

# Peripheral blood lymphocyte responses to cytomegalovirus seropositivity after allogeneic-hematopoietic stem cell transplantation

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**Background:** The main purpose of this study was to investigate the relationship among cytomegalovirus (CMV) viremia, peripheral immune cells alternations, and leukemia prognosis.

**Patients and methods:** We studied 90 leukemia patients who underwent allogeneic hematopoietic stem cell transplantation (allo-HSCT) from 2008 to 2015. Their complete clinical laboratory data were collected until 1 year after transplantation.

**Results:** All patients were serum CMV negative before allo-HSCT. After transplantation, the CMV reactivation group showed increased peripheral CD8<sup>+</sup> T cells and decreased CD4<sup>+</sup> T cells and B cells. However, CD8/CD4 ratio and B cells restored by control of CMV infection due to 2 months maximum course of ganciclovir treatment. CMV seropositivity was positively related to leukemia-free survival (LFS) of all recruited leukemia types.

**Conclusion:** In summary, CMV drives immune cell post-transplantation fluctuation, which also favors LFS of leukemia partly resulted from CD8<sup>+</sup> T cells.

**Keywords:** cytomegalovirus, leukemia, allo-HSCT, CD8<sup>+</sup> T cells, leukemia-free survival

## Introduction

Cytomegalovirus (CMV) is a common double-stranded DNA herpes virus. Infection by CMV induces few, if any, clinical symptoms in healthy people.<sup>1</sup> This virus may not ever be reactivated except for immune suppression circumstances, such as stem cell transplantation or human immunodeficiency virus infection. CMV can be detected from serum of leukemia patients early after transplantation, which are susceptible to CMV infection, and develop CMV pneumonia with highly mortality.<sup>2-5</sup>

Allogeneic hematopoietic stem cell transplantation (allo-HSCT) has been widely applied as a recommended first-line therapy for leukemia. Before transplantation, patients receive chemotherapy to destroy tumor cells as well as immune system to make room for new stem cells for restoring hematopoiesis. To prevent CMV infection during therapy, patients are simultaneously designated antiviral drug, such as ganciclovir (GCV). Nevertheless, CMV still emerges in serum as early as a few days after transplantation. Moreover, some studies indicated that pre-surgery chemotherapy and GCV administration will impel patients more frail to CMV infection due to its side effects of myelosuppression, and postpone reconstitution of CMV-specific cellular immunity by suppressing proliferation of T cells as well as CMV-specific T-cell precursors.<sup>6,7</sup>

Immune system plays a significant role in controlling virus infection, survival, and proliferation. For example, CD4<sup>+</sup> T-helper (Th) cells secrete interferon- $\gamma$ , tumor necrosis factor- $\alpha$ , and interleukin-2 to exert a Th1-type immune reaction to eliminate

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virus.<sup>8</sup> Natural killer (NK) cell-expressed killer cell Ig-like receptor and NKG2C subsets contribute to graft versus leukemia effect.<sup>9</sup> It has been reported that CMV reactivation reduces the risk of relapse in acute myeloid leukemia (AML) patients until 100 days after transplantation.<sup>10</sup> These studies prompted us to further investigate the interplay between CMV post-surgery seropositivity and immune status in leukemia patients who undergo allo-HSCT and to discuss the factors that may influence their clinical prognosis.

## Materials and methods

### Patient characteristics

This research was authorized by the Ethical Committee of the First Affiliated Hospital of Nanjing Medical University (Nanjing, China). All patients provided written informed consent. Patients diagnosed with AML, acute lymphoblastic leukemia (ALL), chronic granulocytic leukemia (CML), lymphoma, chronic lymphocytic leukemia (CLL), myelodysplastic syndrome (MDS), and other undefined leukemia in 2008–2015 were identified. Key exclusion criteria included allo-HSCT more than one time and serum CMV positive before allo-HSCT. All enrolled 90 leukemia patients were divided into CMV+ group and CMV– group according to post-transplantation serum CMV DNA load. Follow-up ended on April 30, 2016, with a survey range of 1.5–98.6 months after transplantation (median: 15.9 months); 25 patients were finally lost to follow-up.

### Fluorescence-activated cell sorting (FACS)

FACS detection was carried out on peripheral blood samples on the very first day of admission till 1 year after allo-HSCT. Whole blood cells were conjugated to antibodies including CD3, CD4, CD8, CD16, CD19, and CD56 (BioLegend, San Jose, CA, USA) at room temperature. Samples were incubated for 20 minutes in the dark. Then red blood cells were lysed by lysis buffer (BD Biosciences, San Jose, CA, USA). The remaining cells were washed with PBS for three times and were measured by flow cytometry (BD Calibur; BD Biosciences). Different gate settings were used to distinguish lymphocyte subsets. The FACS results indicated the percentages of CD4<sup>+</sup> T cells, CD8<sup>+</sup> T cells, NK cells, and B cells.

### CMV detection

We detected serum CMV DNA load via quantitative polymerase chain reaction following manufacturer's instructions (Daan Gene, Sun Yat-Sen University, China). This experiment was applied for each patient before surgery and weekly after surgery until CMV seroconversion. The lower threshold of this assay is 500 genome copies per mL blood

(c/mL); the upper limit is 50,000,000 c/mL. Serum CMV DNA load <500 c/mL was defined CMV negative.

### GVHD prophylaxis

GVHD prophylaxis primarily comprises cyclosporine A (CSA) and methotrexate (MTX), or CSA + MTX with other immunosuppressants (mycophenolate mofetil, basiliximab). All patients received GVHD prophylaxis before transplantation. Diagnosis of acute GVHD was based on clinical signs and symptoms and laboratory studies. Acute GVHD was graded using accepted criterion;<sup>11</sup> chronic GVHD was graded according to NIH criterion.<sup>12</sup>

### Clinical definition

Overall survival (OS) was defined as the number of days from the date of transplantation to the date of death from any cause or the last date of follow-up. Leukemia-free survival (LFS) was counted from the date of surgery to the date of leukemia relapse or to the date of follow-up without relapse.

### Statistical analysis

Fisher's exact test or the chi-square test was used to analyze the clinical parameters. Multivariate analyses were conducted using the Cox proportional hazards model to evaluate the risk factors for LFS. Kaplan–Meier survival analysis was used to estimate the rates of OS, and the log-rank test was applied to each group. All data were analyzed with IBM SPSS Statistics for Windows 17.0. *P*-values less than 0.05 were considered significant.

## Results

### Patient characteristics

Serum CMV DNA load of all 90 patients before allo-HSCT was beyond the detection limit, among which 54 patients (60%) acquired CMV infection after transplantation. Their other clinical parameters are shown in Table 1. Patients with HLA-matched siblings donors received a stronger immunosuppression drug treatment, or patients who had acute GVHD (aGVHD) were more susceptible to CMV post-surgery infection.

### CMV seropositivity after allo-HSCT alters peripheral immune cell distribution

We analyzed percentages of peripheral CD4<sup>+</sup> T cells, CD8<sup>+</sup> T cells, NK cells, and B cells in the CMV+ and CMV– groups, respectively. As shown in Figure 1, CD4<sup>+</sup> T cells were significantly downregulated and CD8<sup>+</sup> T cells were highly upregulated in the CMV+ group, which led to obviously upregulated CD8/CD4 ratio. B cells were lower in the CMV+ group compared with the CMV– group. No difference was

**Table 1** Clinical characteristics of 90 leukemia patients in the CMV+ and CMV- groups

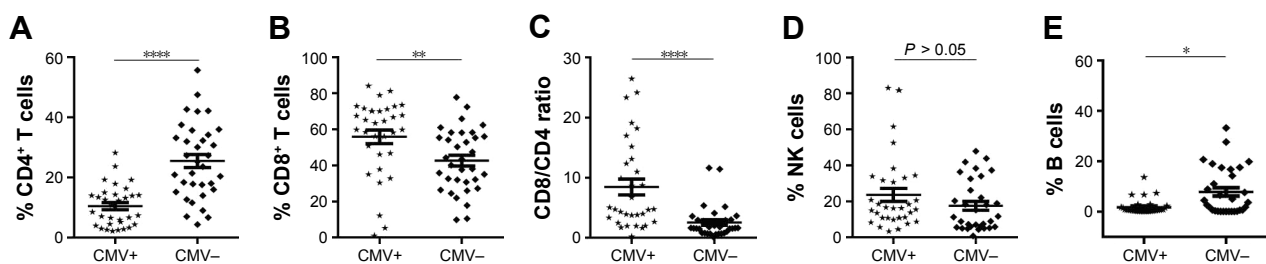
Variables	Patients	CMV+ group	CMV- group	P-value
Patients	90	54 (60%)	36 (40%)	
Sex				0.7
Female/male	46/44 (51%)	27/27 (50%)	19/17 (53%)	
Age	35.5 (6–65) years	35 (6–60) years	41 (9–65) years	0.9
Diagnosis				
AML	36 (40%)	22 (40.7%)	14 (38.9%)	
ALL	14 (15%)	10 (18.5%)	4 (11.1%)	
CML	8 (9%)	4 (7.4%)	4 (11.1%)	
RBC leukemia	6 (7%)	4 (7.4%)	2 (5.5%)	
Lymphoma	14 (16%)	5 (9.3%)	9 (25%)	
CLL	2 (2%)	2 (3.7%)	0 (0%)	
MDS	9 (10%)	7 (13%)	2 (5.6%)	
Others	1 (1%)	0 (0%)	1 (2.8%)	
Conditioning regimen				0.1
MAC	68 (75.5%)	45 (83.3%)	23 (63.9%)	
RIC	15 (16.7%)	7 (13%)	8 (22.2%)	
Others	7 (7.8%)	2 (3.7%)	5 (13.9%)	
Donors				0.003
HLA-matched siblings	62 (68.9%)	33 (61.1%)	29 (80.5%)	
Unrelated matched	20 (22.2%)	18 (33.3%)	2 (5.6%)	
Unknown	8 (8.9%)	3 (5.6%)	5 (13.9%)	
Stem cell source				0.143
PB + CB	10 (11%)	7 (12.9%)	3 (8.3%)	
PB	76 (84%)	42 (77.8%)	33 (91.7%)	
CB	3 (3%)	3 (5.6%)	0 (0%)	
Unknown	2 (2%)	2 (3.7%)	0 (0%)	
GVHD prophylaxis				0.03
CSA/MTX	82 (91.1%)	52 (96.3%)	30 (83.3%)	
ATG or other	8 (8.9%)	2 (3.7%)	6 (16.7%)	
GVHD				0.009
Acute GVHD	43 (47.8%)	34 (62.9%)	9 (25%)	
Chronic GVHD	16 (17.8%)	7 (12.9%)	9 (25%)	
Status				0.19
Relapse	13 (14.4%)	7 (7.8%)	6 (6.7%)	
Dead	16 (17.8%)	7 (7.8%)	9 (6.7%)	
Missing	24 (26.7%)	16 (17.8%)	8 (8.9%)	

**Abbreviations:** ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; ATG, Anti-human Thymocyte Globulin; CB, stem cells from cord blood; CLL, chronic lymphocytic leukemia; CML, chronic granulocytic leukemia; CMV, cytomegalovirus; CSA, Cyclosporin A; GVHD, graft-versus-host disease; HLA, human lymphocyte antigen; MAC, Myeloablative stem cell transplantation; MDS, myelodysplastic syndrome; MTX, Methotrexate; PB, stem cells from peripheral blood; RIC, Reduce-intensity conditioning.

found in NK cells. We further analyzed the data of AML patients and found similar trends, as shown in Figure 2. These results suggest that CMV affects immune cells post-transplantation distribution, and that CD8<sup>+</sup> T cells dominate in peripheral blood.

## Control of CMV infection induces peripheral immune cell re-distribution

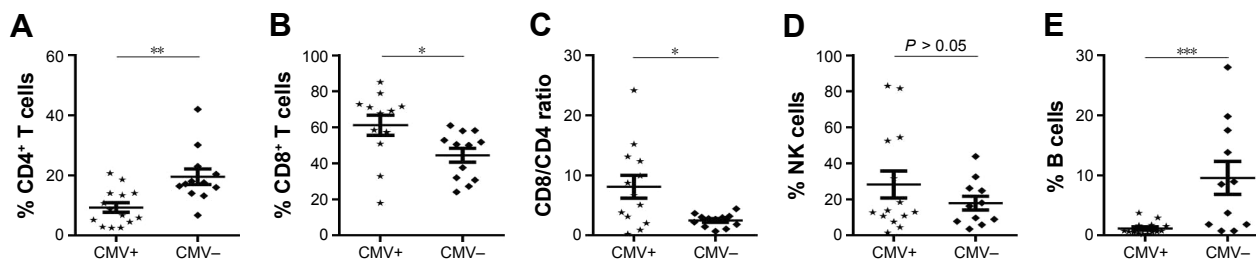
Severe CMV infection often occurred within the first 100 days after allo-HSCT.<sup>13</sup> The median time of CMV seropositivity in our leukemia patients was 38.5 days (range: 2–102 days)



**Figure 1** CMV seropositivity after allo-HSCT alters peripheral immune cells distribution.

**Notes:** Serum CMV DNA load was detected via real-time PCR 1 week after allo-HSCT. Patients suffered from CMV seropositivity defined as CMV+ group, otherwise CMV- group. The percentages of peripheral CD4<sup>+</sup> T cells (A), CD8<sup>+</sup> T cells (B), ratio of CD8/CD4 (C), NK cells (D), and B cells (E) of leukemia patients in the CMV+ and CMV- groups. Graphs show mean  $\pm$  SEM. \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\*\* $P < 0.0001$ .

**Abbreviations:** allo-HSCT, allogeneic hematopoietic stem cell transplantation; CMV, cytomegalovirus; NK, natural killer.



**Figure 2** Peripheral immune cell distributions in AML patients with CMV seropositivity after allo-HSCT.

**Notes:** Representative data of circulating CD4<sup>+</sup> T cells (A), CD8<sup>+</sup> T cells (B), ratio of CD8/CD4 (C), NK cells (D), and B cells (E) in AML patients with CMV infection after transplantation. All the graphs show mean  $\pm$  SEM. \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ .

**Abbreviations:** allo-HSCT, allogeneic hematopoietic stem cell transplantation; AML, acute myeloid leukemia; CMV, cytomegalovirus; NK, natural killer.

within the CMV+ group. We weekly monitored peripheral CMV genome copies of these patients after allo-HSCT and documented the first date of CMV seropositivity and 2 months after the date, respectively, to investigate immune cell responses to GCV administration. We successively collected results of 18 patients in the CMV+ group. As shown in Figure 3, CD8<sup>+</sup> T cells, CD4<sup>+</sup> T cells, and B cells were restored when serum CMV was negatively converted, and the CD8/CD4 ratio dropped back to the baseline.

Next, we extended the follow-up period to 12 months after allo-HSCT and analyzed flow cytometry data of all patients in the CMV+ group. Their serum CMV all turned negative (if infection occurred) within 2 months after courses of GCV administration. Alteration of CD4<sup>+</sup> T cells, CD8<sup>+</sup> T cells, and B cells significantly occurred from 1 month to 3 months post-surgery (Figure 4). However, these differences between the CMV+ and CMV- groups became less pronounced with elongated follow-up time. Distribution of peripheral immune cells restored to pre-transplantation at as early as 6 months. These results indicated that CMV drives immune cells' post-transplantation fluctuation.

## CMV seropositivity affects leukemia prognosis after allo-HSCT

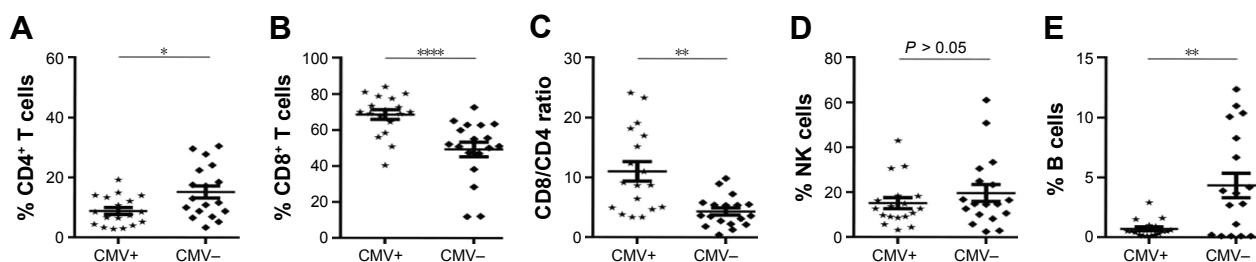
Totally, 13 patients suffered leukemia relapse after allo-HSCT. The median relapse time was 262 days (8.7 months).

As shown in Table 2, both prophylactic GVHD treatment and occurrence of aGVHD were risk factors of LFS. As reported, CMV reactivation protected AML patients from relapse within 100 days post-transplantation.<sup>10</sup> Therefore, we excluded the data of AML patients and further investigated the influence of CMV on other leukemia types. As shown in Table 3, CMV seropositivity was positively correlated with their LFS, indicating the favorable effect of CMV on leukemia overall prognosis.

CMV seropositivity, as well as stem cell sources, was positively correlated with LFS, although the overall survival of the CMV+ group was approximately to that of the CMV- group (53.10 months vs 59.21 months;  $P = 0.24$ ; Figure 5). It indicated that CMV seropositivity may rescue leukemia patients, but it cannot extend their length of survival probably due to its control failure of peripheral immune system in the long-term.

## Discussion

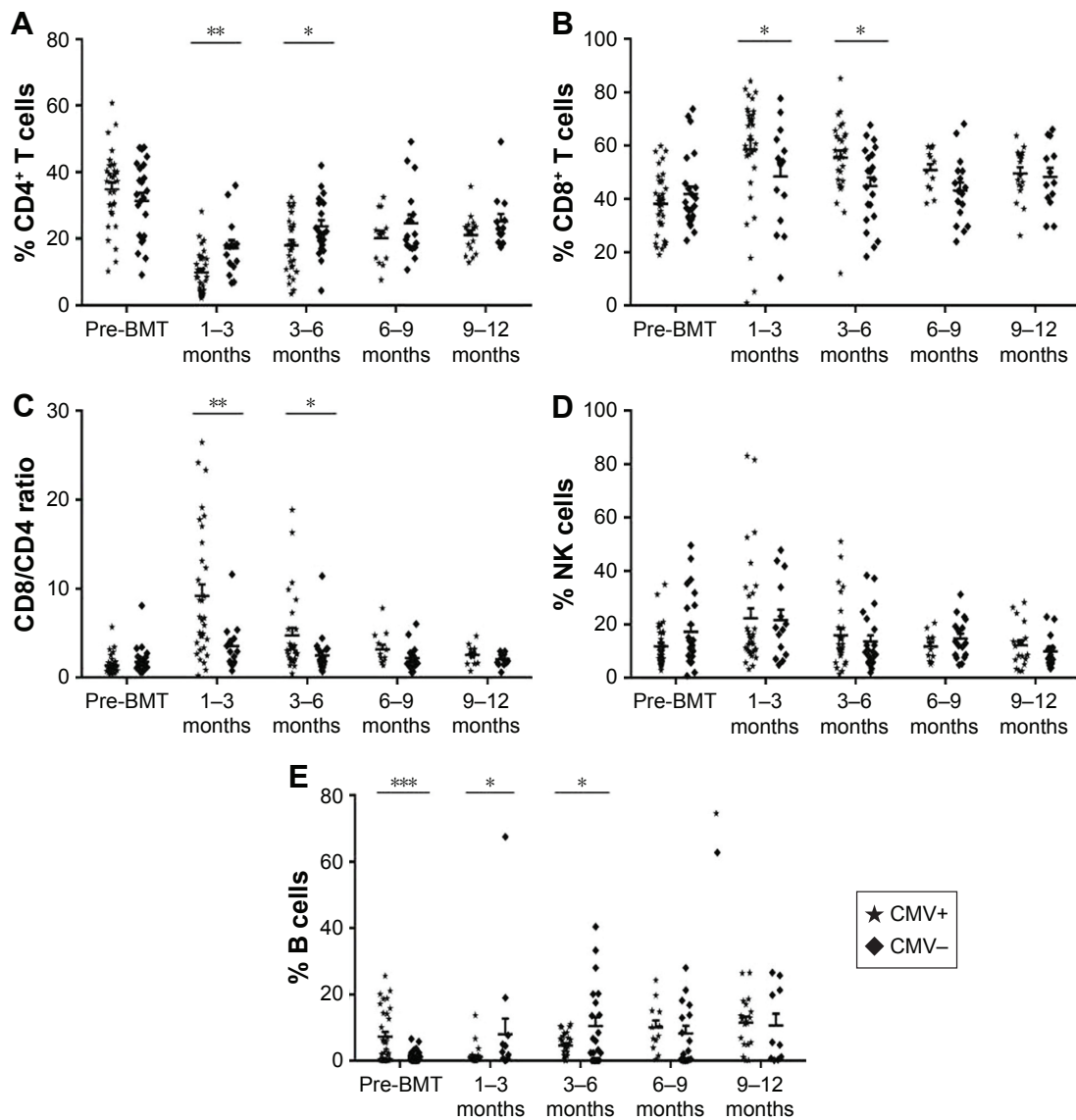
CMV often "hides" in hematopoietic progenitors.<sup>14</sup> Along with the differentiation and maturation of these progenitors, CMV amplifies. Some suggested that latent virus can be reactivated by the allogeneic monocyte-derived macrophages.<sup>15</sup> Therefore, peripheral blood mononuclear cells are believed to serve as CMV carriers.<sup>16</sup> In this study, we elaborately collected data of 90 leukemia patients who were diagnosed



**Figure 3** Control of CMV infection induces peripheral immune cell re-distribution.

**Notes:** In total, 54 leukemia patients had CMV reactivation after allo-HSCT, among which 18 patients' cytometry data were continuously collected after their serum CMV-negative conversion 2 months later by GCV administration. Statistical analysis represented their peripheral CD4<sup>+</sup> T cells (A), CD8<sup>+</sup> T cells (B), CD8/CD4 ratio (C), NK cells (D), and B cells (E). \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\*\* $P < 0.0001$ . All the graphs show mean  $\pm$  SEM.

**Abbreviations:** allo-HSCT, allogeneic hematopoietic stem cell transplantation; CMV, cytomegalovirus; NK, natural killer.



**Figure 4** Distribution of peripheral immune cells during 12 months' follow-up in all 90 leukemia patients after allo-HSCT.

**Notes:** We recorded their serum CMV load and correspondent flow cytometry data. Those with incomplete data were removed from the pool. Lymphocyte distribution was described in the time points of pre-BMT, 1–3 months (CMV seropositivity), 3–6 months (CMV negatively converted by GCV), 6–9 months, and 9–12 months. Representative dot plots of the percentages with circulating CD4<sup>+</sup> T cells (A), CD8<sup>+</sup> T cells (B), CD8/CD4 ratio (C), NK cells (D), and B cells (E) in CMV+ group and CMV– group. All the statistical graphs show mean ± SEM. \*P < 0.05; \*\*P < 0.01; \*\*\*P < 0.001.

**Abbreviations:** allo-HSCT, allogeneic hematopoietic stem cell transplantation; BMT, bone marrow transplantation; CMV, cytomegalovirus; GCV, Ganciclovir; NK, natural killer.

**Table 2** Clinical parameters related to LFS in 65 leukemia patients by multivariate variable Cox regression

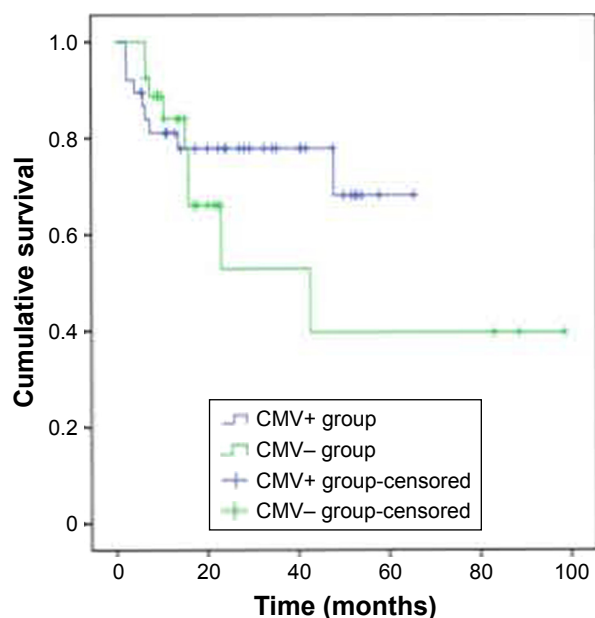
Parameters	Hazard ratio	95% CI	P-value
CMV seropositivity	2.293	1.136–4.629	0.021
Age	1.196	0.662–2.300	0.591
Sex	0.627	0.348–1.130	0.120
Diagnosis	1.214	0.976–1.510	0.081
Conditioning regimen	0.810	0.456–2.736	0.810
Stem cell sources	2.006	1.253–3.211	0.004
GVHD prophylactic	0.097	0.011–0.884	0.038
aGVHD	0.526	0.280–0.991	0.047

**Abbreviations:** aGVHD, acute GVHD; CMV, cytomegalovirus; GVHD, graft-versu-host disease; LFS, leukemia-free survival.

**Table 3** Clinical parameters associated with LFS in other leukemia patients, except for AML, by multivariate Cox regression analysis

Parameters	Hazard ratio	95% CI	P-value
CMV seropositivity	3.384	1.196–9.575	0.022
Age	1.123	0.411–3.072	0.821
Sex	1.083	0.424–2.765	0.868
Conditioning regimen	0.754	0.240–2.367	0.629
Stem cell sources	2.550	0.816–7.969	0.108
GVHD prophylactic	0.126	0.006–2.535	0.176
aGVHD	0.367	0.132–1.021	0.055

**Abbreviations:** aGVHD, acute GVHD; AML, acute myeloid leukemia; CMV, cytomegalovirus; GVHD, graft-versu-host disease; LFS, leukemia-free survival.



**Figure 5** Overall survival curve of 65 leukemia patients.  
**Abbreviation:** CMV, cytomegalovirus.

with AML, ALL, CML, CLL, MDS, and other undefined leukemia and had been determined CMV-free to standardize comparison criteria before allo-HSCT. Our data showed that the number of CD8<sup>+</sup> T cells and the CD8/CD4 ratio were statistically higher in the CMV+ group, consistent with other study referring to CMV reactivation after transplantation, suggesting that post-surgery serum CMV status is closely related to peripheral immune cell alternation. CD8<sup>+</sup> T-cell and CD4<sup>+</sup> T-cell restoration after subsequent CMV seroconversion by GCV administration further confirms that CMV had impact on the human peripheral immune system in our study.

Another important finding in our study is that CMV seropositivity was positively related to LFS of patients regardless of leukemia types. It was supposed to be related to functional CD8<sup>+</sup> T cells for exerting cytotoxic effects in resisting chronic virus invasion and expansion of the renewed clone,<sup>17,18</sup> and likewise for its peripheral domination after CMV seropositivity in our study. Higher CD8<sup>+</sup> T cells have been reported, but with increased CD4<sup>+</sup> T cells in CLL patients.<sup>19</sup> CMV-specific CD8<sup>+</sup> T cells express relatively lower inhibitory markers PD-1 and CD160, and they maintain conventional virus-specific cytotoxicity by CD45RA<sup>+</sup> CD27<sup>+</sup> phenotype.<sup>17,20</sup> CMV-specific CD4<sup>+</sup> T cells promote cytotoxic T-cell activity through Th1 cytokines and activation of antigen-presenting cells.<sup>21,22</sup> They also play an important role in CMV-specific CD8<sup>+</sup> cytotoxic T-lymphocyte differentiation and expansion via dendritic cell stimulation.<sup>23,24</sup> Considering

CD4<sup>+</sup> T cells were not numerically increased in the CMV+ group in this study, it is worth exploring whether ratios of each subtype of CD4<sup>+</sup> T cells alter correspondingly. Recently, Scheper et al found that gamma delta T cells ( $\gamma\delta$ T) initiated by CMV infection after allo-HSCT cross-recognized both CMV-infected cells and primary leukemia blasts, indicating that CMV is a warrior against leukemia.<sup>25</sup> The interplay between  $\gamma\delta$ T cells and CMV infection seems to be a plausible explanation for the advantageous clinical prognosis.<sup>26</sup> Mycophenolate mofetil affects viral replication by altering cytokine profiles and modulating adhesion-related molecules mediated by CMV, but it impairs both T and B cells' proliferation.<sup>27,28</sup> Still, fewer CD4<sup>+</sup> T cells can induce T-B collaboration and reduce chronic GVHD (cGVHD) incidence. Moreover, CMV seropositivity increases the expression of leukocyte fixation antigen-3 on CMV-infected leukemia blasts and enhances NK cell-mediated blasts lysis without an increase in amount.<sup>29,30</sup> Our data that CD19<sup>+</sup> B cells reconstituted until 6 months after transplantation in the CMV+ group are consistent with the opinion that both aGVHD and cGVHD inhibit B cell reconstitution and delay B-cell immune recovery.<sup>6</sup>

Leukemia patients who receive peripheral blood stem cells will acquire donor-derived passive immunity, whereas cord blood stem cells are immunological naïve, and upon activation, donor-derived T cells will aim at CMV lesion and directly exert cytotoxic effects towards leukemia blasts expressing CMV-related antigens. Unfortunately, the wide use of ATG in leukemia patients who are ready to receive cord blood stem cell transplantation messes with the recipient-derived immune cells, and this may be vital to post-surgery immune response especially encountering CMV infection.<sup>31</sup>

## Conclusion

Here, we reported the alternations of peripheral immune cells, including CD4<sup>+</sup> T cells, CD8<sup>+</sup> T cells, NK cells, and B cells, responses to CMV seropositivity of leukemia patients after allo-HSCT. Leukemia patients may benefit from CMV seropositivity on LFS aspect, probably due to the immune constitution of peripheral CD8<sup>+</sup> T cells. Further, how to maintain functional and long-run peripheral CD8<sup>+</sup> T cells after allo-HSCT may be a promising alternative therapeutic regimen in individualized prognosis.

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## Disclosure

The authors report no conflicts of interest in this work.

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