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Research Paper

Prognostic implication of leucocyte subpopulations in diffuse large B-cell lymphoma

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ABSTRACT

Background: Recent studies have suggested that variables related to host adaptive immunity and the tumor microenvironment may predict the outcome in patients with non-Hodgkin's lymphoma. This study was undertaken to determine the prognostic value of peripheral blood leucocyte subpopulations in diffuse large-B-cell lymphoma patients.

Methods: We prospectively analyzed the 16 leukocyte subpopulations using Cytodiff flow cytometric technique in a cohort of 45 diffuse large-B-cell lymphoma patients at a single institution between February and December 2014. The Cox proportional hazards model was used to evaluate prognostic factors for overall survival and progression free survival.

Results: Diffuse large-B-cell lymphoma patients had decreased cytotoxic and non-cytotoxic NK&T cells as well as increased CD16+ monocytes, CD16- monocytes and mature neutrophils. The decreased CD16- monocyte/CD16+ monocyte ratio and increased mature neutrophil/cytotoxic NK&T cell ratio were related to poor progression-free and overall survival outcome in single and multivariate analysis. The co-constructed model using International Prognostic Index and mature neutrophil/cytotoxic NK&T cell ratio can also help discriminate the clinical outcome.

Conclusions: The decreased CD16-monocyte/CD16+monocyte ratio and increased mature neutrophil/cytotoxic NK&T cell ratio predict poor prognosis in diffuse large-B-cell lymphoma patients. This finding provides a strong rationale for the study of cellular immunotherapy in B-cell lymphoma.

INTRODUCTION

Diffuse large B-cell lymphoma (DLBCL) is the most common type of non-Hodgkin lymphoma (NHL). It is aggressive while potentially curable [1]. DLBCL is a heterogeneous entity with highly variable clinical outcomes [2]. The International Prognostic Index (IPI) and the Revised International Prognostic Index (R-IPI) are commonly used to evaluate the prognosis of DLBCL, even though they are still imprecise especially in the post-rituximab era [3, 4]. Therefore, other approaches are needed to help better identify high-risk DLBCL patients. Previous studies have shown that peripheral blood absolute lymphocyte count (ALC), absolute monocyte count (AMC) and the ALC/AMC ratio are related to clinical outcomes in DLBCL and classical Hodgkin lymphoma [5]. Recently, Park et al. demonstrated that lymphocyte subpopulations determined by the Cytodiff flow cytometric system are associated with the prognosis of metastatic gastric and lung cancers [5].

The Cytodiff flow cytometric system is able to automatically detect and separate 16 leukocyte subpopulations in the peripheral blood, including B lymphocytes, CD16+ cytotoxic NK&T lymphocytes, CD16- non-cytotoxic NK&T lymphocytes, CD16- monocytes, CD16+ monocytes, and neutrophils, generating a result called the extended leukocyte differential count [6]. The Cytodiff flow cytometric system has been recognized as a reliable and accurate way to count peripheral blood blasts [7-9]. It has also been used in research areas such as prognostic prediction of metastatic carcinoma, laboratory evaluation of sepsis severity [10] and monitoring of anti-viral therapy in HIV patients [11]. To the best of our knowledge, this detection system has not been used to evaluate the prognostic implication of changes in peripheral blood leukocyte subpopulations in NHL patients. In this prospective cohort study, we recruit 45 DLBCL patients and collect the 16 leukocyte subpopulations at the time of diagnosis to investigate the prognostic value of the leucocyte subpopulations.

RESULTS

Patient characteristics

There were 45 patients that met the study criteria and were included in this study. All 45 patients were first diagnosed with DLBCL without any treatment in PUMCH. The basic characteristics were presented in Table 1. RCHOP (rituximab, cyclophosphamide, doxorubicin, vincristine, prednisone) or RCHOP-like treatment was given to each patient. Five patients received autologous stem cell transplantation. As of January, 2016, the median follow-up was 16 months (range: 2-24months), the estimated 2-year overall survival (OS) was 0.763, and 2-year progression free survival (PFS) was 0.606 for the whole cohort. At the end of 16-month follow-up, 25 patients (55.6%) reached complete remission (CR), 8 (17.8%) reached partial remission (PR), 3 (6.7%) had progression disease (PD) and 9 (20%) died from disease progression. Eight patients had experienced disease relapse or refractory.

Leukocyte subpopulations are related to the prognosis of DLBCL

The numbers of leukocyte subsets and their differences between patients and healthy controls were shown in Table 2. The percentage and absolute number of all lymphocyte subpopulations including B lymphocytes, cytotoxic and non-cytotoxic NK&T cells in DLBCL patients significantly decreased compared to the controls. On the other hand, the patients had significantly greater relative and absolute mature neutrophils numbers as well as increased percentage of CD16+ monocytes while no significant difference was found in the absolute monocyte numbers. We performed single factor analysis comparing overall survival (OS) and progression-free survival (PFS) with all leukocyte subpopulations and other known prognostic variables including IPI (Supplementary Table 1). The decreased percentages of total NK&T lymphocytes, especially cytotoxic NK&T lymphocytes, were related to worse OS. Increased absolute numbers of both CD16+ and CD16- monocytes and the absolute number of mature neutrophils was associated to poor outcomes. In addition, all above changes except for the percentage of CD16+ monocytes were related to PFS. As for the well-known prognostic factors, only the number of extra-nodal sites, ECOG and IPI were found to be significantly related to patient outcomes.

Multivariate analysis was also analyzed for those factors with good performance in univariate analysis (Supplementary Table 2). Considering the sample size and event number of our study and the association between leucocyte subpopulations, the results of univariate analyses were adjusted by bone marrow involvement, IPI, ECOG and number of involved extra nodal sites. We found that only the absolute number of CD16+ monocytes and mature neutrophils were independently related to DLBCL outcomes (Table 3). As IPI is currently considered to be the most important clinical index for predicting DLBCL prognosis, we also constructed models that combined IPI with the absolute number of CD16+ monocytes or mature neutrophils (respectively) but the results accounting for IPI were insignificant.

CD16- monocyte/CD16+ monocyte and mature neutrophil/cytotoxic NK&T cell ratios can predict the prognosis of DLBCL with high specificity

Several ratios between the leukocyte subsets were evaluated for OS and PFS with cox analysis. Decreased CD16-/CD16+ monocyte ratio and increased mature neutrophil/cytotoxic NK&T cell ratio were found to be associated with shorter overall survival time in both single-factor and multi-factor analysis. Mature neutrophil/cytotoxic NK&T cell ratio was also related to PFS in Cox analysis (Table 2).

To further determine the prognostic value of CD16-/CD16+ monocyte ratio and mature neutrophil/cytotoxic NK&T cell ratio, the time-dependent receiver operating characteristic (ROC) curve of 2-year-survival was performed (Figure 1). The CD16-/CD16+ monocyte ratio seemed to be the most specific index to predict the overall survival of DLBCL patients. Using a cut-off value of 7.5 resulted in diagnostic specificity being 93% and sensitivity being 52% (area under curve (AUC) = 0.755). A mature neutrophil/cytotoxic NK&T cell ratio cut-off value of 46 resulted in diagnostic sensitivity of 50% and specificity of 93% (AUC = 0.763). The time-dependent AUCs of CD16- monocyte/CD16+ monocyte ratio and neutrophil/cytotoxic NK&T cell ratios were both significantly higher than IPI in our study ($p<0.01$).

The Kaplan–Meier analysis of overall survival was also performed for these two ratios with the cut-off value determined by the survival ROC analysis. The estimated 2-year overall survival rate was 0.313 and 0.859 for

Table 1: Basic characteristics of diffuse B cell lymphoma patients

Characteristics	Number	Percentage(%)
Age		
<60	19	42.22
≥60	26	57.78
Sex		
Female	23	51.11
Male	22	48.89
Subtype		
DLBCL nos	35	77.78
DLBCL GCB	18	40.00
DLBCL ABC	17	37.78
PCNSL	4	8.89
ALK+DLBCL	1	2.22
DLBCL/BL	2	4.44
DLBCL of unknown	3	6.67
Ann Arbor Stage		
I,II	21	46.67
III,IV	24	53.33
B symptoms		
No	20	44.44
Yes	21	46.67
NA	4	8.89
Number of extra nodal sites		
0,1	29	64.44
>1	16	35.56
ECOG		
0,1	25	55.56
2,3,4	20	44.44
IPI		
0,1	19	42.22
2	10	22.22
3	6	13.33
4,5	10	22.22

Abbreviations: GCB DLBCL, germinal center B-cell-like diffuse large B-cell lymphoma; ABC DLBCL, activated B-cell-like diffuse large B-cell lymphoma; PCNSL, primary central nervous system lymphoma; ALK+DLBCL, anaplastic lymphoma kinase positive diffuse large B-cell lymphoma; DLBCL/BL, B-cell lymphoma, unclassifiable, with features intermediate between diffuse large B-cell lymphoma and Burkitt lymphoma; ECOG, Eastern Cooperative Oncology Group performance status; IPI, the international prognostic index.

CD16-/CD16+ monocyte ratio <7.5 and ≥7.5, respectively ($p<0.05$). For mature neutrophil/cytotoxic NK&T cell ratio of <46 and ≥46, the 2-year overall survival rate was 0.851 and 0.333, respectively ($p<0.05$) (Figure 2).

Finally, we evaluated models that combined IPI with either CD16-/CD16+ monocyte ratio or mature neutrophil/cytotoxic NK&T cell ratio for estimating the overall survival of patients with DLBCL (Table 4). We found that the model constructed using IPI and CD16-/CD16+ monocyte ratio gave an AUC of 0.702, with the cutoff value of 13, there is no statistical significance in Kaplan–Meier analysis ($p = 0.115$). In contrast, the model combining IPI with mature neutrophil/cytotoxic NK&T cell ratio showed great performance with an AUC of 0.781 and 2-year overall survival rate of 0.321 and 0.894 in the two groups respectively ($p=0.004$, Figure 3).

DISCUSSION

Previous studies have shown that low absolute lymphocytes and NK cells, along with increased monocytes and mature neutrophils all indicate a poor outcome and shorter OS and/or PFS [12-18]. However, the cytotoxic T cells play an uncertain role in predicting the survival of patients with DLBCL [19-21]. The current study aims to further elucidate the roles of peripheral leukocytes in prognosis of DLBCL by investigating the prognostic significance of changes in 16 leukocyte subpopulations, including the subsets of lymphocytes and monocytes.

We demonstrated that the DLBCL patients had decreased number and percentage of all lymphocyte subsets. This phenomenon may suggest an immune defect in DLBCL patients [21] or the antitumor response to a high tumor burden [17]. In addition, there is an increase in mature neutrophils and the percentage of CD16+ monocytes, which may be due to tumor-derived chemokines that can induce proliferation of neutrophils and monocytes, thus promoting tumor growth [22-24]. Furthermore, we are the first to demonstrate that CD16-monocyte/CD16+monocyte and mature neutrophil/cytotoxic NK&T cell ratios are both independent indicators for OS in DLBCL patients based on multi-factor and ROC analyses. These ratios may provide additional prognostic information when used in conjunction with the International Prognostic Index in the post-rituximab era.

It has been shown that increased neutrophil/lymphocyte ratio at diagnosis of DLBCL independently represents poor prognosis [25, 26]. The lymphocyte/monocyte ratio [5, 27-29] is also a useful prognostic indicator for DLBCL patients in several studies. The lymphocyte/monocyte ratio was also tested in our study. No statistical significant results were found which may due to different populations or insufficient samples.

According to the 2010 Nomenclature Committee of the International Union of Immunological Societies,

Table 2: The level of subsets of leucocyte and their difference between patients and healthy controls

Clinical factors and leucocyte subsets	Controls		DLBCL patients		P value
	Mean	SD	Mean	SD	
Sex(male:female)	153:116		22:23		
Age(y)	41.95	13.49	60.89	11.02	*<0.05*
Lymphocytes($X10^9$)	2.07	0.61	1.37	0.78	*<0.001*
Lymphocytes(%)	33.96	7.20	21.99	12.69	*<0.001*
B lymphocytes($X10^9$)	0.21	0.09	0.14	0.17	*0.018*
B lymphocytes(%)	3.41	1.29	2.27	2.41	*0.003*
NK&T lymphocytes($X10^9$)	1.86	0.56	1.22	0.69	*<0.001*
NK&T lymphocytes(%)	30.54	6.77	19.72	11.51	*<0.001*
CD16+ cytotoxic NK&T lymphocytes($X10^9$)	0.50	0.27	0.28	0.25	*<0.001*
CD16+ cytotoxic NK&T lymphocytes(%)	8.29	3.94	4.26	3.23	*<0.001*
CD16- non-cytotoxic NK&T lymphocytes($X10^9$)	1.36	0.46	0.94	0.50	*<0.001*
CD16- non-cytotoxic NK&T lymphocytes(%)	22.25	5.68	15.45	9.67	*<0.001*
Monocytes($X10^9$)	0.41	0.11	0.56	0.71	0.183
Monocytes(%)	6.79	1.32	7.37	3.48	0.274
CD16- monocytes($X10^9$)	0.39	0.10	0.50	0.62	0.226
CD16- monocytes(%)	6.37	1.23	6.68	2.98	0.493
CD16+ monocytes($X10^9$)	0.03	0.02	0.06	0.10	0.061
CD16+ monocytes(%)	0.42	0.23	0.69	0.87	*0.049*
Mature neutrophils($X10^9$)	3.38	0.78	5.11	3.85	*0.004*
Mature neutrophils(%)	55.37	7.27	66.55	13.88	*<0.001*
Eosinophils($X10^9$)	0.17	0.11	0.13	0.17	*0.029*
Eosinophils(%)	2.77	1.55	2.04	2.63	*0.001*
Basophils($X10^9$)	0.05	0.02	0.06	0.20	0.618
Basophils(%)	0.80	0.39	0.67	1.02	0.408
Immature granulocytes($X10^9$)	0.01	0.01	0.12	0.51	0.141
Immature granulocytes(%)	0.19	0.16	0.89	2.03	*0.025*
CD16-monocytes/ CD16+monocytes	19.05	10.15	16.63	15.08	0.172
Lymphocytes/monocytes	5.22	1.64	3.51	2.36	*<0.001*
Cytotoxic NK&T lymphocytes /CD16+ monocyte	24.46	18.14	10.43	8.18	*<0.001*
Cytotoxic NK&T lymphocytes /CD16- monocyte	1.37	0.75	0.74	0.60	*<0.001*
Mature neutrophils /monocyte	8.48	2.13	13.01	14.95	*0.049*

(*Continued*)

Clinical factors and leucocyte subsets	Controls		DLBCL patients		P value
	Mean	SD	Mean	SD	
Mature neutrophils / CD16+monocyte	172.50	103.93	197.68	200.26	0.413
Mature neutrophils / cytotoxic NK&T lymphocytes	8.46	4.86	40.30	57.85	*0.001*
Mature neutrophils / noncytotoxic NK&T lymphocytes	2.73	1.04	8.86	14.67	*0.008*

Table 3: univariate and multivariate analysis for overall survival

Prognostic factors	Univariate analysis		Multivariate analysis[1]	
	HR[2] (95% CI)	P value	HR (95%CI)	P value
Lymphocytes(%)	0.873(0.794-0.961)	0.005	0.917(0.823-1.021)	0.114
NK&T lymphocytes(%)	0.846(0.756-0.948)	0.004	0.896(0.787-1.021)	0.100
CD16- non-cytotoxic NK&T lymphocytes(%)	0.819(0.712-0.943)	0.005	0.882(0.752-1.033)	0.119
Monocytes($X10^9$)	16.75(2.413-116.3)	0.005	24.07(0.913-634.5)	0.057
CD16- monocytes($X10^9$)	42.58(2.236-810.9)	0.013	29.48(0.749-1160.5)	0.071
CD16+ monocytes($X10^9$)	5643 (74.33-4.3X105)	<0.001	2.53E9(11.27-5.67E17)	0.027
Mature neutrophils($X10^9$)	1.477(1.217-1.792)	<0.001	1.822(1.246-2.662)	0.002
Mature neutrophils(%)	1.07(1.008-1.135)	0.026	1.036(0.971-1.105)	0.280
CD16-monocytes/ CD16+monocytes	0.862(0.761-0.976)	0.019	0.857(0.759-0.967)	0.012
Cytotoxic NK&T lymphocytes /CD16+ Monocyte	0.859(0.747-0.986)	0.031	0.907(0.791-1.039)	0.160
Mature neutrophils/ cytotoxic NK&T lymphocytes	1.016 (1.006-1.025)	0.001	1.018(1.003-1.034)	0.019
Mature neutrophils/ noncytotoxic NK&T lymphocytes	1.03 (1.009-1.051)	0.006	0.983(0.931-1.037)	0.526

[1]Multivariate analysis was adjusted by IPI, ECOG, number of extra nodal involvement and bone marrow.
[2]HR: Hazard Ratio

monocytes are divided into 3 types [30], the classical CD14++CD16- monocytes (about 91% of the blood monocytes), intermediate CD14+CD16+ cells and non-classical CD14+CD16++ monocytes [31]. The latter two were previously called CD16+ monocytes. Once emigrated to the target tissue, circulating monocytes differentiate into macrophages and polarize into distinct subtypes depending on the microenvironment [24] While the classical monocytes have high phagocytic and antimicrobial activity, CD16+ monocytes have been shown

to participate in anti-tumor responses [32]. However, in some disease states, circulating CD16+ monocytes in peripheral blood have been shown to promote tumor growth [33] and indicate the worse prognosis. In current study, we found a small increase in CD16- monocytes and a greater (1.8-fold on average) increase in CD16+monocytes in DLBCL patients compared to healthy controls. The decreased CD16-monocyte/CD16+monocyte ratio is associated with poor prognosis. Subimerb et al found that in cholangiocarcinoma patients, the peripheral

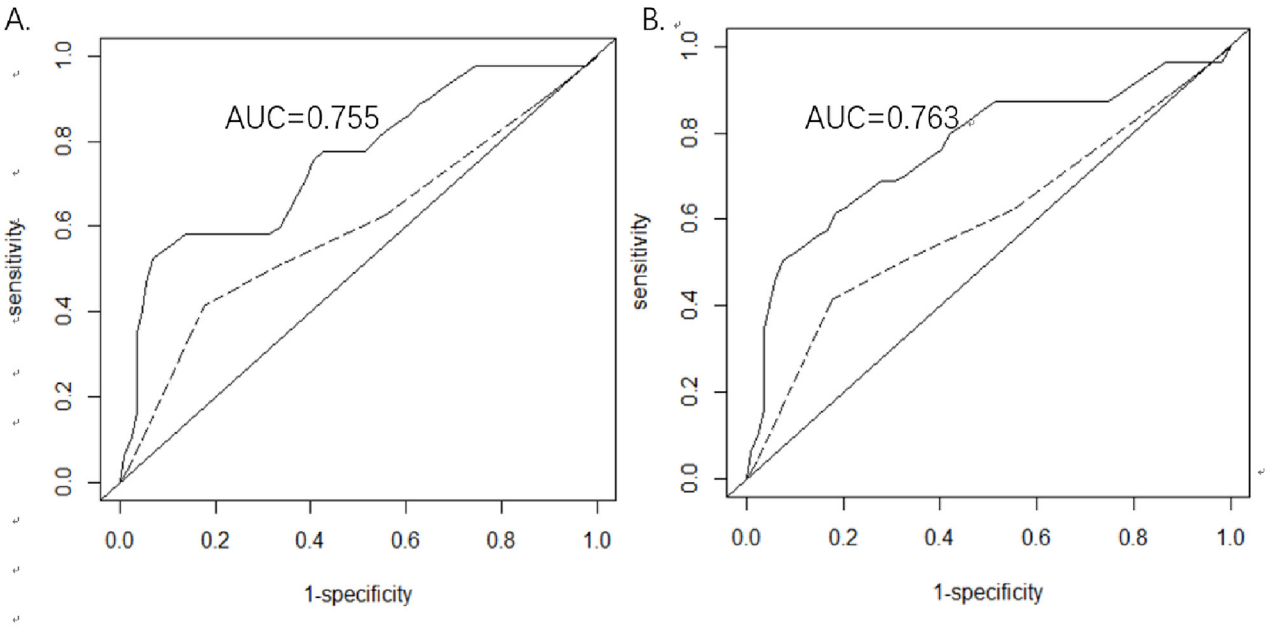

Figure 1: (A) The receiver operating characteristic (ROC) curve of 2-year-survival based on the CD16-monocytes/CD16+monocyte ratio. (B) The ROC curve of 2-year-survival based on mature neutrophils/cytotoxic NK&T cell ratio. The dashed line represents the ROC curve of 2-year-survival for IPI. Abbreviations: AUC, area under ROC curve.

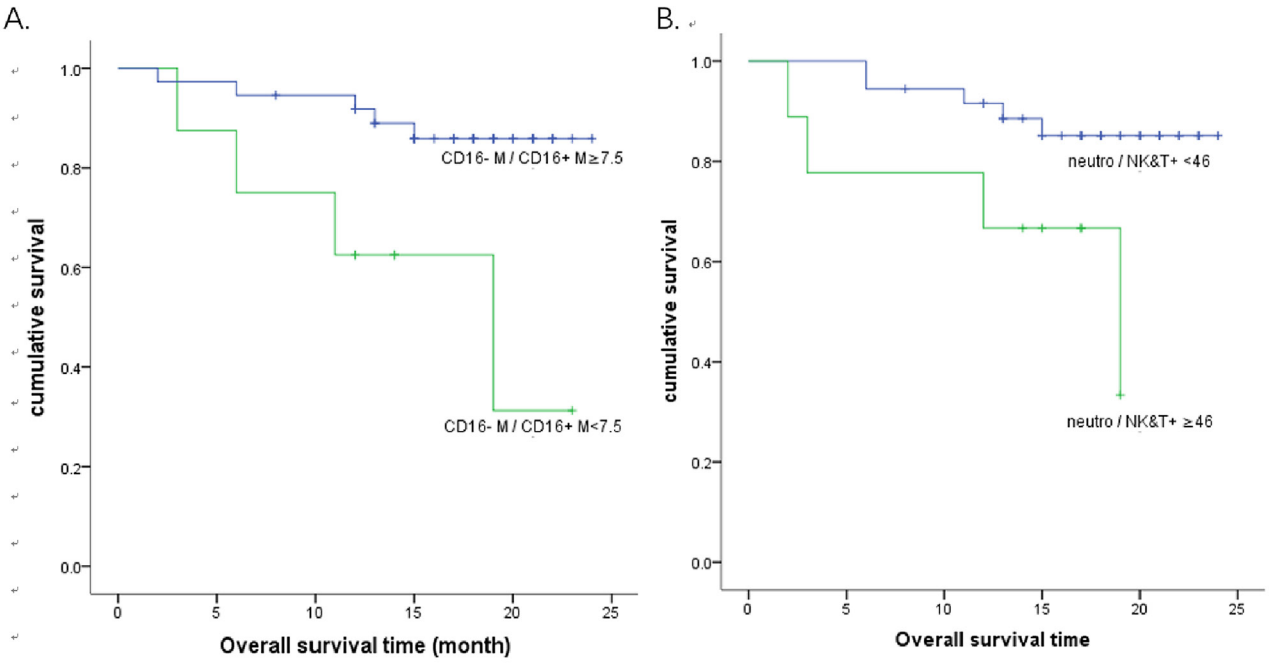

Figure 2: Kaplan-Meier survival curves for overall survival. (A) The estimated 2-year overall survival rate was 0.313 and 0.859 for CD16- monocyte/CD16+ monocyte ratio <7.5 and ≥7.5, respectively ($p<0.05$). **(B)** The estimated 2-year overall survival rate was 0.851 and 0.333 for mature neutrophil/cytotoxic NK&T cell ratio of <46 and ≥46, respectively ($p<0.05$).

Table 4: Prognostic models for overall survival constructed with IPI, CD16-/CD16+ monocyte ratio, and mature neutrophil/cytotoxic NK&T cell ratio

	β	95% CI	P value
Model 1			
IPI	2.025	1.114-3.683	0.021
CD16-M/CD16+M	0.887	0.788-0.998	0.048
Model 2			
IPI	1.904	1.068-3.397	0.029
Neutro/ NK&T	1.014	1.004-1.024	0.008

Abbreviations: C16-M/CD16+M, CD16- monocytes/CD16+ monocytes; Neutro/ NK&T, mature neutrophils /cytotoxic NK&T lymphocytes.

CD16+ monocytes had tumor-promoting characteristics and expressed higher levels of growth factor (EREG) and angiogenic chemokines (CXCL3), these cytokines corresponded to a M2 pro-tumorigenic phenotype [34]. M2-like macrophages have been shown to develop pro-angiogenic functions and promote angiogenesis which is associated with poor OS in NHL including DLBCL in several studies [35-37]. It is possible that with increased circulating CD16+ monocytes, more M2-like macrophages may infiltrate the tumor microenvironment and promote angiogenesis while suppressing host antitumor immunity, resulting in worsened clinical outcomes.

We have also shown that an increased mature neutrophil/cytotoxic NK&T cell ratio was associated with poor prognosis. Two factors may contribute to this observation including either increase in neutrophils or the decrease in NK&T cells. The increased inflammatory cells including mature neutrophils can release growth and survival factors, stimulating DNA damage, promoting angiogenesis and tumor evasion of the host defense system [38, 39]. On the other hand, the majority of the CD16+ cytotoxic NK&T cells are NK cells according to the Cytodiff system [40], which are important effectors in antitumor immunity [41-43]. Studies have shown that low NK cells are associated with worse prognosis in DLBCL patients [17].

Our study has some limitations. The predictive value of these ratios for overall survival and progression-free survival and the dynamic change of the ratios during disease improvement or progression and relapse should

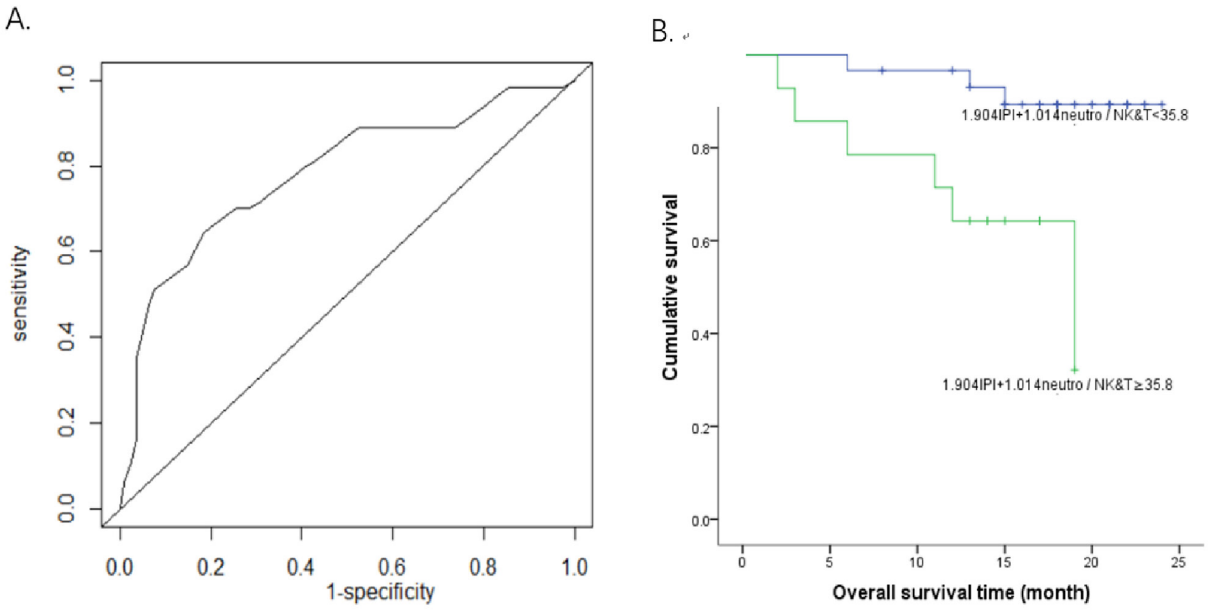

Figure 3: Survival analysis using a model constructed by mature neutrophils/cytotoxic NK&T cells and IPI. (A) The survival receiver operating characteristic (ROC) curve of 2-year-survival for mature neutrophils/cytotoxic NK&T cells and IPI model. AUC was 0.781. **(B)** The overall survival curve for 1.904IPI+1.014 mature neutrophils/cytotoxic NK&T cells <35.8 and ≥35.8 showed the 2 year overall survival rate was 0.894 and 0.321 respectively in the two groups (p=0.004).

also be further confirmed in a larger population and a longer follow-up. Regular white blood cell subsets measurement should be done in the future to examine whether effective treatment can normalize the white blood cell subsets. Furthermore, the NK cells and T cells cannot be separated by this system and restricts further investigation into the roles played by these immune cells.

In conclusion, we are the first to demonstrate that the CD16-monocyte/CD16+monocyte and mature neutrophil/cytotoxic NK&T cell ratios are valuable indices to discriminate the overall survival of DLBCL patients in post-rituximab era. This study may also help us better understand the mechanism especially the roles of host immune homeostasis and tumor microenvironment in lymphoma development and progression.

MATERIALS AND METHODS

Patient and controls

Patients diagnosed with DLBCL, according to the 2008 World Health Organization (WHO) classification of hematopoietic malignancies [44], at Peking Union Medical College Hospital (PUMCH) between February and December 2014 were included in this study. All patients were over 18 years old and were first diagnosed without any prior treatment. Pregnant patients were excluded. 269 healthy volunteers without known diseases aged 18-80 years old were also recruited from the physical examination center of PUMCH at the same time. All patients and volunteers provided written informed consent and the study was approved by the Ethics Committee of Peking Union Medical College Hospital.

Data collection and follow-up

The following parameters were collected: age, sex, subtype, Eastern Cooperative Oncology Group (ECOG) performance status (PS), Ann Arbor stage (I-IV), absence or presence of B symptoms, number and type of involved sites, prognostic index including International Prognostic Index (IPI) for DLBCL based on medical record review. The peripheral blood from all 45 patients and 269 controls were also collected for further flow cytometric analysis upon admission in the study. All patients received regular R-CHOP treatment and follow-up in our facility. During the follow up, treatment response was evaluated by enhanced computed tomography or PET-CT. Complete remission (CR), relapse and progression of lymphoma were evaluated according to previously published reports [45, 46].

Flow cytometry analysis

The extended blood leukocyte differential count was determined using the flow cytometer (FC500) in conjunction with premixed CytoDiff™ reagent and analysis software (Beckman Coulter, USA). Antibodies used in CytoDiff™ include CD36-FITC, CD2-PE, CD294-PE, CD19-ECD, CD16-PC5, and CD45-PC7. The leukocytes were differentiated into 16 cell populations (B-lymphocytes, CD16- T-lymphocytes, CD16+ T and NK cells, T and NK lymphocytes, total lymphocytes, CD16 monocytes, CD16+ monocytes, total monocytes, immature granulocytes [IGs], total eosinophil, mature neutrophils, total neutrophils, B blasts, T blasts, non-B-non-T blasts, and total basophils). Analysis procedures were conducted according to the manufacturer's instructions. Briefly, 100 μL of whole blood samples was mixed with 10 μL of CytoDiff reagent. After 20 minutes of incubation at room temperature, the red blood cells were then lysed by Versalyse solution (Beckman Coulter) for 15 min. Approximately 20,000 cells were acquired and analyzed automatically by the analysis software. The analysis software is self-gating and separates populations by automatic logic pathways. [6]

Statistical analysis

The correlation between the possible prognostic value and clinical parameters was assessed by the chi-square test. Progression-free survival (PFS) and overall survival (OS) were estimated using the Kaplan–Meier method and two-tailed log-rank test. The Cox proportional hazards model was used to evaluate the lymphocyte subtypes as prognostic factors for PFS and OS and to adjust for other known prognostic variables included in the IPI. All two-sided P values <0.05 were considered to be statistically significant. All these statistical tests were carried out using SPSS 19.0 software (SPSS Inc., Chicago. USA).

Specificity, sensitivity and cut-off were established using time-dependent receiver operating characteristic (ROC) curve analysis. As proposed by previous publication [47], we considered that area under curve (AUC) values >0.7 indicate that the parameter can be used for diagnosis, with values >0.9 indicating high clinical accuracy. We used R software version 3.3.1 including the "time ROC" package and the "survival ROC" package to do the time-dependent AUC comparison and ROC curve analysis [48].

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CONFLICTS OF INTEREST

The authors declare there are no conflicts of interest to disclose.

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