Cellular Physiology and Biochemistry

Cell Physiol Biochem 2018;45:951-961
DOI: 10.1159/000487288
Published online: February 08, 2018
Accepted: January 16, 2018

Original Paper

Ratio of Immune Response to Tumor Burden Predicts Survival Via Regulating Functions of Lymphocytes and Monocytes in Diffuse Large B-Cell Lymphoma

Hongfei Ji[a,b] Xingjian Niu[c] Lei Yin[a,b] Yiran Wang[a,b] Lan Huang[c] Qijia Xuan[c] Liru Li[d] Han Zhang[c] Jingtong Li[c] Yue Yang[a,b] Weiwei An[a,b] Qingyuan Zhang[a,d]

[a]Institute of Cancer Prevention and Treatment, Harbin Medical University, Harbin, Heilongjiang, [b]Heilongjiang Academy of Medical Sciences, Harbin, Heilongjiang, [c]Department of Medical Oncology, Harbin Medical University Cancer Hospital, [d]Precision Medicine Center, Harbin Medical University Cancer Hospital, Harbin, Heilongjiang, China

Key Words
Diffuse large B-cell lymphoma• Lymphocyte to monocyte ratio• Tumor burden• LDH• Prognosis

Abstract
Background/Aims: Diffuse large B-cell lymphoma (DLBCL) is an aggressive disease, and is the most common type of lymphoma in adults. Although significant progress in treatment has been made using chemotherapy combinations, there exist a large amount of relapse or refractory cases. Thus, effective clinical biomarkers for DLBCL are urgently needed. Our study aims to explore the predictive significance of using the immune response to tumor burden ratio [defined as the lymphocyte to monocyte ratio (LMR)/lactate dehydrogenase (LDH) levels] in 184 DLBCL patients and the potential mechanism underlying the use of the LMR to tumor burden ratio in predicting patient survival. ***Methods:*** The correlation between serum LDH levels and tumor levels assessed by PET-CT was determined using Spearman's correlation analysis. Clinical data from 184 DLBCL patients was assessed using receiver operating characteristic curve analysis and survival analysis. The potential correlation between tumor burden and lymphocytes or monocytes was analyzed by immunohistochemical staining, flow cytometry, and ELISA analysis of patient samples. In addition, we performed *in vitro* studies to further determine the effects of tumor burden on the anti-tumor activity of T lymphocytes. ***Results:*** We observed that serum LDH was an excellent surrogate marker of tumor burden in DLBCL patients, and that the ratio of LMR to LDH was an independent prognostic biomarker capable of predicting survival in DLBCL patients. Further analysis showed that a high tumor burden was correlated with decreased Ki67 expression in T cells, either in the solid tumor tissue or in the circulating blood. In addition, based on an *in vitro* co-culture study, a higher

H. Ji and X. Niu contributed equally as co-first authors.

Qingyuan Zhang

Department of Medical Oncology, Harbin Medical University Cancer Hospital, Harbin 150081, Heilongjiang Province (China)
Tel. +86-451-86298333, E-Mail zqyHMU1965@163.com

tumor burden led to the suppression of the anti-tumor response of T cells. Furthermore, we found that a higher tumor burden was correlated with the differentiation of monocytes to tumor associated macrophages in the tumor micro-environment. Both results demonstrate the importance of considering both the immune system and tumor burden for prognostic analysis. ***Conclusion:*** Our study has identified a novel clinical biomarker, namely, the immune response to tumor burden ratio, that can be used to distinguish survival outcomes in DLBCL patients, and demonstrated the potential mechanism underlying the use of this biomarker, that incorporates both the immune system and tumor burden, for use in future clinical applications.

Introduction

Non-Hodgkin's lymphomas (NHLs) are a diverse group of diseases originating from lymphoid tissues, consisting of considerable subtypes with heterogeneous biological characteristics and clinical behaviors [1, 2]. Diffuse large B-cell lymphoma (DLBCL), which is the most common type of NHL, is characterized by an aggressive natural history of untreated patients, with a median survival of less than one year [3, 4]. Despite the remarkable success of CHOP (cyclophosphamide, doxorubicin, vincristine, and prednisone) chemotherapy, or rituximab plus CHOP (R-CHOP) chemotherapy, approximately 30% of DLBCL patients suffer relapse or refractory disease [3-6]. Therefore, the identification of effective biomarkers should be helpful in improving the clinical prognosis in DLBCL patients.

Recent breakthroughs in immunotherapy have attracted a broad interest for treating tumor in an effective way [7, 8]. Numerous clinical studies have focused on improving survival outcomes using novel combinations of immunotherapy and chemotherapy in DLBCL patients [9, 10]. Thus, effective immunological biomarkers should be considered when evaluating the immune status of the patients. In this regard, the prognostic value of the lymphocyte to monocyte ratio (LMR), which mirrors the circulating immune status of the host, has been reported for a number of different malignancies [11-13]. In particular, LMR has been suggested as an effective prognostic factor for predicting clinical survival in DLBCL patients [14, 15]. However, a recent study has suggested that an immunological biomarker alone is not sufficient to predict survival and clinical responses, and the immune cell invigoration to tumor burden ratio could be useful as an indicator of clinical response [16].

Tumor burden is thought to be an important risk factor in multiple malignancies, usually reflecting the degree of tumor damage to the body [17, 18]. In solid tumors, such as melanoma, breast cancer, and lung cancer, tumor burden is always represented as the tumor size assessed using a CT scan [17]. However, as a hematopoietic and lymphoid disease, CT scan is not always performed to assess the tumor burden in DLBCL due to the large number of lymph nodes involved, as well as tumors in extra-nodal tissues. Although FDG-PET/CT is more accurate in identifying lesions than a CT scan, its use has been limited because of its cost [19, 20]. Lactate dehydrogenase (LDH) has been recognized to be a potential circulating surrogate for the tumor burden; it is inexpensive and can easily be assessed clinically [16, 21]. Therefore, in the present study, we investigated whether LDH was able to reflect the tumor burden in DLBCL patients, and performed a retrospective analysis to determine the prognostic value of the LMR to tumor burden ratio and the potential mechanism underlying this ratio to allow for its future application in DLBCL.

Materials and Methods

Patient selection

In this retrospective study, we reviewed a database of 184 patients from East Asia who were diagnosed as *denovo* DLBCL patients. All the patients were selected from January 2008 to December 2012 from the Harbin Medical University Cancer Hospital and met the inclusion criteria, including pathological

confirmation of DLBCL, no previous treatment, and no prior history of other malignancies. All patients were treated with CHOP or R-CHOP chemotherapy regimens, with or without involved-field radiation therapy. This study protocol was performed in accordance with the principles of the Declaration of Helsinki, and approved by the Institutional Review Board of Harbin Medical University Cancer Hospital. Written informed consent was obtained from every participant, and informed consent from patients less than 16 years old was obtained from their guardians. All methods were performed in accordance with the relevant guidelines and regulations.

Laboratory data

Absolute lymphocyte and monocyte counts were obtained from the peripheral blood samples of DLBCL patients before treatment, and standard complete blood cell count (CBC) data were obtained using a Sysmex XT-1800 Automated Hematology System (Shanghai, China). Each LMR was calculated by dividing the lymphocyte count by the monocyte count. Serum LDH levels were measured with an Olympus® AU640 clinical chemistry analyzer. We defined "*R*" to represent the LMR to LDH ratio as R = LMR/LDH×100 (%).

FDG-PET/CT scanning

Whole-body ^{18}F-FDG PET/CT studies were performed before the start of treatment. After a 6-hour fasting period, the patients were intravenously injected with approximately 4-5 MBq/kg body weight of ^{18}F-FDG. Following this, at 60 minutes post ^{18}F-FDG injection, the patients were scanned from the skull base to the knees.PET scanning was performed in the same position in the 2-dimensional mode (4 minutes per bed position, and 6-7 bed positions for the emission scans). CT scanning was performed at 120 kV and 30 mA for the attenuation correction of the FDG signal. The FOV and pixel size of PET images were analyzed using a computer workstation (Xeleris; General Electric Medical System).

Immunohistochemical (IHC) staining

The IHC staining was performed to detect the expression of Ki67 and CD163 in tumor tissue sections. Briefly, slides were dried overnight at 60°C followed by de-paraffinized in xylene and rehydrated through graded ethanol. Next, antigens were retrieved using citrate buffer (pH 6.0) by microwaves and then incubated with 3% H_2O_2 in methanol for 10 minutes to quench endogenous peroxidase activity. Subsequently, the sections were incubated with 5% bovine serum albumin for 30 minutes and then incubated with primary antibody [anti-Ki67 antibody (dilution 1:200; ab15580, Abcam, Cambridge, USA); anti-CD163 antibody (dilution 1:300; ab182422, Abcam, Cambridge, USA)] at 4°C overnight. Later, the sections were incubated with HRP-labelled goat anti-rat secondary antibody (ab97057, Abcam, Cambridge, USA) for 20 minutes at 37°C. Then the slides were counterstained with instant hematoxylin, and then dehydrated, cleared, and mounted. For the negative control, phosphate buffered saline was used instead of the primary antibody.

Cell preparation

Peripheral blood mononuclear cells (PBMCs) were isolated by density-gradient centrifugation using Ficoll-Hypaque (GE HealthCare, Helsinki, Finland) according to the manufacturer's protocols. T-cells were separated from the PBMCs and expanded using an interleukin-2 (IL-2)-containing culture media. The human diffuse large B cell line, SU-DHL-6, was purchased from the China Center for Type Culture Collection (Wuhan, China). All the cells were incubated in RPMI-1640 medium supplemented with 10% fetal bovine serum and antibiotics (10000U/mL penicillin and 10μg/mL streptomycin) and maintained in a humidified incubator at 37°C with 5% CO_2.

Flow cytometry

$CD8^+$ T cells were isolated from PBMCs using Manual MACS Cell Separation (Miltenyi Biotech; Auburn, CA, USA). Sorted cells were stained with a FITC-labeled anti-CD8antibody (RPA-T8, ebioscience, San Diego, CA, USA), and then fixed with 4% fixation buffer for 10 minutes. Following this, cells were washed with staining buffer and re-suspended in 100 μL of permeabilization buffer (BD Biosciences, San Diego, CA, USA) and incubated for 15 minutes. After the cells were washed with staining buffer, Ki-67-PE (20Raj1, ebioscience, San Diego, CA, USA) was added and incubated at 4°C for 30 minutes. Finally, the stained cells were re-suspended in 100 μL of 1% fixation buffer and analyzed using a BD Biosciences LSRFortessa, and the data obtained was analyzed using BD Accuri C6 software (FlowJo, San Jose, CA, USA).

The T cells, expanded as described above, were co-cultured with1 × 10^5 SU-DHL-6 cells [pre-treated with mitomycin C (10μg/mL) for 2hours] at T cell/tumor cell culture ratios of 20:1, 10:1, 5:1, and 1:1. SU-DHL-6 cells were sorted from the co-culture system by fluorescence activated cell sorting (FACS) using flow cytometry at 0, 24, 48, and 60 hours, respectively. The cells were stained with an FITC-labeled anti-CD20antibody (BD Bioscience, San Diego, CA, USA), and then analyzed using an FACS Calibur 1 (Becton Dickinson, San Jose, CA, USA). Finally, the cells were evaluated by cell counting using a TM10™ automated cell counter (Bio-Rad Laboratories, Hercules, CA, USA).

Real-time PCR

Total mRNA was extracted using TRIZOL Reagent (Invitrogen, Carlsbad, CA, USA), and cDNA was generated using the Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics). Real-time RT-PCR for granzyme B (forward primer5′-TCCTAAGAACTTCTCCAACGACATC-3′, reverse primer 5′-GCACAGCTCTGGTCCGCT-3′) was performed using SYBR Premix Ex Taq II (TaKaRa, Ohtsu, Japan). The relative quantification of mRNA expression was calculated by the $2^{-\Delta\Delta Ct}$ method.

Enzyme-linked immunosorbent assay (ELISA)

Peripheral blood serum samples (for CCL3 and IL-10 levels) and cell supernatants(for IFN-γ levels) were analyzed using the appropriate Quantikine Kit (R&D Systems, Minneapolis, MN, USA), according to the manufacturer's instructions. The absorbance was determined at a wavelength of 450nm using a Bio-Rad Model 680 Microplate Reader (BioRad Laboratories, Tokyo, Japan).

Statistical analysis

A Spearman correlation analysis was performed to determine the correlation between serum LDH and the maximum standardized uptake value (SUVmax) measured using FDG-PET/CT. The best cut-off points for LMR and *R* were determined using a receiver operating characteristics (ROC) curve analysis, and were considered as having the highest Youden index (sensitivity + specificity-1). The significance of associations between groups with clinicopathological variables was determined using Chi-squared and Fisher's Exact tests. Overall survival (OS) was defined as the time from the date of diagnosis until death from any cause, or the last follow-up. Progression-free survival (PFS) was calculated from the day of treatment until relapse, disease progression, death from any cause, or the last follow-up. OS and PFS curves were determined using the Kaplan-Meier method, and were compared using the log-rank test. Multivariate modeling was assessed using a Cox regression analysis. IHC, flow cytometry, RT-PCR, and ELISA data were analyzed and are shown as mean ± standard deviation (SD). $P < 0.05$ was considered as being statistically significant. All statistical analyses were performed using IBM SPSS 20.0 statistics software (IBM, Armonk, NY, USA).

Results

Clinical characteristics of 184 patients with DLBCL

Table 1 shows the clinical data from the 184 DLBCL patients enrolled in this study. There were 106 (57.61%) males and 78 (42.39%) females, with a mean age of 54.0 (range from 13.0 to 82.0) years. The majority of the patients (120 cases, 65.22%) had localized disease (Ann Arbor stage I-II). Based on the Eastern Cooperative Oncology Group performance status (ECOG PS), 149 (80.98%) patients were in group 0-1. In addition, 49 (26.63%) patients had positive B symptoms, and more than one-half of the patients (108 cases, 58.69%) were in the low risk group. At diagnosis, the median LDH was 230.79 U/L (range from 70-1026 U/L).

Serum LDH levels reflect tumor burden

To evaluate whether the serum LDH level is able to reflect the tumor burden in DLBCL patients, we performed a Spearman correlation analysis evaluating the relationship between serum LDH and SUVmax for all lesions measured by FDG-PET/CT in patients. The data shown in Fig. 1a indicated a significantly positive correlation between serum LDH levels and FDG uptake based on PET/CT ($P < 0.01$), with a correlation coefficient of 0.78. Using the ROC curve, we selected the best cut-off value for LDH levels in order to divide the patients

Table 1. Correlation with clinicopathological characteristics based on LMR and the LMR/LDH ratio. *Mean with standard deviationsshown in parentheses. R = LMR/LDH×100 (%) [a]Chi-square test by two-sided Pearson's exact test. [b]Wilcoxon rank-sum test

Characteristics		LMR			R		
	Overall (n = 184)	≤ 3.14 (n = 86)	> 3.14 (n = 98)	P-value	≤ 1.26 (n = 69)	>1.26 (n = 115)	P-value
Sex							
Male	106 (57.61)	51	55	0.663[a]	39	67	0.817[a]
Female	78 (42.39)	35	43		30	48	
Age, y							
≤ 60	110 (59.78)	49	61	0.467[a]	41	69	0.938[a]
> 60	74 (40.22)	37	37		28	46	
Ann Arbor stage							
I	55 (29.89)	19	36	0.031[a]	12	43	0.002[a]
II	65 (35.33)	29	36		22	43	
III	40 (21.74)	26	14		20	20	
IV	24 (13.04)	12	12		15	9	
ECOG PS							
0-1	149 (95.02)	64	85	0.034[a]	49	100	0.008[a]
≥ 2	35 (4.98)	22	13		20	15	
B symptoms							
-	135 (73.37)	57	78	0.042[a]	42	93	0.003[a]
+	49 (26.63)	29	20		27	22	
IPI score							
0	47 (25.54)	16	31	0.052[a]	11	36	0.002[a]
1	61 (33.15)	26	35		20	41	
2	44 (23.91)	24	20		17	27	
≥ 3	32 (17.39)	20	12		21	11	
LDH (U/L)		267.15 (140.77)*	199.91 (147.73)*	0.008[b]	362.05 (191.17)*	167.32 (51.15)*	0.000[b]

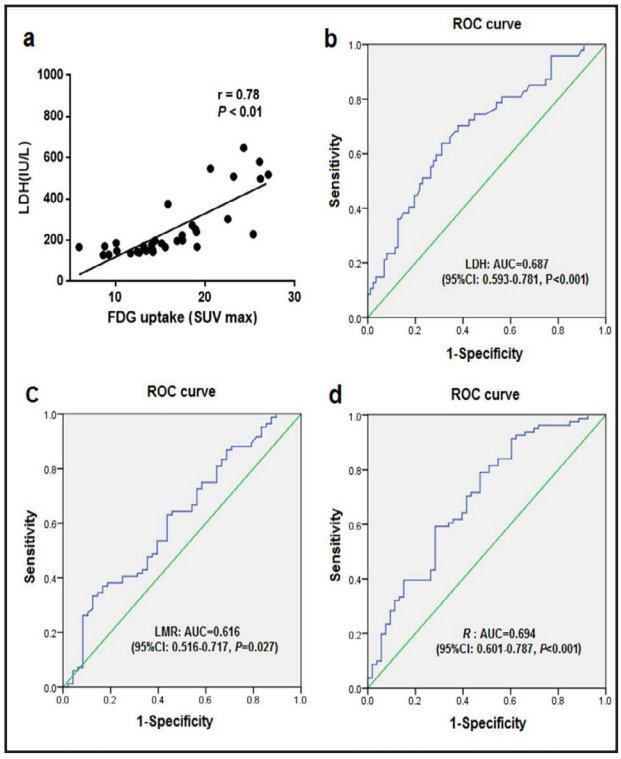

Fig. 1. Spearman correlation analysis of the relationship between serum LDH and SUVmax from FDG-PET/CT of DLBCL patients at diagnosis (a); ROC and AUC for LDH (b), ROC and AUC for LMR (c), and ROC and AUC for the LMR/LDH ratio (d).

into high or low tumor burden groups based on their LDH levels. The area under the curve (AUC) was 0.687 [95% confidence interval (CI): 0.593-0.781, $P < 0.001$, Fig. 1b], with an LDH value of 193.35. Thus, we defined LDH > 193.35 U/L as being the high LDH group, and LDH ≤ 193.35 U/L as being the low LDH group.

LMR/LDH ratio shows a higher significant correlation with the clinical characteristics of DLBCL patients than LMR

The ROC curves were also generated to select the appropriate cut-off values of LMR and the LMR/LDH ratio based on the survival analysis. For LMR, the AUC was 0.616 (95% CI: 0.516-0.717, $P = 0.027$) in all patients (Fig. 1c), with an LMR value of 3.14. With respect to the LMR/LDH ratio, the AUC was calculated to be 0.694 (95% CI: 0.601-0.787, $P < 0.001$) in patients (Fig. 1d), with an LMR/LDH value of 1.26. The relationships between LMR or LMR/LDH and baseline clinical features are listed in Table 1. Patients with an LMR ≤ 3.14 had a higher incidence of advanced Ann Arbor stage ($P = 0.031$), ECOG PS ($P = 0.034$), B symptoms ($P = 0.042$), and elevated LDH level ($P = 0.008$); however, the IPI score was not statistically significant ($P = 0.054$). For the LMR/LDH ratio, this was more significantly related to the Ann Arbor stage ($P = 0.002$), ECOG PS ($P = 0.008$), B symptoms ($P = 0.003$), IPI score ($P = 0.002$), as well as the LDH level ($P < 0.001$).

LMR/LDH ratio demonstrates a greater prognostic significance in DLBCL patients

Kaplan-Meier curves were generated to evaluate the OS and PFS rates based on LMR and LMR/LDH, as shown in Fig. 2. Patients with LMR ≤ 3.14 had significantly lower OS (P = 0.024; Fig. 2a) and PFS (P = 0.002; Fig. 2b) compared to those with LMR > 3.14. With respect to the LMR/LDH ratio, the survival curves showed more prognostic significance; patients with $R \leq 1.26$ had much lower OS ($P < 0.001$; Fig. 2c) and PFS ($P < 0.001$; Fig. 2d)comparing with values $R > 1.26$.

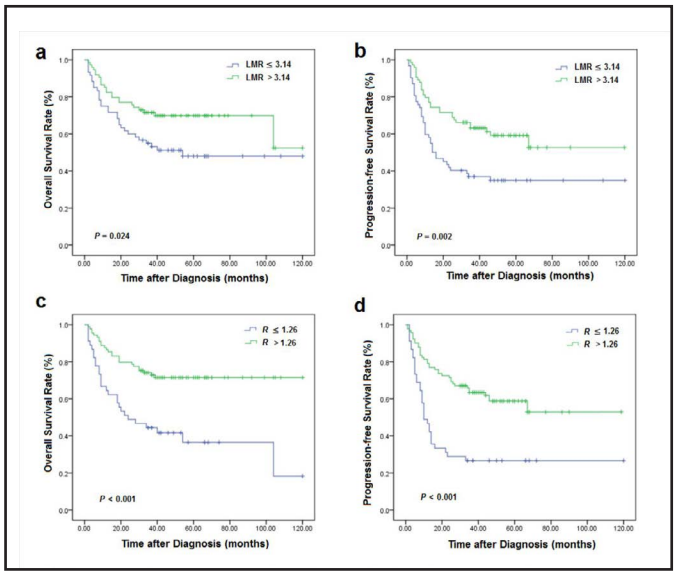

Fig. 2. Kaplan-Meier survival analysis estimates of the OS and PFS according to baseline LMR (a, b) and the LMR/LDH ratio (c,d) in DLBCL patients.

LMR/LDH ratio is an independent prognostic marker in DLBCL patients

We used a Cox Regression model to evaluate the prognostic impact of the LMR/LDH ratio at diagnosis on the survival of DLBCL patients. In the multivariate analysis, the LMR/LDH ratio was identified as being an independent prognostic marker for OS (P = 0.037, HR = 2.543; 95%CI: 1.056-6.125) and PFS (P = 0.018, HR = 2.807; 95%CI: 1.196-6.589); however, the results were not significant for LMR or LDH alone, either in OS (P = 0.605, P = 0.142) or PFS (P = 0.965, P = 0.654). Moreover, OS and PFS were also significantly associated with age (P = 0.004, HR = 0.429; 95%CI: 0.243-0.759 for OS; P = 0.015, HR = 0.541; 95%CI: 0.330-0.888 for PFS) and IPI score (P = 0.003, HR = 0.068; 95%CI: 0.011-0.398 for OS; P = 0.017, HR = 0.150; 95%CI: 0.032-0.716 for PFS). Among the other variables studied, Ann Arbor stage, and B symptoms were shown to be independent prognostic factors for OS (P = 0.045, HR = 2.420; 95%CI: 1.018-5.749), while sex independently predicted PFS (P = 0.039, HR = 1.753; 95%CI: 1.030-2.983) (Table 2).

Table 2. Multivariate analysis of prognostic factors for survival. [a]Cox analysis

Covariate	OS			PFS		
	HR	95%CI	P-value[a]	HR	95%CI	P-value[a]
Sex	1.802	0.961-3.378	0.066	1.753	1.030-2.983	0.039
Age, y	0.429	0.243-0.759	0.004	0.541	0.330-0.888	0.015
Ann Arbor stage	0.203	0.053-0.784	0.021	0.513	0.157-1.671	0.268
ECOG PS	0.540	0.251-1.160	0.114	0.710	0.359-1.405	0.325
B symptoms	2.420	1.018-5.749	0.045	1.947	0.920-4.119	0.082
IPI score	0.068	0.011-0.398	0.003	0.150	0.032-0.716	0.017
LMR	0.826	0.402-1.701	0.605	0.986	0.516-1.882	0.965
LDH	0.567	0.266-1.209	0.142	0.850	0.416-1.734	0.654
R	2.543	1.056-6.125	0.037	2.807	1.196-6.589	0.018

Tumor burden correlates with T cell proliferation

We then assessed whether tumor burden was correlated with T cell proliferation in DLBCL patients. Ki67 is a marker of T cell proliferation in tumor masses, as well as in peripheral blood samples. Thus we analyzed changes in Ki67 expression in $CD8^+$ T cells in patients with different tumor burdens as shown by high or low LDH levels. Tissue samples from patients with low LDH levels showed a positive expression of Ki67 in the tumor infiltrating lymphocytes (TILs), whereas in patients in the high LDH group, Ki67 expression was almost absent (Fig. 3a). From the flow cytometry analysis, Ki67 expression in the $CD8^+$ T cells persisted at low levels in DLBCL patients with higher LDH levels. In contrast, Ki67 expression was remarkably increased in patients with low LDH levels ($P < 0.001$, Fig 3c and 3d), suggesting a negative correlation between T cell proliferation and tumor burden in peripheral blood samples of DLBCL patients.

A higher tumor burden decreases the anti-tumor effects of T lymphocytes

To further evaluate whether tumor burden would affect the anti-tumor function of T lymphocytes, we co-cultured T cells with SU-DHL-6 cells at different T cell/tumor cell ratios of 20:1, 10:1, 5:1 and 1:1. Fig. 4a shows the number of SU-DHL-6 cells following incubation with different amounts of T cells for 0, 24, 48, and 60 hours. We found a significant decrease in the SU-DHL-6 cell count at lower tumor burden ratios (T cell/tumor cell ratio of 20:1) compared to higher tumor burden ratios (T cell / tumor cell ratio of 1:1) (P = 0.003 and P = 0.002 respectively). In addition, the expression of granzyme B in T cells and IFN-γ in cell supernatants in the coculture with a 1:1 T cell/tumor cell ratio was significantly lower than in the coculture with a 20:1 T cell/tumor cell ratio (P < 0.001, Fig. 4b; P = 0.006, Fig. 4c). Taken together, these observations suggest a weaker anti-tumor effect of the T cells at higher tumor burdens.

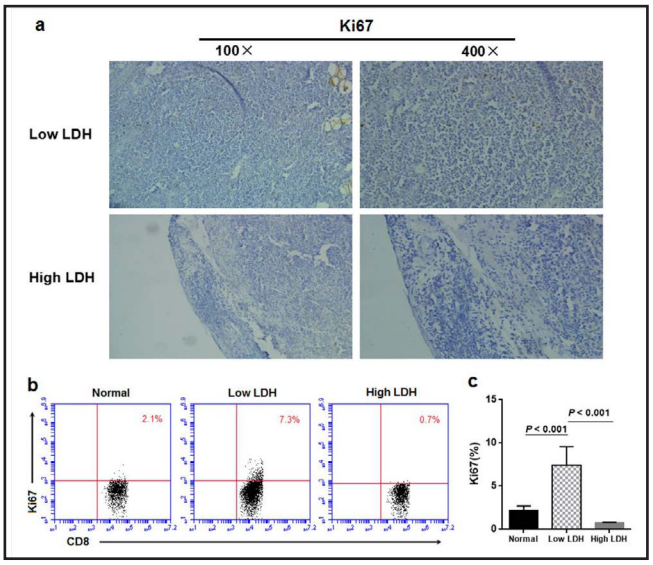

Fig. 3. The correlation between tumor burden and the expression of Ki67 using IHC (a) and flow cytometry (b, c) in DLBCL patients.

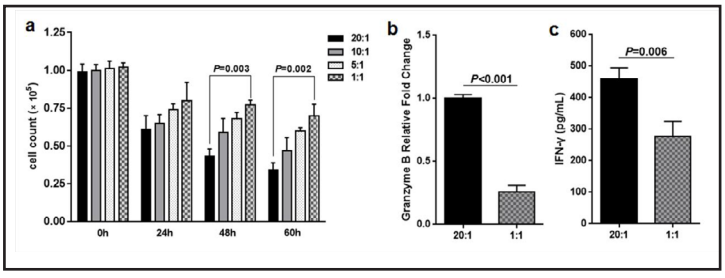

Fig. 4. SU-DHL-6 cell count in co-cultures with different T cell/tumor cell ratios(a); the expression of granzyme B in T cells (b) and IFN-γ in cell supernatants (c) in cultures with high or low tumor burden ratios.

Higher tumor burden contributes to the differentiation of monocytes to tumor associated macrophages (TAMs)

It has been demonstrated that peripheral blood monocytes can be recruited to tumors and differentiated into TAMs in response to the chemokines or cytokines released by the tumor cells. Thus, we examined the serum levels of CCL3 and IL-10 expression in DLBCL patients by ELISA analysis and examined the differentiation of monocytes into TAMs. The data showed that the serum CCL3 and IL-10 levels were significantly increased in patients with high LDH levels (P = 0.002, Fig. 5a; P = 0.03, Fig. 5b). In parallel, we detected

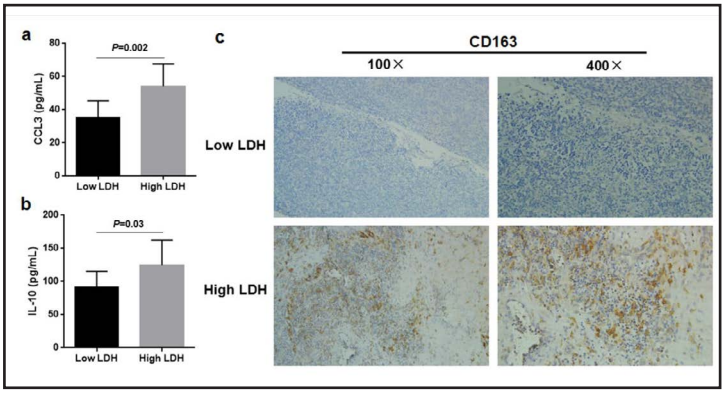

Fig. 5. Serum expression of CCL3 (a) and IL-10 (b) and tissue expression of CD163 (c) in DLBCL patients with high or low LDH levels.

higher expression levels of CD163 in tissue samples from patients with high LDH levels (Fig. 5c), which suggests that a higher tumor burden is able to promote the differentiation of monocytes to TAMs.

Discussion

In this study, we report the predictive significance of the LMR to tumor burden ratio (LMR/LDH) in 184 DLBCL patients and investigated the potential correlation of tumor burden with immune system status. There were several findings relevant to the understanding of the LMR/LDH ratio in predicting clinical outcomes more significantly. To our knowledge, this is the first large-scale study to evaluate the prognostic value of the LMR/LDH ratio in DLBCL patients. Our study demonstrated the utility of using this novel predictive biomarker, which assesses both the immune system and tumor burden, in DLBCL patients, thus opening avenues for future mechanistic exploration.

Recently, the lymphocyte to monocyte ratio has been reported to be an important prognostic indicator for many malignancies, including DLBCL [11-15]. The levels of circulating lymphocytes and monocytes are reflective of both the immunological function in the peripheral blood as well as in the tumor micro-environment [22, 23]. Lymphocytes have an important anti-tumor role in tumor immunity, and a low lymphocyte level might weaken the host immunological response to malignancy [24]. In contrast, monocytes are considered to be negative factors for malignancy as they may infiltrate into tumor sites and differentiate into TAMs, which can promote angiogenesis, tumor proliferation, and metastasis by releasing growth factors and immune-suppressive cytokines [25, 26]. Thus, the lymphocyte-monocyte ratio reflects the immune status of the host, but more importantly, it might be able to predict the survival outcomes of patients with malignancies, as has been reported in several studies [11-15]. However, there are several limitations in using only LMR to determine patient prognosis: first of all, the survival outcomes of patients are not only influenced by the immune system, but also by some tumors' intrinsic characteristics, such as the precise genetic mutation, type of pathology, and tumor size [27, 28]; second, treatment with either chemotherapy or radiation therapy will affect the function of immune cells [29-31]; last, but not the least, the levels and functions of lymphocytes or monocytes can be regulated by tumor cells [32]. Therefore, LMR alone is probably not a perfect predictor of the clinical survival of patients, and consideration of the tumor burden might be more valuable [16].

Unlike other solid malignancies, DLBCL is a hematopoietic and lymphoid disease, which has a wide distribution in lymph nodes and extra-nodal tissues. PET/CT is recognized as being the most sensitive and specific imaging modality used to determine tumor burden in DLBCL patients [19, 20, 33]. However, the use of PET/CT scans in patients is often very limited before treatment. In our study, we found that serum LDH levels were significantly related to the SUVmax in PET/CT scans in DLBCL patients, which is consistent with a previous study that evaluated LDH as a potential circulating proxy for tumor burden [16]. Thus, we developed the ratio of LMR to LDH [defined as "*R*" = LMR/LDH ×100 (%)] in order to examine the influence of both the peripheral immune response and tumor burden on survival outcomes in DLBCL patients. Comparing the LMR/LDH ratio with LMR alone, we found that the LMR/LDH ratio was more significantly correlated with clinical characteristics, and showed more prognostic significance for OS and PFS in the 184 DLBCL patients evaluated in this study. In addition, a multivariate analysis demonstrated that the LMR/LDH ratio was an independent prognostic biomarker, compared to LMR or LDH alone. These data suggest that the ratio of LMR to tumor burden could be a better prognostic indicator in DLBCL patients compared to LMR alone.

Moreover, we investigated the potential mechanism behind using the LMR/LDH ratio in patients with DLBCL. It is well known that Ki67 is a biomarker of cellular proliferation in tumors, and is associated with poor prognosis [34, 35]. In T lymphocytes, Ki67 expression is

also a good indicator of T cell activation and reinvigoration [16]. Here, we examined changes in Ki67 expression in TILs, as well as in peripheral $CD8^+$ T cells to assess T cell responses to different tumor burdens. Our data showed that a larger tumor burden was correlated with decreased Ki67 expression in T cells, in either the solid tumor or in the circulating blood. Meanwhile, based on the data from the tumor cell/T cell co-culture system, we found that the anti-tumor responses of T cells were suppressed at high tumor burdens. In contrast, a lower tumor burden was correlated with enhanced anti-tumor effects of T cells. In addition, we found that tumor burden was associated with the generation of TAMs in the tumor micro-environment. We detected higher expression levels of CCL3, IL-10, and CD163 in patients with high LDH, indicating that a higher tumor burden induces the release of large amounts of chemokines and cytokines, which promote the recruitment of monocytes and their differentiation into TAMs, eventually leading to an immune suppressive environment in the tumor. On the basis of these observations, it might be possible to understand the role of the tumor burden on the immune system, and furthermore they raise the possibility of using the LMR to tumor burden ratio to predict survival. The relationship between LMR and tumor burden incorporates the relative balance between immune response and tumor burden. That is, if the tumor burden is high, a robust immune response might be clinically effective. Thus, it will be important to test both the immune factors and the tumor burden to define valuable predictors.

Conclusion

In conclusion, our study has identified a novel clinical biomarker, the LMR/LDH ratio, that can be used to distinguish survival outcome in DLBCL patients, and demonstrated the potential mechanism behind the successful use of the immune system combined with tumor burden. More studies are required to further understand the mechanisms underlying the use of the LMR to tumor burden ratio to facilitate its use in future clinical applications.

Acknowledgements

This study was supported by the National Natural Science Foundation of China (Grant No. 81372839 & No. 81672599), and the National Science Foundation of Heilongjiang Province of China for Returnees (Grant No. LC2017037).

Disclosure Statement

The authors have declared that no competing interests exist.

References

1 Armitage JO, Gascoyne RD, Lunning MA, Cavalli F: Non-Hodgkin lymphoma. Lancet 2017;390:298-310.
2 Morton LM, Wang SS, Cozen W, Linet MS, Chatterjee N, Davis S, Severson RK, Colt JS, Vasef MA, Rothman N, Blair A, Bernstein L, Cross AJ, De Roos AJ, Engels EA, Hein DW, Hill DA, Kelemen LE, Lim U, Lynch CF, Schenk M, Wacholder S, Ward MH, Hoar Zahm S, Chanock SJ, Cerhan JR, Hartge P: Etiologic heterogeneity among non-Hodgkin lymphoma subtypes. Blood 2008;112:5150-5160.
3 Flowers CR, Sinha R, Vose JM: Improving outcomes for patients with diffuse large B-cell lymphoma. CA Cancer J Clin 2010;60:393-408.
4 Nastoupil LJ, Rose AC, Flowers CR: Diffuse large B-cell lymphoma: current treatment approaches. Oncology (Williston Park) 2012;26:488-495.

5 Boyle J, Beaven AW, Diehl LF, Prosnitz LR, Kelsey CR: Improving outcomes in advanced DLBCL: systemic approaches and radiotherapy. Oncology (Williston Park) 2014;28:1074-81, 1084.
6 Cheson BD: Therapy for diffuse large B-cell lymphoma: getting personal. Lancet 2013;381:1793-1794.
7 Couzin-Frankel J: Breakthrough of the year 2013. Cancer immunotherapy. Science 2013;342:1432-1433.
8 Grivennikov SI, Greten FR, Karin M: Immunity, inflammation, and cancer. Cell 2010;140:883-899.
9 Juárez-Salcedo LM, Sandoval-Sus J, Sokol L, Chavez JC, Dalia S: The role of anti-PD-1 and anti-PD-L1 agents in the treatment of diffuse large B-cell lymphoma: The future is now. Crit Rev Oncol Hematol 2017;113:52-62.
10 Hude I, Sasse S, Engert A, Bröckelmann PJ: The emerging role of immune checkpoint inhibition in malignant lymphoma. Haematologica 2017;102:30-42.
11 Xue P, Hang J, Huang W, Li S, Li N, Kodama Y, Matsumoto S, Takaori K, Zhu L, Kanai M: Validation of Lymphocyte-to-Monocyte Ratio as a Prognostic Factor in Advanced Pancreatic Cancer: An East Asian Cohort Study of 2 Countries. Pancreas DOI: 10.1097/MPA.0000000000000891.
12 Peng J, Li H, Ou Q, Lin J, Wu X, Lu Z, Yuan Y, Wan D, Fang Y, Pan Z: Preoperative lymphocyte-to-monocyte ratio represents a superior predictor compared with neutrophil-to-lymphocyte and platelet-to-lymphocyte ratios for colorectal liver-only metastases survival. Onco Targets Ther 2017;10:3789-3799.
13 Ji H, Xuan Q, Yan C, Liu T, Nanding A, Zhang Q: The prognostic and predictive value of the lymphocyte to monocyte ratio in luminal-type breast cancer patients treated with CEF chemotherapy. Oncotarget 2016;7:34881-34889.
14 Katoh D, Ochi Y, Yabushita T, Ono Y, Hiramoto N, Yoshioka S, Yonetani N, Matsushita A, Hashimoto H, Kaji S, Imai Y, Ishikawa T: Peripheral Blood Lymphocyte-to-Monocyte Ratio at Relapse Predicts Outcome for Patients With Relapsed or Refractory Diffuse Large B-cell Lymphoma in the Rituximab Era. Clin Lymphoma Myeloma Leuk 2017;17:e91-e97.
15 Zhao P, Zang L, Zhang X, Chen Y, Yang H, Zhao H, Yu Y, Wang Y, Zhang Y, Wang X: The Lymphocyte-Monocyte Ratio and the Platelet-Lymphocyte Ratio at Diagnosis as Independent Prognostic Factors in Primary Gastrointestinal Diffuse Large B Cell Lymphoma. Indian J Hematol Blood Transfus2017;33:333-341.
16 Huang AC, Postow M, Orlowski RJ, Mick R, Bengsch B, Manne S, Xu W, Harmon S, Giles JR, Wenz B, Adamow M, Kuk D, Panageas KS, Carrera C, Wong P, Quagliarello F, Wubbenhorst B, D'Andrea K, Pauken KE, Herati RS, Staupe RP, Schenkel JM, McGettigan S, Kothari S, George SM, Vonderheide RH, Amaravadi RK, Karakousis GC, Schuchter LM, Xu X, Nathanson KL, Wolchok JD, Gangadhar TC, Wherry EJ: T-cell invigoration to tumour burden ratio associated with anti-PD-1 response. Nature 2017;545:60-65.
17 Sasaki K, Margonis GA, Andreatos N, Zhang XF, Buettner S, Wang J, Deshwar A, He J, Wolfgang CL, Weiss M, Pawlik TM: The prognostic utility of the "Tumor Burden Score" based on preoperative radiographic features of colorectal liver metastases. J Surg Oncol 2017;116:515-523.
18 Sasaki K, Morioka D, Conci S, Margonis GA, Sawada Y, Ruzzenente A, Kumamoto T, Iacono C, Andreatos N, Guglielmi A, Endo I, Pawlik TM: The Tumor Burden Score: A New "Metro-ticket" Prognostic Tool For Colorectal Liver Metastases Based on Tumor Size and Number of Tumors. Ann Surg 2018;267:132-141.
19 Barrington SF, Kluge R: FDG PET for therapy monitoring in Hodgkin and non-Hodgkin lymphomas. Eur J Nucl Med Mol Imaging 2017;44:97-110.
20 Valls L, Badve C, Avril S, Herrmann K, Faulhaber P, O'Donnell J, Avril N: FDG-PET imaging in hematological malignancies. Blood Rev 2016;30:317-331.
21 Balch CM, Gershenwald JE, Soong SJ, Thompson JF, Atkins MB, Byrd DR, Buzaid AC, Cochran AJ, Coit DG, Ding S, Eggermont AM, Flaherty KT, Gimotty PA, Kirkwood JM, McMasters KM, Mihm MC Jr, Morton DL, Ross MI, Sober AJ, Sondak VK: Final version of 2009 AJCC melanoma staging and classification. J Clin Oncol 2009;27:6199-6206.
22 Kim DH, Baek JH, Chae YS, Kim YK, Kim HJ, Park YH, Song HS, Chung JS, Hyun MS, Sohn SK: Absolute lymphocyte counts predicts response to chemotherapy and survival in diffuse large B-cell lymphoma. Leukemia 2007;21:2227-2230.
23 Wilcox RA, Wada DA, Ziesmer SC, Elsawa SF, Comfere NI, Dietz AB, Novak AJ, Witzig TE, Feldman AL, Pittelkow MR, Ansell SM: Monocytes promote tumor cell survival in T-cell lymphoproliferative disorders and are impaired in their ability to differentiate into mature dendritic cells. Blood 2009;114:2936-2944.
24 Wei X, Huang F, Wei Y, Jing H, Xie M, Hao X, Feng R: Low lymphocyte-to-monocyte ratio predicts unfavorable prognosis in non-germinal center type diffuse large B-cell lymphoma. Leuk Res 2014;38:694-698.

25 Noy R, Pollard JW: Tumor-associated macrophages: from mechanisms to therapy. Immunity 2014;41:49-61.
26 Wada N, Zaki MA, Hori Y, Hashimoto K, Tsukaguchi M, Tatsumi Y, Ishikawa J, Tominaga N, Sakoda H, Take H, Tsudo M, Kuwayama M, Morii E, Aozasa K; Osaka Lymphoma Study Group: Tumour-associated macrophages in diffuse large B-cell lymphoma: a study of the Osaka Lymphoma Study Group. Histopathology 2012;60:313-319.
27 Reddy A, Zhang J, Davis NS, Moffitt AB, Love CL, Waldrop A, Leppa S, Pasanen A, Meriranta L, Karjalainen-Lindsberg ML, Nørgaard P, Pedersen M, Gang AO, Høgdall E, Heavican TB, Lone W, Iqbal J, Qin Q, Li G, Kim SY, Healy J, Richards KL, Fedoriw Y, Bernal-Mizrachi L, Koff JL, Staton AD, Flowers CR, Paltiel O, Goldschmidt N, Calaminici M, Clear A, Gribben J, Nguyen E, Czader MB, Ondrejka SL, Collie A, Hsi ED, Tse E, Au-Yeung RKH, Kwong YL, Srivastava G, Choi WWL, Evens AM, Pilichowska M, Sengar M, Reddy N, Li S, Chadburn A, Gordon LI, Jaffe ES, Levy S, Rempel R, Tzeng T, Happ LE, Dave T, Rajagopalan D, Datta J, Dunson DB, Dave SS: Genetic and Functional Drivers of Diffuse Large B Cell Lymphoma. Cell 2017;171:481-494.e15.
28 Leivonen SK, Taskinen M, Cervera A, Karjalainen-Lindsberg ML, Delabie J, Holte H, Lehtonen R, Hautaniemi S, Leppä S: Alternative splicing discriminates molecular subtypes and has prognostic impact in diffuse large B-cell lymphoma. Blood Cancer J 2017;7:e596.
29 Zhou S, Xu L, Ma Y, Tang L, Zhang Y, Shi Y, Sun L, Chen Y, Liang B, Zhou Y, Yu K, Shen J: Peripheral blood lymphocyte to monocyte ratio recovery from low levels at diagnosis after completion of first line therapy predicts good clinical outcomes in patients with diffuse large B-cell lymphoma. Oncotarget 2017;8:19556-19565.
30 Porrata LF, Ristow KM, Habermann TM, Macon WR, Witzig TE, Colgan JP, Inwards DJ, Ansell SM, Micallef IN, Johnston PB, Nowakowski G, Thompson CA, Markovic SN: Peripheral blood absolute lymphocyte/monocyte ratio recovery during ABVD treatment cycles predicts clinical outcomes in classical Hodgkin lymphoma. Blood Cancer J 2013;3:e110.
31 Lin GN, Liu PP, Liu DY, Peng JW, Xiao JJ, Xia ZJ: Prognostic significance of the pre-chemotherapy lymphocyte-to-monocyte ratio in patients with previously untreated metastatic colorectal cancer receiving FOLFOX chemotherapy. Chin J Cancer 2016;35:5.
32 Su S, Liu Q, Chen J, Chen J, Chen F, He C, Huang D, Wu W, Lin L, Huang W, Zhang J, Cui X, Zheng F, Li H, Yao H, Su F, Song E: A positive feedback loop between mesenchymal-like cancer cells and macrophages is essential to breast cancer metastasis. Cancer Cell 2014;25:605-620.
33 Haioun C, Itti E, Rahmouni A, Brice P, Rain JD, Belhadj K, Gaulard P, Garderet L, Lepage E, Reyes F, Meignan M: [^{18}F]fluoro-2-deoxy-D-glucose positron emission tomography (FDG-PET) in aggressive lymphoma: an early prognostic tool for predicting patient outcome. Blood 2005;106:1376-1381.
34 Penault-Llorca F, Radosevic-Robin N: Ki67 assessment in breast cancer: an update. Pathology 2017;49:166-171.
35 Pascale M, Aversa C, Barbazza R, Marongiu B, Siracusano S, Stoffel F, Sulfaro S, Roggero E, Bonin S, Stanta G: The proliferation marker Ki67, but not neuroendocrine expression, is an independent factor in the prediction of prognosis of primary prostate cancer patients. Radiol Oncol 2016;50:313-320.