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Original Article

Prognostic Impact of Lymphocyte Subpopulations in Peripheral Blood after Hematopoietic Stem Cell Transplantation for Hematologic Malignancies

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Background: We prospectively evaluated prognostic value of lymphocyte subpopulations in peripheral blood of allogeneic hematopoietic stem cell transplant (HSCT) recipients.

Methods: 113 allogeneic HSCT (47 sibling matched, 37 unrelated matched, 29 haploidentical)-performed patients diagnosed as AML (n = 66), ALL (n = 28), and MDS (n = 19) were prospectively enrolled. 14 lymphocyte subpopulations were quantified by flow cytometry of PB at specific time-points after HSCT, and their prognostic impacts were analyzed.

Results: At 1, 2, and 3 months post-HSCT, significant adverse impact on overall survival (OS) and/or event free survival (EFS) was exhibited by low levels of natural killer (NK) cells ($\leq$32 and $\leq$90/μL at 1 and 2 months on OS and EFS); regulatory T cells ($\leq$1/μL) on EFS at 2 months; and B cells ($\leq$19 and $\leq$92/μL for OS and EFS at 3 months). At 12 months, low levels of T cells ($\leq$1180/μL), helper/inducer (H/I) T cells ($\leq$250/μL), cytotoxic/suppressor (C/S) T cells ($\leq$541/μL), and NK cells ($\leq$138/μL) were associated with significantly higher risk of relapse. Low levels of T cells ($\leq$879/μL) and C/S T cells ($\leq$541/μL), and high level of naïve thymic T cells ($>$115/μL) showed a significant association with poor OS; low levels of C/S T cells ($\leq$541/μL) and NK cells ($\leq$138/μL) showed a significant adverse impact on EFS.

Conclusions: Low levels of NK cells, regulatory T cells, and B cells at early stage post-HSCT are adverse prognostic indicators. At late stage, low levels of T cells and their subpopulations, NK cells, and high level of naïve thymic T cells are adverse prognostic indicators. © 2017 International Clinical Cytometry Society

Key terms: hematologic malignancies; hematopoietic stem cell transplantation; lymphocyte subpopulations; peripheral blood; prognosis

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Additional Supporting Information may be found in the online version of this article.

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INTRODUCTION

Allogeneic hematopoietic stem cell transplantation (HSCT) is a curative treatment for hematologic malignancies such as acute myeloid leukemia (AML), acute lymphoblastic leukemia (ALL), and myelodysplastic syndrome (MDS). Owing to pre-HSCT conditioning chemotherapy, HSCT recipients are vulnerable to serious infections or relapse based on the strength of pretransplant conditioning regimen (myeloablative or nonmyeloablative) and graft-versus-leukemia effect induced by donor origin T or natural killer (NK) cells (1). Therefore, in addition to graft-versus-host disease (GvHD) which is an important factor determining outcome of HSCT, the extent of immune reconstitution in peripheral blood (PB) after HSCT may also be a potential predictor of outcomes in these recipients.

The time span required for reconstitution of different lymphocyte subpopulations post-HSCT tends to vary (2–4). A recent study reported that white blood cell (WBC) count recovered within 1 month after HSCT, while T and B lymphocyte counts recovered at 2 months, and that NK-T cells, effector memory T cells, and NK cell subpopulations showed recovery in 1–2 months after HSCT, but the increase in regulatory T cells initiated at 3 months post-HSCT (5). Rapid lymphocyte recovery post-HSCT was reported to be associated with superior outcomes (6–9). A low lymphocyte count at 3–4 weeks after HSCT was reported to be an adverse prognostic factor in HSCT recipients from both matched sibling and matched unrelated donors, irrespective of graft manipulation (10–13). In addition, early CD4+ T cell reconstitution correlates with superior survival (14), while high NK cell counts at 1 month post-HSCT predict better outcomes in adult patients with myeloid malignancies who received T-cell depleted, HLA-matched HSCT (15), as well as in pediatric allogeneic HSCT recipients (16). However, the prognostic impact of other lymphocyte subpopulations has not been investigated in allogeneic HSCT recipients.

In the present study, we prospectively evaluated the prognostic value of lymphocyte subpopulations in PB at each time point (1, 2, 3, 6, and 12 months) after allogeneic HSCT in patients with hematologic malignancies. We aimed to identify specific lymphocyte subpopulations at each time point post-HSCT, which predict clinical outcomes irrespective of the primary disease and donor subtype.

MATERIALS AND METHODS

Patients Characteristics

A total of 113 patients (AML [n = 66], ALL [n = 28], and MDS [n = 19]) who underwent allogeneic HSCT in Asan Medical Center between January 2008 and July 2010 were prospectively enrolled. 19 MDS patients included 1 patient with refractory cytopenia with multilineage dysplasia (RCMD), 7 patients with refractory anemia with excess blasts (RAEB)−1 and 11 patients with RAEB-2. This study was approved by the institutional review board of corresponding author's hospital. There were three subtypes of HSCT according to the donor relationship and status of HLA mismatch (sibling matched [n = 47], unrelated matched [n = 37], and haploidentical [n = 29]). All patients received one of the following three pretransplant conditioning regimens: Bu–Cy (busulfan intravenously (iv) 3.2 mg/kg/day for 4 days plus cyclophosphamide iv 60 mg/kg/day for 2 days); Bu–Flu (busulfan iv 3.2 mg/kg/day for 4 days plus fludarabine iv 30 mg/m^2/day for 5 days); or Bu–Flu–ATG (busulfan iv 3.2 mg/kg/day for 2 days, fludarabine iv 30 mg/m^2/day for 6 days, plus anti-thymocyte globulin iv 3 mg/kg/day for 3–4 days). Of the 47 patients who received sibling matched HSCT, 19, 9, and 19 patients received Bu–Cy, Bu–Flu, and Bu–Flu–ATG conditioning therapy, respectively. Of the 37 patients who received unrelated matched HSCT, 6 and 30 patients received Bu–Cy and Bu–Flu–ATG conditioning therapy, respectively and only 1 received Bu–Flu conditioning therapy. All the 29 patients who received haploidentical HSCT received Bu–Flu–ATG conditioning therapy. Post-transplant immunosuppressive regimens used for GvHD prophylaxis are described in the previous study (5), and only 3 AML patients still received immunosuppression at 12 months after HSCT due to chronic GvHD. Also, we disclose that some patients in the current study were represented in the previous foundational study (5) of "event-free patients".

Sampling Strategy and Flow Cytometric Analysis

Pre-HSCT PB samples obtained 7 days prior to HSCT and at 1, 2, 3, 6, and 12 months post-HSCT were collected; complete blood cell counts were measured using automated hematology analyzer Sysmex XE-2100 (Sysmex, Kobe, Japan). Assessment of lymphocyte immunophenotyping and quantification was performed by dual platform strategy (DPT), using the samples obtained within 6 h after the sample collection. After erythrocyte lysis, the samples were labeled with specific antibodies. All antibodies except CD45 (BD Bioscience, San Jose, CA, USA) were obtained from Beckman Coulter (Fullerton, CA, USA). Three- to five-color flow cytometric immunophenotypic analysis was performed using a FACSCanto II flow cytometry system (BD Bioscience) and analyzed using the FACSDiva software (BD Bioscience). Total 14 lymphocyte subpopulations [T cells, helper/inducer (H/I) T cells, cytotoxic/suppressor (C/S) T cells, naive thymic T cells, naïve central T cells, NK cells, CD56bright$^+$/CD16$^-$ NK cells, CD56dim$^+$/CD16$^+$ NK cells, CD56bright$^+$/CD16$^+$ NK cells, NK-T cells, central memory T cells, effector memory T cells, regulatory T cells, and B cells) were quantified. All flow cytometric analyses were conducted in duplicate and mean values were used.

Monoclonal antibodies were labeled by fluorescein isothiocyanate (FITC), phycoerythrin (PE), allophycocyanin (APC), peridinin chlorophyll protein (PerCP), PE-cyanine 7 (PE-Cy7), and Alexa fluorophores. Gating strategies and fluorophores used for immunophenotypic determination

Table 1
Summary of Immunophenotypes for the Determination of Total 14 Lymphocyte Subpopulations

Lymphocyte subpopulations	Immunophenotype
T cells	$CD3^+$
Helper/inducer T cells	$CD3^+CD4^+$
Cytotoxic/suppressor T cells	$CD3^+CD8^+$
Naive thymic T cells	$CD3^+CD4^+CD31^+$
Naive central T cells	$CD3^+CD4^+CD31^-$
NK cells	$CD3^-CD16^+$ and/or $CD56^+$
CD56bright$^+$/CD16$^-$ NK cells	$CD56^{bright}CD16^-CD3^-$
CD56dim$^+$/CD16$^+$ NK cells	$CD56^{dim}CD16^+CD3^-$
CD56bright$^+$/CD16$^+$ NK cells	$CD56^+CD16^+CD3^-$
NK-T cells	$CD3^+CD161^+$
Central memory T cells	$CD8^+CD45RA^-CCR7^+CD62L^+$
Effector memory T cells	$CD8^+CD45RA^-CCR7^-CD62L^-$
Regulatory T cells	$CD4^+CD25^{high}FoXP3^+$
B cells	$CD19^+$

NK, natural killer; CD, cluster of differentiations.

of lymphocyte subpopulations were as follows: CD4-FITC/CD8-PE/CD3-PerCP/CD31-PE-Cy7/CD45-APC for T cells and their subsets (H/I T cells, C/S T cells, naïve thymic T cells and naïve central T cells); CD19-PE/CD45-APC for B cells; CD56-FITC/CD16-PE/CD3-PerCP/CD45-APC for NK cells and their subsets; CD161-FITC/CD3-PE/CD45-APC for NK-T cells; CD45RA-FITC/CD8-PE/CCR7-PE-Cy7/CD45-PerCP/CD62L-APC for memory T cell subsets; CD4-FITC/CD25-PE/CD45-PerCP/FOXP3-ALEXA 647 for regulatory T cells; IgG-FITC/IgG-PE/IgG-PerCP (or IgG-PE-Cy7, IgG-ALEXA647)/IgG-APC for isotype control. Total five tubes (CD4/CD8/CD3/CD31/CD45 for T cells and their subsets, CD56/CD16/CD3/CD19/CD45 for B cell, NK cell and their subsets CD161/CD3/−/−/CD45 for NK-T cells, CD45RA/CD8/CCR7/CD62L/CD45 for memory T cell subsets, and CD25/CD4/−/Foxp3/CD45 for regulatory T cells) were tested in the present study and we gated at least 30,000 lymphocytes on CD45 versus side-scatter plots. Prior to lymphocyte gating, forward-scatter/side-scatter plot was initially obtained to remove debris and noncellular components. We rejected duplication of sample analysis and excluded the results from our present study, if the acquired lymphocyte counts were <30,000 cells to remove samples with too large variations from our present study results.

We defined T cell as $CD3^+$, helper/inducer T cell; $CD3^+$ $CD4^+$, suppressor/cytotoxic T cell; $CD3^+$ $CD8^+$, and B cell as $CD19^+$. NK cell subsets which express CD56bright$^+$/CD16$^+$, CD56bright$^+$/CD16$^-$ and CD56dim$^+$/CD16$^+$ NK cells were gated in CD3 negative cell population. NK-T cells were positive for CD3 and CD161, and regulatory T cells were positive for CD4, $CD25^{high}$ and intracellular Foxp3. We measured naïve thymic T cells ($CD3^+$ $CD4^+$ $CD31^+$) and naïve central T cells ($CD3^+$ $CD4^+$ $CD31^-$) with indirect method using another tube. Among $CD45RA^-CD8^+$ cells, $CCR7^+$ $CD62L^+$ cells were classified to central memory T cells and $CCR7^-CD62L^-$ cells were classified to effector memory T cells. Detailed immunophenotyping strategy and schemes for the determination of 14 lymphocyte subpopulations are summarized in Table 1 and Figure 1.

Assessment of Clinical Characteristics and Comparison of Lymphocyte Subpopulations at Each Time Point after HSCT between Different Prognosis Groups

Nonrelapse-related mortality (NRM, defined as death due to graft rejection/failure, infection, acute respiratory distress syndrome, GvHD, or organ failure), relapse, and event (defined as death, relapse/progression, graft failure, or administration of donor lymphocyte infusion due to suspected relapse) during follow-up period were investigated in all patients. Initially, both absolute numbers and proportions of 17 leukocyte populations in the PB (14 lymphocyte subpopulations, WBC, lymphocytes, and monocytes) obtained at pre-HSCT were compared among the three patient subgroups (AML, ALL, and MDS) to evaluate whether the difference of primary diagnosis would affect lymphocyte subpopulations at the time of pre-HSCT, which may introduce a potential bias affecting prognosis. These results are summarized in Supporting Information Table 1.

Subsequently, two comparisons of these 17 cell populations at 1, 2, 3, 6, and 12 months after HSCT were performed between two patient subgroups categorized by outcomes (NRM and relapse) on follow-up (Supporting Information Tables 2–5). In addition, identical comparisons were performed between two patient subgroups categorized by outcomes (death and events) (Supporting Information Tables 6–9).

Prognostic Impact of Lymphocyte Subpopulations at Each Time Point

Receiver operating characteristic (ROC) curve analysis was performed for each lymphocyte subpopulation which showed significant difference between the two patient groups in the previous comparison. The area-under-the-curve (AUC), 95% confidence interval (CI), best cutoff values applicable to each lymphocyte subpopulation, sensitivity, and specificity for the discrimination of two patient subgroups when the predefined best cutoff values were applied were calculated. The prognostic impact of calculated best cutoff values in each lymphocyte subpopulation on the NRM event, relapse, overall survival (OS), and event-free survival (EFS) were assessed by logistic regression analyses (for NRM and relapse) and Cox's proportional hazard models (for OS and EFS). Since the proportion of lymphocyte subpopulations did not show significant differences between the two patient subgroups in the majority of comparisons, the absolute numbers of lymphocyte subpopulations were only included as variables in further prognostic evaluation. Results at 6 months after HSCT did not show any significant differences and were excluded from the prognostic evaluations. All the results at 1, 2, 3, and 12 months post-HSCT are summarized in Tables (2–5), respectively. Although the statistical power is limited due to small patient numbers in each patient subgroup, the multivariate analysis was also additionally performed to investigate independent prognostic impact of each

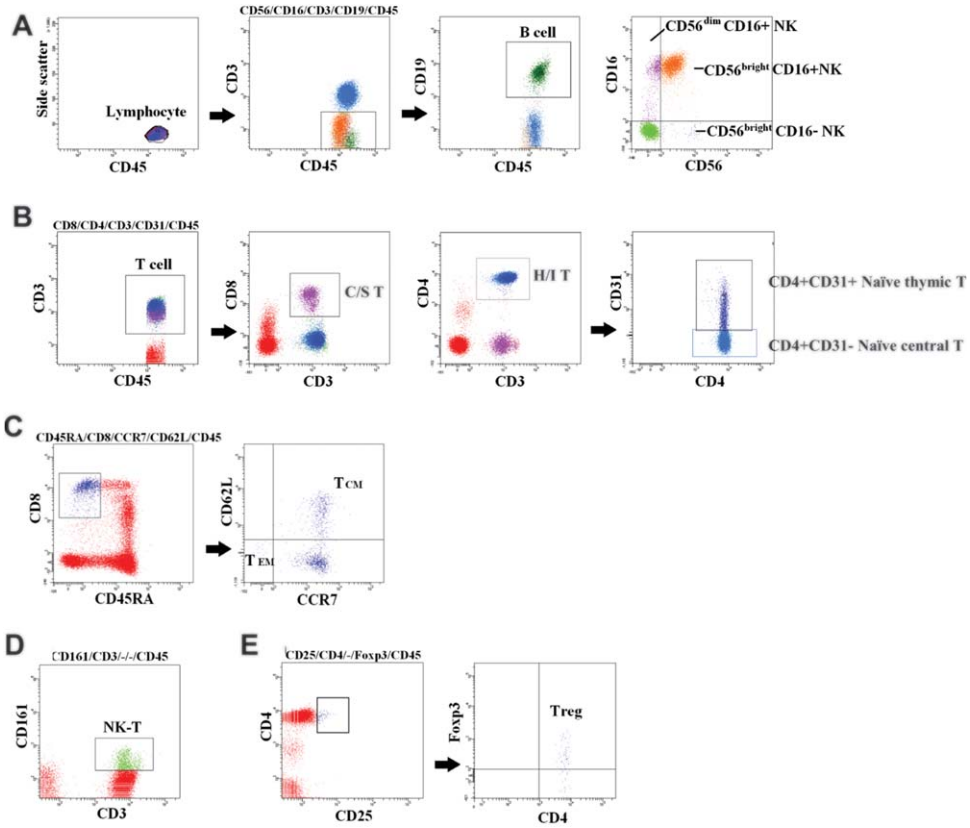

FIG. 1. Schematic illustrations of immunophenotyping strategy for the determination of 14 lymphocyte subpopulations. (**A**) Gates for lymphocytes were applied on $CD45^{high}$ and low side-scatter plots. Among $CD3^-$ cells, we measured $CD19^+$ B cells and NK cell subsets which express $CD56bright^+/CD16^-$, $CD56dim^+/CD16^+$, and $CD56bright^+/CD16^+$. (**B**) T cells were gated as $CD3^+$ cells, and among the $CD3^+$ population, cytotoxic/suppressor T cells (C/S T cells) with $CD3^+CD8^+$, helper/inducer T cells (H/I T cells) with $CD3^+CD4^+$, naïve thymic T cells with $CD3^+CD4^+CD31^+$, and naïve central T cells with $CD3^+CD4^+CD31^-$ were measured. (**C**) Among $CD45RA^-CD8^+$ cells, $CCR7^+CD62L^+$ cells were classified to central memory T cells (T_{CM}) and $CCR7^-CD62L^-$ cells were classified as effector memory T cells (T_{EM}). (**D**) NK-T cells were positive for CD3 and CD161. (E) Regulatory T cells (*T*reg) were positive for CD4, $CD25^{high}$, and Foxp3. Abbreviations: CD, cluster of differentiation; NK, natural killer; C/S, cytotoxic/suppressor; H/I, helper/inducer; EM, effector memory; CM, central memory. [Color figure can be viewed at wileyonlinelibrary.com]

lymphocyte subpopulation if multiple candidates are presented in the univariate analysis.

Subsequently, survival analyses were performed to validate prognostic impacts of variables included in the previous assessment of NRM, relapse, OS, and EFS. These results are represented in Figure 2 (at early stage, defined as 1, 2, and 3 months post-HSCT) and Figure 3 (at late stage, defined as 12 months post-HSCT).

In addition, serial follow-up data of seven lymphocyte subpopulations (T cells, H/I T cells, C/S T cells, NK cells, B cells, naïve thymic T cells, and regulatory T cells), which showed significant prognostic impact in the previous analyses, were analyzed. Examples from two AML patients who showed different outcomes are shown in Figure 4.

Statistical Analysis

Between-group differences with respect to continuous variables were assessed by Mann–Whitney *U* test. ROC analysis was performed to calculate AUC values and best cutoff values for each lymphocyte subpopulation for discrimination of patient subgroups with different outcomes. Both logistic regression analysis and Cox's proportional hazard model were performed to evaluate the independent prognostic effect of each lymphocyte subpopulation on NRM, relapse, OS, and EFS (in both univariate and multivariate analyses). Estimates of survival were generated using the Kaplan–Meier method, and between-group differences compared using log-rank test. In all comparisons except for those among three different disease subgroups, two-tailed analyses were applied and *P*-values <0.05 were considered statistically significant. In comparisons among three different disease subgroups, the Bonferroni correction method was performed in the subsequent two subgroups analysis and *P* values <0.0167 were considered statistically significant. SPSS software version 13.0.1 (SPSS, Chicago, IL, USA) was used for all statistical analyses.

RESULTS

Comparison of Pre-HSCT Lymphocyte Subpopulations in Different Diagnosis Subgroups

Patients with MDS showed significantly lower WBC and monocyte counts than those in patients with AML.

Table 2
Effects of Each Candidate Item Obtained at 1 Month After Stem Cell Transplantation on the Prediction of Prognosis

Items	AUC (95% CI)	Best cutoff (/μL)	Sensitivity (%)	Specificity (%)	HR (*P* values, 95% CI)
For NRM event					
Lymphocytes	0.667 (0.570–0.754)	≤792	73.3	67.4	5.677 (0.005*, 1.673–19.271)
Helper/inducer T cells	0.671 (0.575–0.757)	≤36	53.3	88.4	6.682 (0.002*, 2.026–22.036)
NK cells	0.712 (0.617–0.794)	≤25	80.0	57.9	3.781 (0.032*, 1.122–12.740)
$CD56dim^+/CD16^+$ NK cells	0.675 (0.561–0.776)	≤36	84.6	50.0	3.333 (0.087*, 0.841–13.216)
NK-T cells	0.812 (0.708–0.891)	≤22	76.9	72.7	6.000 (0.007*, 1.641–21.937)
Regulatory T cells	0.723 (0.630–0.804)	≤1	93.3	46.3	4.085 (0.038*, 1.083–15.408)
For relapse					
Naïve thymic T cells	0.637 (0.533–0.732)	>35	73.1	56.3	3.502 (0.013*, 1.307–9.382)
For OS					
NK cells	0.662 (0.566–0.750)	≤32	75.6	58.0	2.633 (0.006**, 1.318–5.261)
For EFS					
NK cells†	0.648 (0.552–0.737)	≤32	71.7	57.8	2.409 (0.006**, 1.284–4.523)
NK-T cells	0.633 (0.517–0.738)	≤64	68.6	54.5	1.860 (0.082**, 0.924–3.745)

Hazard ratio of each item were calculated when the best cutoff values of each item were applied.
AUC, area under the curve; CI, confidence interval; HR, hazard ratio; NK, natural killer; CD, cluster of differentiations; NRM, non-relapse related mortality; OS, overall survival; EFS, event free survival.
***P* values were obtained from the logistic regression analysis and from the *Cox's proportional hazard model.
†Items with independent adverse prognostic impact from the multivariate analysis were presented.

MDS patients also had significantly lower WBC and monocyte counts than those in patients with ALL. The statistical significance of *P* values obtained from these four comparisons were maintained after the application of Bonferroni correction method. Other variables did not significantly differ between the three disease subgroups (Supporting Information Table 1).

Comparison of Post-HSCT Lymphocyte Subpopulations Between Patients Who Experienced NRM Event or Relapse and Those Who Did Not

At 1 month post-HSCT, patients who experienced NRM event showed significantly lower lymphocyte counts, H/I T cells, NK cells, $CD56dim^+/CD16^+$ NK cells, NK-T cells, and regulatory T cells than those who did not. Patients

Table 3
Effects of Each Candidate Item Obtained at 2 Months After Stem Cell Transplantation on the Prediction of Prognosis

Items	AUC (95% CI)	Best cutoff (/μL)	Sensitivity (%)	Specificity (%)	HR (*P* values, 95% CI)
For NRM event					
NK cells	0.767 (0.673–0.846)	≤37	83.3	67.4	6.207 (0.010*, 1.562–24.666)
NK-T cells	0.822 (0.712–0.903)	≤45	83.3	72.4	7.875 (0.005*, 1.889–32.838)
B cells	0.775 (0.660–0.865)	≤24	75.0	78.0	7.077 (0.004*, 1.837–27.269)
For OS					
NK cells	0.628 (0.526–0.722)	≤90	76.5	53.7	2.518 (0.018**, 1.175–5.399)
For EFS					
NK cells†	0.617 (0.515–0.712)	≤90	75.0	55.7	2.413 (0.013**, 1.204–4.837)
Regulatory T cells†	0.624 (0.522–0.718)	≤1	65.0	60.7	1.932 (0.039**, 1.035–3.606)

Hazard ratio of each item were calculated when the best cutoff values of each item were applied.
AUC, area under the curve; CI, confidence interval; HR, hazard ratio; NK, natural killer; NRM, non-relapse related mortality; OS, overall survival; EFS, event free survival.
**P* values were obtained from the logistic regression analysis and **from the Cox's proportional hazard model.
†Items with independent adverse prognostic impact from the multivariate analysis were presented.

Table 4
Effects of Each Candidate Item Obtained at 3 Months After Stem Cell Transplantation on the Prediction of Prognosis

Items	AUC (95% CI)	Best cutoff (/μL)	Sensitivity (%)	Specificity (%)	HR (*P* values, 95% CI)
For NRM event					
Helper/inducer T cells	0.758 (0.652–0.845)	≤160	77.8	70.3	4.727 (0.039*, 1.084–20.618)
NK cells	0.718 (0.608–0.811)	≤22	66.7	83.8	6.458 (0.012*, 1.511–27.611)
NK-T cells	0.739 (0.603–0.848)	≤62	87.5	55.3	3.714 (0.130*, 0.678–20.342)
B cells	0.840 (0.717–0.924)	≤48	87.5	72.9	9.000 (0.013*, 1.598–50.691)
For OS					
B cells	0.695 (0.558–0.811)	≤19	38.9	94.7	4.686 (0.002**, 1.741–12.611)
For EFS					
B cells	0.702 (0.564–0.816)	≤92	69.6	63.6	3.043 (0.012**, 1.284–7.213)

Hazard ratio of each item were calculated when the best cutoff values of each item were applied.
AUC, area under the curve; CI, confidence interval; HR, hazard ratio; NK, natural killer; NRM, non-relapse related mortality; OS, overall survival; EFS, event free survival.
**P* values were obtained from the logistic regression analysis and **from the Cox's proportional hazard model.

who experienced relapse showed significantly higher naïve thymic T cells as compared to those who did not. At 2 months, patients who experienced NRM event demonstrated significantly lower NK cells, NK-T cells, and B cells than those who did not; but status of relapse was not significantly different. At 3 months, patients who experienced NRM event showed significantly lower helper/inducer T cells, NK cells, NK-T cells, and B cells than those who did not; however, status of relapse was not significantly different.

At 12 months post-HSCT, patients who experienced relapse showed significantly lower T cells, H/I T cells, C/S T cells, and NK cells than those who did not (Supporting Information Tables 2–5).

Table 5
Effects of Each Candidate Item Obtained at 12 Months After Stem Cell Transplantation on the Prediction of Prognosis

Items	AUC (95% CI)	Best cutoff (/μL)	Sensitivity (%)	Specificity (%)	HR (*P* values, 95% CI)
For relapse					
T cells	0.762 (0.655–0.849)	≤1,180	75.0	79.7	6.556 (0.017*, 1.406–30.563)
Helper/inducer T cells	0.723 (0.613–0.816)	≤250	62.5	83.8	5.167 (0.034*, 1.133–23.564)
Cytotoxic/suppressor T cells	0.742 (0.633–0.832)	≤541	62.5	91.9	11.333 (0.003*, 2.248–57.146)
NK cells†	0.792 (0.688–0.874)	≤138	87.5	79.7	11.800 (0.004*, 2.161–64.444)
For OS					
T cells	0.837 (0.738–0.909)	≤879	75.0	88.5	7.017 (0.042**, 1.182–50.137)
Cytotoxic/suppressor T cells	0.824 (0.724–0.899)	≤541	75.0	89.7	6.501 (0.047**, 1.113–46.293)
Naïve thymic T cells	0.823 (0.718–0.901)	>115	100.0	61.1	6.447 (0.044**, 1.022–22.578)
NK cells	0.926 (0.847–0.972)	≤138	100.0	76.9	6.254 (0.115**, 0.640–61.085)
For EFS					
T cells	0.736 (0.627–0.827)	≤1,545	80.0	61.1	3.199 (0.093**, 0.825–12.402)
Cytotoxic/suppressor T cells	0.704 (0.593–0.800)	≤541	50.0	91.7	4.495 (0.021**, 1.248–16.185)
NK cells†	0.749 (0.641–0.838)	≤138	80.0	80.6	5.212 (0.020**, 1.290–21.056)
Effector memory T cells	0.696 (0.584–0.793)	≤336	80.0	56.9	3.390 (0.078**, 0.872–13.176)

Hazard ratio of each item were calculated when the best cutoff values of each item were applied.
AUC, area under the curve; CI, confidence interval; HR, hazard ratio; NK, natural killer; OS, overall survival; EFS, event free survival.
***P* values were obtained from the logistic regression analysis and *from the Cox's proportional hazard model.
†Items with independent adverse prognostic impact from the multivariate analysis were presented.

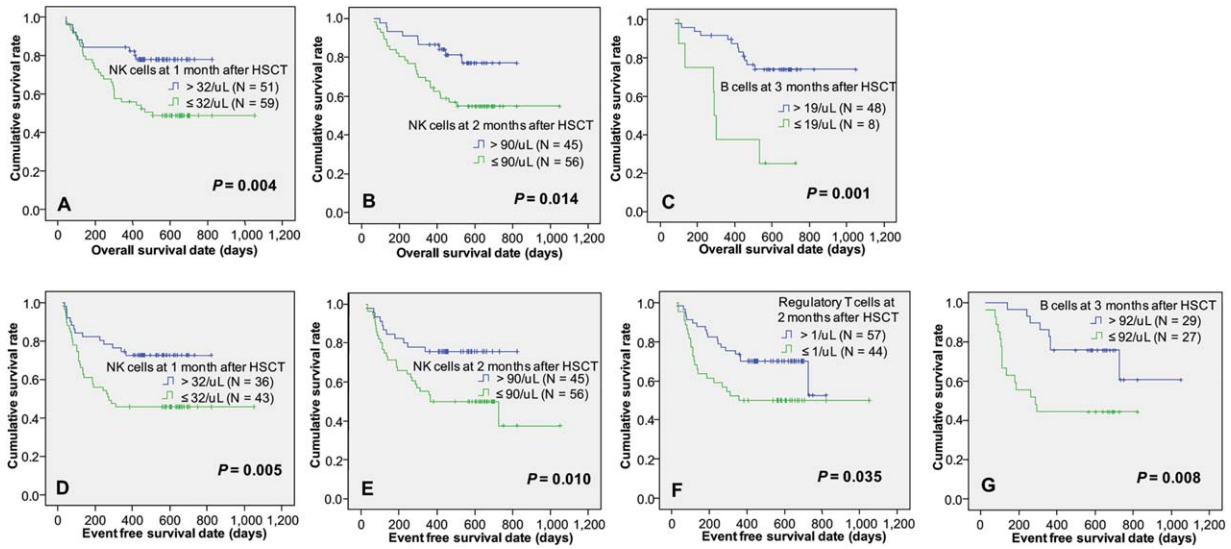

FIG. 2. Survival analysis results of lymphocyte subpopulations at 1, 2, and 3 months post-HSCT which demonstrated significant prognostic values on overall survival (**A**–**C**) and event free survival (**D**–**G**). Abbreviations: HSCT, hematopoietic stem cell transplantation; NK, natural killer. [Color figure can be viewed at wileyonlinelibrary.com]

Association of Post-HSCT Lymphocyte Subpopulations with Overall Survival or Event-Free Survival

At 1 month after HSCT, patients who did not survive had significantly lower NK cells than those who did, and patients who experienced events showed significantly lower NK cells and NK-T cells than those who did not. At 2 months, NK cell levels were significantly lower in patients with adverse prognosis (both OS and

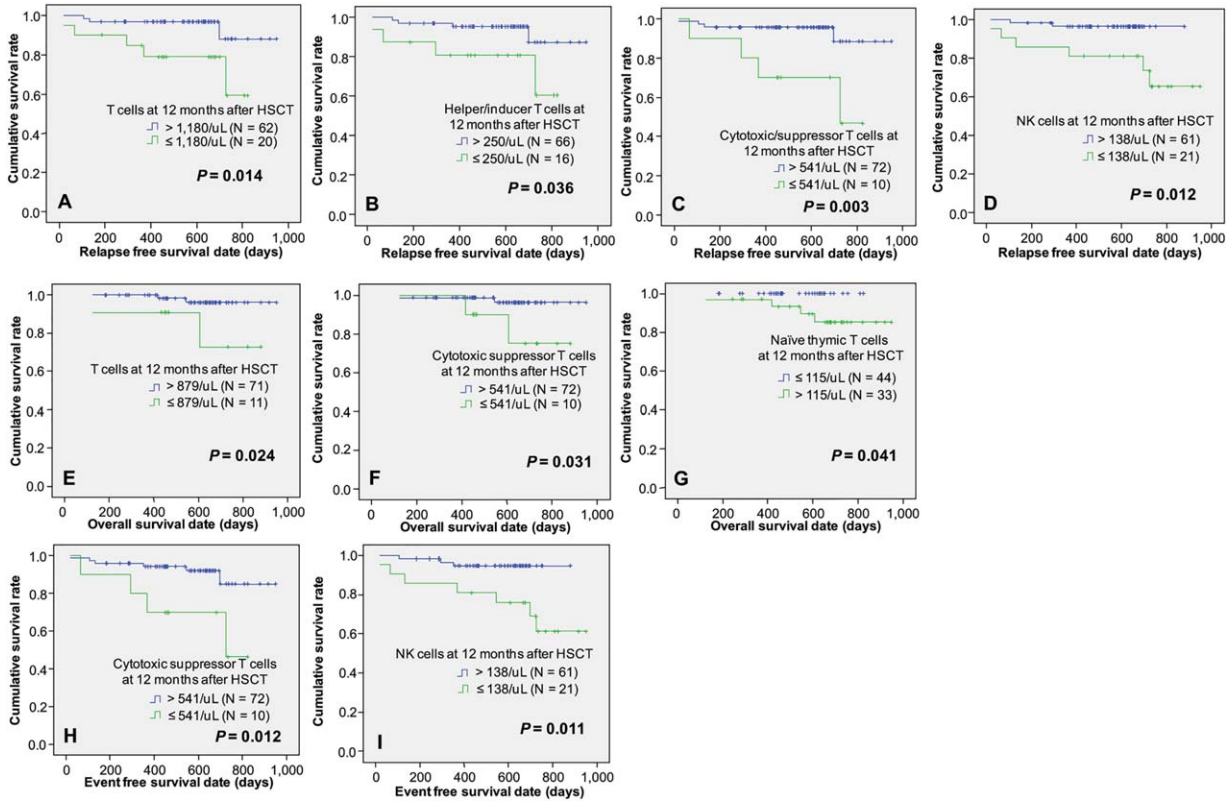

FIG. 3. Survival analysis results of lymphocyte subpopulations at 12 months post-HSCT which demonstrated significant prognostic values on relapse free survival (**A**–**D**), overall survival (**E**–**G**), and event free survival (**H**–**I**). Abbreviations: HSCT, hematopoietic stem cell transplantation; NK, natural killer. [Color figure can be viewed at wileyonlinelibrary.com]

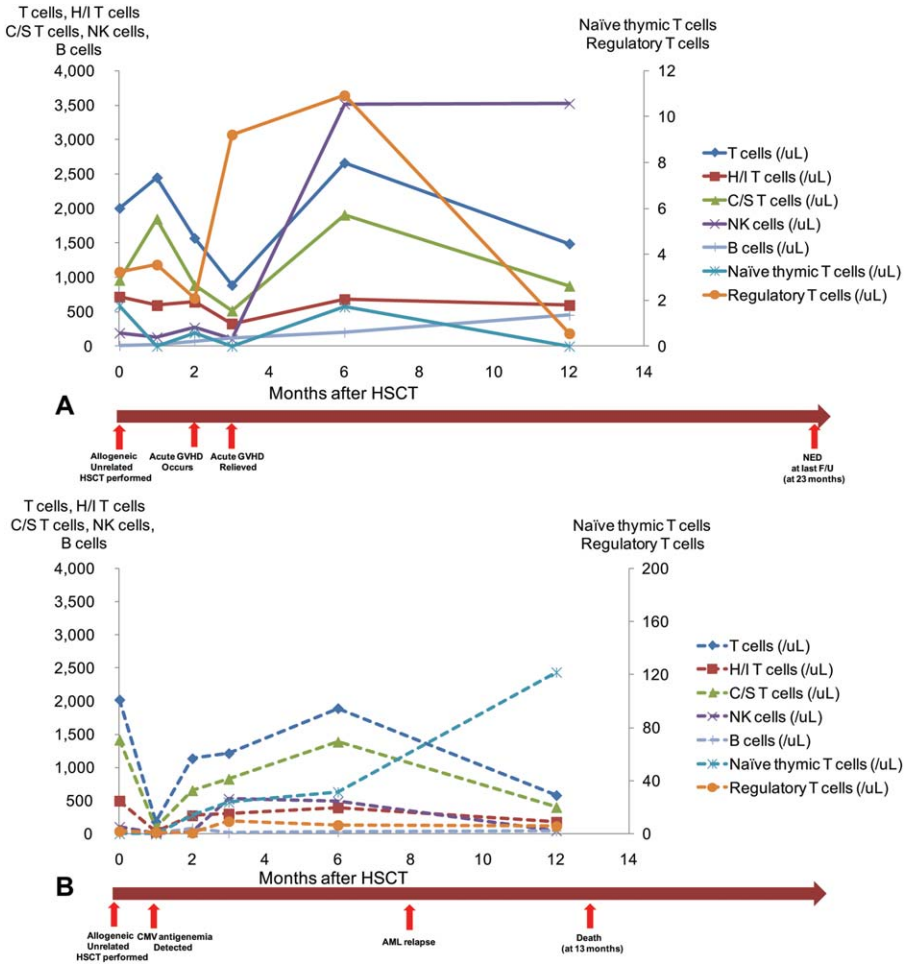

FIG. 4. Serial follow-up data on seven lymphocyte subpopulations from two representative AML cases with favorable (**A**) and adverse outcome (**B**). Abbreviations: H/I, helper/inducer; C/S, cytotoxic/suppressor; NK, natural killer; HSCT, hematopoietic stem cell transplantation; GvHD, graft-versus-host disease; NED, no evidence of disease; F/U, follow-up; CMV, cytomegalovirus; AML, acute myeloid leukemia. [Color figure can be viewed at wileyonlinelibrary.com]

event) as compared to those in patients with favorable prognosis; further, lower regulatory T cells also appeared to confer a poor prognosis in terms of events. At 3 months, B cells showed significantly lower values in patients with adverse prognosis (both OS and event) than those with favorable prognosis.

At 12 months, patients who did not survive had significantly lower T cells, C/S T cells, NK cells, and significantly higher naïve thymic T cells, as compared to those in survivors. Of these, T cells, C/S T cells, and NK cells maintained significantly lower values in patients who experienced events than those who did not. Patients who experienced events also showed significantly lower effector memory T cells than those who did not (Supporting Information Tables 6–9).

Effect of Each Candidate Item on the Prediction of Prognosis

At 1 and 2 months post-HSCT, six and three items showed significant impact on NRM and relapse event, respectively, but survival analysis failed to confirm their prognostic impact, and subsequent multivariate analysis confirmed the lack of significant prognostic impact in all these items. However, low levels of NK cells ($\leq$32/μL at 1 month and $\leq$90/μL at 2 months) showed significant adverse association with OS and EFS (Tables 2 and 3; Fig. 2) and multivariate analysis confirmed the adverse prognostic impact of low levels of NK cells [at 1 month: hazard ratio (HR) 3.281, $P = 0.031$; at 2 months: HR 2.221, $P = 0.026$; data not shown]. In addition, low levels of regulatory T cells ($\leq$1/μL) at 2 months also had a significant adverse impact on EFS (Table 3; Fig. 2), and multivariate analysis confirmed the adverse prognostic impact of regulatory T cells (HR 2.726, $P = 0.040$, data not shown). At 3 months, four variables showed significant impact on NRM event; however, survival analysis and multivariate analysis did not demonstrate their prognostic impact. However, low levels of B cells ($\leq$19 and $\leq$92/μL) were significantly associated with adverse OS and EFS, respectively (Table 4; Fig. 2).

At 12 months post-HSCT, low levels of T cells (≤1180/μL), H/I T cells (≤250/μL), C/S T cells (≤541/μL), and NK cells (≤138/μL) were significantly associated with risk of relapse but their adverse prognostic impacts were not confirmed by the multivariate analysis except for NK cells (HR 9.221, $P = 0.017$, data not shown). In terms of OS, low levels of T cells (≤879/μL) and C/S T cells (≤541/μL) and high level of naïve thymic T cells (>115/μL) were significantly associated with poor outcomes, but their adverse prognostic impacts were not confirmed by the multivariate analysis. In terms of EFS, low levels of C/S T cells (≤541/μL) and NK cells (≤138/μL) showed a significant association with poor outcomes (Table 5; Fig. 3), and the adverse prognostic impact of low levels of NK cells was confirmed by the multivariate analysis (HR 4.783, $P = 0.041$, data not shown).

Serial Follow-up of Seven Lymphocyte Subpopulations in Two Representative Cases with Contrasting Prognosis

Serial follow-up data of seven lymphocyte subpopulations from one AML patient who received allogeneic unrelated HSCT at first complete remission state and demonstrated no evidence of disease/relapse/event at 23 months post-HSCT, showed relatively preserved NK cells (129 and 272/μL) and regulatory T cells (4 and 2/μL) at 1 and 2 months after HSCT, respectively. This patient also showed relatively high B cells (117/μL) at 3 months post-HSCT, and had high T cells, H/I T cells, C/S T cells, and NK cells (1488, 596, 876, and 3524/μL, respectively) and no naïve thymic T cells at 12 months after HSCT (Fig. 4A).

In contrast, the corresponding data from the other AML patient who also received allogeneic unrelated HSCT at first complete remission state, experienced relapse at 8 months after HSCT and died at 13 months after HSCT, showed low NK cells (5 and 26/μL) and regulatory T cells (1 and 1/μL) at 1 and 2 months post-HSCT, respectively. This patient also showed low B cells (14/μL) at 3 months after HSCT, and had low T cells, H/I T cells, C/S T cells, and NK cells (582, 180, 402, and 49/μL, respectively) and high naïve thymic T cells (122/μL) at 12 months after HSCT (Fig. 4B).

DISCUSSION

Recent studies reported that the recovery of NK cells, which confer innate immunity, occurs in the early phase after HSCT (5,17), and high NK cell counts at 1 month post-HSCT have been shown to be associated with superior outcomes in recipients of HLA-matched HSCT (15). In the present study, low levels of NK cells in PB at early stage (1–2 months) post-HSCT were associated with adverse prognosis, which is consistent with findings from previous studies (15). Regulatory T cells are known to recover at a relatively later stage (3 months) after HSCT (5); we observed that low levels of regulatory T cell at an early stage (2 months) post-HSCT were also related to poor prognosis. These results suggest that the successful recovery of adaptive $CD4^+$ regulatory T cells in PB, which is indicative of an early institution of adequate defense mechanism against aggressive immune activation, may also be associated with favorable prognosis in the early phase after HSCT, similar to that observed in the case of NK cells. These results are consistent with a previous study which reported a high proportion of regulatory T cells maintained after HSCT in event-free patients (5).

Circulating NK cells are composed of major $CD56dim^+/CD16^+$ NK cells (at least 90% of total NK cells) and minor $CD56bright^+/CD16^-$ NK cells (<10% of total NK cells), and the latter is the first lymphocyte subpopulation that appears in PB after HSCT (18–21). Our present study showed that the number of $CD56bright^+/CD16^-$ NK cells increase (median 35/μL) at 1 month post-HSCT as compared to their pretransplant levels (median 12/μL); however, the number of $CD56dim^+/CD16^+$ NK cells was similar (median 39 and 35/μL at 1 month after HSCT and pretransplant, respectively) at both the time points. These results also support the previous study results (18–21). However, in our study, the prognostic impact of $CD56bright^+/CD16^-$ NK cells was not evident despite an increased number of these cells at 1 month post-HSCT; further, we did not observe an adverse prognostic impact of decreased $CD56dim^+/CD16^+$ NK cells on both OS and EFS at 1 month post-HSCT. These results suggest that although NK cells are important prognostic indicators in the early phase post-HSCT, the specific association of their subpopulations with prognosis was not clearly observed, and needs to be investigated in further studies.

Our study showed a significant adverse prognostic impact of low levels of B cells in the early stage (3 months) after HSCT. These results partly support the previous study results which demonstrated relatively late recovery of B cells post-HSCT as compared to that of NK cells (5,22,23), and may also underscore the prognostic role of humoral immunity in the early stage post-HSCT. In addition, our present study also demonstrated a significant adverse prognostic impact of low levels of T cells and their subpopulations as well as NK cells at late phase (12 months). These results are consistent with those from a previous study which revealed relatively late recovery of T cell and rapid expansion of NK cells in the haploidentical HSCT setting (5,24). Our study results also demonstrate that low level of $CD4^+$ T cells at 12 months after HSCT predicts poor prognosis, and these results are consistent with those reported earlier (14). In contrast with NK cells, our present study could not confirm the adverse prognostic impacts of T cells and their subpopulations at late phase after HSCT, from the multivariate analyses. However, the statistical significance of multivariate analysis performed in our present study should be limited due to small patient numbers of each patient subgroup, and this issue should be confirmed from more comprehensive study in the future.

Since there were no significant differences in any lymphocyte subpopulations between the diagnostic subgroups at baseline prior to HSCT, our results can be summarized as showing that, irrespective of primary disease, recovery of sufficient innate immunity (NK cells), immune defence mechanism (regulatory T cells) and humoral immunity (B cells) in the early phase post-HSCT, and recovery of cellular immunity (T cells) and innate immunity (NK cells) in the late phase after HSCT impact favorably on prognosis. Interestingly, our present study also identified the poor prognostic impact of a high level of naïve thymic T cells at 12 months post-HSCT. Since the increase in naïve thymic T cells may indicate an increase in under-educated T cells with deficient immunity, these results suggest the importance of T cell education as cells with effector immune function in HSCT patients and support previous studies which reported that early thymic reconstitution suggesting sufficient T cell maturation/education, is associated with favorable outcomes after umbilical cord blood transplantation or HSCT (25–28).

In our present study, we could not perform single platform strategy (SPT), because commercially available kits which can measure detailed 14 lymphocyte subpopulations, were not present at the time of analysis, and commercially available kits and reagents such as Multitest 6-color TBNK and Trucount (BD Bioscience) can apply SPT strategy to measure only simple lymphocyte subsets (B cells, helper T cells, cytotoxic T cells, and NK cells). Recently introduced SPT can generally produce greater precision in all T-cell subset measurements, as well as greater accuracy on helper T cells and cytotoxic T cells than DPT, as reported in a previous study (29). But this study also mentioned that there is a high degree of interlaboratory variability in DPT and some DPT laboratories outperformed all SPT laboratories on T-cell subset percentages, and interval between sampling and analysis (<6 h) would be an important factor to minimize overestimation of lymphocytes due to degranulation of granulocytes in DPT laboratories. Our present study performed assessment of lymphocyte subsets using samples obtained within 6 h after the collection. We gated at least 30,000 acquired lymphocytes on CD45 versus side-scatter plots and we rejected duplication of sample analysis and excluded the results from our present study, if the acquired lymphocyte counts were <30,000 cells to remove samples with too large variations from the analysis results. These points can partly justify the appropriateness of DPT in terms of accuracy in our present study.

Since our present study included heterogeneous patient subgroups with three different HSCT settings (sibling/unrelated matched/haploidentical), three different disease MDS subtypes (RCMD, RAEB-1, and −2), and three different pretransplant conditioning regimens in the total study population, we could expect that the heterogeneity of patient population may affect the results. However, we could not analyze the impact of this issue on the main results of our present study, because the division of such small study population would result in the lack of sufficient statistical power. More comprehensive study with sufficient number of each study subpopulation should be required to confirm and enrich our main results. In addition, as mentioned above, the multivariate analysis performed in our present study has limited value in terms of low statistical power due to small patient numbers of each patient subgroup, and the results of multivariate analyses in our present study should be confirmed from the comprehensive study in the future.

In conclusion, low levels of NK cells, regulatory T cells, and B cells in PB measured at early stage (1–3 months) after HSCT are adverse prognostic indicators irrespective of the disease subtype in HSCT recipients with hematologic malignancies. At late stage (12 months) after HSCT, low levels of T cells and their subpopulations as well as NK cells, and high level of naïve thymic T cells in PB are also adverse prognostic indicators in these patients.

ACKNOWLEDGMENTS

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