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Human cytomegalovirus control in allogeneic stem cell transplant recipients in the letermovir era – emerging humoral and cellular players

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Author Contributions

The study was conceived by C.D.L., H.E., S.W. and S.K. Patient enrollment and clinical documentation were performed by S.K. Experiments were planned and performed by C.D.L., H.G., K.K., B.W. and C.K. Data were analyzed by C.D.L., H.G., N.I., M.H., H.H, S.W. and SK. Data were visualized by C.D.L. Project administration and supervision were led by C.D.L, L.D., H.E., S.W. and S.K. Funding was acquired by L.D., H.E., and S.K. The original draft was written by C.D.L., S.W. and S.K. All co-authors reviewed, edited, and approved the manuscript.

Conflict of Interest

The authors declare no conflicts of interest.

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Abstract (249 words)

Allogeneic hematopoietic stem cell transplant (alloSCT) recipients frequently experience late-onset human cytomegalovirus (HCMV) reactivations following termination of letermovir prophylaxis. Letermovir prophylaxis extends the window for protective B- and T-cell reconstitution; however, our understanding of humoral responses and their contribution to HCMV immune control remains limited. Combining serological and flow cytometric analyses in 42 HCMV-seropositive alloSCT recipients, we herein provide the first comprehensive longitudinal (days 90-270 post-transplant) characterization of HCMV-specific humoral responses, natural killer (NK)-cell phenotypes, and γδ T cells in the letermovir era. HCMV controllers showed predominantly HCMV-specific IgG-driven responses, higher pre-reactivation $V\delta1^+$ γδ T-cell frequencies, and stronger expansion of "memory-like" NK cells than patients with clinically significant CMV infection (csCMVi). In contrast, csCMVi patients showed delayed HCMV-specific IgG production, IgM-skewed responses, and stronger post-reactivation expansion of memory B cells and $V\delta1^+$ γδ T cells. Early (day 90) γδ T-cell reconstitution was associated with subsequent HCMV control. HCMV-specific IgG levels correlated only weakly with γδ T cells but showed distinct associations with "memory-like" NK-cell reconstitution in HCMV controllers, suggesting synergisms between humoral and cellular immunity. Collectively, these findings highlight a need to study anti-HCMV immune protection beyond type-1 T cells and refine risk stratification models in alloSCT patients by inclusion of novel immune markers such as γδ T-cell frequencies and phenotypes. Leveraging the extended B-cell reconstitution window created by letermovir, novel immunotherapies (e.g., therapeutic antibodies) and future vaccines might boost humoral anti-HCMV immunity and benefit from synergisms with γδ T cells and "memory-like" NK cells in improving HCMV control.

Introduction (290 words)

Reactivation of human cytomegalovirus (HCMV) remains a major complication following allogeneic hematopoietic stem cell transplantation (alloSCT)[1-3]. With late-onset HCMV reactivations after letermovir cessation leading to clinically significant CMV infections (csCMVi) in 25 to 57% of cases[4-7], further investigation into the immune mechanisms controlling HCMV is critically needed.

While T cells are well-established as critical players in HCMV immunity[8-10], the role of humoral immunity has been more scarcely studied. Historically, this was due to the slow reconstitution of B cells and the predominance of early-onset HCMV reactivations before day 100 post-transplant[11]. However, in the letermovir era, late-onset HCMV reactivations often occur when B-cell reconstitution is more advanced[12], and antibody responses may play a crucial role in HCMV control through viral neutralization, CD16-mediated recognition of virions, antibody-dependent cellular cytotoxicity (ADCC), and cytokine secretion[13-15].

Furthermore, other immune cell populations that are poorly explored in the letermovir era, such as "memory-like" NK cells and γδ T cells, have known anti-HCMV properties and interact with HCMV-specific antibodies via the IgG-Fc receptor CD16[13-15], suggesting a potential interplay between humoral and cellular immunity that warrants further investigation.

To address these knowledge gaps, we conducted a longitudinal analysis of peripheral blood mononuclear cell (PBMC) and serum samples from 42 HCMV-seropositive alloSCT patients at four post-transplant timepoints: day 90 (prior to letermovir cessation), day 150 (around the median time of first HCMV reactivation), and days 210 and 270 (following HCMV reactivation). We compared patients who controlled HCMV reactivation with those who developed csCMVi to investigate the roles of humoral anti-HCMV responses, "memory-like" NK cells, and γδ T cells in HCMV defense (**Figure 1A**). Thereby, we provide novel insights into HCMV immune control in the letermovir era beyond antigen-specific T cells, informing improved strategies for risk stratification, vaccination, and immunotherapy.

Methods

Ethics approval

This study was approved by the Ethics Committees of the University of Wuerzburg, Germany (protocol code 17/19-sc). Written informed consent was obtained from all patients.

Study population

Forty-two HCMV-seropositive alloSCT recipients (Recipient[R]+Donor[D]+, R+D-) were enrolled at the University Hospital of Wuerzburg (Germany) between December 2019 and October 2023. All patients received letermovir prophylaxis at 480 mg (240 mg when co-administered with cyclosporine A) once daily from day 1 to day 100 post-transplant. HCMV DNAemia was monitored weekly until day 100 post-transplant and biweekly thereafter using a clinically validated real-time PCR from plasma samples, which is described in more detail in the **Supplementary Materials.** Preemptive systemic antiviral therapy with (val)ganciclovir was initiated whenever HCMV DNA levels exceeded 1,000 copies/mL. If this occurred before day 100, letermovir prophylaxis was discontinued. Additionally, cryopreserved samples from 15 patients from the pre-letermovir era without letermovir prophylaxis (2015-2019) were used for comparative analyses.

Definitions

During a 365-day follow-up, subjects were classified as HCMV controllers (DNA <1 000 copies/mL without antiviral therapy) or csCMVi patients (DNA >1 000 copies/mL requiring therapy). Refractory HCMV infection (Rf-csCMVi) was defined by Chemaly et al[16].

Cell populations were compared pre-csCMVi (last available timepoint before therapy initiation) and post-csCMVi (first available timepoint after HCMV DNA levels returned below 300 copies/mL, corresponding to the lower limit of quantification of the PCR assay). In controllers, pre-reactivation measurements were taken before DNA first rose into 1–1 000 copies/mL, and post-reactivation once levels returned to zero, reflecting a more stringent threshold used for this group due to their typically low-level, self-limiting DNAemia without antiviral therapy.

Immunoassays

EDTA blood and serum samples were collected on days 84-106 (day 90), 141-167 (day 150), 200-233 (day 210), and 262-309 (day 270) post-transplant. Serum HCMV-specific IgM and IgG were measured using Architect CMV kits (Abbott Ireland). PBMCs were isolated and cryopreserved for flow cytometry of γδ T-, B-, and NK-cell phenotypes and quantification of HCMV-specific B cells via biotinylated gB, trimer and pentamer protein probes. Detailed protocols and gating strategies (**Figures S1-S4**) are provided in **Supplementary Materials**. Frequencies of immune populations were calculated using a combination of flow cytometry data and absolute lymphocyte counts (ALCs).

Statistical analysis and software applications

Statistical significance was assessed using the Mann-Whitney U test, paired Wilcoxon test, Kruskal-Wallis test, or Fisher's exact test as detailed in the figure legends. Data were compiled, visualized, and analyzed using Microsoft Excel (Microsoft, Redmond, WA, USA), Prism v.10.2 (GraphPad Software, La Jolla, California, USA), and R v.4.2.1 (R Core Team). ChatGPT-4o (OpenAI, San Francisco, California, USA) was used to assist with language editing, improving readability, and condensing text for clarity. The AI did not contribute to the study's conceptualization, analysis, or interpretation. Final responsibility for the text rests with the authors.

Results

Patient characteristics

Forty-two HCMV-seropositive alloSCT patients receiving letermovir prophylaxis until day 100 post-transplant were included in the study. Of these, 25 (60%) controlled HCMV until day 365, while 17 (40%) developed csCMVi. Patient characteristics were similar between controllers and csCMVi patients, except for a higher incidence of grade ≥2 acute graft-versus-host disease in csCMVi patients (p=0.018, **Table 1**). To preclude a significant impact of pharmacologic immunosuppression on our immunologic analyses, we specifically analyzed the number and dosage of immunosuppressants administered during and after letermovir prophylaxis. Slightly more csCMVi patients than controllers (18% vs. 8%) received systemic high-dose glucocorticosteroids (GCS, 2 mg/kg prednisolone), while use of non-GCS immunosuppressive agents was similar (**Table 2**).

Median first HCMV reactivation occurred on day 146. In five controllers, no quantifiable HCMV DNAemia was detected. Eight csCMVi patients and five controllers experienced minimal and transient HCMV DNAemia during letermovir prophylaxis. Except for one csCMVi case that required preemptive therapy, these episodes likely do not represent true viral reactivation but rather the detection of non-infectious viral DNA, commonly referred to as "blips", a well-documented phenomenon during letermovir prophylaxis[17]. Consequently, these events are not classified and shown as reactivations (**Figure 1B**). Peak median HCMV DNA loads were significantly higher in csCMVi patients than in controllers (6 100 copies/mL vs. 180 copies/mL, p<0.001, **Figure 1C**). Notably, absolute lymphocyte counts (ALC) were similar between controllers and csCMVi patients across all timepoints (**Figure 1D**).

AlloSCT recipients in the letermovir era mount robust humoral responses by the time of HCMV reactivation and develop memory B cells after reactivation events

Before the approval of letermovir, there was no routine prophylaxis against HCMV. HCMV reactivations mostly occurred early (median day 26 in our pre-letermovir cohort) before B-cell reconstitution ($CD20^+$), resulting in minimal humoral responses at time of reactivation (**Figure 2A-B**). In contrast, in our contemporary cohort, HCMV reactivation occurred significantly later (median, day 146). By that time, we expectedly observed more readily reconstituted B-cell numbers (median, 97.5) and frequencies (median, 6.8% of total lymphocytes) (**Figure 2A**).

In the contemporary cohort from the letermovir era, HCMV-specific IgG and IgM increased between days 150 and 210 (**Figure 2C**), coinciding with reactivation. HCMV-specific IgM (p=0.370) and IgG (p=0.506) levels were comparable between the two cohorts on day 90, thereby precluding bias from differences in immunoglobulin levels. Notably, patient characteristics of the pre-letermovir cohort were also largely comparable to those of our cohort from the letermovir era (**Table S1**). Comparisons of pre- and post-reactivation measurements in the letermovir cohort further confirmed significant expansion of HCMV-specific IgG (131 vs. 809 AU/mL, p=0.009) and IgM (0.24 vs. 1.84 IgM Index, p<0.001) during reactivation (**Figure 2D**).

Total B-cell numbers and frequencies of csCMVi patients and controllers in the cohort from the letermovir era were largely comparable, except for higher frequencies in controllers at day 210 (**Figure 2E**). In both groups, IgM^+ B cells decreased from day 90 to day 270, whereas IgG^+ B cells increased, consistent with expected patterns of immune reconstitution and memory formation including antibody class switching (**Figure 2F-G**). Furthermore, the ratio of IgG^+ to IgM^+ memory B cells (MBCs, $CD20^+IgD^-$) increased from pre- to post-HCMV events (p=0.006, **Figure 2H**), a trend that was observed in both controllers (p=0.040) and csCMVi patients (p=0.085).

Although global B-cell frequencies were largely comparable, the time-dependent evolution of B-cell phenotypes differed significantly between HCMV controllers and csCMVi patients. Specifically, those with csCMVi displayed significantly higher day-270 frequencies of MBCs (14.0 vs. 5.7% among B cells, p=0.032) and lower frequencies of naïve B cells ($CD20^+IgD^+$, 84.8 vs. 93.0% among B cells, p=0.018) than controllers (**Figure 2I-K**).

In summary, alloSCT patients in the letermovir era mount robust HCMV-specific humoral responses by the time of HCMV reactivation, suggesting a potential novel role of the humoral response in anti-HCMV immunity. Furthermore, the time-dependent shift in B-cell phenotypes gives rise to the hypothesis that occurrence and severity of HCMV reactivations may shape the B-cell repertoire.

HCMV reactivation triggers the expansion of HCMV-specific B cells

To test this hypothesis, we longitudinally analyzed the magnitude and quality of HCMV-specific humoral response in our letermovir cohort in more detail. Therefore, HCMV-specific MBCs were detected using fluorescent protein probes targeting three key HCMV-derived antigens eliciting neutralizing antibody responses, the gH/gL/gO trimer, the gH/gL/UL128/UL130/UL131A pentamer, and glycoprotein B (gB) (**Figure 3A-B**). Quantifying these antigens individually and in combination showed high specificity, with no HCMV-specific MBCs detected in HCMV-seronegative alloSCT recipients (**Figure S5A**).

Notably, csCMVi patients had higher counts and frequencies of gB-, trimer-, and pentamer-specific MBCs than controllers on day 270 (**Figure 3C**). Given the low frequencies of HCMV-specific MBCs early after transplantation and limited cell availability, we combined the probes for all three antigens for subsequent longitudinal quantification. This approach revealed strongly increasing HCMV-specific MBCs in both HCMV controllers and csCMVi patients between days 90 and 210. However, by day 270, csCMVi patients had developed higher HCMV-specific MBC counts (35.1 vs. 16.8, p=0.064) and frequencies (0.33% vs. 0.10%, p=0.009) than controllers (**Figure 3D)**. To determine whether the increase in HCMV-specific MBCs coincided with HCMV reactivation, we further compared their frequencies before and after reactivation in patients with detectable HCMV viral load, regardless of csCMVi status. Indeed, HCMV-specific MBC frequencies significantly increased from a median of 0.00% pre-reactivation to 0.13% post-reactivation (p=0.001) (**Figure 3E**). Overall, these results suggest that HCMV reactivation drives HCMV-specific MBC development in seropositive alloSCT recipients.

csCMVi patients display an IgM-skewed HCMV-specific response during reactivation and a delayed IgG response compared to controllers

Given the disparate affinity and antiviral effector responses of IgG and IgM[18], we next examined differences in HCMV-specific IgG and IgM serum concentration in controllers and csCMVi patients. On day 150, i.e., around the median time of HCMV reactivation (**Figure 1B**), 100% of the detected HCMV-specific MBCs in controllers showed an IgG phenotype. In contrast, HCMV-specific MBCs in csCMVi patients were predominantly IgM-positive (70% IgM^+, 30% IgG^+) (**Figure 3F**).

These differences were also reflected in longitudinal serum levels of HCMV-specific IgG and IgM. HCMV controllers showed a non-significant trend toward higher HCMV-specific IgG levels on day 150 (median 212 vs. 82 AU/mL, median-to-median ratio (MMR)=2.6) and day 210 (median 1 363 vs. 163 AU/mL, MMR=8.4). Conversely, csCMVi patients had significantly higher HCMV-specific IgM indices on day 210 (median 3.3 vs. 0.6, MMR=5.5, p=0.029) and day 270 (1.1 vs. 0.4, MMR=2.8, p=0.046) (**Figure 3G**).

Moreover, controllers showed a robust increase in HCMV-specific IgG levels between days 90 and 210 (147 vs. 1 363 AU/mL, p<0.001), but only a small increase in HCMV-specific IgM indices (0.2 vs. 0.6, p<0.001). Inversely, csCMVi patients mounted a strongly increasing HCMV-specific IgM response during that timeframe (0.3 vs. 3.3, p=0.015), but showed no significant increase in HCMV-specific IgG levels (130 vs. 164 AU/mL, p=0.421) (**Figure 3H**). Instead, csCMVi patients had a delayed rise in HCMV-specific IgG levels between day 210 and day 270, resulting in comparable IgG levels between the two groups by day 270 (median 1 523 vs. 1 500 AU/mL) (**Figure 3I**).

Overall, time-dependent HCMV-specific B cell responses differed significantly between controllers and csCMVi patients. Controllers displayed modest, early IgG-tilted development, whereas csCMVi patients exhibited a comparatively more IgM-skewed humoral response upon viral reactivation. This suggests a delayed IgM-to-IgG class switch in csCMVi patients.

csCMVi events drive the expansion of $V\delta1^+$ γδ T cells and shift the $V\delta1^+/V\delta2^+$ ratio

In addition to directly neutralizing virions, HCMV-specific IgG can enhance other determinants of immune defense[13-15]. Specifically, immune cells with anti-HCMV properties, including γδ T cells and memory-like NK cells, can interact in part with HCMV-specific IgGs via the IgG receptor CD16[13-15]. To investigate potential associations between humoral and cellular immunity, we also characterized the reconstitution of these immune cell populations.

Firstly, we evaluated the reconstitution of γδ T cells ($TCR\gamma\delta^+$), a cell population that is protective against HCMV reactivation in alloSCT patients (**Figure 4A**)[19-21]. Both controllers and csCMVi patients had similar γδ T-cell kinetics and significantly expanded their total γδ T-cell numbers (**Figure 4B-C**). However, sub-populations differed (**Figure 4D**). Specifically, controllers had higher $V\delta1^+$ cell numbers ($TCR\gamma\delta^+TCRV\delta1^+$, 3.7 vs. 1.8 cells/µL, MMR=2.1) and frequencies (40.7 vs. 19.6% Vδ1+ cells among γδ T cells, p=0.050) than csCMVi patients before letermovir cessation (day 90) (**Figure 4E**). In fact, high day 90 $V\delta1^+$ cell frequencies were associated with subsequent HCMV control, as confirmed by receiver-operating characteristics (ROC) analysis (area under the curve=0.688, p=0.049,

95% confidence interval: 0.4967-0.8793, **Figure 4F**). Inversely, csCMVi patients showed higher $V\delta1^+$ frequencies among γδ T cells than controllers from day 210 onward (day 210: 72.0 vs. 56.0%, p=0.032; day 270: 73.6 vs. 59.9%, p=0.021) (**Figure 4E**). $V\delta2^-$ ($TCR\gamma\delta^+TCRV\delta2^-$) cells followed the trend of $V\delta1^+$ cells (**Figure S5B-E**).

$V\delta2^+$ frequencies ($TCR\gamma\delta^+TCRV\delta2^+$) showed minimal differences between HCMV controllers and csCMVi patients. Likewise, comparable numbers and frequencies of the $V\delta2^+V\delta9^+$ and $V\delta2^+V\delta9^-$ subpopulations were found in both groups (**Figure S5F-Q**).

In csCMVi patients, these trends collectively led to a marked shift in $V\delta1^+/V\delta2^+$ ratios over time, which were initially higher (day 90: $V\delta2^+/V\delta1^+$ 4.0 vs. 1.2, p=0.038) and later lower (day 270: $V\delta2^+/V\delta1^+$ 0.2 vs. 0.4, p=0.015) than in controllers (**Figure 4G**). Both groups expanded $V\delta1^+$ cells post-reactivation/csCMVi, but csCMVi patients showed significantly greater expansion from day 90 to 210 than controllers (median fold changes, 22.3 vs. 4.3, p=0.007, **Figure 4H-J**).

Furthermore, differences in $V\delta1^+$ memory populations were observed between the two cohorts from day 210 onward. Specifically, csCMVi patients had significantly higher numbers of effector memory (day 270: 1.4 vs. 0.5 $CD45RA^-CD27^-$ $V\delta1^+$ cells/μL, p=0.003) and central memory $V\delta1^+$ cells (day 210: 4.6 vs. 1.8 $CD45RA^-CD27^+$ $V\delta1^+$ cells/μL, p=0.023) than controllers (**Figure 4K**). In contrast, naïve and terminally differentiated effector cells remained comparable (**Figure S5R-S**).

In summary, low $V\delta1^+$ frequencies before letermovir cessation were a marker of increased csCMVi risk. csCMVi events were, in turn, associated with significant expansion of $V\delta1^+$ γδ T cells, resulting in an increased $V\delta1^+/V\delta2^+$ ratio compared to controllers, partially due to expansion of effector and central memory phenotypes.

Lack of "memory-like" NK-cell expansion is associated with refractory csCMVi

NK and "memory-like" NK cells are key for HCMV control and IgG-dependent ADCC[14, 22]. Therefore, we also analyzed NK-cell reconstitution in our cohort. Consistent with prior evidence[8], there were no differences in total NK-cell numbers or distribution of $CD56^{dim}$ and $CD56^{bright}$ NK-cell

subpopulations between HCMV controllers and csCMVi patients (**Figure 5A-B**). Likewise, there was no major change in the expansion of these cell populations before and after csCMVi except for a minor shift toward $CD56^{dim}$ post-reactivation (**Figure 5C**).

Next, we specifically focused on ($NKG2C/CD159c^{+}$) "memory-like" NK-cell reconstitution (**Figure 5D**). Although not statistically significant due to considerable inter-individual variation, controllers consistently showed a trend toward higher "memory-like" $CD56^{dim}$ (NK^{dim}) NK-cell numbers (MMR 1.6-2.0) and frequencies (MMR: 1.2-1.4) than csCMVi patients (**Figure 5E**). Similarly, mature ($CD57^{+}$) "memory-like" NK^{dim}-cell counts tended to be higher in controllers than in csCMVi patients from day 210 onwards (**Figure 5F**).

We previously reported that low numbers and frequencies of "memory-like" NK cells were associated with severe and refractory csCMVi events[8]. To corroborate that trend in our current cohort, we again subdivided csCMVi patients into those with and without Rf-csCMVi. Indeed, patients with Rf-csCMVi had markedly lower numbers and frequencies of "memory-like" NK^{dim} cells and mature "memory-like" NK^{dim} cells compared to both no Rf-CMVi patients and controllers (**Figure 5G-H**).

Notably, controllers demonstrated significant expansion of "memory-like" NK^{dim} cells from day 90 to day 210 (16.0 vs. 35.8, median fold change=2.2, p=0.006) and from pre- to post-HCMV reactivation (15.4 vs. 50.0, median fold change=3.2, p=0.003). Although not reaching significance due to the smaller group size, similar changes were seen in non-Rf-csCMVi patients (day 90 to day 210: 8.9 vs. 36.3, median fold change=4.1, p=0.052; pre- to post-csCMVi: 24.2 vs. 36.3, median fold change=1.5, p=0.206). In contrast, median "memory-like" NK^{dim} -cell counts remained consistently <10 cells/μL in Rf-csCMVi patients (**Figure S6A**). Similar trends and differences between the groups were observed when specifically analyzing mature "memory-like" NK cells (**Figure S6B**).

In summary, consistent with prior evidence, controllers had higher numbers and frequencies of (mature) "memory-like" NK cells compared to csCMVi patients, especially those with Rf-csCMVi events. These differences increased over time and after reactivation events due to significant expansion of these cells in controllers and some patients with non-Rf-csCMVi, contrasting very limited expansion in those with Rf-csCMVi.

HCMV-specific humoral IgG response correlates with the expansion of $CD56^{dim}$ NK-cell subpopulations in controllers but not in csCMVi patients.

Lastly, we correlated HCMV-specific IgG levels with γδ T cells and "memory-like" NK cells, both key IgG-interacting populations. Across all patients, correlations between HCMV-specific IgG levels and γδ T-cell subpopulations were generally weak and non-significant. However, the $V\delta1^+$ γδ T-cell population showed a slight positive correlation with HCMV-specific IgG levels aggregated across all timepoints (rho=0.22, p=0.005, **Figure S7A–B**). Notably, this correlation was confined to the effector $V\delta1^+$ γδ T-cell population, where weak to moderate correlations were observed both on aggregate across all timepoints (rho=0.38, p<0.001) and specifically on day 150 (rho=0.32, p=0.046) (**Figure S7A, C**), shortly after the median time of HCMV reactivation (**Figure 1B**). These trends were largely comparable among controllers and csCMVi patients.

Interestingly, $CD56^{dim}$ NK cells (rho=0.27, p=0.008), "memory-like" NK^{dim} cells (rho=0.27, p=0.007), and mature "memory-like" NK^{dim} cells (rho=0.33, p=0.001) showed significant correlations with HCMV-specific IgG levels on aggregate across all timepoints only in controllers but not in csCMVi patients. Positive correlation between HCMV-specific IgG levels and these cell populations in controllers was most pronounced on day 150, near the median time of HCMV reactivation ($CD56^{dim}$: rho=0.55, p=0.005; "memory-like" NK^{dim} cells: rho=0.31, p=0.136; mature "memory-like" NK^{dim} cells: rho=0.41, p=0.046). In contrast, csCMVi patients showed an increasing disconnection between HCMV-specific IgG formation and memory-like" NK^{dim}-cell reconstitution, resulting in a marked negative correlation by day 270 (mature "memory-like" NK^{dim} cells: rho=-0.47, p=0.076) (**Figure 6A-C**).

Overall, HCMV-specific IgG production correlated weakly with γδ T-cell reconstitution, with minimal differences between controllers and csCMVi patients. In contrast, $CD56^{dim}$ NK-cell subpopulations, including (mature) "memory-like" NK cells correlated significantly with HCMV-specific IgG levels only in controllers, whereas these responses were disconnected in csCMVi patients.

Discussion

Extending letermovir prophylaxis from day 100 to day 200 did not substantially improve post-prophylaxis csCMVi incidence or survival[12]. Therefore, alternative management strategies and improved risk stratification are urgently needed. Late-onset reactivation is driven by slow anti-HCMV immune recovery[9]. While T cells are well-recognized as central to HCMV immunity[8-10], other key effectors, including HCMV-specific B cells, antibodies, "memory-like" NK cells, and γδ T cells[8], had been scarcely studied.

Before letermovir, early-onset HCMV reactivation often preceded significant B-cell reconstitution. In contrast, we hypothesized that more readily reconstituted humoral immunity might play a more important role in controlling late-onset HCMV reactivation in the letermovir era. Indeed, in our cohort, B cells were moderately reconstituted at the time of the first HCMV reactivation, with significant expansion of HCMV-specific humoral immunity between days 150 and 210. These observations suggest that letermovir creates a window for B-cell reconstitution, facilitating robust humoral responses following HCMV stimulation. Reactivation in csCMVi patients drove MBC expansion, exceeding that in controllers by the end of follow-up, likely in response to elevated viral replication and antigen presentation. These prominent humoral responses highlight the potential of future HCMV vaccines to leverage enhanced B-cell reconstitution, promoted by the shift to late-onset csCMVi in alloSCT recipients receiving letermovir.

Interestingly, we found a noticeable difference in HCMV-specific IgG and IgM responses dynamics between HCMV controllers and csCMVi patients. Specifically, csCMVi patients showed a delayed onset of HCMV-specific IgG production and a stronger HCMV-specific IgM response than controllers. Consistent with this observation, others had reported that HCMV-specific IgG levels >400 mg/dL at the time of letermovir cessation were associated with reduced risk of HCMV reactivation[7]. More frequent quantification of HCMV-specific IgM and IgG levels in future studies may enable a more detailed characterization of the humoral response, especially considering the short half-life of IgM[23]. The delayed switch from HCMV-specific IgM to IgG may be attributable to a deficiency of

HCMV-specific T helper (Th) cells. Th cells are known to support class switch recombination (CSR) through mechanisms such as CD40L-CD40 interaction and cytokine secretion[24]. We have previously shown that the kinetics of HCMV-specific Th cell reconstitution differ significantly between controllers and csCMVi patients. While controllers often develop HCMV-specific Th cells early, even during letermovir prophylaxis, csCMVi patients harbor significantly lower frequencies of these cells for an extended period following transplantation[8-10]. Thus, the presence of HCMV-specific Th cells in controllers may facilitate an early Ig class switch, whereas in csCMVi patients, the switch may be delayed until these cells are reconstituted. Therefore, future research exploring the interaction between HCMV-specific Th responses and humoral immunity could inform strategies to enhance vaccine efficacy or develop targeted immunotherapies.

Humoral responses provide sustained antibody production, but effective viral control after alloSCT also depends on γδ T cells[25] and "memory-like" NK cells[22]. Both Vδ1$^+$ and Vδ2$^+$ γδ T-cell subpopulations are protective against infections[26]. In the context of HCMV, Vδ1$^+$ cells play a key role by lysing HCMV-infected cells and secreting IFNγ[19-21], while Vδ2$^+$ cells appear to have limited anti-HCMV activity[26]. In the pre-letermovir era, HCMV reactivation primarily induced expansion of Vδ2$^-$ cells, especially Vδ1$^+$ cells[19, 27]. This resulted in an increased Vδ2$^-$/Vδ2$^+$ ratio, which may benefit anti-HCMV immunity[28]. Our new data suggests that this trend persists even in the letermovir era since both controllers and csCMVi patients showed an expansion of their Vδ2$^-$ and Vδ1$^+$ γδ T-cell populations following letermovir cessation. However, Vδ1$^+$ γδ T-cell expansion was more pronounced in csCMVi patients, resulting in an increased Vδ1$^+$/Vδ2$^+$ ratio after reactivation events compared to controllers. This is likely a function of longer and more intense HCMV reactivation in csCMVi patients.

Notably, HCMV controllers already had elevated Vδ1$^+$ γδ T-cell frequencies prior to letermovir cessation, underscoring the significance of Vδ1$^+$ γδ T cells in effective HCMV control. While the predictive performance of Vδ1$^+$ γδ T-cell frequencies in our study would be insufficient for use as a standalone risk stratification marker, quantification of these cells might be of interest for combinatorial immune-guided risk stratification. However, the higher incidence of aGvHD and the associated

immunosuppression in csCMVi patients may have influenced the reconstitution of key immune populations, such as γδ T cells, and could have biased their predictive value at the end of letermovir prophylaxis.

HCMV also reshapes the NK-cell repertoire, leading to the emergence of “memory-like” NK cells[29, 30]. These cells have adaptive features, including clonal expansion, long-term persistence, and enhanced recall responses[22, 31]. Their significant anti-HCMV effects are well-documented[22, 31] and are further supported by the reported association of low numbers of “memory-like” NK cells with an increased risk of future HCMV reactivation in alloSCT recipients[32]. In both our previous[8] and current studies, controllers showed higher frequencies and counts of "memory-like" NK cells than csCMVi patients, particularly those with Rf-csCMVi. However, even patients with higher "memory-like" NK cell frequencies can develop csCMVi, suggesting that these cells alone are insufficient for viral control. This conclusion is supported by data from kidney transplant recipients, indicating that "memory-like" NK cells can suppress HCMV replication after its onset but cannot prevent its initiation[33].

Both γδ T cells and "memory-like" NK cells are linked to the humoral immune response through their receptor CD16 (FcγRIII), an Fc receptor for IgG that mediates key immune functions such as ADCC[13-15]. Consequently, we investigated whether the more advanced humoral response in the letermovir era correlated with an expansion of these immune cell populations. We found that HCMV-specific IgG positively correlated with the expansion of effector γδ T cells in both controllers and csCMVi patients. $CD16^{+}$ effector γδ T cells are known to expand following stimulation by stress-induced signals from HCMV-infected cells and persist long-term[20], a process potentially supported by HCMV-specific IgGs. This likely enhances protection via IFNγ production upon CD16 engagement with immunoglobulin-coated virions[13].

The expansion of “memory-like” NK cells *in vitro* has been linked to polymorphic signal sequences of HCMV UL40 which can bind to HLA-E serving as a ligand for CD159c[34]. However, it appears that the most effective CD159c ligands of UL40 are relatively rare among clinical HCMV isolates[34].

Moreover, a link between the pool size of “memory-like” NK cells and HCMV-IgG was previously reported in healthy seropositive individuals[35]. Our observation of $CD56^{dim}$ “memory-like” NK cell expansion correlating with HCMV-specific IgG suggests that humoral immune responses may play a role in driving this NK-cell phenotype *in vivo*. Notably, "memory-like" NK cells have enhanced ADCC capacity and superior functionality compared to conventional NK cells, responding robustly to antibody-coated HCMV-infected cells[22]. The observed differences in the co-evolution of these cells and humoral immunity between controllers and csCMVi patients suggest that IgG-dependent "memory-like" NK cell-mediated anti-HCMV immunity may constitute a thus far scarcely understudied protective mechanism against csCMVi in the letermovir era, warranting further investigation. While beyond the scope of the present study, future functional analyses are needed to characterize the interactions between patient-derived specific IgGs and "memory-like" NK cells or γδ T cells in greater detail.

This study has some limitations. Firstly, HCMV-specific IgM and IgG levels were measured by a commercial immunoassay without distinguishing neutralizing from non-neutralizing antibodies or identifying antigen-specific targets. Secondly, although we captured key timepoints in HCMV immune reconstitution, the 60-day intervals between our immune analyses led to variable gaps around reactivation or csCMVi events. Thirdly, the use of cryopreserved PBMCs may have influenced phenotypic analyses. Future studies should address these limitations and incorporate multivariate analyses integrating the combined impact of clinical and immunological variables on HCMV control in larger cohorts.

Despite these limitations, our study highlights how the shift from early- to late-onset HCMV reactivation driven by letermovir prophylaxis has increased the impact of humoral immune responses in anti-HCMV defense. We further provided additional evidence for the involvement of “memory-like” NK cells and the first comprehensive analysis of γδ T-cell reconstitution in the context of letermovir prophylaxis. Investigating the interplay between humoral and cellular immunity will be essential to optimize vaccination timing and develop effective immunotherapies. Moreover, early

tracking of HCMV-specific B cells, immunoglobulins, and/or γδ T-cell reconstitution may refine prognostic immune monitoring and risk stratification strategies beyond the assessment of type-1 T cells.

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Tables

Table 1. Characteristics of alloSCT recipients who successfully controlled HCMV (controllers) and those who developed clinically significant HCMV infection (csCMVi).

Variables	Total (n=42)	HCMV controllers (n=25)	csCMVi (n=17)	*p**
Age, median (range)	62	62 (22-73)	61 (33-71)	0.995
Sex, n (%)				
Male	21 (50)	14 (56)	7 (41)	0.530
Female	21 (50)	11 (44)	10 (59)	
Underlying disease, n (%)				
Chronic leukemia	1 (2)	1 (4)	0 (0)	
Multiple myeloma	2 (5)	1 (4)	1 (6)	
Acute leukemia	25 (60)	14 (56)	11 (65)	-
Lymphoma	0 (0)	0 (0)	0 (0)	
Others	14 (33)	9 (36)	5 (29)	
HLA matching, n (%)				
Matched related	5 (12)	1 (4)	4 (24)	
Matched unrelated	24 (57)	19 (76)	5 (29)	-
Haploidentical	0 (0)	0 (0)	0 (0)	
Mismatch	13 (31)	5 (20)	8 (47)	
Stem cell source, n (%)				
Bone marrow	1 (2)	1 (4)	0 (0)	>0.999
PBSC	41 (98)	24 (96)	17 (100)	
Conditioning Regimen, n (%)				
Reduced intensity (RIC)	39 (93)	23 (92)	16 (94)	>0.999
Myeloablative (MAC)	3 (7)	2 (8)	1 (6)	
Antithymocyte globulin, n (%)				
No	5 (12)	1 (4)	4 (24)	0.140
Yes	37 (88)	24 (96)	13 (76)	
HCT-CI, n (%)				
0-2	30 (71)	19 (76)	11 (65)	
3-4	9 (21)	4 (16)	5 (29)	-
≥5	3 (7)	2 (8)	1 (6)	
aGvHD, n (%)				
0-1	29 (69)	21 (84)	8 (47)	0.018*
2-4	13 (31)	4 (16)	9 (53)	
cGvHD, n (%)				
No	27 (64)	16 (64)	11 (65)	>0.999
Yes	15 (36)	9 (36)	6 (35)	
HCMV serostatus (R/D), n (%)				
+/+	26 (62)	17 (68)	9 (53)	0.353
+/-	16 (38)	8 (32)	8 (47)	
One-year mortality, n (%)				
Alive	35 (83)	21 (84)	14 (82)	>0.999
Dead	7 (17)	4 (16)	3 (18)	

Table 2. Immunosuppressant administration during and after letermovir prophylaxis in controllers and csCMVi patients.

	Total		HCMV controllers		csCMVi	
	on LVR	after LVR	on LVR	after LVR	on LVR	after LVR
Steroid use*						
None	36 (86)	37 (88)	24 (96)	23 (92)	12 (71)	14 (82)
>0 to ≤1 mg/kg/d	2 (5)	0 (0)	1 (4)	0 (0)	1 (6)	0 (0)
2 mg/kg/d	4 (10)	5 (12)	0 (0)	2 (8)	4 (24)	3 (18)
Immunosuppressants[#]						
0	0 (0)	39 (93)	0 (0)	23 (92)	0 (0)	16 (94)
1	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
2	40 (95)	2 (5)	25 (100)	2 (8)	15 (88)	0 (0)
> 2	2 (5)	1 (2)	0 (0)	0 (0)	2 (12)	1 (6)

* Divided into 0, >0 to ≤1, or 2 mg/kg/d based on maximum steroid dose during the study period.

[#] Not including steroids (e.g. ruxolitinib, tacrolimus, sirolimus etc.)

Table Legends

Table 1. Characteristics of alloSCT recipients who successfully controlled HCMV (controllers) and those who developed clinically significant HCMV infection (csCMVi).

Mann-Whitney U test or Fisher's exact test was applied as appropriate. Abbreviations: aGvHD = acute graft versus host disease, cGvHD = chronic graft versus host disease, HCT-CI = hematopoietic cell transplantation comorbidity index, HLA = human leukocyte antigen, PBSC = peripheral blood stem cells, R/D = recipient / donor.

Table 2. Immunosuppressant administration during and after letermovir prophylaxis in controllers and csCMVi patients.

Abbreviations: csCMVi = clinically significant HCMV infection, LVR = Letermovir

Figure Legends

Figure 1. HCMV reactivation timing, HCMV viral load, and absolute lymphocyte counts.

(**A**) Study flowchart. (**B**) Median day of first quantifiable HCMV load in controllers (blue, N = 25) and csCMVi patients (brown), as well as the day of csCMVi onset in affected patients (N = 17). Transient low-level HCMV DNAemia ("blips") occurred in some patients during letermovir prophylaxis but are not shown, as they likely reflect non-infectious viral DNA. No HCMV viral load was detected in five controllers throughout the observation period. (**C**) Peak HCMV DNA copies/mL until day 365 (measured by PCR). Mann-Whitney U test. (**D-E**) ALC kinetics for controllers and csCMVi patients. Mann-Whitney U test. * $p < 0.05$, *** $p < 0.001$**.** Abbreviations: ALC = absolute lymphocyte counts, cryopres. = cryopreservation, csCMVi = clinically significant HCMV infection, HCMV = human cytomegalovirus, PCR = polymerase chain reaction.

Figure 2. HCMV reactivation is associated with a robust humoral response and memory B-cell formation.

Samples from alloSCT patients receiving letermovir prophylaxis (N = 42) or historic samples from the pre-letermovir era (N = 15, A and B only) were analyzed using flow cytometry for cellular markers and CMIA for serological markers. (**A**) B-cell counts ($CD20^+$) and frequencies around the median time of first HCMV reactivation in the pre-letermovir era (day 26) and in patients receiving letermovir prophylaxis (day 150). Mann-Whitney U test. (**A–D**) Kinetics of HCMV-specific IgM and IgG levels in the pre- (**B)** and post-letermovir era (**C-D**) around the time of HCMV reactivation (**A-D**). N = 40. (**E**) B-cell counts and frequencies in controllers and csCMVi patients. Mann-Whitney U test. (**F–G**) Evolution of IgM^+ (**F**) and IgG^+ (**G**) MBC ($CD20^+IgD^-$) frequencies between day 90 and day 270 in controllers and csCMVi patients. Paired Wilcoxon test. (**H**) Ratio of IgG^+/IgM^+ MBC frequencies before and after HCMV reactivation. Paired Wilcoxon test. (**I**) Representative flow cytometry plot showing differentiation of naïve B cells ($CD20^+IgD^+$) and MBCs. (**J-K**) Kinetics of MBC (**J**) and naïve B-cell (**K**) frequencies in controllers and csCMVi patients. Mann-Whitney U test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Interquartile ranges are shown where applicable. Abbreviations: alloSCT =

allogeneic stem cell transplantation, CMIA = chemiluminescent microparticle immunoassay, csCMVi = clinically significant HCMV infection, d = day, HCMV = human cytomegalovirus, Ig = immunoglobulin, LVR = letermovir, MBCs = memory B cells, rct = reactivation, PT = preemptive therapy.

Figure 3. csCMVi patients show an IgM-skewed HCMV-specific response with delayed IgG kinetics compared to controllers.

Reconstitution of the HCMV-specific humoral immune response was analyzed by flow cytometry and CMIA. (**A**) Flowchart illustrating the generation of HCMV-specific protein probes to detect HCMV-specific B cells. (**B**) Representative flow cytometry plot showing the detection of HCMV-specific MBCs ($CD20^+IgD^-$). (**C**) Absolute numbers and frequencies of HCMV-specific MBCs targeting glycoprotein B (gB), the pentamer complex (gH/gL/UL128/UL130/UL131A), and the trimer complex (gH/gL/gO) on day 270. N = 20. Mann-Whitney U test. (**D**) Kinetics of absolute numbers and frequencies of combined gB-, pentamer-, and trimer-specific MBCs. N = 20. Mann-Whitney U test. (**E**) HCMV-specific MBC frequencies before and after HCMV reactivation in patients with detectable HCMV viral load. N = 12. Paired Wilcoxon test. (**F**) Normalized median frequencies of IgG^+ and IgM^+ HCMV-specific MBCs over time. N = 20. (**G**) Kinetics of HCMV-specific IgG levels and IgM indices. N = 40. Mann-Whitney U test. (**H-I**) Evolution of serologic responses between days (**H**) 90 and 210 and (**I**) day 210 and 270, respectively. N = 40; 25 controllers, 15 csCMVi. Paired Wilcoxon test. *p < 0.05, **p < 0.01, ***p < 0.001. Interquartile ranges are shown where applicable. Abbreviations: alloSCT = allogeneic stem cell transplantation; CMIA = chemiluminescent microparticle immunoassay; csCMVi = clinically significant HCMV infection; d = day; HCMV = human cytomegalovirus; Ig = immunoglobulin; MBCs = memory B cells; rct = reactivation.

Figure 4. csCMVi events promote $V\delta1^+$ γδ T cell expansion and alter the $V\delta1^+/V\delta2^+$ ratio.

Reconstitution of γδ T cells in 42 alloSCT recipients was analyzed by flow cytometry. (**A**) Representative flow cytometry plot for quantification of γδ T cells. (**B**) Kinetics of γδ T-cell ($TCR\gamma\delta^+$)

numbers. Mann-Whitney U test. (**C**) Comparison of γδ T-cell numbers on days 90 and 210. Paired Wilcoxon test. (**D**) Representative flow cytometry plot illustrating γδ T-cell subpopulations. (**E**) Kinetics of absolute numbers and frequencies of $V\delta1^+$ γδ T cells ($TCR\gamma\delta^+TCRV\delta1^+$). Mann-Whitney U test. (**F**) ROC analysis for prediction of csCMVi events based on $V\delta1^+$ γδ T-cell frequencies on day 90. (**G**) Longitudinal assessment of the $V\delta2^+$ ($TCR\gamma\delta^+TCRV\delta2^+$)/$V\delta1^+$ ratio. Mann-Whitney U test. (**H**) Quantification of $V\delta1^+$ γδ T cells before and after HCMV reactivation/csCMVi events. Paired Wilcoxon test. (**I**) Comparison of $V\delta1^+$ γδ T-cell counts at days 90 and 210. Paired Wilcoxon test. (**J**) Intra-individual fold changes (FC) of $V\delta1^+$ γδ T-cell counts from day 90 until day 210. Mann-Whitney U test. (**K**) Kinetics of effector ($CD45RA^-CD27^-$) and central memory ($CD45RA^-CD27^+$) $V\delta1^+$ γδ T-cell counts. Mann-Whitney U test. *p < 0.05, **p < 0.01, ***p < 0.001. Interquartile ranges are shown where applicable. Abbreviations: alloSCT = allogeneic stem cell transplantation; AUC = area under the curve; csCMVi = clinically significant HCMV infection; d = day; FC = fold changes; rct = reactivation; ROC = receiver operating characteristic curve.

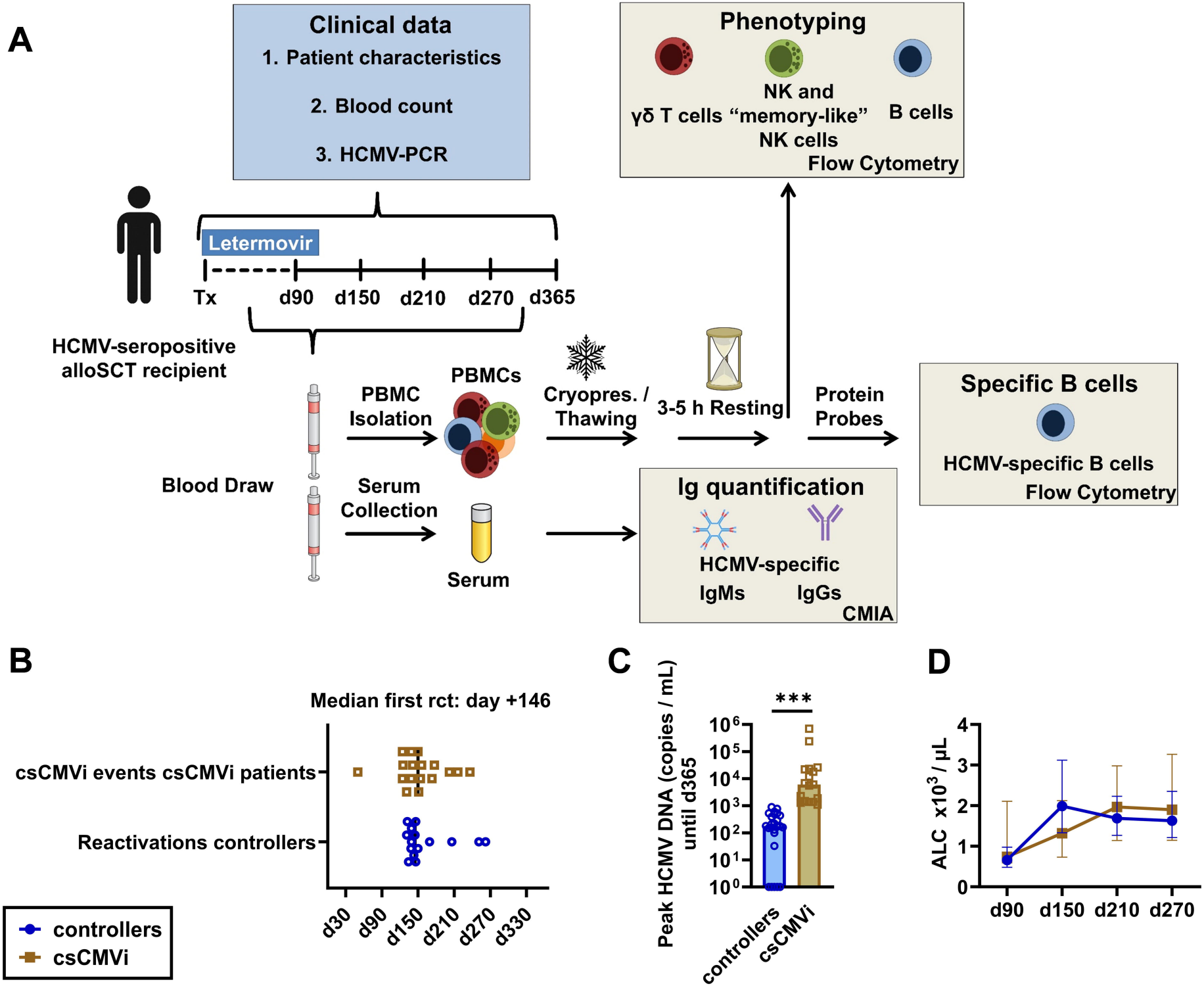
Figure 5. Failure to expand "memory-like" NK cells is linked to refractory csCMVi.

Reconstitution of NK cells in alloSCT recipients (N = 42) was analyzed by flow cytometry. (**A**) Representative flow cytometry plot of NK-cell subpopulations. (**B**) Kinetics of total NK cells ($CD56^+$) and the frequencies of $CD56^{dim}$ and $CD56^{bright}$ NK-cell subpopulations. Mann-Whitney U test. (**C**) Absolute numbers of NK cells and $CD56^{dim}$ and $CD56^{bright}$ NK-cell subpopulations before and after HCMV reactivation or csCMVi events. Paired Wilcoxon test. (**D**) Representative flow cytometry plot showing the gating strategy for "memory-like" NK cells based on CD159c expression. (**E, G**) Kinetics of absolute numbers and frequencies of "memory-like" ($CD159c^+$) NK $cells^{dim}$ ($CD56^{dim}$) (**F, H**) and mature ($CD57^+$) "memory-like" NK $cells^{dim}$ according to HCMV control, using two-group comparisons of controllers and csCMVi patients (**E-F**, Mann-Whitney U test) or three-group comparisons of controllers, no Rf-csCMVi, and Rf-csCMVi patients (**G-H**, Kruskal-Wallis). *p < 0.05, **p < 0.01, ***p < 0.001. Interquartile ranges are shown where applicable. Abbreviations: alloSCT = allogeneic stem cell transplantation; csCMVi = clinically significant HCMV infection; d = day; rct = reactivation; Rf-csCMVi = refractory clinically significant HCMV infection.

Figure 6. HCMV-specific humoral IgG response is associated with the expansion of CD56^{dim} NK-cell subpopulations in controllers but not in csCMVi patients.

Correlations between HCMV-specific IgG and NK cells were analyzed across all timepoints (TPs) and at individual timepoints. N = 40 patients; 25 controllers, 15 csCMVi. (**A**) Heatmap summarizing correlations of HCMV-specific IgG levels with numbers of NK cells and "memory-like" (CD159c^{+}) NK cells. (**B-C**) Correlation of HCMV-specific IgG with total CD56^{dim} NK cells (**B**) and mature (CD57^{+}) "memory-like" CD56^{dim} NK cells (**C**) across all timepoints (large panels), and at individual timepoints (small panels). Data for HCMV controllers (left) and csCMVi patients (right) are displayed separately. All analyses were performed using Spearman's rank correlation test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Abbreviations: alloSCT = allogeneic stem cell transplantation; csCMVi = clinically significant HCMV infection; HCMV = human cytomegalovirus; Ig = immunoglobulin; TPs = timepoints.

Figure 1


A
Clinical data
1. Patient characteristics
2. Blood count
3. HCMV-PCR
Phenotyping
NK and
γδ T cells "memory-like"
NK cells
B cells
Flow Cytometry
Letermovir
Tx
d90
d150
d210
d270
d365
HCMV-seropositive
alloSCT recipient
PBMCs
PBMC
Isolation
Cryopres. /
Thawing
3-5 h Resting
Protein
Probes
Specific B cells
HCMV-specific B cells
Flow Cytometry
Blood Draw
Serum
Collection
Serum
Ig quantification
HCMV-specific
IgMs
IgGs
CMIA
B
Median first rct: day +146
csCMVi events csCMVi patients
Reactivations controllers
d30
d90
d150
d210
d270
d330
controllers
csCMVi
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Peak HCMV DNA (copies / mL)
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ALC x10³ / µL
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d150
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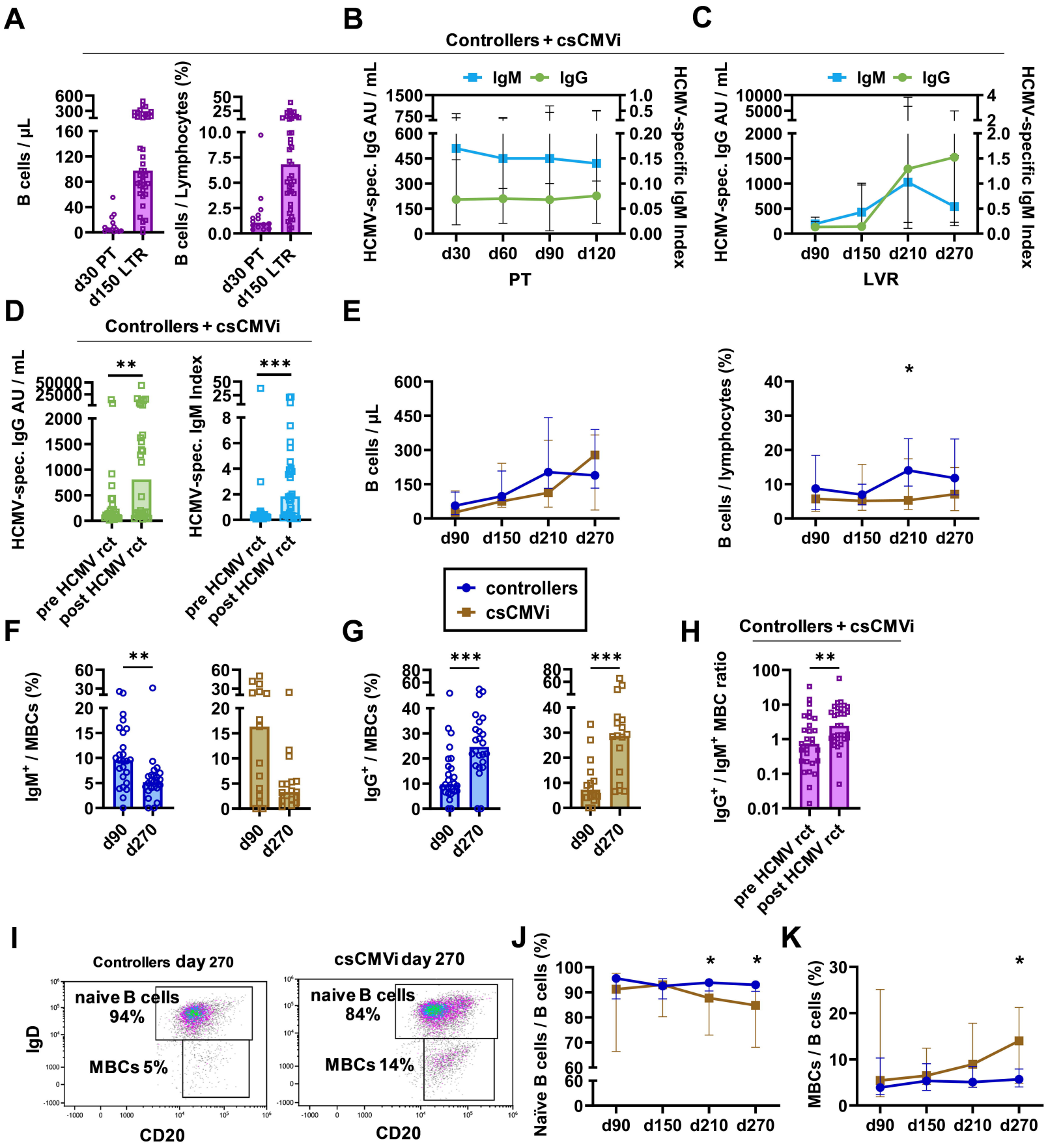
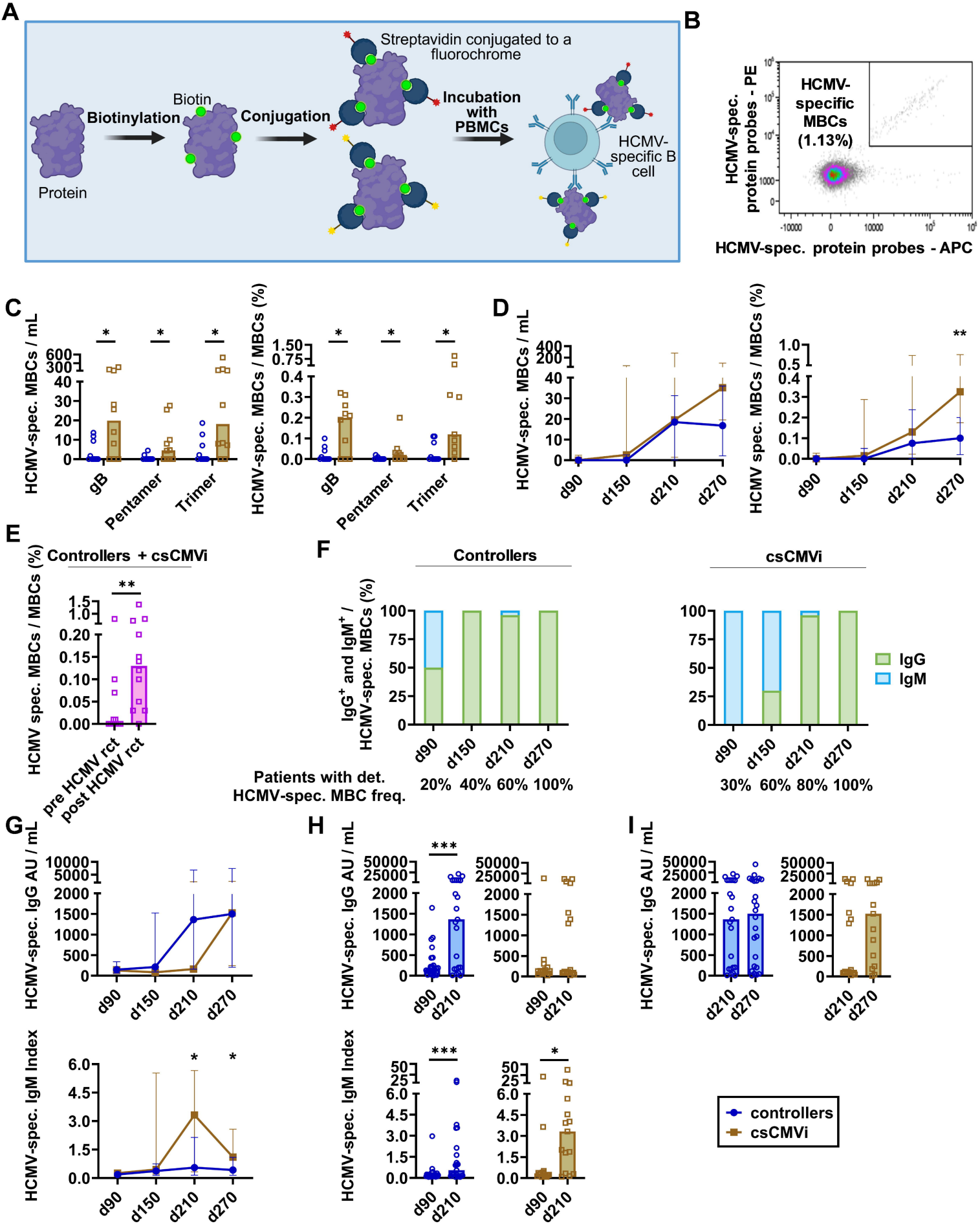
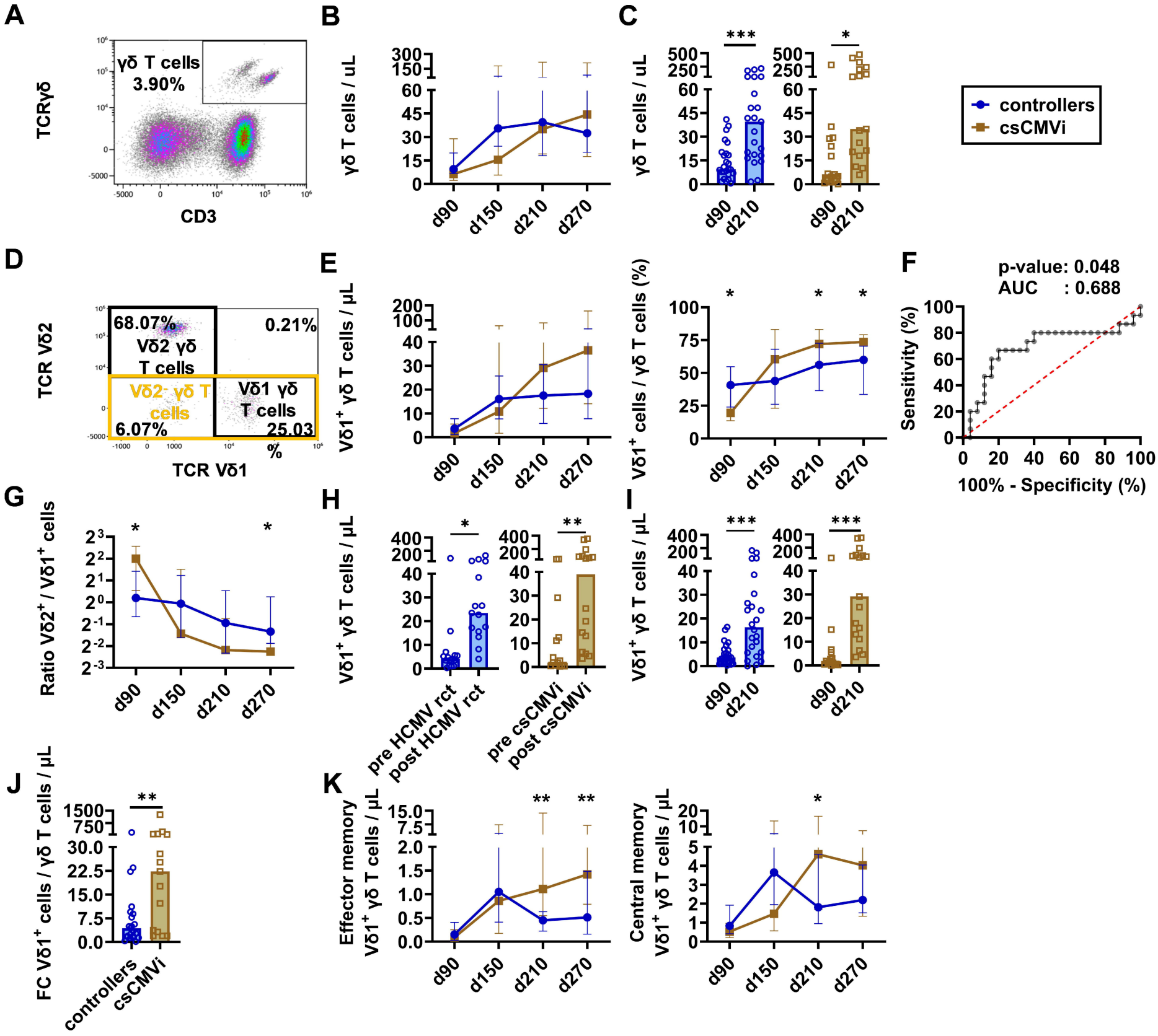
Figure 2


A
B
C
Controllers + csCMVi
B cells / µL
B cells / Lymphocytes (%)
d30 PT
d150 LTR
IgM
IgG
HCMV-spec. IgG AU / mL
HCMV-specific IgM Index
d30
d60
d90
d120
PT
d90
d150
d210
d270
LVR
D
E
HCMV-spec. IgM Index
pre HCMV rct
post HCMV rct
controllers
csCMVi
F
G
H
IgM+ / MBCs (%)
IgG+ / MBCs (%)
IgG+ / IgM+ MBC ratio
I
Controllers day 270
csCMVi day 270
naive B cells
94%
84%
MBCs 5%
MBCs 14%
IgD
CD20
J
K
Naïve B cells / B cells (%)
MBCs / B cells (%)


A
Biotinylation
Biotin
Protein
Conjugation
Streptavidin conjugated to a fluorochrome
Incubation with PBMCs
HCMV-specific B cell
B
HCMV-specific MBCs (1.13%)
HCMV-spec. protein probes - PE
HCMV-spec. protein probes - APC
C
HCMV-spec. MBCs / mL
HCMV-spec. MBCs / MBCs (%)
gB
Pentamer
Trimer
D
HCMV-spec. MBCs / mL
HCMV spec. MBCs / MBCs (%)
d90
d150
d210
d270
E
Controllers + csCMVi
HCMV spec. MBCs / MBCs (%)
pre HCMV rct
post HCMV rct
F
Controllers
csCMVi
IgG+ and IgM+ / HCMV-spec. MBCs (%)
IgG
IgM
Patients with det. HCMV-spec. MBC freq.
20% 40% 60% 100%
30% 60% 80% 100%
G
HCMV-spec. IgG AU / mL
HCMV-spec. IgM Index
H
HCMV-spec. IgG AU / mL
HCMV-spec. IgM Index
I
HCMV-spec. IgG AU / mL
controllers
csCMVi


A
TCRγδ
γδ T cells
3.90%
CD3
B
γδ T cells / uL
d90
d150
d210
d270
C
γδ T cells / uL
***
*
d90
d210
controllers
csCMVi
D
TCR Vδ2
68.07%
Vδ2 γδ
T cells
0.21%
Vδ2⁻ γδ T
cells
6.07%
Vδ1 γδ
T cells
25.03
%
TCR Vδ1
E
Vδ1⁺ γδ T cells / µL
Vδ1⁺ cells / γδ T cells (%)
F
p-value: 0.048
AUC : 0.688
Sensitivity (%)
100% - Specificity (%)
G
Ratio Vδ2⁺ / Vδ1⁺ cells
H
Vδ1⁺ γδ T cells / µL
pre HCMV rct
post HCMV rct
pre csCMVi
post csCMVi
I
Vδ1⁺ γδ T cells / µL
J
FC Vδ1⁺ cells / γδ T cells / µL
controllers
csCMVi
K
Effector memory
Vδ1⁺ γδ T cells / µL
Central memory
Vδ1⁺ γδ T cells / µL

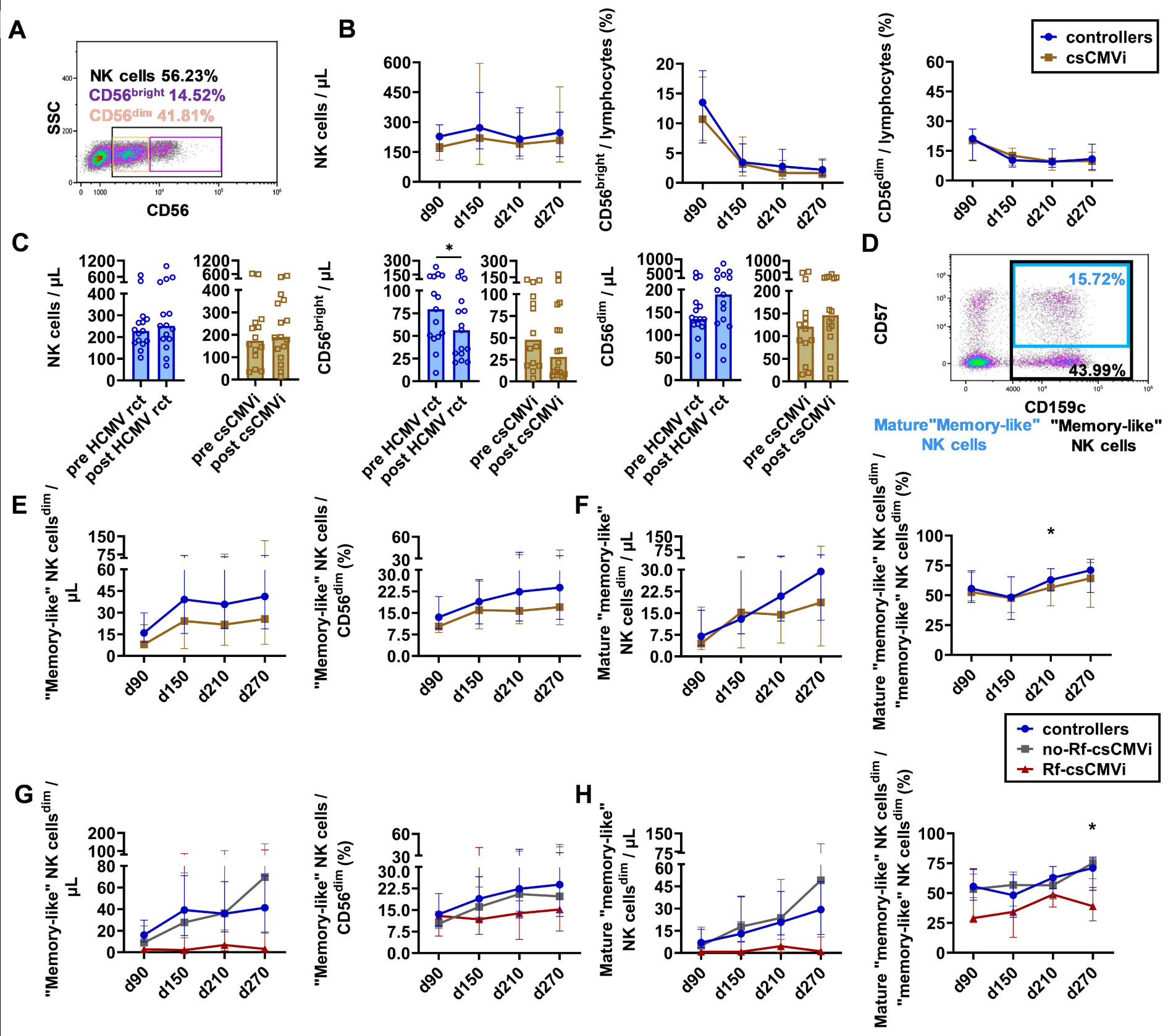
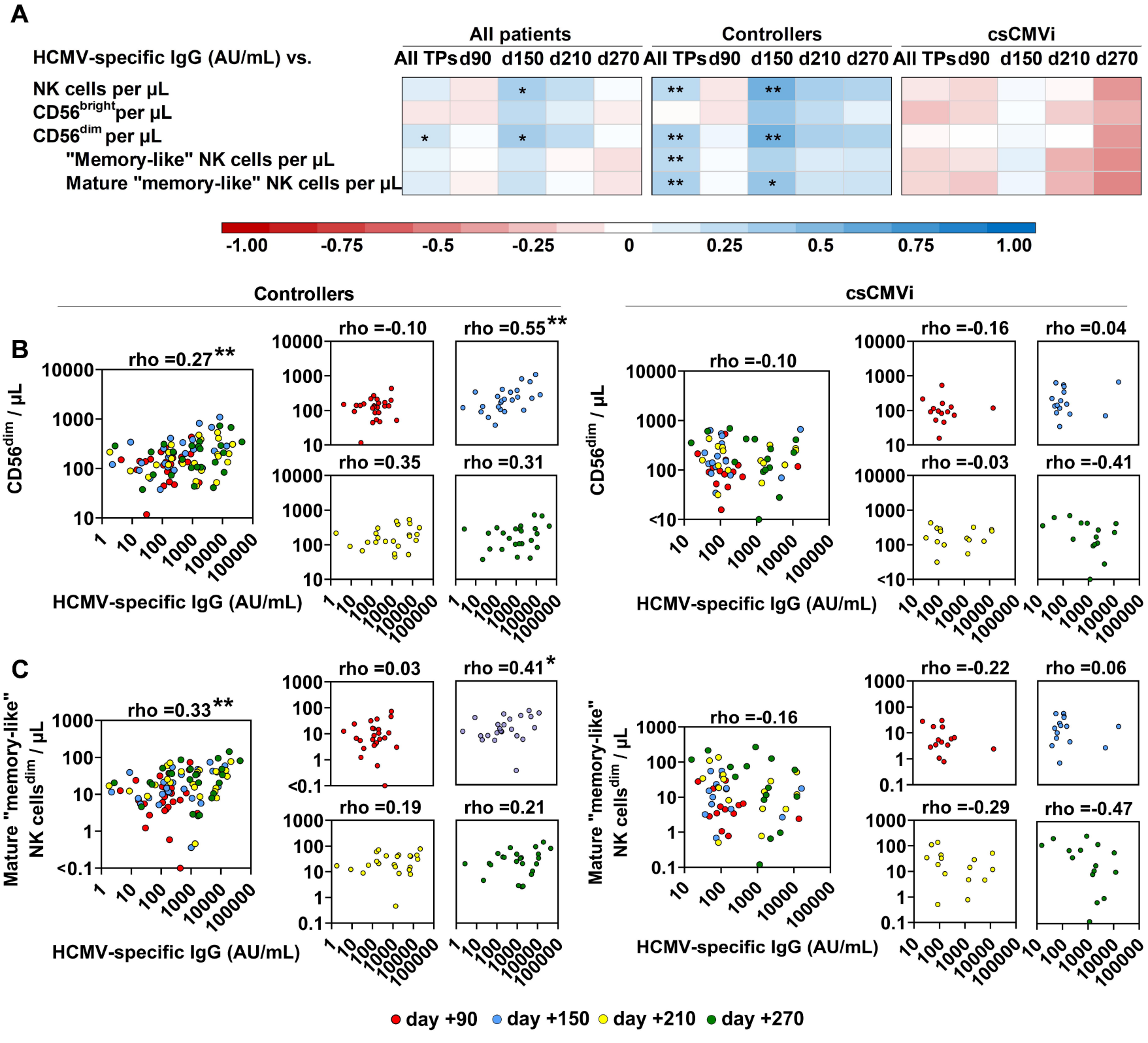

A
NK cells 56.23%
CD56bright 14.52%
CD56dim 41.81%
SSC
CD56
B
NK cells / µL
CD56bright / lymphocytes (%)
CD56dim / lymphocytes (%)
d90
d150
d210
d270
controllers
csCMVi
C
NK cells / µL
CD56bright / µL
CD56dim / µL
pre HCMV rct
post HCMV rct
pre csCMVi
post csCMVi
*
D
CD57
CD159c
15.72%
43.99%
Mature"Memory-like" NK cells
"Memory-like" NK cells
E
"Memory-like" NK cellsdim / µL
"Memory-like" NK cells / CD56dim (%)
F
Mature "memory-like" NK cellsdim / µL
Mature "memory-like" NK cellsdim / "memory-like" NK cellsdim (%)
controllers
no-Rf-csCMVi
Rf-csCMVi
G
"Memory-like" NK cellsdim / µL
"Memory-like" NK cells / CD56dim (%)
H
Mature "memory-like" NK cellsdim / µL
Mature "memory-like" NK cellsdim / "memory-like" NK cellsdim (%)

Figure 6


A
All patients
Controllers
csCMVi
HCMV-specific IgG (AU/mL) vs.
All TPs d90 d150 d210 d270
NK cells per µL
CD56bright per µL
CD56dim per µL
"Memory-like" NK cells per µL
Mature "memory-like" NK cells per µL
-1.00 -0.75 -0.5 -0.25 0 0.25 0.5 0.75 1.00
B
Controllers
csCMVi
CD56dim / µL
rho =0.27**
rho =-0.10
rho =0.55**
rho =0.35
rho =0.31
rho =-0.10
rho =-0.16
rho =0.04
rho =-0.03
rho =-0.41
HCMV-specific IgG (AU/mL)
C
Mature "memory-like" NK cellsdim / µL
rho =0.33**
rho =0.03
rho =0.41*
rho =0.19
rho =0.21
rho =-0.16
rho =-0.22
rho =0.06
rho =-0.29
rho =-0.47
HCMV-specific IgG (AU/mL)
day +90 day +150 day +210 day +270

HCMV control in allogeneic stem cell transplant recipients in the letermovir era - emerging humoral and cellular players

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Supplementary Methods

HCMV viral load measurements

HCMV DNA was quantified from EDTA plasma samples using a clinically validated real-time PCR assay, performed by the Institute of Virology at the University of Wuerzburg as part of routine diagnostics. The following primers and probe were used: CMV31 (AAgCggCCTCTgATAACCAAg), CMV32 (gAgCAgACTCTCAgAggATCgg), and CMV-TM46FAM (catgcagatctcctcaatgcggcg). A pCMV8 plasmid cloned into the pCR2.1-TOPO vector served as the positive control. PCR amplification was carried out on an ABI 7500 Real-Time PCR System (Life Technologies).

HCMV-specific IgG and IgM measurements

Serum HCMV-specific IgM and IgG levels were measured using clinically validated ARCHITECT CMV assays (Abbott, Ireland) on the ARCHITECT i2000SR system, performed by the Institute of Virology at the University of Wuerzburg. IgG samples exceeding the upper detection limit of 250 AU were automatically diluted 1:10 by the instrument and reanalyzed. In rare cases where this dilution was insufficient to bring values within range, a 1:100 dilution was prepared manually and subsequently analyzed on the same system.

Flow cytometry

After thawing, global γδ T-, B-, and NK-cell phenotypes were analyzed, and HCMV-specific B cells were quantified using flow cytometry. For γδ T- and B-cell phenotyping, 5×10^5 PBMCs per panel were stained using specific antibodies.

γδ T-cell staining consisted of TCR Vδ1 FITC, TCR γ/δ PE, CD27 PE-Vio615, TCR Vδ2 APC, TCR Vγ9 VioBlue, CD45RA VioGreen (Miltenyi, Bergisch Gladbach, Germany), CD3 AF700 (BD, Franklin Lakes, New Jersey, USA), and Fixable Viability Dye eFluor780 (Invitrogen, Waltham, Massachusetts) in 90 µL Brilliant Stain Buffer (BD). B-cell staining utilized IgG FITC, CD27 PE-Vio615 (Miltenyi), IgM BV421, IgD BV605, CD20 BV650, CD21 Alexa Fluor700 (BioLegend, San Diego, California, USA), and Fixable Viability Dye eFluor780 in 90 µL Brilliant Stain Buffer.

For the NK-cell phenotype, 5×10^5 PBMCs were washed in wash buffer (PBS + 1% FCS), blocked for 10 minutes at 4°C with 95 µL Brilliant Stain Buffer and 5 µL Blocking Reagent (Miltenyi), and stained for 30 minutes at 4°C in the same buffer. Antibodies used were CD159c PE-Vio770, CD57 APC (Miltenyi), CD56 BV510 BioLegend, CD3 AF700 (BD) and Fixable Viability Dye eFluor780.

HCMV glycoproteins were used as probes to identify specific memory B cell populations. HCMV trimer (gH/gL/gO complex, The Native Antigen Company, Kidlington, UK), pentamer (gH, gL, UL128, UL130, UL131A complex, The Native Antigen Company), and glycoprotein B (gB, SinoBiological, Beijing, China) were biotinylated with EZ-Link Sulfo-NHS-Biotin (Thermo Scientific, Waltham, Massachusetts), incubated for 30 minutes at room temperature with a 20-fold molar excess of biotin reagent, and desalted using Zeba Spin Columns (Thermo Scientific). Biotinylated proteins were enriched with 15% glycerol (final concentration) and stored at 4°C. Before use, streptavidin PE and APC (Miltenyi) were bound to the probes, and 5×10^6 PBMCs per protein/protein complex were stained. Additional antibodies used consisted of IgG FITC, CD27 PE-Vio615 (Miltenyi), IgM BV421, IgD BV605, CD20 BV650, CD21 AF700 (BioLegend), and Fixable Viability Dye eFluor780. Streptavidin BV510 (BioLegend) was used as a decoy probe to exclude non-specific B cells, with only double-positive (PE and APC) B cells considered specific. For a comprehensive, step-by-step protocol for detecting antigen-specific memory B cells, please refer to Weskamm et al[1].

Flow cytometric analysis was performed using a CytoFLEX cytometer and CytExpert v.2.4 software (Beckman Coulter, Brea, California). Data were analyzed in Kaluza v.2.1, and cell subpopulation frequencies were calculated by multiplying flow cytometry-derived percentages by absolute lymphocyte counts per µL of whole blood, as determined by clinical hematology.

References

1. Weskamm LM, Dahlke C, Addo MM. Flow cytometric protocol to characterize human memory B cells directed against SARS-CoV-2 spike protein antigens. STAR Protoc. 2022;3(4):101902.

Table S1. AlloSCT recipient characteristics: historical preemptive therapy cohort vs. current letermovir cohort

Variables	Preemptive therapy cohort (n=15)	Letermovir cohort (n=42)	*p**
Age, median (range)	55 (31-78)	62 (22-73)	0.925
Sex, n (%)			
Male	14 (93)	21 (50)	0.004**
Female	1 (7)	21 (50)	
Underlying disease, n (%)			
Chronic leukemia	0 (0)	1 (2)	
Multiple myeloma	2 (13)	2 (5)	
Acute leukemia	7 (47)	25 (60)	-
Lymphoma	0 (0)	0 (0)	
Others	6 (40)	14 (33)	
HLA matching, n (%)			
Matched related	3 (20)	5 (12)	
Matched unrelated	10 (67)	24 (57)	-
Haploidentical	0 (0)	0 (0)	
Mismatch	2 (13)	13 (31)	
Stem cell source, n (%)			
Bone marrow	0 (0)	1 (2)	>0.999
PBSC	15 (100)	41 (98)	
Conditioning Regimen, n (%)			
Reduced intensity (RIC)	14 (92)	39 (93)	>0.999
Myeloablative (MAC)	1 (8)	3 (7)	
Antithymocyte globulin, n (%)			
No	2 (13)	5 (12)	>0.999
Yes	13 (87)	37 (88)	
HCT-CI, n (%)			
0-2	8 (53)	30 (71)	
3-4	3 (20)	9 (21)	-
≥5	4 (27)	3 (7)	
aGvHD, n (%)			
0-1	11 (73)	29 (69)	>0.999
2-4	4 (27)	13 (31)	
cGvHD, n (%)			
No	9 (60)	27 (64)	0.766
Yes	6 (40)	15 (36)	
HCMV serostatus (R/D), n (%)			
+/+	14 (93)	26 (62)	0.025*
+/-	1 (7)	16 (38)	
One-year mortality, n (%)			
Alive	12 (80)	35 (83)	0.713
Dead	3 (20)	7 (17)	
HCMV reactivation, n (%)			
Controllers	11 (73)	25 (60)	0.534
csCMVi	4 (27)	17 (40)	

Figure S1.

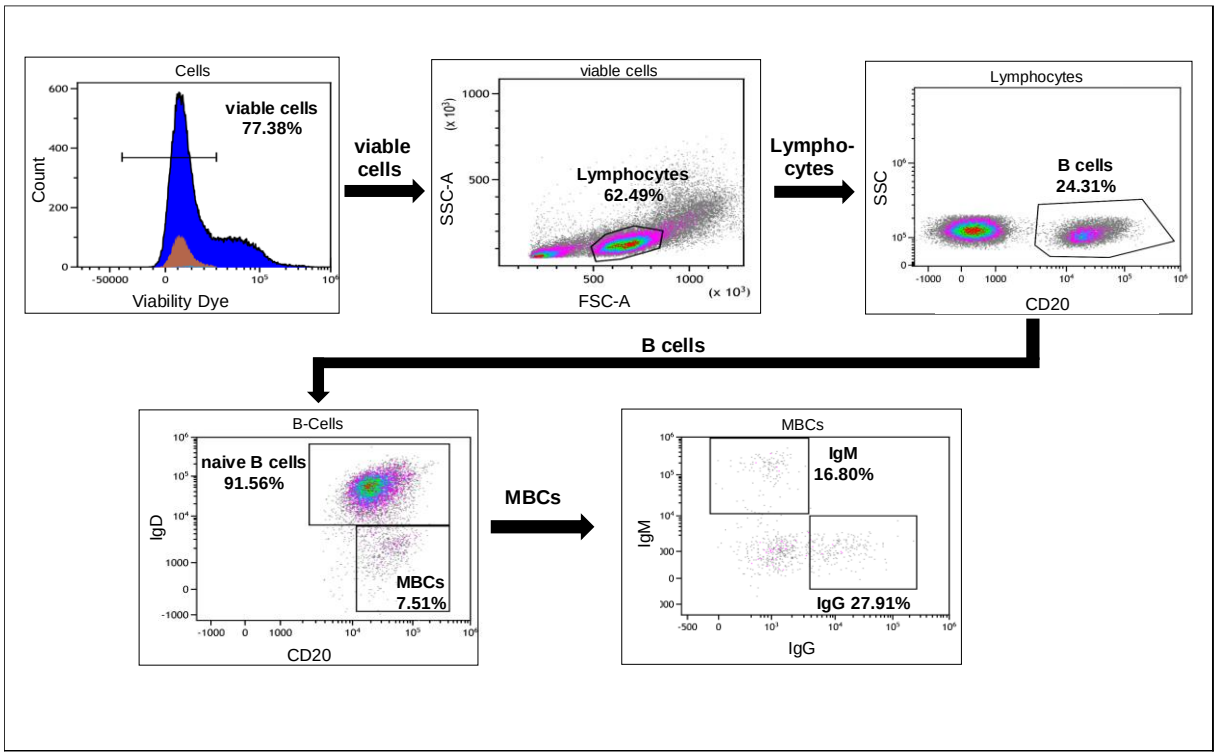


Figure S2.

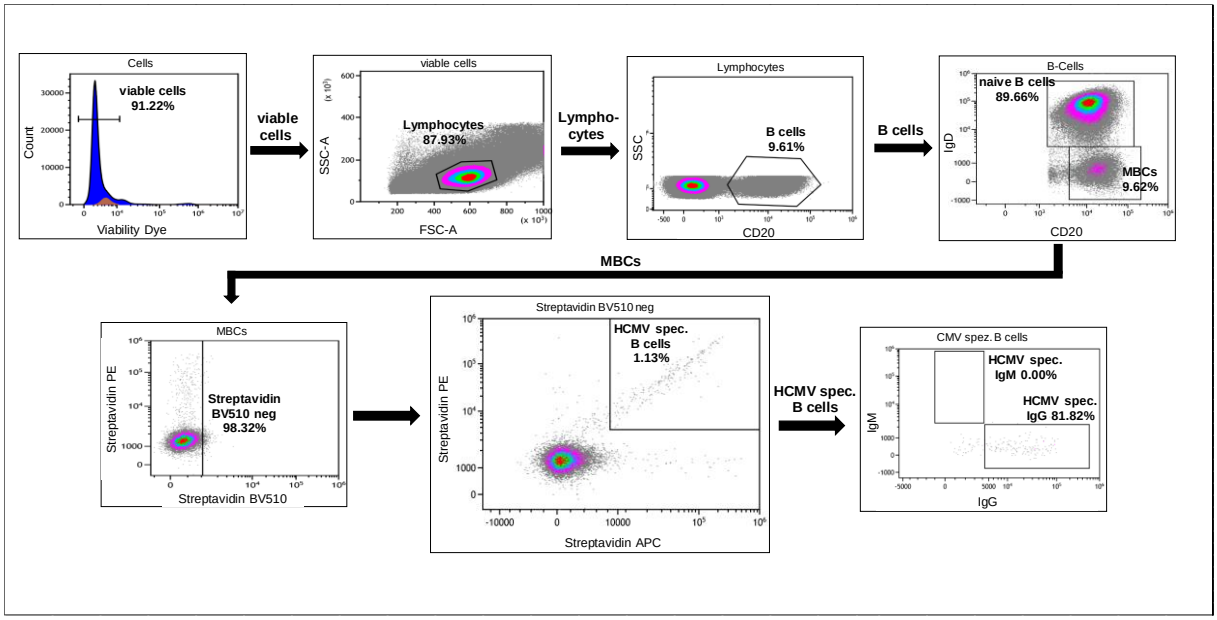


Figure S3.

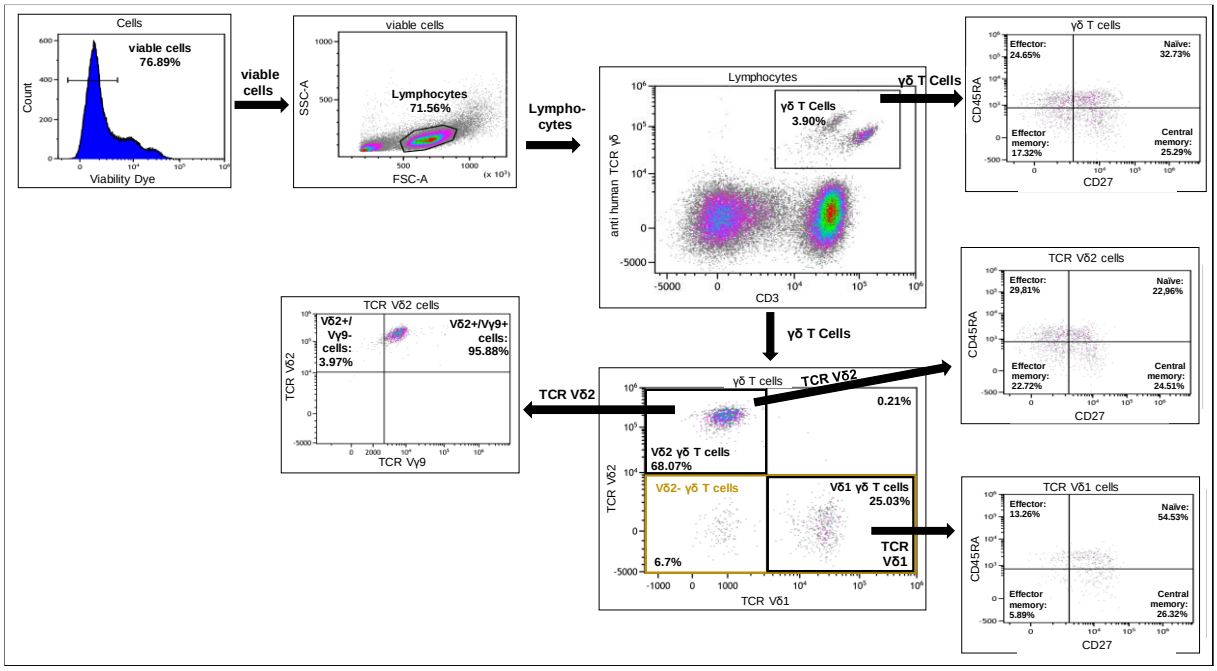


Figure S4.

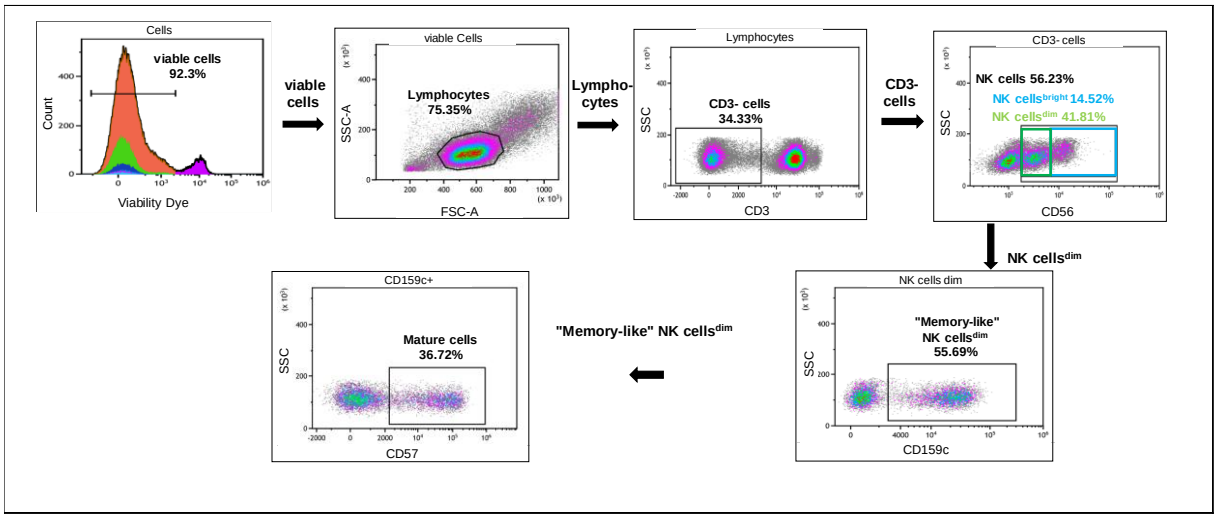


Figure S5.

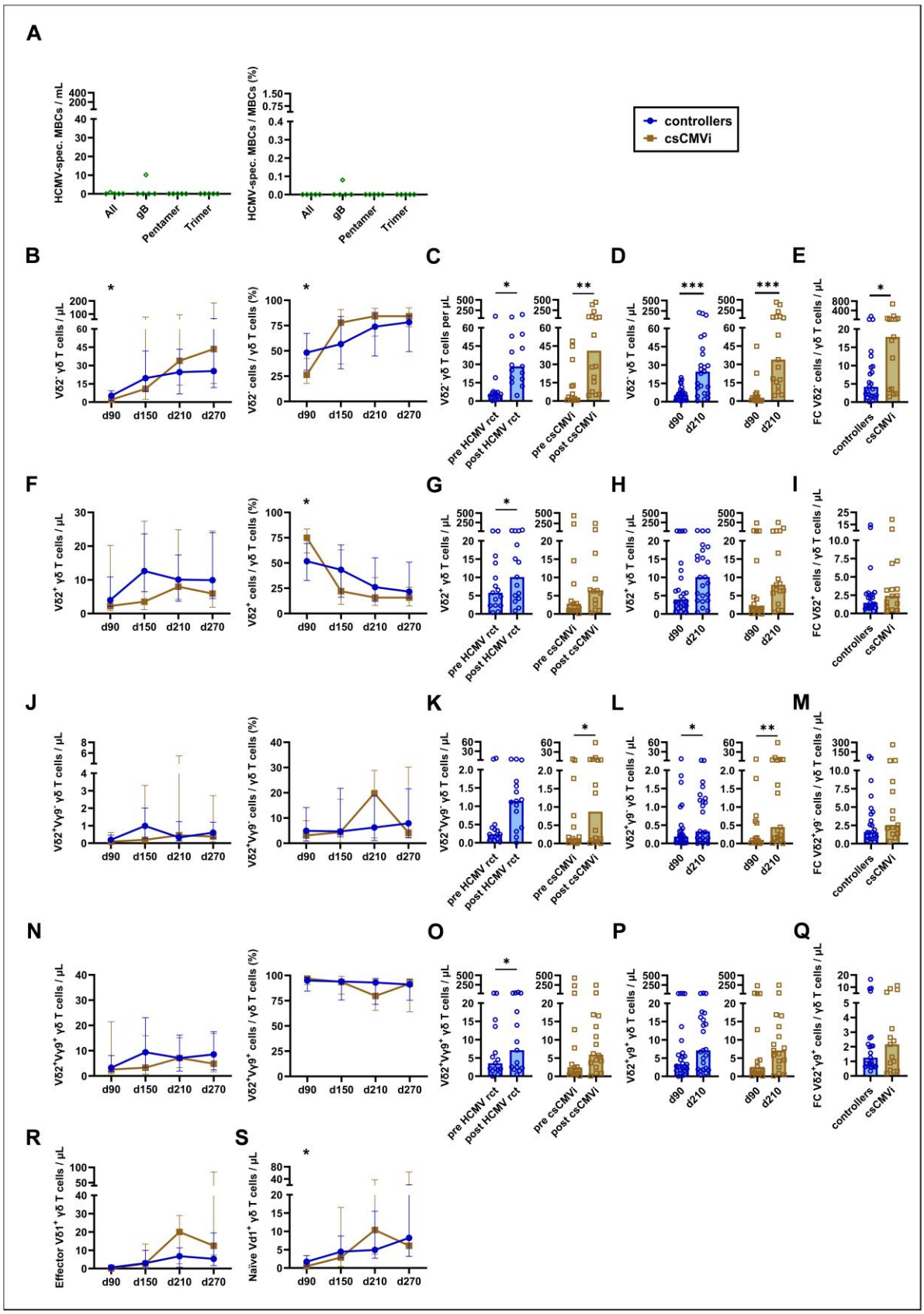

A
HCMV-spec. MBCs / mL
HCMV-spec. MBCs / MBCs (%)
All
gB
Pentamer
Trimer
controllers
csCMVi
B
Vδ2⁻ γδ T cells / µL
Vδ2⁻ cells / γδ T cells (%)
C
Vδ2⁻ γδ T cells per µL
pre HCMV rct
post HCMV rct
pre csCMVi
post csCMVi
D
Vδ2⁻ γδ T cells / µL
d90
d210
E
FC Vδ2⁻ cells / γδ T cells / µL
controllers
csCMVi
F
Vδ2⁺ γδ T cells / µL
Vδ2⁺ cells / γδ T cells (%)
G
Vδ2⁺ γδ T cells / µL
H
Vδ2⁺ γδ T cells / µL
I
FC Vδ2⁺ cells / γδ T cells / µL
J
Vδ2⁺Vγ9⁻ γδ T cells / µL
Vδ2⁺Vγ9⁻ cells / γδ T cells (%)
K
Vδ2⁺Vγ9⁻ γδ T cells / µL
L
Vδ2⁺γ9⁻ γδ T cells / µL
M
FC Vδ2⁺γ9⁻ cells / γδ T cells / µL
N
Vδ2⁺Vγ9⁺ γδ T cells / µL
Vδ2⁺Vγ9⁺ cells / γδ T cells (%)
O
Vδ2⁺Vγ9⁺ γδ T cells / µL
P
Vδ2⁺γ9⁺ γδ T cells / µL
Q
FC Vδ2⁺γ9⁺ cells / γδ T cells / µL
R
Effector Vδ1⁺ γδ T cells / µL
S
Naïve Vd1⁺ γδ T cells / µL
d90
d150
d210
d270

Figure S6.

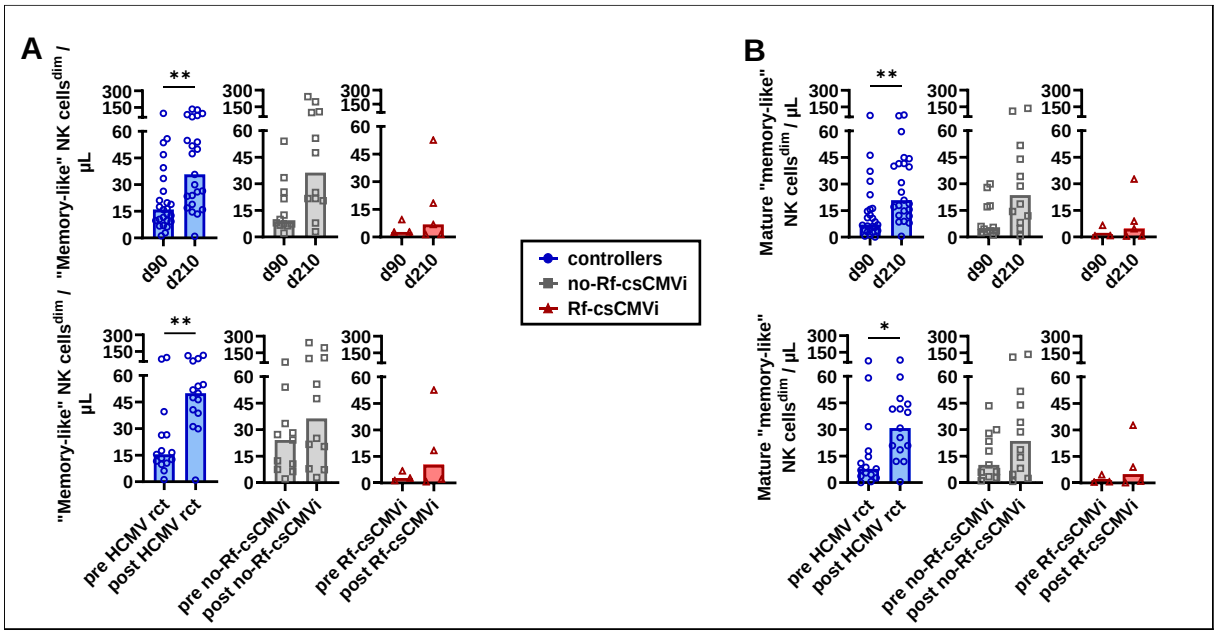
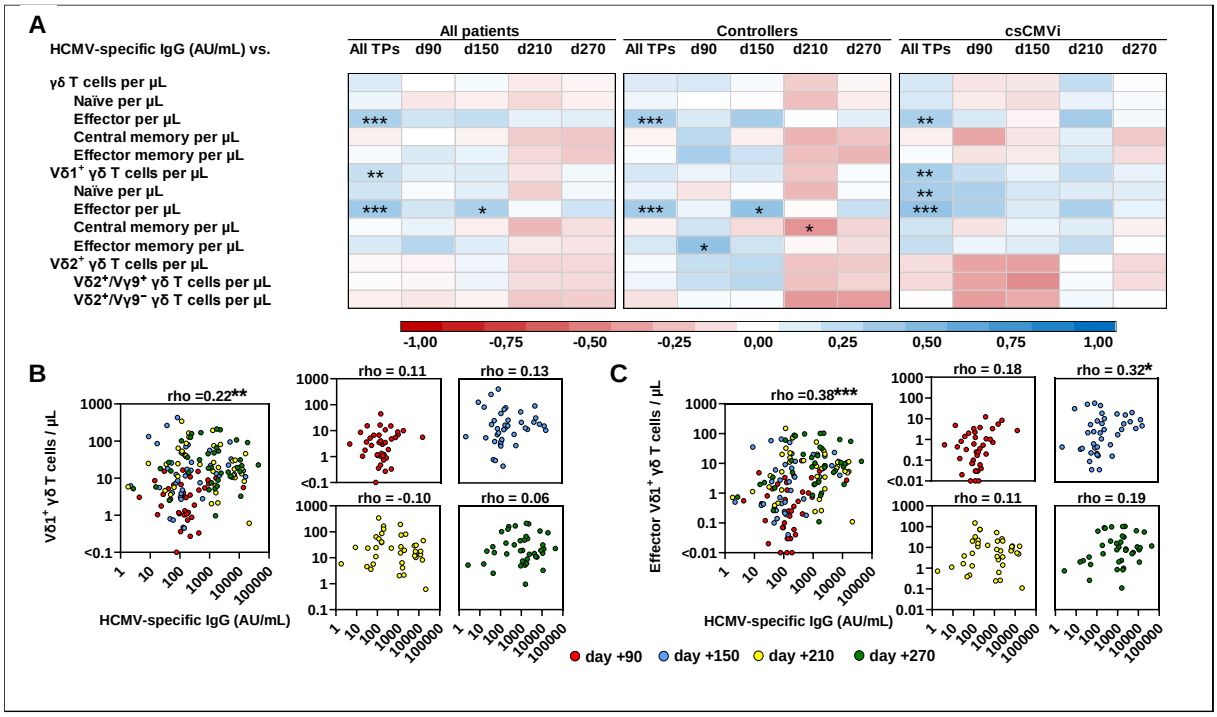

A
B
"Memory-like" NK cells^dim / µL
Mature "memory-like" NK cells^dim / µL
**
*
300
150
60
45
30
15
0
d90
d210
pre HCMV rct
post HCMV rct
pre no-Rf-csCMVi
post no-Rf-csCMVi
pre Rf-csCMVi
post Rf-csCMVi
controllers
no-Rf-csCMVi
Rf-csCMVi

Figure S7.


A
HCMV-specific IgG (AU/mL) vs.
All patients
Controllers
csCMVi
All TPs d90 d150 d210 d270
γδ T cells per µL
Naïve per µL
Effector per µL
Central memory per µL
Effector memory per µL
Vδ1+ γδ T cells per µL
Naïve per µL
Effector per µL
Central memory per µL
Effector memory per µL
Vδ2+ γδ T cells per µL
Vδ2+/Vγ9+ γδ T cells per µL
Vδ2+/Vγ9− γδ T cells per µL
-1,00 -0,75 -0,50 -0,25 0,00 0,25 0,50 0,75 1,00
B
rho =0.22**
rho = 0.11
rho = 0.13
rho = -0.10
rho = 0.06
Vδ1+ γδ T cells / µL
HCMV-specific IgG (AU/mL)
C
rho =0.38***
rho = 0.18
rho = 0.32*
rho = 0.11
rho = 0.19
Effector Vδ1+ γδ T cells / µL
HCMV-specific IgG (AU/mL)
day +90 day +150 day +210 day +270

Supplementary Table Legend

AlloSCT recipient characteristics: historical preemptive therapy cohort vs. current letermovir cohort

Mann-Whitney U test or Fisher's exact test was applied as appropriate. Abbreviations: aGvHD = acute graft versus host disease, cGvHD = chronic graft versus host disease, csCMVi = clinically significant CMV infection, HCT-CI = hematopoietic cell transplantation comorbidity index, HLA = human leukocyte antigen, PBSC = peripheral blood stem cells, R/D = recipient / donor.

Supplementary Figure Legends

Figure S1. Gating strategy for the identification of B cells.

The gating strategy used to identify B cells is depicted. Abbreviations: CD = cluster of differentiation; FSC = forward scatter; MBCs = memory B cells; IgG = immunoglobulin G; IgM = immunoglobulin M; SSC = side scatter.

Figure S2. Gating strategy for the identification of HCMV-specific memory B cells.

The gating strategy used to identify HCMV-specific memory B cells is depicted. Abbreviations: CD = cluster of differentiation; FSC = forward scatter; HCMV = human cytomegalovirus; MBCs = memory B cells; HCMV-spec. = HCMV-specific; IgG = immunoglobulin G; IgM = immunoglobulin M; SSC = side scatter.

Figure S3. Gating strategy for the identification of γδ T cells.

The gating strategy used to identify γδ T cells is depicted. Abbreviations: CD = cluster of differentiation; FSC = forward scatter; TCR = T-cell receptor; SSC = side scatter.

Figure S4. Gating strategy for the identification of ("memory-like") NK cells.

The gating strategy used to identify ("memory-like") NK cells is depicted. Abbreviations: CD = cluster of differentiation; FSC = forward scatter; TCR = T-cell receptor; SSC = side scatter.

Figure S5. HCMV-specific B cells are undetectable in HCMV-seronegative patients, and HCMV reactivation is primarily associated with alterations in the Vδ2$^-$ γδ T cell subset.

(A) Absolute numbers and frequencies of HCMV-specific memory B cells ($CD20^+IgD^-$, MBCs) targeting glycoprotein B (gB), the pentamer complex (gH/gL/UL128/UL130/UL131A), the trimer complex (gH/gL/gO), and all three antigens combined in HCMV-seronegative alloSCT recipients on day 270 (N = 5). **(B–Q)** Reconstitution of Vδ2$^-$ ($TCR\gamma\delta^+TCRV\delta2^-$) (**B-E**), Vδ2$^+$ ($TCR\gamma\delta^+TCRV\delta2^+$) (**F-I**), $V\delta2^+V\gamma9^+$ (**J-M**), and $V\delta2^+V\gamma9^-$ (**N-Q**) γδ T cells in 42 alloSCT recipients analyzed by flow cytometry. Absolute cell counts and frequencies are shown in **B**, **F**, **J**, and **N**. Mann-Whitney U test.

Cell counts before and after HCMV reactivation or clinically significant CMV infection (csCMVi) are displayed in **C**, **G**, **K**, and **O**. Paired Wilcoxon test. Changes in cell numbers between day 90 and day 210 are shown in **D**, **H**, **L**, and **P**. Paired Wilcoxon test. Intra-individual fold changes (FC) from day 90 to day 210 are presented in **E**, **I**, **M**, and **Q**. Mann-Whitney U test. (**R-S**) Absolute cell counts of effector ($CD45RA^+CD27^-$) (**R**) and naive ($CD45RA^+CD27^+$) (**S**) $V\delta1^+$ γδ T cells ($TCR\gamma\delta^+TCRV\delta1^+$) were also analyzed (paired Wilcoxon test). *p < 0.05, **p < 0.01, ***p < 0.001. Interquartile ranges are shown where applicable. Abbreviations: csCMVi = clinically significant HCMV infection; d = day; FC = fold changes; HCMV = human cytomegalovirus; rct = reactivation.

Figure S6. Failure to expand "memory-like" NK cells is linked to refractory csCMVi.

Reconstitution of NK cells in alloSCT recipients (N = 42) was analyzed by flow cytometry. (**A-B**) Evolution of "memory-like" NK $cells^{dim}$ (**A**) and mature "memory-like" NK $cells^{dim}$ (**B**) counts between days 90 and 210 (top panels), or before and after HCMV reactivation/csCMVi events (bottom panels). Paired Wilcoxon test. *p < 0.05, **p < 0.01. Abbreviations: alloSCT = allogeneic stem cell transplantation; csCMVi = clinically significant HCMV infection; d = day; rct = reactivation; Rf-csCMVi = refractory clinically significant HCMV infection.

Figure S7. HCMV-specific humoral IgG response shows a slight positive correlation to $V\delta1^+$ γδ T cells.

Correlations between HCMV-specific IgG and γδ T cells were analyzed across all timepoints (TPs) and at individual timepoints. N = 40 patients; 25 controllers, 15 csCMVi. (**A**) Heatmap summarizing correlations of HCMV-specific IgG levels with numbers of γδ T cells ($TCR\gamma\delta^+$) and their subpopulations. (**B-C**) Correlation of HCMV-specific IgG with total $V\delta1^+$ γδ T-cell ($TCR\gamma\delta^+TCRV\delta1^+$) (**B**) and effector $V\delta1^+$ γδ T-cell ($CD45RA^+/CD27^-$) numbers (**C**) in 40 patients across all timepoints (large panels) and at individual timepoints (small panels). All analyses were performed using Spearman's rank correlation test. *p < 0.05, **p < 0.01, ***p < 0.001. Abbreviations: alloSCT =

allogeneic stem cell transplantation; csCMVi = clinically significant HCMV infection; HCMV = human cytomegalovirus; Ig = immunoglobulin; TPs = timepoints.