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ORIGINAL RESEARCH

Prognostic implications of tumor-infiltrating macrophages, M2 macrophages, regulatory T-cells, and indoleamine 2,3-dioxygenase-positive cells in primary diffuse large B-cell lymphoma of the central nervous system

Soo Jeong Nam[a,b], Sehui Kim[a], Dohee Kwon [a], Hannah Kim[a], Soyeon Kim[c], Eunyoung Lee[d], Tae Min Kim[c,d], Dae Seog Heo[c,d], Sung Hye Park[a], Megan S. Lim[e], Chul Woo Kim[a,c], and Yoon Kyung Jeon [a,c]

[a]Department of Pathology, Seoul National University Hospital, Seoul National University College of Medicine, Seoul, Republic of Korea; [b]Department of Pathology, Asan Medical Center, University of Ulsan College of Medicine, Seoul, Republic of Korea; [c]Cancer Research Institute, Seoul National University, Seoul, Republic of Korea; [d]Department of Internal Medicine, Seoul National University Hospital, Seoul National University College of Medicine, Seoul, Republic of Korea; [e]Department of Pathology and Laboratory Medicine, University of Pennsylvania Perelman School of Medicine, Philadelphia, PA, USA

ABSTRACT

Primary diffuse large B-cell lymphoma of the central nervous system (CNS-DLBCL) is an aggressive disease with a poor prognosis. The status of the tumor immune microenvironment in CNS-DLBCL remains unclear. We investigated the prognostic implications of tumor-associated macrophages (TAMs), regulatory T-cells (Tregs), and indoleamine 2,3-dioxygenase (IDO)$^+$ cells in primary CNS-DLBCL (n = 114) by immunohistochemical analysis. The numbers of tumor-infiltrating immune cells, including CD68$^+$ TAMs, CD163$^+$ or CD204$^+$ M2 macrophages, FOXP3$^+$ Tregs, and IDO$^+$ cells were all significantly lower in CNS-DLBCL versus systemic DLBCL (n = 165; all $P < 0.001$), but with little difference in the ratio of CD163$^+$/CD68$^+$ or CD204$^+$/CD68$^+$ cells. An increase in CD68$^+$ cell numbers was significantly associated with prolonged progression-free survival (PFS) and overall survival in patients with CNS-DLBCL ($P = 0.004$ and 0.021, respectively). In contrast, an increase in CD204$^+$ cell numbers or a higher ratio of CD204$^+$/CD68$^+$ cells was related to a shorter PFS ($P = 0.020$ and 0.063, respectively). An increase in IDO$^+$ cell numbers was associated with a significantly longer PFS ($P = 0.019$). In combination, the status of low IDO$^+$ cell numbers combined with low CD68$^+$ cell numbers, high CD204$^+$ cell numbers, or a high CD204$^+$/CD68$^+$ cell ratio all predicted poor PFS in multivariate analyses. This study showed that an increase in CD204$^+$ cell numbers, suggestive of M2 macrophages, was associated with poor clinical outcome in CNS-DLBCL, whereas increased CD68$^+$ or IDO$^+$ cell numbers were related to a favorable prognosis. The analysis of tumor-infiltrating immune cells could help in predicting the prognosis of CNS-DLBCL patients and determining therapeutic strategies targeting tumor microenvironment.

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Introduction

Primary central nervous system lymphoma (PCNSL) is one of the most aggressive malignant lymphomas, accounting for 2–3% of non-Hodgkin lymphoma (NHL) cases. More than 90% of PCNSLs are diffuse large B-cell lymphoma (DLBCL), which is a clinicopathologically unique entity, distinct from systemic DLBCL.[1] Previous studies have demonstrated that primary CNS-DLBCL has a distinct gene expression signature and mutation status,[2–6] including features involved in CNS tropism, B-cell migration, activated B cell-like (ABC) subtype, and activation of B-cell receptor and toll-like receptor signaling. Patients with primary CNS-DLBCL often show refractoriness to chemotherapy and aggressive clinical behavior and poor clinical outcomes,[1,7] demanding novel therapeutic approach and risk stratification. Recently, immunotherapy targeting the programmed cell death-1 (PD-1)/PD-1 ligand (PD-L) immune checkpoint pathway has shown clinical benefit in patients with some malignant lymphomas.[8] Of note, primary CNS-DLBCL frequently showed alterations in PD-L genes and increased expression of PD-Ls, and PD-1 blockade demonstrated efficacy in patients with primary CNS-DLBCL.[6,9] Thus, understanding the tumor immune microenvironment of primary CNS-DLBCL is of importance.

The tumor immune microenvironment is complex and plays an important role in the development and progression of tumors.[10] Immunosuppressive mechanisms of tumors include cellular components, such as regulatory T cells (Tregs), myeloid-derived suppressor cells, and M2 macrophages, immunosuppressive cytokines, metabolic enzymes, such as indoleamine 2,3-dioxygenase (IDO), and immune checkpoints, mediated via receptor-ligand interaction.[10,11] In many solid tumors, an

CONTACT Yoon Kyung Jeon, MD, PhD. ykjeon@snu.ac.kr Department of Pathology, Seoul National University College of Medicine, 101 Daehak-ro, Jongno-gu, Seoul 03080, Republic of Korea.

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immunosuppressive microenvironment has been associated with tumor aggressiveness and poor clinical outcome.[11] However, the prognostic implications of the immune microenvironment in lymphomas has been conflicting, likely attributed to the fact that lymphoma cells can be influenced by the immunosuppressive mechanisms.[11,12] Tumor-associated macrophages (TAMs) produce growth factors, cytokines, and proteases and contribute to tumor initiation and progression.[13] Macrophages are classified into two lineages, M1 and M2, which differ in phenotype and function.[14] M1 macrophages produce pro-inflammatory cytokines and function primarily as effecter cells that kill invading pathogens. They also exhibit tumor-suppressive functions and stimulate anti-tumor immune responses. In contrast, M2 macrophages expressing the mannose receptor and scavenger receptors (e.g., CD163 and CD204) promote tumor cell survival, invasion, metastasis, and angiogenesis. M2 macrophages also play an immunosuppressive role by downregulating the anti-tumor immune responses of M1 and Th1 cells and recruiting and activating Tregs and Th2 cells.[14]

In solid tumors, tumor-infiltrating Tregs hamper effective anti-tumor immunity, thereby contributing to tumor progression and poor prognosis in patients.[15] However, in several hematolymphoid malignancies, increased quantities of tumor-infiltrating Tregs were associated with favorable clinical outcomes.[12,16–20] IDO is a tryptophan-catabolizing enzyme expressed by diverse cells, including myeloid, stromal, and epithelial cells, and functions as a metabolic regulator of immune responses.[21,22] IDO exerts its immunosuppressive effects by depleting local tryptophan stores and producing kynurenine, a tryptophan metabolite.[23] Tryptophan depletion inhibits the clonal expansion of T cells, leads to T-cell anergy and apoptosis,[24] promotes the conversion of naive $CD4^+$ T cells to Tregs, and activates Tregs function.[25]

Taken together, it seems conceivable that crosstalk among TAMs, Tregs and IDO may affect the constitution of the tumor immune microenvironment and biology of tumor. The CNS microenvironment is unique in that it is in many ways an immunologically privileged site. The blood brain barrier (BBB) limits the entry of immune cells and immune mediators into the CNS.[26] However, the immune microenvironment of primary CNS-DLBCL remains unclear. Thus, here, we analyzed the statuses of TAMs, M2 macrophages, Tregs, and IDO in primary CNS-DLBCL to investigate their prognostic implications.

Results

Characteristics of patients with primary CNS-DLBCL

The clinicopathological characteristics of the patients with primary CNS-DLBCL are summarized in Table 1. The male to female ratio was 1.7:1, and the age of the patients ranged from 10 to 82 (mean, 58.7±13.9; median 61) years. Most patients had an Eastern Cooperative Oncology Group (ECOG) performance status of 0, 1 (65.8%), a lack of B symptoms (97.4%), involvement deep brain structures (72.6%), multifocal diseases (61.1%), and ABC immunophenotype (84.8%). The International Extranodal Lymphoma Study Group (IELSG) prognostic index and Nottingham/Barcelona score were 0–2 in 56.1% of patients and 0–2 in 83.3% of patients, respectively. *MYD88* mutation (all L265P mutations) was observed in 38.1% and *CD79B* mutation (all involving Y196) was observed in 23%, of which 52.2% had concomitant *MYD88* mutation. Most patients were treated with high-dose methotrexate-containing regimens including combined high-dose methotrexate, vincristine and procarbazine chemotherapy (MVP) (57.9%) or high-dose methotrexate (17.5%).

Table 1. Clinicopathological features of patients with primary CNS-DLBCL

Variables		No. (%)
Age	≤60 yr	53 (46.5)
	>60 yr	61 (53.5)
Sex	Male	71 (62.3)
	Female	43 (37.7)
Initial symptom*	H & V	27 (25.0)
	Seizure	7 (6.5)
	Neurologic deficit	74 (68.5)
ECOG performance status	0, 1	75 (65.8)
	≥ 2	39 (34.2)
B symptom	Absent	111 (97.4)
	Present	3 (2.6)
Serum LDH*	Normal	67 (62.6)
	Elevated	40 (37.4)
CSF cytology*	Negative	84 (87.5)
	Positive	12 (12.5)
CSF protein*	Normal	45 (48.9)
	Elevated	47 (51.1)
Ocular disease*	Absent	101 (89.4)
	Present	12 (10.6)
Involvement of deep structures†*	Absent	31 (27.4)
	Present	82 (72.6)
Extent of disease*	Unifocal	44 (38.9)
	Multifocal	69 (61.1)
IELSG prognostic index	0 ~ 2	64 (56.1)
	3 ~ 5	50 (43.9)
Nottingham/Barcelona score	0 ~ 2	95 (83.3)
	3	19 (16.7)
Radiation	Not done	40 (35.1)
	Done	74 (64.9)
Chemotherapy	MVP	66 (57.9)
	HD-MTX	20 (17.5)
	Others††	5 (4.4)
	No chemotherapy	23 (20.2)
Rituximab	Not done	104 (91.2)
	Done	10 (8.8)
IT-MTX	Not done	91 (79.8)
	Done	23 (20.2)
Immunophenotype*	GCB	14 (15.2)
	ABC	78 (84.8)
MYD88 mutation*	Absent	52 (61.9)
	Present	32 (38.1)
CD79B mutation*	Absent	77 (77.0)
	Present	23 (23.0)
	- concomitant with *MYD88* mutation	12/23 (52.2)

No., number; H&V, Headache and Vomiting; ECOG, Eastern Cooperative Oncology Group; LDH, lactate dehydrogenase; CSF, cerebrospinal fluid; IELSG, International Extranodal Lymphoma Study Group; MVP, combined chemotherapy regimen of high-dose methotrexate, vincristine and procarbazine; HD-MTX, high-dose methotrexate; IT-MTX, intrathecal methotrexate; GCB, germinal center B cell-like; ABC, activated B cell-like;

†Involvement of deep structures of the brain, i.e., basal ganglia and/or corpus callosum and/or brain stem and/or cerebellum.; ††Others of chemotherapy includes CHOP, COPADM, etc.; *These variables contain missing values that lacked information about variables.

Quantitative analysis of tumor-infiltrating $CD68^+$, $CD163^+$, and $CD204^+$ TAMs, $FOXP3^+$ Tregs, and IDO^+ cells in primary CNS-DLBCL

CD68, CD163, and CD204 immunostaining showed a cytoplasmic and/or membranous pattern in cells presumed to be

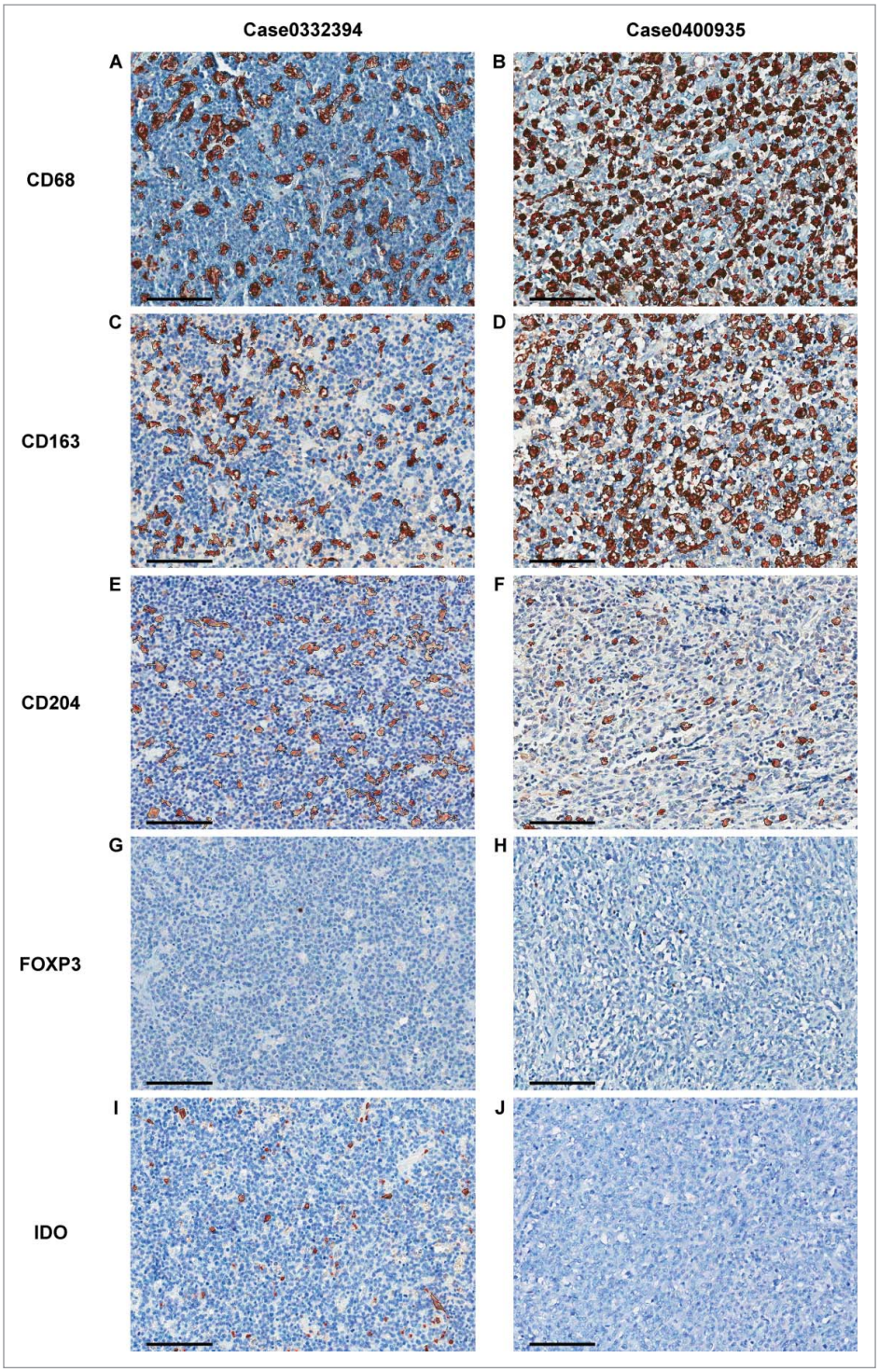

Figure 1. Representative images from the automated enumeration of tumor-infiltrating CD68$^+$, CD163$^+$, CD204$^+$, FOXP3$^+$, and IDO$^+$ cells. Representative images of immune cells from two patients with primary CNS-DLBCL are demonstrated. CD68, CD163, and CD204 were expressed in a granular cytoplasmic pattern by macrophages. FOXP3 showed a nuclear pattern in small lymphoid cells. IDO was expressed in a granular cytoplasmic pattern by suspected macrophages, dendritic cells, small plasmacytoid dendritic cells, and vascular endothelial cells. Images were captured by virtual microscopy and submitted to an image analyzer, which delineated the positive cells by thin black lines, as seen in (A—F), (I) and (J). In the first case, the counts of CD68$^+$ cells (A), CD163$^+$ cells (C), and CD204$^+$ cells (E) were 134, 115, and 115, respectively, per unit area (0.28 mm^2). The count of FOXP3$^+$ cells was 1 per unit area (0.28 mm^2) (G). The count of IDO$^+$ cells was 75 per unit area (0.28 mm^2) (I). In the second case, the counts of CD68$^+$ cells (B), CD163$^+$ cells (D), and CD204$^+$ cells were 294, 257, and 57, respectively, per unit area (0.28 mm^2). The count of FOXP3$^+$ cells in this case was 11 per unit area (0.28 mm^2) (H). No IDO$^+$ cell was observed in this case (J). (Scale bar, 100 μm, in all images).

macrophages (Fig. 1A-F). The mean numbers of tumor-infiltrating CD68$^+$, CD163$^+$, and CD204$^+$ cells in primary CNS-DLBCL were 145.42±70.55 (range, 5.67–385.00; median, 132.00), 149.67±67.76 (range, 21.00–282.67; median, 146.33), and 65.51±61.64 (range, 2.00–278.00; median, 42.00) per unit area, respectively. The mean ratios of CD163$^+$/CD68$^+$ cells and CD204$^+$/CD68$^+$ cells were estimated to be 1.32±1.76 (range, 0.19–17.47; median, 1.06) and 0.46±0.42 (range, 0.02–3.06; median, 0.36), respectively. Overall, the numbers of CD68$^+$ versus CD163$^+$ cells CD68$^+$ versus CD204$^+$ cells, and

$CD163^+$ versus $CD204^+$ cells showed significant positive correlations with each other (R = 0.416, 0.552, and 0.656, respectively; all P < 0.001; Fig. 2).

FOXP3 immunostaining was detected in the nuclei of tumor-infiltrating small lymphocytes (Fig. 1G-H). The mean number of $FOXP3^+$ cells per unit area was 21.44±26.24 (range, 0.00–109.0; median, 8.5). The number of $FOXP3^+$ cells showed a positive correlation with the number of $CD68^+$ and $CD204^+$ cells (R = 0.327 and 0.329, respectively; both P = 0.001; Fig. 2), but no correlation with the number of $CD163^+$ cells (Supplementary Fig. S1).

IDO was not expressed in tumor cells. Based on morphology and double immunostaining in representative cases (Supplementary Fig. S2), the IDO^+ cells were suspected to be mostly macrophages, dendritic cells, plasmacytoid dendritic cells, or vascular endothelial cells (Fig. 1I-J). Overall, the mean number of IDO^+ cells per unit area was 30.88±53.21 (range, 0–340.0; median, 10.0). The number of IDO^+ cells was positively correlated with the numbers of $FOXP3^+$ cells and $CD68^+$ cells (R = 0.419; P < 0.001 and R = 0.217; P = 0.058, respectively; Fig. 2 and Supplementary Fig. S1).

Comparative analysis of tumor-infiltrating $CD68^+$, $CD163^+$, and $CD204^+$ TAMs, $FOXP3^+$ Tregs, and IDO^+ cells between primary CNS-DLBCL and systemic DLBCL

To compare the numbers of tumor-infiltrating immune cells between primary CNS-DLBCL and systemic DLBCL, systemic DLBCL data from our previous study were used.[27, 28] The numbers of tumor-infiltrating $CD68^+$ TAMs, $CD163^+$ or $CD204^+$ M2 TAMs, $FOXP3^+$ Tregs, and IDO^+ cells were all significantly lower in primary CNS-DLBCL than in systemic DLBCL (all P < 0.001; Table 2). However, there was no significant difference in the ratio of $CD163^+/CD68^+$ cells or $CD204^+/CD68^+$ cells between primary CNS-DLBCL and systemic DLBCL.

Relationships between clinicopathological features and the numbers of TAMs, M2 macrophages, Tregs, and IDO^+ cells in primary CNS-DLBCL

The associations between clinicopathological features and the number of tumor-infiltrating immune cells are summarized in Supplementary Table S1. Briefly, the $CD163^+$ cells was higher in patients with advanced IELSG prognostic index (P = 0.013). The number of $FOXP3^+$ cells was much lower in patients with a higher ECOG performance status (P = 0.039). IDO^+ cells were also decreased in number in patients with multifocal diseases (P = 0.002) and in those with involvement of deep structure (P = 0.043). Otherwise, there were no significant associations between the status of tumor-infiltrating immune cells and other clinicopathological and genetic features.

Numbers of tumor-infiltrating TAMs, M2 macrophages, Tregs, and IDO^+ cells and the survival of patients with primary CNS-DLBCL

In total, 104 patients with primary CNS-DLBCLs who did not receive rituximab were classified into two groups according to the quantity and status of tumor-infiltrating immune cells, as described in the Materials and Methods, and subjected to a survival analysis. In a Kaplan-Meier analysis, patients with increased $CD68^+$ cell numbers showed better progression-free survival (PFS) (P = 0.004; Fig. 3A) and overall survival (OS) (P = 0.021; Supplementary Fig. S3A). In contrast, an increased

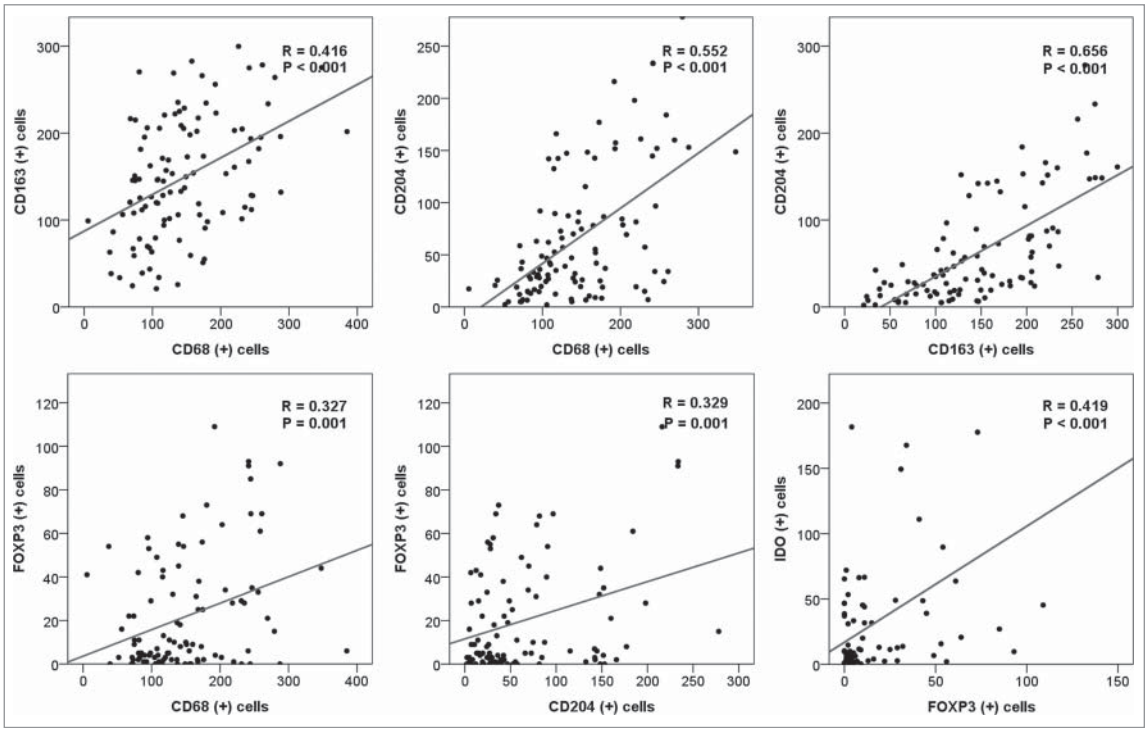

Figure 2. Correlations between the tumor-infiltrating $CD68^+$, $CD163^+$, $CD204^+$, $FOXP3^+$, and IDO^+ cells in primary CNS-DLBCL. The counts of $CD68^+$, $CD163^+$, $CD204^+$, $FOXP3^+$, and IDO^+ cells for each case were plotted, and correlations between the values were analyzed.

Table 2. Comparison of tumor-infiltrating $CD68^+$ cells, $CD163^+$ cells, $CD204^+$ cells, $FOXP3^+$ cells, and IDO^+ cells between primary CNS-DLBCL and systemic DLBCL

Variables	Primary CNS (n = 114)	Systemic (n = 165)	P
Age†† (n (%))			
≤60 years	53 (46.5)	68 (41.2)	n.s.
> 60 years	61 (53.5)	97 (58.8)	
Sex†† (n (%))			
Male	71 (62.3)	91 (55.2)	n.s.
Female	43 (37.7)	74 (44.8)	
ECOG performance status* (n (%))			
0, 1	75 (65.8)	125 (76.2)	0.059
≥ 2	39 (34.2)	39 (23.8)	
B symptoms* (n (%))			
Absent	111 (97.4)	125 (77.6)	<0.001
Present	3 (2.6)	36 (22.4)	
LDH* (n (%))			
Normal	67 (62.6)	64 (43.5)	0.003
Elevated	40 (37.4)	83 (56.5)	
Immunophenotype by the Hans classification* (n (%))			
GCB	14 (15.2)	58 (35.8)	<0.001
ABC	78 (84.8)	104 (64.2)	
CD68†			
mean ± SD	145.42 ± 70.55	239.18 ± 77.96	<0.001
CD163†*			
mean ± SD	149.67 ± 67.76	293.99 ± 222.19	<0.001
CD163/CD68†*			
mean ± SD	1.32 ± 1.76	1.21 ± 0.92	n.s.
CD204†*			
mean ± SD	65.51 ± 61.64	122.44 ± 104.20	<0.001
CD204/CD68†*			
mean ± SD	0.46 ± 0.42	0.51 ± 0.43	n.s.
FOXP3†*			
mean ± SD	21.44 ± 26.24	96.18 ± 109.95	<0.001
IDO†*			
mean ± SD	30.88 ± 53.21	112.64 ± 117.27	<0.001

SD, standard deviation; ECOG, Eastern Cooperative Oncology Group; LDH, lactate dehydrogenase; GCB, germinal center B cell-like; ABC, activated B cell-like; n.s., not significant; †These variables were compared using Student's t-test; ††These variables were compared using χ^2 tests.
*These variables contain missing values.

number of $CD204^+$ cells and a higher ratio of $CD204^+/CD68^+$ cells were associated with worse PFS (P = 0.020 and 0.063, respectively; Fig. 3B, C). Patients with a higher ratio of $CD163^+/CD68^+$ cells also showed significantly poor OS (P = 0.029; data not shown). An increased number of $FOXP3^+$ cells tended to be related to prolonged OS (P = 0.057; Supplementary Fig. S4B). An increased number of IDO^+ cells was associated with significantly longer PFS (P = 0.019; Fig. 3D) and tended to be related to prolonged OS (Supplementary Fig. S3D).

When considering the quantities of IDO^+ cells and TAMs (Fig. 4), patients with a low number of IDO^+ cells combined with a low number of $CD68^+$ cells, a high number of $CD204^+$ cells, or a high ratio of $CD204^+/CD68^+$ cells showed the worst PFS (P < 0.001, = 0.002, and 0.002, respectively; Fig. 4A, C, E). The prognostic significance of the combination of IDO^+ with TAM or $CD204^+$ cell numbers was maintained in patients treated with combined MVP and radiotherapy (Fig. 4B, D, F). Patients with high numbers of both IDO^+ cells and $FOXP3^+$ cells showed a tendency towards better PFS and OS (Supplementary Fig. S4C, D). The above associations between TAMs, M2 macrophages, Tregs, IDO^+ cells and the survival of patients with primary CNS-DLBCL were maintained in ABC group (Supplementary Fig. S6).

In an independently evaluated validation cohort (n = 40) composed of patients with primary CNS-DLBCL and homogeneously treated with rituximab-MVP (Supplementary Table S2), comparable findings were observed in terms of prognostic significance of $CD68^+$, $CD204^+$ and IDO^+ cell counts and the ratio of $CD204^+/CD68^+$ cells (Fig. 5 and Supplementary Fig. S7). Briefly, increased $CD68^+$ cell numbers were related with better PFS (P = 0.030; Fig. 5A), whereas a higher ratio of $CD204^+/CD68^+$ cells was associated with worse PFS (P = 0.017; Fig. 5C). Patients with high $CD204^+$ cells or low IDO^+ cells also tended to have poor prognosis, although statistical significance was not reached (Fig. 5B, D).

Multivariate survival analysis of tumor-infiltrating TAMs, M2 TAMs, Tregs, and IDO^+ cells in patients with primary CNS-DLBCL

To further examine the prognostic implications of TAMs, M2 TAMs, and IDO^+ cells in primary CNS-DLBCL, we performed univariate and multivariate survival analyses using the Cox proportional hazard model. As summarized in Supplementary Table S3, univariate Cox analysis revealed that the numbers of $CD68^+$ cells and $CD204^+$ cells, the ratio of $CD204^+/CD68^+$ cells, and the number of IDO^+ cells were predictive of PFS. In multivariate Cox regression analysis, integrating risk factors including the LDH level and multifocal disease, a low $CD68^+$ cell number, a high $CD204^+$ cell number, and a low IDO^+ cell number were independent predictors of poor PFS (P = 0.017, 0.032, and 0.035, respectively; Supplementary Table S4). When incorporating the Nottingham/Barcelona score, a low $CD68^+$ cell number and low IDO^+ cell number were independent poor prognostic factors for poor PFS (P = 0.011 and 0.030, respectively; Supplementary Table S4).

When incorporating the numbers of IDO^+ cells and $CD68^+$ or $CD204^+$ cells, a low IDO^+ cell number combined with a low $CD68^+$ cell number, high $CD204^+$ cell number, or a high $CD204^+/CD68^+$ cell ratio was found to be an independent predictor of poor PFS (Table 3).

Discussion

In this study, we demonstrated that the numbers of tumor-infiltrating immune cells affect the clinical outcome of patients with primary CNS-DLBCL. Briefly, an increased number of $CD68^+$ TAMs and an increased number of IDO^+ cells were associated with a favorable prognosis, whereas an increased number of $CD204^+$ cells and a high ratio of $CD204^+/CD68^+$ cells, suggestive of M2 polarization, were associated with a poor prognosis in primary CNS-DLBCL.

In this study, the numbers of tumor-infiltrating immune cells, including $CD68^+$ TAMs, $CD163^+$ or $CD204^+$ M2 TAMs, $FOXP3^+$ Tregs, and IDO^+ cells, were significantly lower in primary CNS-DLBCL than in systemic DLBCL. Supporting this, it was recently reported that infiltration of immune cells, including dendritic cells and effector or cytotoxic T cells, was decreased in CNS-DLBCL compared with

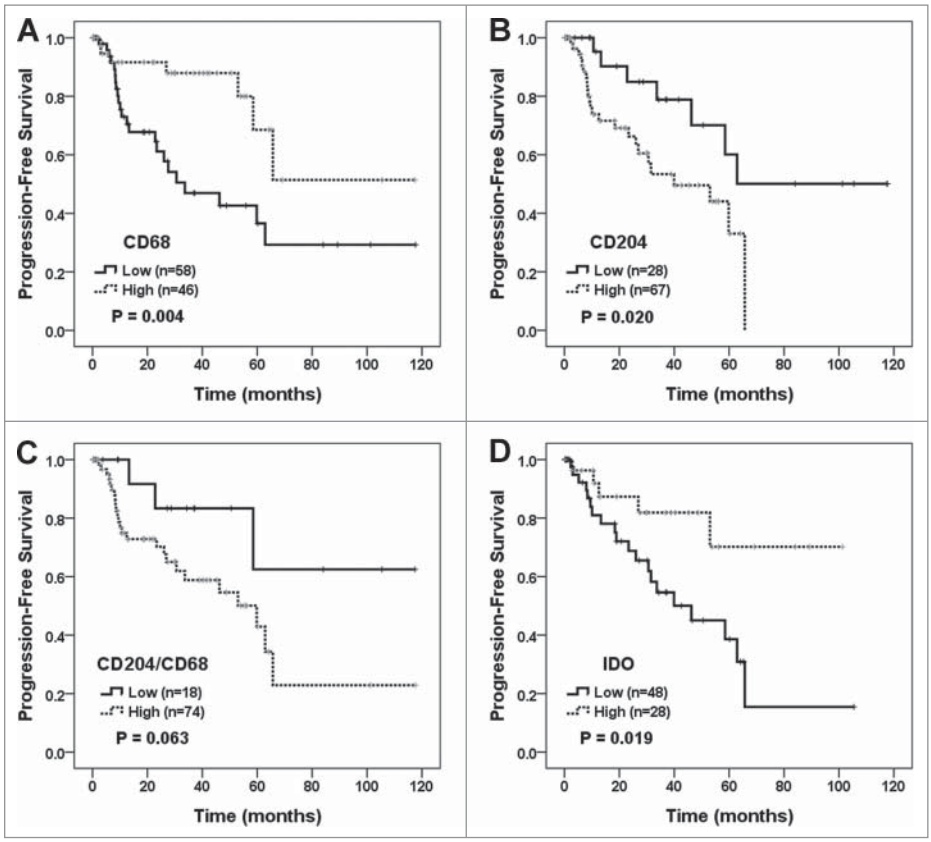

Figure 3. Survival analysis according to the numbers of tumor-infiltrating $CD68^+$, $CD204^+$, and IDO^+ cells and the ratio of $CD204^+/CD68^+$ cells. PFS of primary CNS-DLBCL patients were evaluated according to the tumor-infiltrating $CD68^+$ cell number (A), $CD204^+$ cell number (B), the ratio of $CD204^+/CD68^+$ cells (C), and IDO^+ cell number (D). Kaplan-Meier curves are shown with P values generated by log-rank test.

non-CNS-DLBCL.[29] The BBB separates the CNS from the systemic circulation, and thus the recruitment of circulating immune cells is relatively restricted,[26] which may account for the decreased number of tumor-associated immune cells in primary CNS-DLBCL. However, the ratio of $CD163^+/CD68^+$ cells or $CD204^+/CD68^+$ cells, suggestive of M2 polarization of TAMs, was not altered compared with systemic DLBCL, suggesting that the qualitative composition of TAMs may actually be similar between primary CNS and systemic DLBCLs.[27,28]

In chronic lymphocytic leukemia (CLL), *MYD88*-mutated CLL cells secreted higher levels of cytokines, involving recruitment of macrophages and T cells, upon toll-like receptor stimulation, compared with *MYD88*-unmutated CLL cells.[30] Although the frequencies of *MYD88* and *CD79B* mutation detected by direct sequencing using formalin-fixed, paraffin-embedded (FFPE) tissue in this study were lower than those previously reported in PCNSL,[6] the statuses of tumor-infiltrating immune cells were not significantly different according to the *MYD88* and *CD79B* mutation status in primary CNS-DLBCL.

Increases in TAM numbers have been associated with poor prognoses in patients with solid tumors.[13] This prognostic relationship was also observed in several types of malignant lymphomas, including follicular lymphoma and Hodgkin lymphoma.[31,32] We previously reported that an increased $CD163^+$ cell number and a high $CD163^+/CD68^+$ cell ratio predicted a poor prognosis in patients with systemic DLBCL treated with R-CHOP.[27] An increase in the IDO^+ cell number was associated with a favorable prognosis in patients with systemic DLBCL.[28] Moreover, an integrative analysis of IDO and TAM statuses revealed that a low IDO^+ cell count combined with either a high $CD163^+$ cell count or a high $CD163^+/CD68^+$ cell ratio was a strong independent predictor of a poor prognosis in systemic DLBCL.[28] In this study, an increased $CD204^+$ cell number and a higher ratio of $CD204^+/CD68^+$ cells were predictors of a poor prognosis in patients with primary CNS-DLBCL. These findings indicate that M2 TAMs may contribute to lymphoma progression and, consequently, poor prognosis in patients with primary CNS-DLBCL. However, the M2 marker having prognostic significance was different between primary CNS-DLBCL (CD204) and systemic DLBCL (CD163), suggesting a need to use multiple markers when evaluating TAMs. In an intracranial B-cell lymphoma xenograft model, expression of interleukin-4 by malignant B cells potentiated B-cell survival and mediated the polarization of TAMs to M2 TAMs, which was associated with enhanced tumorigenesis.[33] In contrast, a favorable prognostic implication of increased $CD68^+$ TAMs might suggest their lymphoma-suppressive function in primary CNS-DLBCL, a finding consistently observed in systemic DLBCL.[27] Together, these findings suggest that both the phenotype and number of TAMs may influence the prognosis of patients with primary CNS-DLBCL. Targeting TAM using various approaches are under investigation for cancer

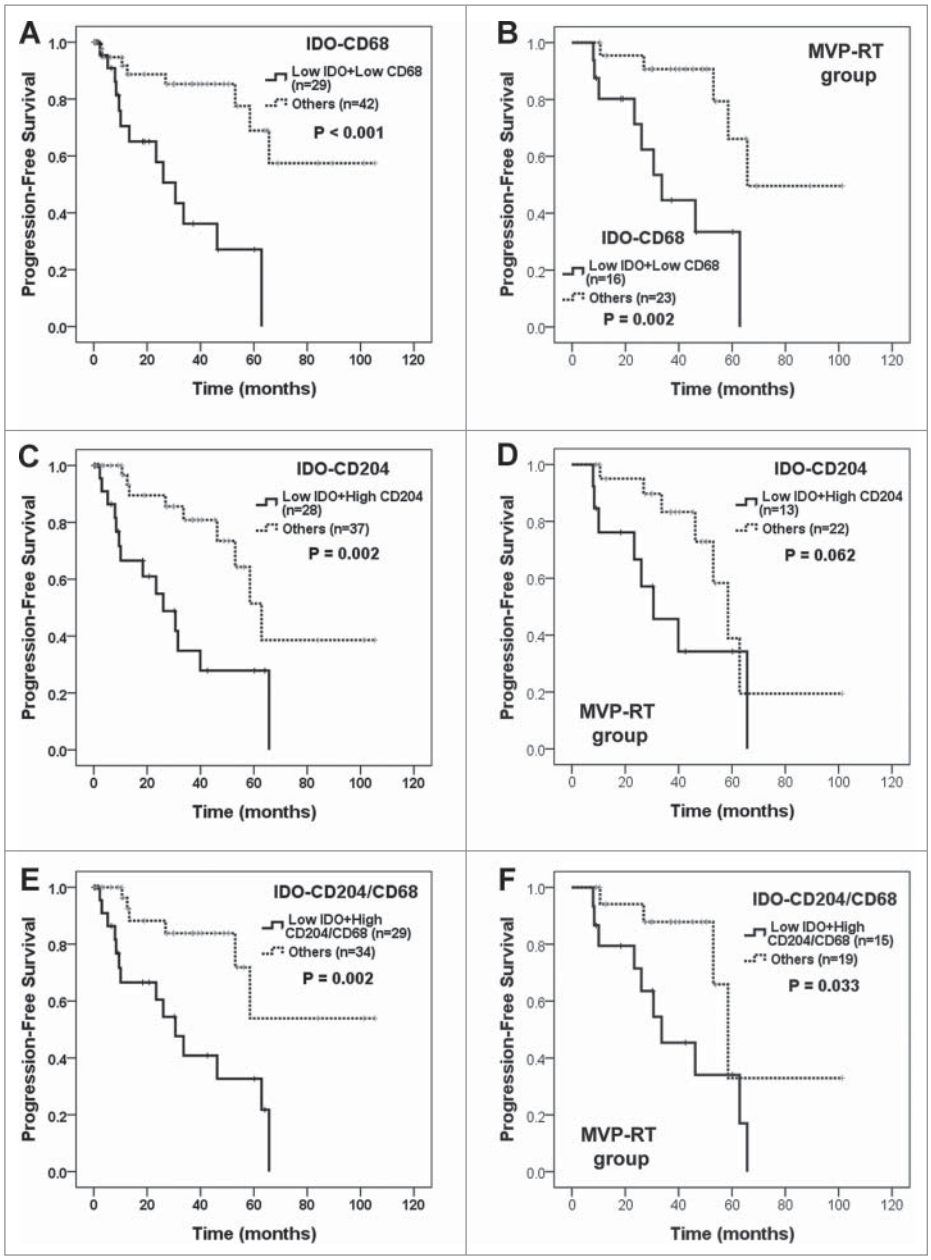

Figure 4. Survival analysis of primary CNS-DLBCL patients according to the combination of IDO$^+$, CD68$^+$, and CD204$^+$ cell numbers and the CD204$^+$/CD68$^+$ cell ratio. Primary CNS-DLBCL patients were classified into two groups: (A and B) low IDO$^+$ and CD68$^+$ cell numbers versus others, (C and D) low IDO$^+$ and high CD204$^+$ cell numbers versus others, (E and F) low IDO$^+$ cell number and high ratio of CD204$^+$/CD68$^+$ cells versus others. Kaplan-Meier curves for PFS in all patients (A, C and E) and in those treated with combined MVP and radiotherapy (MVP-RT) (B, D and F) are shown with P values generated by log-rank test.

therapy, including depletion of TAM, suppression of M2 polarization, and repolarization of TAM from M2 to M1 phenotype.[34] Moreover, reprogramming of M2 TAM into M1 TAM enhanced the efficacy of immune checkpoint blockade in preclinical model.[35] This study support that targeting M2 TAM may have clinical relevance combined with conventional therapy or immunotherapy in primary CNS-DLBCL.

In a study using NHL tissues, FOXP3 levels were increased markedly alongside elevated IDO levels, suggesting that upregulation of IDO in NHL tissues induces local immune tolerance by favoring development and infiltration of FOXP3$^+$ Tregs.[36] Consistently, in this study, the numbers of IDO$^+$ cells and Tregs showed a positive correlation with each other in primary CNS-DLBCL. IDO contributed to tumor progression and metastasis *in vivo*, and increased IDO expression was associated with a poor prognosis in patients with solid tumors.[37] In this study, an increased number of tumor-infiltrating IDO$^+$ cells was associated with a favorable prognosis in patients with primary CNS-DLBCL. Consequently, a decrease in the IDO$^+$ cell number and a concomitant increase in the CD204$^+$ M2 macrophage number in the tumor microenvironment were significant predictors of a poor prognosis in patients with primary CNS-DLBCL. These findings were also consistent with those observed in systemic DLBCL.[28] The seemingly paradoxical association of increased IDO$^+$ cell numbers with a favorable prognosis in primary CNS-DLBCL may be explained by

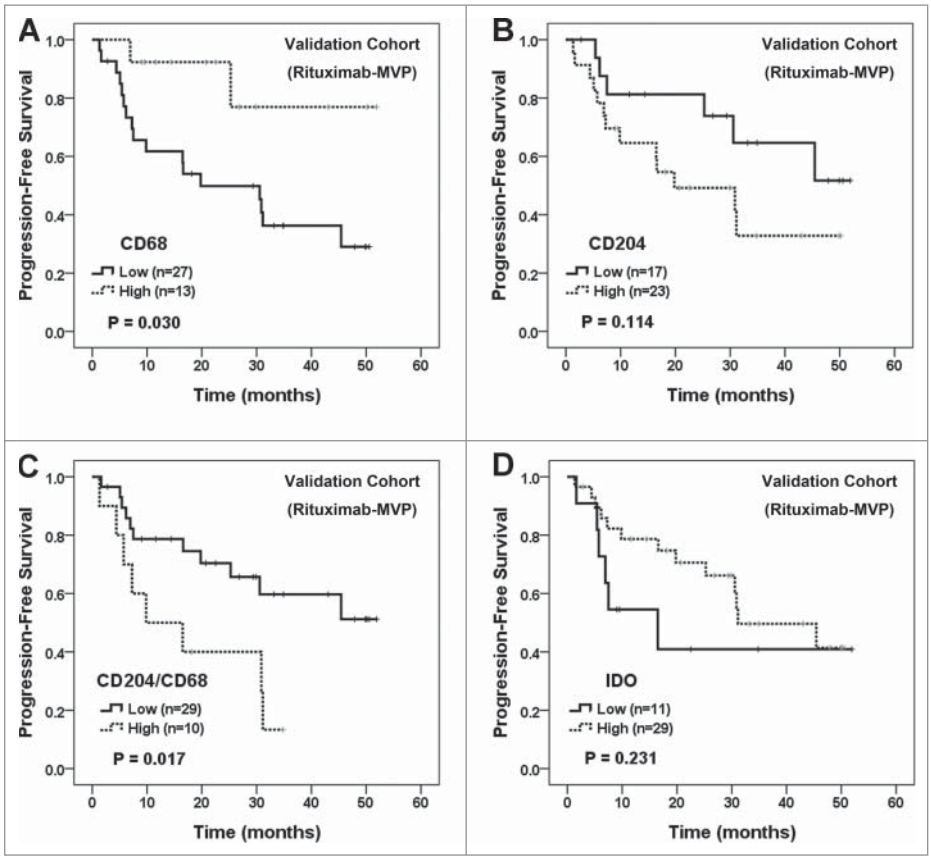

Figure 5. Survival analysis of independent validation cohort of primary CNS-DLBCL patients treated with rituximab-MVP according to $CD68^+$, $CD204^+$, and IDO^+ cells and the ratio of $CD204^+/CD68^+$ cells. PFS of patients were evaluated according to the tumor-infiltrating $CD68^+$ cell number (A), $CD204^+$ cell number (B), the ratio of $CD204^+/CD68^+$ cells (C), and IDO^+ cell number (D). Kaplan-Meier curves are shown with P values generated by log-rank test.

previous reports that IDO can suppress B cells in addition to T cells and induce growth arrest and apoptosis of lymphoma B cells.[38,39] This hypothesis needs to be addressed by further *in vitro* and *in vivo* studies.

Only a few previous studies have reported the status of tumor-infiltrating immune cells in primary CNS-DLBCL. In one, the numbers of $CD68^+$, $CD163^+$, and $CD204^+$ TAMs in primary CNS-DLBCL were not correlated with prognosis.[40] However, decreased numbers of tumor-infiltrating $S100^+$ dendritic cells and granzyme B^+ cytotoxic T cells were related to poor prognoses in patients with PCNSL,[29] suggesting a prognostic role for the tumor microenvironment in PCNSL. Our present study also suggests that TAMs, Tregs, and IDO may influence the biology of primary CNS-DLBCL cooperatively and have prognostic implications. Furthermore, our study has some additional merits in that a relatively large number of patients was included, automatic cell counting was used rather than manual counting, and diverse tumor-infiltrating immune cells were analyzed together. Of note, the combined status of immune cells, rather than their individual statuses, showed stronger association with prognosis in primary CNS-DLBCL. Specifically, combined low IDO^+ with low $CD68^+$ cell numbers and a combined low IDO^+ cell number with high $CD204^+/CD68^+$ cell ratio were related with a poor prognosis.

This study has some limitations. First, it was a retrospective study, and thus the treatment modalities of the patients were not homogeneous. However, subgroup analyses according to treatment modality demonstrated that the prognostic significance of TAMs, M2 macrophages, and IDO^+ cells was maintained; particularly, combined low IDO^+ with low $CD68^+$ cell numbers and a combined low IDO^+ cell numbers with high $CD204^+/CD68^+$ cell ratio were significantly associated with poor prognoses in patients homogeneously treated with combined MVP chemotherapy and radiotherapy (Fig. 4 and Supplementary Fig. S5). Moreover, in an independently evaluated validation cohort of patients treated with rituximab-MVP, low $CD68^+$ cell numbers and high $CD204^+/CD68^+$ cell ratio were significantly related with poor prognosis (Fig. 5). Second, the prognostic significance of immune cells was observed mainly in terms of PFS, not OS, which may restrict the value of immune cells as a prognostic marker but instead help establish management strategies for patients. Third, some of the patients were administered with steroid before biopsy/surgery, which could affect the viability of tumor cells and immune responses. Fourth, because we did not evaluate the entire area of tumor due to small biopsied samples and capturing representative areas, the heterogeneous distribution of infiltrating immune cells in the tumor could not be fully addressed in this study.

In summary, a decreased number of $CD68^+$ cells, an increased number of $CD204^+$ cells or an increased ratio of $CD204^+/CD68^+$ cells, and a decreased number of IDO^+ cells

Table 3. Multivariate analysis of PFS according to clinicopathological parameters and combined IDO$^+$ cell and TAM numbers in patients with primary CNS-DLBCL

	PFS		
Variables	HR	95% CI	P
Low IDO$^+$ and CD68$^+$ cell numbers			
Comparison with risk factors			
Normal LDH	3.586	1.187–10.837	0.024
Multifocal disease	1.091	0.370–3.215	0.875
Low IDO$^+$ and CD68$^+$ cell numbers	7.618	2.506–23.160	<0.001
High CD204$^+$ cell numbers	3.223	1.043–9.958	0.042
Comparison with Nottingham/Barcelona score			
Nottingham/Barcelona score 3	3.785	1.170–12.246	0.026
Low IDO$^+$ and CD68$^+$ cell numbers	6.276	2.324–16.945	<0.001
High CD204$^+$ cell numbers	2.151	0.772–5.993	0.143
Low IDO$^+$ and high CD204$^+$ cell numbers			
Comparison with risk factors			
Normal LDH	2.923	1.017–8.399	0.046
Multifocal disease	1.542	0.474–5.018	0.472
Low IDO$^+$ and high CD204$^+$ cell numbers	3.429	1.143–10.287	0.028
Low CD68$^+$ cell numbers	3.156	1.075–9.267	0.037
Comparison with Nottingham/Barcelona score			
Nottingham/Barcelona score 3	4.016	1.244–12.971	0.020
Low IDO$^+$ and high CD204$^+$ cell numbers	3.251	1.314–8.045	0.011
Low CD68$^+$ cell numbers	3.329	1.168–9.484	0.024
Low IDO$^+$ cell number and high CD204$^+$/CD68$^+$ cell ratio			
Comparison with risk factors			
Normal LDH	3.213	1.129–9.146	0.029
Multifocal disease	1.369	0.486–3.856	0.552
Low IDO$^+$ cell number and high CD204$^+$/CD68$^+$ cell ratio	4.290	1.529–12.040	0.006
Comparison with Nottingham/Barcelona score			
Nottingham/Barcelona score 3	5.187	1.609–16.716	0.006
Low IDO$^+$ cell number and high CD204$^+$/CD68$^+$ cell ratio	4.730	1.786–12.524	0.002

PFS, progression-free survival; HR, hazard ratio; CI, confidence interval; LDH, lactate dehydrogenase.

were associated with a poor prognoses in patients with primary CNS-DLBCL. An evaluation of the combined statuses of IDO and TAM may be helpful for risk stratification of patients with primary CNS-DLBCL. This study also provides valuable information for developing therapeutic strategies targeting TAMs and IDO in patients with primary CNS-DLBCL.

Materials and methods

Patients

In total, 114 patients who were diagnosed with primary CNS-DLBCL at Seoul National University Hospital (SNUH) between 1996 and 2012 were included in this study. Pathological materials were reviewed by hematopathologists (NSJ, YKJ, and CWK) according to current World Health Organization criteria.[41] Clinical data were obtained from the medical records, reviewed by hemato-oncologists (EL, TMK, and DSH). The follow-up periods ranged from 0.2 to 178 months, with a median of 31.35 months.

As an independent validation cohort, a total of 40 patients who were diagnosed at SNUH between 2013 and 2015, and homogeneously treated with rituximab-MVP, were collected. The age of patients ranged from 30 to 81 years and the follow-up duration was from 3 to 55.43 months.

This study followed the World Medical Association Declaration of Helsinki recommendations and the institutional review board at SNUH approved this study (No. 1012-053-344).

Immunohistochemistry

Whole sections of representative FFPE tumor tissue blocks were submitted for immunohistochemistry. The immunohistochemical subgroup of DLBCL was determined to be ABC or GCB type according to Hans' criteria.[42] CD68 was used as a marker of TAMs, CD163 or CD204 as a marker of M2 macrophages, and FOXP3 as a marker of Tregs. Immunostaining for CD68 (PG-M1, DakoCytomation, Copenhagen, Denmark), CD163 (10D6, Novocastra, Newcastle Upon Tyne, UK), CD204 (SRA-E5, Transgenic, Kumamoto, Japan) and IDO (1F8.2; Millipore, Billerica, MA, USA) was performed using the Bond-Max autostainer (Leica Microsystems, Melbourne, Australia. Immunostaining for FOXP3 (236 A/E7, Abcam, Cambridge, UK) was performed using the BenchMark XT autostainer (Ventana Medical Systems, Inc., Tucson, AZ, USA).

Double immunostainings for IDO and CD68, IDO and CD204, IDO and CD163, IDO and CD123 (a marker of plasmacytoid dendritic cell) (BR4MS, Novocastra), and IDO and FOXP3 were performed using a BenchMark XT Slide automated system (Ventana Medical Systems, Inc., Tucson, AZ) in representative cases.

Automated quantitation of TAMs, Tregs and IDO$^+$ cells by image analysis

To obtain an unbiased analysis of tumor-infiltrating immune cells with objective and reproducible data, automated enumeration of immune cells was performed using

an image analyzer. Briefly, all immunostained slides were subjected to virtual microscope scanning under high-power magnification using the ScanScope CS2 eSlide (Aperio Technologies, Vista, CA, USA). Three different fields of the intratumoral area, excluding necrotic or squeezed area, were captured from virtual microscopic images, and the numbers of $CD68^+$, $CD163^+$, $CD204^+$, and IDO^+ cells were counted using ImageJ software (National Institutes of Health, Bethesda, MD, USA), as described previously.[27] $FOXP3^+$ cells were enumerated using the nuclear v9 algorithm of the ImageScope software (Aperio Technologies). The average numbers of positive cells per unit area (0.28 mm^2) were calculated from values obtained from three areas for each case and used for further statistical analyses.

Direct sequencing for MYD88 and CD79B

Genomic DNA was extracted from 10 μm-thick sections of FFPE tumor tissue using a Maxwell 16 FFPE Plus Tissue LEV DNA Purification kit (Promega, Madison, WI, USA). *MYD88* exon 5 and *CD79B* exon 5 were amplified by PCR using the following primers: for *MYD88*, forward 5′-CTGGGGTTGAAGACTGGGCT-3′ and reverse 5′-TTGGTGTAGTCGCAGACAGTGA-3′; for *CD79B*, forward 5′-GGGCTGGGGGACACTAACACTC-3′ and reverse 5′-TGGGTGCTCACCTACAGACCAC-3′. The PCR reactions were performed with EconoTaq PLUS GREEN 2X premix (Lucigen, Middleton, WI, USA) and conditions of 95°C for 5 min followed by 38 cycles of 95°C for 30 seconds, 60°C for 30 seconds and 72°C for 30 seconds. After purifying the PCR products, bi-directionally Sanger sequencing was performed using the ABI3730 DNA analyzer (Applied Biosystems, Carlsbad, CA, USA).

Statistical analysis

All statistical analyses were performed using SPSS (ver. 21; IBM Corp., New York, NY, USA) and R' software (ver. 2.14.0; The R Foundation for Statistical Computing). Non parametric Wilcoxon rank-sum test was used to assess differences in the numbers and ratios of immune cells according to the clinicopathological variables. For survival analysis, we fitted a Kaplan-Meier model after dichotomizing the cases into two groups by cut-off values. The values that maximized the survival difference, including OS and PFS, between the groups according to log-rank test were chosen as the cut-off values. This approach resulted in the following cut-off values: 145.00 for the $CD68^+$ cell number, 120.00 for the $CD163^+$ cell number, 0.81 for the $CD163^+/CD68^+$ cell ratio, 27.4 for the $CD204^+$ cell number, 0.169 for the $CD204^+/CD68^+$ cell ratio, 24.00 for the $FOXP3^+$ cell number, and 16.00 for the IDO^+ cell number. Univariate and multivariate survival analyses were performed using Cox-proportional hazard models. A two-sided P value < 0.05 was considered statistically significant in all analyses.

Abbreviations

ABC	activated B cell-like
BBB	blood brain barrier
CSF	cerebrospinal fluid
CNS	central nervous system
DLBCL	diffuse large B-cell lymphoma
ECOG	Eastern Cooperative Oncology Group
GCB	germinal center B cell-like
IDO	indoleamine 2,3-dioxygenase
IELSG	International Extranodal Lymphoma Study Group
IT-MTX	intrathecal methotrexate
LDH	lactate dehydrogenase
MVP	combined chemotherapy regimen of high-dose methotrexate, vincristine and procarbazine
NHL	non-Hodgkin lymphoma
OS	overall survival
PCNSL	primary CNS lymphoma
PFS	progression-free survival
R-CHOP	combined immunochemotherapy regimen of rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisolone
TAM	tumor-associated macrophage

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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ORCID

Dohee Kwon http://orcid.org/0000-0002-4725-3852
Yoon Kyung Jeon http://orcid.org/0000-0001-8466-9681

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