ORIGINAL ARTICLE

Circulating cytokine and chemokine patterns associated with cytomegalovirus reactivation after stem cell transplantation

Lauren Stern[1,2] (iD), Helen M McGuire[1,2] (iD), Selmir Avdic[3], Emily Blyth[3,4,5] (iD), David Gottlieb[3,4,5], Ellis Patrick[3,6], Allison Abendroth[1,2,a] & Barry Slobedman[1,2,a] (iD)

[1]Infection, Immunity and Inflammation, School of Medical Sciences, Faculty of Medicine and Health, The University of Sydney, Sydney, NSW, Australia
[2]Charles Perkins Centre, The University of Sydney, Sydney, NSW, Australia
[3]Westmead Institute for Medical Research, The University of Sydney, Sydney, NSW, Australia
[4]Blood Transplant and Cell Therapies Program, Department of Haematology, Westmead Hospital, Sydney, NSW, Australia
[5]Faculty of Medicine and Health, Sydney Medical School, The University of Sydney, Sydney, NSW, Australia
[6]School of Mathematics and Statistics, The University of Sydney, Sydney, NSW, Australia

Correspondence

B Slobedman, Level 5E Charles Perkins Centre D17, The University of Sydney, Sydney, NSW 2006, Australia.
E-mail: barry.slobedman@sydney.edu.au

[a]Equal contributors.

Abstract

Objectives. Human cytomegalovirus (HCMV) reactivation is the leading viral complication after allogeneic haematopoietic stem cell transplantation (allo-HSCT). Understanding of circulating cytokine/chemokine patterns which accompany HCMV reactivation and correlate with HCMV DNAemia magnitude is limited. We aimed to characterise plasma cytokine/chemokine profiles in 36 allo-HSCT patients (21 with HCMV reactivation and 15 without HCMV reactivation) at four time-points in the first 100-day post-transplant. **Methods.** The concentrations of 31 cytokines/chemokines in plasma samples were analysed using a multiplex bead-based immunoassay. Cytokine/chemokine concentrations were compared in patients with high-level HCMV DNAemia, low-level HCMV DNAemia or no HCMV reactivation, and correlated with immune cell frequencies measured using mass cytometry. **Results.** Increased plasma levels of T helper 1-type cytokines/chemokines (TNF, IL-18, IP-10, MIG) were detected in patients with HCMV reactivation at the peak of HCMV DNAemia, relative to non-reactivators. Stem cell factor (SCF) levels were significantly higher before the detection of HCMV reactivation in patients who went on to develop high-level HCMV DNAemia (810–52 740 copies/mL) *vs.* low-level HCMV DNAemia (< 250 copies/mL). High-level HCMV reactivators, but not low-level reactivators, developed an elevated inflammatory cytokine/chemokine profile (MIP-1α, MIP-1β, TNF, LT-α, IL-13, IL-9, SCF, HGF) at the peak of reactivation. Plasma cytokine concentrations displayed unique correlations with circulating immune cell frequencies in patients with HCMV reactivation. **Conclusion.** This study identifies distinct circulating cytokine/chemokine signatures associated with the magnitude of HCMV DNAemia and the progression of HCMV reactivation after

allo-HSCT, providing important insight into immune recovery patterns associated with HCMV reactivation and viral control.

Keywords: allo-HSCT, chemokines, cytokines, human cytomegalovirus, transplant, viral reactivation

INTRODUCTION

Human cytomegalovirus (HCMV) reactivation is a common complication causing morbidity and mortality after allogeneic haematopoietic stem cell transplantation (allo-HSCT). Opportunistic HCMV reactivation occurs in up to two-thirds of seropositive allo-HSCT patients and is associated with negative impacts including the development of end-organ HCMV disease (seen in ~10% of patients), toxicities from antiviral drugs and increased risks of acute graft-*vs*.-host disease (aGvHD) and microbial infections.[1–5] Despite the availability of prophylactic and pre-emptive antiviral therapies,[6] HCMV reactivation continues to have a significant negative impact on transplant outcomes,[7,8] underlining the need for improved understanding of the immune parameters associated with HCMV reactivation and its impacts on immune recovery after allo-HSCT.

Cytokines and chemokines are secreted effector proteins with diverse roles in regulating inflammation, maintaining homeostasis and directing immune cell differentiation, proliferation, migration and function.[9] The circulating cytokine environment that accompanies immune cell reconstitution after allo-HSCT is highly dynamic,[10,11] with alterations in the cytokine milieu associated with post-transplant complications, including HCMV reactivation.[12–15] Previous studies have identified elevated levels of circulating pro-inflammatory cytokines such as interleukin (IL)-6, IL-8 and tumor necrosis factor (TNF) in allo-HSCT recipients with HCMV reactivation.[13–15] Inflammatory signalling is recognised as an important factor in promoting HCMV reactivation and replication,[16–19] and levels of TNF,[14] IL-6[9] or transforming growth factor-β1 (TGF-β1)[20] have been reported to predict HCMV reactivation or disease post-HSCT. Additionally, elevated plasma C-reactive protein (CRP) levels were reported to predict HCMV DNAemia episodes requiring pre-emptive antiviral therapy (PET).[20] While circulating cytokines may serve as clinically relevant biomarkers to predict reactivation, understanding of how cytokine patterns evolve during the course of HCMV reactivation post-HSCT and correlate with the magnitude of HCMV DNAemia is limited.

Here, we analysed the concentrations of 31 cytokines, chemokines and growth factors in plasma samples taken from allo-HSCT recipients at four time-points (before detection, at the initial detection, at the peak, and near the resolution, of HCMV DNAemia) in the first 100 days post-transplant (d.p.t.), using a multiplex bead-based immunoassay. We compared plasma cytokine/chemokine profiles between patients with high-level HCMV DNAemia (HR), low-level HCMV DNAemia (LR) or no HCMV reactivation, identifying an elevated T helper 1 (Th1)-associated inflammatory cytokine/chemokine profile in HR patients at the peak of HCMV DNAemia. We also investigated correlations between circulating cytokine levels and peripheral blood mononuclear cell (PBMC) subset frequencies in patients with and without HCMV reactivation. Our results contribute to a greater understanding of plasma cytokine dynamics over the course of HCMV reactivation and reveal distinct circulating cytokine/chemokine patterns associated with the magnitude of HCMV DNAemia after allo-HSCT.

RESULTS

Patient population

Plasma samples from 21 allo-HSCT recipients with HCMV reactivation (R) and 15 allo-HSCT recipients without HCMV reactivation (NR) in the first 100 days post-HSCT (Figure 1a) were studied. Patient and transplant characteristics, including donor type, donor/recipient HCMV serostatus, age, stem cell source, conditioning intensity, aGvHD incidence and HCMV reactivation parameters, are shown in Table 1. The median day of HCMV DNAemia detection was 31 (range 12–45) d.p.t. and peak HCMV DNA copies mL^{-1} in the cohort ranged from 150 to 52 740 (median 2690) copies mL^{-1} (Table 1). No patient had documented HCMV end-organ disease. Aside from recipient HCMV-serostatus, there were no significant differences in demographic or baseline transplant characteristics between R and NR groups (Table 1).

Emergence of elevated Th1-associated cytokine profile in allo-HSCT patients with HCMV reactivation

A 48-plex bead-based immunoassay was utilised to measure the concentrations of cytokines, chemokines and growth factors in plasma samples from four time-points in patients with HCMV reactivation (T1, before detection of HCMV reactivation; T2, initial detection of HCMV DNAemia; T3, peak of HCMV DNAemia; T4, near resolution of HCMV DNAemia) (Figure 1b), or from equivalent days post-transplant from patients without HCMV reactivation (Figure 1c; Supplementary figure 1). The timing of samples relative to transplant is shown in Figure 1c. A total of 138 plasma samples from 36 patients (3 or 4 samples per patient) were analysed over four assay plates. Intra-assay precision calculated from sample duplicates was < 15% coefficient of
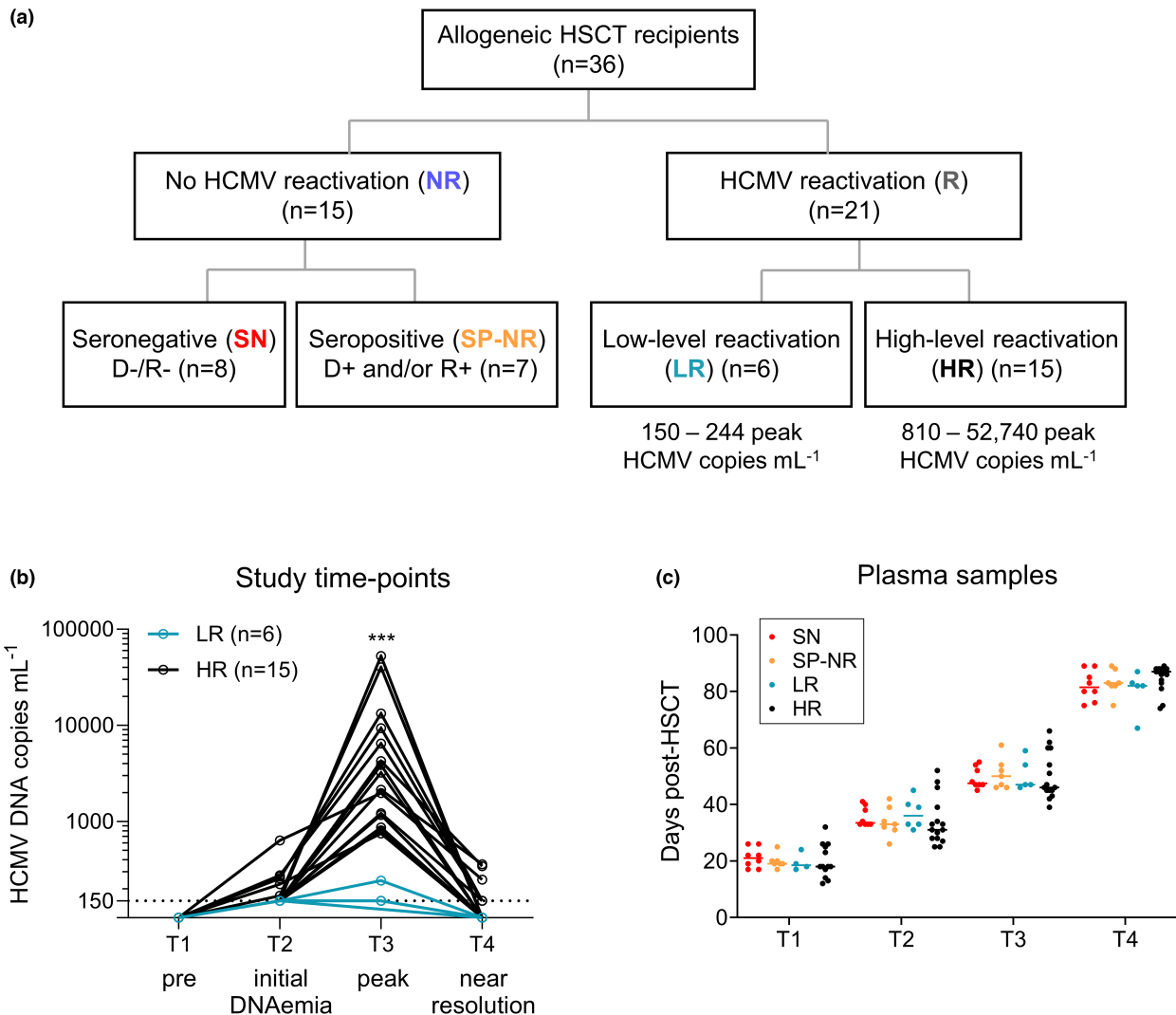

Figure 1. Outline of patient groups and plasma sample time-points. **(a)** Flowchart of patient subgroups analysed. **(b)** Human cytomegalovirus (HCMV) DNA copy numbers in plasma at the four time-points (T1-4) analysed with a bead-based multiplex plasma cytokine assay: T1, before detection of HCMV reactivation; T2, initial detection of HCMV DNAemia; T3, peak of HCMV DNAemia; T4, near the resolution of HCMV DNAemia. Statistical significance evaluated using two-tailed Mann Whitney *U*-tests comparing low-level HCMV reactivators (LR, blue) and high-level HCMV reactivators (HR, black) per time-point. ***P < 0.0001. T1, LR *n* = 4, HR *n* = 13. T2, LR *n* = 6, HR *n* = 15. T3, LR *n* = 5, HR *n* = 15. T4, LR *n* = 5, HR *n* = 15. **(c)** Plasma samples studied with the multiplex cytokine assay. Lines display the median. One-way ANOVA used to compare groups at each time-point (all not significant, *P* > 0.05). Median number of days between patient samples was 19 (range 5–49) days. SN, D−/R− HCMV seronegative (red); SP-NR, HCMV seropositive non-reactivators (orange); D, donor; R, recipient; +, HCMV seropositive; −, HCMV seronegative; HCMV, human cytomegalovirus; HSCT, haematopoietic stem cell transplant.

Table 1. Characteristics of HSCT recipients

Characteristic	All patients (n = 36)	SN (n = 8)	SP-NR (n = 7)	LR (n = 6)	HR (n = 15)	R vs. NR P-value	LR vs. HR P-value
Demographics							
Age, years	52 (18–70)	43.5 (31–70)	55 (19–60)	42.5 (24–62)	59 (18–70)	0.85	0.33
Sex (M:F)	17:19	7:1	3:4	2:4	5:10	0.090	> 0.9999
HCMV serostatus (n)							
D−/R−	8	8	0	0	0	ND	0.12
D+/R−	4	0	4	0	0		
D−/R+	6	0	0	0	6		
D+/R+	18	0	3	6	9		
Recipient HCMV serostatus (n)							
R+	24	0	3	6	15	< 0.0001	> 0.9999
R−	12	8	4	0	0		
Donor HCMV serostatus (n)							
D+	22	0	7	6	9	0.18	0.12
D−	14	8	0	0	6		
Underlying disease (n)							
AML	21	7	4	4	6	ND	ND
ALL	7	0	2	1	4		
MDS	3	0	1	0	2		
SAA	3	0	0	1	2		
MPD	2	1	0	0	1		
Transplant characteristics							
Conditioning (n)							
Myeloablative	13 (36%)	4 (50%)	3 (43%)	3 (50%)	3 (20%)	0.31	0.29
Reduced intensity	23 (64%)	4 (50%)	4 (57%)	3 (50%)	12 (80%)		
Stem cell source (n)							
Peripheral blood	33 (92%)	8 (100%)	7 (100%)	5 (83%)	13 (87%)	0.25	> 0.9999
Bone marrow	3 (8%)	0	0	1 (17%)	2 (13%)		
Donor type (n)							
Matched unrelated	24 (67%)	5	5	3	11	ND	ND
Haploidentical	3 (8%)	1	1	0	1		
HLA-identical related	9 (25%)	2	1	3	3		
T cell depletion (n)	21 (58%)	3 (38%)	3 (43%)	4 (67%)	11 (73%)	0.090	> 0.9999
ATG	18	3	3	3	9		
Alemtuzumab	3	0	0	1	2		
Post-transplant events							
HCMV reactivation (n)	21 (58%)	0	0	6 (100%)	15 (100%)	ND	ND
EBV reactivation (n)	17 (47%)	4 (50%)	2 (29%)	1 (17%)	10 (67%)	0.52	0.064
Acute GvHD (n)							
Overall (grade II-IV)	9 (25%)	2 (25%)	1 (14%)	0	6 (40%)	0.71	0.12
Severe (grade III-IV)	2 (6%)	1 (13%)	0	0	1 (7%)		
Relapse (n)	11 (31%)	3 (38%)	2 (29%)	1 (17%)	5 (33%)	> 0.9999	0.62
Death, first-year post-HSCT (n)	4 (11%)	0	1 (14%)	0	3 (20%)	0.63	0.53
HCMV reactivation characteristics							
Group	**All HCMV reactivators (n = 21)**	n/a	n/a	**LR (n = 6)**	**HR (n = 15)**	n/a	**LR vs. HR P-value**
Day post-HSCT of HCMV reactivation detection	31 (12–45)	n/a	n/a	35 (12–45)	29 (17–45)	n/a	0.56
Magnitude of first detected HCMV DNAemia (copies mL^{-1})	150 (150–269)	n/a	n/a	150 (150–150)	150 (150–269)	n/a	0.54
Peak HCMV DNA copies mL^{-1}	2690 (150–52740)	n/a	n/a	150 (150–244)	3380 (810–52740)	n/a	**< 0.0001**
HCMV DNAemia duration, days	33 (7–121)	n/a	n/a	21.5 (7–49)	38 (25–121)	n/a	**0.035**
$\log_{10}$ HCMV DNA AUC$_{0-100}$	4.46 (3.32–5.54)	n/a	n/a	3.69 (3.32–3.87)	4.72 (4.02–5.54)	n/a	**< 0.0001**
Resolution of HCMV DNAemia by day 100 post-HSCT	13 (62%)	n/a	n/a	6 (100%)	8 (53%)	n/a	0.061
Pre-emptive antiviral therapy for HCMV reactivation	16 (76%)	n/a	n/a	1 (17%)	15 (100%)	n/a	**0.0003**

variation (CV) for all analytes. Inter-assay precision, calculated from the kit's quality control (QC) sample across the four plates, was median 12.1 %CV (range 2.7–100.1 %CV). For QC purposes, analytes with an inter-assay %CV $\geq$ 30 (*n* = 7) (Supplementary table 1) were excluded from analysis. A further 10 analytes which were not detected in any plasma sample on one or more plates were also excluded (Supplementary table 1), resulting in the retention of 31/48 analytes for further analysis.

We first compared plasma concentrations of the 31 analytes that passed QC between patients with (R) and without (NR) HCMV reactivation. While no differences in cytokine concentrations were seen at T1 (Figure 2), a collection of five cytokines/chemokines (TNF, IL-18, IFN-γ-induced protein 10 kDa [IP-10], monokine induced by IFN-γ [MIG], IL-2) associated with T helper type 1 (Th1) responses became elevated in R compared to NR patients at T2 or T3 (Figure 2). Higher levels of TNF (*P* = 0.019), IL-2 (*P* = 0.028) and IP-10 (*P* = 0.012) were seen at T2 in R compared to NR patients (Figure 2). TNF and IP-10 concentrations further increased in R patients at T3, and together with IL-18 (*P* = 0.0027) and MIG (*P* = 0.0044) were significantly higher in R compared to NR at T3 (Figure 2). Mean levels of TNF, MIG and IP-10 declined in R patients at T4, with only MIG (*P* = 0.030) and cutaneous T-cell-attracting chemokine (CTACK) (*P* = 0.025) significantly higher in R *vs.* NR patients at T4 (Figure 2). Overall, these results illustrate the emergence of an elevated Th1-like cytokine profile in allo-HSCT patients experiencing HCMV reactivation.

Early plasma cytokine patterns differ in low-level HCMV reactivators and high-level HCMV reactivators

To investigate plasma cytokine patterns associated with the magnitude of HCMV DNAemia, patients with HCMV reactivation were divided into LR

(*n* = 6), who maintained HCMV DNAemia levels near to or below the lower limit of quantitation (LLQ) (150 [150–244] peak copies mL^{-1}) and largely resolved HCMV DNAemia without PET; and HR (*n* = 15), who developed clinically significant infection with higher peak viral loads (3380 [810–52 740] copies mL^{-1}; *P* < 0.0001) and a longer duration of HCMV DNAemia (*P* = 0.035) (Figure 1a; Table 1). There were no significant differences in baseline transplant characteristics between the HR and LR subgroups (Table 1). Non-reactivators were divided by pre-transplant HCMV-serostatus into seronegative (SN; D−/R−; *n* = 8) and seropositive (SP-NR; D+ and/or R+; *n* = 7) groups (Figure 1a; Table 1).

Analyte concentrations were compared between LR and HR at each time-point (Supplementary figure 2b). The earliest difference detected was lower levels of stem cell factor (SCF) in LR compared to HR at T1 (28.7 [7.1–67.3] *vs.* 79.2 [30.1–169.1] pg mL^{-1}; *P* = 0.042) (Figure 3a). Mean SCF levels appeared elevated in the HR group at all time-points examined, and were significantly higher in HR relative to LR (*P* = 0.040) and SP-NR (*P* = 0.031) at T3 (Figure 3a). No other analytes differed significantly between LR and HR at T1, and no differences between LR and HR were detected at T2 (Supplementary figures 2b, 3). As SCF is a haematopoietic cytokine which can promote thymopoiesis,[21] we analysed the relationship between plasma SCF levels and naïve T cell counts at corresponding timepoints in 31/36 of the current patients, for which immune cell frequencies were measured in a previous study by mass cytometry.[22] Naïve CD4+ T cell counts were higher in non-reactivators compared to HCMV reactivators, although SCF levels were not found to correlate with naïve T cell counts and there were no significant differences in naïve T cell counts between LR and HR (Supplementary figure 4).

To further understand early plasma cytokine dynamics in LR and HR patients, changes in
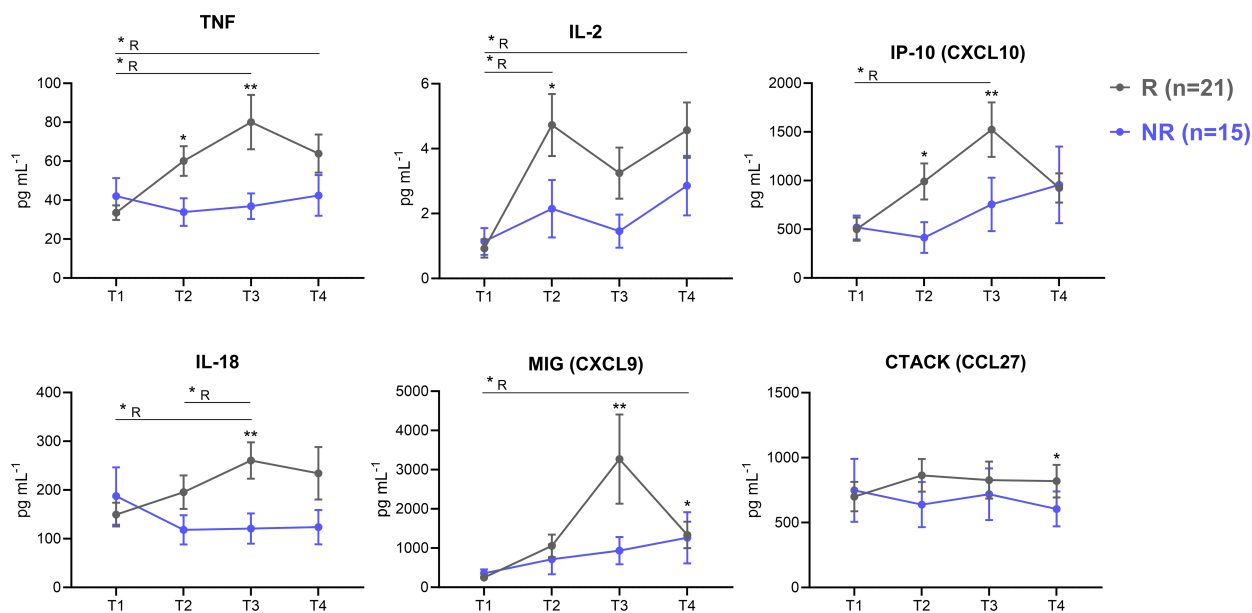
Figure 2. Development of an elevated Th1 associated plasma cytokine profile in allo-HSCT patients with HCMV reactivation. Concentrations (pg mL^{-1}) of 31 analytes in plasma samples from allo-HSCT recipients who experienced human cytomegalovirus (HCMV) reactivation (R; grey, $n = 21$) or who did not experience HCMV reactivation (NR; blue, $n = 15$) were measured using a multiplex bead-based assay. Analytes found to be significantly different between the R group and NR group at one or more time-points are shown. The time-points assessed were: T1, before detection of HCMV reactivation; T2, initial detection of HCMV DNAemia; T3, peak of HCMV DNAemia; T4, near the resolution of HCMV DNAemia; or equivalent days post-transplant from NR patients. Graphs display mean ± SEM. Statistical significance between patient groups was determined by Mann–Whitney *U*-tests. Comparisons between time-points were evaluated using two-way mixed effects models with Tukey's multiple comparisons test. $P < 0.05$ was considered significant. *$P < 0.05$; **$P < 0.01$. CCL27, C-C motif chemokine ligand 27; CTACK, cutaneous T cell-attracting chemokine; CXCL10, C-X-C motif chemokine ligand 10; CXCL9, C-X-C motif chemokine ligand 9; IL-18, interleukin 18; IL-2, interleukin 2; IP-10, interferon-γ induced protein 10 kDa; MIG, monokine induced by interferon-γ; TNF, tumor necrosis factor.

circulating cytokine concentrations between T1 and T2 were explored. Three analytes (TNF, IL-2, macrophage colony-stimulating factor [M-CSF]) exhibited significant increases between T1 and T2 in the HR group only ($P = 0.028$, $P = 0.016$ and $P = 0.0003$, respectively; Figure 3b). No significant changes in cytokine concentrations between T1 and T2 in the LR group were detected. Of note, however, TNF levels increased between T1 and T2 in both LR and HR (mean fold increase 2.3 ± 0.8 and 1.8 ± 0.3 per patient, respectively), while decreasing in the SP-NR group between T1 and T2 (Figure 3c). TNF levels at T2 were significantly higher in HR ($P = 0.048$), but not LR ($P = 0.18$), relative to SP-NR (Figure 3b). Previous studies have noted an association between elevated circulating TNF levels and HCMV reactivation in HSCT patients,[13,14] and proposed high TNF levels as a predictive marker for HCMV reactivation.[14] While TNF levels did not differ between patient groups before the detection of HCMV DNAemia (T1) in the current cohort (Figures 2 and 3b), these findings suggest an early rise in

plasma TNF levels may be an indicator of the development of HCMV DNAemia in HCMV-seropositive (D+ and/or R+) patients.

Inflammatory plasma cytokine/chemokine profile at the peak of HCMV DNAemia in high-level reactivators

At the peak of HCMV DNAemia (T3), HR patients developed a markedly distinct inflammatory plasma cytokine/chemokine profile with significantly elevated concentrations of chemokines (macrophage inflammatory protein 1α [MIP-1α], MIP-1β), cytokines (TNF, lymphotoxin-α [LT-α], IL-13, IL-9) and growth factors (SCF, hepatocyte growth factor [HGF]) relative to LR (Figure 4a and c). Many of these factors, as well as MIG, IL-18 and IP-10 (Figure 4b), were also higher in HR relative to patients without reactivation (SN and/or SP-NR) (Figure 4a–c). In contrast, relatively few differences in analyte concentrations were seen between LR and SP-NR; lower levels of
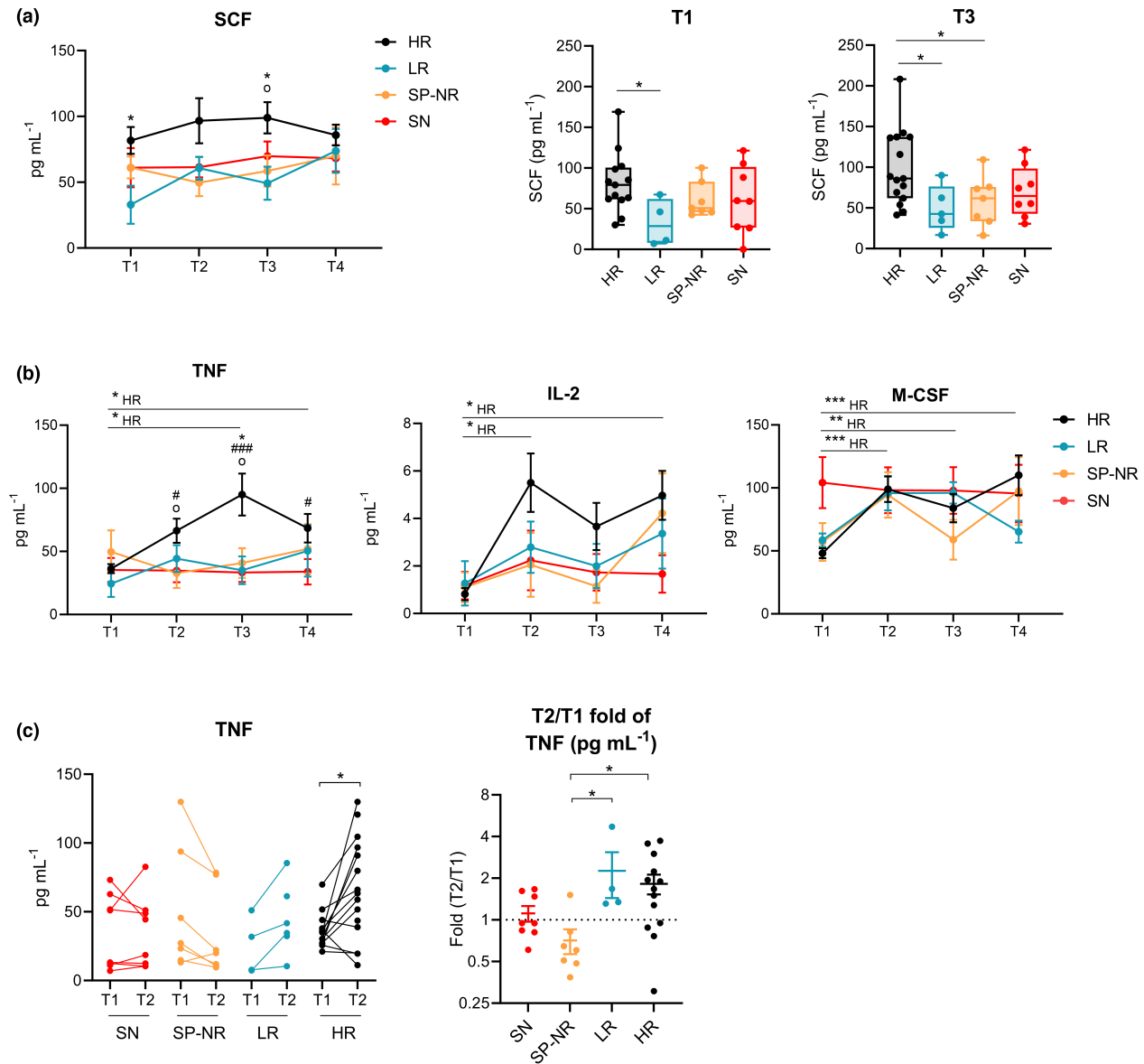
Figure 3. Early plasma cytokine profiles in low-level HCMV reactivators and high-level HCMV reactivators. **(a)** Stem cell factor (SCF) concentrations (pg mL^{-1}) in plasma samples from allo-HSCT patients with high-level HCMV reactivation (HR; black, $n = 15$), low-level HCMV reactivation (LR; blue, $n = 6$), D−/R− HCMV seronegative patients (SN; red, $n = 8$) and HCMV seropositive (D+ and/or R+) patients without HCMV reactivation (SP-NR; orange, $n = 7$). *Left*: Mean ± SEM is shown. Significance determined by Mann–Whitney *U*-tests. *$P < 0.05$ for HR *vs.* LR; $^{\circ}P < 0.05$ for HR *vs.* SP-NR. *Right*: Box plot lines display min-max with interquartile range and median. Each symbol represents one patient sample. Significance determined by Kruskal–Wallis tests; *$P < 0.05$. **(b)** Plasma concentrations of tumor necrosis factor (TNF), interleukin 2 (IL-2) and macrophage colony-stimulating factor (M-CSF). Mean ± SEM is shown. For symbols directly above time-points: *$P < 0.05$ for HR *vs.* LR; $^{\circ}P < 0.05$ for HR *vs.* SP-NR; $^{\#}P < 0.05$ and $^{\#\#\#}P < 0.001$ for HR *vs.* SN, determined by Mann–Whitney *U*-tests. Horizontal lines show significant differences between time-points in the indicated patient group, as determined by two-way mixed effects models with Tukey's multiple comparisons test; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. **(c)** *Left*: TNF plasma concentrations at T1 and T2. Lines connect samples from individual patients. Significance was determined by the two-tailed Wilcoxon test. *Right*: Fold change of plasma TNF levels (pg mL^{-1}) between T1 and T2. Lines show mean ± SEM. Significance determined by Kruskal–Wallis with Dunn's multiple comparisons test; *$P < 0.05$. T1, before detection of HCMV reactivation; T2, initial detection of HCMV DNAemia; T3, peak of HCMV DNAemia; T4, near the resolution of HCMV DNAemia. D, donor; HCMV, human cytomegalovirus; R, recipient.
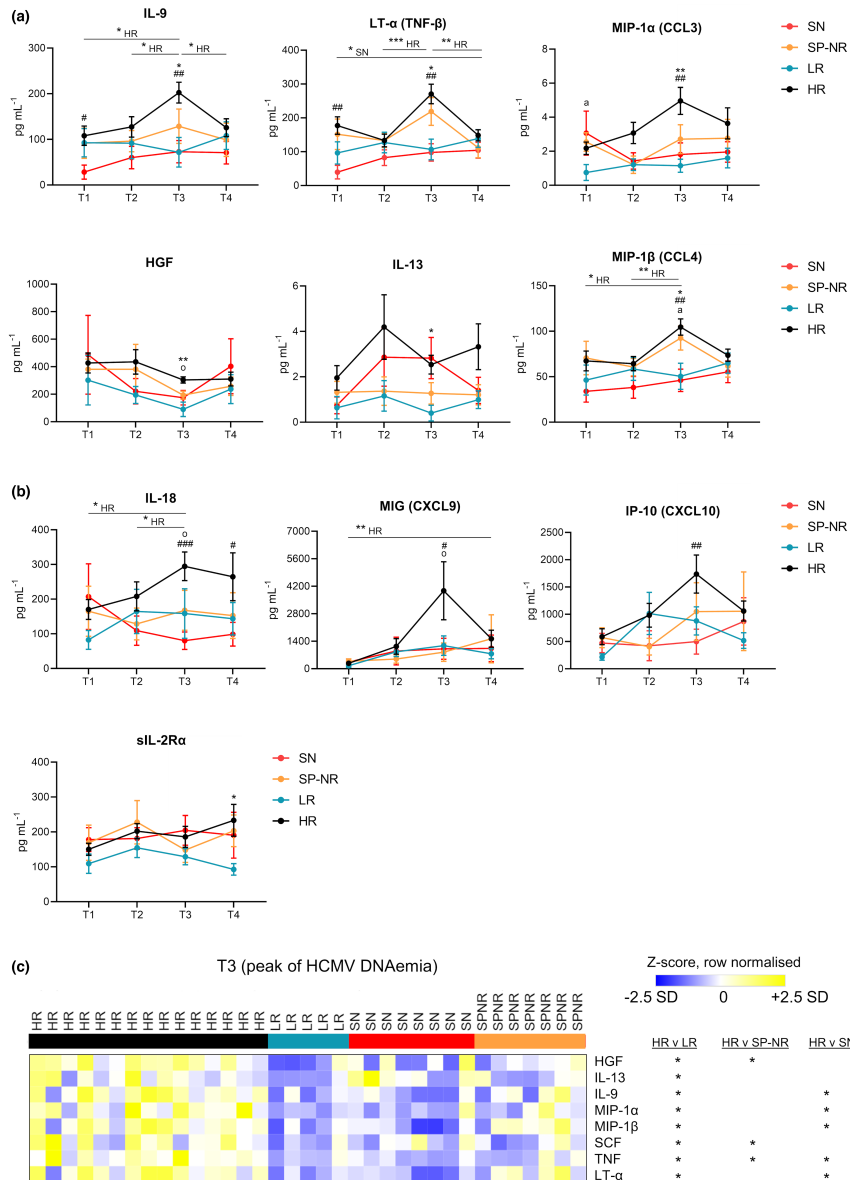
Figure 4. Elevated plasma cytokine profile at peak of HCMV DNAemia in patients with high-level HCMV reactivation. **(a)** Concentrations (pg mL^{-1}) of interleukin 9 (IL-9), lymphotoxin α (LT-α)/tumor necrosis factor β (TNF-β), macrophage inflammatory protein 1α (MIP-1α)/C-C motif chemokine ligand 3 (CCL3), hepatocyte growth factor (HGF), interleukin 13 (IL-13), macrophage inflammatory protein 1β (MIP-1β)/C-C motif chemokine ligand 4 (CCL4) in plasma samples from allo-HSCT patients with high-level HCMV reactivation (HR; black, $n = 15$), low-level HCMV reactivation (LR; blue, $n = 6$), D−/R− HCMV seronegative patients (SN; red, $n = 8$) and HCMV seropositive (D+ and/or R+) patients without HCMV reactivation (SP-NR; orange, $n = 7$). **(b)** Plasma concentrations of interleukin 18 (IL-18), monokine induced by interferon-γ (MIG)/C-X-C motif chemokine ligand 9 (CXCL9), interferon-γ induced protein 10 kDa (IP-10) and soluble IL-2 receptor α chain (sIL-2Rα). For **a** and **b**, mean ± SEM is shown. Significance between patient groups determined by Mann–Whitney U-tests: *$P < 0.05$ and **$P < 0.01$ for HR *vs.* LR; #$P < 0.05$, ##$P < 0.01$ and ###$P < 0.001$ for HR *vs.* SN; °$P < 0.05$ for HR *vs.* SP-NR; ^{a}P < 0.05$ for LR *vs.* SP-NR. Horizontal lines show significant differences in analyte concentrations between time-points in the indicated patient group, as determined by two-way mixed effects models with Tukey's multiple comparisons test; *$P < 0.05$, **$P < 0.01$. **(c)** Heatmap illustrating analytes which differed significantly in plasma concentration between HR and LR at T3, as determined by Mann–Whitney U-tests. Rows show analytes and columns represent individual patients, labelled and ordered by patient group. Heatmap displays the Z-score normalised plasma cytokine concentrations per row. Heatmap generated using the Morpheus web tool (Broad Institute; https://software.broadinstitute.org/morpheus). On the right, * indicates statistical significance ($P < 0.05$; Mann–Whitney U-tests) per analyte between HR and the listed patient groups. At T3: HR $n = 15$, LR $n = 5$, SN $n = 8$, SP-NR $n = 7$. T1, before detection of HCMV reactivation; T2, initial detection of HCMV DNAemia; T3, peak of HCMV DNAemia; T4, near the resolution of HCMV DNAemia. D, donor; HCMV, human cytomegalovirus; R, recipient.

MIP-1α at T1 (*P* = 0.024) and MIP-1β levels at T3 (*P* = 0.048) were the only differences observed in LR compared to SP-NR (Figure 4a; Supplementary figure 2d). A suite of cytokines/chemokines (TNF, IL-9, MIP-1β, MIP-1α, LT-α, MIG, IP-10) exhibited similar dynamics in the HR group, increasing to their highest concentration at T3 and subsequently declining at T4 (Figures 3b and 4a and b). This same pattern was not evident in the LR group (Figures 3b and 4a and b) and no analyte was detected at higher levels in LR relative to HR at any time-point (Supplementary figure 2b). Finally, at T4, lower concentrations of soluble IL-2 receptor α-chain (sIL-2Rα; CD25) were identified in LR *vs*. HR (*P* = 0.012) (Figure 4b), representing the only difference detected between these groups at T4. Together, these data reflect an elevated inflammatory plasma cytokine profile in HR patients at the peak of HCMV DNAemia, and indicate no wide-ranging modulation of the plasma cytokine environment in LR patients relative to non-reactivators.

Correlations between plasma cytokine concentrations and peak HCMV viral load

To further explore the relationship between plasma cytokine concentrations and HCMV DNAemia magnitude, Spearman correlations between peak HCMV DNA copies mL^{-1} and concentrations of the 31 analytes at each time-point were assessed (Figure 5a). Significant positive correlations were detected between peak HCMV copies mL^{-1} and a range of analytes (sIL-2Rα, IL-8, IP-10, monocyte chemoattractant protein 1 [MCP-1], M-CSF, MIP-1α, TNF), predominantly at T4 (Figure 5a). sIL-2Rα concentrations at T1 (*r* = 0.5077, *P* = 0.039), T2 (*r* = 0.5616, *P* = 0.0081), and most strongly at T4 (*r* = 0.7660, *P* < 0.0001) were significantly positively correlated with peak HCMV DNA copies mL^{-1} (Figure 5a and b). A negative correlation between peak HCMV copies mL^{-1} and platelet-derived growth factor BB (PDGF-BB) levels at T4 (*r* = −0.6868, *P* = 0.0008) was also detected (Figure 5a and c).

Relationships between circulating immune cell frequencies and plasma cytokine concentrations in patients with HCMV reactivation

To obtain a more global view of immune profiles in allo-HSCT recipients with or without HCMV reactivation, correlations between plasma cytokine concentrations and PBMC frequencies, measured using mass cytometry in our previous study,[22] at matched time-points were evaluated. Immune cell frequency measurements were available for 31 of 36 patients in the current cohort. Pairwise correlations of plasma cytokines (*n* = 31) with PBMC subset percentages (*n* = 88) and absolute cell counts (×10^9/L blood, *n* = 77) were performed for each patient group, using a false discovery rate (FDR) of 1% to minimise false-positive associations.

A suite of significant correlations between plasma cytokines (predominantly MIG and sIL-2Rα) and immune cell percentages (mainly γδ T-cells, CD3$^+$CD56$^+$ cells and additional T-cell subsets) were detected in the HR group (Supplementary table 2). Two of the most prominent correlations in HR were positive correlations of MIG (*r* = 0.6770, *q* = 0.0012) (Figure 6a) and sIL-2Rα (*r* = 0.6593, *q* = 0.0016) (Figure 6b) with γδ T-cell percentages (Figure 6a and b). In the LR group, a collection of lymphocyte subset counts (×10^9/L) positively correlated with IP-10 and MIG plasma concentrations (Supplementary table 3). The strongest correlation in LR was between IP-10 and ICOS$^+$HLA-DR$^+$CD38$^+$CD4$^+$ T-cell counts (*r* = 0.8632, *q* = 0.0023) (Figure 6c). No significant correlations between immune cell frequencies and plasma cytokines were detected in the SN or SP-NR groups. Overall, distinct correlation patterns between plasma cytokines and PBMC subset frequencies are evident in allo-HSCT patients experiencing HCMV reactivation, suggestive of different underlying immune patterns in HR, LR and non-reactivators.

DISCUSSION

Here, we show that dynamic changes in plasma cytokine/chemokine concentrations occur during the course of HCMV DNAemia in allo-HSCT patients, and report the development of an elevated inflammatory, Th1-associated cytokine/chemokine profile in individuals who experience high-level HCMV reactivation. Our results reveal circulating cytokine patterns which correlate with the progression of HCMV reactivation and the magnitude of viral load, providing insight into immune biomarkers which can be harnessed to better understand the pathogenesis of HCMV reactivation and immune responses during active HCMV DNAemia episodes following allo-HSCT.

Previous work has identified circulating inflammatory markers (*e.g.* TNF or IL-6) as predictive
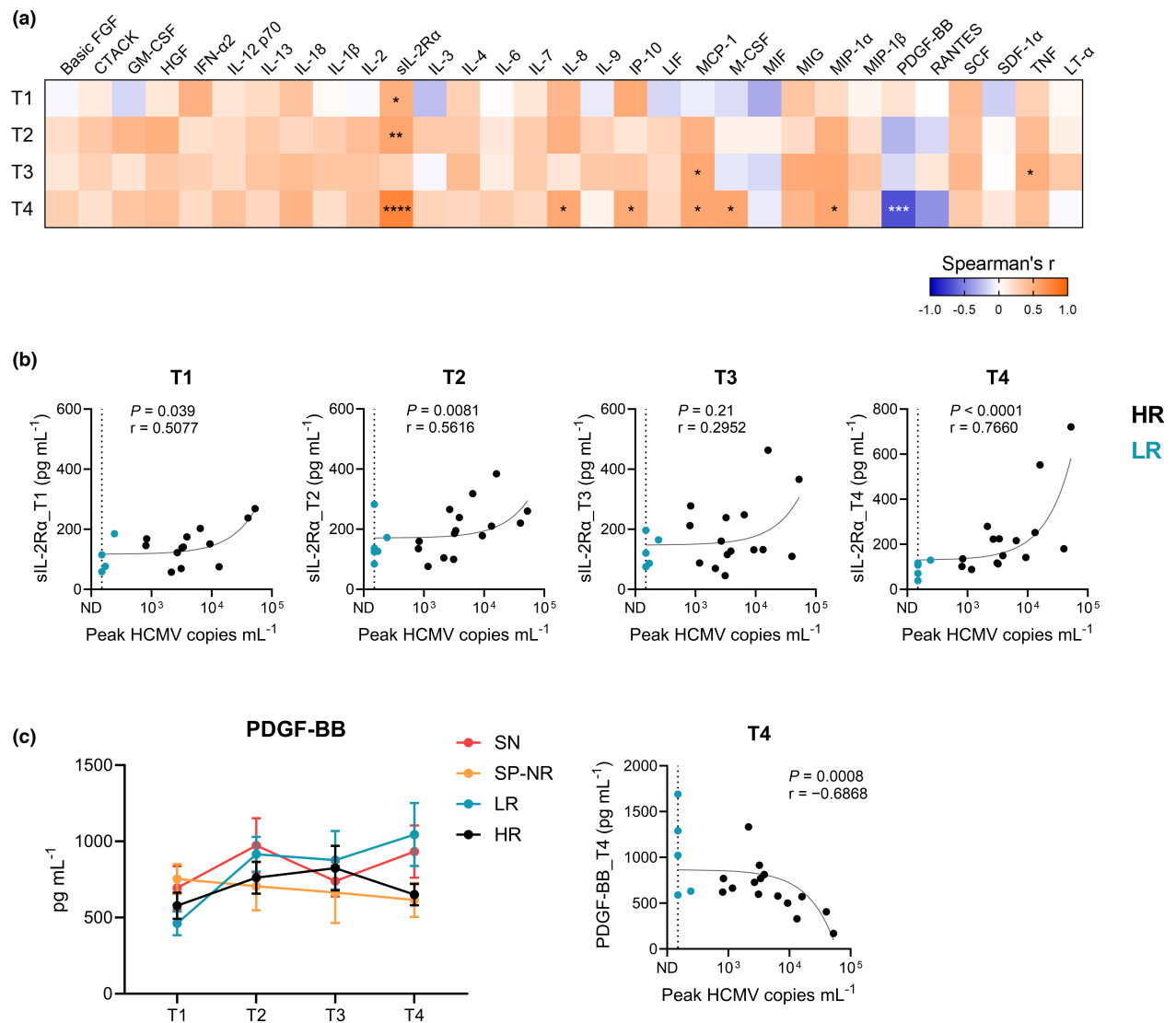
Figure 5. Correlations between plasma cytokine concentrations and HCMV viral load in allo-HSCT patients with HCMV reactivation. **(a)** Spearman's rank correlation coefficients (r) between peak human cytomegalovirus (HCMV) DNA copies mL^{-1} and the plasma concentrations (pg mL^{-1}) of 31 analytes in patients with HCMV reactivation ($n = 21$). Significant correlations are indicated by $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$. **(b)** Two-tailed Spearman correlations between soluble IL-2 receptor α chain (sIL-2Rα) concentrations (pg mL^{-1} in plasma) and peak HCMV DNA copies mL^{-1} in allo-HSCT patients who experienced HCMV reactivation: high-level reactivators (HR, black symbols) and low-level reactivators (LR, blue symbols). A simple linear regression line is shown. Vertical black dotted line indicates the lower limit of quantitation of HCMV DNAemia (150 copies mL^{-1}). **(c)** *Left*: Platelet-derived growth factor BB subunits (PDGF-BB) concentrations (pg mL^{-1}) in plasma samples from HR ($n = 15$, black), LR ($n = 6$, blue), HCMV seropositive (D+ and/or R+) patients without HCMV reactivation (SP-NR, orange; $n = 7$) and D−/R− HCMV seronegative patients (SN, red; $n = 8$). Mean ± SEM is shown. *Right*: Two-tailed Spearman correlation between PDGF-BB concentrations at T4 and peak HCMV DNA copies mL^{-1} in reactivators (LR and HR). A simple linear regression line is shown. Vertical black dotted line indicates the lower limit of quantitation (150 HCMV DNA copies mL^{-1}). r denotes the Spearman correlation coefficient. T1, before detection of HCMV reactivation; T2, initial detection of HCMV DNAemia; T3, peak of HCMV DNAemia; T4, near the resolution of HCMV DNAemia. D, donor; R, recipient.

of HCMV reactivation or disease post-HSCT.[13,14] HCMV infection in solid organ transplant (SOT) recipients and healthy seropositive individuals is associated with the induction of systemic

inflammation and Th1-polarised cytokine responses.[23] HCMV-induced immunomodulatory effects, partly through the provocation of inflammatory cytokine responses and the production of virally encoded

(a)

MIG vs. γδ-T cells (% live)

(b)

sIL-2Rα vs. γδ-T cells (% live)

(c)

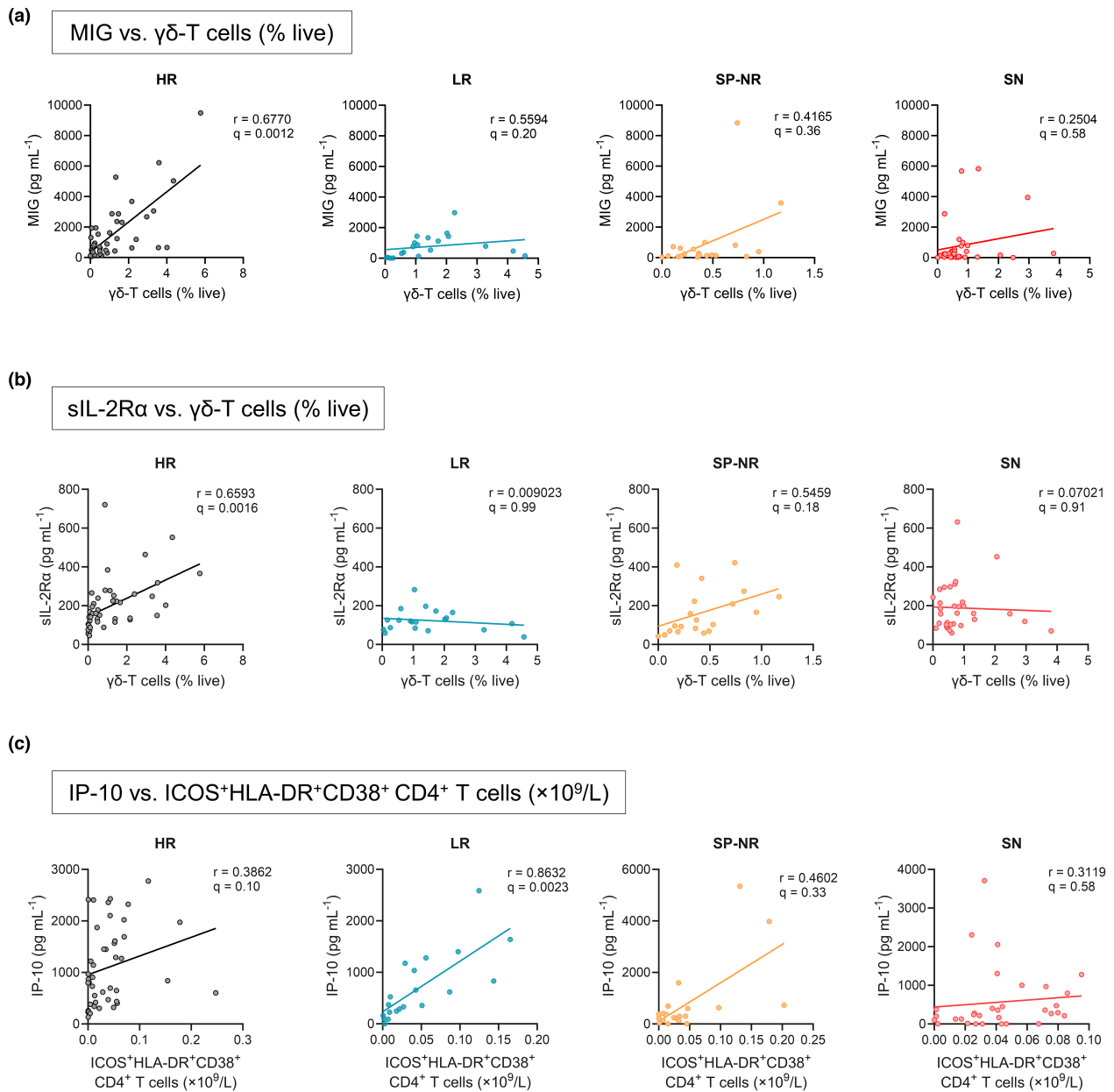IP-10 vs. ICOS⁺HLA-DR⁺CD38⁺ CD4⁺ T cells (×10⁹/L)

Figure 6. Correlations between plasma cytokine concentrations and immune cell subset frequencies in patients with HCMV reactivation. Two-tailed Spearman correlations between plasma cytokine concentrations (pg mL^{-1}) ($n = 31$ analytes) and paired peripheral blood mononuclear cell frequencies (percentages [$n = 88$] or absolute counts [$n = 77$]) were performed in each patient group, all time-points combined. Immune cell frequency measurements were available for 31 patients (HR $n = 12$, LR $n = 6$, SP-NR $n = 5$, SN $n = 8$). Plasma cytokine concentrations were measured by multiplex bead-based assay, and immune cell frequencies were measured by mass cytometry. False discovery rate (FDR) adjusted *P*-values (q) < 0.01 were considered significant. Spearman correlations between **(a)** monokine induced by interferon-γ (MIG) plasma concentration and γδ-T cell percentages (% of live cells), **(b)** soluble IL-2 receptor α chain (sIL-2Rα) plasma concentration and γδ-T cell percentages (% of live cells), and **(c)** interferon-γ induced protein 10 kDa (IP-10) plasma concentration and ICOS⁺HLA-DR⁺CD38⁺ CD4⁺ T cell counts (×10⁹ L^{-1}), in each patient group. Each symbol represents one plasma sample. r denotes the Spearman correlation coefficient. Simple linear regression lines are shown. High-level HCMV reactivators (HR, black; $n = 44$ samples), low-level HCMV reactivation (LR, blue; $n = 21$ samples), HCMV seropositive (D+ and/or R+) patients without HCMV reactivation (SP-NR, orange; $n = 20$ samples) and D−/R− HCMV seronegative patients (SN, red; $n = 32$ samples). HCMV, human cytomegalovirus.

human cytokine/chemokine homologues,[24] are thought to be linked to a variety of negative impacts on the host,[25] such as allograft rejection, aGvHD and bacterial/fungal infections in transplant recipients. Few studies have mapped the expression patterns of multiple circulating cytokines/chemokines across the course of HCMV DNAemia and investigated their relationship with viral load after allo-HSCT.[13] HCMV viral load magnitude and kinetics are of clinical significance as higher viral loads are associated with prolonged viraemia, higher antiviral therapy requirements, and increased risks of end-organ disease and non-relapse mortality.[7,26–28]

Here, we identified that while high-level HCMV reactivation is associated with the appearance of a heightened inflammatory cytokine/chemokine signature, seen prominently at the peak of HCMV DNAemia, allo-HSCT patients who effectively control HCMV DNAemia to low levels (< 250 copies mL^{-1}) do not exhibit a similar inflammatory profile. Instead, they display cytokine profiles more akin to patients without detectable HCMV DNAemia. The recovery of polyfunctional HCMV-specific T-cells expressing Th1 cytokines is associated with protection from clinically significant HCMV reactivation post-HSCT,[29,30] and it may be that in low-level reactivators, viral replication is able to be effectively controlled locally in the absence of an elevated systemic inflammatory response. In addition to Th1 cytokines (*e.g.* TNF, LT-α, IP-10, MIP-1β), elevated levels of IL-9 and IL-13, cytokines associated with type 2 inflammatory responses,[31,32] were found in high-level reactivators at the peak of HCMV DNAemia. It will be important to determine whether the heightened cytokine/ chemokine profile seen in HR is reflective of immune activation favorable for viral control, or might represent a maladaptive, immunopathological response to HCMV reactivation or tissue damage resulting from lytic HCMV replication. In this respect, inflammation is known to enhance HCMV replication and promote reactivation,[18,19] and many of the circulating mediators identified at increased levels in patients with high-level HCMV DNAemia, including TNF, LT-α, MIP-1α, MIP-1β, IP-10, MIG and HGF, have been identified in the secretomes of fibroblasts and/ or endothelial cells productively infected with HCMV.[33,34]

Early differences in circulating cytokine profiles were identified in this study in patients who developed HCMV reactivation. This included increased TNF levels between T1 and T2, supporting the findings of previous studies demonstrating a relationship between increased TNF levels and HCMV reactivation.[13,14] The identification of a circulating cytokine signature allowing early recognition of clinically significant reactivation would be of great clinical value, as this could assist clinicians in optimising antiviral treatment decisions to minimise HCMV-related morbidity.[20] Here, we found significantly higher SCF levels prior to the detection of reactivation in patients who developed high-level *vs.* low-level HCMV DNAemia. SCF is a cytokine which promotes haematopoiesis and thymopoiesis,[21,35] and is also of interest given a potential relationship between SCF, thymopoiesis and HCMV reactivation. Elevated plasma SCF levels were found to be associated with higher mortality in double umbilical cord blood transplant recipients, and inversely correlated with naïve CD4$^+$ T-cell recovery and T cell receptor excision circle (TREC) counts.[36] We previously observed slow CD4$^+$ T-cell reconstitution in high-level HCMV reactivators in the first 100 days post-HSCT,[22] and HCMV reactivation has been associated with reduced naïve T-cell recovery and impaired thymopoiesis after allo-HSCT, perhaps as a consequence of thymic damage from HCMV infection or GvHD.[37] Given the observation of elevated SCF levels in high-level HCMV reactivators in this study, further investigation into the functional relevance and importance of SCF in HCMV infection after allo-HSCT is warranted. Assessment of TREC levels (a marker of recent thymic output[38]) in parallel with SCF in allo-HSCT patients who develop HCMV reactivation would be of interest in future studies.

Our study also reveals correlation patterns of soluble mediators with peak viral copy numbers and PBMC subset frequencies in allo-HSCT patients with HCMV reactivation. While correlations do not imply direct relationships, these findings suggest distinct expression patterns of cellular and soluble immune factors are associated with HCMV DNAemia magnitude in the first 100 d.p.t. A strong relationship between peak HCMV copy numbers and sIL-2Rα concentrations at T4 was observed in patients with reactivation. IL-2Rα (CD25) is predominantly expressed on the surface of activated T-cells and Tregs, and increased shedding of surface IL-2Rα is observed in inflammatory conditions involving T-cell activation, including viral infections and GvHD.[23,39–43] Also at T4, PDGF-BB levels were found to negatively correlate with peak HCMV load. PDGF-BB, a mesenchymal cell mitogen, is among a suite of proteins associated with

angiogenesis and wound healing found in the secretome of HCMV-infected fibroblasts *in vitro*.[33]

Interestingly, LR and HR patients exhibited differing immune cell-cytokine correlation patterns, which included relationships between $\gamma\delta$ T-cell percentages and plasma sIL-2Rα and MIG concentrations in the HR group, and between activated (ICOS$^+$HLA-DR$^+$CD38$^+$) CD4$^+$ T-cell counts and plasma IP-10 concentrations in the LR group. IP-10 and MIG are both IFN-γ-inducible CXCR3 ligands which act as chemoattractants for T-cells and NK-cells.[44,45] While correlations do not imply direct relationships, these distinct correlations in LR and HR warrant further investigation to establish the extent to which these factors contribute to the control of reactivation, and whether these immune patterns could be modulated to achieve favorable immune recovery outcomes. Further studies using intracellular cytokine staining and flow or mass cytometry[46,47] will be informative in defining the cytokine expression profiles of specific lymphocyte subsets at the single-cell level in these samples.

A number of cytokines were excluded from analysis (*e.g.* IFN-γ and IL-10) because of poor detectability and/or inter-plate variability in analyte quantitation within the QC sample. IL-10, in particular, should be further investigated as increased IL-10 levels are reported to correlate with HCMV reactivation and disease post-SOT,[41,48] and may function to counterbalance the inflammatory cytokine profile seen in HR patients. Strategies applied in the current study to minimise the potential influences of technical and/or inter-plate variability included the use of internal standards, exclusion of analytes with high inter-assay variation and use of a statistical batch adjustment method. The probability of all analytes simultaneously meeting QC specifications has been noted to be lower in multiplex assays than in a single-plex assay,[49,50] and the requirement to use a single sample dilution factor in multiplex assays may contribute to limitations in detecting analytes present in low concentrations.

As a subset of HR (6/15), but no LR, patients also had aGvHD, future studies with larger patient numbers should compare plasma cytokine profiles in high-level reactivators with and without aGvHD, to determine specific signatures associated with these complications. It should also be noted that the viraemia threshold used to define high-level HCMV reactivation varies between studies.[26,51,52] Finally, to minimise the number of statistical comparisons, we did not compare all pairs of patient groups (*e.g.* SN and SP-NR). Analysis of cytokine/chemokine profiles in allo-HSCT patients stratified by recipient and donor HCMV-serostatus[53] may also be important to consider further in future cohorts.

In conclusion, this study shows that high-level HCMV reactivation in the first 100 days after allo-HSCT is associated with a marked modulation of circulating cytokine/chemokine profiles, including the development of an elevated, Th1-like inflammatory cytokine profile at the peak of HCMV DNAemia, which was not seen in low-level reactivators. Further characterisation of immune patterns which correlate with the progression of HCMV reactivation and viral load magnitude may provide insight into immune recovery profiles and biomarker pathways which could be targeted to improve viral control and limit high-level HCMV reactivation after allo-HSCT.

METHODS

Study subjects

Thirty-six adults undergoing allo-HSCT for haematological diseases at Westmead Hospital (Sydney, Australia) between 2015 and 2017 were studied. Patients were recruited sequentially, and plasma specimens prospectively collected weekly until 100 d.p.t. Patients with at least three plasma samples available for study were analysed. Patient clinical characteristics are shown in Table 1. The patients included were representative of the overall group of patients enrolled during the study interval. While those who had a very poor trajectory and early death were not analysed because of limited sample collection, this was a minority of patients. GvHD prophylaxis strategy was as per institutional practice and all patients having transplant for malignant and non-malignant diseases were eligible for this observational study. No aspect of the transplant procedure was mandated by this study. For matched related and unrelated donors patients received methotrexate and calcineurin inhibitors and haploidentical donors received post-transplant cyclophosphamide and calcineurin inhibitors. The study had ethical approval from the University of Sydney and Western Sydney Local Health District ethics committees (2014/440). Written informed consent was obtained from all participants in accordance with the Declaration of Helsinki.

Isolation of plasma samples

Peripheral blood collected in ethylenediaminetetraacetic acid vacutainers was centrifuged at 800 × g for 5 min at room temperature (RT). Plasma was removed and immediately frozen at −80°C in aliquots.

HCMV DNAemia monitoring and treatment

Patients who were HCMV-seropositive prior to transplant (R+) and/or had a HCMV-seropositive donor (D+) underwent weekly plasma HCMV DNAemia surveillance until 100 d.p.t. using quantitative polymerase chain reaction (qPCR) (COBAS® AmpliPrep/COBAS® TaqMan® CMV Test; Roche). The LLQ is 150 HCMV DNA copies mL^{-1} (1 copy = 0.91 International Units).[54] HCMV reactivation was defined as the detection of HCMV DNA in plasma. Clinically significant infection was defined as HCMV DNAemia requiring PET. Patients with HCMV DNAemia were treated with PET using (val)ganciclovir or foscarnet, initiated as previously described.[22] Pre-emptive therapy was initiated when two consecutive HCMV DNA qPCR results had increasing copy numbers, if a single result was > 1000 copies mL^{-1}, or according to physician discretion if a patient with HCMV DNAemia was considered at high risk of uncontrolled HCMV replication. First-line therapy was ganciclovir or valganciclovir. Foscarnet was used if (val)ganciclovir was contraindicated. No patient received pre-emptive antiviral therapy at/or before T2. Of the 16 patients treated with pre-emptive antiviral therapy, 10/16 patients received pre-emptive therapy at or before T3, and 16/16 received pre-emptive therapy by T4. No patient received Letermovir prophylaxis.

Sample time-points and patient groups

Plasma samples from four time-points per patient (range three to four samples), where available, were studied with a multiplex cytokine immunoassay (Supplementary figure 1). For patients with HCMV reactivation, the time-points analysed were as follows: T1, before detection of HCMV reactivation; T2, initial detection of HCMV DNAemia; T3, peak of HCMV DNAemia; T4, near resolution of HCMV DNAemia (Figure 1b; Supplementary figure 1; Supplementary table 4). Samples from equivalent days relative to transplant were analysed from patients without HCMV reactivation (Figure 1c). If samples from the specific timepoints for T2, T3 or T4 were unavailable, the closest available samples were used (see Supplementary table 4): for T2, the next available sample following the detection of HCMV DNAemia was selected; for T3, the most proximate sample preceding the peak of HCMV DNAemia was selected. For T4 samples, HCMV DNAemia was undetectable in 8/15 HR and 6/6 LR patients, had declined to detectable levels below the lower limit of quantitation (< 150 copies mL^{-1}) in 4/15 HR patients, or had declined substantially (by 83–98%) from peak levels but was still quantifiable in 3/15 HR patients (Figure 1b).

Patients were retrospectively divided into four analysis groups: Seronegative (SN), HCMV-seronegative recipients with seronegative donors (D−/R−); Seropositive non-reactivators (SP-NR), patients with a HCMV-seropositive donor (D+) and/or recipient (R+), without detected post-transplant HCMV DNAemia; LR, who experienced HCMV DNAemia levels near to or below the LLQ (range 150–244 peak copies mL^{-1}); and HR, who developed clinically significant infection with high viral loads (range 810–52 740 peak copies mL^{-1}) (Figure 1a; Supplementary figure 5).

Multiplex cytokine assay

Plasma concentrations of 48 analytes were measured using a multiplex magnetic bead-based sandwich immunoassay (Bio-Plex Pro™ 48-plex Human Cytokine Screening Panel; BioRad). Plasma samples (n = 138) were assayed in duplicate, with the mean used for analysis. Four assay kits/96-well plates from the same manufacturing lot were used. Each plate included plasma samples from at least two patient groups and time-points, a 9-point standard curve series, blank and QC sample from the kit, all in duplicate.

The assay was performed according to the manufacturer's protocol. Briefly, plasma samples were thawed on wet ice, pulse vortexed and centrifuged at 10 000 × g for 10 min at 4°C. All samples were used first-thaw. Plasma (30 μL) was extracted from the middle aqueous layer, avoiding the platelet pellet (below) or lipid layer (above) if present. Plasma was equilibrated to RT and diluted fourfold with the provided sample diluent. Samples (50 μL) were added to coupled magnetic capture beads in a 96-well flat-bottom plate and incubated on a horizontal plate shaker at 850 rpm for 30 min at RT protected from light. After three washes in wash buffer (using a BioTek 405™ LS microplate washer with magnetic carrier for all washes), biotin-labelled detection antibodies were added and the plate incubated for 30 min at RT with shaking as above. After three washes with wash buffer, streptavidin-phycoerythrin conjugate was added, with shaking for 10 min at RT protected from light. Finally, after three washes with wash buffer, beads were resuspended in assay buffer (125 μL) with shaking for 10 min at RT protected from light. The plate was read immediately on a Luminex MAGPIX® with xPONENT software 4.2.1324.0 (Luminex Corp), acquiring a minimum of 50 beads per analyte. MAGPIX® calibration and performance verification routines were run prior to reading plates.

Standard curves for each analyte were generated using five-parameter logistic regression in MILLIPLEX® Analyst 5.1 (Merck, VigeneTech). Curves were generated using the average MFI (median fluorescence intensity) of standard duplicates at each dilution, minus the blank. Standard replicates were excluded if percentage recovery was outside the 70–130% range. Sample measurements with a MFI below the blank were assigned a concentration of 0 pg mL^{-1}. Analyte concentrations below the minimum detectable concentration (minDC) (Supplementary table 5) were assigned a value equal to half the minDC.

Data quality control

Intra-assay precision and inter-assay precision were evaluated for all analytes. Intra-assay %CV calculated from sample duplicates was < 15% for all analytes (median 3.5%, range 0.6–14.0%), meeting the representative performance characteristics of the kit (< 15% intra-assay %CV). Inter-assay %CV per analyte calculated from the QC sample over the four plates was median 12.1% (range 2.7–100.1%). To minimise plate-specific bias in the data set, analytes with an inter-assay CV ≥ 30%[55] (n = 7) and/or those which were not detected in any plasma sample on ≥ 1 plates (n = 15) (Supplementary table 1) were excluded from analysis. All analytes were detected in the QC sample on every plate, except for vascular endothelial growth factor A (VEGF-A; not

detected in plates 1 and 4), eotaxin (not detected in plate 4) and growth-regulated oncogene α (GRO-α; not detected in plate 1), which were excluded from analysis. The median intra-assay and inter-assay %CVs of the analytes retained for analysis ($n = 31$) were 3.4% and 10.4%, respectively.

The analytes retained for analysis (31/48) were: interferon (IFN)-α2, IL-1β, IL-2, IL-3, IL-4, IL-6, IL-7, IL-8, IL-9, IL-12 p70, IL-13, IL-18, monocyte chemoattractant protein (MCP)-1, macrophage migration inhibitory factor (MIF), TNF-α (referred to as TNF[56]), LT-α (TNF-β), leukaemia inhibitory factor (LIF), CTACK, IP-10, MIG, MIP-1α, MIP-1β, regulated on activation normal T-cell expressed and secreted (RANTES), stromal cell-derived factor 1α (SDF-1α), basic fibroblast growth factor (FGF), HGF, PDGF-BB, SCF, M-CSF, granulocyte-macrophage colony-stimulating factor (GM-CSF) and sIL-2Rα. The analytes excluded from analysis were: stem cell growth factor β (SCGF-β), eotaxin, TNF-related apoptosis-inducing ligand (TRAIL), MCP-3, granulocyte colony-stimulating factor (G-CSF), growth-regulated oncogene (GRO-α), vascular endothelial growth factor A (VEGF-A), IL-10, IL-15, IL-16, IL-5, IL-17A, IL-12 p40, β nerve growth factor (β-NGF), IL-1 receptor antagonist (IL-1RA), IFN-γ and IL-1α (see Supplementary table 1).

Batch effect adjustment

To adjust for potential batch effects between the four assay plates, raw MFI values were $\log_2$-transformed and a robust linear model constructed for each analyte using limma[57] in R/Bioconductor, with plate, timepoint, patient and patient group as covariates. The fitted plate effect was then subtracted from the data.

Measurement of immune cell frequencies

PBMC subset frequencies (percentages and absolute counts $\times 10^9$/L) from matched time-points in 31/36 patients in the current study (Supplementary figure 6) were measured previously using mass cytometry.[22] For pairwise cytokine-immune cell Spearman correlations, all time-points were analysed together and a FDR of 1% (Benjamini, Krieger and Yekutieli two-stage linear step-up procedure) employed to identify significant correlations in each patient group. Cytokine correlations with PBMC percentages (%; $n = 88$ subsets) and absolute counts ($\times 10^9$/L; $n = 77$ subsets) were analysed separately.

Statistical analysis

Statistical tests were performed in GraphPad Prism v.9.2 (GraphPad Software, LLC) and are indicated in Figure legends. Median with range is reported in text. Patient characteristics were summarised using descriptive statistics, using two-tailed Mann–Whitney U-tests or Fisher's exact tests. Correlations were evaluated using two-tailed Spearman correlations. $P < 0.05$ was considered significant for all tests, unless indicated otherwise.

For inter-group and inter-timepoint comparisons, parallel statistical tests were performed in the primary dataset and batch-adjusted dataset to identify robust comparisons that were maintained across both sets of data. Only comparisons demonstrating significance ($P < 0.05$) in both data sets were reported (Supplementary figure 2). Analyte concentrations between time-point pairs were compared using two-way mixed-effects models with Geisser–Greenhouse correction and Tukey's multiple comparisons test. Analyte concentrations between patient groups were compared using two-tailed Mann–Whitney U-tests. Figures presented are derived from the primary (non-batch-adjusted) dataset. Comparisons performed included: R *vs.* NR, HR *vs.* LR, HR *vs.* SP-NR, LR *vs.* SP-NR, HR *vs.* SN. Comparisons not studied: LR *vs.* SN, SN *vs.* SP-NR.

AUTHOR CONTRIBUTIONS

Lauren Stern: Formal analysis; investigation; methodology; writing – original draft; writing – review and editing. **Helen M McGuire:** Formal analysis; methodology; supervision; writing – original draft; writing – review and editing. **Selmir Avdic:** Resources; supervision; writing – review and editing. **Emily Blyth:** Resources; writing – review and editing. **David Gottlieb:** Resources; writing – review and editing. **Ellis Patrick:** Formal analysis; writing – review and editing. **Allison Abendroth:** Conceptualization; funding acquisition; supervision; writing – original draft; writing – review and editing. **Barry Slobedman:** Conceptualization; funding acquisition; supervision; writing – original draft; writing – review and editing.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Original data are available from the corresponding author, Barry Slobedman (barry.slobedman@sydney.edu.au), on reasonable request.

REFERENCES

1. Stern L, Withers B, Avdic S *et al.* Human cytomegalovirus latency and reactivation in allogeneic hematopoietic stem cell transplant recipients. *Front Microbiol* 2019; **10**: 1186.

2. Ljungman P, Hakki M, Boeckh M. Cytomegalovirus in hematopoietic stem cell transplant recipients. *Hematol Oncol Clin North Am* 2011; **25**: 151–169.

3. Cantoni N, Hirsch HH, Khanna N *et al*. Evidence for a bidirectional relationship between cytomegalovirus replication and acute graft-versus-host disease. *Biol Blood Marrow Transplant* 2010; **16**: 1309–1314.

4. Nichols WG, Corey L, Gooley T, Davis C, Boeckh M. High risk of death due to bacterial and fungal infection among cytomegalovirus (CMV)—Seronegative recipients of stem cell transplants from seropositive donors: Evidence for indirect effects of primary CMV infection. *J Infect Dis* 2002; **185**: 273–282.

5. Chuleerarux N, Nematollahi S, Thongkam A *et al*. The association of cytomegalovirus infection and cytomegalovirus serostatus with invasive fungal infections in allogeneic haematopoietic stem cell transplant recipients: A systematic review and meta-analysis. *Clin Microbiol Infect* 2022; **28**: 332–344.

6. Jakharia N, Howard D, Riedel DJ. CMV infection in hematopoietic stem cell transplantation: Prevention and treatment strategies. *Curr Treat Options Infect Dis* 2021; **13**: 123–140.

7. Green ML, Leisenring W, Xie H *et al*. Cytomegalovirus viral load and mortality after haemopoietic stem cell transplantation in the era of pre-emptive therapy: A retrospective cohort study. *Lancet Haematol* 2016; **3**: e119–e127.

8. Teira P, Battiwalla M, Ramanathan M *et al*. Early cytomegalovirus reactivation remains associated with increased transplant-related mortality in the current era: A CIBMTR analysis. *Blood* 2016; **127**: 2427–2438.

9. Commins SP, Borish L, Steinke JW. Immunologic messenger molecules: Cytokines, interferons, and chemokines. *J Allergy Clin Immunol* 2010; **125**: S53–S72.

10. Melenhorst JJ, Tian X, Xu D *et al*. Cytopenia and leukocyte recovery shape cytokine fluctuations after myeloablative allogeneic hematopoietic stem cell transplantation. *Haematologica* 2012; **97**: 867–873.

11. DiCarlo J, Agarwal-Hashmi R, Shah A *et al*. Cytokine and chemokine patterns across 100 days after hematopoietic stem cell transplantation in children. *Biol Blood Marrow Transplant* 2014; **20**: 361–369.

12. Lakshmikanth T, Olin A, Chen Y *et al*. Mass cytometry and topological data analysis reveal immune parameters associated with complications after allogeneic stem cell transplantation. *Cell Rep* 2017; **20**: 2238–2250.

13. Humar A, St. Louis P, Mazzulli T *et al*. Elevated serum cytokines are associated with cytomegalovirus infection and disease in bone marrow transplant recipients. *J Infect Dis* 1999; **179**: 484–488.

14. Reddy V, Meier-Kriesche HU, Greene S, Schold JD, Wingard JR. Increased levels of tumor necrosis factor alpha are associated with an increased risk of cytomegalovirus infection after allogeneic hematopoietic stem cell transplantation. *Biol Blood Marrow Transplant* 2005; **11**: 698–705.

15. Kato T, Nishida T, Ito Y, Murase M, Murata M, Naoe T. Correlations of programmed death 1 expression and serum IL-6 level with exhaustion of cytomegalovirus-specific T cells after allogeneic hematopoietic stem cell transplantation. *Cell Immunol* 2014; **288**: 53–59.

16. Fietze E, Prösch S, Reinke P *et al*. Cytomegalovirus infection in transplant recipients. The role of tumor necrosis factor. *Transplantation* 1994; **58**: 675–680.

17. Söderberg-Nauclér C, Fish KN, Nelson JA. Interferon-gamma and tumor necrosis factor-alpha specifically induce formation of cytomegalovirus-permissive monocyte-derived macrophages that are refractory to the antiviral activity of these cytokines. *J Clin Invest* 1997; **100**: 3154–3163.

18. Forte E, Swaminathan S, Schroeder MW, Kim JY, Terhune SS, Hummel M. Tumor necrosis factor alpha induces reactivation of human cytomegalovirus independently of myeloid cell differentiation following posttranscriptional establishment of latency. *mBio* 2018; **9**: e01560-01518.

19. Reeves MB, Compton T. Inhibition of inflammatory interleukin-6 activity via extracellular signal-regulated kinase-mitogen-activated protein kinase signaling antagonizes human cytomegalovirus reactivation from dendritic cells. *J Virol* 2011; **85**: 12750–12758.

20. Talaya A, Giménez E, Vinuesa V *et al*. Kinetics of inflammatory biomarkers in plasma predict the occurrence and features of cytomegalovirus DNAemia episodes in allogeneic hematopoietic stem cell transplant recipients. *Med Microbiol Immunol* 2019; **208**: 405–414.

21. Wils EJ, Rombouts EJ, van Mourik I *et al*. Stem cell factor consistently improves thymopoiesis after experimental transplantation of murine or human hematopoietic stem cells in immunodeficient mice. *J Immunol* 2011; **187**: 2974–2981.

22. Stern L, McGuire HM, Avdic S *et al*. Immunoprofiling reveals cell subsets associated with the trajectory of cytomegalovirus reactivation post stem cell transplantation. *Nat Commun* 2022; **13**: 2603.

23. van de Berg PJ, Heutinck KM, Raabe R *et al*. Human cytomegalovirus induces systemic immune activation characterized by a type 1 cytokine signature. *J Infect Dis* 2010; **202**: 690–699.

24. McSharry BP, Avdic S, Slobedman B. Human cytomegalovirus encoded homologs of cytokines, chemokines and their receptors: Roles in immunomodulation. *Viruses* 2012; **4**: 2448–2470.

25. Varani S, Landini MP. Cytomegalovirus-induced immunopathology and its clinical consequences. *Herpesviridae* 2011; **2**: 6.

26. Leserer S, Bayraktar E, Trilling M *et al*. Cytomegalovirus kinetics after hematopoietic cell transplantation reveal peak titers with differential impact on mortality, relapse and immune reconstitution. *Am J Hematol* 2021; **96**: 436–445.

27. Duke ER, Williamson BD, Borate B *et al*. CMV viral load kinetics as surrogate endpoints after allogeneic transplantation. *J Clin Invest* 2021; **131**: e133960.

28. Tan SK, Waggoner JJ, Pinsky BA. Cytomegalovirus load at treatment initiation is predictive of time to resolution of viremia and duration of therapy in hematopoietic cell transplant recipients. *J Clin Virol* 2015; **69**: 179–183.

29. Camargo JF, Wieder ED, Kimble E *et al*. Deep functional immunophenotyping predicts risk of cytomegalovirus reactivation after hematopoietic cell transplantation. *Blood* 2019; **133**: 867–877.

30. Lilleri D, Fornara C, Chiesa A, Caldera D, Alessandrino EP, Gerna G. Human cytomegalovirus-specific CD4^{+} and CD8^{+} T-cell reconstitution in adult allogeneic hematopoietic stem cell transplant recipients and immune control of viral infection. *Haematologica* 2008; **93**: 248–256.

31. Chakraborty S, Kubatzky KF, Mitra DK. An update on interleukin-9: From its cellular source and signal transduction to its role in immunopathogenesis. *Int J Mol Sci* 2019; **20**: 2113.

32. Wynn TA. Type 2 cytokines: Mechanisms and therapeutic strategies. *Nat Rev Immunol* 2015; **15**: 271–282.

33. Dumortier J, Streblow DN, Moses AV *et al*. Human cytomegalovirus secretome contains factors that induce angiogenesis and wound healing. *J Virol* 2008; **82**: 6524–6535.

34. Luganini A, Terlizzi ME, Gribaudo G. Bioactive molecules released from cells infected with the human cytomegalovirus. *Front Microbiol* 2016; **7**: 715.

35. Broudy VC. Stem cell factor and hematopoiesis. *Blood* 1997; **90**: 1345–1364.

36. Politikos I, Kim HT, Nikiforow S *et al*. IL-7 and SCF levels inversely correlate with T cell reconstitution and clinical outcomes after cord blood transplantation in adults. *PLoS One* 2015; **10**: e0132564.

37. Suessmuth Y, Mukherjee R, Watkins B *et al*. CMV reactivation drives posttransplant T-cell reconstitution and results in defects in the underlying TCRβ repertoire. *Blood* 2015; **125**: 3835–3850.

38. Douek DC, Vescio RA, Betts MR *et al*. Assessment of thymic output in adults after haematopoietic stem-cell transplantation and prediction of T-cell reconstitution. *Lancet* 2000; **355**: 1875–1881.

39. Xie M, Yunis J, Yao Y *et al*. High levels of soluble CD25 in COVID-19 severity suggest a divergence between anti-viral and pro-inflammatory T-cell responses. *Clin Transl Immunology* 2021; **10**: e1251.

40. Damoiseaux J. The IL-2 – IL-2 receptor pathway in health and disease: The role of the soluble IL-2 receptor. *Clin Immunol* 2020; **218**: 108515.

41. Sadeghi M, Daniel V, Naujokat C *et al*. Dysregulated cytokine responses during cytomegalovirus infection in renal transplant recipients. *Transplantation* 2008; **86**: 275–285.

42. Grimm J, Zeller W, Zander AR. Soluble interleukin-2 receptor serum levels after allogeneic bone marrow transplantation as a marker for GVHD. *Bone Marrow Transplant* 1998; **21**: 29–32.

43. Kobayashi S, Imamura M, Hashino S, Tanaka J, Asaka M. Clinical relevance of serum soluble Interleukin-2 receptor levels in acute and chronic graft-versus-host disease. *Leuk Lymphoma* 1997; **28**: 159–169.

44. Groom JR, Luster AD. CXCR3 ligands: Redundant, collaborative and antagonistic functions. *Immunol Cell Biol* 2011; **89**: 207–215.

45. Tokunaga R, Zhang W, Naseem M *et al*. CXCL9, CXCL10, CXCL11/CXCR3 axis for immune activation – a target for novel cancer therapy. *Cancer Treat Rev* 2018; **63**: 40–47.

46. Van Beek JJP, Puccio S, Roberto A *et al*. Single-cell profiling reveals the dynamics of cytomegalovirus-specific T cells in haploidentical hematopoietic stem cell transplantation. *Haematologica* 2021; **106**: 2768–2773.

47. Newell EW, Sigal N, Bendall SC, Nolan GP, Davis MM. Cytometry by time-of-flight shows combinatorial cytokine expression and virus-specific cell niches within a continuum of CD8^{+} T cell phenotypes. *Immunity* 2012; **36**: 142–152.

48. Cervera C, Filella X, Linares L *et al*. Th1/Th2 cytokine release pattern during in vivo cytomegalovirus disease in solid organ transplantation. *Transplant Proc* 2007; **39**: 2233–2235.

49. Ellington AA, Kullo IJ, Bailey KR, Klee GG. Measurement and quality control issues in multiplex protein assays: A case study. *Clin Chem* 2009; **55**: 1092–1099.

50. Tighe PJ, Ryder RR, Todd I, Fairclough LC. ELISA in the multiplex era: Potentials and pitfalls. *Proteomics Clin Appl* 2015; **9**: 406–422.

51. Krawczyk A, Ackermann J, Goitowski B *et al*. Assessing the risk of CMV reactivation and reconstitution of antiviral immune response post bone marrow transplantation by the QuantiFERON-CMV-assay and real time PCR. *J Clin Virol* 2018; **99–100**: 61–66.

52. Szmit Z, Frączkiewicz J, Salamonowicz-Bodzioch M *et al*. The impact of high CMV viral load and refractory CMV infection on pediatric HSCT recipients with underlying non-malignant disorder. *J Clin Med* 2022; **11**: 5187.

53. Yeh AC, Varelias A, Reddy A *et al*. CMV exposure drives long-term CD57^{+} CD4 memory T-cell inflation following allogeneic stem cell transplant. *Blood* 2021; **138**: 2874–2885.

54. Hirsch HH, Lautenschlager I, Pinsky BA *et al*. An international multicenter performance analysis of cytomegalovirus load tests. *Clin Infect Dis* 2012; **56**: 367–373.

55. Defawe OD, Fong Y, Vasilyeva E *et al*. Optimization and qualification of a multiplex bead array to assess cytokine and chemokine production by vaccine-specific cells. *J Immunol Methods* 2012; **382**: 117–128.

56. Grimstad Ø. Tumor necrosis factor and the tenacious α. *JAMA Dermatol* 2016; **152**: 557.

57. Ritchie ME, Phipson B, Wu D *et al*. Limma powers differential expression analyses for RNA-sequencing and microarray studies. *Nucleic Acids Res* 2015; **43**: e47.

Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.