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Early Cytomegalovirus Reactivation and Expansion of $CD56^{bright}CD16^{dim/-}DNAM1^{+}$ Natural Killer Cells Are Associated with Antileukemia Effect after Haploidentical Stem Cell Transplantation in Acute Leukemia

Ji Eun Jang[1], Doh Yu Hwang[2], Haerim Chung[1], Soo-Jeong Kim[1], Ju-In Eom[3], Hoi-Kyung Jeung[3], Jaewoo Song[4], Jin Seok Kim[1], June-Won Cheong[1], Yoo Hong Min[1,*]

[1] *Department of Internal Medicine, Yonsei University College of Medicine, Seoul, Korea*
[2] *Department of Internal Medicine, Catholic Kwandong University College of Medicine, Incheon, Korea*
[3] *Avison Biomedical Research Center, Yonsei University College of Medicine, Seoul, Korea*
[4] *Department of Laboratory Medicine, Yonsei University College of Medicine, Seoul, Korea*

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A B S T R A C T

Cytomegalovirus (CMV) infection is a major complication after allogeneic hematopoietic stem cell transplantation but is suggested to exert a strong antileukemia effect in part due to alterations in the composition of natural killer (NK) cells. We evaluated the impact of early CMV reactivation and changes in NK cell subset recovery on relapse rate and survival after haploidentical stem cell transplantation (haploSCT) for acute leukemia. Fifty patients with acute leukemia who received haploSCT were analyzed. Expression of T cells and specific receptors (NKG2A, NKG2D, DNAM1, and CD57) on circulating NK cells ($CD56^{bright}CD16^{dim/-}$ or $CD56^{dim}CD16^{+}$ cells) was serially measured using multiparametric flow cytometry. CMV reactivation during the first 100 days was observed in 41 patients (82%) at a median of 23 days after haploSCT. The incidence of acute graft-versus-host disease (GVHD) and chronic GVHD tended to be higher in patients with CMV reactivation, although this difference was not statistically significant. Multivariate analysis showed that CMV reactivation ($P = .011$) and a dose of infused T cells $> 3.2 \times 10^8$/kg ($P = .027$) were independent predictors of a reduced relapse risk and only CMV reactivation ($P = .029$) was an independent predictor of improved leukemia-free survival. $CD56^{bright}CD16^{dim/-}DNAM1^{+}$NK cell counts increased from day 30 to 90 in patients with CMV reactivation but decreased after day 30 in patients without CMV reactivation. An increase in $CD56^{bright}CD16^{dim/-}DNAM1^{+}$ NK cells was not associated with the occurrence of chronic GVHD but was associated with a reduced cumulative relapse rate (16.4% versus 58.0%, $P = .019$). Multivariate analysis indicates that an increase in the $CD56^{bright}CD16^{dim/-}DNAM1^{+}$NK cell count was an independent predictor of reduced relapse risk. Our study demonstrates a significant correlation between low relapse rates and CMV reactivation as well as the recovery of $CD56^{bright}CD16^{dim/-}DNAM1^{+}$ NK cells, providing valuable information for understanding the plausible immunologic mechanism of the graft-versus-leukemia effect.

INTRODUCTION

Despite the introduction of preemptive therapy for viral reactivation, cytomegalovirus (CMV) infection remains a major complication after allogeneic hematopoietic stem cell transplantation (alloSCT), causing a variety of end-organ diseases and increased nonrelapse mortality (NRM) [1–3]. This issue is particularly relevant in alternative-donor settings—for instance, haploidentical hematopoietic stem cell transplantation (haploSCT)—in which a greater degree of immunosuppression is induced [4–6]. CMV reactivation has also been associated with graft failure, graft-versus-host disease (GVHD), and other infectious complications [7–9]. However, CMV reactivation during the first 100 days after T cell–replete and natural killer (NK) cell–replete alloSCT was implicated as an independent predictor of a reduced leukemia relapse risk in patients with adult acute myeloid leukemia (AML) [10–13] or chronic myeloid leukemia [14] and in pediatric acute leukemia or myelodysplastic syndrome [15]. CMV reactivation remained an independent predictor of a low relapse rate in AML after multivariate analysis [12,13]. Moreover, we previously

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* Correspondence and reprint requests: Yoo Hong Min, MD, PhD, Division of Hematology, Department of Internal Medicine, Yonsei University College of Medicine, Yonsei-ro 50, Seodaemun-gu, Seoul 03722, Korea.
E-mail address: minbrmmd@yuhs.ac (Y.H. Min).

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demonstrated that when CMV reactivation during the first 100 days precedes chronic GVHD, the relapse rate after alloSCT is significantly reduced in patients with AML [12].

The mechanism underlying this beneficial effect of CMV reactivation on leukemic relapse remains to be elucidated. Because CMV reactivation does not protect against AML relapse when T and/or NK cells are depleted in vivo or in vitro [16–18], the early and effective reconstitution of donor-derived NK cells and/or T cells may be required for the anti-leukemia effect of CMV infection after alloSCT [19–21]. Recently, it was suggested that CMV-driven expansion of donor-derived memory-like NK cells and Vδ2(-)$\gamma\delta$ T cells may be associated with the antileukemia effect of CMV reactivation after alloSCT [22]. It has also been shown that this effect may be mediated by CMV–induced shifts in the composition of NK cell subsets [19,21,23]. NK cells are endowed with a complex system of both activating and inhibitory receptors that either positively or negatively modulate their functions [24–26]. The inhibitory NK cell receptor families include the killer cell immunoglobulin-like receptors (KIRs) and CD94/NKG2A [26,27]. Activating signals are mediated by receptor families such as natural cytotoxicity receptors (NKp46, NKp30, and NKp44), NKG2C, NKG2D, DANAX accessory molecule (DNAM1/CD226), activating KIRs, and the low-affinity Fc-γ receptor IIIA (CD16) [28–30]. It has been shown that the CMV–driven accumulation of NKG2C$^+$/NKG2A$^-$ NK cells is associated with a strong antileukemia effect that is commensurate with the magnitude of leukemia cell HLA–E expression [21]. Thus, the evidence of a protective association between CMV reactivation and leukemic relapse in AML patients appears to be compelling but only under specific transplantation conditions [16–18].

Although immune reconstitution after alloSCT has been studied extensively, the core immune cell subsets responsible for the antileukemia effect of CMV reactivation remain unclear. Whereas Lin et al. [31] reported that CMV infection after haploSCT may likewise reduce relapse risk in leukemia, it is currently unclear whether CMV reactivation exerts a similar antileukemia effect in patients after haploSCT, which often causes delayed immune reconstitution because of more severe immunosuppression [4]. If CMV-specific immune reconstitution is delayed, CMV-induced antileukemia effects may also be impaired. In this study we evaluated the potential antileukemia roles of CMV reactivation in haploSCT and examined the subset of immune cells contributing to the antileukemia effect after CMV reactivation. Our research provides valuable information for understanding the plausible immunologic mechanism of the graft-versus-leukemia (GVL) effect of CMV.

METHODS

Patients

Fifty-five adults with acute leukemia underwent haploSCT at Severance Hospital, Yonsei University College of Medicine, between 2009 and 2018. Five patients with secondary engraftment failure were excluded from this study; thus, 50 patients with successful engraftment were analyzed. All patients were unable to find a suitable HLA-matched donor in their families or on donor registries for alloSCT.

This study was approved by the Severance Hospital institutional review board (4-2010-0669) and carried out in accordance with the Declaration of Helsinki. This prospective and retrospective cohort is registered at ClinicalTrials.gov (NCT02344953). All subjects provided written informed consent, and all patient samples were coded and linked anonymously. Anonymous clinical information of the linked samples was provided to the researchers. Clinical data from patients with AML (n = 23), acute lymphoblastic leukemia (n = 24), and mixed phenotype acute leukemia (n = 3) were retrospectively analyzed. Adverse risk cytogenetics and molecular abnormality were distinguished on the basis of the National Comprehensive Cancer Network Guidelines (version 1.2018).

Transplantation

In accordance with institutional practice for haploSCT, most of the 50 patients (n = 35) received a preparative regimen consisted of fludarabine (30 mg/m^{2}i.v. on days −7 to −2), busulfan (3.2 mg/kg i.v. on days −7 and −6), and rabbit antithymocyte globulin (2.5 mg/kg i.v. on days −4 to −1) according to a modified reduced-intensity conditioning regimen [32]. For GVHD prophylaxis, patients were administered cyclosporine A (1.5 mg/kg i.v. every 12 hours starting on day −1). In addition, patients received methotrexate (15 mg/m^2 i.v. on day 1 and 10 mg/m^2 on days 3, 6, and 11). Cyclosporine A was progressively tapered beginning on day 90 by 10% every 2 to 4 weeks. Eight patients received fludarabine (30 mg/m^2 i.v. on days −6 to −2), cyclophosphamide (14.5 mg/kg i.v. on days −6 and −5), and total body irradiation (200 cGy on day −1) according to the Baltimore protocol [33]. GVHD prophylaxis in these patients involved cyclophosphamide (50 mg/kg i.v. on days 3 and 4) and cyclosporine A plus mycophenolate mofetil (15 mg/kg/day) starting on day 5 [33]. In the absence of GVHD, mycophenolate mofetil was discontinued on day 35. Regardless of conditioning regimens, graft sources were mobilized peripheral blood stem cells for 44 patients and harvested bone marrow cells for 6 patients. Peripheral blood stem cells were harvested from donors by large-volume leukapheresis after 4 and/or 5 days of treatment with recombinant human granulocyte colony-stimulating factor (filgrastim, 10 μg/kg/day s.c.) with the goal of collecting at least 5×10^6 CD34$^+$ cells/kg of recipient body weight. Donated cells were infused to recipients without further manipulation.

All patients received i.v. granulocyte colony-stimulating factor from day 5 until engraftment. The first 3 consecutive days on which absolute neutrophil counts were ≥500/μL and the first 7 consecutive days on which unsupported platelet counts were ≥20,000/μL were recorded as the times of neutrophil and platelet engraftment, respectively. Full donor chimerism was defined as ≥95% leukocytes of donor origin in peripheral blood samples, as measured by short tandem repeat PCR.

Prophylaxis, Monitoring, and Preemptive Therapy for CMV Infection

Acyclovir (250 mg i.v. 3 times daily) was administered to all patients prophylactically from day 1 of conditioning chemotherapy to day 14, followed by oral dosing at 400 mg twice daily for 9 months. All patients received i.v. polyvalent immunoglobulin (.5 g/kg) once every 2 weeks for 3 months, followed by once every 4 weeks for an additional 6 months. Patients were monitored for CMV using CMV-specific quantitative PCR with a CMV real-time PCR kit (Biocore Inc., Seoul, Korea) weekly during the first month after transplantation and then at 2- to 4-week intervals until either the end of treatment with immunosuppressive agents or the resolution of GVHD. The detection threshold of real-time PCR for measurement of CMV was 500 copies/mL.

To determine “true” increases and avoid unnecessary treatment of patients at low risk of progression to disease, CMV reactivation was defined as increasing 2 consecutive positive PCR tests or a viral load > 5000 copies/mL, which is $\log_{10}$ times the detection threshold. In patients with CMV reactivation, preemptive therapy with ganciclovir (5 mg/kg i.v. every 12 hours) was administered for 14 days. If CMV viremia persisted after 14 days, the treatment was continued for an additional 7 to 14 days at the same dose. CMV disease was defined as CMV reactivation accompanied by disease-associated clinical manifestations, radiographic findings, or histologic documentation [34]. For patients with symptoms of CMV syndrome or diagnosed CMV disease, concurrent immunoglobulin was administered.

GVHD, NRM, and Relapse

The diagnosis and clinical grading of acute and chronic GVHD conformed to the National Institutes of Health consensus criteria [35]. Donor chimerism was evaluated on days 14, 28, 90, 120, 180, and 365 using short tandem repeat analysis. Analysis was performed more frequently when mixed chimerism was present or leukemia relapse was suspected. If applicable, the diagnosis of leukemic relapse was also evaluated using disease-specific cytogenetic and/or molecular genetic anomalies of bone marrow or peripheral blood cells. NRM was defined as death from causes other than relapse.

Patient Samples and Flow Cytometry Analyses

Blood samples were collected from patients before conditioning therapy and on days 30 and 90 after haploSCT. Mononuclear cells were isolated by Ficoll-Hypaque (GE Healthcare Bio-Sciences, Buckinghamshire, UK) density gradient centrifugation and then cryopreserved. For immunophenotypic analysis, peripheral blood mononuclear cells were evaluated by flow cytometry on a FACSVerse (BD Biosciences, San Jose, CA) using FACSuite software (BD Biosciences) as previously reported [36]. Lymphocyte subpopulations were evaluated using a CD45 gate and side scatter according to the manufacturer's instructions.

The following monoclonal antibodies were used (Supplementary Table S1): CD3-PC7, CD4-FITC, CD8-FITC, CD57-PE, and PD-1-PE from BD Biosciences; Tim-3-PerCP, CD56-FITC, CD16-APC, NKG2A-PE, and DNAM1-PE from BioLegend (San Diego, CA); and NKG2D-PE from R&D Systems (Minneapolis,

MN). All patients were checked for differential count results of WBCs, including absolute lymphocyte counts, before conditioning therapy and at days 30 and 90 after haploSCT. The absolute cell counts of specific NK cell subsets were calculated by assessing the percentage of the respective cell population as determined by flow cytometry. At least 30,000 lymphocytes were analyzed to ensure adequate subset evaluation.

Statistical Methods

Statistical analyses were performed using SPSS version 23.0 (SPSS Inc., Chicago, IL). Variables were compared between groups using the 2-sided Fisher exact test. The cause-specific cumulative incidences of relapse, leukemia-free survival (LFS), and overall survival (OS) were compared using Kaplan-Meier estimates and the log-rank test. The Cox regression hazards model was used for univariate and multivariate analysis of the endpoints of leukemic relapse risk, OS, and LFS. A hazard ratio with a 95% confidence interval is reported for each significant ($P < .05$) covariate included in the final model.

RESULTS

Patient Characteristics

Clinical characteristics and transplant demographics of the 50 enrolled patients are shown in Table 1. Median age was 38 years (range, 21 to 62), and 24 patients (48%) were men. Thirty-five patients (70%) received transplantation during their first complete remission. Of the remaining patients, 7 were in their second complete remission and 8 were not in remission when they received haploSCT. Of the 8 patients who were not in remission, 1 patient had refractory disease and 7 were in relapsed disease. All patients were CMV-seropositive before transplant. The HLA-haploidentical family donors were offspring (median age, 21 years; range, 15 to 33; n = 13), parents (median age, 56 years; range, 46 to 73; n = 15), and siblings (median age, 38 years; range, 23 to 69; n = 22) of patients.

Transplantation

The median number of $CD34^+$ cells transplanted was 6.9×10^6/kg of recipient body weight (range, 1.3 to 14.1). Neutrophil engraftment was documented in all patients, and platelet engraftment was documented in 47 patients (94%) at a median of 11 and 13 days after haploSCT. No recipient experienced primary engraftment failure. Early transplant-related mortality and disease progression before day 100 occurred in 4 and 3 patients, respectively. Acute GVHD occurred in 32 patients (64%) at a median of 22 days (range, 6 to 91) after haploSCT. Of these cases, 22 patients had grades II to IV acute GVHD. Chronic GVHD occurred in 16 patients (37.2%) at a median of 6.4 months (range, 3.1 to 39.3) after haploSCT, with 5 patients (11.6%) experiencing severe chronic GVHD.

Characteristics of CMV Reactivation after HaploSCT

CMV reactivation was first diagnosed in 41 patients at a median of 23 days after haploSCT (range, 1 to 49), requiring

Table 1
Patient Characteristics

Variable	Total (n = 50)	With CMV Reactivation (n = 41)	Without CMV Reactivation (n = 9)	*P*
Median age, yr (range)	38.5 (21-62)	37 (21-62)	45.5 (35-52)	NS
Male patients	24 (48)	19 (45)	5 (63)	NS
Disease etiology				NS
AML	23	22	1	
ALL	24	17	7	
MPAL	3	3	0	
Adverse cytogenetics	8 (19)	6 (17.1)	2 (28.6)	NS
Disease status				NS
CR1	35	31	4	
≥CR2	7	4	3	
Not in remission	8	7	1	
Recipient/donor CMV serology				NS
R+/D−	1	1	0	
R+/D+	49	40	9	
Median donor age, yr (range)	38 (15-73)	38.5 (15-73)	21 (17-58)	NS
Conditioning				NS
MAC	7	6	1	
RIC	43	36	7	
GVHD prophylaxis				NS
Antithymocyte globulin/CSA/low-dose MTX	42	34	8	
CSA/PTCy/MMF	8	8	0	
Stem cell source				NS
PB	45	38	7	
BM	5	4	1	
Median infused CD34 cells, $\times 10^6$/kg (range)	6.9 (1.3-14.1)	6.8 (1.3-12.8)	7.7 (1.6-14.1)	NS
Median infused T cells, $\times 10^8$/kg (range)	3.0 (.3-13.7)	2.9 (.3-6.1)	3.2 (.9-13.7)	NS
$CD56^{bright}CD16^{dim/-}DNAM1^+$ NK cells				.013
Increase	15	15	0	
Decrease	13	8	5	
Acute GVHD	32 (64)	29 (69)	3 (37.5)	.118
Chronic GVHD	16 (37.2)	16 (42.1)	0 (0)	.139

Values are n or n (%) unless otherwise indicated. ALL indicates acute lymphoblastic leukemia; MPAL, mixed phenotype acute leukemia; CR1, first complete remission; CR2, second complete remission; MAC, myeloablative conditioning; RIC, reduced-intensity conditioning; CSA, cyclosporine A; MTX, methotrexate, PTCy; post-transplant cyclophosphamide, MMF; mycophenolate mofetil; PB, peripheral blood; BM, bone marrow; NS, not significant.

Table 2
Univariate Analyses for Relapse Rate and LFS

	No. of Cases	3-Year CIR (%)	*P*	3-Year LFS (%)	*P*
Age					
≤50 yr	40	42	.926	48.2	.864
>50 yr	10	62		50.0	
Conditioning regimen					
MAC	7	39.0	.306	14.3	.033
RIC	43	73.3		53.3	
Disease status					
First remission	35	33.5	.072	61.9	.040
≥2nd remission or not remission	15	69.7		25.0	
Infused T cells					
$\leq 3.2 \times 10^8$/kg	27	60.0	.022	31.7	.024
$> 3.2 \times 10^8$/kg	22	18.3		66.2	
Stem cell source					
BM	5	20.0	.478	60.0	.656
PB	45	46.0		46.0	
CMV reactivation					
Yes	42	36.4	<.001	53.5	<.001
No	8	83.3		15.6	
Acute GVHD					
Present	32	40.4	.471	49.0	.498
Absent	18	50.4		46.2	
Chronic GVHD					
Present	16	6.2	.006	87.5	.005
Absent	27	54.5		37.9	
Donor age					
≤27 yr	13	71.4	.018	35.9	.391
>27 yr	34	23.1		57.9	
$CD56^{bright}CD16^{dim/-}DNAM1^+$ NK cell					
Increase	14	16.4	.019	70.7	.096
Decrease	13	58.0		42.0	

CIR indicates cumulative incidence of relapse.

preemptive treatment with ganciclovir. All patients who showed CMV reactivation, except 4 patients who had spontaneous resolution while waiting to be hospitalized, were treated. The median peak viral load during CMV reactivation was 53,750 copies/mL (range, 1690 to 5,000,000). More than 2 episodes of CMV reactivation were observed in 14 patients throughout the follow-up period. Nine patients ultimately developed CMV disease, including 4 cases of CMV pneumonitis, 3 cases of CMV gastroenteritis, 1 case of CMV retinitis, and 1 case of CMV cystitis. Two patients died from CMV pneumonia. On grouping the patients according to the presence of CMV reactivation, several clinical parameters showed no significant difference between patients with and without CMV reactivation (Table 1). However, the incidence of acute GVHD by day 100 and chronic GVHD tended to be higher in patients with CMV reactivation (Table 1).

Transplant Outcomes

Median follow-up duration was 34.8 months (range, 3.2 to 122.2). Of the 50 patients, 31 were alive, 9 had died from relapse, and 10 had died from NRM at the cut-off point for the study; the latter category included 6 patients who died from infections and 4 who died from GVHD (n = 3) or hemorrhage (n = 1). The 3-year rates of cumulative incidence of relapse, LFS, and OS were 39.3%, 47.8%, and 65.3%, respectively. Based on univariate analysis, the factors predicting a reduced relapse risk were a dose of infused T cells $> 3.2 \times 10^8$/kg ($P = .022$), chronic GVHD ($P = .006$), an increase in the number of $CD56^{bright}CD16^{dim/-}DNAM1^+$ NK cells ($P = .016$), and CMV reactivation ($P < .001$) (Table 2). The factors predicting an improved LFS included a dose of infused T cells $> 3.2 \times 10^8$/kg ($P = .024$), reduced-intensity conditioning ($P = .033$), first remission status at transplantation ($P = .04$), chronic GVHD ($P = .005$), and CMV reactivation ($P < .001$). There was no significant difference in HLA alleles according to CMV viremia, CMV disease, or relapse in this study. Multivariate analysis showed that CMV reactivation ($P = .011$) and a dose of infused T cells $> 3.2 \times 10^8$/kg ($P = .027$) were independent predictors of a reduced relapse risk, and only CMV reactivation ($P = .029$) was an independent predictor of improved LFS (Table 3). The 3-year OS rate for patients with CMV reactivation was superior to that observed in patients without CMV reactivation, although this difference was not statistically significant (64% versus 43.8%, $P = .163$) (Figure 1A).

CMV Load and Survival Rate

Patients with CMV reactivation were divided into 2 groups based on viral load at the start of antiviral treatment, as determined by receiver operating characteristic curve analysis. The relapse rate was not significantly different between the groups with the higher (≥55,000 copies/mL, n = 17) and lower (<55,000 copies/mL, n = 25) viral loads (35.3% versus 20.0%, $P = .305$). However, the 3-year OS rate of the group with the lower viral load was significantly higher than that of the group with the higher viral load (80.0% versus 33.1%, $P = .014$). Of note, the 3-year OS rate of the lower viral load group was also

Table 3
Multivariate Analyses for Relapse Rate and LFS

	Relapse Rate		LFS	
	HR (95% CI)	*P*	HR (95% CI)	*P*
CMV reactivation				
Yes	.06 (.010-.558)	.011	.15 (.026-.819)	.029
No				
Donor age				
>27 yr	.35 (.090-1.376)	.133	–	
≤27 yr				
Chronic GVHD				
Present	.23 (.028-1.929)	.177	.29 (.061-1.355)	.115
Absent				
Infused T cells				
$>3.2 \times 10^8$/kg	.12 (.019-.784)	.027	.34 (.089-1.287)	.112
$\leq 3.2 \times 10^8$/kg				
Disease status				
First remission	–		.57 (.164-1.991)	.380
≥2nd remission or not remission				
Conditioning regimen				
RIC	–		.49 (.119-1.990)	.316
MAC				

HR indicates hazard ratio; CI, confidence interval.

higher than that of patients without CMV reactivation (43.8%, *P* = .048) (Figure 1B). The 3-year cumulative incidence of NRM in the lower viral load group was lower than that of the higher viral load group, although this difference was not statistically significant (8.3% versus 31.2%, *P* = .093) (Supplementary Figure S1).

Immune Cell Recovery

To assess NK and T cell recovery after haploSCT, we analyzed peripheral blood collected from the 50 patients before conditioning therapy and on days 30 and 90 after transplantation. Median numbers of infused cells were 3.0×10^8/kg for $CD3^+$ T cells (range, .3 to 13.7) and 2.3×10^8/kg for $CD3^-CD56^+CD16^{dim/-}$ NK cells (range, .03 to 14.2). The median numbers of infused $CD4^+$ and $CD8^+$ T cells were 3.4×10^8/kg (range, .2 to 6.7) and 1.4×10^8/kg (range, .1 to 2.2), respectively. The numbers of infused cells in each subset were similar between patients with and without CMV reactivation. There was no significant difference in the T and NK cell recovery between patients who received peripheral blood and bone marrow grafts. There was no significant difference in the expansion of CD4 or CD8 T cells at day 30 or 90 according to CMV reactivation. Moreover, PD-1 and Tim-3 expression on CD4 or CD8 T cells did not differ based on CMV reactivation. Counts of $CD56^{bright}CD16^{dim/-}$ cells and $CD56^{dim}CD16^+$cells at days 30 and 90 were not significantly different between patients with and without CMV reactivation. Additionally, NKG2A, NKG2D, and CD57 expression on $CD56^{bright}CD16^{dim/-}$ and $CD56^{dim}CD16^+$ cells was not associated with CMV reactivation.

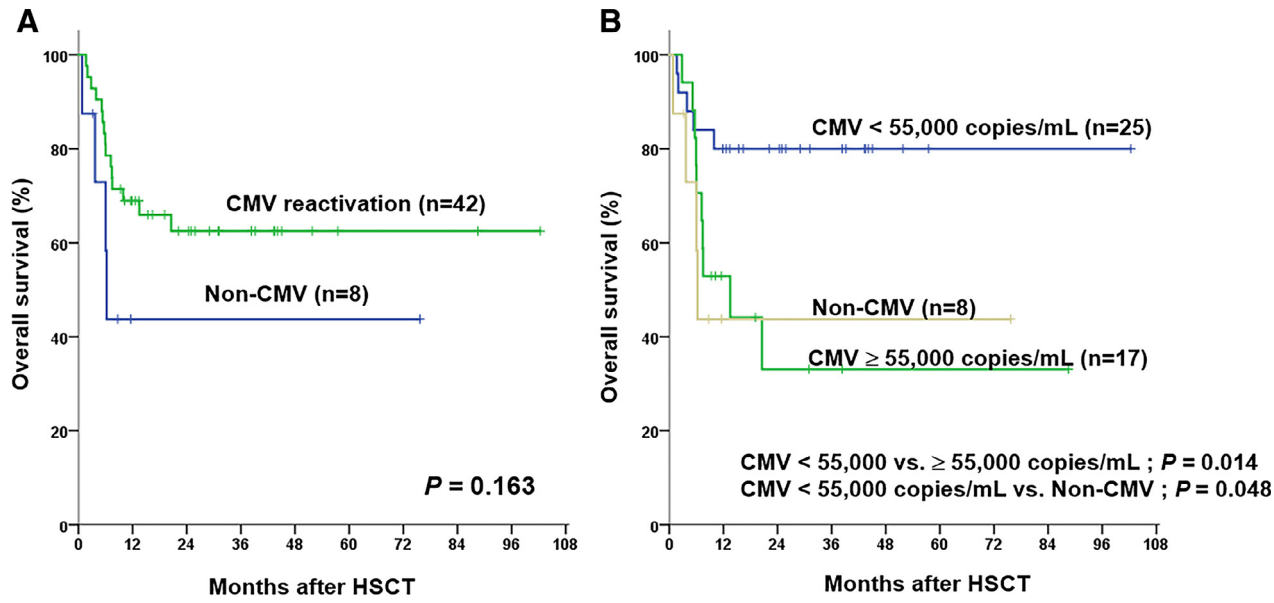

Figure 1. Kaplan-Meier estimates of OS according to (A) CMV reactivation and (B) viral load.

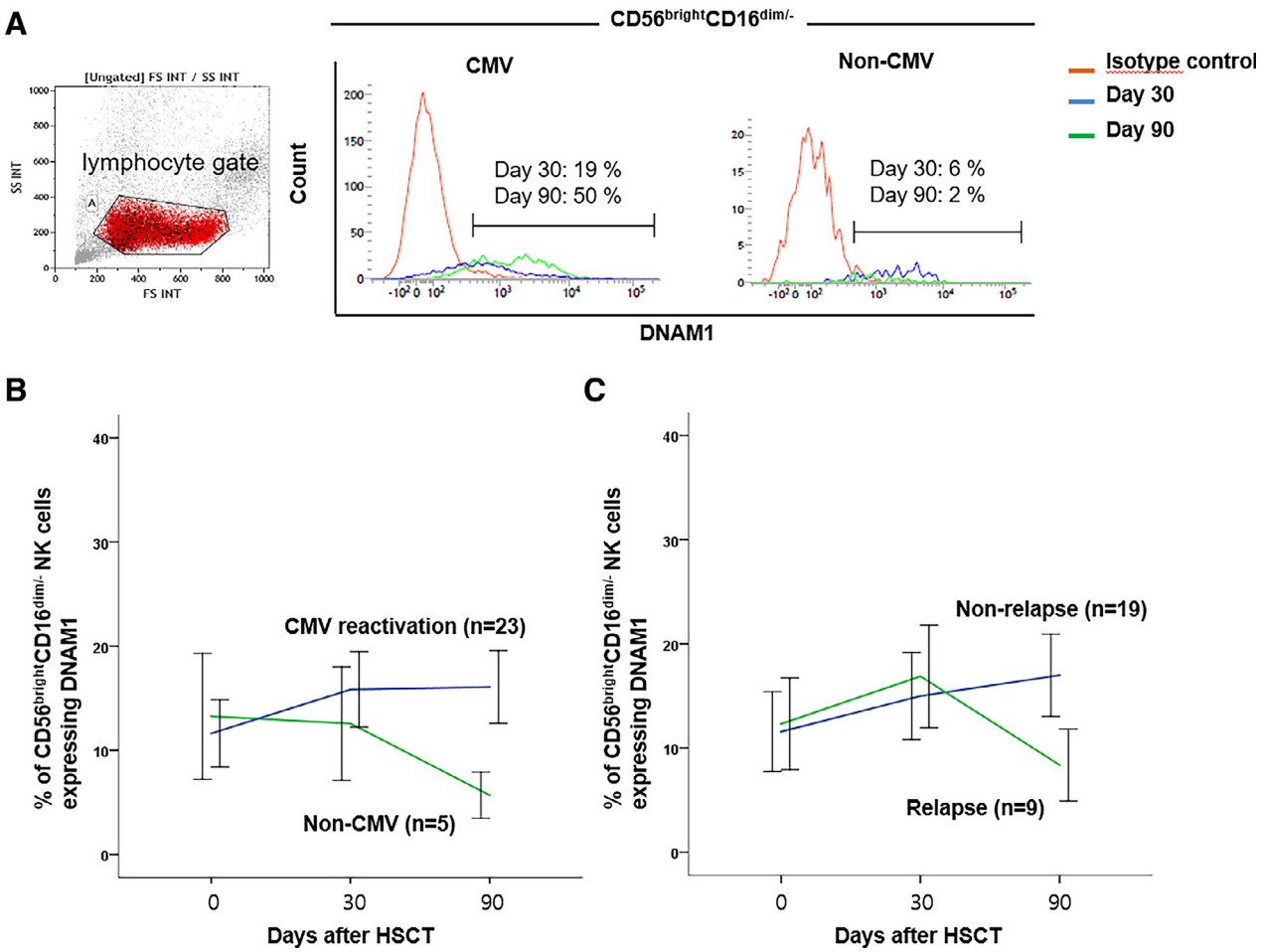

Figure 2. Correlation between increase in CD56^{bright}CD16$^{dim/-}$DNAM1^{+} NK cell count and reduced relapse associated with CMV reactivation. (A) Flow cytometry was performed on total peripheral blood mononuclear cells, and CD56^{bright}CD16$^{dim/-}$ NK cells were gated and analyzed for expression of DNMA1. Histograms for patients with or without CMV reactivation are shown. Red and black dots show lymphocyte gate and CD56^{bright}CD16$^{dim/-}$ NK cells, respectively. (B and C) Kinetics of CD56^{bright}CD16$^{dim/-}$DNAM1^{+} NK cell subset reconstitution after haploSCT according to (B) CMV reactivation and (C) relapse. Error bars show SEM. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

The type of conditioning regimen and GVHD prophylaxis did not affect the recovery of NK cell subsets. When we performed 4-color flow cytometric analysis of mononuclear cells of 8 samples with remaining frozen cells using CD3-PC7/CD56-FITC/ DNMA1-PE/CD16-APC, we found that CD56^{bright}CD16$^{dim/-}$ cells were mostly CD3^{-}cells, and the percentage of the CD3^{+} marker was negligible in CD56^{bright}CD16$^{dim/-}$ cells (median, .84%, range, .03% to 3.95%) (Supplementary Figure S2). These findings indicate that it is reasonable to designate CD56^{bright}CD16$^{dim/-}$ cells as NK cells.

In patients with CMV reactivation, CD56^{bright}CD16$^{dim/-}$ NK cell counts tended to be higher at day 90 than at day 30. In contrast, the number of CD56^{bright}CD16$^{dim/-}$ NK cells at day 30 was higher without CMV reactivation but decreased after day 30. Similarly, CD56^{bright}CD16$^{dim/-}$ NK cell counts tended to be higher at day 90 than at day 30 in patients without relapse but decreased after day 30 in patients with relapse. There was no significant difference in NK subset kinetics according to CMV viremia or disease. When patients with CMV reactivation were divided into 2 groups based on the date of CMV reactivation development after haploSCT (before versus after day 22), there was no significant difference in CD56^{bright}CD16$^{dim/-}$ NK cell counts between the 2 groups at days 30 and 90. The number of CD56^{dim}CD16^{+} cells at day 30 tended to be lower in patients who relapsed (18.8/μL [range, 1.0 to 113.1] versus 78.2/μL [range, .4 to 915.1], P = .062). However, there was no significant difference in CD56^{dim}CD16^{+} cell counts at day 90 according to the relapse development.

CD56^{bright}CD16$^{dim/-}$DNAM1^{+} NK CeAls associated with CMV and Relapse Rate

CMV reactivation was associated with expansion of the CD56^{bright}CD16$^{dim/-}$DNAM1^{+} NK cell subset between days 30 and 90 after haploSCT (Table 1 and Figure 2A). When patients were stratified according to an increase or decrease in the number of CD56^{bright}CD16$^{dim/-}$DNAM1^{+} NK cells from day 30 to 90, there were more patients with an increase in CD56^{bright}CD16$^{dim/-}$DNAM1^{+} NK cell counts in the CMV group (Table 1). An increase in the CD56^{bright}CD16$^{dim/-}$DNAM1^{+} NK cell counts was defined as an increase in the absolute cell count between days 30 and 90. Patients with an increase in CD56^{bright}CD16$^{dim/-}$DNAM1^{+} NK cell counts also had a significantly higher CMV viral load (111,000 copies/mL versus 9950 copies/mL, P = .004). We compared DNAM1 expression (%) on CD56^{bright}CD16$^{dim/-}$ NK cells on days 30 and 90. The extent of the increase in DNAM1 expression (%) between days 30 and 90 was greater in patients with CMV reactivation (1.3-fold versus .6-fold, P = .06) (Figure 2B). An increase in CD56^{bright}CD16$^{dim/-}$DNAM1^{+} NK cell count was not associated with the occurrence of chronic GVHD. However, the expansion

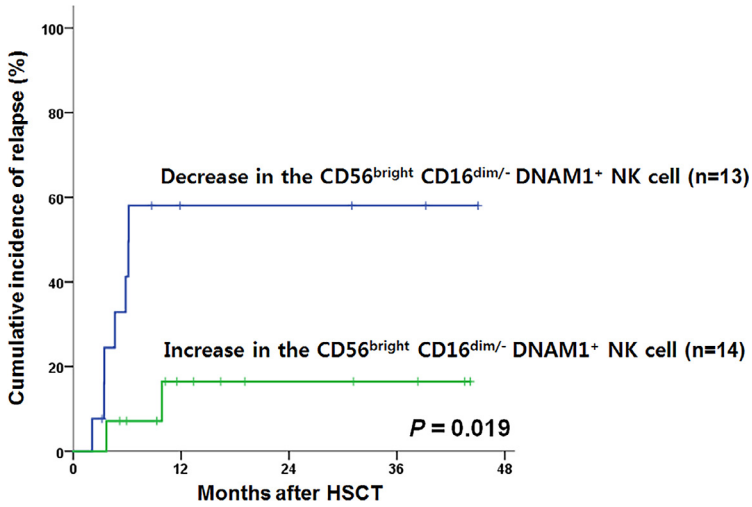

Figure 3. Kaplan-Meier estimate of cumulative incidence of relapse according to increase/decrease in $CD56^{bright}CD16^{dim/-}DNAM1^{+}$ NK cells from day 30 to 90.

of the $CD56^{bright}CD16^{dim/-}DNAM1^{+}$ NK cell subset was associated with the relapse rate. The rate of the increase in DNAM1 expression on $CD56^{bright}CD16^{dim/-}$ NK cells between days 30 and 90 was significantly lower in patients who relapsed than in those who did not (mean rate of increase, .5-fold versus 1.5-fold; $P = .026$) (Figure 2C). The 3-year cumulative incidence of relapse was significantly lower in patients with an increase in the $CD56^{bright}CD16^{dim/-}DNAM1^{+}$ NK cell counts from day 30 to 90 (16.4% versus 58.0%, $P = .019$) (Table 2 and Figure 3). Multivariate analysis indicated that an increase in the $CD56^{bright}CD16^{dim/-}DNAM1^{+}$ NK cell counts was an independent predictor of low relapse risk (Table 4). When patients with CMV reactivation were divided into 2 groups based on the median day of 22 of CMV reactivation development after haploSCT, there was no significant difference in $CD56^{bright}CD16^{dim/-}$ $DNAM1^{+}$ NK cell counts between the 2 groups at days 30 and 90.

Subgroup Analysis

Among 50 patients, 8 received post-transplant cyclophosphamide, a treatment protocol that is expected to result in substantial differences in immune reconstitution, compared with patients who received a T cell–replete graft in the absence of post-transplant cyclophosphamide. Therefore, we performed subgroup analyses for 42 patients who received a T cell–replete graft without post-transplant cyclophosphamide. The incidence of CMV reactivation was 80.5%, similar to the overall incidence. In subgroup analyses CMV reactivation ($P = .004$ for relapse rate and $P = .014$ for LFS), a dose of infused T cells $> 3.2 \times 10^8$/kg ($P = .01$ for relapse rate and $P = .016$ for LFS), and the first remission status ($P = .03$ for relapse rate and $P = .038$ for LFS) were the independent predictors for reduced relapse risk and improved LFS (Supplementary Table S2 and S3). No patient with an increase in $CD56^{bright}CD16^{dim/-}DNAM1^{+}$ NK cell counts from day 30 to 90 showed relapse, whereas the 3-year cumulative relapse rate was 59.6% in patients with a decrease in $CD56^{bright}CD16^{dim/-}DNAM1^{+}$ NK cell counts ($P = .005$) (Supplementary Table S2). In subgroup analyses, because the relapse rate for patients with an increase in $CD56^{bright}CD16^{dim/-}DNAM1^{+}$ NK cell counts was zero, quasi-separation occurred, and statistical estimation using the Cox regression hazard model was not possible.

Table 4
Multivariate Analysis for Relapse Rate

	Relapse Rate	
	HR (95% CI)	*P*
$CD56^{bright}CD16^{dim/-}DNAM1^{+}$ NK cell		
Increase	.12 (.019-.822)	.031
Decrease		
Donor age		
>27 yr	.07 (.006-.823)	.035
≤27 yr		
Chronic GVHD		
Present	.21 (.020-2.201)	.193
Absent		
Infused T cells		
$>3.2 \times 10^8$/kg	.07 (.005-.884)	.040
$\leq 3.2 \times 10^8$/kg		

DISCUSSION

Several studies have reported that CMV reactivation may reduce leukemia relapse after alloSCT [11–13,16,20]. Investigations into how the immune system changes after CMV reactivation are currently underway to understand the underlying mechanism of this antileukemia effect of CMV. Such analyses are especially important for Korean patients, because CMV seroprevalence is nearly 100% in this population [37]. Here, in a CMV-seropositive population, we identified an antileukemia effect of CMV reactivation in the haploSCT setting. We additionally found that $CD56^{bright}CD16^{dim/-}DNAM1^{+}$ NK cells may play an important role in linking CMV reactivation and the GVL effect.

The antileukemia effect of CMV in the haploSCT setting was previously unclear. We have shown that CMV reactivation is an important prognostic factor, predicting a reduced relapse rate and improved LFS among the CMV-seropositive population receiving T cell–replete haploSCT. However, CMV infection remained an important cause of death and did not improve OS. The identification of the immune cell subsets that contribute to the prevention of relapse of leukemia after CMV reactivation may provide a basis for the development of immunotherapy that prevents leukemia relapse without the risk of NRM because of viral infection. Early in primary CMV infection, nonspecific cellular immune defenses, such as NK cells, are essential in limiting viral reactivation [38]. It was

reported that CMV reactivation promotes a more rapid reconstitution of mature functional NK cells with potential memory-like characteristics [19,39]. After CMV reactivation in patients who received CMV-naive umbilical cord blood or matched unrelated donor HSCT, a subset of reconstituting NK cells displaying an increased density of surface NKG2C expand [19]. This subset has been characterized by predominant expression of NKG2C and the inhibitory KIR specific for self-HLA but lack NKG2A, a phenotype required for robust IFN-γ production. After haploSCT, a subset of NK cells with the $CD56^{dim}$, $NKG2C^{+}$, and self-KIR^{+} phenotype expands and is responsible for IFN-γ production during CMV infection [39]. However, there is no direct evidence of a specific NK cell subset that reduces leukemia relapse among the CMV-engineered NK cell receptor repertoires.

Independent of the graft source, NK cells typically regenerate within the first month after alloSCT [40]. Moreover, there is an over-representation of $CD56^{bright}CD16^{dim/-}$ NK cells in the early phase post-transplant compared with that in healthy individuals [41,42]. $CD56^{bright}CD16^{dim/-}$ NK cells are known to be a noncytotoxic cell subset that plays immunoregulatory roles through cytokine production. Our findings indicated the important role of $CD56^{bright}CD16^{dim/-}$ NK cells, which secrete immunoregulatory cytokines, in the antileukemia effect in patients with CMV reactivation. DNAM1 (DNAX accessory molecule 1, also known as CD226), a costimulatory adhesion molecule expressed by T cells and NK cells, has a crucial function in tumor immune surveillance [43–45]. DNAM1 was shown to co-stimulate the proliferation and IFN-γ production of alloantigen-specific $CD8^{+}$ T cells [46]. Furthermore, DNAM1 is essential for the optimal function and differentiation of memory NK cells during mouse CMV infection [47]. We showed that in patients with CMV reactivation, $DNAM1^{+}$ NK cells, in particular $CD56^{bright}CD16^{dim/-}DNAM1^{+}$ NK cells, steadily increased until day 90. In patients without CMV reactivation, however, the increase in $CD56^{bright}CD16^{dim/}DNAM1^{+}$ NK cell counts was not maintained after day 30, and these cells decreased in number.

To determine whether this NK cell subset expansion could recapitulate the effect of CMV reactivation in reducing the relapse rate, we included an increase in the $CD56^{bright}CD16^{dim/-}DNAM1^{+}$ NK cell count as a variable in the multivariate analysis model instead of CMV reactivation. The results confirmed that an increase in $CD56^{bright}CD16^{dim/-}DNAM1^{+}$ NK cell counts was an independent factor predicting a reduced rate of leukemia relapse. However, in the multivariate analysis of factors predicting an improved LFS, CMV reactivation, but not an increase in $CD56^{bright}CD16^{dim/-}DNAM1^{+}$NK cell counts, was an independent factor. This may be because the increase in $CD56^{bright}CD16^{dim/-}DNAM1^{+}$ NK cell counts is highly correlated with a high viral load of CMV. NRM was more closely related to CMV viral load than to CMV proliferation, as shown in Supplementary Figure S1. A higher peak viral load has been linked to reduced OS [48]. Further investigation of the effects of cytokine production by this cell subtype on the antileukemia effect will improve our knowledge of the mechanism underlying the GVL effect of this NK cell subset associated with CMV reactivation. Although we have demonstrated for the first time a significant correlation between CMV reactivation, relapse rate, and increase in $CD56^{bright}CD16^{dim/-}DNAM1^{+}$ NK cell counts from day 30 to 90, no correlation between the expansion of $CD56^{bright}CD16^{dim/-}DNAM1^{+}$ NK cells and CMV viremia duration or peak viral load was observed. This may result from a variety of factors affecting CMV kinetics such as immunosuppressive therapy, GVHD treatment, and preemptive therapy during the first 3 months after transplantation.

Chronic GVHD is a well-known factor preventing leukemia relapse [49,50]. Thus, the reduction in relapse risk may be secondary to the immune-mediated GVL effect associated with chronic GVHD. However, the protective effect of chronic GVHD has not been confirmed after haploSCT, and, recently, multiple studies have reported a reduced incidence of chronic GVHD after haploSCT [51,52]. In haploSCT, NK cells may express inhibitory KIRs that are not engaged by any of the HLA class I alleles present on recipient cells. Such “alloreactive” NK cells greatly contribute both to the eradication of leukemia blasts escaping the preparative regimen and to the clearance of residual host dendrite cells and T lymphocytes (thus preventing GVHD and graft rejection, respectively) [53]. In this study chronic GVHD was associated with a reduced relapse rate in the univariate analysis but was not found to be an independent factor in the multivariate analysis. Therefore, in the haploSCT setting, immune-mediated GVL may be more associated with CMV reactivation than with chronic GVHD.

The current study has some limitations. This analysis was retrospective and the sample size small. However, because patients were recruited from a single transplant center for chart review, it was difficult to recruit a large number of patients. Further research with a larger sample size could provide more definitive evidence. In addition, all patients and donors except 1 in this study demonstrated CMV seropositivity. Thus, a comparison with CMV-seronegative patients is needed.

In conclusion, CMV reactivation was identified as an independent prognostic factor for LFS in the haploSCT setting. Maintained expansion of $CD56^{bright}CD16^{dim/-}DNAM1^{+}$ NK cells until day 90 was associated with CMV reactivation and a low relapse rate. Our findings suggest the possibility that $CD56^{bright}CD16^{dim/-}DNAM1^{+}$ NK cells may play a leading role in linking CMV reactivation and the GVL effect. Therefore, we suggest that additional studies are required for elucidating the mechanism of the GVL effect of this NK cell subset associated with CMV reactivation.

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SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found, in the online version at doi:10.1016/j.bbmt.2019.06.008.

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