ORIGINAL RESEARCH

Protective Effects of Cytomegalovirus DNA Copies ≧1000/mL for AML Patients in Complete Remission After Single Cord Blood Transplantation

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Introduction: Current consensus recommends a protective effect of cytomegalovirus (CMV) infection on relapse after peripheral blood or bone marrow hematopoietic stem cell transplantation. However, in cord blood transplantation (CBT), studies of CMV infection, especially CMV viral load, on relapse are limited.

Patients and Methods: Wct e retrospectively analyzed the effect of CMV infection on 3-year outcomes in 249 AML patients according to CMV DNA load (DNA copies <1000/mL and DNA copies ≧1000/mL) within 100 days after CBT. Furthermore, eight-colour flow cytometry was used to detect peripheral blood lymphocyte subsets in 38 patients who received CBT in the last year, and 10 healthy volunteers were included as controls.

Results: The results showed that CMV DNA load did not affect the cumulative incidence of relapse in the whole study population. However, in patients with complete remission status before transplantation, the high CMV DNA load group showed a significantly reduction of relapse than the low CMV DNA load group (3.9% vs 14.6%, p=0.012, respectively), which was confirmed by multivariate analysis (HR 0.23; 95% CI, 0.07–0.73, p = 0.012). Surprisingly, high or low CMV DNA load did not significantly affect non-relapse mortality or overall survival (18.0% vs 17.0%, p=0.777 and 79.0% vs 74.6%, p=0.781, respectively). Besides, the absolute number of $CD8^+$ T cells were increased in the high CMV DNA load group compared with the low DNA load group 1 month after CBT (0.20×10^9/L vs 0.10×10^9/L, p=0.021, respectively).

Conclusion: DNA copies ≧1000/mL for AML patients in complete remission was associated with a lower incidence of relapse after CBT, which might partly result from the expansion of CMV-related $CD8^+$ T cells.

Keywords: cord blood transplantation, cytomegalovirus, DNA load, relapse, acute myeloid leukemia, complete remission

Introduction

Hematopoietic stem cell transplantation (HSCT) is a curative approach for hematologic malignancies. In general, cord blood transplantation (CBT) is a safe and effective alternative for patients lacking an HLA-matched related or unrelated donor.[1–3] In recent years, with the improvement of transplantation procedures, the use of unrelated cord blood (CB) has dramatically increased,[4] whereas immunodeficiency during the transplantation procedures can provide opportunities for cytomegalovirus (CMV) infection.

CMV infection is a major cause of morbidity and mortality related to CMV disease and non-relapse mortality (NRM) in HSCT.[5,6] With the improvement of

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CMV management, severe CMV infection and death has significantly reduced.[7,8] However, recent studies reported that CMV infection was associated with reduced relapse in AML recipients receiving peripheral blood (PB) or bone marrow (BM) HSCT but not CBT.[9–14] And the protective effects appeared to be related to myeloablative conditioning (MAC) HSCT[12] and whether or not to use ATG.[14] Additionally, Michael Koldehoff et al suggested that CMV might initiate viral anti-leukemia functions to promote apoptosis in AML or BCR-ABL-positive ALL cells.[15] However, more studies have theorized that CMV exerted anti-leukemia effects through indirect modulation of innate and adaptive immune responses.[16–19]

All of the above has provoked a reassessment of CMV infection on relapse after CBT. In this paper we attempt to defend the view that the degree of CMV infection might be related to the strength of the anti-leukemia effect. Thus, we retrospectively analyzed the relationship between CMV infection and 3-year outcomes according to CMV DNA load. In order to identify the mechanisms of CMV on outcomes, a group of patients were asked to analyze the immunological responses caused by CMV.

Patients and Methods
Study Design and Patients
This study was performed at the Anhui Provincial Hospital in Hefei, China. All patients who received a single unrelated CB graft between August 2008 and October 2018 were invited to participate in the study. The enrolment criteria were as follows: 1) Patients diagnosed with AML; 2) Patients with no vital organs seriously injured before transplantation, such as the brain, heart, lungs and abdominal organs et al; 3) Patients who had neutrophil engraftments; and 4) Patients who received MAC without depleted-T cells. We captured data for CMV infection before day 100 post transplantation.[20] The 3-years outcomes of the high CMV DNA load group (DNA copies $\geqq$1000/mL) and the low CMV DNA load group (DNA copies <1000/mL) were comparatively analyzed after CBT. The negative CMV DNA copy group was merged into the CMV DNA copies <1000/mL group due to the high incidence of CMV infection (85%) in the analysis of survival.

Meanwhile, PB samples from 38 patients of the above populations, receiving a CB graft between January 2017 and February 2018, were collected 1 month after CBT. Correspondingly, 10 healthy physical examination populations were recruited as controls. Our protocol complied with the Declaration of Helsinki and was approved by the Anhui Medical University Institutional Review Board, and all individuals included in the study provided written informed consent.

Study Endpoints and Definitions
The primary endpoint was the cumulative incidence (CI) of relapse that was stratified by CMV DNA load. The secondary endpoints included NRM, disease-free survival (DFS), overall survival (OS), GVHD-free relapse-free survival (GRFS),[21] CMV disease,[22] acute and chronic GVHD status.[23,24] Neutrophil engraftment was defined as the achievement of an ANC $\geqq$ 0.5×10^9/L for 3 consecutive days. The MAC regimen referred to an intravenous busulfan-based regime (Bu, total 12.8 mg/kg, 0.8mg/kg every 6 h, day-7 to day-4) or a total body irradiation-based regime (TBI, total 12 Gy in 4 fractions, day-7 to day-6).[25,26] The NCCN-2018 guideline definitions of disease risk stratification were used.[27] Complete remission (CR) was defined as less than 5% blasts in the bone marrow, the absence of extra-medullary leukemia, and normal values for absolute neutrophil count (>1×10^9/L) and platelet count (>100×10^9/L), and independence from red cell transfusion.[28] Any other disease status was categorized as non-complete remission (NR). Pre-engraftment syndrome (PES) was characterized by a noninfectious fever and various other clinical findings before neutrophil engraftment, including skin rash, pulmonary infiltrates, and weight gain.[29]

CMV Monitoring and Pre-Emptive Therapy
CMV infection was defined as virus isolation or the detection of viral proteins (antigens) or nucleic acids in any body fluid or tissue specimen. In this study, CMV infection referred to CMV viremia, which was defined as the detection of CMV DNA in samples of plasma, serum, whole blood, peripheral blood leukocytes, bronchoalveolar lavage fluid, or tissue.[30] And CMV disease referred to symptomatic CMV infectious disease, which was defined as the quantitation of CMV DNA in bronchoalveolar lavage fluid or tissue combined with attributable symptoms.[22] The CMV nucleic acid testing was performed using the quantitative real-time PCR (RT-PCR) method as we previously reported.[31] CMV monitoring was examined twice per week for at least 100 days and once a week for at least half a year after transplantation.

Recently, letermovir was approved for CMV prophylaxis in allogeneic HSCT recipients[32] but was not yet available in China. Therefore, the enrolled patients did

not receive prophylaxis of CMV. The pre-emptive antiviral therapy (5 mg/kg intravenous ganciclovir every 12 h) was initiated when CMV DNA copies ≧1000/mL of whole blood for at least 7 days, followed by an intravenous or oral maintenance therapy until two consecutive negative CMV DNA copies were obtained. Foscarnet (90 mg/kg every 12 h), with or without intravenous gammaglobulin administration (0.4 g/kg daily, for 3–5 d), was used as an alternative drug for the treatment of ganciclovir intolerance or for resistant and refractory CMV. The same treatment regimen was also adapted to the treatment of CMV disease.

Flow Cytometry Analysis of Peripheral Blood Cells

T cells ($CD3^+$, $CD3^+CD4^+$, $CD3^+CD8^+$), regulatory T (Treg) cells ($CD3^+CD4^+CD25^+CD127^{low}$), B cells ($CD19^+$), regulatory B (Breg) cells ($CD3^-CD19^+CD24^{high}CD38^{high}CD27^-CD5^{dim}$) and NK cells ($CD3^-CD16^+CD56^+$) were assessed via flow cytometry to monitor the recovery of immune reconstruction 1 month after transplantation.

The same specimen was divided into three tubes for detection: 1) TBNK: a multitest six- colour TBNK reagent panel (from Tongsheng Times, Beijing, China) of monoclonal antibodies as used for CD3 (FITC), CD16CD56 (PE), CD45 (PerCP-cy5.5), CD4 (PE-Cy7), CD19 (APC) and CD8 (APC-Cy7); 2) Treg: fluorescein-labelled monoclonal antibodies were used for CD45 (PerCP-cy5.5), CD4 (FITC), CD25 (PE) and CD127 (APC); and 3) Breg: fluorescein-labelled monoclonal antibodies were used for CD45 (PerCP-cy5.5), CD19 (PE-Cy7), CD27 (FITC), CD24 (PE), CD5 (APC) and CD38 (V450). Two hundred microliters of fresh PB from EDTA tubes were added to the above fluorescein-labeled monoclonal antibodies for 30 mins (4°C and protected from light). Then, red blood cell lysates (Beckman Coulter, Miami, FL, USA) were added for 5 mins and then washed three times. And appropriate isotype controls were performed simultaneously. All of the fluorescein-labelled monoclonal antibodies were obtained from BD Biosciences (San Jose, CA, USA), except for CD19, which was obtained from Biolegend (San Diego, CA, USA), and CD25, which was obtained from Beckman Coulter (Miami, FL, USA). Flow cytometry was performed on a FACS Canto II (BD Bioscience) and analyzed using Flowjo V10 software.

Statistical Analysis

The patients and transplantation characteristics of different CMV DNA load groups were compared using the chi-square test or Fisher exact test for categorical variables and the Mann–Whitney Nonparametric test for continuous variables. The probabilities of OS, DFS, and GRFS were analyzed by the Kaplan–Meier method. For the CI analyses of relapse, acute GVHD (aGVHD) and chronic GVHD (cGVHD), death was a competing event, whereas relapse was the competing event for the CI of NRM. Time-to-event analyses were performed using the Gray test for competing events. In multivariate analysis of acute and chronic GVHD, the proportional hazard assumption was examined. If violated, it was compared using the time-dependent cox regression model. A stepwise selection procedure was used. In univariate analysis, the main aim was to investigate the relationship between the outcomes and the CMV DNA load, and a series of other independent variables like recipient's age/gender/weight, donor-recipient ABO/HLA compatibility, disease stage/risk stratification, total number of nucleated cells (TNC), total number of $CD34^+$ cells, conditioning regimes, PES,[26] and acute/chronic GVHD were also taken into consideration. In multivariate analysis, borderline significance ($p < 0.1$) in univariate analysis or more related to the outcomes was analyzed simultaneously. Statistical analyses were performed using SPSS software, version 24.0, and Easy R software, Version 3.3.2. A p-value <0.05 was considered to indicate statistical significance.

Results

Patient Characteristics and CMV Infection

For the entire population, a total of 249 patients participated in the study, among whom 199 (80%) patients achieved CR while 50 (20%) patients in NR before transplantation. With a median follow-up period of 1148 (349–4075) days, CMV infection occurred in 211 (85%) patients at a median time of 31 (15–90) days after CBT. Specifically, 130 (52%) patients had a high CMV DNA load and 119 (48%) patients had low CMV DNA load. Additionally, a total of 202 patients underwent pre-transplantation CMV antibody tests (IgG), most of which were transplanted after 2015. We found that the CMV-IgG positive rate in this group of patients was as high as 98%. The baseline characteristics for the entire population are shown in Table 1.

The baseline characteristics in CR patients are also shown in Table 1, and the patients' ages, gender, weights,

Table 1 Patients' Characteristics for the Groups According to CMV DNA Load

Characteristic	CR+NR		P	CR		P
	High DNA Load	Low DNA Load		High DNA Load	Low DNA Load	
	No. (%)	No. (%)		No. (%)	No. (%)	
Number of patients	130 (52)	119 (48)	–	107 (54)	92 (46)	–
Median age, years (range)	13 (0–55)	14 (1–55)	0.259	13 (0–54)	14 (1–55)	0.196
Median weight, kg (range)	40 (10–82)	45 (9–95)	0.275	40 (10–82)	45 (9–95)	0.159
Gender			0.913			0.791
Male	73 (56)	66 (55)		59 (55)	49 (53)	
Female	57 (44)	53 (45)		48 (45)	43 (37)	
ABO compatibility			0.037			0.072
Match	48 (37)	50 (42)		40 (37)	43 (47)	
Major mismatch	31 (24)	30 (25)		22 (21)	20 (21)	
Minor mismatch	45 (34)	25 (21)		39 (36)	19 (21)	
Major and minor mismatch	6 (5)	14 (12)		6 (6)	10 (11)	
HLA compatibility (A, B, DR)			0.891			0.688
6/6	13 (10)	14 (12)		11 (10)	13 (14)	
5/6	61 (47)	56 (47)		54 (51)	43 (47)	
4/6 or 3/6	56 (43)	49 (41)		42 (39)	36 (39)	
Disease stage			0.760			0.460
First remission	74 (57)	68 (57)		74 (69)	68 (74)	
Second/third remission	33 (25)	24 (20)		33 (31)	24 (26)	
Non-remission	23 (18)	27 (23)		—	—	
Risk stratification			0.759			0.962
Poor	80 (62)	85 (71)		62 (58)	53 (58)	
Intermediate	39 (38)	45 (29)		45 (42)	39 (42)	
TNC ($\times 10^7$/kg)	4.0 (1.0–13.1)	3.6 (0.4–11.0)	0.139	4.0 (1.0–13.1)	3.6 (0.4–11.0)	0.067
CD34 ($\times 10^5$/kg)	2.1 (0.5–10.6)	1.9 (0.2–13.8)	0.318	2.1 (0.5–10.6)	1.9 (0.2–13.8)	0.108
Conditioning regimen			0.002			0.002
BU-based	91 (70)	106 (89)		75 (70)	81 (88)	
TBI-based	39 (30)	13 (11)		32 (30)	11 (12)	
PES			<0.001			<0.001
With	114 (88)	79 (66)		95 (89)	59 (64)	
Without	16 (12)	40 (34)		12 (11)	33 (36)	
aGVHD			0.027			0.054
0 – I	73 (56)	83 (70)		64 (60)	67 (73)	
II–IV	57 (44)	36 (30)		43 (40)	25 (27)	
cGVHD			0.116			0.160
With	26 (20)	15 (13)		22 (21)	12 (13)	
Without	104 (80)	104 (87)		85 (79)	80 (87)	
CMV-IgG			0.001			0.036
Positive	93 (72)	104 (87)		78 (73)	79 (86)	
Negative	2 (1)	3 (3)		2 (2)	2 (2)	
None	35 (27)	12 (10)		27 (25)	11 (12)	

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disease statuses, HLA compatibility, ABO blood compatibility, TNC cell doses, CD34^{+} cell doses, aGVHD and cGVHD statuses exhibited no significant differences between the high and low CMV DNA load groups. However, the Conditioning regimens and incidence of PES were imbalance between the two groups, and all of them were included in the univariate analysis of the outcomes.

CBT Outcomes

For the entire population, there were no statistically significant differences in the CI of relapse, NRM, and the probability of survival between the two different CMV DNA load groups (Table 2), which were confirmed by multivariate analysis (Supporting Information, Table S1). A total of 26 patients in the entire cohort suffered relapse (10 cases in high DNA load group and 16 cases in low DNA load group), and 16 (62%) of them relapsed within 1 year after CBT (data not shown). Besides, in 199 patients with CR status before transplantation, we have seen the following differences.

Relapse and Non-Relapse Mortality

For CR recipients, the high CMV DNA load group had a lower 3-year CI of relapse than the low CMV DNA load group (Figure 1A; 3.9% vs 14.6%, p=0.012, respectively). In detail, a total of 16 patients suffered relapse (4 cases in high DNA load group and 12 cases in low DNA load group). In multivariate analysis, a high CMV DNA load was confirmed to have a protective effect on relapse (Table 3; HR 0.23; 95% CI, 0.07–0.73, p = 0.012). Besides, man recipient had a higher CI of relapse (Table 3; HR: 4.39; 95% CI: 1.24–15.54; p=0.022) in multivariate analysis.

The 3-year NRM showed no significant difference between the high and low CMV DNA load groups (Figure 1B; 18.0% vs 17.0%, p=0.777, respectively). In

multivariate analysis, grade $\text{II}-\text{IV}$ aGVHD was an important risk factor for increasing NRM (Table 3; HR: 6.79; 95% CI: 3.13–14.72; p < 0.001). In detail, 15 (16%) patients in the low DNA load group and 19 (18%) patients in the high DNA load group died without relapse. Pulmonary infection and grade $\text{II}-\text{IV}$ aGVHD were the leading causes of death.

Survival and GVHD

For CR recipients, 3-years DFS was non-significant between the high and low DNA load groups in univariate analysis (Figure 2A; 78.2% vs 69.6%, p=0.277, respectively). However, in multivariate analysis including variables with borderline significance (p < 0.1), high CMV DNA load was associated with the improvement of DFS (HR 0.51, 95% CI, 0.27–0.91, p=0.024). Besides, OS and GRFS showed non-significant between the high and low CMV DNA load groups (Figure 2B and C; 79.0% vs 69.6%, p=0.781 and 53.9% vs 58.3%, p=0.357, respectively). When comparing the aGVHD and cGVHD, there were no significant differences between the two CMV DNA load groups (Table 2).

It was worth noting that grade $\text{II}-\text{IV}$ aGVHD was a remarkable risk factor for reducing OS, and DFS (Table 3; HR, 5.45, 95% CI, 2.85–10.42, p<0.001 and HR, 4.87, 95% CI, 2.70–8.79, p<0.001, respectively) in multivariate analysis. Also, man recipients showed significantly reduced probability of OS and DFS than female recipients (Table 3; HR, 1.94, 95% CI, 1.01–3.71, p=0.046 and HR, 2.21, 95% CI, 1.15–3.87, p=0.016, respectively). In addition, BU-based conditioning regimen presented a better OS than TBI-based conditioning regimen and poor risk stratification was associated with the reduction of OS in multivariate analysis (Table 3; HR, 2.79, 95% CI,

Table 2 Univariate Analysis of 3-Year Outcomes for the Groups According to CMV DNA Load

| Outcome	CR+NR		P	CR		P
	High DNA Load	**Low DNA Load**		**High DNA Load**	**Low DNA Load**	
	% (95% CI)	**% (95% CI)**		**% (95% CI)**	**% (95% CI)**	
Relapse	7.9 (4.0–13.5)	16.7 (9.2–26.0)	0.102	3.9 (1.3–9.0)	14.6 (7.8–23.4)	0.012
NRM	20.9 (14.4–28.4)	19.0 (12.4–26.8)	0.639	18.0 (11.3–25.9)	17.0 (9.9–25.7)	0.777
OS	71.8 (63.1–78.8)	68.8 (56.8–78.1)	0.858	79.0 (69.9–85.7)	74.6 (61.7–83.6)	0.781
DFS	71.2 (62.5–78.2)	65.2 (54.1–74.2)	0.664	78.2 (69.0–84.9)	69.6 (58.2–78.4)	0.277
GRFS	48.9 (40.0–57.2)	50.3 (39.4–60.3)	0.371	53.9 (43.9–62.8)	58.3 (47.0–68.0)	0.357
Grade $\text{II}-\text{IV}$ aGVHD	43.8 (35.2–52.2)	30.3 (20.2–38.7)	0.037	40.2 (30.8–49.3)	27.2 (18.5–36.6)	0.062
cGVHD	20.2 (13.7–27.6)	14.1 (8.1–21.8)	0.143	20.8 (13.6–29.1)	14.1 (7.6–22.5)	0.187
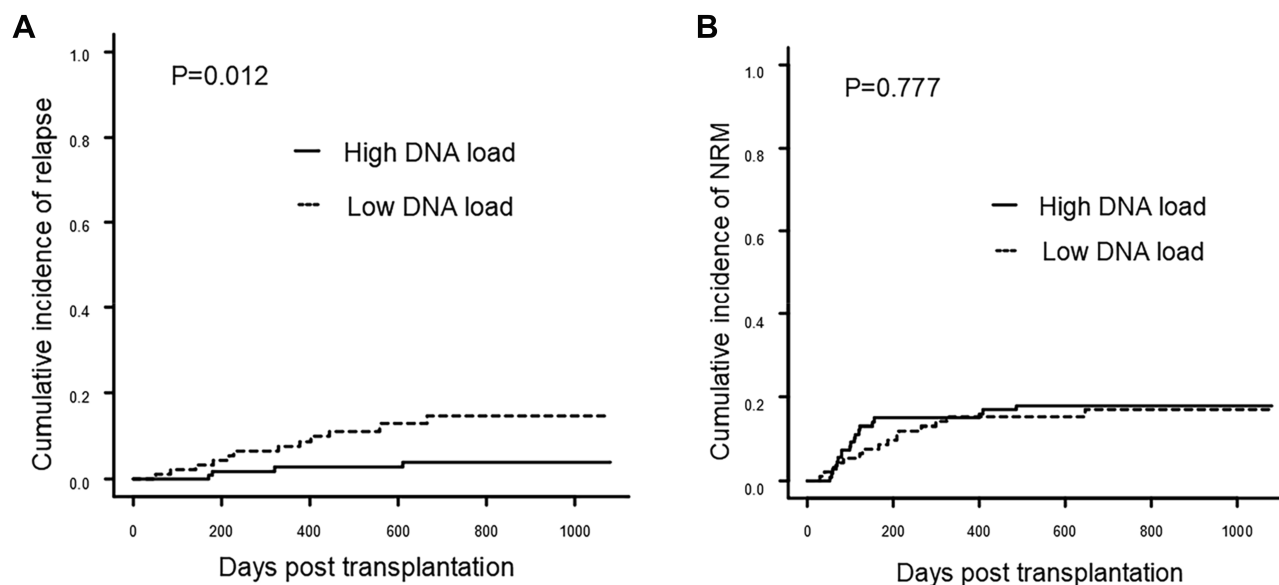
Figure 1 Cumulative incidence of relapse and NRM according to CMV DNA load in CR patients. (**A**) Relapse; (**B**) Non-relapse mortality.

1.43–5.43, p=0.003 and HR, 0.36, 95% CI, 0.17–0.76, p=0.007, respectively).

CMV Disease

In the entire group of patients, precise CMV monitoring and pre-emptive treatments were performed for CMV management. Since the launch of the CMV disease-related examination in 2016, only six (2.4%) cases of CMV disease were diagnosed (one case of CMV pneumonia and three cases of CMV enteritis in low CMV DNA load group while two cases of CMV enteritis in high CMV DNA load group) (data not shown). Fortunately, all of them cured and survived from CMV disease at the end of follow-up.

Immune Reconstruction
High CMV DNA Load Alters Peripheral CD8^{+} T Cells Distribution

A total of 38 patients were included in the immune reconstruction analysis. Among them, 11 (29%) patients were high CMV DNA loads, 27 (71%) patients were low CMV DNA loads. There were no significant differences in baseline characteristics between the two groups (Supporting Information, Table S2). We analyzed the number of T cells, B cells, NK cells, Treg cells and Breg cells according to CMV DNA load.

The data showed that CD3^{+} T cells were significantly increased in the high CMV DNA load group (Figure 3A; 0.40×10^9/L vs 0.27×10^9/L, p=0.010). And NK cells presented no statistical difference between the high and low CMV DNA groups (Figure 3B; 0.20×10^9/L vs 0.21×10^9/L, p=0.657, respectively). However, donor-derived B cells,

along with Breg cells, were not reconstituted 1 month after transplantation.

Upon further analyses of T cell subsets found that CD8^{+} T cells in the high CMV DNA load group were significantly expanded (Figure 4A; 0.20×10^9/L vs 0.10×10^9/L, p=0.021). Nevertheless, no significant differences were found in CD4^{+} T cells and Treg cells between the high and low CMV DNA load groups (Figure 4B and C; 0.20×10^9/L vs 0.10×10^9/L and 0.20×10^7/L vs 0.32×10^7/L; p=0.159 and p =0.612, respectively).

Discussion

Studies have shown that CMV infection for AML patients after PB or BM HSCT has a protective effect on relapse.[11–13] However, in the CB setting, CMV infection was not associated with relapse.[9] Indeed, our previous study has also shown that only infection with CMV for AML patients could not reduce relapse after CBT (data not shown). The results of this study indicated that the CMV DNA copies ≥1000/mL (high CMV DNA load) might be a valuable parameter to that protective purpose.

In our center, up to 98% of the patients receiving pre-transplant antibody testing were positive for CMV-IgG (data not shown). Similarly, previous research has reported that CMV seroprevalence (IgG) tended to be highest in South America, Africa and Asia (>90%).[33] Antiviral prophylaxis has not been a first-line option for CMV prevention in allogeneic HSCT recipients because myelosuppression, particularly leukopenia and neutropenia, is

Table 3 Multivariate Analysis of 3-Year Outcomes in CR Patients

Characteristic	Hazard Ratio	95% CI	P
RELAPSE			
Gender (male vs female)	4.39 vs 1	1.24–15.54	0.022
Risk stratification (intermediate vs poor)	0.33 vs 1	0.11–1.04	0.058
CMV DNA load (high vs low)	0.23 vs 1	0.07–0.73	0.012
Grade Ⅱ–Ⅳ aGVHD (without vs with)	0.99 vs 1	0.90–1.12	0.929
cGVHD (with vs without)	0.30 vs 1	0.04–2.33	0.250
NRM			
Age (years), (≥17 vs <17)	1.21 vs 1	0.56–2.63	0.630
Risk stratification (intermediate vs poor)	0.61 vs 1	0.23–1.60	0.310
Disease stage (second/third vs first remission)	1.52 vs 1	0.63–3.65	0.360
Conditioning regimen (TBI vs BU)	2.45 vs 1	0.99–6.04	0.052
CMV DNA load (low vs high)	0.66 vs 1	0.31–1.38	0.270
PES (with vs without)	1.58 vs 1	0.51–4.96	0.430
Grade Ⅱ–Ⅳ aGVHD (with vs without)	6.79 vs 1	3.13–14.72	<0.001
DFS			
Age (years), (≥17 vs <17)	1.38 vs 1	0.67–2.84	0.385
Gender (male vs female)	2.21 vs 1	1.15–3.87	0.016
Risk stratification (intermediate vs poor)	0.51 vs 1	0.24–1.06	0.073
Disease stage (second/third vs first remission)	1.51 vs 1	0.78–2.90	0.226
Conditioning regimen (TBI vs BU)	2.00 vs 1	0.90–4.43	0.087
CMV DNA load (high vs low)	0.51 vs 1	0.27–0.91	0.024
Grade Ⅱ–Ⅳ aGVHD (with vs without)	4.87 vs 1	2.70–8.79	<0.001
OS			
Gender (male vs female)	1.94 vs 1	1.01–3.71	0.046
Risk stratification (intermediate vs poor)	0.36 vs 1	0.17–0.76	0.007
Conditioning regimen (TBI vs BU)	2.79 vs 1	1.43–5.43	0.003
CMV DNA load (high vs low)	0.60 vs 1	0.32–1.13	0.115
Grade Ⅱ–Ⅳ aGVHD (with vs without)	5.45 vs 1	2.85–10.42	<0.001
GRFS			
CD34 ($\times 10^5$/kg), (≥2.0 vs <2.0)	1.40 vs 1	0.91–2.17	0.126
CMV DNA load (high vs low)	1.01 vs 1	0.65–1.60	0.934
PES (with vs without)	1.63 vs 1	0.89–2.97	0.111
aGVHD			
Recipient weight (kg), (≥40 vs <40)	0.62 vs 1	0.32–1.22	0.170
TNC ($\times 10^7$/kg), (≥3.7 vs <3.7)	0.97 vs 1	0.48–1.96	0.920
CD34 ($\times 10^5$/kg), (≥2.0 vs <2.0)	1.20 vs 1	0.70–2.04	0.520
CMV DNA load (high vs low)	1.27 vs 1	0.74–2.17	0.390
PES (with vs without)	2.20 vs 1	0.96–4.94	0.058
cGVHD			
Gender (male vs female)	0.52 vs 1	0.26–1.04	0.063
CMV DNA load (high vs low)	1.47 vs 1	0.72–3.00	0.290
Grade Ⅱ–Ⅳ aGVHD (with vs without)	2.45 vs 1	1.25–4.82	0.009

commonly observed when using intravenous ganciclovir and valganciclovir.[32] In this study, the CMV infection rate was as high as 88% after CBT (data not shown). Considering the reasons, the high serological positive rate before transplantation and the absence of CMV prevention after transplantation might contribute to this result.

Further in-depth analysis found that high CMV DNA load was associated with a significant reduction of relapse
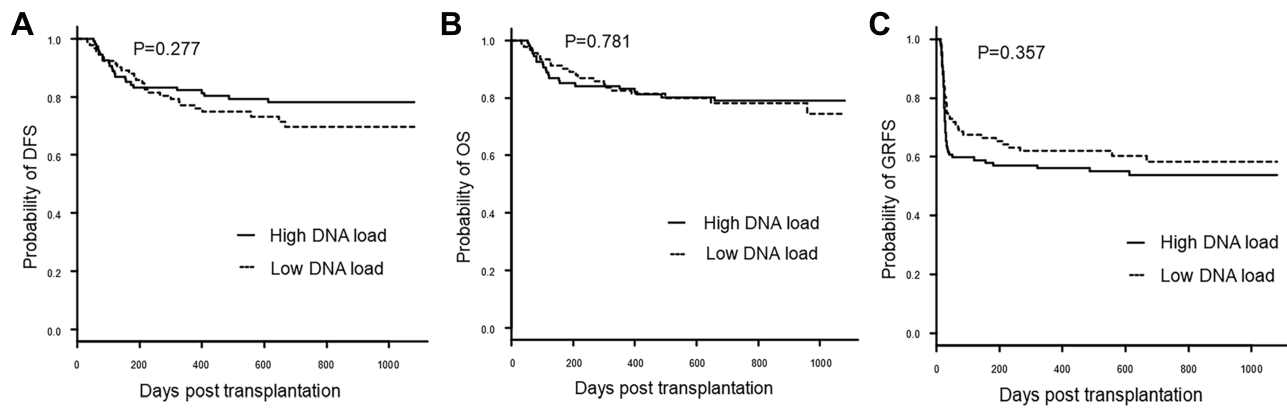
Figure 2 Probability of survival according to CMV DNA load in CR patients. (**A**) Disease-free survival; (**B**) Overall survival; (**C**) GVHD-free, relapse-free survival.
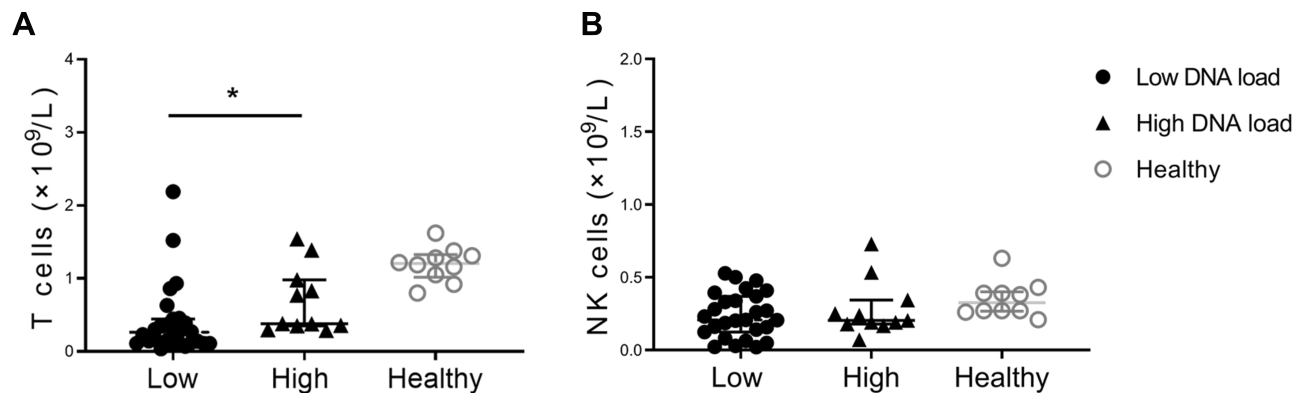

Figure 3 Lymphocytes reconstitution according to CMV DNA load in CR patients. (**A**) Absolute number of T cells; (**B**) Absolute number of NK cells; Notes: All of the statistical graphs show median±interquartile range. *P $\leqq$ 0.05.
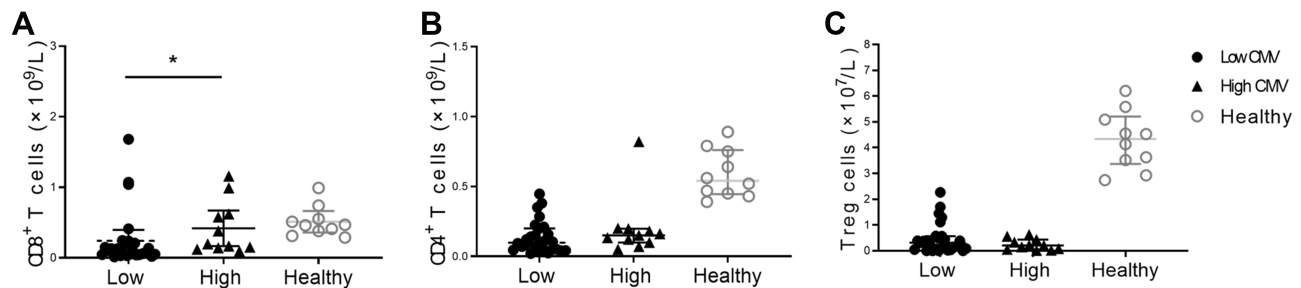

Figure 4 T cell subsets reconstitution according to CMV DNA load in CR patients. (**A**) Absolute number of CD8$^+$ T cells; (**B**) Absolute number of CD4$^+$ T cells; (**C**) Absolute number of Treg cells.
Notes: All of the statistical graphs show median±interquartile range. *P $\leqq$ 0.05.

after CBT in CR recipients (p=0.012). The protective effect was confirmed and independent of acute/chronic GVHD in multivariate analyses (Table 3). However, patients who did not achieve disease remission before transplantation could not benefit from the high CMV DNA load (Table 2). Through the above, we could summarize the following two points. Firstly, this meant that the effect of CMV on relapse was related to the level of

DNA replication of CMV, and the high CMV DNA load group had a lower CI of relapse than the low CMV DNA load group. Secondly, the high CMV DNA load played apositive role in relapse for patients who achieved CR before transplantation, but was not strong enough to reduce relapse in NR patients. For patients who did not achieve CR before HSCT, relapse was the predominant cause of treatment failure.[34,35] So, the intensity of CMV on

relapse might be so limited that high CMV DNA load failed to reverse the natural characteristics of high risk of relapse in NR recipients after transplantation. Considering the other factors, male gender was an independent risk predictor for relapse in multivariate analysis (p=0.022). This negative effect of male gender on relapse has been reported in studies of diffuse large B-cell lymphoma, but has not been reported in HSCT.[36]

In the study, pre-emptive therapy was initiated at the CMV level threshold of DNA copies ≧1000/mL.[5] Under the CMV management, the results suggested an improvement of DFS in the high DNA load group (Table 3). And the NRM, OS, or GRFS showed non-significant between the two groups (Table 3). Considering the other risk factors, grade II–IV aGVHD was an independent risk factor in increasing NRM and reducing OS and DFS.[37] Besides, the high DNA load group contained more patients with II–IV aGVHD in baseline data, which might explain why a high CMV viral load reduced relapse but did not translate into OS benefits in the study.

As mentioned above, through the analysis of CMV DNA load and disease remission status, we first reported this protective effect of CMV DNA copies ≧1000/mL on relapse after CBT, while not affected NRM or OS. The finding was similar to previous studies demonstrating a protective effect of CMV infection on relapse after PB and BM transplantation.[11,38] However, another study reported that under the era of pre-emptive therapy, a CMV DNA load of ≧250 IU/mL (four DNA copies=1 IU) was associated with increased risk of NRM and overall mortality within one-year post HSCT.[5] Considering the reasons for different results, only 11% of them received CBT without indicating single or double CBT, and 40% of them received reduced intensity conditioning, which were different from us. In the study, all patients received MAC without depleted-T cells treatment. In general, MAC was correlated with a lower incidence of relapse[39,40] and depleted-T cells treatment indicated a better outcome due to the faster and more complete T cells recovery. Overall, the high CMV DNA load in our transplant central indicated a better outcome, probably due to the strong clearance of leukemia cells by MAC and immune protection without T-cell depletion.

Amplification of T cells and NK cells stimulated by CMV was considered to play a role in the protection of relapse after HSCT.[16–18] Studies in both humans and animal models have indicated the protective effect of CD8^{+} T cell expansion on tumor regression.[41,42] In HSCT, it has also been reported that

CMV-related or CMV-specific CD8^{+} T-cells were associated with a lower CI of relapse.[43,44] To understand the immunological basis of high CMV DNA on relapse, a study involving seven lymphocyte subsets according to CMV DNA load had been conducted. The results showed that the high CMV DNA load group presented a significant CD8^{+} T cells expansion 1 month after CBT, which was similar to the previous report.[44] Moreover, the infusion of CMV-specific T cells indicated that T cells are required for CMV to exert anti-tumour effects after transplantation.[45] Consequently, all of the above data demonstrated the expansion of CD8^{+} T cells induced by high CMV DNA load was partly associated with the reduction of relapse.

In conclusion, in CB setting, this study indicated the correlation of a significant reduction of relapse with CMV DNA copies ≧1000/mL in patients with CR. What's more, the high CMV DNA load promoted peripheral CD8^{+} T cells expansion 1 month after CBT. Therefore, patients with AML, achieving CR status before transplantation can benefit from CMV DNA copies ≧1000/mL after CBT, and partly from CMV-related CD8^{+} T cells expansion. However, we failed to further define whether the populations of CD8^{+} T cells were specific for CMV. Further serological status of CMV analysis was limited because of the imbalance sample side between the negative and positive CMV-IgG groups in the study. Meanwhile, the number of relapse events could not reach 10 times the number of variables in the multivariate analysis. Previous studies have reported that the probability of relapse was lower in the CBT than other unrelated PB or BM stem cell transplantation, which might account for the low probability of relapse in the study.[46] The causative conclusions were also limited by the limited involved cases and the inherent defects of retrospective clinical studies. It is recommended that further larger sample size of studies be undertaken.

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Disclosure

The authors declare no competing financial interests in this work.

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