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Effect of changes in lymphocyte subsets at diagnosis in acute myeloid leukemia on prognosis: association with complete remission rates and relapse free survivals

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Abstract

We prospectively investigated whether the characteristics of lymphocyte subsets at diagnosis in acute myeloid leukemia (AML) patients are different from healthy controls and affect treatment outcomes. A total of 91 AML patients classified into 3 genetic risk subgroups (favorable/intermediate/poor) according to 2022 NCCN guidelines were enrolled. We measured lymphocyte subsets by flow cytometry with peripheral blood samples at diagnosis and compared results with healthy controls. Influences of lymphocyte subsets on complete remission (CR) rates and survivals were also evaluated. AML patients had significantly lower numbers and proportions of $CD56^{dim}CD16^+$ natural killer (NK) cells, central memory T cells, and regulatory T cells than healthy controls. Higher proportion of helper/inducer T cells, $CD4^+CD31^+$ naïve T cells, and decreased proportion of NK cells significantly increased CR rates in 65 non-promyelocytic leukemia patients ($P = 0.034$, 0.027, and 0.019, respectively), and it was also significant in multivariable analysis with age/risk adjusted ($P = 0.014$, 0.016, and 0.045, respectively). NK cells < 4.8% of lymphocytes demonstrated significantly shorter relapse free survivals (RFS) in both univariate and multivariate analyses with risk adjusted ($P = 0.006$ and 0.037, respectively). AML patients showed significant lower numbers of $CD56^{dim}CD16^+$ NK cells, central memory T cells, and regulatory T cells than healthy controls at diagnosis. Higher proportion of helper/inducer T cells and $CD4^+CD31^+$ naïve T cells and decreased proportion of NK cells at diagnosis were independent factor of increasing probability of CR, and proportion of NK cells < 4.8% at diagnosis had adverse impact in RFS.

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Introduction

Acute myeloid leukemia (AML) is one of the most common hematologic malignancies in adult patients. The only curative treatment for AML is an intensive chemotherapy that can be combined with hematopoietic stem cell transplantation (HSCT). However, this therapeutic strategy can be performed in younger patients below 60–65 years of age, and HSCT is an aggressive therapy which is associated with severe complications such as graft-versus-host disease and opportunistic infections.

Physiologic function of the immune system in the tumor is to recognize and destroy clonally transformed cells before they grow into tumors and to kill tumors after they are formed. It is now clear that the immune system does react against many tumors including AML in vivo. The function of the immune system to cure the leukemia is already

apparent in the setting of allogeneic HSCT with the graft-versus-leukemia (GVL) effect [1]. Subsequent studies in the immunotherapy using GVL effect such as donor T lymphocyte infusion after HSCT [2] and recipient T lymphocyte infusion after MHC matched HSCT provided successful results [3], and donor natural killer (NK) cell infusion after allogeneic HSCT in AML was associated with better outcomes [4]. Although AML patients showed increased NK phenotype lymphocytes, especially immature NK cells lacking CD16 antigen, and increased $CD3^+CD56^+$ lymphocytes, but normal T cell distribution [5–7], anti-leukemic effects, or subset alteration of lymphocytes at the time of diagnosis is not well established, and there are few reports about lymphocyte subset distribution and their prognostic impact in AML patients.

In this study, we prospectively studied the distribution of lymphocyte subsets in peripheral blood (PB) obtained from AML patients at diagnosis and investigated whether the characteristics of lymphocyte subsets at diagnosis in AML patients are different from healthy controls and affect treatment outcomes, to know whether the difference of lymphocyte subsets had the prognostic implication.

Material and methods

Patients and treatments

A total of 91 consecutive patients who were newly diagnosed as AML based on bone marrow (BM) study, immunophenotyping, and cytogenetic and molecular studies at the Asan Medical Center from July 2017 to May 2019 were enrolled prospectively in this study. The diagnosis of AML was defined according to the 2016 World Health Organization (WHO) classifications. Genetic risk status was classified as favorable, intermediate, and poor according to the recently introduced 2022 National Comprehensive Cancer Network (NCCN) guidelines [8].

Karyotyping, reverse transcriptase-real time polymerase chain reaction [Hemavision (DNA Diagnostic, Risskov, Denmark)] for the detection of frequently identified chromosomal rearrangements in AML, and molecular studies including next-generation sequencing for the detection of frequently identified molecular abnormalities in AML including *FLT3*, *NPM1*, and *CEBPA* mutations were performed in all patients at diagnosis.. In terms of genetic risk status, 33, 28, and 30 patients were classified as favorable, intermediate, and poor risk according to genetic risk classifications presented in 2022 National Comprehensive Cancer Network (NCCN) guidelines [8]. Patient details are given in Supplemental Table 1.

Among the 91 AML patients, 5 patients received low-dose cytarabine or low-dose azacitidine therapy, and 5

patients received only supportive treatment. Eighty-one patients were eligible for intensive chemotherapy, but 3 of them died before initiation of induction treatment. Forty-three patients received induction chemotherapy with cytarabine and daunorubicin combination and 22 patients with cytarabine and idarubicin. Eleven patients with AML with t(15;17) received induction chemotherapy with idarubicin and all-trans retinoic acid. Three patients received chemotherapy with cytarabine and etoposide combination. We analyzed treatment outcomes with 65 patients who were eligible for cytarabine plus anthracycline-based induction chemotherapy. After the initial induction chemotherapy, the patients without blast clearance in BM on the 14th day evaluation received a reinduction chemotherapy. Patients who did not achieve complete remission (CR) after the initial chemotherapy or reinduction were offered alternative therapies. A total of 41 patients received HSCT. Patients who achieved CR after induction or reinduction chemotherapy received two cycles of consolidation therapies, and 30 patients subsequently received allogeneic or autologous HSCT. Eleven patients received HSCT without evidence of induction remission. Among 65 patients in whom treatment outcome evaluated, 33 (50.8%) patients achieved CR after initial induction chemotherapy, and 39 (60.0%) patients achieved CR after initial or reinduction chemotherapy. Eleven patients relapsed, and 17 patients died including 1 patient who expired before completion of initial induction chemotherapy.

CR was defined as less than 5% of blast cells with more than > 20% cellularity in a standardized BM aspirate and biopsy in patients who underwent induction chemotherapy and exhibiting hemogram results with absolute neutrophil counts $> 1.0 \times 10^9$/L and platelet counts $> 100.0 \times 10^9$/L. Relapse was defined as the reappearance of more than 5% leukemic blasts of BM aspirates in patients with CR state. This study was approved by the institutional review board of Asan Medical Center and was performed in accordance with the Declaration of Helsinki. Treatment characteristics and responses in 65 AML patients are summarized in the Supplemental Table 2.

Flow cytometric analysis

PB samples from 91 AML patients at diagnosis and 10 healthy controls [median age 45 years (25 ~ 65); 5 males and 5 females] were obtained. Assessment of lymphocyte subsets was performed using the samples within 48 h after the sample was drawn. After erythrocyte lysis, the samples were labeled with specific antibodies. An allophycocyanin (APC)-Cy7-conjugated anti-CD45 antibody; a peridinin-chlorophyll protein (PerCP)-conjugated anti-CD3 antibody; fluorescein isothiocyanate (FITC)-conjugated anti-CD8, anti-CD56, anti-CD161, anti-CD45RA, and

anti-CD25 antibodies; APC conjugated anti-CD31, anti-CD19, anti-CD18, anti-CD62L, and intracellular anti-Foxp3 antibodies; phycoerythrin (PE)-conjugated anti-CD4, anti-CD16, anti-CD3, anti-CD45RO, and anti-CD8 antibodies; and a PE-Cy7- conjugated anti-CCR7 antibody were used. All antibodies except CD45 (BD Bioscience, San Jose, CA, USA) were obtained from Beckman Coulter (Beckman Coulter, Fullerton, CA, USA). Three- to five-color flow cytometric immunophenotypic analysis was performed using a FACSCanto™ II flow cytometry system (BD Bioscience, USA) and analyzed using the FACSDiva software (BD Bioscience, USA).

A total of 6 tubes were tested with combinations of antibodies CD8/CD4/CD3/CD31/CD45, CD56/CD16/CD3/CD19/CD45, CD161/CD3/CD45, CD45RA/CD45RO/CCR7/CD28/CD45, CD45RA/CD8/CCR7/CD62L/CD45, and CD25/CD4/Foxp3/CD45. We gated 30,000 lymphocytes on CD45 versus side-scatter plots. 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 $CD56^{bright}CD16^+$, $CD56^{bright}CD16^-$, and $CD56^{dim}CD16^+$ 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 T cells with direct method ($CD45RA^{high}CDRO^-CCR7^+CD28^+$) and indirect method ($CD3^+CD4^+CD31^+$) 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. The definition of each lymphocyte subset is given in the Table 1, and schematic illustrations representing gating strategies for measurement of each lymphocyte subset are described in the Fig. 1.

Comparison of lymphocyte subset distribution at diagnosis in AML patients and healthy controls

Both proportions and absolute numbers of lymphocyte subsets were compared between PB samples obtained from AML patients at diagnosis and healthy controls. These results are summarized in Table 2.

Comparison of lymphocyte subset distribution at diagnosis in AML patients with respect to three subgroups defined by risk status

Both proportions and absolute numbers of lymphocyte subsets were compared among PB samples obtained from

Table 1 Phenotypes of lymphocyte subsets in flow cytometric immunophenotyping analysis

| Lymphocyte subsets	Phenotype
T cells	$CD3^+$ cells
Helper/inducer T cells	$CD3^+CD4^+$ cells
Suppressor/cytotoxic T cells	$CD3^+CD8^+$ cells
NK cells	$CD3^-CD16^+$ and/or $CD56^+$
$CD56^{bright}CD16^+$ NK cells	$CD3^-CD56^{bright}CD16^+$
$CD56^{bright}CD16^-$ NK cells	$CD3^-CD56^{bright}CD16^-$
$CD56^{dim}CD16^+$ NK cells	$CD3^-CD56^{dim}CD16^+$
NK-T cells	$CD3^+CD161^+$
Naïve T cells	$CD45RA^{high}CD45RO^-CCR7^+CD28^+$
$CD4^+CD31^+$naïve T cells	$CD3^+CD4^+CD31^+$
Memory T cells	
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	$CD19^+$

CD, cluster of differentiations; NK, natural killer

AML patients at diagnosis with respect to three genetic risk statuses. These results are summarized in Table 3.

Lymphocyte subsets at diagnosis as a predictor of complete remission

The CR achievement rates after induction or reinduction chemotherapy were estimated in 65 non-promyelocytic leukemia patients who received cytarabine + anthracycline-based induction chemotherapy. The univariate and multivariate logistic regression analysis was performed to predict whether characteristics of lymphocyte subset expressions would influence CR achievement rates or not after the initial induction or reinduction chemotherapy. In multivariate analysis, the age, genetic risk status, and lymphocyte subset were included as covariables. These results are summarized in Table 4.

Effect of lymphocyte subsets at diagnosis on the survival

The Kaplan–Meier method and univariate, multivariate analysis using a Cox's proportional hazards model were performed to predict whether characteristics of lymphocyte subset expressions would influence overall survival (OS, defined as the interval from the time of diagnosis to the time of death or last follow-up) and relapse free survival (RFS, defined as the interval from the time of CR to the time of relapse, death or last follow-up) or not
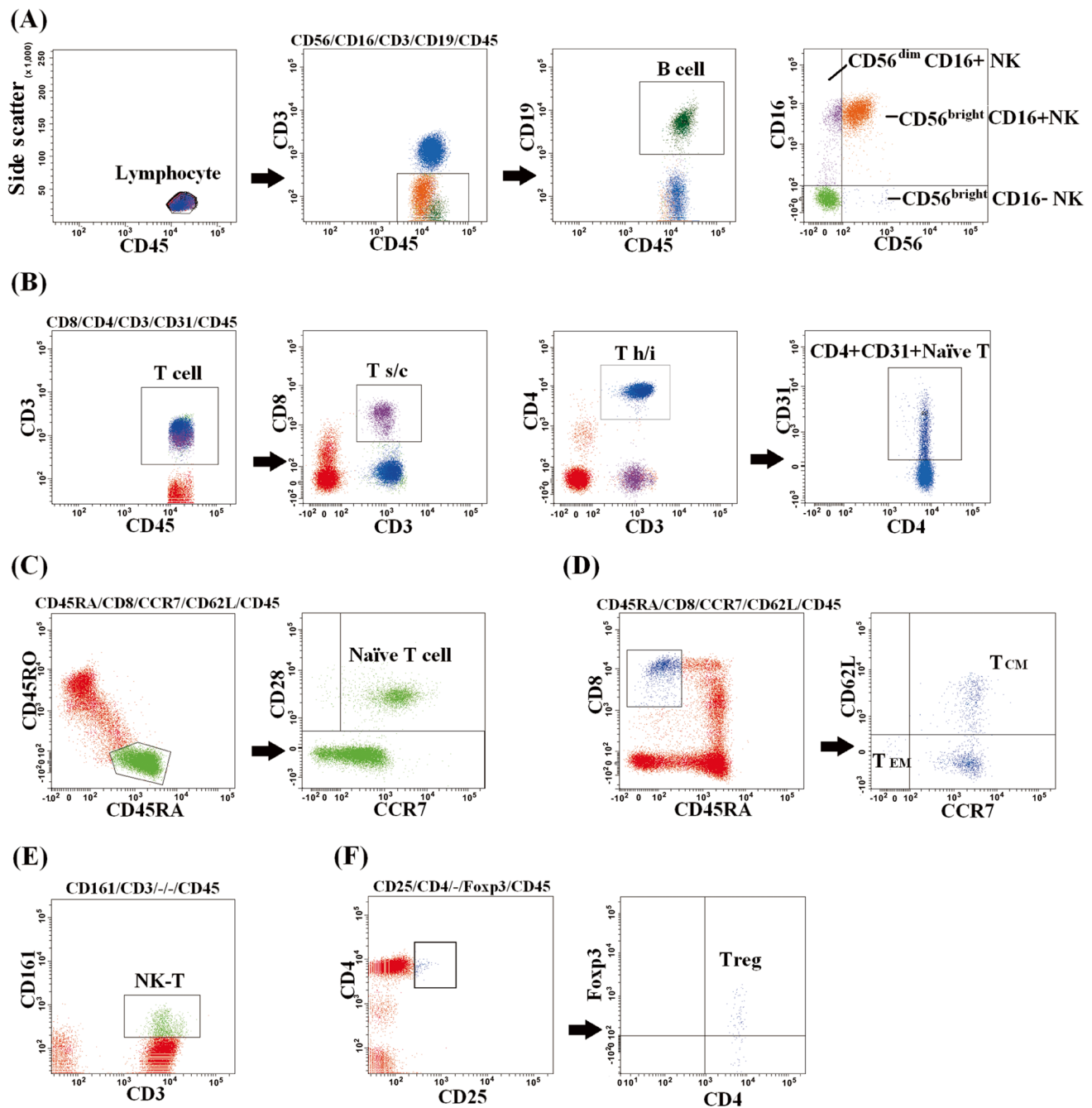
Fig. 1 Schematic illustrations representing flow cytometric gating strategies for the determination of each lymphocyte subset. **A** Gates for lymphocytes were applied on CD45^{high} and low side-scatter plots. Among CD3$^-$ cells, we measured CD19$^+$ B cells and NK cell subsets which express CD56^{bright}CD16$^-$, CD56^{dim}CD16$^+$, and CD56^{bright}CD16$^+$. **B** T cells were gated as CD3$^+$ cells and among the CD3$^+$ population suppressor/cytotoxic T cells (T s/c) and CD3$^+$CD8$^+$ and helper/inducer T cells (T h/i); CD3$^+$CD4$^+$ and CD4$^+$CD31$^+$ naïve T cells were measured. **C** CD45RA^{high}CD45RO$^-$CCR7$^+$CD28$^+$ cells were assigned for direct measurement of naïve T cells. **D** 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$_{EM}$). **E** NK-T cells were positive for CD3 and CD161. **F** Regulatory T cells (Treg) were positive for CD4, CD25^{high}, and Foxp3. Abbreviations and definitions: CD, cluster of differentiations; NK, natural killer; s/c, suppressor/cytotoxic; h/i, helper/inducer; CD45RO, 180-kilodalton (kDa) isoform of CD45 leukocyte common antigen; CD45RA, 220-kilodalton (kDa) isoform of the leukocyte common antigen; CCR7, C–C motif chemokine receptor 7; L, ligand; EM, effector memory; CM, central memory; reg, regulatory; Foxp3, forkhead box P3

Table 2 Comparison of lymphocyte subset distribution at diagnosis in AML patients and healthy controls

Lymphocyte subsets	Proportions (%), median (range)		P values	Absolute numbers (x 10^6/L), median (range)		P values
	AML (N=91)	Healthy controls (N=10)		AML (N=91)	Healthy controls (N=10)	
T cells	70.40 (10.80–91.20)	74.05 (59.30–87.00)	NS	1,440 (85–28,800)	1,590 (740–2010)	NS
Helper/inducer T cells	39.70 (4.40–72.50)	39.65 (33.60–51.20)	NS	760 (34–15,600)	950 (350–1250)	NS
Suppressor/cytotoxic T cells	20.60 (5.30–39.10)	21.25 (10.50–35.70)	NS	380 (41–9840)	390 (260–810)	NS
Total NK cells	9.22 (0.17–55.47)	15.94 (6.88–26.47)	NS	200 (1–5410)	270 (130–770)	NS
$CD56^{bright}$ $CD16^-$ NK cells	0.23 (0.00–13.18)	0.45 (0.09–0.76)	NS	5 (0–506)	7 (1–22)	NS
$CD56^{bright}CD16^+$ NK cells	6.72 (0.10–55.35)	8.09 (0.33–21.32)	NS	160 (1–4820)	140 (7–620)	NS
$CD56^{dim}$ $CD16^+$ NK cells	0.83 (0.00–13.76)	4.49 (0.21–18.00)	0.005	26 (0–1110)	88 (4–370)	0.030
NK-T cells	7.10 (0.30–28.50)	6.45 (4.10–10.90)	NS	150 (2–1930)	150 (64–250)	NS
Naïve T cells	27.11 (0.00–56.77)	24.26 (16.25–37.23)	NS	460 (0–18,760)	540 (240–940)	NS
$CD4^+CD31^+$ naïve T cells	9.35 (0.33–56.74)	12.54 (7.20–29.38)	NS	200(4–7160)	270 (110–740)	NS
Central memory T cells	2.09 (0.00–18.76)	4.18 (2.05–5.69)	0.007	40 (0–930)	76 (40–170)	0.027
Effector memory T cells	0.04 (0.00–3.42)	0.04 (0.00–0.25)	NS	1 (0–56)	1 (0–6)	NS
Regulatory T cells	0.17 (0.00–6.60)	0.92 (0.39–1.30)	0.019	4 (0–1090)	22 (6–31)	0.008
B cells	10.40 (0.60–79.20)	10.60 (3.50–15.90)	NS	230 (4–8300)	210 (57–350)	NS

CD, cluster of differentiations; NK, natural killer; NS, not significant

All P values were obtained from the Mann–Whitney U test

Statistical analysis

Statistical analysis was performed using the Statistical Package for the Social Sciences (version 18.0; SPSS, Chicago, IL, USA). For continuously distributed variables, the Mann–Whitney U test was used to analyze differences of expression levels between two subgroups. Spearman's correlation analysis method was used to estimate relationships between two subgroups. We used univariate and multivariate logistic regression analysis to predict whether characteristics of lymphocyte subset expressions would influence CR achievement rates or not after the initial or second induction chemotherapy. Kaplan–Meier method was used to estimate OS and RFS, and survival curves were compared using a log-rank test. Univariate and multivariate analyses of OS and RFS with respect to lymphocyte subset expressions at diagnosis were performed using a Cox's proportional hazards model. For all analyses, tests were two-tailed and P values ≤ 0.05 were considered statistically significant.

after the initial induction or reinduction chemotherapy. In multivariate analysis, the age, genetic risk status, and lymphocyte subset were included as covariables. These results are summarized in the Tables 5 and 6 and Fig. 2.

Results

Comparison of lymphocyte subset distribution at diagnosis in AML patients and healthy controls

AML patients showed lower lymphocyte subset proportions of $CD56^{dim}$ $CD16^+$ NK cells, central memory T cells, and regulatory T cells at diagnosis than healthy controls (P = 0.005, 0.007, 0.019, respectively) (Table 4). Proportion of total NK cells of patients was lower than healthy control, but there was no statistical significance. The absolute number of white blood cells (WBC) and lymphocytes of patients with AML at diagnosis were not significantly different from that of healthy individuals (data not shown), but AML patients had significantly lower number of $CD56^{dim}$ $CD16^+$ NK cells, central memory T cells, and regulatory T cells than those of healthy controls (P = 0.030, 0.027, and 0.008, respectively) (Table 2).

Comparison of lymphocyte subset distribution at diagnosis in AML patients with respect to three genetic risk statuses

Poor risk patients had significantly lower proportions of NK-T cells than favorable risk patients (P = 0.008) and higher proportion of naïve T cells than favorable and intermediate risk patients (P = 0.005 and 0.001, respectively).

Table 3 Comparison of clinical variables and lymphocyte subset distribution at diagnosis in AML patients with respect to three genetic risk status

Variables	Favorable (N=33)	Intermediate (N=28)	Poor (N=30)	P values		
				Favorable *vs.* intermediate	Favorable *vs.* poor	Intermediate *vs.* poor
Age, median (range)	51 (20–80)	54 (20–81)	51 (17–81)	NS	NS	NS
WBC count, median (range), x10^9/L	7.6 (0.7–104.9)	11.8 (0.9–430.0)	4.8 (1.2–284.6)	NS	NS	NS
Lymphocyte count, median (range), x10^9/L	1.9 (0.4–14.8)	2.4 (0.6–41.2)	1.8 (0.2–25.6)	NS	NS	NS
Lymphocyte subsets	Proportions (%), median (range)					
	Favorable (N=33)	Intermediate (N=28)	Poor (N=30)			
T cells	70.40 (24.90–86.30)	66.75 (22.00–90.00)	72.85 (10.80–91.20)	NS	NS	NS
Helper/inducer T cells	36.40 (15.30–57.60)	38.15 (5.60–72.50)	42.60 (4.40–63.20)	NS	NS	NS
Suppressor/cytotoxic T cells	22.40 (6.40–36.10)	20.80 (6.50–39.10)	17.80 (5.30–36.10)	NS	NS	NS
Total NK cells	11.23 (2.82–50.75)	8.72 (0.17–55.47)	7.18 (0.66–28.36)	NS	NS	NS
CD56^{bright} CD16$^-$ NK cells	0.24 (0.05–3.94)	0.27 (0.00–6.08)	0.19 (0.04–13.18)	NS	NS	NS
CD56^{bright}CD16$^+$ NK cells	9.56 (0.86–49.67)	6.48 (0.10–55.35)	5.41 (0.44–27.12)	NS	NS	NS
CD56^{dim} CD16$^+$ NK cells	0.68 (0.12—8.96)	0.98 (0.00–13.76)	0.81 (0.10–7.10)	NS	NS	NS
NK-T cells	8.30 (3.20–26.80)	6.50 (2.60–28.50)	6.20 (0.30–18.90)	NS	0.008	NS
Naïve T cells	23.01 (2.19–45.87)	22.80 (0.00–9.49)	33.08 (1.44–56.77)	NS	0.005	0.001
CD4$^+$CD31$^+$ naïve T cells	8.64 (2.02–56.74)	9.46 (0.33–55.32)	11.96 (0.50–27.74)	NS	NS	NS
Central memory T cells	2.59 (0.00–18.76)	1.97 (0.00–9.49)	1.86 (0.03–7.97)	NS	NS	NS
Effector memory T cells	0.04 (0.00–0.37)	0.12 (0.00–3.42)	0.02 (0.00–0.61)	NS	NS	NS
Regulatory T cells	0.12 (0.00–5.60)	0.41 (0.02–6.60)	0.11 (0.01–1.94)	0.019	NS	NS
B cells	10.50 (3.20–36.80)	9.18 (0.60–32.70)	14.82 (3.00–79.20)	NS	NS	0.019

CD, cluster of differentiations; NK, natural killer; NS, not significant

All *P* values were obtained from the Mann–Whitney *U* test

Intermediate risk patients had higher proportion of regulatory T cells than favorable risk patients ($P = 0.019$) and lower proportions of B cells than poor risk patients ($P = 0.019$). The proportions of CD56^{dim}CD16$^+$ NK cells in poor risk subgroups tended to increase than those in favorable risk subgroups (median 0.81% *vs.* 0.68%, differences 0.13%), and when the intermediate risk subgroup was included in the poor risk subgroup, the differences tended to increase slightly. In addition, as the genetic risk increased from favorable to poor risk, the sum of CD56^{bright}CD16$^-$ and CD56^{bright}CD16$^+$ NK cells which reflect CD56$^+$ NK cells tends to decrease from 9.80% in favorable risk, 6.75% in intermediate risk to 5.60% in poor risk (Table 3).

In the comparison of absolute number of lymphocyte subset, no significant differences were found between the two subgroups categorized by genetic risk in all lymphocyte subsets, and therefore, raw data was not presented in the following tables for simplicity. In correlation analysis, proportions of NK cells and effector memory T cells were higher with increasing age ($r = 0.274$, and 0.248, respectively), but naïve T cells and B cells were significantly lower in old age ($r = -0.360$ and -0.318, respectively). Numbers of suppressor/cytotoxic T cells, B cells, NK-T cells, and naïve T cells decreased with increasing age ($r = -0.255$, -0.240, -0.226, and -0.334, respectively) (data not shown).

Lymphocyte subsets at diagnosis as a predictor of complete remission

In univariate analysis, higher proportions of helper/inducer T cells and CD4$^+$CD31$^+$ naïve T cells and lower proportion of total NK cells and CD56^{bright}CD16$^+$ NK cells increased probability of CR with statistical significance ($P = 0.034$ and 0.027 and 0.019 and 0.040, respectively). In multivariate analysis, elevated proportion of helper/inducer T cells and CD4$^+$CD31$^+$ naïve T cells and lower proportions of total NK cells independently increased probability of CR with statistical significance ($P = 0.014$, 0.016, and 0.045, respectively).

Table 4 The univariate and multivariate logistic regression analysis results to evaluate the capability of lymphocyte subset expressions as a predictor of CR achievement rates after the initial induction or reinduction chemotherapy

Prognostic marker	Univariate			Multivariate*		
	HR	95% CI	P	HR	95% CI	P
Age	0.968	0.934–1.004	NS	0.950	0.909–0.994	0.025
Genetic risk status	-	-		-	-	
Favorable *vs.* poor	4.916	1.298–22.176	0.018	6.758	1.583–32.165	0.010
Favorable *vs.* intermediate	3.187	0.811–13.087	NS	3.618	0.798–17.173	NS
Poor *vs.* intermediate	0.618	0.153–1.789	NS	0.516	0.128–1.769	NS
Lymphocyte subsets (%)						
Helper/inducer T cells	1.044	1.003–1.087	0.034	1.062	1.012–1.114	0.014
$CD4^+CD31^+$ naïve T cells	1.109	1.012–1.215	0.027	1.141	1.025–1.270	0.016
Total NK cells	0.912	0.845–0.985	0.019	0.915	0.839–0.998	0.045
$CD56^{bright}CD16^+$ NK cells	0.911	0.834–0.996	0.040	0.918	0.832–1.012	NS
T cells	1.018	0.984–1.053	NS	NP	NP	NP
Suppressor/cytotoxic T cells	0.966	0.910–1.026	NS	NP	NP	NP
$CD56^{bright} CD16^-$ NK cells	0.720	0.453–1.143	NS	NP	NP	NP
$CD56^{dim} CD16^+$ NK cells	0.975	0.720–1.319	NS	NP	NP	NP
NK-T cells	0.927	0.832–1.033	NS	NP	NP	NP
Naïve T cells	1.041	0.995–1.089	NS	NP	NP	NP
Central memory T cells	0.923	0.744–1.145	NS	NP	NP	NP
Effector memory T cells	1.943	0.009–418.602	NS	NP	NP	NP
Regulatory T cells	0.987	0.695–1.403	NS	NP	NP	NP
B cells	1.054	0.980–1.133	NS	NP	NP	NP

CD, cluster of differentiations; NK, natural killer; NS, not significant; NP, not performed; HR, hazard ratio; CI, confidence interval

*Multivariate analysis results in combination with the age, genetic risk status, and lymphocyte subsets as covariables

Lower proportion of $CD56^{bright}CD16^+$ NK cells increased probability of CR with statistical significance in univariate analysis, but it lost statistical significance in multivariate analysis (Table 4).

Effect of lymphocyte subsets at diagnosis on the survival

In the OS, old age group (≥ 65 years) showed significantly shorter OS than younger age group ($P = 0.011$) (Fig. 2A). In univariate analysis for OS, old age showed adverse prognostic impact in OS ($P = 0.017$), and it was also significant in multivariate analysis with the risk status and lymphocyte subset adjusted ($P = 0.028$) (Table 5). In the RFS, proportion of total NK cells (range 0.17–55.47%) were divided into dichotomous variable by the point of upper quartile value (4.8%). Patients with low proportion of NK cell < 4.8% was associated with significantly shorter RFS than patients with NK cells $\geq 4.8\%$ ($P = 0.009$) (Fig. 2B). In univariate analysis, lower proportion of NK cells < 4.8% indicated adverse prognostic impact on RFS ($P = 0.006$); it was also significant in multivariate analysis with the age and risk status adjusted ($P = 0.037$) (Table 6). Although elevated proportion of helper/inducer T cells and $CD4^+CD31^+$ naïve T cells and

lower proportions of total NK cells independently increased probability of CR with statistical significance, these three lymphocyte subsets did not significantly affect the OS and RFS (Tables 5 and 6).

Discussion

In the present study, we have shown that AML patient with higher proportion of helper/inducer T cells and $CD4^+CD31^+$ naïve T cells and lower proportion of NK cells at diagnosis have significantly enhanced CR probability, but NK cells < 4.8% at diagnosis shows significantly poor prognosis in RFS. Previous studies have shown that patients achieving higher absolute lymphocyte count recovery after autologous HSCT or induction chemotherapy had superior survival [7, 9]. Another study of regulatory T cell in AML patients indicated that regulatory T cell frequency at diagnosis was lower in patient group who achieved CR than in patient groups who had persistent leukemia or died respectively [10]. Two recent studies focused on the distribution of T cell subsets in patients with newly diagnosed AML showed that the proportion of regulatory T cells in AML patients is higher than in

Table 5 The univariate and multivariate analysis results of lymphocyte subset expressions on the effect to OS using a Cox's proportional hazards model

Prognostic marker	Univariate			Multivariate*		
	HR	95% CI	P	HR	95% CI	P
Age	1.045	1.008–1.084	0.017	1.047	1.005–1.091	0.028
Genetic risk status	-	-		-	-	
Favorable *vs.* poor	0.287	0.075–1.113	NS	0.243	0.061–1.042	NS
Favorable *vs.* intermediate	0.563	0.127–2.283	NS	0.675	0.143–2.621	NS
Poor *vs.* intermediate	1.987	0.691–6.225	NS	2.672	0.823–8.153	NS
Lymphocyte subsets (%)						
Total NK cells	1.041	0.999–1.084	NS	1.018	0.973–1.066	NS
CD56^{bright} CD16$^-$ NK cells	0.997	0.749–1.329	NS	NP	NP	NP
CD56^{bright}CD16$^+$ NK cells	1.038	0.997–1.082	NS	NP	NP	NP
CD56^{dim} CD16$^+$ NK cells	1.138	0.916–1.415	NS	NP	NP	NP
T cells	0.980	0.954–1.006	NS	NP	NP	NP
Helper/inducer T cells	0.997	0.960–1.035	NS	NP	NP	NP
Suppressor/cytotoxic T cells	0.958	0.900–1.018	NS	NP	NP	NP
NK-T cells	1.079	0.993–1.172	NS	-NP	NP	NP
Naïve T cells	0.986	0.945–1.029	NS	-NP	NP	NP
CD4$^+$CD31$^+$ naïve T cells	0.958	0.888–1.034	NS	-NP	NP	NP
Central memory T cells	1.082	0.889–1.317	NS	NP	NP	NP
Effector memory T cells	2.844	0.028–285.841	NS	NP	NP	NP
Regulatory T cells	0.866	0.507–1.481	NS	NP	NP	NP
B cells	0.983	0.920–1.049	NS	NP	NP	NP

CD, cluster of differentiations; NK, natural killer; NS, not significant; NP, not performed; HR, hazard ratio; CI, confidence interval

*Multivariate analysis results in combination with the age, genetic risk status and lymphocyte subsets as covariables

healthy donors [11, 12] and our present study support this result. Recent study with 28 de novo AML patients disclosed that a number of invariant NK-T cells < 0.2/μL conferred shorter OS [13]. Immune cell functions to hematologic malignancy were noted in NK cells and CD8$^+$ T cells in vivo, and they became target for immunotherapy [2, 4], although the importance of CD4$^+$ helper T cells in cancer immunity is not well established. One of the principal mechanism of adaptive cancer immunity by CD8$^+$ cytotoxic T cells is direct recognition of antigens on the surface of malignant cells through MHC class I molecule, and the other is cross-presentation of the antigens by antigen presenting cells (APC), particularly dendritic cells. In cross-presentation pathway, the APCs express costimulators that provide the signals needed for differentiation of CD8$^+$ T cells into anti-tumor cytotoxic T cells, and the APCs express class II MHC molecules that may present internalized tumor antigens and activate CD4$^+$ helper T cells as well. Helper T cells may promote CD8$^+$ T cell activation by several mechanisms such as secreting cytokines that stimulate the differentiation of CD8$^+$ T cells. Antigen-stimulated helper T cells express CD40 ligand (CD40L), which binds to CD40 on APCs and activates these APCs to make them more efficient at stimulating the differentiation

of CD8$^+$ T cells. In our study, proportion of helper/inducer T cells was independent prognostic factor in increasing probability of CR. However, we did not find significant correlation between proportion of suppressor/cytotoxic T cells and responses for induction therapy. Because we did not distinguish functional effector T cells from CD8$^+$ population of suppressor/cytotoxic T cells, the functional studies might be able to reveal the detailed correlation between helper/inducer T cells and suppressor/cytotoxic T cells in clinical outcome.

Expression of CD31 by leukocytes and endothelial cells are associated with regulation of T lymphocyte trafficking. Endothelial cell interactions mediated by CD31 molecules are required for efficient localization of naïve T cells to secondary lymphoid tissue and constitutive recirculation of primed T cells to nonlymphoid tissues. In inflammatory conditions, T cell:endothelial cell CD31-mediated interactions facilitate T cell recruitment to antigen-rich sites [14]. In present study, we measured naïve T cells subset which expressed CD4 and CD31 to estimate functional activity of naïve cells and thymic function. Our present study demonstrated that higher proportion of CD4$^+$CD31$^+$ naïve T cells significantly increased CR probability, and this result may suggest that these cells could be a marker of proper reactivity of immune system in AML patients.

Table 6 The univariate and multivariate analysis results of lymphocyte subset expressions on the effect to RFS using a Cox's proportional hazards model

Prognostic marker	Univariate			Multivariate*		
	HR	95% CI	P	HR	95% CI	P
Age	0.991	0.951–1.032	NS	-	-	-
Genetic risk status	-	-		-	-	
Favorable *vs.* poor	0.075	0.008–0.612	0.016	0.106	0.012–1.538	NS
Favorable *vs.* intermediate	0.158	0.013–1.385	NS	0.218	0.021–2.023	NS
Poor *vs.* intermediate	2.423	0.712–8.168	NS	2.134	0.598–8.014	NS
Lymphocyte subsets (%)						
NK cells < 4.8% *vs.* ≥ 4.8%	5.686	1.659–19.486	0.006	4.052	1.090–15.058	0.037
T cells	0.965	0.928–1.003	NS	NP	NP	NP
Helper/inducer T cells	0.967	0.913–1.025	NS	NP	NP	NP
Suppressor/cytotoxic T cells	0.961	0.882–1.046	NS	NP	NP	NP
Total NK cells	0.954	0.845–1.077	NS	NP	NP	NP
$CD56^{bright} CD16^-$ NK cells	0.011	0.000–5.576	NS	NP	NP	NP
$CD56^{bright}CD16^+$ NK cells	0.962	0.844–1.097	NS	NP	NP	NP
$CD56^{dim} CD16^+$ NK cells	0.896	0.578–1.391	NS	-NP	NP	NP
NK-T cells	0.845	0.695–1.027	NS	-NP	NP	NP
Naïve T cells	1.011	0.956–1.069	NS	-NP	NP	NP
$CD4^+CD31^+$ naïve T cells	0.982	0.910–1.059	NS	NP	NP	NP
Central memory T cells	1.146	0.867–1.516	NS	NP	NP	NP
Effector memory T cells	0.465	0.001–314.361	NS	NP	NP	NP
Regulatory T cells	0.875	0.452–1.694	NS	NP	NP	NP
B cells	1.017	0.948–1.092	NS	NP	NP	NP

CD, cluster of differentiations; NK, natural killer; NS, not significant; NP, not performed; HR, hazard ratio; CI, confidence interval

*Multivariate analysis results in combination with the age, genetic risk status and lymphocyte subsets as covariables
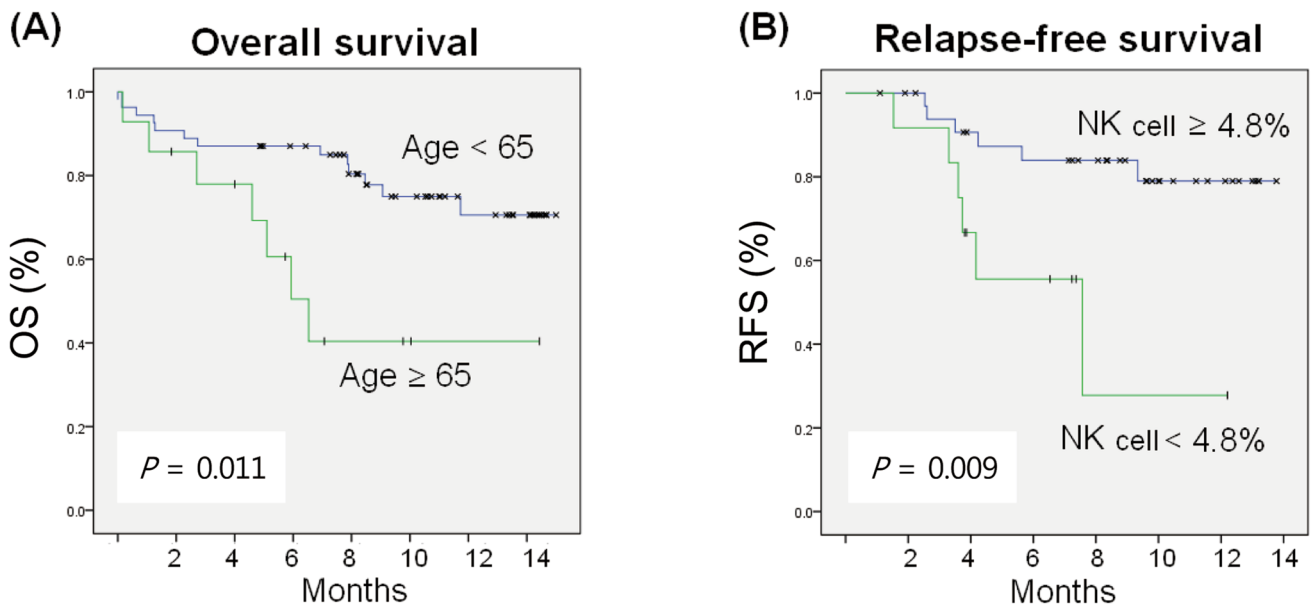
Fig. 2 Kaplan–Meier curves demonstrating clinical outcome with respect to age and NK cell proportion. **A** Estimates of overall survival with respect to age and **B** relapse free survival with respect to proportion of NK cells are represented. Abbreviations: OS, overall survival; RFS, relapse free survival

NK cells, as central players of the innate immune system, can exert direct anti-tumor effects via their cytotoxic and cytokine-secreting capacity and indirectly contribute to tumor control by communicating with dendritic cells and other immune cells, supporting the development of an efficient adaptive anti-tumor immune response [15]. The capability of NK cells to eliminate leukemic cells has been established in the setting of HSCT for AML patients that donor-versus-recipient NK cell alloreactivity reduced the risk of leukemia relapse, showing better engraftment as well as protection from graft-versus-host disease [4]. In our present study, lower NK cell proportion at diagnosis led to significantly higher CR probability. We thought that NK cells were irrelevant to offer sufficient anti-leukemic effect in the setting of heavy tumor burden and higher proportion of NK cells might be related to lower proportion of other lymphocyte subsets, particularly helper/inducer T cells and CD4+CD31+ naïve T cells, whereas we revealed that lower proportion of NK cells < 4.8% in PB from AML patient at diagnosis was associated with shorter RFS. Our present study result supports a recent study data which showed that a low proportion of NK cells (≤ 9.4%) was associated with tendency to higher relapse rates [16], and we think that the result can contribute to developing immunotherapeutic strategies such as combination of immune cell infusion with chemotherapy.

Antigen stimulated naïve T cells change the expression of various surface molecules as well as the secretion of cytokines and the expression of cytokine receptors. These are followed by proliferation of the antigen-specific cells, driven in part by the secreted cytokines, and then by differentiation of the activated cells into effector and memory T cells. The mechanisms that determine whether an individual antigen-stimulated T cell will become a short-lived effector cell or enter the long-lived memory cell pool are not established. Memory T cells can be divided into two subsets as central memory T cell and effector memory T cell by their anatomical location and effector function [17]. Central memory T cells express molecules such as CD62L (L-selectin) and CCR7 which allow efficient homing to the lymph node (LN), whereas effector memory T cells lack the expression of these LN homing receptors and are located in nonlymphoid tissues especially mucosal tissues. On repeated antigenic stimulation, effector memory T cells produce effector cytokines such as IFN-γ or rapidly become cytotoxic, but they do not proliferate much. Complete eradication of the antigen may also require large numbers of effectors generated from the central memory T cells. Memory T cells may survive for months or years. Thus, as humans age in an environment in which they are constantly exposed to and responding to infectious agents, the proportion of memory cells induced by these microbes compared with naive cells progressively increases. In individuals older than 50 years or so, half or more of circulating T cells may be memory cells. In our present study, increasing age were associated with higher proportion of effector memory T cells and lower proportion of naïve T cells, but proportion of total memory T cells was not significant. Effector memory T cells specific for leukemic antigen were expected to have powerful anti-leukemic effect; however, we did not find the prognostic impact in this study. It might happen because very small proportion of effector memory T cells made it difficult to measure quantitatively within 30,000 lymphocytes. It should be investigated by further experiments that accompany acquisition of more lymphocytes.

Compared to the absolute number and/or proportions of lymphocyte subsets and their potential prognostic impact, researches focused on the impact of changes in absolute number and/or proportions of lymphocyte subsets circulating in PB on their functions have not been sufficiently conducted. Lower absolute numbers and/or proportions of a particular lymphocyte subset in PB may indicate reduced functionality of those cells that could be connected to immune response to AML seems to be the underlying assumption. Recent study which evaluated the expression and function of NK cells in AML patients reported that compared with healthy controls, the proportion of CD56bright NK cells in AML patients increased, while the number and proportion of NK cells and proportion of CD56dim NK cells significantly decreased, and the expression of DNAM-1 in CD56bright NK cells, NKG2D, DNAM-1, and perforin in CD56dim NK cells decreased significantly [18]. This study suggested that compared with healthy controls, the number and expression ratio of NK cells in AML patients decrease, and the expression of functional markers is abnormal, indicating that its function is impaired [18]. These results are concordant to our study and would provide supporting evidence to the previously mentioned assumption made by authors.

When focused on the NK cells and their subsets, our present study showed decreased CD56dim CD16+ NK cells in AML patients compared to healthy controls. Although an old study showed increased NK cells in AML patients [5], our present study results are consistent with the majority of more recent studies on this topic [19, 20]. In terms of the relationship between the proportions of NK cells, their subsets, and outcomes, our present study showed that decreased proportions of PB NK cells was associated with decreased RFS and this result is similar with previous study which reported decreased proportion of PB NK cells being associated with higher rate of relapse [16]. However, conflicting results have been also reported such as decreased PB NK cells associated with better OS [20]. Prior studies correlating lymphocyte subsets with outcomes in AML have also reported that the proportion of NK cells or subsets of NK cells have the most significant

impact on outcomes, but inconsistency exists in terms of whether there is an association with better or worse outcomes, especially when the BM NK cells are investigated [21, 22]. Although our present study showed that lower proportions of total NK cells independently increased probability of CR with statistical significance, the hazard ratios (HR) of these effects are close to 1, and therefore, the impact of the proportions of total NK cells on the outcomes in AML needs to be more investigated. Different methods for defining NK cells and subsets of NK cells may be one of possible mechanisms reflecting inconsistency between studies.

A recent study which analyzed four NK cell subsets defined by high-dimensional mass cytometry reported that a high proportion of $CD56^-CD16^+$ NK cells at diagnosis was associated with an adverse clinical outcome and the frequency of them retained statistical significance [23]. In our study, we did not use a gating strategy that separately analyzed $CD56^-CD16^+$ NK cells, but these cells were included in the part of $CD56^{dim}CD16^+$ NK cells. It is expected that the results obtained from $CD56^{dim}CD16^+$ NK cells partially reflect the results of $CD56^-CD16^+$ NK cells in our study and we found that the proportions of $CD56^{dim}CD16^+$ NK cells in poor risk subgroups tended to increase than those in favorable risk subgroups (median 0.81% vs. 0.68%, differences 0.13%), and when the intermediate risk subgroup was included in the poor risk subgroup, the differences tended to increase slightly. These results may support partly with previous study [23]. However, we could not identified independent prognostic impact of the proportions of $CD56^{dim}CD16^+$ NK cells. Considering that the gating strategy for obtaining pure $CD56^-CD16^+$ NK cells itself was not used in our study, direct comparison between the previous study is judged to have limitations. In our study, the sum of $CD56^{bright}CD16^-$ and $CD56^{bright}CD16^+$ NK cells reflects the results of $CD56^+$ NK cells. We found that as the genetic risk increased from favorable to poor risk, the sum of $CD56^{bright}CD16^-$ and $CD56^{bright}CD16^+$ NK cells tends to decrease from 9.80% in favorable risk, 6.75% in intermediate risk to 5.60% in poor risk. These results support the previous study results [23].

In conclusion, out present study showed that AML patients harbor significant lower numbers of $CD56^{dim}CD16^+$ NK cells, central memory T cells, and regulatory T cells in the PB at diagnosis than healthy controls. Higher proportion of helper/inducer T cells and $CD4^+CD31^+$ naïve T cells and decreased proportion of NK cells in the PB at diagnosis were independent prognostic factor of increasing probability of CR, and proportion of NK cells < 4.8% in the PB at diagnosis was also independent adverse prognostic impact in RFS.

Declarations

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