Standardized monitoring of cytomegalovirus-specific immunity can improve risk stratification of recurrent cytomegalovirus reactivation after hematopoietic stem cell transplantation

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Supplemental Methods

Study design and participants

A prospective, longitudinal, observational, multicenter study was conducted at 10 German medical centers between June 2014 and April 2018 in a cohort of 175 intermediate- and high-risk (donor (D) / recipient (R) CMV serostatus: D+/R+, D+/R-, D-/R+) allogeneic hematopoietic stem cell transplantation (HSCT) recipients. Recipients of a first-time bone marrow or peripheral blood transplantation from either a matched sibling, matched unrelated or mismatched unrelated donor, of any gender and race, aged at least 18 years were recruited in the study. Patients scheduled for preemptive antiviral therapy were eligible for study participation. Patients receiving Alemtuzumab as induction therapy, recipients of a haploidentical HSCT or of a cord-blood transplantation, as well as patients suffering from uncontrolled or chronic infections (e.g. HIV, chronic hepatitis) were ineligible for study participation. Patients were initially scheduled for three post-transplantation visits at days 45, 60 and 80. Unscheduled visits were planned at the starting day of CMV reactivations requiring antiviral treatment and following discontinuation of antiviral treatment (up to 3 visits at days 0, 7 and 14 relative to end of therapy). The observational period was of six months after the initial visit, thus from day 45 to day 225 after transplantation. Starting November 2015, and according to results of a planned interim analysis, only high-risk (D-/R+) patients were recruited as the main subpopulation affected by recurrent CMV reactivations, and two additional visits at days 100 and 120 post-transplantation were implemented for all patients under observation (see study flow chart in Supplemental Table 1).

Patients received a standard conditioning regimen (myeloablative, non-myeloablative or reduced-intensity), with or without anti-thymocyte globulin (ATG), per institutional guidelines. All patients received herpes simplex virus/varicella zoster virus (HSV/VZV) antiviral prophylaxis (acyclovir or valacyclovir) from the time of conditioning or at transplantation, and according to center-specific guidelines. Upon CMV reactivation requiring treatment, Valgancyclovir, Gancyclovir or Foscarnet was used as first-line drug for preemptive anti-CMV therapy. Patients receiving anti-CMV therapy without evidence of treatment-requiring CMV reactivation were excluded from the analysis. Graft-versus-host disease (GvHD) was treated using steroids (mainly prednisolone or methylprednisolone) or calcineurin inhibitors (CNI).

The study was registered at the German Institute of Medical Documentation and Information (DIMDI). Patient enrollment was initiated after receiving the exemption of the permit requirement by the Federal Institute for Drugs and Medical Devices (BfArM) and approval by the ethics committees (DIMDI's registration number 00008544; leading institutional ethics board of the University of Regensburg's approval number 13-122-0282). All subjects gave written informed consent, in accordance with the Declaration of Helsinki. The study was registered at clinicaltrials.gov (Identifier: NCT02156479).

Viral load measurement

CMV load was measured by quantitative PCR (CMV DNAemia) either from whole blood (nine centers) or plasma (one center) using either a commercial assay (Abbott RealTime CMV*;* two centers) or validated in-house protocols and equipment (eight centers). In three centers, phosphoprotein 65 (pp65) antigenemia was also measured in some patients. Accordingly, treatment-requiring viral load thresholds were center-specific and preemptive antiviral treatment followed institutional guidelines.

CMV-specific cell-mediated immunity (CMV-CMI) measurement and analysis

Blood collection and peripheral blood mononuclear cell (PBMC) isolation (within 8 hours of blood collection) were performed as previously described.[1–3] Counting of PBMC was performed using an automated cell counting device (Hem-o-test 2000, BioGen Technologies, Germany or Sysmex KX-21N, Sysmex Deutschland GmbH, Germany; LYM# and WBC parameters for lymphocytes and total white blood cells respectively).

IFN-γ ELISpot assays (T-Track® CMV, Lophius Biosciences GmbH, Regensburg, Germany) were performed as previously described.[1–3] Briefly, PBMC were stimulated with T-activated® CMV-specific immediate-early 1 (IE-1) and pp65 proteins[1,4] for 19 hours at 37°C. ELISpot assays were performed using four-replicate measurements for unstimulated and CMV-specific antigen stimulated conditions, and duplicate measurements for the positive control (stimulation with staphylococcal enterotoxin B [SEB]) and operator control. Spot-forming cells (SFC) were enumerated on the same automated reader (Bioreader® 5000 Pro-Eα, BIO-SYS GmbH, Germany). Test results were interpreted on the basis of square-root-transformed SFC (sqrt-SFC) values. Square-root transformation of data that follow a Poisson distribution, as assumed for ELISpot counts, is commonly used to reduce right skewness and to stabilize variance.[5–7] A test was considered positive if the mean of four replicate sqrt-SFC for 200,000 cells (SRM) resulting from IE-1 and/or pp65 stimulation was ≥ 3.16 [where 3.16 = sqrt(10)] and if the difference of the mean of sqrt-SFC (for 200,000 cells) of the stimulated condition to that of the unstimulated condition (SRM[stimulated] - SRM[unstimulated]) was ≥ 0.742 (based on a one-sided z-test). An analysis based on the geometric mean (GM) of four replicate SFC counts, as previously reported,[1–3] showed comparable results (Supplemental Table 6 and Supplemental Table 7). SRM SFC values from unstimulated conditions were subtracted from those of the respective IE-1- and pp65-stimulated conditions. SRM values normalized to 200,000 lymphocytes (LYM# parameter) were used for statistical analysis. SRM SFC values normalized to PBMC (WBC parameter) yielded comparable results (Supplemental Table 6 and Supplemental Table 7). Participants with at least one visit with a valid ELISpot test contributed to the analysis. Investigators and caregivers were blinded in regard to the ELISpot results, to avoid influencing their decision of when and how to treat patients.

For quality-control purposes, six inter-center ELISpot and/or cell counting tests were conducted between July 2015 and February 2018, and yielded comparable results. It should be noted that between May 2014

and August 2015 one cell counting device revealed a progressive deviation in its counting capability, which was estimated to decrease linearly by a factor two (bias factor 0.5) and was corrected accordingly by a reciprocal bias factor. In the presented analyses, 147 out of 647 assays were adjusted in 52 out of 154 patients.

A post-hoc analysis normalizing SRM SFC values to absolute lymphocyte counts (as SRM/µl blood) was also conducted based on the peripheral blood absolute lymphocyte count (PBALC) determined at the same visit using the formula: SRM SFC/µl blood = SRM SFC for 200,000 lymphocytes x 5 x 10^{-6} x PBALC (lymphocytes/µl blood).[8] When no PBALC was available at the corresponding visit, the next available PBALC (±14 days) was used for calculation. In the presented analysis, 5 out of 63 test results normalized to absolute lymphocyte counts were derived from a PBALC measured at a different visit (+6 to +14 days deviation).

Lymphocyte subpopulation count determination

Lymphocyte subpopulations were characterized by multicolor flow cytometry from the same PBMC as those used for the ELISpot assays, when available. Remaining PBMC were frozen at 2.5 x 10^6 cells per cryogenic vial in 500 µl RPMI 1640 (Gibco 61870-010) containing 10% dimethyl sulfoxide (DMSO; SIGMA-ALDRICH D2650) and 40% fetal bovine serum (FBS; Gibco 10270-106), and stored in liquid nitrogen until processed for flow cytometry analysis. Samples from different centers were collected and processed in batches at one center and analyzed on the same flow cytometer (FACSCanto II, BD Biosciences). Samples were gently thawed one at a time, washed twice in 10 ml RPMI 1640 containing 10% FBS and 1% penicillin-streptomycin (PAN P06-07100), and resuspended in 1 ml AIM-V medium (Gibco 31035-025). 10^6 cells were washed once in 1x phosphate-buffered saline (PBS; Lonza BE17-516F) containing 0.5% bovine serum albumin (BSA; AppliChem A1391) and resuspended in a final volume of 100 µl 1x PBS / 0.5% BSA containing 0.1-2 µg of either cell-surface-marker-specific conjugated antibodies (anti-CD3 APC-H7, clone SK7, BD 560176; anti-CD4 FITC, clone RPA-T4, BioLegend 300506; anti-CD8 PerCP, clone RPA-T8, BioLegend 301030; anti-CD45RA BV510, clone HI100, BD 563031; anti-CD45RO eFluor 450, clone UCHL1, eBiosciences 48-0457-42; anti-CD56 PE, clone HCD56, BioLegend 318306) or the same amount of the respective isotype controls (IgG1-κ APC-H7, clone X40, BD 641401; IgG1-κ FITC, clone MOPC-21, BioLegend 400110; IgG1-κ PerCP, clone MOPC-21, BioLegend 400148; IgG2b-κ BV510, clone 27-35, BD 563025; IgG2a-κ eFluor 450, clone eBM2a, eBiosciences 48-4724-82; IgG1-κ PE, clone MOPC-21, BioLegend 400114). Cells were stained for 20 minutes at room temperature, protected from light, washed once with 1x PBS / 0.5% BSA and resuspended in 500 µl 1x PBS containing 1% paraformaldehyd (PFA; Merck 104005). Samples were kept at 2-8 °C until data acquisition. Flow cytometry data were analyzed from 100,000 acquired events using the BD FACSDiva 7.0 software. Briefly, lymphocytes were first gated on Side Scatter-Area (SSC-A) vs. Forward Scatter-Area (FSC-A). Natural killer (NK) and T cells were gated on CD56 vs. CD3 ($CD56^+CD3^-$ and $CD56^-CD3^+$, respectively). T cells were further gated on CD4- vs. CD8-

positive T cells ($CD3^+CD4^+CD8^-$ and $CD3^+CD4^-CD8^+$, respectively), and finally separated in CD45RA vs. CD45RO for naïve ($CD45RA^+CD45RO^-$) and memory ($CD45RA^-CD45RO^+$) phenotypes. A representative gating strategy is shown in Supplemental Figure 1. T and NK cell subpopulation levels were expressed as a percentage (%) of the gated lymphocytes. Absolute cell counts (in cells/µl blood) were calculated using the peripheral blood absolute lymphocyte count (PBALC) determined at the same visit using the formula: Absolute cell count (cells/µl) = cell count in percent of gated lymphocytes (expressed as a decimal) x PBALC (lymphocytes/µl).[9,10] When no PBALC were available at the corresponding visit, the next available PBALC (±14 days) were used for calculation. In the presented analysis, 7 out of 47 absolute count values were derived from a PBALC measured at a different visit (-5 to +14 days deviation).

Other variables

Successive episodes of treatment-requiring CMV reactivation following transplantation, CMV disease (as defined by Ljungman et al.),[11] presence and severity of graft-versus-host disease (GvHD), occurrence of infections other than CMV, and death were documented.

Definitions

CMV reactivation was defined as CMV viral load requiring antiviral treatment based on center-specific guidelines and/or physician's decision. Accordingly, the term "CMV reactivation" will refer to preemptively treated CMV reactivation throughout the present manuscript. Primary aim of the study was to determine the suitability of T-Track® CMV measured following the end of treatment for a first CMV reactivation to predict recurrence of CMV reactivation. A minimum of 30-day follow up was considered for the group of patients with no further CMV reactivation. Secondary aims included the comparison of T-Track® CMV performance to that of T cell count determination by flow cytometry and to that of multimer staining (PRO5 MHC class I pentamers for pp65(465-504)-specific CD8+ T cells in 40 patients with HLA allele A*02:01) to predict recurrent CMV reactivation. The suitability of multimer staining to predict CMV recurrence could not be addressed due to the absence of respective measurements in the group of patients experiencing recurrent CMV reactivation. The technical performance of multimer staining compared to that of T-Track® CMV will be described elsewhere (manuscript in preparation). A possible association between IFN-γ ELISpot results and the occurrence and severity of GvHD was also investigated. Analysis of ELISpot results in relation to GvHD severity (i.e. acute GvHD grade I, II, III, IV and chronic GvHD mild, moderate, severe) could not be addressed due to too low case numbers in each category. A post-hoc analysis considering the suitability of T-Track® CMV measured between day 80 and day 100 post-transplantation to predict occurrence (and recurrence) of late (post day 100) CMV reactivation was also performed. Prerequisite for this analysis was the absence of ongoing CMV reactivation at time of IFN-γ ELISpot measurement and up to day 100, as well as the absence of existing viral load below threshold but

on its way to treatment-requiring CMV reactivation. A minimum of 30-day follow up was requisite for patients with no late CMV reactivation.

Statistical analysis

Calculations were performed with SAS 9.4 Software and figures were generated using GraphPad Prism 5.04. Unless otherwise stated, the mean of sqrt-SFC values (SRM) normalized to 200,000 lymphocytes was used for statistical analyses. For a better display of IE-1- and pp65-specific SFC levels, however, SRM SFC values are presented as squared (SRM^2) SFC values (i.e. as "spot count-equivalent"), and in a log10 scale. SRM^2 SFC values are depicted as scattered plots showing median values (horizontal line). Statistical analyses are presented for high-risk (D-/R+) and all patients only, due to the small size of the other two CMV-serostatus groups (D+/R+, D+/R-) in most analyses. Differences in IE-1- and pp65-specific SFC distributions between groups were tested using the non-parametric two-sided Mann-Whitney U (MWU) test. As categorical variables, qualitative (positive vs. negative) test results were compared using a chi-square test. Cumulative probability of (recurrent or late) CMV reactivation between patients with a positive or negative ELISpot test result was estimated using Kaplan-Meier failure curves. Hazard ratio (HR) estimates were obtained by Cox Regression and differences in CMV reactivation probability between both groups were tested using a log-rank test. Receiver operating characteristic (ROC) curves were generated to test the performance of the ELISpot assay in predicting recurrent or late CMV reactivation. Area under the curve (AUC) estimates were obtained by Logistic Regression. Two-sided p-values < 0.05 were considered statistically significant, without adjustment for multiple hypothesis testing. P-values were reported according to the American Statistical Association's statement on p values.[12]

Limitations

Several potential biases should be considered when interpreting the presented data. The first bias concerns the possible deviance in cell counting between May 2014 and August 2015 at one measuring center, despite correction with a reciprocal bias factor of 147 out of the 647 measurements that are part of the final analysis. On the other hand, inter-center ELISpot quality control tests showed good comparability of results between centers (not shown). The second bias relates to the non-standardized CMV viral load measurements and the definition of center-specific viral load thresholds for initiation of preemptive therapy, and thus the potential deviance in definition of CMV reactivation between centers. In that regard, out of 157 antiviral treatments, 115 (73%) were initiated based on PCR-determined CMV DNAemia ≥ defined threshold, and 42 (27%) were initiated based on other validated viral load measurement methods or criteria. The third bias relates to the differences in conditioning regimen between centers, in particular in regard to the administration or not of ATG, which might differentially influence clinical outcome and results of viral load and immune-based tests. The fourth potential bias relates to the calculation of absolute lymphocyte subpopulations (number of cells/µl blood) based on flow

cytometry data (% of lymphocytes in the isolated PBMC) normalized to the absolute lymphocyte count measured in blood (PBALC). In 7/47 cases, absolute cell counts were calculated using the PBALC from a prior (down to 5 days) or subsequent (up to 14 days) visit. The last bias is in regard to the change in study design implemented in November 2015. Due to the focus on high-risk D-/R+ patients from that time on, these patients were over-represented in the total population and respective analyses. Similarly, the additional visits scheduled at days 100 and 120 introduced a potential bias in some of the analyses (such as the post-hoc analysis of prediction of late CMV reactivation based on ELISpot measurement at day 80-100).

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Supplemental Tables

Supplemental Table 1. Study flow chart

	Scheduled visits (study inclusion)					Unscheduled visits (at every CMV reactivation requiring antiviral therapy)
Visit	1	2	3	4[a]	5[a]	Unscheduled visits A-D (6 months observational period) A: diagnosis of CMV reactivation B: withdrawal of antiviral therapy (d0) C: withdrawal of antiviral therapy (d7) D: withdrawal of antiviral therapy (d14)
Day post-HSCT	45	60	80	100	120	At any time between d45-d225
Deviation (in days)	±7	±7	±7	±7	±7	Visit A: +10 Visit B-D: ±3
Informed consent	X					
Demography and medical history	X					
T-Track® CMV	X	X	X	X	X	Day of CMV reactivation diagnosis or at the next possible visit with routine blood draw (up to 10d after diagnosis of CMV reactivation); days 0, 7, 14 (±3d) after withdrawal of anti-CMV therapy
MHC-I CMV-specific multimers staining; lymphocyte sub-population counts	X	X	X	X	X	
CMV DNAemia (qPCR)	X	X	X	X	X	As defined in the respective guidelines of the participating institutes, at least in parallel with T-Track® CMV
CMV complications	X	X	X	X	X	X
Adverse events	X	X	X	X	X	X
Laboratory parameters	X	X	X	X	X	X

[a]Additional visits were implemented from November 2015 until end of the study (April 2018)

Supplemental Table 2. Time to CMV disease per CMV serostatus of HSCT recipient

CMV disease	all patients		D-/R+		D+/R+		D+/R-	
	N[a]	Median days (range)	N[a]	Median days (range)	N[a]	Median days (range)	N[a]	Median days (range)
First CMV disease	8[c]	88 (28-189)	6[d]	66 (28-189)	2[e]	130 (113-146)	0	-
Second CMV disease[b]	1[e]	102	1[e]	102	0	-	0	-

[a]Number of patients; [b]First documented CMV disease for this patient started at day 47 and ended at day 77 post-HSCT; [c]7/8 biopsy-proven and organ-involved; [d]5/6 biopsy-proven and organ-involved; [e]biopsy-proven and organ-involved.

Supplemental Table 3. Percentile rank of SFC values[a] related to T-Track® CMV measured after end of a first CMV reactivation in patients with or without a future recurrent CMV reactivation (primary aim analysis; see Figure 2B)

Percentile rank of SFC values (SRM^2)	IE-1				pp65			
	all patients		D-/R+ patients		all patients		D-/R+ patients	
	No recurrent CMV	Recurrent CMV	No recurrent CMV	Recurrent CMV	No recurrent CMV	Recurrent CMV	No recurrent CMV	Recurrent CMV
Minimum	0	0	0	0	0.25	0	0.25	0
P10%	0.31	0	0.01	0	5.14	0	1.34	0
P25%	1.99	0.11	0.31	0.09	29.53	0.11	9.08	0.09
Median	13.9	1.35	1.81	1.1	155.8	3.55	36.7	3.55
P75%	52.41	6.2	4.67	5.72	594.6	15.85	152.8	15.34
P90%	176.3	16.79	42.31	16.26	814.7	277.9	616.5	250.8
Maximum	868.7	772.5	178.3	70.69	924.1	1066	813.4	386.5

[a]SFC (SRM^2)/200,000 lymphocytes (stimulated minus unstimulated condition). Abbreviations: CMV, cytomegalovirus; D/R, donor/recipient CMV serostatus; Max, maximum SFC; Min, minimum SFC; P10%, 10th percentile SFC; P25%, 25th percentile SFC; P75%, 75th percentile SFC; P90%, 90th percentile SFC.

Supplemental Table 4. Percentile rank of SFC values[a] related to T-Track® CMV measured at day 100 in patients with prior CMV reactivation, in relation to occurrence of late (after day 100) recurrent CMV reactivation (post-hoc analysis; see Figure 3B)

Percentile rank of SFC values (SRM^2)	IE-1				pp65			
	all patients		D-/R+ patients		all patients		D-/R+ patients	
	No recurrent CMV	Recurrent CMV	No recurrent CMV	Recurrent CMV	No recurrent CMV	Recurrent CMV	No recurrent CMV	Recurrent CMV
Minimum	0	0	0	0	0.29	0	0.29	0
P10%	0.09	0	0.01	0	26.7	0.14	4.62	0.14
P25%	1.53	1.21	0.06	1.21	32.46	1.12	26.49	1.12
Median	21.76	2.02	0.97	2.02	255.9	5.25	32.11	5.25
P75%	64.92	8.48	5.62	8.48	706.2	8.67	142.4	8.67
P90%	525	13.07	56.84	13.07	815.4	221.4	735.1	221.4
Maximum	682.8	13.94	62.42	13.94	859.4	283.6	813.4	283.6

[a]SFC (SRM^2)/200,000 lymphocytes (stimulated minus unstimulated condition). Abbreviations: CMV, cytomegalovirus; D/R, donor/recipient CMV serostatus; Max, maximum SFC; Min, minimum SFC; P10%, 10th percentile SFC; P25%, 25th percentile SFC; P75%, 75th percentile SFC; P90%, 90th percentile SFC.

Supplemental Table 5. Diagnostic accuracy. Sensitivity and specificity in identifying patients with and without late (after day 100 post-HSCT) CMV reactivation, regardless of occurrence of CMV events prior to day 100, based on CMV-specific negative and positive ELISpot test results at ~day 100, respectively.

Population	Marker	Sensitivity	Specificity	Chi-square	PPV[b]	NPV[b]
all patients	IE-1	76.9% (10/13) [95% CI: 46.2-95.0%]	46.8% (22/47) [95% CI: 32.1-61.9%]	p=0.125	-	-
	pp65	75.0% (9/12) [95% CI: 42.8-94.5%]	76.6% (36/47) [95% CI: 62.0-87.7%]	**p=0.001**	-	-
	IE-1, pp65[a]	69.2% (9/13) [95% CI: 38.6-90.9%]	78.7% (37/47) [95% CI: 64.3-89.3%]	**p=0.001**	-	-
D-/R+ patients	IE-1	76.9% (10/13) [95% CI: 46.2-95.0%]	12.5% (2/16) [95% CI: 1.6-38.3%]	p=0.453	41.7% (10/24)	40.0% (2/5)
	pp65	75.0% (9/12) [95% CI: 42.8-94.5%]	58.8% (10/17) [95% CI: 32.9-81.6%]	p=0.071	56.3% (9/16)	76.9% (10/13)
	IE-1, pp65[a]	69.2% (9/13) [95% CI: 38.6-90.9%]	58.8% (10/17) [95% CI: 32.9-81.6%]	p=0.127	56.3% (9/16)	71.4% (10/14)

[a]T-Track® CMV assay: test is positive when at least one of the IE-1- and/or pp65-specific response is positive, and test is negative when both IE-1- and pp65-specific responses are negative; [b]PPV and NPV were not calculated in the "all patients" population due to the imbalance toward D-/R+ patients in that group. Abbreviations: CI, confidence interval; CMV, cytomegalovirus; D/R, donor/recipient CMV serostatus; NPV, negative predictive value; PPV, positive predictive value.

Supplemental Table 6. AUC estimates (ROC analysis) according to the method of normalization of T-Track® CMV test results (bold: p<0.05)

Analysis	all patients (N=74 to 77)		D-/R+ patients (N=46 to 50)	
	IE-1	pp65	IE-1	pp65
SRM/LYM#	**0.741**	**0.840**	0.532	**0.780**
SRM/WBC	**0.750**	**0.825**	0.555	**0.759**
GM/LYM#	**0.733**	**0.839**	0.520	**0.770**
GM/WBC	**0.739**	**0.827**	0.541	**0.753**

Abbreviations: SRM, mean of quadruplicate square-root-transformed spot-forming cell (SFC) counts; GM, geometric mean of quadruplicate SFC counts; LYM#, normalization to 200,000 lymphocytes in the PBMC preparation, as determined by the automated cell counter; WBC, normalization to 200,000 white-blood cells in the PBMC preparation, as determined by the automated cell counter

Supplemental Table 7. Diagnostic accuracy (% sensitivity and specificity) according to the method of normalization of T-Track® CMV test results (chi-square test; bold: p<0.05)

Analysis	all patients (N=74 to 78)						D-/R+ patients (N=46 to 50)					
	IE-1		pp65		IE-1, pp65		IE-1		pp65		IE-1, pp65	
	Sensi.	Speci.	Sensi.	Speci.	Sensi.	Speci.	Sensi.	Speci.	Sensi.	Speci.	Sensi.	Speci.
SRM/LYM#	**73.3**	**56.8**	**58.1**	**88.4**	**53.3**	**93.2**	75.9	17.7	**58.6**	**77.8**	**55.2**	**83.3**
SRM/WBC	**78.8**	**52.3**	**67.7**	**86.1**	**58.8**	**90.9**	83.9	11.8	**68.8**	**72.2**	**62.5**	**77.8**
GM/LYM#	**80.0**	**56.8**	**58.1**	**88.4**	**53.3**	**93.2**	82.8	17.7	**58.6**	**77.8**	**55.2**	**83.3**
GM/WBC	**78.8**	**52.3**	**67.7**	**86.1**	**58.8**	**90.9**	83.9	11.8	**68.8**	**72.2**	**62.5**	**77.8**

Abbreviations: Sensi., sensitivity; Speci., specificity; SRM, mean of quadruplicate square-root-transformed spot-forming cell (SFC) counts; GM, geometric mean of quadruplicate SFC counts; LYM#, normalization to 200,000 lymphocytes in the PBMC preparation, as determined by the automated cell counter; WBC, normalization to 200,000 white-blood cells in the PBMC preparation, as determined by the automated cell counter

Legends to Supplemental Figures

Supplemental Figure 1. Gating strategy of the flow cytometry-based analysis of lymphocyte subpopulations. As detailed in the Methods section, lymphocytes were gated on SSC-A vs. FSC-A channels. NK and T cells were gated based on $CD56^+$ vs. $CD3^+$ staining, respectively. Cytotoxic T cells and T helper cells were further gated according to $CD8^+$ vs. $CD4^+$ staining, respectively. Finally, naïve and memory $CD8^+$ and $CD4^+$ T cells were gated based on $CD45RA^+$ vs. $CD45RO^+$ staining, respectively.

Supplemental Figure 2. Study flow diagram. 175 patients were enrolled in the study. Twenty-one patients were excluded based on inclusion and/or exclusion criteria, as well as due to missing or invalid T-Track® CMV tests. Altogether, 154 patients were included in the final analysis. Twenty-seven patients discontinued the study before the end of observational period, mainly due to death (21 patients).

Supplemental Figure 3. Median time to the first, second and third CMV reactivation post-HSCT. (A) Schematic representation and definitions of treatment-requiring CMV reactivation post-HSCT, including respective number (N) of patients affected. **(B)** Median time (range) of occurrence of CMV reactivation (in days post-HSCT), according to the number of CMV reactivations (one, two or three) and to patients' donor (D)/recipient (R) CMV serostatus (all patients vs. high-risk D-/R+ patients).

Supplemental Figure 4. Quantitative and qualitative ELISpot test results at successive visits in all and D-/R+ patients. (A) IE-1- and pp65-specific IFN-γ ELISpot test results were evaluated on the basis of the mean of square-root-transformed (SRM) spot-forming cells (SFC), as described in the Methods section, at the indicated periods post-transplantation. In case of patients with several measurements in a given period, only the first available test was considered (to avoid a bias of multiple measurements per patient). For the sake of simplicity, scatter plots are depicted as squared SRM values (SRM^2). A Kruskal-Wallis test using the Dunn's multiple comparison test showed statistically significant differences in pp65-specific SFC distributions in D-/R+ patients between periods 1 and 6 (***$p<0.001$), 2 and 6 (**$p<0.01$), and 3 and 6 (*$p<0.05$). **(B)** Proportion of positive test results at the indicated periods post-transplantation for IE-1- and pp65-specific test results, as well as according to T-Track® CMV test results (IE-1 and pp65 tests combined, using the following definition: T-Track® CMV test is positive when at least one of the IE-1- and/or pp65-

specific response is positive, and T-Track® CMV test is negative when both IE-1- and pp65-specific responses are negative). **(C)** SFC distribution was evaluated as in panel A, this time relative to the start of the first CMV reactivation. In case of multiple measurements per patient in a given period, only the first measurement was considered. SFC distribution relative to the start of the first CMV reactivation showed low median spot counts in D-/R+ patients at start of CMV reactivation in response to both IE-1 and pp65 antigens, progressively increasing over time. A Kruskal-Wallis test with the Dunn's multiple comparison test showed statistically significant differences in IE-1- and pp65-specific SFC distributions in D-/R+ patients between period 1 and period 6 (**p<0.01).

Supplemental Figure 5. Performance of CMV-CMI measured after end of a first CMV reactivation to predict recurrence of CMV reactivation, following normalization of ELISpot results to absolute lymphocyte counts. (A) IFN-γ ELISpot was performed after end of antiviral therapy for a first CMV reactivation, at up to three time points relative to 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 Figure 2, and then further normalized to absolute lymphocyte counts, as described in the Methods section. ELISpot results were expressed as SFC/µl blood. Differences in absolute SFC distribution between patients with only one CMV reactivation and those with recurrent CMV reactivation were evaluated using a Mann-Whitney-U (MWU) test. Respective p-values are shown under each graph. For the sake of simplicity, scatter plots are depicted as squared SRM values (SRM^2). Due to log scale representation, values of zero SRM^2 were replaced by low values (y-axis), meaning that depicted baseline values are actually equal to zero. **(C)** Prediction of CMV reactivation recurrence based on IE-1- and pp65-specific absolute SFC counts measured at end of treatment of a first CMV reactivation was evaluated by ROC analysis. Area under the curve (AUC), 95% confidence intervals (CI) and respective p-values are indicated within each graph. In B and C, statistically significant p-values are in bold.

Supplemental Figure 6. Spot-forming cell distribution measured after end of a first CMV reactivation in patients with and without recurrent CMV reactivation, according to the conditioning regimen (post-hoc analysis). IFN-γ ELISpot results shown in Figure 2B were analyzed according to patients' conditioning regimen (non-myeloablative vs. myeloablative). Differences in SFC distribution were evaluated using a Kruskal-Wallis (K-W) test with Dunn's multiple comparison test. Respective p-values are shown under each graph (ns p>0.05; *p<0.05; **p<0.01; ***p<0.001). Scatter plots are depicted as squared SRM values (SRM^2). Median and interquartile range (IQR) of SRM^2 SFC are shown above each graph. Due to 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 depict negative and positive tests respectively, defined according to the rules described in the Methods section. SFC distributions were comparable in patients who received myeloablative and non-myeloablative conditioning.

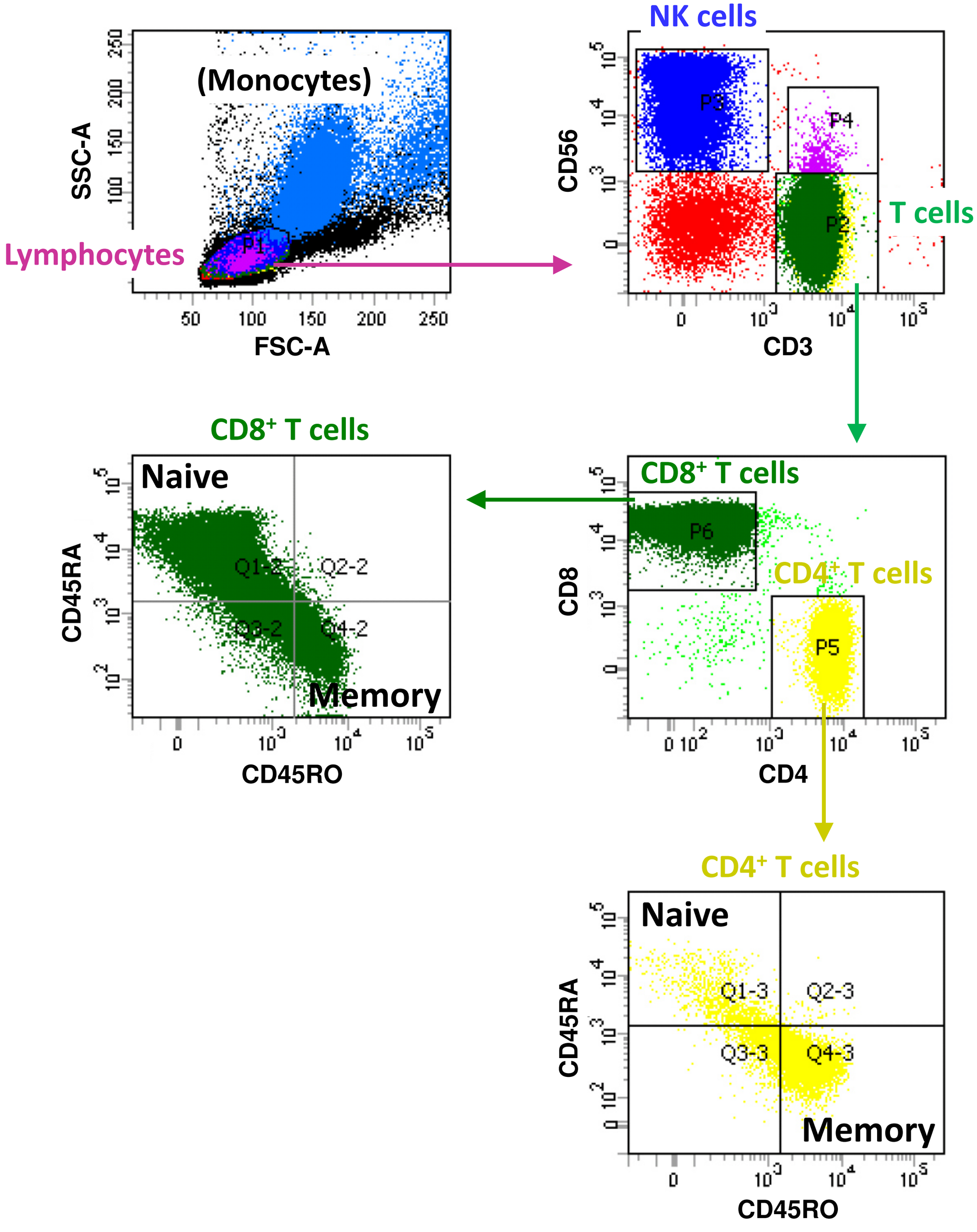
Supplemental Figure 7. Spot-forming cell distribution measured after end of a first CMV reactivation in patients with and without recurrent CMV reactivation, according to the GvHD status (post-hoc analysis). IFN-γ ELISpot results shown in Figure 2B were analyzed according to whether or not patients experienced GvHD prior to and/or at current visit of T-Track® CMV. Differences in SFC distribution were evaluated using a Kruskal-Wallis (K-W) test with Dunn's multiple comparison test. Respective p-values are shown under each graph (ns $p>0.05$; *$p<0.05$; ***$p<0.001$). Scatter plots are depicted as squared SRM values (SRM^2). Median and interquartile range (IQR) of SRM^2 SFC are shown above each graph. Due to 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 depict negative and positive tests respectively, defined according to the rules described in the Methods section. Significant differences in SFC distribution between patients without and with recurrent CMV was apparent only in patients with no prior and/or current GvHD, in line with reports from the literature.[13] This observation highlights the clinical benefit of monitoring CMV-CMI in patients with no prior GvHD, who are usually less closely monitored under standard care.

Supplemental Figure 8. Performance of absolute count of lymphocyte subpopulations measured after end of a first CMV reactivation to predict recurrent CMV reactivation. (A) Lymphocyte cell subpopulations were quantified by flow cytometry after cell-surface staining of remaining PBMC, and normalized to absolute lymphocyte counts, as described in the Methods section. Absolute cell counts measured after the first CMV reactivation (first available measurement of day 0, day 7 and day 14 post-end of antiviral therapy) were plotted according to the existence of subsequent recurrent CMV reactivation (No vs. Yes), in all and D-/R+ patients. Differences in absolute cell count between patients with only one CMV reactivation (No) and those with recurrent CMV reactivation (Yes) were evaluated using a Mann-Whitney-U (MWU) test. Respective p-values are shown above each graph. **(B)** Prediction of future recurrent CMV reactivation based on absolute lymphocyte subpopulations' count measured after end of antiviral therapy for a first CMV reactivation was evaluated per ROC analysis in the total population (ALL) and in D-/R+ patients. Area under the curve (AUC), 95% confidence intervals (CI) and respective p-values are indicated. Statistically significant p-values are in bold. **(C)** The difference in predictive value of absolute lymphocyte subsets and pp65-specific IFN-γ ELISpot SFC was evaluated using ROC analyses of

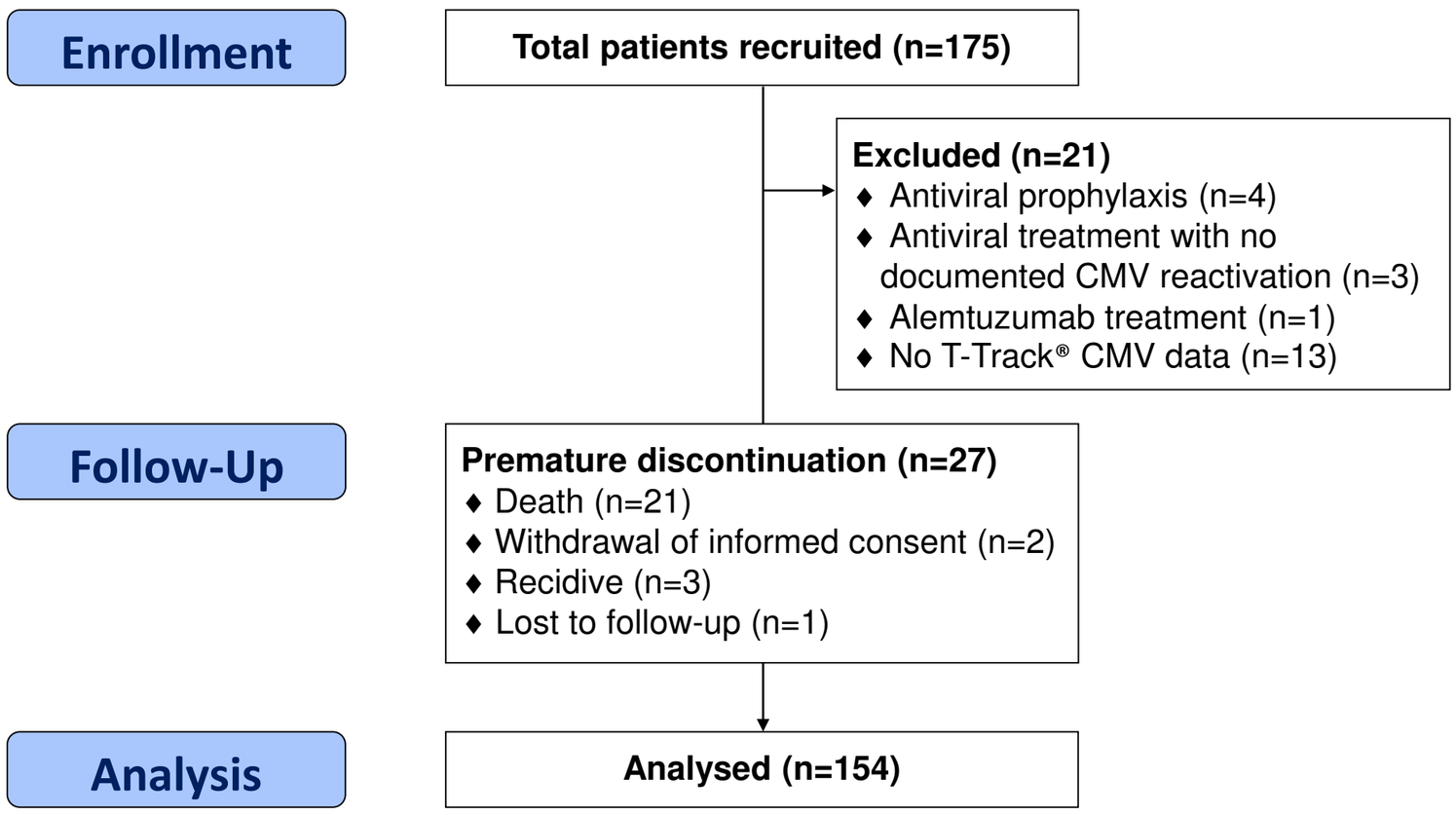
paired measurements (i.e. from the same visit). The difference in the respective AUC estimates (ΔAUC = [AUC absolute cell count] – [AUC pp65-ELISpot]) was calculated and tested for statistical significance. Two analyses were performed, the first one using the mean of square-root-transformed pp65-SFC normalized to 200,000 lymphocytes (SRM[pp65-SFC]/200,000 lymphocytes; left part of the table), the second one using the mean of square-root-transformed pp65-SFC normalized to absolute lymphocytes (SRM[pp65-SFC]/µl blood; right part of the table). Statistically significant p-values are in bold.

Supplemental Figure 9. Performance of CMV-CMI measured around day 100 post-HSCT to predict freedom from and/or occurrence of late (after day 100) CMV reactivation, regardless of existing early (before day 100) CMV reactivation. (A) IFN-γ ELISpot performed between day 80 and day 100 post-HSCT, regardless of whether patients experienced an earlier CMV reactivation, were used for the analysis. In case of several available measurements, the one closer to day 100 was considered. **(B)** Quantitative ELISpot results in response to CMV proteins IE-1 and pp65 were evaluated as detailed in legend to Figure 2. Due to 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. Differences in SRM SFC distribution between patients with and without a late (after day 100) CMV reactivation were evaluated using a Mann-Whitney-U (MWU) test. Orange-labelled dots represent SFC counts of the 17 patients (6 D-/R+) with no CMV reactivation prior to the day 100 measurement. Note that none of these patients experienced a late CMV reactivation either (all in the "No late CMV reactivation" group), meaning that these patients did not develop treatment-requiring CMV reactivation during the entire observational period. **(C)** Prediction of late CMV reactivation based on IE-1- and pp65-specific SFC counts measured around day 100 was evaluated by ROC analysis, as detailed in legend to Figure 2. **(D)** Probability of late CMV reactivation based on IE-1- and pp65-specific qualitative test results around day 100, was evaluated as detailed in legend to Figure 2.

Supplemental Figure 1

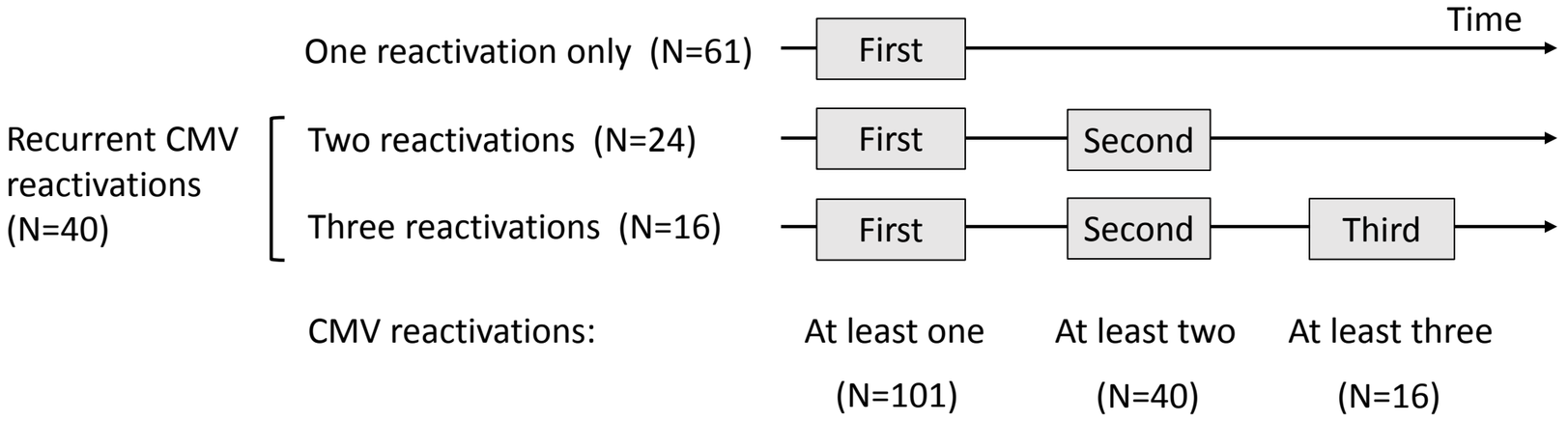


Supplemental Figure 2



Supplemental Figure 3

A



B

Time to CMV reactivation (in days) per CMV serostatus of HSCT recipient

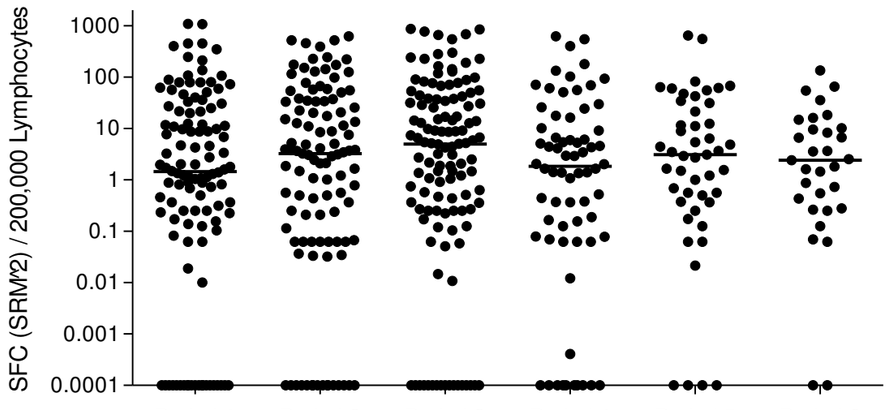
Number of CMV reactivations	all patients		D-/R+		D+/R+		D+/R-	
	N[a]	Median days (range)	N[a]	Median days (range)	N[a]	Median days (range)	N[a]	Median days (range)
At least one CMV reactivation	101	39 (3-112)	65	37 (19-58)	33	42 (3-112)	3	57 (41-58)
At least two CMV reactivations	40	108 (63-188)	37	109 (63-188)	3	97 (88-146)	0	-
At least three CMV reactivations	16	174 (119-219)	16	174 (119-219)	0	-	0	-

[a]Number of patients

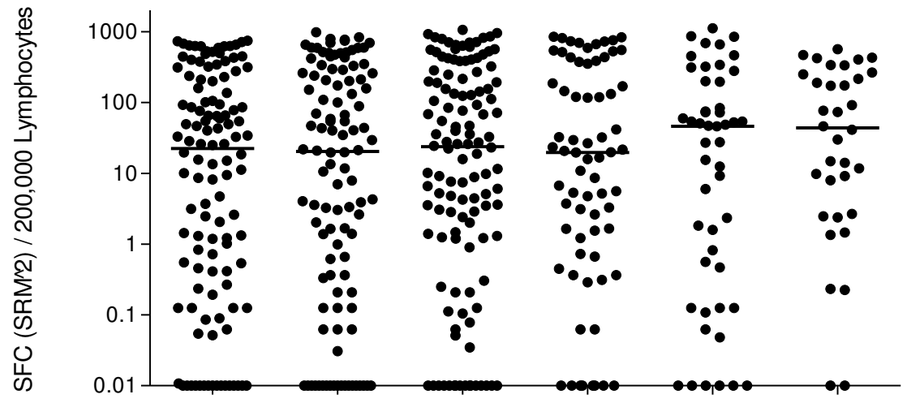
Supplemental Figure 4

A

all patients

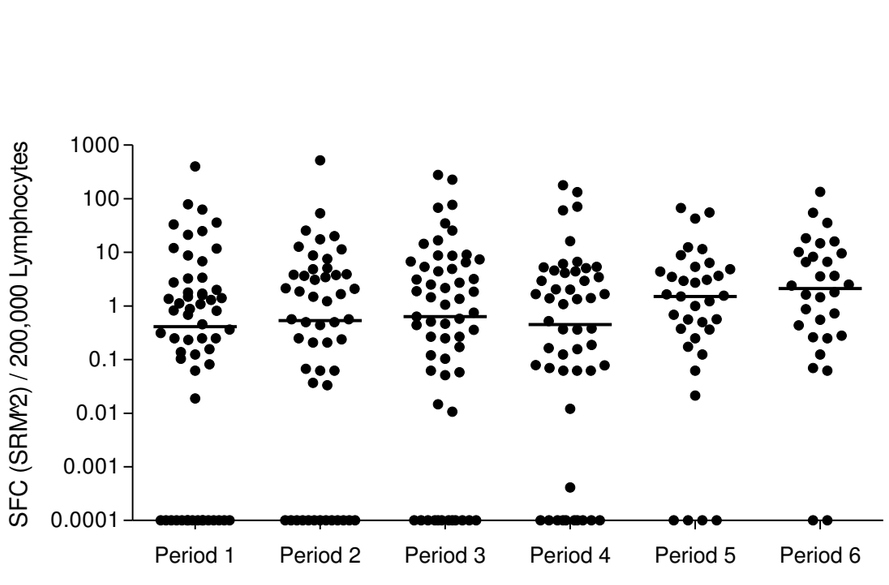


N	112	105	120	68	47	31
Median SFC (interquartile range)	1.4 (0.1-24)	3.2 (0.1-34)	5.0 (0.3-38)	1.8 (0.1-15)	3.1 (0.4-31)	2.4 (0.3-10)
Median day post-HSCT (range)	46 (33-52)	60 (44-67)	77 (61-86)	90 (81-105)	118 (101-126)	143 (124-211)

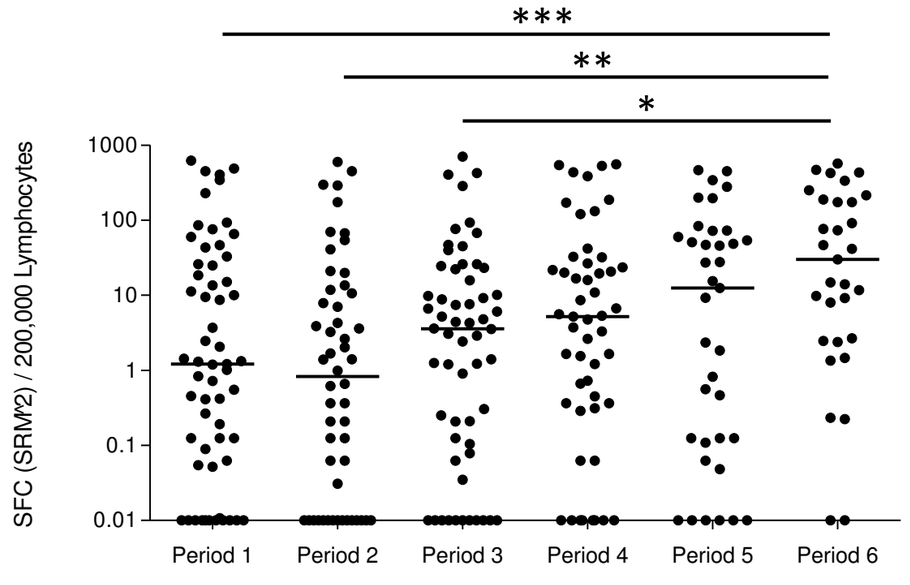


116	108	120	69	49	34
22.4 (0.3-207)	20.4 (0.2-261)	23.8 (1.2-240)	19.6 (1.0-365)	46.0 (0.3-198)	43.9 (2.6-255)
46 (33-52)	60 (44-67)	77 (61-86)	90 (81-105)	118 (101-126)	143 (124-211)

D-/R+ patients



N	58	50	57	50	35	30
Median SFC (interquartile range)	0.4 (0.0-2.2)	0.5 (0.0-3.9)	0.6 (0.1-5.9)	0.5 (0.1-4.2)	1.5 (0.3-4.8)	2.1 (0.3-9.7)
Median day post-HSCT (range)	47 (36-52)	60 (48-66)	77 (61-86)	90 (81-105)	119 (102-126)	145 (124-211)



59	52	58	51	37	31
1.2 (0.1-26)	0.8 (0.1-12)	3.6 (0.1-23)	5.2 (0.4-27)	12.5 (0.1-66)	30.1 (2.5-189)
47 (36-52)	60 (48-66)	77 (61-86)	90 (81-105)	119 (102-126)	145 (124-211)

B

Proportion of positive test results over time after HSCT, % (N)

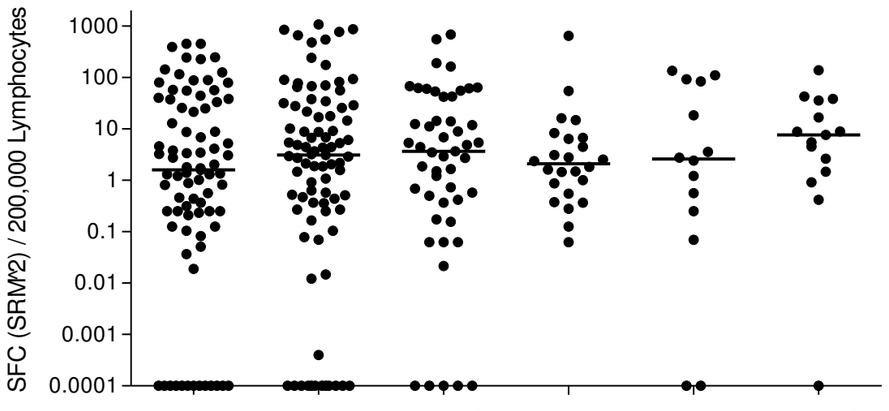
Population	Marker	Period 1	Period 2	Period 3	Period 4	Period 5	Period 6
all patients	IE-1	36.6% (41/112)	40.0% (42/105)	45.8% (55/120)	29.4% (20/68)	40.4% (19/47)	35.5% (11/31)
	pp65	57.8% (67/116)	57.4% (62/108)	60.8% (73/120)	59.4% (41/69)	63.3% (31/49)	67.6% (23/34)
	IE-1, pp65[a]	62.1% (72/116)	59.8% (64/107)	68.3% (82/120)	60.3% (41/68)	73.5% (36/49)	79.4% (27/34)
D-/R+ patients	IE-1	20.7% (12/58)	20.0% (10/50)	24.6% (14/57)	12.0% (6/50)	22.9% (8/35)	33.3% (10/30)
	pp65	39.0% (23/59)	32.7% (17/52)	39.7% (23/58)	47.1% (24/51)	54.1% (20/37)	64.5% (20/37)
	IE-1, pp65[a]	44.1% (26/59)	35.3% (18/51)	50.9% (29/57)	47.1% (24/51)	63.9% (23/36)	77.4% (24/31)

[a]T-Track® CMV assay: test is positive when at least one of the IE-1- and/or pp65-specific response is positive; test is negative when both IE-1- and pp65-specific responses are negative. Abbreviations: D/R, donor/recipient cytomegalovirus serostatus.

C

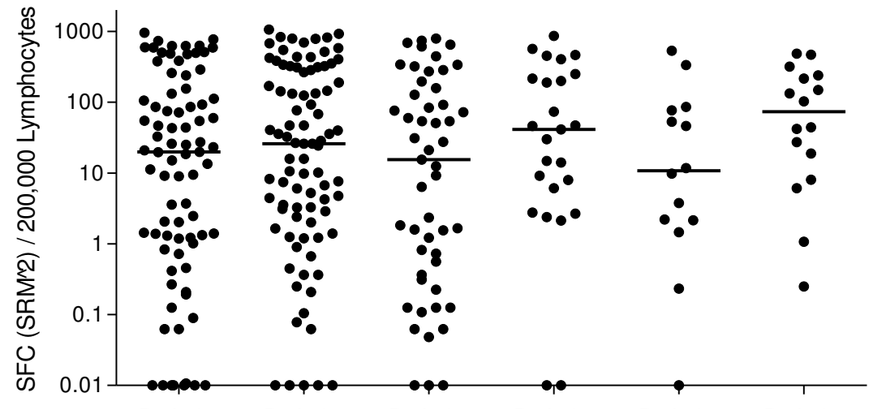
all patients

IE-1



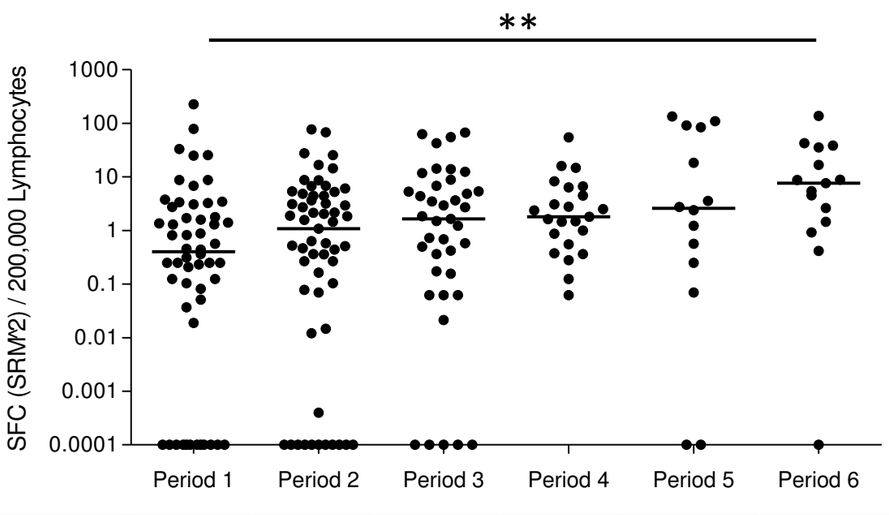
N	81	85	49	24	14	15
Median SFC (interquartile range)	1.6 (0.2-35)	3.1 (0.3-28)	3.7 (0.4-42)	2.1 (0.6-6.6)	2.6 (0.2-86)	7.6 (1.5-36)
Median (range) day relative to start of first CMV reactivation	11 (0-30)	39 (31-56)	69 (61-87)	99 (91-118)	131 (121-145)	174 (153-186)

pp65

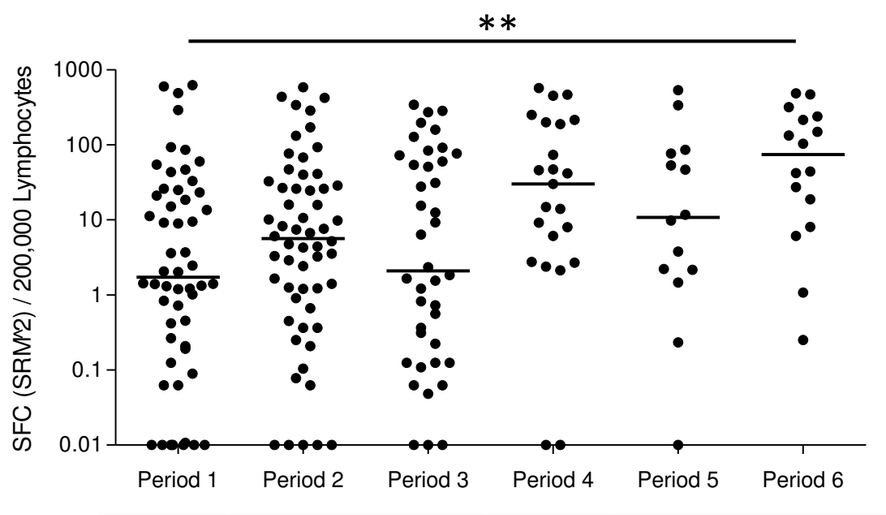


181	87	51	25	14	16
19.8 (1.1-144)	26.0 (2.4-311)	15.5 (0.4-158)	41.5 (4.4-233)	10.8 (2.0-79)	73.7 (11-233)
11 (0-29)	39 (31-57)	69 (61-87)	98 (91-118)	137.5 (121-145)	173.5 (151-186)

D-/R+ patients



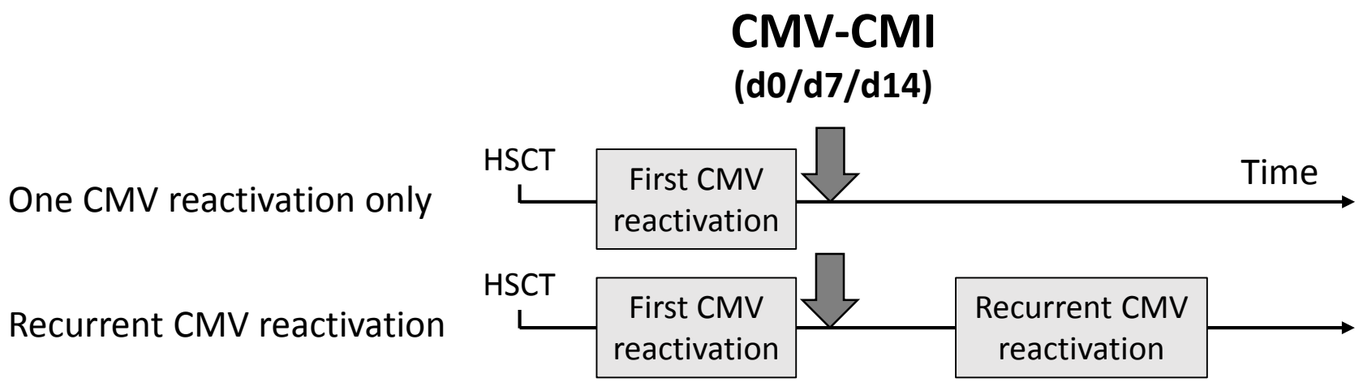
N	54	57	39	23	14	15
Median SFC (interquartile range)	0.4 (0.0-2.8)	1.1 (0.0-4.6)	1.6 (0.2-6.9)	1.8 (0.5-6.4)	2.6 (0.2-86)	7.6 (1.5-36)
Median (range) day relative to start of first CMV reactivation	13 (0-30)	41 (31-56)	69 (61-87)	98 (91-118)	131 (121-145)	174 (153-186)



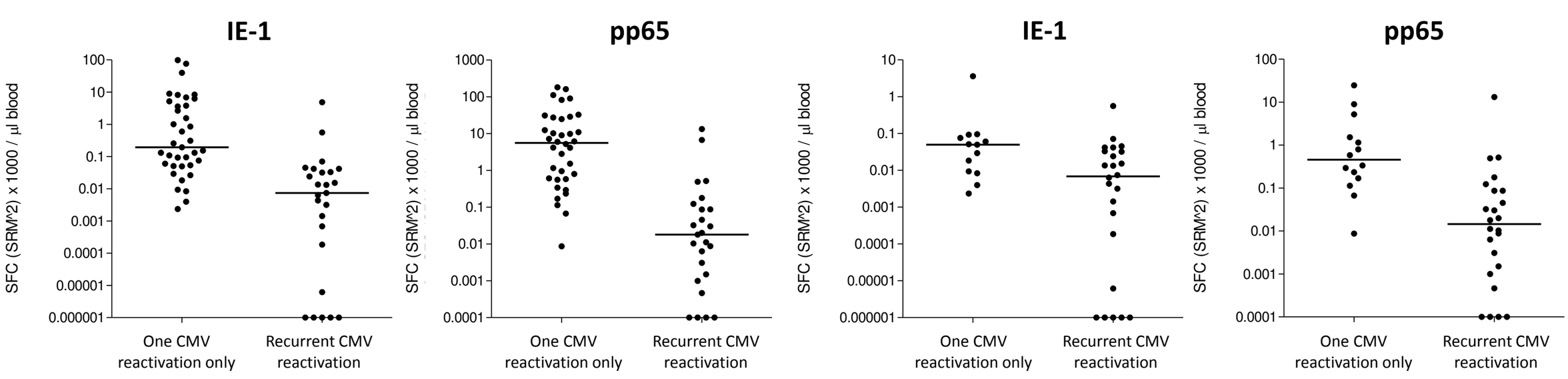
54	58	40	23	14	16
1.7 (0.2-24)	5.6 (0.8-30)	2.1 (0.1-69)	30.1 (2.8-199)	10.8 (2.0-79)	73.7 (11-233)
13 (0-29)	41 (31-57)	69 (61-87)	98 (91-118)	137.5 (121-145)	173.5 (151-186)

Supplemental Figure 5

A



B



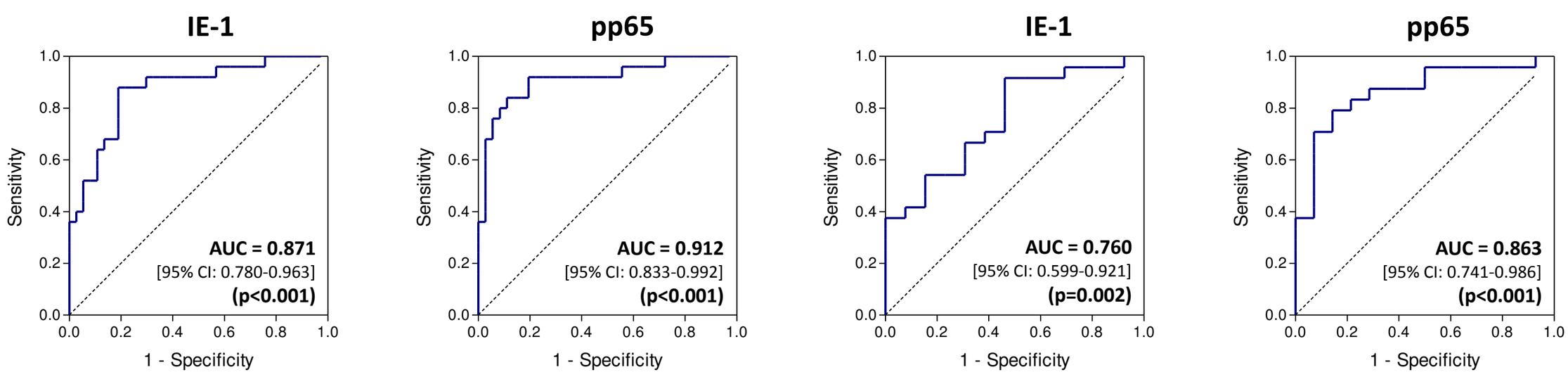
N	37	25
MWU	**p<0.001**	

N	36	25
MWU	**p<0.001**	

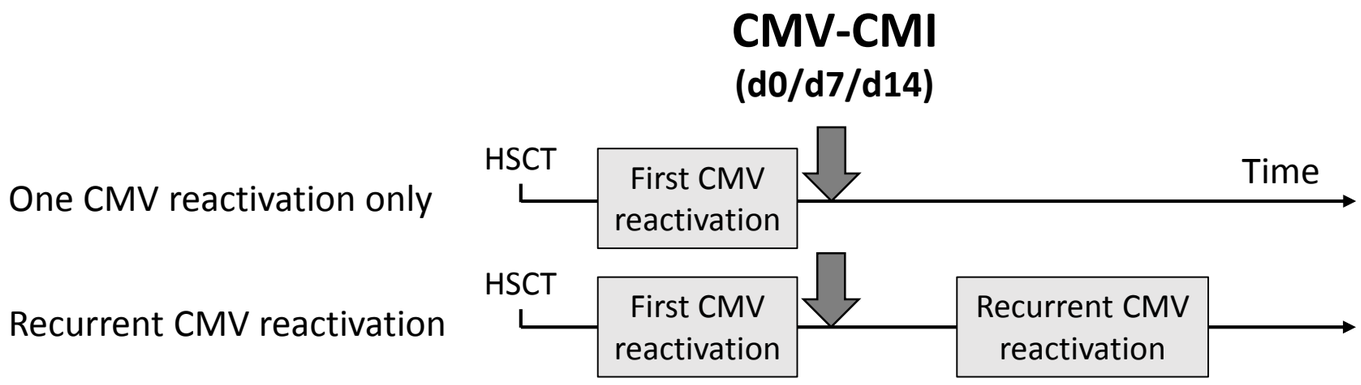
N	13	24
MWU	**p=0.010**	

N	14	24
MWU	**p<0.001**	

C

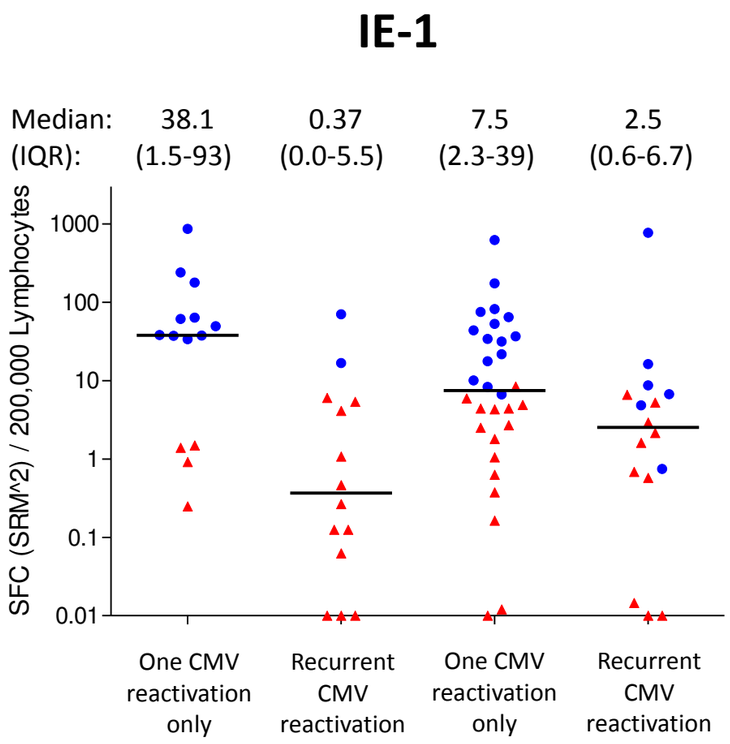


A

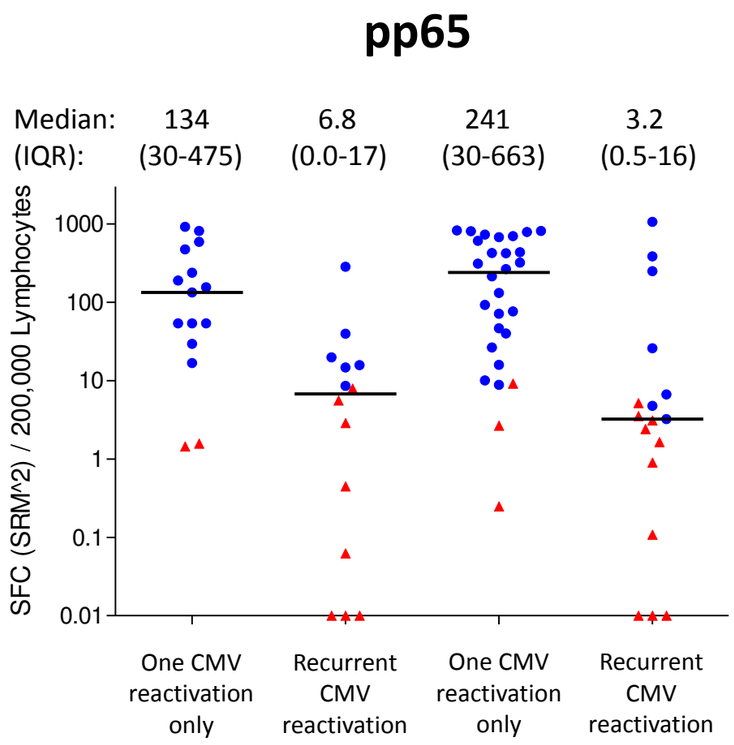


B

all patients

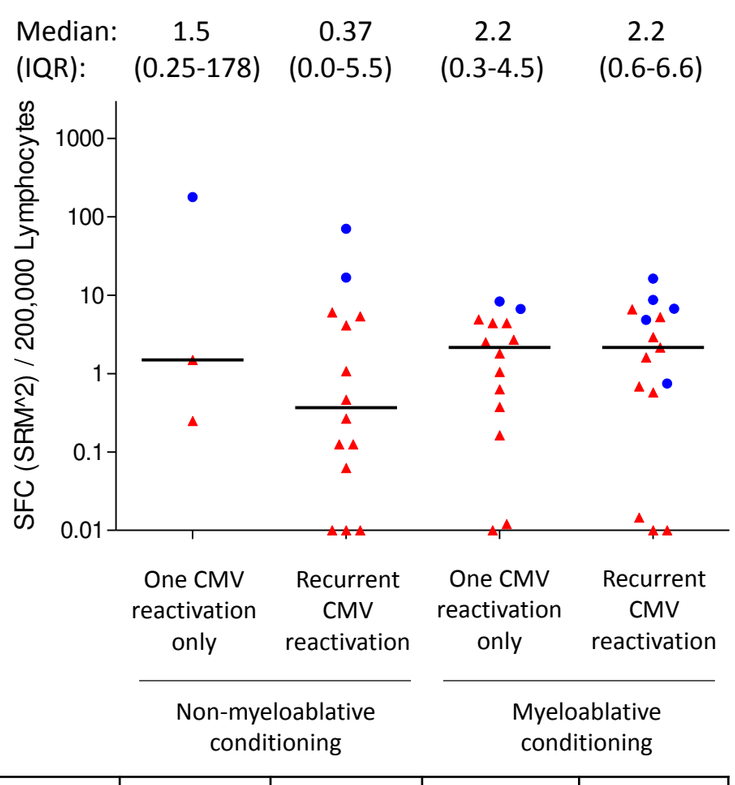


N	14	14	30	16
K-W (**p=0.002**)	**		ns	

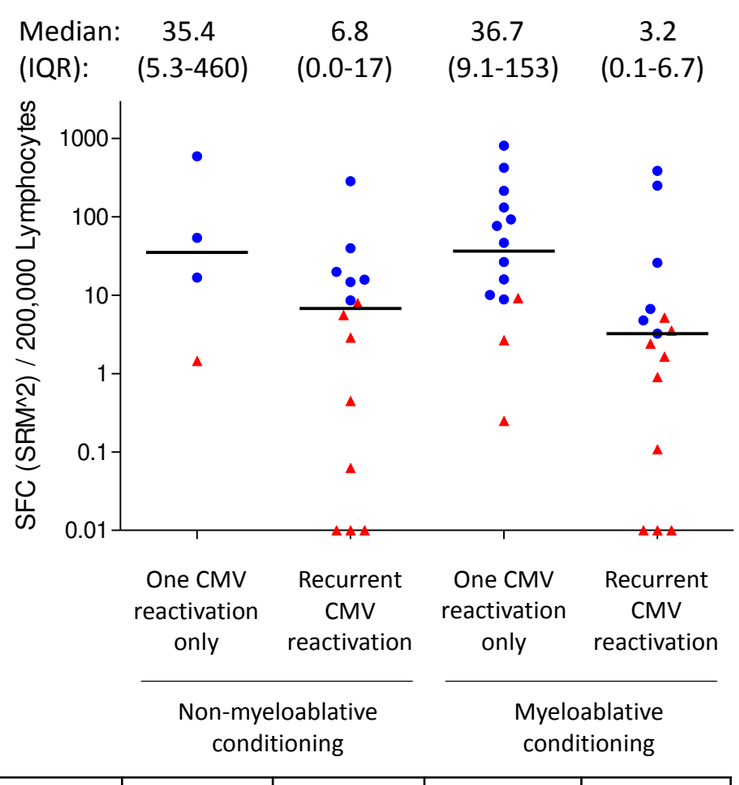


N	15	14	28	17
K-W (**p<0.001**)	*		***	

D-/R+ patients

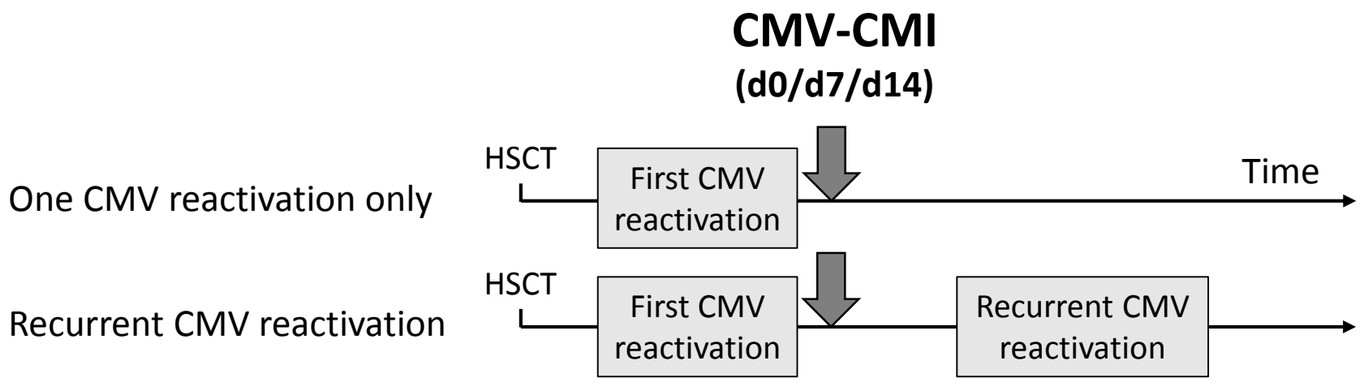


N	3	14	14	15
K-W (p=0.719)	ns		ns	



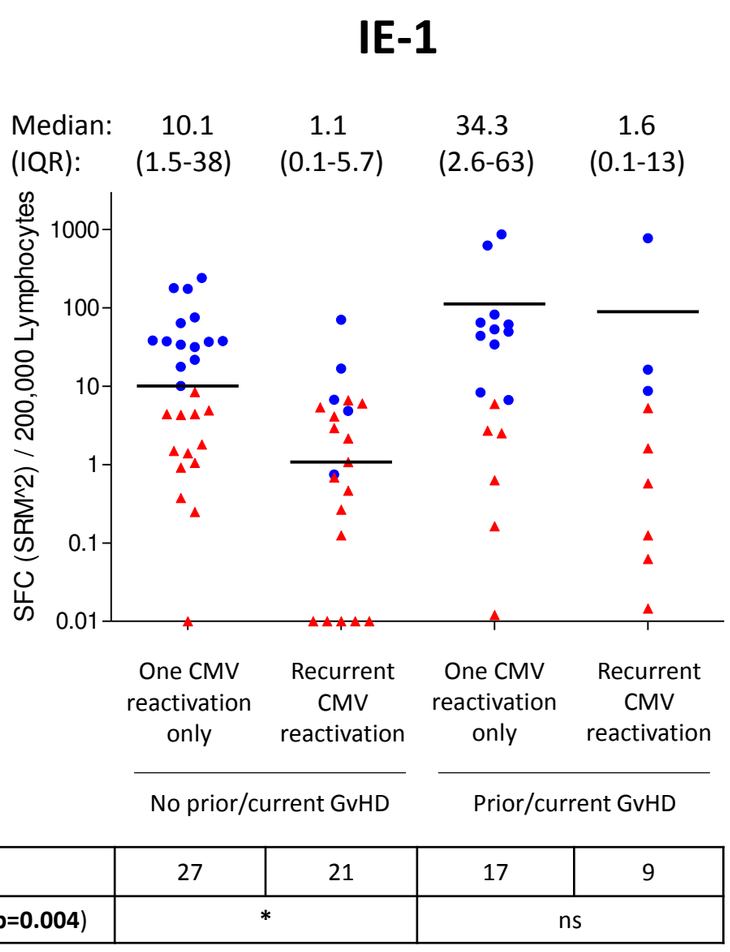
N	4	14	14	15
K-W (**p=0.016**)	ns		*	

A

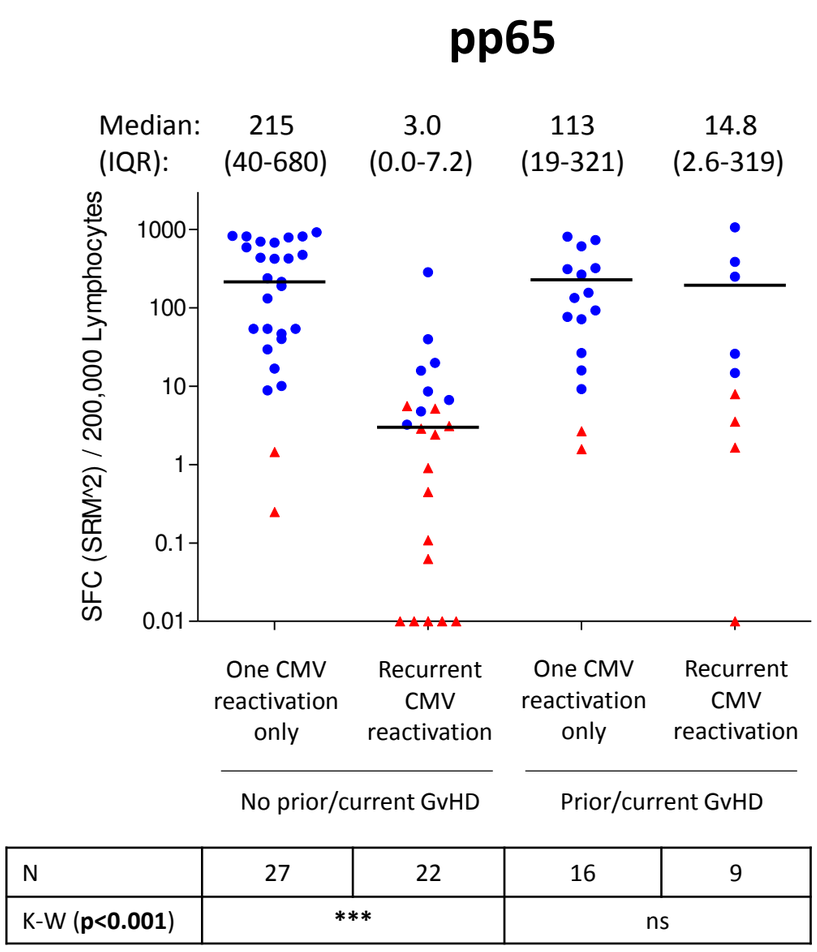


B

all patients

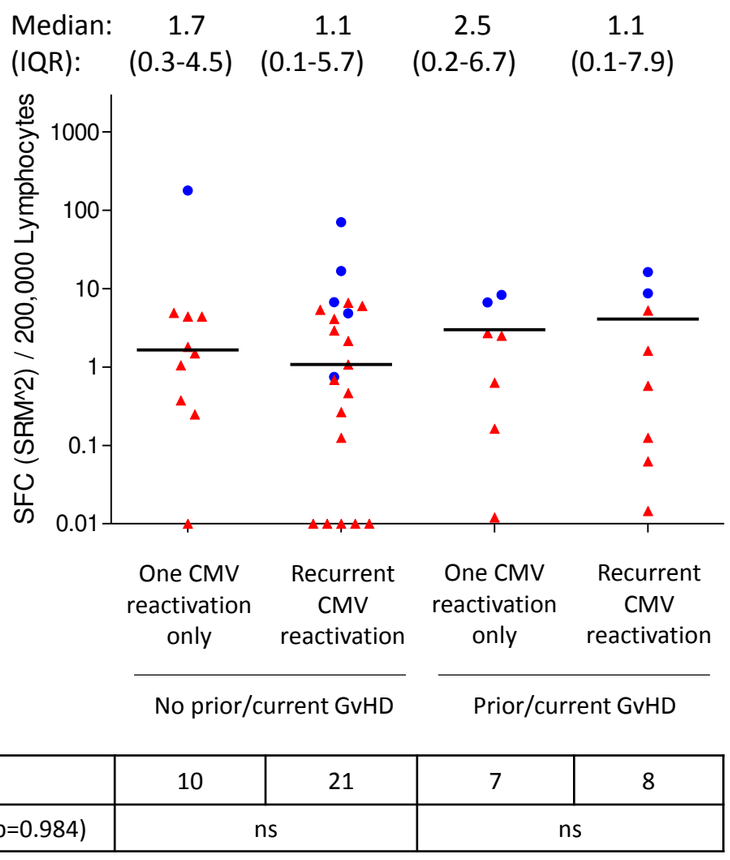


N	27	21	17	9
K-W (**p=0.004**)	*		ns	

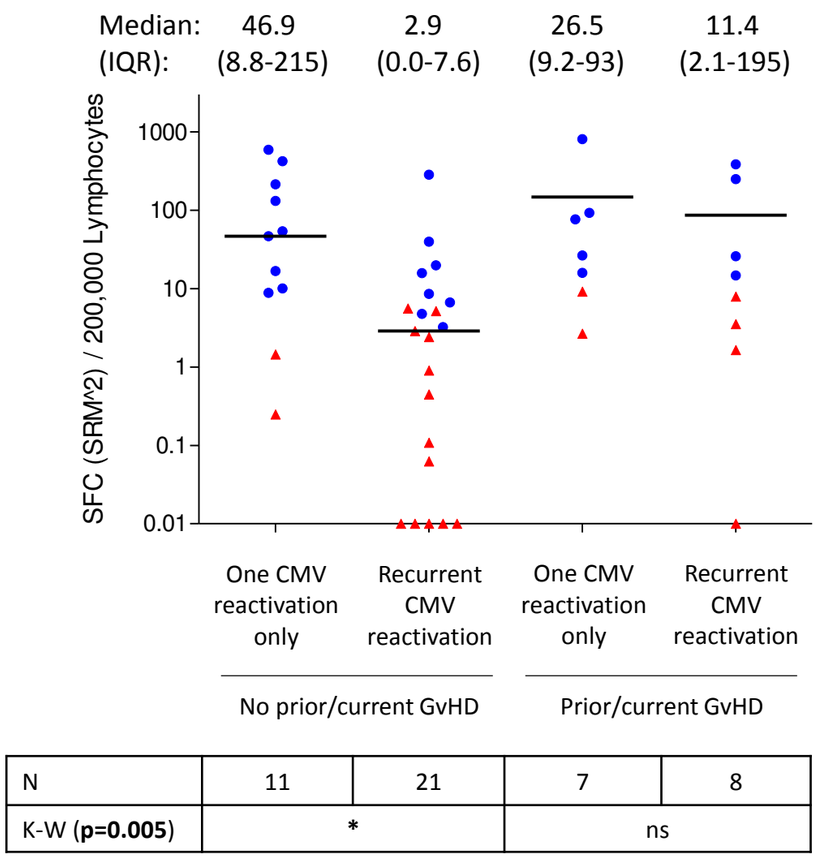


N	27	22	16	9
K-W (**p<0.001**)	***		ns	

D-/R+ patients



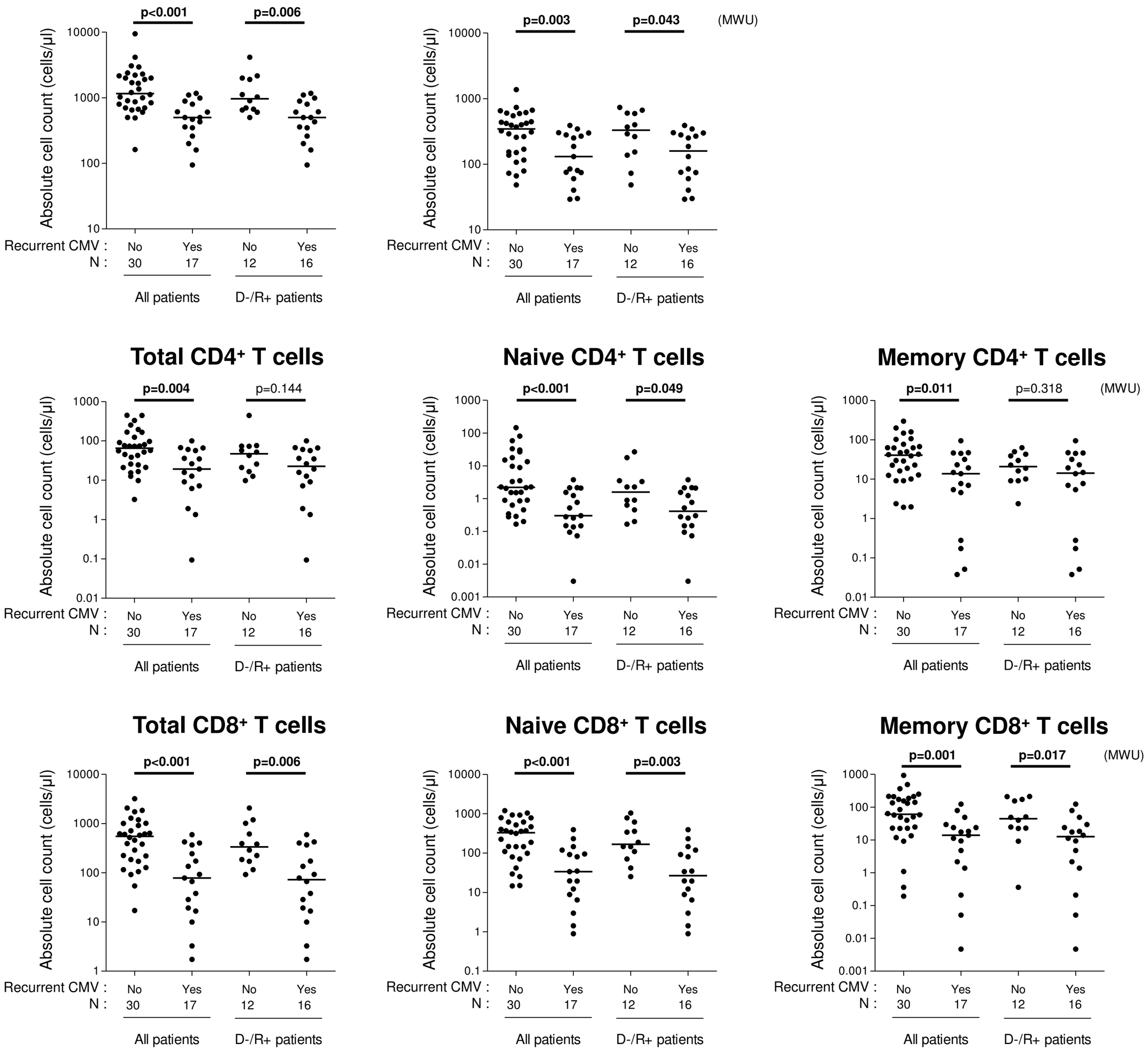
N	10	21	7	8
K-W (p=0.984)	ns		ns	



N	11	21	7	8
K-W (**p=0.005**)	*		ns	

Supplemental Figure 8

A



B

AUC estimates (ROC analysis) for the prediction of future recurrent CMV reactivation based on absolute lymphocyte subpopulations' count after end of antiviral therapy for a first CMV reactivation, measured on isolated PBMC using flow cytometry (p<0.05 are in bold)

Absolute cell count (cells/µl blood)	all patients (n=47)			D-/R+ patients (n=28)		
	AUC	95% CI	p-value	AUC	95% CI	p-value
Total lymphocytes	0.844	0.732-0.957	**<0.001**	0.813	0.655-0.970	**<0.001**
Total CD4+ T cells	0.759	0.618-0.900	**<0.001**	0.667	0.461-0.872	0.112
Naive CD4+ T cells	0.794	0.664-0.924	**<0.001**	0.724	0.529-0.919	**0.025**
Memory CD4+ T cells	0.727	0.577-0.878	**0.003**	0.615	0.401-0.829	0.294
Total CD8+ T cells	0.831	0.715-0.947	**<0.001**	0.813	0.652-0.973	**<0.001**
Naive CD8+ T cells	0.835	0.720-0.951	**<0.001**	0.833	0.684-0.983	**<0.001**
Memory CD8+ T cells	0.788	0.659-0.917	**<0.001**	0.771	0.588-0.954	**0.004**
NK cells	0.761	0.625-0.897	**<0.001**	0.729	0.529-0.930	**0.025**

Abbreviations: AUC, area under the curve; CI, confidence interval; CMV, cytomegalovirus; D/R, donor/recipient CMV serostatus; NK, natural killer; PBMC, peripheral blood mononuclear cells; ROC, receiver operating characteristic.

C

Difference in AUC estimates (ΔAUC) for absolute lymphocyte counts and pp65-specific ELISpot results (paired measurements post end of antiviral therapy for a first CMV reactivation; p<0.05 are in bold)

Absolute cell count (cells/µl blood)	Analysis 1: SRM(pp65-SFC)/200,000 lymphocytes				Analysis 2: SRM(pp65-SFC)/µl blood			
	all patients (n=40)		D-/R+ patients (n=23)		all patients (n=40)		D-/R+ patients (n=23)	
	ΔAUC[a]	p-value	ΔAUC[a]	p-value	ΔAUC[a]	p-value	ΔAUC[a]	p-value
Total lymphocytes	0.080	0.383	0.269	0.095	0.017	0.800	0.085	0.473
Total CD4+ T cells	0.006	0.955	0.123	0.428	-0.058	0.437	-0.062	0.586
Naive CD4+ T cells	0.060	0.504	0.208	0.159	-0.003	0.970	0.023	0.831
Memory CD4+ T cells	-0.039	0.717	0.039	0.840	-0.102	0.229	-0.146	0.302
Total CD8+ T cells	0.063	0.443	0.254	0.122	0.000	1.000	0.069	0.561
Naive CD8+ T cells	0.071	0.388	0.285	**0.050**	0.008	0.885	0.100	0.325
Memory CD8+ T cells	0.006	0.954	0.162	0.377	-0.058	0.424	-0.023	0.863
NK cells	-0.011	0.916	0.154	0.424	-0.074	0.357	-0.031	0.839

[a]A negative ΔAUC indicates that pp65-specific ELISpot is superior to the respective absolute lymphocyte count. Abbreviations: AUC, area under the curve; CMV, cytomegalovirus; D/R, donor/recipient CMV serostatus; NK, natural killer; SRM, mean of quadruplicate square-root-transformed pp65-specific SFC counts

Supplemental Figure 9