)	47.2 (16.1-73.2)	0.415
N of CRE at D90 (%)			0.016*
0	16 (59.3)	15 (100.0)	
1	10 (37.0)	0	
2	1 (3.7)	0	
N of CRE at D180 (%)			0.024*
0	14 (51.9)	14 (93.3)	
1	12 (44.4)	1 (6.7)	
2	1 (3.7)	0	
N of CMV reactivations at D90 (%)			<0.001*
0	10 (37.0)	15 (100.0)	
1	15 (55.6)	0	
2	2 (7.4)	0	
N of CMV reactivations at D180 (%)			0.053
0	8 (29.6)	10 (66.7)	
1	11 (40.7)	5 (33.3)	
2	7 (25.9)	0	
3	1 (3.7)	0	

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Characteristic	No-LTV N=27	LTV N=15	*P*
N of CMV reactivations including blips at D90 (%)			
0	9 (33.3)	14 (93.3)	0.007*
1	12 (44.4)	1 (6.7)	
2	4 (14.8)	0	
3	1 (3.7)	0	
4	1 (3.7)	0	
N of CMV reactivations including blips at D180 (%)			
0	6 (22.2)	7 (46.7)	0.128
1	9 (33.3)	7 (46.7)	
2	5 (18.5)	1 (6.7)	
3	4 (14.8)	0	
4	3 (11.1)	0	
Steroids ≥0.5 mg/kg/day at D90, N (%)			
No	25 (92.6)	15 (100)	0.280
Yes	2 (7.4)	0	
Steroids ≥0.5 mg/kg/day at D180, N (%)			
No	23 (85.2)	15 (100)	0.117
Yes	4 (14.8)	0	

Statistical analyses used χ^2 test for categorical variables and Spearman's correlation for continuous variables. AML: acute myeloid leukemia; ALL: acute lymphoblastic leukemia; BM: bone marrow; CBU: cord blood unit; CMV: cytomegalovirus; CRE: clinically relevant reactivations; D: day; F: female; GvHD: graft-*versus*-host disease; HSCT: hematopoietic stem cell transplantation; LTV: letermovir; M: male; MAC: myeloablative conditioning; MDS: myelodysplastic syndrome; MMF: mycophenolate mofetil; MPN: myeloproliferative neoplasm; MRD: matched related donor; MMRD: mismatched related donor; MUD: matched unrelated donor; N: number; neg: negative; PBSC: peripheral blood stem cells; pos: positive; PTCy: post-transplant cyclophosphamide; RIC: reduced-intensity conditioning. Values may not add up to 100% due to rounding. *Statistically significant.

also observed a reduction in moderate-to-severe cGvHD (*P*=0.009) and a trend towards a lower incidence of any grade aGvHD (*P*=0.06) and cGvHD (*P*=0.07) (*Online Supplementary Figure 1C-E*).

In our cohort, absolute numbers of CMV-specific CD8$^+$ T cells were enriched from D90 to D180 (*P*=0.02) (*Online Supplementary Figure 2A*). Analyzing separately the two different CMV prophylaxis groups, this increase was evident only in patients receiving LTV (*P*=0.03) (Figure 1A). Accordingly, the absolute numbers of CMV-specific T lymphocytes and the percentages of patients harboring protective levels of CMV-specific CD8$^+$ T cells were lower in LTV compared to no-LTV patients at D90 (*P*=0.009 and *P*=0.04 respectively), but these values are comparable in the two groups at D180 as a consequence of CMV-specific immunity restoration in LTV patients (Figure 1B, C). Interestingly, the incidence of cGvHD (both overall and moderate-to-severe) was not affected by the presence of CMV-specific T cells (Figure 1D, *Online Supplementary Figure 2B*) but was increased in patients experiencing CMV reactivations (including not-CRE) before either D90 or D180, especially when considering only moderate-to-severe cGvHD (Figure 1E, *Online Supplementary Figure 2C*). These data suggest that, while CMV-specific lymphocytes do not seem to be involved in driving cGvHD onset and progression, its occurrence might be influenced by the increased inflammation associated with viral reactivation. Larger-scale investigations are needed to substantiate this hypothesis.

In bivariate analyses, the variables associated with the presence of protective CMV-specific immunity at D90 were: absence of LTV prophylaxis, the percentage of CD8$^+$ T cells, and the number and occurrence of CMV reactivations (including those not-clinically relevant) before D90. (See Table 2 for further details.) For D180, statistically significant correlation was found only for viral reactivations before D180, with a trend towards positive correlation with absolute CD3$^+$ T-cell counts and donor CMV serostatus (Table 2). Of note, CMV-specific T cells were not affected by either the type of transplant or the intensity of the conditioning regimen, which are the other two variables that are significantly different between LTV and no-LTV groups (Tables 1 and 2). Multivariate binomial logistic regression confirmed for both timepoints a positive correlation between the presence of CMV-specific T cells above the protective threshold and previous CMV reactivation, regardless of their number (Table 2 and *data not shown*). Interestingly, at D180 this correlation was significant only including the occurrence of CMV blips in the analysis: D90, no blips *P*=0.010 and with blips *P*=0.007; D180, no blips *P*=0.159 and with blips *P*=0.028. Furthermore, at D90, also the percentage of CD8$^+$ T cells was significantly correlated with CMV-specific immunity (*P*=0.025), which is possibly dependent on the type of assay, while in this cohort with good immune-reconstitution, absolute T-cell counts had no impact (Table 2).

In this study we quantified CMV-specific CD8$^+$ T cells by a flow cytometry IVDR kit. Compared to the detection of interferon(IFN)-γ producing CMV-specific T cells by flow cytometry, ELISpot or QuantiFERON, previously used to evaluate CMV-specific immunity in this setting,[6-8,16] the use of this kit strongly reduces the analysis time and is characterized by standardization, ease of use, and high reproducibility, in agreement with the recent European Regulation IVD-R 2017/746. Furthermore, we have recently demonstrated that the quantification of CMV-specific T cells by Dextramer staining correlates with functional assays such as IFN-γ ELISpot and QuantiFERON.[10]

The GvHD prophylaxis regimen in this cohort was CNI-free. Instead of the more traditional tacrolimus or cyclosporine

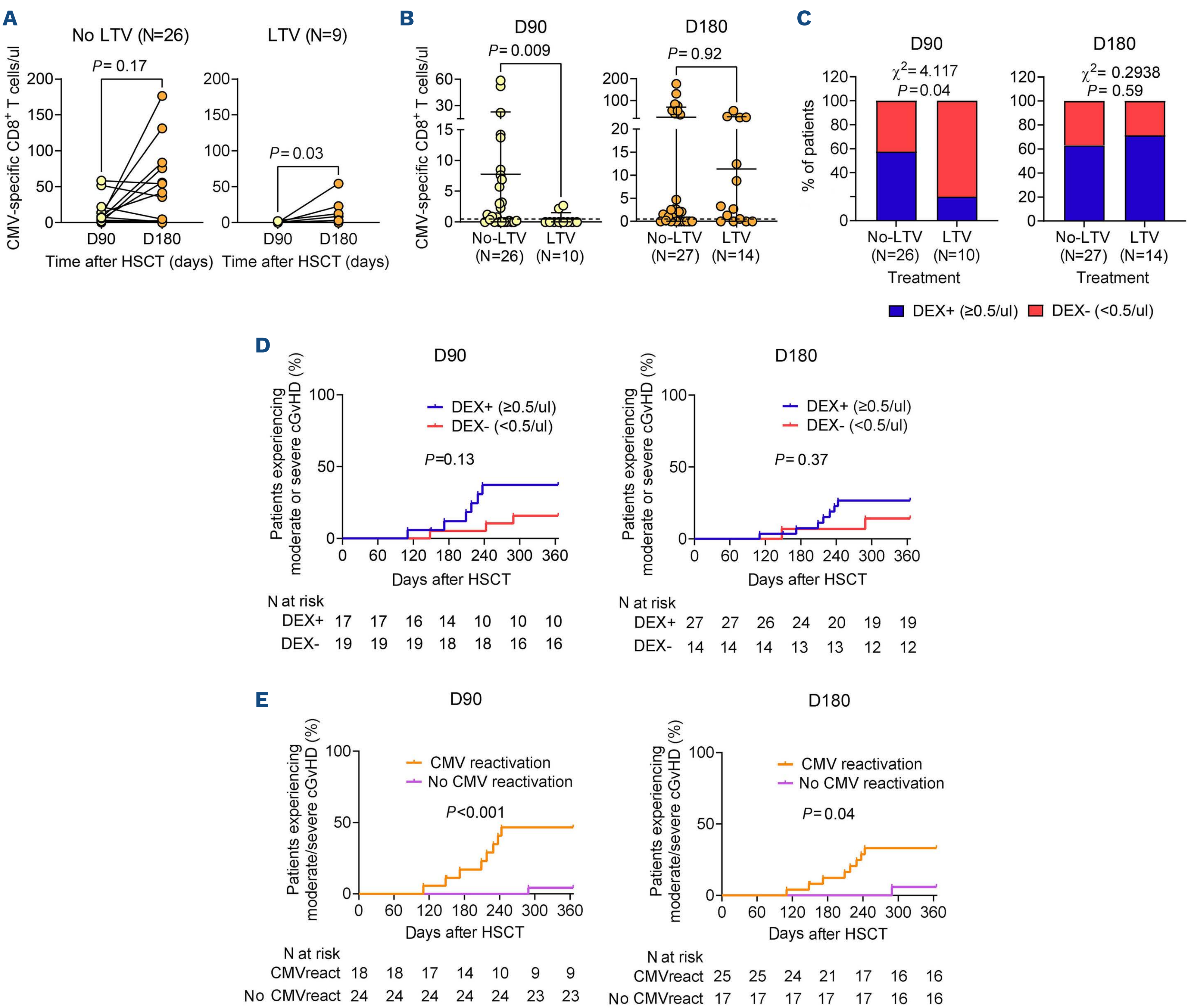

Figure 1. Dynamics of cytomegalovirus-specific T cells differ according to letermovir treatment without impact on moderate-to-severe chronic graft-*versus*-host disease. (A) Longitudinal variation in the absolute values of cytomegalovirus (CMV)-specific CD8+ T cells between Day (D)90 and D180 in patients receiving (right) or not receiving (left) letermovir (LTV) as CMV prophylaxis. Only patients with evaluable samples (≥15,000 CD8+ T cells acquired) at both D90 and D180 were included. Means of CMV-specific T cells: no-LTV, 7.8 cells/μL at D90 and 26.0 cells/μL at D180; LTV, 0.5 cells/μL at D90 and 9.6 cells/μL at D180. Statistical analyses by Wilcoxon test. (B and C) Comparison of the absolute counts of CMV-specific lymphocytes (B) and of the percentages of patients with levels of CMV-specific T cells above the protective threshold of 0.5/μL (C) at D90 and D180 after allogeneic hematopoietic stem cell transplantation (allo-HSCT) between patients treated or not treated with LTV. Only patients with evaluable samples (≥15,000 CD8+ T cells acquired) at either D90 or D180 were included. Means of CMV-specific T cells in (B): D90, 7.8 cells/μL in no-LTV and 0.5 cells/μL in LTV; D180, 25.0 cells/μL in no-LTV and 11.3 cells/μL in LTV. Statistical analyses by Mann-Whitney test in (B) and χ^2 test in (C). (D) Incidence of moderate-to-severe chronic graft-*versus*-host disease (cGVHD) according to the presence of protective levels of CMV-specific T cells (DEX+ ≥0.5/μL) at D90 (left) or D180 (right). (E) Incidence of moderate-to-severe cGvHD according to the occurrence of CMV reactivations before D90 (left) or D180 (right). (D and E) Statistical analyses by Log-rank test. Lines indicate censored data.

Table 2. Correlation between the presence of protective cytomegalovirus-specific T cells and clinical/biological variables.

	Bivariate analyses									
	D90					D180				
	DEX+ <0.5/µL	DEX+ ≥0.5/µL	*P*	χ^2	Odds Ratio (95% CI)	DEX+ <0.5/µL	DEX+ ≥0.5/µL	*P*	χ^2	Odds Ratio (95% CI)
Treatment No LTV LTV	 11 8	 15 2	0.042*	4.12	0.183 (0.032-1.04)	 10 4	 17 10	0.588	0.294	1.47 (0.363-5.95)
Disease Myeloid Lymphoid	 17 2	 14 3	0.537	0.380	0.549 (0.080-3.76)	 13 1	 23 4	0.477	0.507	0.442 (0.0446-4.39)
Type of HSCT MUD MRD MMRD CBU	 13 4 2 0	 11 0 5 1	0.095	6.36	NA	 9 3 1 1	 18 1 6 2	0.240	4.21	NA
Donor CMV serostatus Negative Positive	 9 10	 5 12	0.270	1.22	2.16 (0.544-8.57)	 9 5	 9 18	0.058	3.59	3.60 (0.929-14.0)
Conditioning intensity MAC RIC	 14 5	 12 5	0.836	0.043	1.17 (0.271-5.02)	 10 4	 16 11	0.443	0.588	1.72 (0.428-6.90)
aGvHD No Yes	 11 8	 9 8	0.765	0.089	1.22 (0.327-4.56)	 7 7	 17 10	0.424	0.638	0.588 (0.159-2.17)
Absolute CD3+ counts Mean (range)	592 (237-1,510)	802 (110-2,226)	0.476	NA	NA	844 (406-2,571)	1198 (89-3,068)	0.050	NA	NA
% CD8+/CD3+ Mean (range)	33.0 (11.5-61.6)	46.7 (19.8-67.5)	0.022*	NA	NA	35.4 (15.2-71.6)	48.1 (13.3-77.8)	0.061	NA	NA
CMV reactivation before No Yes	 15 4	 4 13	< 0.001*	11.1	12.2 (2.53-58.7)	 9 5	 7 20	0.017*	5.70	5.14 (1.28-20.7)
N of CMV reactivations before 0 1 2 3	 16 2 1 0	 4 12 1 0	< 0.001*	14.3	NA	 9 3 2 0	 8 13 5 1	0.174	4.97	NA
CMV reactivation before including blips No Yes	 15 4	 3 14	< 0.001*	13.5	17.5 (3.31-92.5)	 8 6	 4 23	0.005*	7.98	7.67 (1.71-34.3)

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Bivariate analyses										
	D90					D180				
	DEX+ <0.5/μL	DEX+ ≥0.5/μL	*P*	χ^2	Odds Ratio (95% CI)	DEX+ <0.5/μL	DEX+ ≥0.5/μL	*P*	χ^2	Odds Ratio (95% CI)
N of CMV reactivations before including blips										
0	15	3				8	4			
1	3	9	0.008*	13.9	NA	4	12	0.023*	11.4	NA
2	1	3				0	6			
3	0	1				2	2			
4	0	1				0	3			

Multivariate analyses					
Variable	*P*	Odds Ratio (95% CI)	Variable	*P*	Odds Ratio (95% CI)
DEX+ ≥0.5/μL D90					
CMV reactivations before (no blips) 1-0	0.010*	53.575 (2.545-1,127.726)	CMV reactivations before (including blips) 1-0	0.007*	54.979 (2.922-1,034.328)
Treatment 1-0	0.862	0.817 (0.084-7.950)	Treatment 1-0	0.975	0.963 (0.092-10.113)
% CD8+/CD3+	0.017*	1.115 (1.020-1.219)	% CD8+/CD3+	0.025*	1.106 (1.013-1.208)
DEX+ ≥0.5/μL D180					
CMV reactivations before (no blips) 1-0	0.159	3.004 (0.651-13.86)	CMV reactivations before (including blips) 1-0	0.028*	6.702 (1.233-36.416)
Donor CVM status pos-neg	0.137	3.169 (0.694-14.47)	Donor CVM status pos-neg	0.060	4.569 (0.936-22.299)
Absolute CD3+ counts	0.246	1.001 (0.999-1.00)	Absolute CD3+ counts	0.356	1.001 (0.999-1.002)

Bivariate statistical analyses used χ^2 test for categorical variables and Spearman's correlation for continuous variables; multivatiate statistical analysis used binomial logistic regression. Only patients with evaluable samples (≥15,000 CD8+ T cells acquired) at either Day (D) 90 or D180 were included. The columns "DEX+" <0.5/μL" and "DEX+ ≥0.5/μL" contain the number of patients in each group. aGvHD: acute graft-*versus*-host disease; CBU: cord blood unit; CI: Confidence Intervals; CMV: cytomegalovirus; CRE: clinically relevant reactivations; D: day; DEX: Dextramer+CD8+ T cells; HSCT: hematopoietic stem cell transplantation; LTV: letermovir; MAC: myeloablative conditioning; MRD: matched related donor; MMRD: mismatched related donor; MUD: matched unrelated donor; N: number; NA: not applicable; neg: negative; pos: positive; RIC: reduced-intensity conditioning. *Statistically significant.

backbones, it relied predominantly on PT-Cy (omitted only in patients receiving cord blood units as graft source), sirolimus and mycophenolate mofetil (MMF). The strategy of omitting CNI aims to reduce nephrotoxicity, neurotoxicity and metabolic complications, while still harnessing the potent *in vivo* T-cell modulation activity of PT-Cy and the synergistic immunoregulation of sirolimus plus MMF to prevent both acute and chronic GvHD.

Our findings reinforce the protective role of LTV against clinically relevant CMV-CRE and confirm that LTV prophylaxis is associated with a delayed reconstitution of CMV-specific $CD8^+$ T cells compared to no-LTV patients. Importantly, our data underscore the pivotal role of antigen exposure (even transient and at low levels, such as during CMV blips) in promoting the expansion of protective levels of CMV-specific T lymphocytes. This suggests that minimal antigenic stimulation is sufficient to boost protective CMV-specific immune responses in the context of ongoing reconstitution. Moreover, in this cohort, 4 patients (N=3 LTV; N=1 no-LTV) achieved protective levels of CMV-specific T cells by D180 despite the absence of previous CMV positivity (*data not shown*). Although we cannot exclude transient CMV reactivation escaping our monitoring schedule, this observation suggests that, while antigen exposure facilitates expansion, the recovery of CMV-specific immunity is also dependent on the broader trajectory of immune reconstitution, which improves at late post-transplant timepoints. A limitation of the method used in this study is that only patients with good immune reconstitution can be evaluated, due to the requirement of at least 15,000 $CD3^+CD8^+$ T cells.

These findings have several implications. Firstly, a clearer definition of the stimuli that promote CMV-specific T-cell emergence (whether through subclinical antigen exposure or endogenous immune recovery) could help refine patients' stratification strategies. Our work supports the potential utility of combining longitudinal immune monitoring with a CMV-specific Dextramer-based assay to identify patients with suboptimal CMV-specific immune reconstitution. Moreover, in the setting of extended prophylaxis that is currently being explored in clinical trials, Dextramer-based assays could distinguish those patients that could safely shorten the duration of therapy or who, on the other hand, would need even longer prophylaxis, allowing for better protection during the window of immune vulnerability.

Additionally, consistent with prior observations,[5] our data suggest that the incidence of GvHD is lower in patients receiving LTV, but increases after CMV reactivation. This supports the hypothesis that reduced CMV reactivation and associated inflammation may contribute to a more favorable GvHD profile. Furthermore, our data provide indirect evidence that CMV-specific T cells are not major contributors to the pathogenesis of cGvHD, since we observed no increased risk of cGvHD in patients with higher levels of CMV-specific T cells.

Taken together, our study provides new insights into the dynamics of CMV-specific immunity under LTV prophylaxis and highlights actionable items for personalized antiviral strategies after allo-HSCT.

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Disclosures

CB has been member of Advisory Board and Consultant for Intellia, TxCell, Novartis, GSK, Allogene, Kite/Gilead, Kiadis, Evir, Janssen, Genyo, Epsilen, and Alia, and received research support from Intellia Therapeutics. She is inventor of different patents on cancer immunotherapy and genetic engineering. RG declares speaker honoraria for educational events from Biotest, Pfizer, Medac, Quiagen, Kyverna MDS and Magenta, and advisory board from Pfizer and BMS. All the other authors have no conflicts of interest to disclose.

Contributions

ET designed the study, conducted laboratory experiments, analyzed and interpreted data, and wrote the paper. GO designed the study, provided clinical data and samples, analyzed and interpreted data, and wrote the paper. VB and VV conducted laboratory experiments and analyzed data. MN designed the study and participated in the data interpretation. GMB, EX, MD, AAc, LL, FG, SP, ED, AB, FF, RD, LV, AAs, AR, DC, CC and MTLS provided clinical data and samples, and contributed to data interpretation. FC designed and supervised the study. CB and RG designed and supervised the study, and wrote the paper.

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Data-sharing statement

The source data that support the findings of this study are openly available in the San Raffaele Open Research Data Repository, DOI: 10.17632/nx2t36ng7s.1.

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