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Prognostic implications of tumor-infiltrating immune cells in primary diffuse large B-cell lymphoma of the central nervous system

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종양관련면역세포의 임상, 병리학적,
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원발성 중추신경 광범위 큰B세포 림프종에서 종양관련 면역세포의 임상, 병리학적, 예후적 의미

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Prognostic implications of tumor-infiltrating immune cells in primary diffuse large B-cell lymphoma of the central nervous system

by

Soo Jeong Nam

(Directed by Prof. Chul Woo Kim, M.D., Ph.D.)

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Abstract

Prognostic implications of tumor-infiltrating immune cells in primary diffuse large B-cell lymphoma of the central nervous system

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Background: Primary diffuse large B-cell lymphoma (DLBCL) of the central nervous system (CNS) is a distinct clinicopathologic entity having poor prognosis. Tumor-associated macrophages/microglial cells (TAMs) and regulatory T-cells (Tregs) play an important role in tumor microenvironment. Macrophages can differentiate to M1 or M2 phenotype. However, little has been known about prognostic factors and role of tumor-infiltrating immune cells in primary CNS DLBCL and systemic DLBCL. Thus I investigated the prognostic implications of tumor-associated macrophages (TAMs) and regulatory T-cells (Tregs) in primary CNS DLBCL and systemic DLBCL.

Methods: Immunohistochemistry was performed for CD68, CD163, CD204, and FOXP3 in 129 primary CNS DLBCL and 165 DLBCL cases. The number of positive cells was determined using image analyzer after virtual microscope scanning.

Results: All of tumor-associated immune cells, tumor-infiltrating CD68 (+) TAMs, CD163 (+) or CD204 (+) M2 macrophages, and FOXP3 (+) Tregs were significantly decreased in primary CNS DLBCL compared with systemic DLBCL. However, the ratio of CD163/CD68 (+) cells or CD204/CD68 (+) cells had no significant difference according to primary site. CD163 (+) cells were tend to greater in primary CNS DLBCL associated with advanced ECOG performance status (PS, $P = 0.052$). FOXP3 (+) cells were decreased in advanced PS ($P = 0.015$), decreased IELSG prognostic index ($P = 0.011$), decreased Nottingham/Barcelona score ($P = 0.010$) and had a tendency to decrease in elevated CSF protein ($P = 0.064$). In systemic DLBCL, CD68 (+), CD163 (+) and CD204 (+) cells and the ratio of CD163/CD68 (+) and CD204/CD68 (+) cells were significantly greater in systemic DLBCLs associated with EBV ($P = 0.005$, 0.046, 0.028, 0.004 and 0.007, respectively). CD68 (+) cells were increased in systemic DLBCL patients with the age of ≤ 60 years ($P = 0.015$). Survival analysis was performed among 100 primary CNS DLBCL patients. An increase in CD68 (+) cells was significantly associated with prolonged overall survival (OS) and progression-free survival (PFS) ($P = 0.014$ and 0.001, respectively). In contrast, an increase of CD204 (+) cells was related with shorter OS and PFS ($P = 0.057$ and 0.012, respectively). In multivariate

analysis, a decreased CD68 (+) cells and increased CD204 (+) cells were independent predictors of shorter PFS. In 109 DLBCLs treated with R-CHOP, an increase in CD68 (+) cells was related to improved OS ($P = 0.033$). By contrast, an increased number of CD163 (+) cells and a higher ratio of CD163/CD68 (+) cells were significantly associated with shorter OS ($P = 0.041$ and 0.003) and PFS ($P < 0.001$ and 0.002). DLBCL patients with increased Tregs tended to have better prognosis. In multivariate analysis, an increased ratio of CD163/CD68 (+) cells was an independent predictor of shorter OS and PFS.

Conclusions: These results suggest that M2 macrophages might have a lymphoma-promoting function in both primary CNS DLBCL and systemic DLBCL, and predict poor clinical outcome. In contrast, CD68 (+) TAMs might have a lymphoma-suppressor function in primary CNS DLBCL and systemic DLBCL treated with R-CHOP, and predict favorable clinical outcome. CD68 (+) TAMs plays an opposite pro-tumoral function in systemic DLBCL in absence of rituximab. The analysis of TAM profiles could be helpful for predicting prognosis of primary CNS and systemic DLBCL patients.

Key words: tumor microenvironment, tumor-associated macrophages, M2 macrophages, primary central nervous system lymphoma, diffuse large B-cell lymphoma, regulatory T-cells

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Introduction

Diffuse large B-cell lymphoma (DLBCL) is the most common malignant lymphoma worldwide accounting for about 30-40% of non-Hodgkin lymphoma. DLBCL is a biologically and clinically very heterogeneous tumor with variable clinical outcomes. Therefore many efforts have been made to classify this entity into more homogenous subgroup, as representative by molecular subtyping into germinal center B-cell-like (GCB) and non-GCB/ activated B-cell-like (ABC) DLBCL reflecting cell of origin (1). In addition, several unique clinicopathologic entities or variants such as DLBCL of central nervous system (CNS) (2).

Primary central nervous system lymphoma (PCNSL) is rare, accounting for only 2–3% of non-Hodgkin lymphoma cases and one of the most aggressive malignant lymphomas. More than 90% of PCNSLs are DLBCL (3). It originates in the brain, spinal cord, or eyes and rarely spreads outside the CNS.

A tumor microenvironment plays an important role in the biology of tumors, and is comprised of proliferating tumor cells, the tumor stroma, blood vessels, infiltrating inflammatory cells and a variety of associated tissue cells. A tumor microenvironment acts as loss of interconnection with the microenvironmental network or dysfunction of interactions with the supportive stroma that provide the malignant cells with growth and drug-resistance signals. In lymphoid malignancy,

the interactions between the malignant cells and the microenvironment are coexisting and largely resemble the pattern that the normal counterpart B cells engage in with their respective microenvironment (4).

Immune cells play a variable and important role in tumor biology by regulating immune responses and shaping the pro- or anti-tumorigenic nature of the tumor microenvironment. Tumor-associated macrophages/microglial cells (TAMs) produce growth factors, cytokines, and proteases that initiate tumorigenesis, enhance tumor progression, promote angiogenesis and metastasis. Accordingly, increases in TAMs have been associated with poor prognosis in solid tumor patients (5). This prognostic relationship has also been observed in several types of malignant lymphomas, including follicular lymphoma (6-8), Hodgkin lymphoma (9, 10), and mature T and NK-cell lymphomas (11-14).

Monocytes can be differentiated into two functionally distinctive subtypes of macrophages, *i.e.*, M1 and M2 TAM, depending on the cytokine milieu during inflammation or tumor development and progression. M1 and M2 TAM express different cell surface molecules and produce different cytokines. In brief, classically activated or M1 TAM produce pro-inflammatory cytokines and primarily function as effector cells that kill invading pathogens. They also exhibit a tumor-suppressive function and stimulate the anti-tumor immune response. By contrast, alternatively activated or M2 TAM are differentiated by Th2 cytokines such as IL-4, IL-13 and TGF-β and characterized by high expression of the mannose receptor and scavenger

receptors, including CD163 and CD204. They secrete a large amount of IL-10, CCL17, and CCL20, and are involved in wound healing and promote tumor cell survival, invasion, metastasis, and angiogenesis. M2 TAM also play an immunosuppressive role by down-regulating anti-tumor immune responses mediated by M1 and Th1 cells and recruiting and activating Tregs and Th2 cells (15). TAMs tend to be skewed to the M2 phenotype in many solid tumors, thereby contributing to tumor progression and poor patient prognosis (16). However, a few reports are available on the prognostic implication of TAMs in the context of M1 and M2 TAM in malignant lymphoma (13, 17, 18).

Tregs inhibit many immune cells including T-, B-, NK-cells, and antigen presenting cells via variable mechanisms, and are characterized by CD4(+)CD25 high expression on the cell surface and FOXP3 expression in the nucleus (19). In solid tumors, Tregs in tumor microenvironment hamper the effective anti-tumor immunity of host, thereby contributing to tumor growth and progression, and a poor prognosis of patients. However, in hematolymphoid malignancies, increased quantity of tumor-infiltrating Tregs was rather associated with favorable outcome of patients with follicular lymphoma, Hodgkin lymphoma, and extranodal NK/T-cell lymphoma (20-23). However, the tumor-infiltrating Tregs in DLBCL showed conflicting influence on the patients' prognosis (24). Moreover considering the immunologic function of macrophages and Tregs, it'd be likely that crosstalk between TAM and Tregs may affect the constitution of tumor microenvironment and the biology of tumor cells. However, there has been no comparative analysis of

TAMs and Tregs in DLBCL or PCNSL.

CNS 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. Microglia are essential components of CNS innate immunity, immune defense mechanisms independent of antigens, and can respond to environmental or endogenous insults by changing the balance between pro-inflammatory and anti-inflammatory macrophages (25).

Recent studies of genome-wide gene expression in PCNSL compared with non-CNS DLBCL indicate that PCNSL associated with specific microenvironmental features to be distinguished from non-CNS DLBCL. The extracellular matrix- and adhesion-related pathways may determine some of the biologic characteristics of PCNSL (26-28).

Therefore, this study was intended to comprehensively investigate the tumor-infiltrating TAMs, M2 TAM, and Tregs in primary CNS DLBCL and systemic DLBCL tissues.

Materials and Methods

Patients

One hundred fourteen patients who were diagnosed with primary CNS DLBCL and one hundred sixty-five patients who were diagnosed with systemic DLBCL at Seoul National University Hospital (SNUH) between 1996 and 2012 were retrieved excluding primary mediastinal large B-cell lymphoma. Pathological materials were reviewed according to current World Health Organization (WHO) criteria. Clinical data were obtained from the medical records reviewed by hemato-oncologists (T.M.K. and D.S.H). Follow-up periods ranged from 0.2 to 178 months, with a median of 31.35 months. Of the 165 systemic DLBCL patients, 109 were treated with R-CHOP. Overall survival (OS) was measured from the date of diagnosis to the date of death or to the date of the last follow-up visit. Progression-free survival (PFS) was calculated from the date chemotherapy treatment began to the date of disease progression, death, or the last follow-up visit. The Institutional Review Board at SNUH approved this study (1012-053-344).

Immunohistochemistry

Whole section of representative formalin-fixed paraffin-embedded tissue blocks

of primary CNS DLBCL patients' tumor were used for further immunohistochemical analysis. Two-mm-diameter core tissues were taken from representative formalin-fixed paraffin-embedded tissue blocks of systemic DLBCL patients' tumor, and tissue microarrays were manufactured for further immunohistochemical analysis.

To determine immunohistochemical subgroups of cases of systemic DLBCL using Choi classifications, immunostaining for CD10, bcl-6, MUM1, GCET1 and FoxP1 was performed with a Bond-Max autostainer (Leica Microsystems, Wetzlar, Germany) under the following conditions: CD10 (56C6, ER2, ready-to-use; Novocastra, Newcastle Upon Tyne, UK); bcl-6 (LN22, ER2, 1:300; Novocastra); MUM1 (MUM1P, bond, 1:100; Dako, Glostrup, Denmark); GCET1 (*P*olyclonal, ER2, 1:50; Abcam, Cambridge, UK); FoxP1 (*P*olyclonal, bond ER2 1:2000; Abcam). Using immunohistochemistry results, Choi algorithms were applied to each case. Immunohistochemistry for GCB or non-GCB/ABC phenotype was interpreted as positive and negative, with cut-off values according to criteria from previous studies as follows: GCET1, MUM1 and FoxP1, 80% cut-off , and CD10 and bcl-6, 30% cut-off..

CD68 was used as a TAM marker, CD163 or CD204 were used as M2 TAM markers, and FOXP3 was used as a Tregs marker. Immunohistochemical staining for CD68, CD163 and CD204 was performed using Bond-Max autostainer (Leica Microsystems, Wetzlar, Germany) and the following conditions: CD68 (*P*G-M1, ER1, 1:50, DakoCytomation, Copenhagen, Denmark), CD163 (10D6, ER2, 1:200,

Novocastra, Newcastle Upon Tyne, UK), and CD204 (SRA-E5, ER2, 1:200, Transgenic, Kumamoto, Japan). Immunohistochemical staining for FOXP3 was performed using a BenchMark XT Slide Preparation System (Ventana Medical Systems, Inc., Tucson, AZ, USA) and FOXP3 (236A/E7, Tris/EDTA, 1:50, Abcam, Cambridge, UK).

Quantitative analysis of TAMs and Tregs by image analysis

All immunostained slides were submitted to virtual microscope scanning under high-power magnification (x200) using ScanScope CS2 eSlide (Aperio Technologies, Vista, CA, USA). For enumeration of immune cells, three different fields were captured from virtual microscopic images (an area of 0.28 mm2), excluding necrotic or squeezed area. The numbers of CD68, CD163, and CD204 (+) cells were counted using ImageJ software (National Institutes of Health, Bethesda, MD, USA). To establish the optimal setting for automatic enumeration, I calibrated and educated the ImageJ program using a series of ten reference cases by comparing the data with manual counts within 90 percent accuracy. The reference cases included four CD68-, three CD163- and three CD204-stained cases. Accuracy values for each cases was 96.2% (101/105; automatic count/manual count), 95.2% (118/124), 99.1% (139.67/141), 96.1% (182.67/190), 95.2% (190.33/200), 107.1% (220.67/206), 107.8% (239.33/222), 104.8% (273.5/261), 108.6% (316/291), and 109.2% (420.33/385). The average numbers of positive cells per unit area (0.28

mm2) were calculated from values obtained from three areas for each case and used for further statistical analysis.

The number of FOXP3 (+) cells was estimated using the nuclear v9 algorithm of ImageScope software (Aperio Technologies). The counts of positive nuclei in unit area were used for statistical analysis.

Statistical analysis

All statistical analyses were performed with SPSS 21 (IBM Corp., New York, USA) and R 2.14.0 (The R Foundation for Statistical Computing). Student's t-test was performed to assess the differences in the numbers of CD68 (+), CD163 (+), CD204 (+) and FOXP3 (+) cells as well as the CD163/CD68 (+) cell ratio and the CD204/CD68 (+) cell ratio according to the clinicopathological variables. Survival analysis was performed for 100 primary CNS DLBCL in the absence of rituximab, 109 systemic DLBCL treated with R-CHOP and 51 systemic DLBCL in the absence of rituximab. For this analysis, I fitted a Kaplan-Meier model after dichotomizing the cases into two groups by serial numbers of cut points. The values that maximized the difference mainly between the groups by a log-rank test were chosen as the cut-offs. For this I compared OS and PFS between groups to determine the cut-off values predicting both OS and PFS most well.

This approach resulted in cut-off values of primary CNS DLBCL: 145.00 for

CD68, 120.00 for CD163, 0.81 for the ratio of CD163/CD68, 27.4 for CD204, 0.185 for the ratio of CD204/CD68 and 24 for FOXP3 and cut-off values of both systemic DLBCL treated with R-CHOP and absence of rituximab: 300.33 for CD68, 132.33 for CD163, 0.877 for the ratio of CD163/CD68, 219 for CD204, 0.7 for the ratio of CD204/CD68 and 27.67 for FOXP3 per unit area. Two-sided P values < 0.05 were considered statistically significant for all analyses. In addition, univariate and multivariate survival analysis were performed using the Cox-proportional hazard model with enter or backward conditional stepwise method for multivariate analysis. I applied the numbers of CD68 (+), CD163 (+), CD204 (+) and FOXP3 (+) cells as categorical variables with cut-off values for Kaplan-Meier analysis.

Results

Quantitative analysis of tumor-infiltrating CD68, CD163, and CD204 (+) TAMs and FOXP3 (+) Tregs and a comparison between primary CNS DLBCL and systemic DLBCL

CD68, CD163, and CD204 were immunostained with a granular cytoplasmic and/or membranous pattern in cells presumed to be macrophages based on morphology. Images from representative cases with increased or decreased CD163 and CD204 (+) cells relative to CD68 (+) cells are presented in Figure 1A-F. Of note, aberrant CD163 expression in lymphoma cells was observed in 24.6% of DLBCLs with strong diffuse pattern. Automatic enumeration of CD163 (+) macrophages excluding tumor cells was difficult in these cases, which therefore were excluded from further analysis.

Overall, the amounts of CD68 versus CD163, CD68 versus CD204, and CD163 versus CD204 (+) cells were well-correlated in a positive manner (Figure 2A-C and 3A-C). In primary CNS DLBCL, median count of CD68 (+) cells, CD163 (+) cells, and CD204 (+) cells were 132.00 (range, 5.67–385.00), 146.33 (21.00–282.67), and 42.00 (2.00-278.00) per unit area, respectively. In systemic DLBCL, median count of CD68 (+) cells, CD163 (+) cells, and CD204 (+) cells were 234.00 (range, 89.0-

594.67), 181.00 (15.0-842.00), and 108.33 (0-499.00) per unit area, respectively.

In primary CNS DLBCL, median ratio of CD163/CD68 (+) cells and CD204/CD68 (+) cells were 1.06 (0.19–17.47) and 0.36 (0.02-3.06), respectively. In systemic DLBCL, median ratio of CD163/CD68 (+) cells and CD204/CD68 (+) cells were 1.01 (0.00-7.23) and 0.43 (0.00-2.24), respectively. There was no statistical difference for the ratio of M2 polarization between CNS and systemic DLBCL.

The count of CD163 (+) cells and CD204 (+) cells was positively correlated with CD68 (+) cells ($P < 0.001$, both, Figure 2A, B and 3A, B). Although, CD163 and CD204 were markers of M2 TAM which is a subtype of macrophage, CD163 (+) and CD204 (+) cells outnumbered the CD68 (+) cells in 51.7% and 10.6% of cases.

FOXP3 staining appeared in a distinct nuclear pattern (Figure 1G-H). The median count of FOXP3 (+) cells to total nuclei were 8.5 (0.00-109.0) for primary CNS DLBCL and 57.0 (0.3-588.3) for systemic DLBCL. The quantity of FOXP3 (+) cells and CD68 (+) cells exhibited a positive correlation (Figure 2D and 3D).

All of tumor-associated immune cells, tumor-infiltrating CD68 (+) TAMs, CD163 (+) or CD204 (+) M2 TAM, and FOXP3 (+) Tregs were significantly decreased in primary CNS DLBCL compared with systemic DLBCL (Table 1 and Figure 4). However, the ratio of CD163/CD68 (+) cells or CD204/CD68 (+) cells had no significant difference according to primary site.

Relationships between clinicopathological features and the number of tumor-infiltrating TAMs, M2 TAM, or Tregs in primary CNS DLBCL

The clinicopathological characteristics of primary CNS DLBCL and their associations with the number of tumor-infiltrating CD68 (+) TAMs, CD163 (+) or CD204 (+) M2 TAM, and FOXP3 (+) Tregs are summarized in Table 2. In brief, CD163 (+) cells were tend to greater in primary CNS DLBCL associated with advanced ECOG performance status (PS, P = 0.052). FOXP3 (+) cells were decreased in advanced PS (P = 0.015), decreased IELSG prognostic index (P = 0.011), decreased Nottingham/Barcelona score (P = 0.010) and had a tendency to decrease in elevated CSF protein (P = 0.064). Otherwise, there were no significant associations between the numbers of CD68 (+), CD204 (+) cells, the ratio of CD163/CD68 (+) cells or the ratio of CD204/CD68 (+) cells and other alleged prognostic factors, including age, B symptom, serum lactate dehydrogenase (LDH) level, cerebrospinal fluid (CSF) cytology or ocular involvement.

Relationships between clinicopathological features and the number of tumor-infiltrating TAMs, M2 TAM, or Tregs in

systemic DLBCLs

The clinicopathological characteristics of systemic DLBCL and their associations with the number of tumor-infiltrating CD68 (+) TAMs, CD163 and CD204 (+) M2 TAM, and FOXP3 (+) Tregs as well as the ratio of CD163/CD68 (+) and CD204/CD68 (+) cells are summarized in Table 3. In brief, CD68 (+), CD163 (+) and CD204 (+) cells and the ratio of CD163/CD68 (+) and CD204/CD68 (+) cells were significantly greater in systemic DLBCLs associated with EBV ($P = 0.005$, 0.046, 0.028, 0.004 and 0.007, respectively). CD68 (+) cells were increased in systemic DLBCL patients with the age of ≤ 60 years ($P = 0.015$). The number of FOXP3 (+) cells was markedly decreased in bulky diseases ($P < 0.001$).

The number of tumor-infiltrating TAMs, M2 TAM, or Tregs and the survival of primary CNS DLBCL patients

A total of 100 primary CNS DLBCL patients in the absence of rituximab were grouped into two tiers according to the quantity of CD68, CD163, a ratio of CD163/CD68, CD204, a ratio of CD204/CD68 and FOXP3 (+) cells, as precisely described in the Materials and Methods. The Kaplan-Meier plots are presented in Figure 5. The numbers of patients grouped into low and high for quantity of each immune cell based on the cut-off values for two tiers system was as follows; for CD68, low (n = 55) vs high (n = 39); for CD163, low (n = 30) vs high (n = 58); for

a ratio of CD163/CD68, low (n = 27) *vs* high (n = 57); for CD204, low (n = 26) vs high (n = 59); for a ratio of CD204/CD68, low (n = 19) *vs* high (n = 63); for FOXP3, low (n = 57) vs high (n = 32). Patients with increased CD68 (+) cells exhibited better OS and PFS (P = 0.057 and 0.007, respectively) (Figure 5A and B). By contrast, the increased number of CD204 (+) cells and the ratio of CD204/CD68 were significantly related to worse PFS (P = 0.007 and 0.023, respectively) (Figure 5H and J). Also, the ratio of CD163/CD68 had a tendency toward worse OS (P = 0.060) (Figure 5E). Otherwise, there were no significant associations between the numbers of CD163 (+) cells (Figure 5C and D). These data suggest that the influence of TAMs on the survival of primary CNS DLBCL differs depending on the macrophage type and M2 TAM rather than total TAMs are associated with poor prognosis.

Meanwhile, patients with increased FOXP3 (+) Tregs exhibited a tendency toward prolonged OS (P = 0.077) (Figure 5K).

Independent prognostic implications of tumor-infiltrating TAMs, M2 TAM, and Tregs in primary CNS DLBCL patients

To further determine the prognostic implication of TAMs and M2 TAM in primary CNS DLBCL patients, I performed univariate and multivariate survival analysis using the Cox proportional hazard model. As summarized in Table 4,

univariate Cox analysis revealed that CD68 (+) cells, CD204 (+) cells and the ratio of CD204/CD68 predicted PFS.

In multivariate Cox regression analysis integrating risk factors including LDH level and multiple disease, a low CD68 (+) cells and high CD204 (+) cells were independently predicted poor PFS (P = 0.013 and 0.023, respectively). These prognostic implications were also significant when compared with the Nottingham/Barcelona score (P = 0.005 and 0.016, respectively) (Table 5). Together, these data demonstrate that to total number of TAMs is an independent favorable prognostic predictor on the contrary M2 TAM in primary CNS DLBCL.

The number of tumor-infiltrating TAMs, M2 TAM, or Tregs and the survival of systemic DLBCL patients treated with R-CHOP

A total of 109 DLBCL patients treated with R-CHOP were grouped into two tiers according to the quantity of CD68, CD163, CD204 and FOXP3 (+) cells and the ratio of CD163/CD68 (+) cells and CD204/CD68 (+) cells, as precisely described in the Materials and Methods. The Kaplan-Meier plots are presented in Figure 6. The numbers of patients grouped into low and high for quantity of each immune cell based on the cut-off values for two tiers system was as follows; for CD68, low (n = 86) *vs* high (n = 23); for CD163, low (n = 51) *vs* high (n = 58); for a ratio of

CD163/CD68, low (n = 64) *vs* high (n = 45); for CD204, low (n = 58) vs high (n = 51); for a ratio of CD204/CD68, low (n = 79) *vs* high (n = 30); for FOXP3, low (n = 26) *vs* high (n = 83). Patients with increased CD68 (+) cells exhibited better OS and PFS ($P = 0.033$ and 0.052, respectively) (Figure 6A and B). By contrast, the increased number of CD163 (+) cells was significantly related to worse OS and PFS ($P = 0.041$ and < 0.001, respectively) (Figure 6C and D). Moreover, patients with a higher ratio of CD163/CD68 (+) cells, suggestive of M2 polarization of TAMs, exhibited a significantly shortened OS and PFS ($P = 0.003$ and 0.002, respectively) (Figure 6E and F). These data suggest that the influence of TAMs on the survival of DLBCLs treated with R-CHOP differs depending on the macrophage type and M2 TAM rather than total TAMs are associated with poor prognosis.

Meanwhile, patients with increased FOXP3 (+) Tregs exhibited a tendency toward prolonged OS and PFS ($P > 0.05$ and 0.045, respectively) (Figure 6K and L).

The number of tumor-infiltrating TAMs, M2 TAM, or Tregs and the survival of systemic DLBCL patients treated with CHOP in the absence of rituximab

I performed survival analysis in 51 DLBCL patients treated with chemotherapy (mostly CHOP) in the absence of rituximab (Figure 7). In contrast to the R-CHOP group, an increase in the number of CD68 (+) cells was related to shortened OS (*P*

= 0.018) (Figure 7A). However, a high ratio of CD163/CD68 (+) cells was also identified as a poor prognostic factor as in the R-CHOP group (P = 0.050) (Figure 7E). However, those with increased Tregs tended to show a shorter survival time, without statistical significance (Figure 7K and L).

Independent prognostic implications of tumor-infiltrating TAMs, M2 TAM, and Tregs in systemic DLBCL patients treated with R-CHOP

To further determine the prognostic implication of TAMs, M2 TAM, and Tregs in DLBCL treated with R-CHOP, I performed univariate and multivariate survival analysis using the Cox proportional hazard model. As summarized in Table 6, univariate Cox analysis revealed that CD68 (+) cells, CD163 (+) cells, and the ratio of CD163/CD68 (+) cells predicted OS and/or PFS.

In multivariate Cox regression analysis integrating risk factors including age, stage, B symptoms, performance status, LDH level, and the number of extranodal sites and the quantity of immune cells, high numbers of CD163 (+) cells were significantly associated with poor PFS (P = 0.011). In addition, a high CD163/CD68 (+) cell ratio independently predicted poor OS (P = 0.003). These prognostic implications were also significant when compared with the IPI score (P = 0.007 and 0.004, respectively) (Table 7). Together, these data demonstrate that

M2 polarization of TAMs, as represented by a higher ratio of CD163/CD68 (+) cells, is an independent poor prognostic predictor in DLBCLs treated with R-CHOP.

Discussion

Primary CNS DLBCL raises a challenge to researchers in view of its increasing incidence, unique clinical behavior and poorly understood pathobiology. Recently, several gene expression profiling studies differ in their results, but tend to demonstrate a distinct signature of primary CNS DLBCL compared with systemic lymphoma of the same histological subtype. This signature includes genes involved in B-cell differentiation, proliferation, apoptosis, cytokine signaling, and highlights the implication of the brain microenvironment in lymphoproliferation and CNS tropism (26-29).

CD68, CD163 and CD204 are widely used to identify TAMs. CD163 and CD204 are members of the scavenger receptor cysteine-rich superfamily and are restricted to the monocyte and macrophage lineage; these receptors are regarded as useful markers of M2 TAM (30, 31).

To perform an unbiased analysis of tumor-infiltrating immune cells and to obtain objective and reproducible data, I used virtual microscopy and automatic enumeration by an image analyzer. Although the automatic enumeration of nuclear staining of FOXP3 was robust, it was challenging to determine an optimal algorithm for counting CD68, CD163 and CD204 (+) cells. To improve the accuracy and consistency of the measurement, I established the most appropriate setting by

comparing the values obtained by automatic and manual counting in pilot cases. The enumeration pattern was also verified in each case. In such a process, there was a difficulty in the specimens of primary CNS lymphoma. The tumor cells are mainly large, with numerous apoptotic cells or widespread necrosis, which commonly hinders analysis. Therefore I used whole sections of representative formalin-fixed paraffin-embedded tissue blocks of primary CNS DLBCL patients' tumor, and captured images excluding necrotic or squeezed area for automatic enumeration from virtual microscopic images carefully.

Overall, strong positive correlations were observed between the numbers of CD68 versus CD163, CD68 versus CD204, and CD163 versus CD204 (+) cells, which led us to conclude that the automatic counting was reliable. The quantity of CD68 (+) or CD163 (+) cells was significantly higher in EBV-associated DLBCL, which is characterized by a prominent histiocytic reaction. Similarly, a positive correlation of high CD68 and CD163-expressing cells and EBV infection has been reported in Hodgkin lymphoma (32).

In this study, all of tumor-associated immune cells, tumor-infiltrating CD68 (+) TAMs, CD163 (+) or CD204 (+) M2 TAM, and FOXP3 (+) Tregs were significantly decreased in primary CNS DLBCL compared with systemic DLBCL. The BBB separates CNS from the systemic circulation, therefore recruiting of circulating immune cells are relatively restricted. Due to the special environment of CNS, the number of tumor-associated immune cells might be decreased. Whereas, the ratio of CD163/CD68 (+) or CD204/CD68 (+) cells, suggestive of M2 polarization of TAMs

were not moved.

I obtained several significant results for the prognostic value of tumor-infiltrating TAMs and Tregs in primary CNS DLBCL and systemic DLBCLs. The greater numbers of CD68 (+) TAMs were strong and independent correlated with prolonged survival. And increased CD163 (+) or CD204 (+) M2 TAM and a higher ratio of CD163/CD68 (+) or CD204/CD68 (+) cells, suggestive of M2 polarization of TAMs, were a poor prognostic factor of primary CNS DLBCL and systemic DLBCLs treated with R-CHOP. These findings signified a subgroup of TAMs excepting M2 TAM, played opposite role in DLBCL microenvironment in primary CNS DLBCL or systemic DLBCLs treated with R-CHOP. However, in DLBCL patients treated without rituximab, although M2 TAM and M2 polarization were a poor prognostic factor equally, an increased number of both CD68 (+) TAMs was related to shortened survival. These data suggest that the prognostic implication of CD68 (+) TAMs can play an opposite role depend on the treatment of rituximab.

Taken together these findings indicate that M2 TAM may contribute to lymphoma progression and the consequent poor prognosis of primary CNS and systemic DLBCLs in common. In contrast, TAMs except M2 TAM plays a lymphoma suppressive function in primary CNS DLBCL on the contrary to systemic DLBCL in the absence of rituximab. Because most of primary CNS DLBCL patients were not treated rituximab, and I analyzed only the no-rituximab treated group of primary CNS DLBCL patients, these findings indicate that TAMs except M2 TAM can perform the different role depending on primary sites in spite of identical phenotype

of malignant lymphoma. And they show that some components of CNS tumor microenvironment may play a different function compared with non-CNS.

The unfavorable outcome of follicular lymphoma with increased CD68 (+) TAM was reportedly circumvented when the patients were treated with rituximab combined chemotherapy (7, 8). In this study, an increase in the number of CD68 (+) cells was related to shortened survival. Although the population of patients without rituximab is small, these data suggest that M2 TAM rather than total TAMs might possess predictive value for the clinical outcome in DLBCL, but the prognostic implication of CD68 (+) TAMs could depend on the treatment modality. This ambivalent characteristic of CD68(+) TAMs was shown in some previous studies of follicular lymphoma, Farinnha *et al*. reported a negative prognostic effect of high TAM in patients treated without rituximab (7). In addition, Taskinen et al. showed that addition of rituximab to chemotherapy reverses the negative prognostic effect of high TAM content to favorable (33). Canioni *et al*. suggested a hypothesis that macrophages may act synergistically with rituximab treatment (8).

In a previous *in vitro* study, M2 TAM were able to phagocyte rituximab-opsonized leukemic B-cells more efficiently than M1 TAM (34). Given these data, the poor prognostic value of M2 TAM in the present study is contradictory. However, in an *in vivo* environment, M2 TAM would exert pleiotropic functions on tumor cells and the adjacent microenvironment to promote tumor progression, which might elicit a synergistic effect toward poor prognosis of DLBCL treated with R-CHOP.

A few previous studies have investigated the specific interaction of TAMs and Tregs in primary CNS and systemic DLBCL. Hasselblom *et al.* found no significant prognostic differences according to the number of CD68 (+) cells in patients treated with CHOP without rituximab (35). However, an association between high CD68 (+) TAMs and poor clinical outcome in CHOP-treated DLBCL has been observed by others (36). Wada *et al.* performed double immunostaining for HLA-DR/CD68 and CD163/CD68 to identify M1 and M2 TAM, respectively. The authors demonstrated that a large number of total TAMs or M2 TAM was associated with shortened survival of patients presumably treated with R-CHOP, and in particular, M2 TAM were an independent prognostic factor (18). Although the influence of total TAMs on survival differed between the study of Wada *et al.* and this data, the significant and independent prognostic implications of M2 TAM for poor clinical outcome were consistent between the studies. Furthermore, this study has some additional merits over previous studies. The follow-up period was much longer; survival analysis was performed in patients who were confirmed to be treated with R-CHOP; automatic cell counting was applied rather than manual evaluation; and the status of Tregs was incorporated.

Reports on the influence of Tregs on the prognosis of DLBCL are conflicting. Increased Tregs were predictive of better prognosis in a few reports (22, 37), but not in others (24). The present study demonstrated that increased levels of Tregs are a favorable prognostic factor in DLBCL treated with R-CHOP, although with marginal significance. Moreover, analyzing TAMs and Tregs together, DLBCLs

with high CD163 (+) macrophages and low Tregs exhibited the worst prognosis. Tregs are presumed to play a lymphoma-suppressive role and to predict favorable clinical outcomes

In intracranial B-cell lymphoma xenograft model, expression of interleukin-4 (IL-4) by malignant B-cells in lymphoma xenografts potentiated B-cell survival and mediated the polarization of TAMs to a M2 TAM associated with enhanced tumorigenesis (38). Additionally, only one study investigated the prognostic value of TAMs in primary CNS DLBCL. In the their report, numbers of CD68+, CD163+, and CD204+ TAMs in primary CNS DLBCL were not correlated with prognosis (39).

In summary, this study reveals that an increased number of CD68 (+) cells and a decreased number of CD204 (+) cells or the ratio of CD204/CD68 (+) cells, which is suggestive of M2 polarization, are a strong and independent favorable prognostic factor of primary CNS DLBCL patients. Also, an increased ratio of CD163/CD68 (+) cells, which is suggestive of M2 polarization, is a strong and independent poor prognostic factor of systemic DLBCL treated with R-CHOP.

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Table 1. Comparison of tumor-infiltrating TAMs, M2 TAM, or Tregs between primary CNS DLBCL and systemic DLBCL

Variables	primary CNS (N=110)	Systemic (N=165)	*P*
Age†† (N (%))			
≤60 yr.	53 (46.5)	68 (41.2)	n.s.
> 60 yr.	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 symptom†† (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 Han's 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

N, number; SD, standard deviation; ECOG, Eastern Cooperative Oncology Group; LDH, lactate dehydrogenase; GCB, germinal center B cell like; ABC, activated B cell-like; †These variables were compared using Student's t-test; ††These variables were compared using chi-square test; *These variables contain missing values that lacked information about the variables.

Table 2. Clinicopathological features of primary CNS DLBCL patients and the associations of these features with tumor-infiltrating immune cells

Variables	No.(%)	CD68 (mean±SD)	*P*	CD163 (mean±SD)	*P*	CD163/CD68 ratio (mean±SD)	*P*	CD204 (mean±SD)	*P*	CD204/CD68 ratio (mean±SD)	*P*	FOXP3 (mean±SD)	*P*
Age													
≤60 yr.	51 (46.4)	147.94±65.99	n.s.	142.19±67.41	n.s.	1.45±2.51	n.s.	59.99±58.19	n.s.	0.45±0.50	n.s.	22.78±28.09	n.s.
> 60 yr.	59 (53.6)	143.25±74.76		156.33±68.01		1.19±0.57		70.33±64.65		0.46±0.35		20.20±24.61	
Sex													
Male	69 (62.7)	144.84±74.68	n.s.	144.22±73.92	n.s.	1.09±0.62	n.s.	60.63±58.90	n.s.	0.40±0.32	n.s.	23.62±28.28	n.s.
Female	41 (37.3)	146.41±63.87		158.13±56.79		1.67±2.71		72.67±65.51		0.53±0.53		17.82±22.32	
Initial symptom													
H & V	26 (25.0)	161.99±84.90	n.s.	141.06±64.37	n.s.	1.75±3.49	n.s.	56.71±61.07	n.s.	0.43±0.61	n.s.	20.48±23.32	n.s.
Seizure	7 (6.7)	161.50±58.13		113.57±48.24		0.64±0.23		53.97±49.35		0.31±0.21		22.67±20.72	
Neurologic deficit	71 (68.3)	140.53±65.47		160.66±66.94		1.26±0.61		74.89±63.39		0.51±0.34		23.33±28.45	
ECOG performance status													
0, 1	73 (66.4)	144.48±76.40	n.s.	140.28±67.48	0.052	1.31±2.11	n.s.	57.19±54.42	n.s.	0.43±0.44	n.s.	25.59±28.09	0.015
≥ 2	37 (33.6)	147.28±58.21		167.67±65.52		1.32±0.74		81.22±71.59		0.51±0.38		13.61±20.48	
B symptom													
Absent	107 (97.3)	144.23±67.20	n.s.	150.26±67.33	n.s.	1.33±1.78	n.s.	66.40±61.93	n.s.	0.46±0.42	n.s.	21.40±26.29	n.s.
Present	3 (2.7)	188.11±170.57		120.33±115.02		0.49±0.05		22.00±12.73		0.24±0.12		23.00±30.32	
LDH*													
Normal	65 (63.1)	145.01±76.66	n.s.	143.76±71.37	n.s.	1.40±2.25	n.s.	64.52±62.83	n.s.	0.46±0.46	n.s.	21.41±25.10	n.s.
Elevated	38 (36.9)	143.74±51.56		159.84±62.29		1.22±0.54		65.08±61.42		0.45±0.35		18.97±27.28	
CSF cytology*													
Negative	80 (87.0)	141.95±69.52	n.s.	150.38±67.46	n.s.	1.40±2.00	n.s.	64.04±58.69	n.s.	0.48±0.45	n.s.	21.24±26.85	n.s.
Positive	12 (13.0)	159.22±71.25		169.00±66.98		1.16±0.80		76.48±75.52		0.44±0.31		14.46±14.93	
CSF protein*													
Normal	42 (47.7)	139.87±77.22	n.s.	145.94±72.15	n.s.	1.14±0.63	n.s.	62.86±65.89	n.s.	0.43±0.35	n.s.	25.70±31.74	0.064
Elevated	46 (52.3)	148.06±66.70		157.70±61.28		1.57±2.51		67.81±58.03		0.51±0.51		15.00±17.38	
Ocular disease*													
Absent	97 (89.0)	143.67±71.27	n.s.	149.60±70.16	n.s.	1.36±1.87	n.s.	65.17±61.80	n.s.	0.46±0.43	n.s.	19.68±24.09	n.s.
Present	12 (11.0)	156.65±68.62		155.02±47.62		1.02±0.35		71.47±65.70		0.45±0.33		32.70±38.51	
Involvement of deep structures*†													
Absent	31 (28.4)	130.55±61.84	n.s.	130.90±75.33	n.s.	1.67±3.28	n.s.	54.37±58.75	n.s.	0.48±0.60	n.s.	19.71±27.91	n.s.

Present	78 (71.6)	152.24±73.17		156.24±64.67		1.18±0.63		70.89±62.59		0.45±0.32		22.25±25.90	
Extent of disease*													
Unifocal	43 (39.4)	141.42±65.84	n.s.	145.70±71.81	n.s.	1.50±2.70	n.s.	53.56±47.88	0.080	0.44±0.53	n.s.	21.07±24.74	n.s.
Multifocal	66 (60.6)	149.11±73.78		152.35±66.03		1.19±0.61		74.12±68.23		0.47±0.34		21.90±27.59	
IELSG prognostic index													
0 ~ 2	63 (57.3)	143.06±79.53	n.s.	136.29±70.30	n.s.	1.35±2.29	0.027	59.12±60.73	n.s.	0.44±0.46	n.s.	26.63±30.12	0.011
3 ~ 5	47 (42.7)	148.59±57.02		165.97±61.41		1.27±0.68		73.48±62.50		0.48±0.37		14.36±17.78	
Nottingham/Barcelona score													
0 ~ 2	92 (83.6)	144.64±71.08	n.s.	145.07±66.66	n.s.	1.33±1.92	n.s.	59.32±55.11	0.094	0.43±0.42	n.s.	23.35±27.75	0.010
3	18 (16.4)	149.45±69.94		171.15±70.69		1.26±0.65		96.13±82.41		0.57±0.43		11.71±13.18	
Radiation													
Not done	40 (36.4)	152.97±75.42	n.s.	154.37±76.09	n.s.	1.08±0.52	n.s.	64.27±61.27	n.s.	0.38±0.28	n.s.	19.70±26.45	n.s.
Done	70 (60.6)	141.11±67.79		147.12±63.24		1.45±2.18		66.21±62.30		0.50±0.48		22.40±26.28	
Chemotherapy													
MOD, MVP	64 (58.2)	142.17±62.14	n.s.	159.63±64.51	n.s.	1.53±2.25	n.s.	75.52±63.01	n.s.	0.54±0.47	n.s.	24.40±29.21	n.s.
HD-MTX	20 (18.2)	139.99±69.84		127.76±76.34		1.05±0.58		59.85±72.80		0.38±0.56		12.85±14.46	
Others††	4 (3.6)	230.29±33.79		169.10±42.09		0.69±0.10		53.60±51.15		0.26±0.23		22.80±31.06	
None	22 (20.0)	144.39±90.78		134.51±71.53		1.07±0.54		47.40±47.25		0.35±0.31		21.14±25.00	
Rituximab													
Not done	100 (90.9)	144.25±71.63	n.s.	150.45±68.86	n.s.	1.34±1.83	n.s.	64.44±61.86	n.s.	0.45±0.43	n.s.	21.54±26.57	n.s.
Done	10 (9.1)	157.22±60.55		140.52±56.20		1.10±0.66		75.33±61.87		0.46±0.29		20.44±23.87	
IT-MTX													
Not done	89 (80.9)	143.45±69.28	n.s.	149.56±67.86	n.s.	1.35±1.94	n.s.	68.57±64.92	n.s.	0.48±0.45	n.s.	21.94±26.59	n.s.
Done	21 (19.1)	153.81±76.91		150.10±69.00		1.18±0.75		53.14±45.28		0.37±0.28		19.59±25.44	
Immunophenotype by Han's classification*													
GCB	14 (15.7)	162.71±54.81	n.s.	149.82±67.61	n.s.	0.99±0.54	n.s.	75.27±53.56	n.s.	0.51±0.43	n.s.	23.36±24.53	n.s.
ABC	75 (84.3)	148.88±71.61		157.97±66.62		1.21±0.64		75.56±66.58		0.47±0.31		21.20±26.98	

No., number; SD, standard deviation; H&V, Headache and Vomiting; ECOG, Eastern Cooperative Oncology Group; LDH, lactate dehydrogenase; CSF, cerebrospinal fluid; GCB, germinal center B cell like; ABC, activated B cell-like; †Involvement of deep structures of the brain, ie, basal ganglia and/or corpus callosum and/or brain stem and/or cerebellum.; ††Others of Chemotherapy, ie, CHOP, COPADM, etc.; *These variables contain missing values that lacked information about variables.

Table 3. Clinicopathological features of systemic DLBCL patients and the associations of these features with tumor-infiltrating immune cells

Variables	No.	CD68 (mean±SD)	*P*	CD163 (mean±SD)	*P*	CD163/CD 68 ratio (mean±SD)	*P*	CD204 (mean±SD)	*P*	CD204/CD 68 ratio (mean±SD)	*P*	FOXP3 (mean±SD)	*P*
Sex													
Male	91 (55.2%)	241.08±73.70	n.s.	247.64±172.25	n.s.	1.09±0.63	n.s.	124.99±102.27	n.s.	0.53±0.43	n.s.	94.53±108.46	n.s.
Female	74 (44.8%)	236.85±83.35		207.41±151.51		0.90±0.52		119.31±107.16		0.49±0.42		98.20±112.46	
Age													
≤60 yr.	68 (41.2%)	256.81±80.66	0.015	250.51±196.24	n.s.	0.99±0.65	n.s.	134.54±117.29	n.s.	0.51±0.40	n.s.	113.48±120.29	n.s.
> 60 yr.	97 (58.8%)	226.82±73.94		214.51±135.29		1.02±0.55		113.96±93.66		0.51±0.44		84.05±100.97	
Primary site													
Nodal	54 (32.7%)	230.01±80.89	n.s.	200.89±118.26	n.s.	0.98±0.52	n.s.	103.13±90.67	n.s.	0.49±0.50	n.s.	105.65±110.00	n.s.
Extranodal	111 (67.3%)	243.65±76.47		242.43±179.72		1.02±0.62		131.84±109.34		0.52±0.39		91.57±110.13	
Ann Arbor Stage*													
I, II	81 (50.3%)	234.98±72.09	n.s.	226.51±160.76	n.s.	1.01±0.61	n.s.	122.39±105.14	n.s.	0.52±0.44	n.s.	86.78±106.67	n.s.
III, IV	80 (49.7%)	244.78±85.11		235.08±169.81		1.00±0.57		123.68±104.41		0.50±0.42		103.96±113.22	
B symptom*													
Absent	125 (77.6%)	240.36±78.60	n.s.	228.17±163.21	n.s.	0.98±0.58	n.s.	128.00±109.33	n.s.	0.53±0.45	n.s.	94.15±103.31	n.s.
Present	36 (22.4%)	232.45±78.97		238.38±178.34		1.12±0.94		104.49±83.11		0.45±0.34		107.33±135.97	
Bulky disease*													
Absent	144 (88.9%)	241.48±80.12	n.s.	232.83±164.59	n.s.	1.01±0.61	n.s.	125.16±105.35	n.s.	0.52±0.43	n.s.	101.61±113.47	<0.001
Present	18 (11.1%)	221.83±64.06		215.31±170.23		0.96±0.47		108.81±96.06		0.48±0.38		43.28±48.45	
BM involvement*													
Absent	128 (85.9%)	236.67±78.97	n.s.	223.66±163.46	n.s.	1.00±0.62	n.s.	118.79±98.71	n.s.	0.52±0.44	n.s.	93.27±109.34	n.s.
Present	21 (14.1%)	239.56±79.44		235.20±137.54		1.02±0.46		126.17±114.96		0.46±0.37		134.56±130.52	
LDH*													
Normal	64 (43.5%)	238.28±83.09	n.s.	240.64±189.60	n.s.	1.04±0.72	n.s.	129.90±117.84	n.s.	0.53±0.44	n.s.	99.84±114.16	n.s.
Elevated	83 (56.5%)	245.68±76.81		232.70±155.01		1.00±0.50		118.07±93.95		0.48±0.39		95.45±112.10	
No. of extranodal sites*													
0, 1	102 (76.7%)	238.61±80.53	n.s.	225.35±155.17	n.s.	1.00±0.58	n.s.	119.84±105.11	n.s.	0.51±0.45	n.s.	93.73±109.80	n.s.
≥2	31 (23.3%)	242.01±74.74		246.53±196.83		1.03±0.64		132.68±104.50		0.52±0.36		102.01±112.51	
ECOG performance status*													
0, 1	125 (76.2%)	236.54±79.35	n.s.	222.65±165.39	n.s.	0.99±0.62	n.s.	124.19±105.00	n.s.	0.52±0.43	n.s.	100.25±110.08	n.s.

≥2	39 (23.8%)	248.85±74.19		255.55±161.49		1.05±0.49		121.31±99.09		0.48±0.35		84.06±111.33	
IPI group*													
Low(0-1)	58 (35.2%)	237.33±85.72	n.s.	243.91±177.67	n.s.	1.06±0.65	n.s.	125.87±108.70	n.s.	0.52±0.41	n.s.	93.39±118.29	n.s.
High(2-5)	92 (55.8%)	242.96±76.05		228.34±164.09		0.99±0.57		121.57±95.94		0.50±0.41		97.92±108.31	
EBV*													
Negative	153 (95.6%)	237.07±72.83	0.005	222.94±156.25	0.046	0.98±0.57	0.004	117.89±101.51	0.028	0.50±0.43	0.007	93.93±109.65	n.s.
Positive	7 (4.4%)	298.74±157.74		452.93±267.02		1.76±0.79		238.48±111.13		0.86±0.25		100.10±105.42	
Immunophenotype by Choi classifier*													
GCB	59 (36.4%)	241.27±72.87	n.s.	211.95±168.10	n.s.	0.91±0.57	n.s.	121.70±89.64	n.s.	0.51±0.37	n.s.	106.18±126.95	n.s.
ABC	103 (63.6%)	238.31±81.94		237.10±161.30		1.04±0.57		125.74±112.18		0.52±0.46		89.64±98.21	

No., number; SD, standard deviation; n.s., not significant; GCB, germinal center B cell like; ABC, activated B cell-like; IPI, International prognostic index; ECOG, Eastern Cooperative Oncology Group; LDH, lactate dehydrogenase; BM, bone marrow; *These variables contain missing values that lacked information about variables or could not be classified into GCB or non-GCB/ABC phenotypes.

Table 4. Univariate analysis of OS and PFS with clinicopathological parameters and tumor-infiltrating immune cells for primary CNS DLBCL patients

Variables	OS			PFS		
	HR	95% CI	*P*	HR	95% CI	*P*
Age > 60 yr.	2.381	0.784-7.232	0.126	1.409	0.668-2.968	0.368
ECOG PS ≥ 2	2.137	0.732-6.237	0.165	1.177	0.557-2.490	0.669
Presence of B symptom	0.048	0.0-456694.0	0.710	1.788	0.241-13.291	0.570
Normal LDH	0.772	0.243-2.451	0.661	2.631	1.193-5.801	0.016
Positive CSF cytology	1.020	0.222-4.692	0.980	0.379	0.089-1.608	0.188
Elevated CSF protein	0.373	0.093-1.496	0.164	0.771	0.360-1.648	0.502
Presence of ocular disease	1.966	0.546-7.081	0.301	1.119	0.390-3.216	0.834
Involvement of deep structures	1.610	0.453-5.724	0.462	1.317	0.614-2.826	0.479
Multiple disease	1.439	0.491-4.215	0.507	2.062	0.984-4.319	0.055
IELSG prognostic index ≥ 3	1.826	0.649-5.138	0.254	0.559	0.272-1.149	0.114
Nottingham/Barcelona score 3	2.887	0.855-9.750	0.088	2.692	1.123-6.451	0.026
ABC phenotype (*vs* GCB)	2.283	0.294-17.748	0.430	1.822	0.549-6.049	0.327
Low CD68 (+) cells	3.921	0.857-17.930	0.078	3.568	1.340-9.499	0.011
High CD163 (+) cells	0.779	0.254-2.387	0.662	0.813	0.368-1.795	0.608
High CD163/CD68 (+) ratio	5.721	0.731-44.777	0.097	1.589	0.621-4.065	0.334
High CD204 (+) cells	2.145	0.596-7.718	0.243	3.707	1.356-10.134	0.011
High CD204/CD68 (+) ratio	1.014	0.274-3.754	0.983	4.653	1.087-19.920	0.038
Low FOXP3 (+) cells	3.559	0.792-15.986	0.098	1.504	0.655-3.454	0.336

ECOG PS, Eastern Cooperative Oncology Group performance status; LDH, lactate dehydrogenase; CSF, cerebrospinal fluid; OS, overall survival; PFS, progression free survival; HR, hazard ratio; CI, confidence interval.

Table 5. Multivariate analysis of OS and PFS with clinicopathological parameters and tumor-infiltrating immune cells for primary CNS DLBCL patients

Variables	OS			PFS		
	HR	95% CI	*P*	HR	95% CI	*P*
Comparison with risk factors						
Normal LDH	-	-	-	2.986	1.121-7.953	0.029
Multiple disease	-	-	-	2.306	0.899-5.921	0.082
Low CD68 (+) cells	-	-	-	4.136	1.345-12.719	0.013
High CD163/CD68 (+) ratio	-	-	-	-	-	-
High CD204 (+) cells	-	-	-	3.590	1.190-10.832	0.023
Low FOXP3 (+) cells	-	-	-	-	-	-
Comparison with Nottingham/Barcelona score						
Nottingham/Barcelona score 3	3.315	0.824-13.331	0.091	3.169	1.096-9.160	0.033
Low CD68 (+) cells	5.779	0.708-47.169	0.101	4.842	1.607-14.588	0.005
High CD163/CD68 (+) ratio	3.476	0.421-28.702	0.247	-	-	-
High CD204 (+) cells	-	-	-	3.943	1.294-12.016	0.016
Low FOXP3 (+) cells	1.043	0.199-5.465	0.961	-	-	-

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

Table 6. Univariate analysis of OS and PFS with clinicopathological variables and tumor-infiltrating immune cells for DLBCL treated with R-CHOP

Variables	OS			PFS		
	HR	95% CI	*P*	HR	95% CI	*P*
Age > 60 yr.	2.686	1.208-5.971	0.015	0.932	0.443-1.960	0.852
Ann Arbor Stage III, IV	2.067	0.987-4.329	0.054	2.983	1.266-7.028	0.012
Presence of B symptoms	2.138	1.069-4.277	0.032	1.832	0.843-3.978	0.126
Elevated LDH	2.479	1.149-5.347	0.021	1.945	0.858-4.410	0.111
ECOG PS ≥ 2	2.190	0.984-4.873	0.055	1.159	0.398-3.375	0.787
High IPI (2-5)	2.880	1.188-6.980	0.019	2.999	1.125-7.997	0.028
Presence of bulky disease	1.573	0.553-4.478	0.396	0.395	0.054-2.910	0.362
No. of extranodal sites ≥ 2	1.052	0.502-2.202	0.894	1.901	0.898-4.024	0.093
BM involvement	1.078	0.414-2.809	0.878	1.259	0.476-3.326	0.642
EBV-positive	0.707	0.096-5.188	0.733	0.652	0.088-4.836	0.676
ABC phenotype (*vs* GCB)	1.048	0.515-2.133	0.896	1.446	0.633-3.304	0.382
Low CD68 (+) cells*	3.369	1.024-11.087	0.046	3.083	0.925-10.276	0.067
High CD163 (+) cells*	2.077	1.010-4.274	0.047	4.888	1.853-12.897	0.001
High CD163/CD68 (+) cell ratio*	2.612	1.194-5.712	0.016	2.820	1.177-6.758	0.020
High CD204 (+) cells	1.021	0.474-2.197	0.958	20.535	0-3223799012	0.754
High CD204/CD68 (+) ratio	1.021	0.474-2.197	0.958	0.701	0.284-1.730	0.441
Low FOXP3 (+) cells*	1.719	0.850-3.477	0.132	2.130	0.994-4.564	0.052

ECOG PS, Eastern Cooperative Oncology Group performance status; LDH, lactate dehydrogenase; IPI, International prognostic index; GCB, germinal center B-cell like; ABC, activated B cell-like; BM, bone marrow; HR, hazard ratio; CI, confidence interval.

Table 7. Multivariate analysis of OS and PFS with clinicopathological parameters and tumor-infiltrating immune cells for DLBCL treated with R-CHOP

Variables	OS			PFS		
	HR	95% CI	P	HR	95% CI	P
Comparison with risk factors						
Age > 60 yr	-	-	-	-	-	-
Stage III, IV	2.846	1.123-7.216	0.028	2.674	0.975-7.336	0.056
Presence of B symptoms	-	-	-	-	-	-
ECOG PS ≥ 2	2.863	1.040-7.884	0.042	-	-	-
Elevated LDH	-	-	-	-	-	-
No. of extranodal sites ≥ 2	0.417	0.163-1.069	0.069	-	-	-
Low CD68 (+) cells*	6.698	1.509-29.730	0.012	5.034	1.145-22.133	0.032
High CD163 (+) cells*	-	-	-	13.989	1.828-107.083	0.011
High CD163/CD68 (+) ratio*	3.907	1.613-9.466	0.003	-	-	-
Low FOXP3 (+) cells	-	-	-	-	-	-
Comparison with IPI score						
High IPI (2-5)	3.632	1.379-9.563	0.009	2.888	1.060-7.867	0.038
Low CD68 (+) cells*	4.974	1.150-21.516	0.032	4.903	1.116-21.538	0.035
High CD163 (+) cells*	-	-	-	16.133	2.128-122.313	0.007
High CD163/CD68 (+) ratio*	3.496	1.495-8.175	0.004	-	-	-
Low FOXP3 (+) cells*	-	-	-	-	-	-

ECOG PS, Eastern Cooperative Oncology Group performance status; LDH, lactate dehydrogenase; No., number; IPI, International prognostic index; HR, hazard ratio; CI, confidence interval. *The quantities of immune cells were submitted to multivariate analyses with the Cox proportional hazard model using backward-conditional stepwise logistic regression

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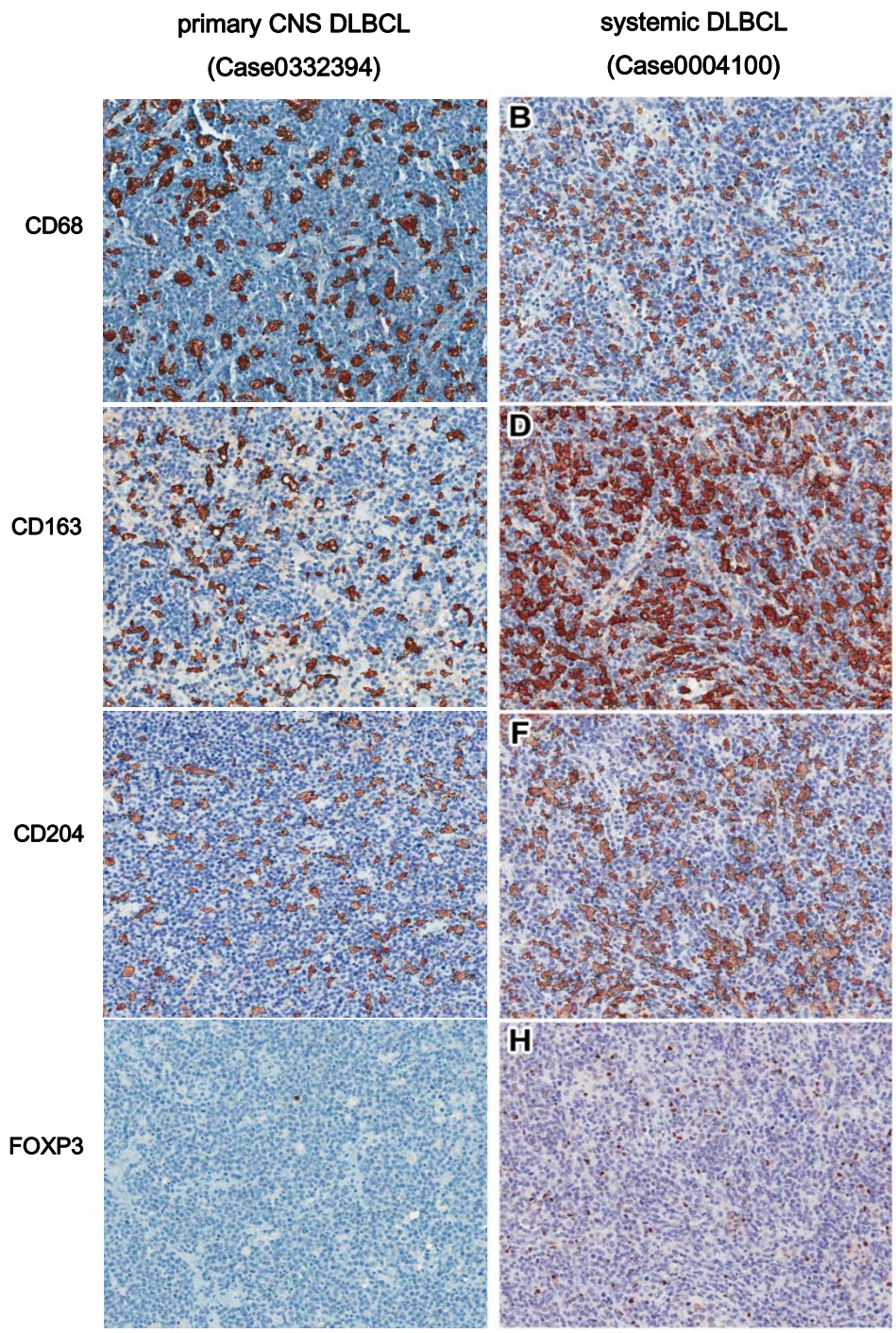

Figure 1. Representative images for automatic enumeration of tumor-

infiltrating CD68, CD163, CD204, and FOXP3 (+) cells.

CD68, CD163, and CD204 were expressed in a granular cytoplasmic pattern by macrophages. FOXP3 staining exhibited a nuclear pattern in small lymphoid 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). (A), (C), and (E) represent a case exhibiting primary CNS DLBCL. The counts of CD68 (+) cells (A), CD163 (+) cells (C), and CD204 (+) cells (E) in this case were 255.67, 182.00, and 24, respectively, per unit area (0.28 mm^2). The count of FOXP3 (+) cells in this case was 30.00 (G). By contrast, (B), (D) and (F) were from systemic DLBCL. The counts of CD68 (+) cells (B), CD163 (+) cells (D), and CD204 (+) cells were 277.33, 520.67, and 270.00, respectively, per unit area. The count of FOXP3 (+) cells in this case was 288.00 (H).

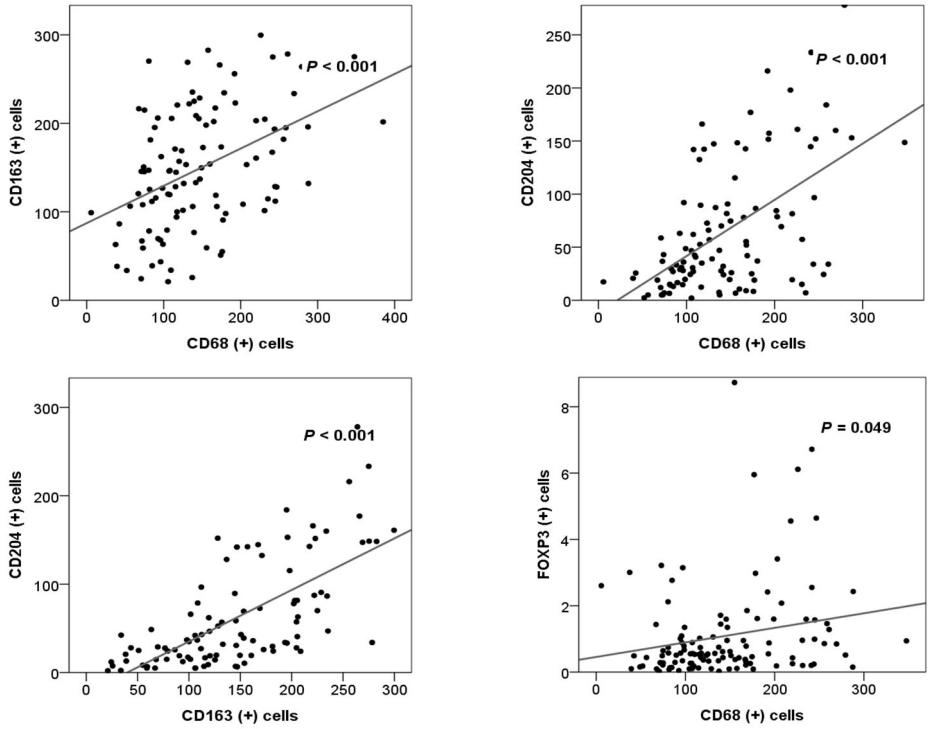

Figure 2. Correlation between the tumor-infiltrating CD68, CD163, CD204, and FOXP3 (+) cells for primary CNS DLBCL.

The counts of CD68, CD163, CD204, and FOXP3 (+) cells and the ratio of CD163/CD68 (+) cells for each case were plotted, and the correlations between these values were analyzed.

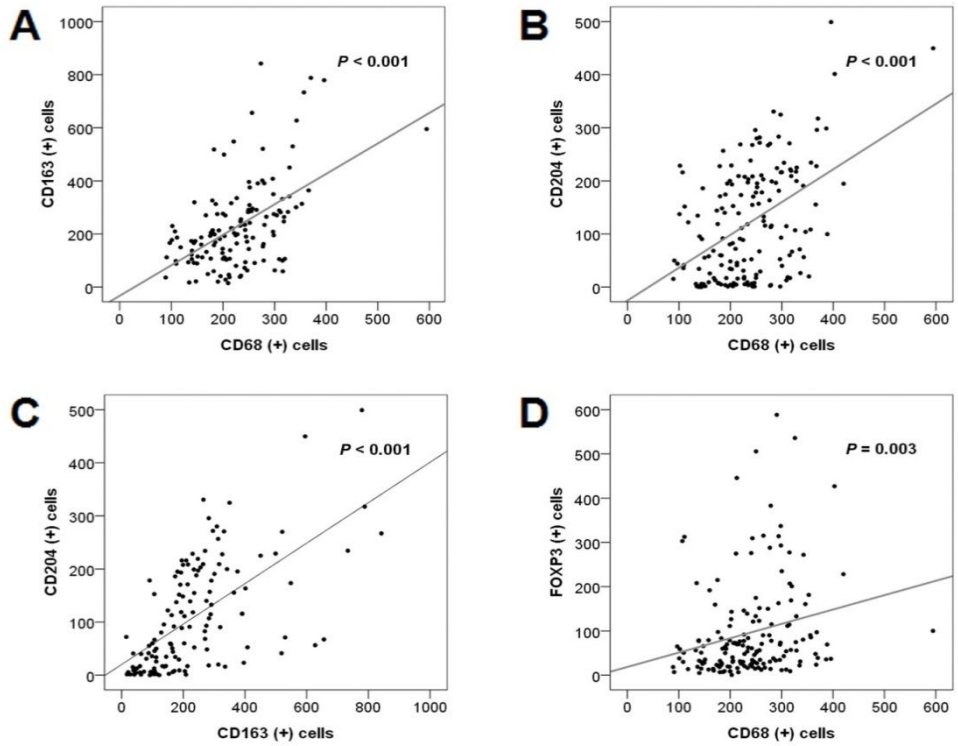

Figure 3. Correlation between the tumor-infiltrating CD68, CD163, CD204, and FOXP3 (+) cells for systemic DLBCL.

The counts of CD68, CD163, CD204, and FOXP3 (+) cells and the ratio of CD163/CD68 (+) cells for each case were plotted, and the correlations between these values were analyzed.

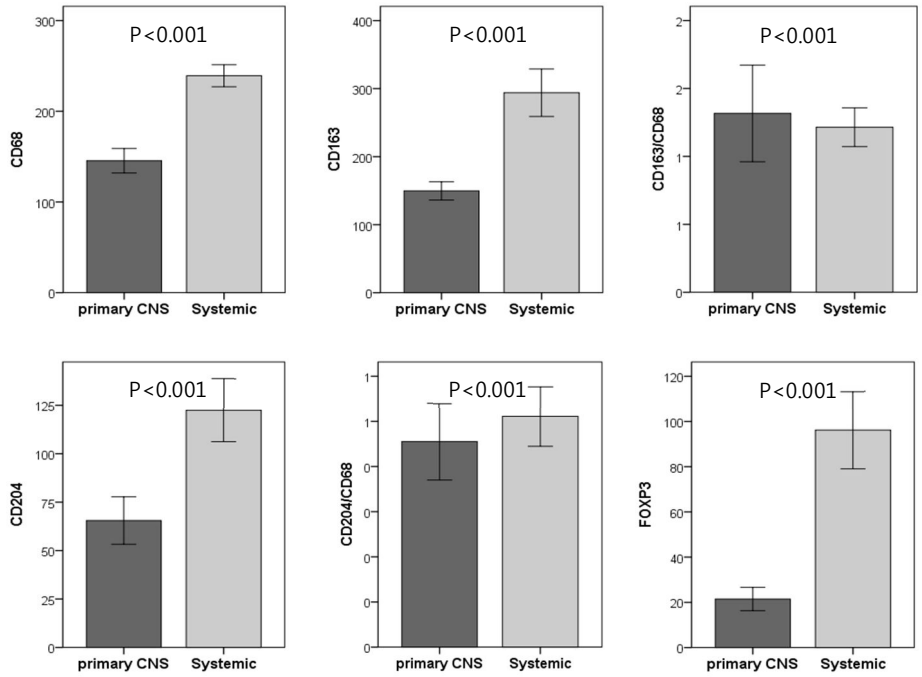

Figure 4. Comparison of clinical variables and tumor-infiltrating CD68, CD163, CD204, and FOXP3 (+) cells according to primary site

The tumor-infiltrating CD68, CD163, CD204, and FOXP3 (+) cells and the ratio of CD163/CD68 and CD204/CD68 (+) cells according to the primary site are plotted and the statistical significance of the correlations were assessed using Pearson's t test. All of tumor-associated immune cells, tumor-infiltrating CD68 (+) TAMs, CD163 (+) or CD204 (+) M2 TAM, and FOXP3 (+) Tregs were significantly decreased in primary CNS DLBCL compared with systemic ($P < 0.001$, respectively). The ratio of CD163/CD68 (+) cells or CD204/CD68 (+) cells had no significant difference according to primary site.

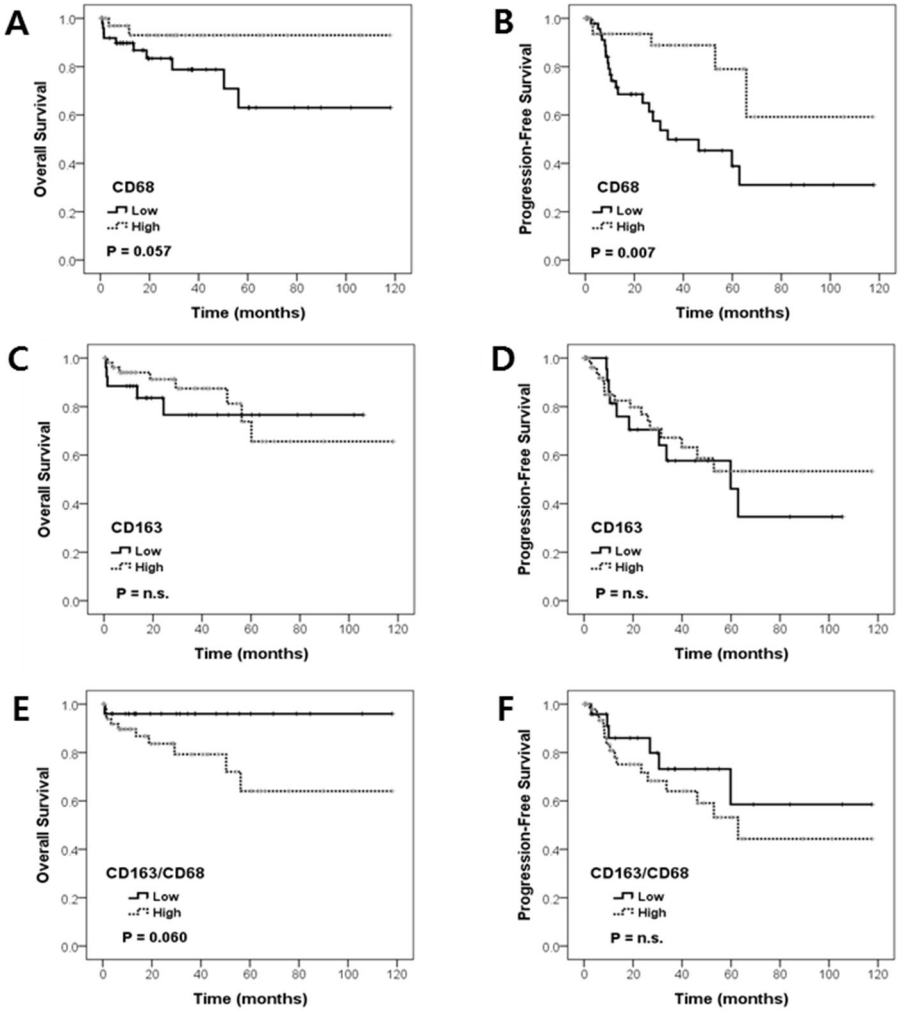

Figure 5-1. Survival analysis in primary CNS DLBCL according to the numbers of CD68, CD163, and FOXP3 (+) cells, and the CD163/CD68 (+) cell ratio

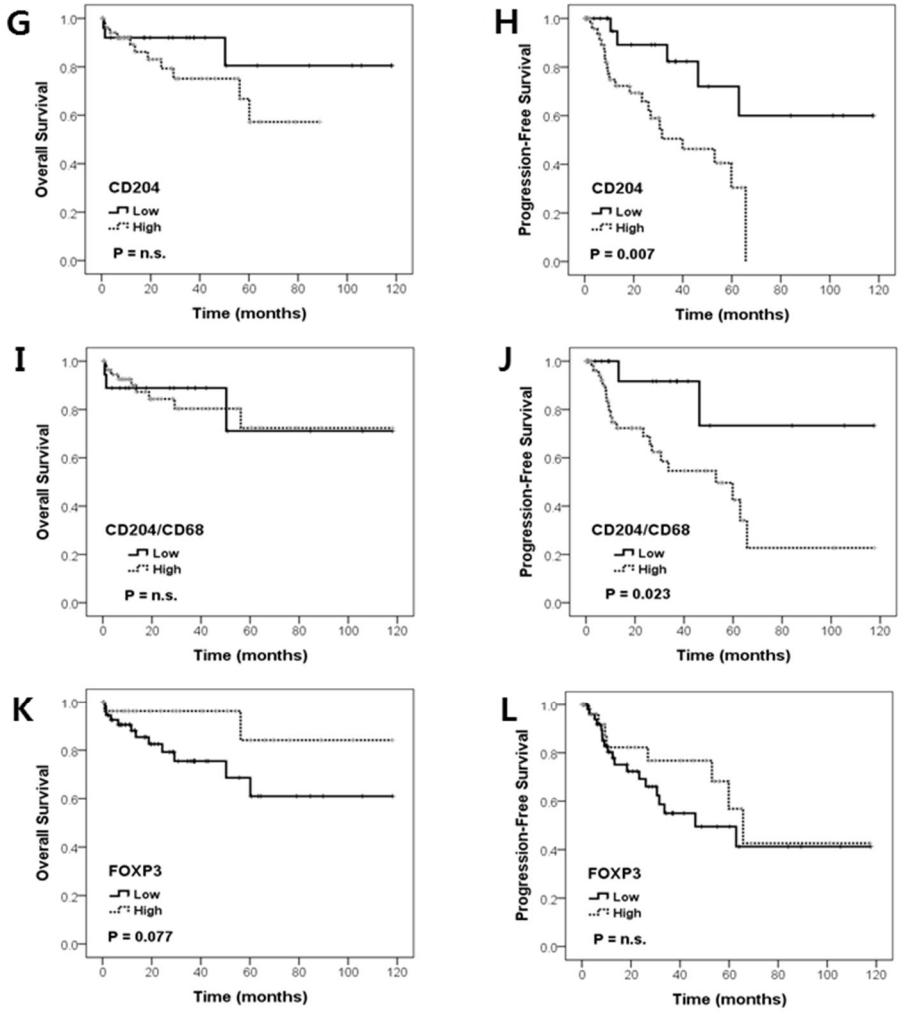

Figure 5-2. Survival analysis in primary CNS DLBCL according to the numbers of CD68, CD163, and FOXP3 (+) cells, and the CD163/CD68 (+) cell ratio.

OS and PFS of primary CNS DLBCL patients were evaluated according to the number tumor-infiltrating of CD68 (+) cells (A, B), CD163 (+) cells (C, D), the

ratio of CD163/CD68 (+) cells (E, F), CD204 (+) cells (G, H), the ratio of CD204/CD68 (+) cells (I, J), and the number of FOXP3 (+) Tregs (K, L). Kaplan-Meier curves for OS (A, C, E, G, I, K) and PFS (B, D, F, H, J, L) are illustrated with the *P* values from the log-rank test.

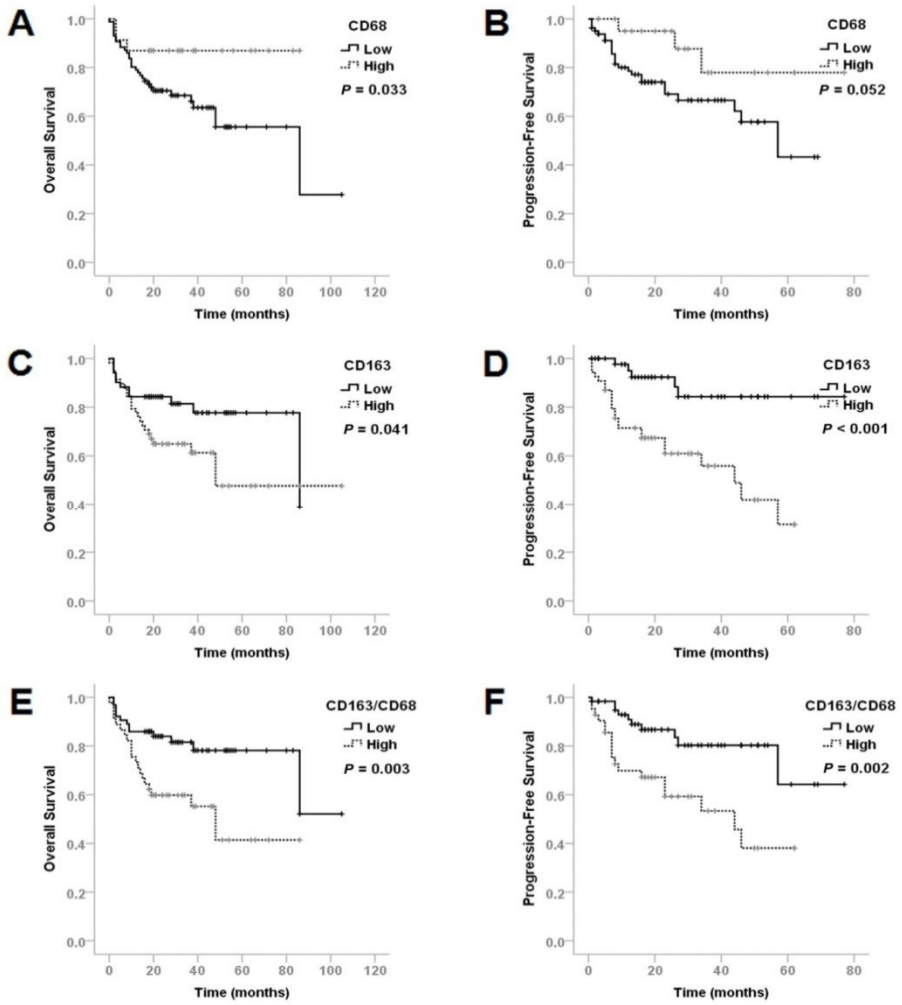

Figure 6-1. Survival analysis in systemic DLBCL patients treated with R-CHOP according to the numbers of CD68, CD163, and FOXP3 (+) cells, and the CD163/CD68 (+) cell ratio.

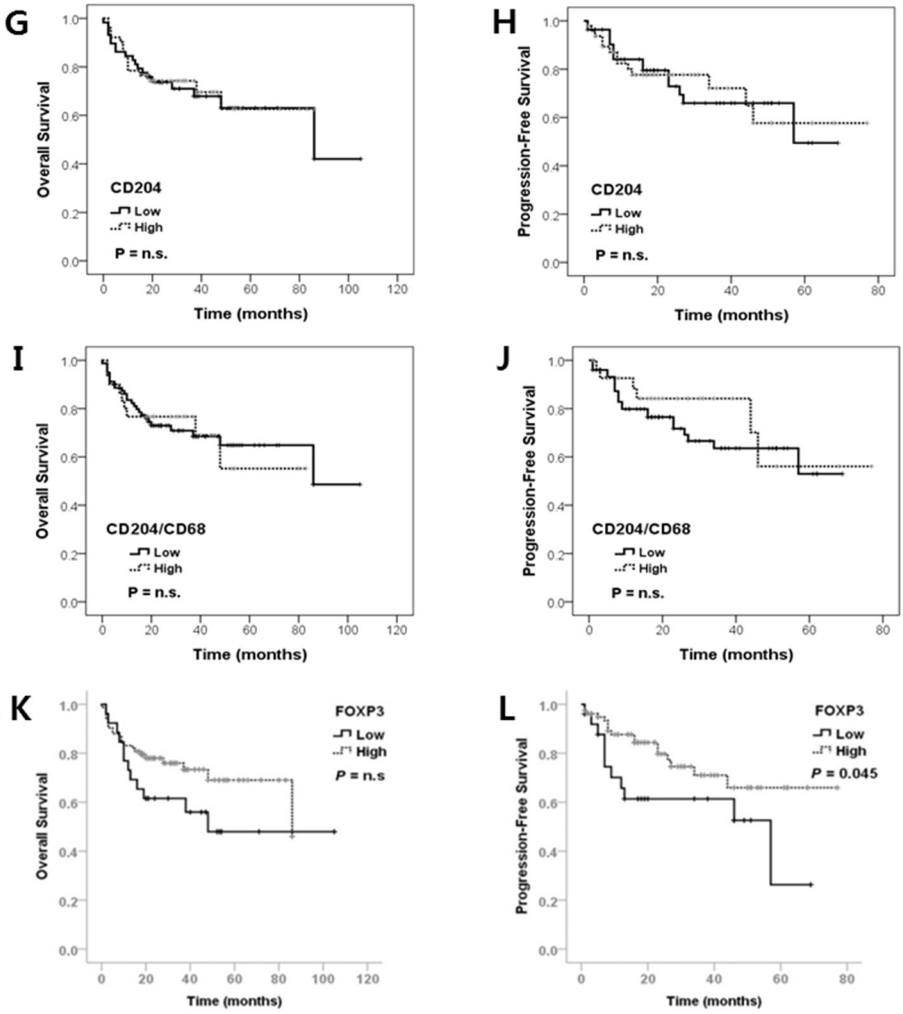

Figure 6-2. Survival analysis in systemic DLBCL patients treated with R-CHOP according to the numbers of CD68, CD163, and FOXP3 (+) cells, and the CD163/CD68 (+) cell ratio.

OS and PFS of systemic DLBCL patients treated with R-CHOP were evaluated according to the number tumor-infiltrating of CD68 (+) cells (A, B), CD163 (+)

cells (C, D), the ratio of CD163/CD68 (+) cells (E, F), CD204 (+) cells (G, H), the ratio of CD204/CD68 (+) cells (I, J), and the number of FOXP3 (+) Tregs (K, L). Kaplan-Meier curves for OS (A, C, E, G, I, K) and PFS (B, D, F, H, J, L) are illustrated with the *P* values from the log-rank test.

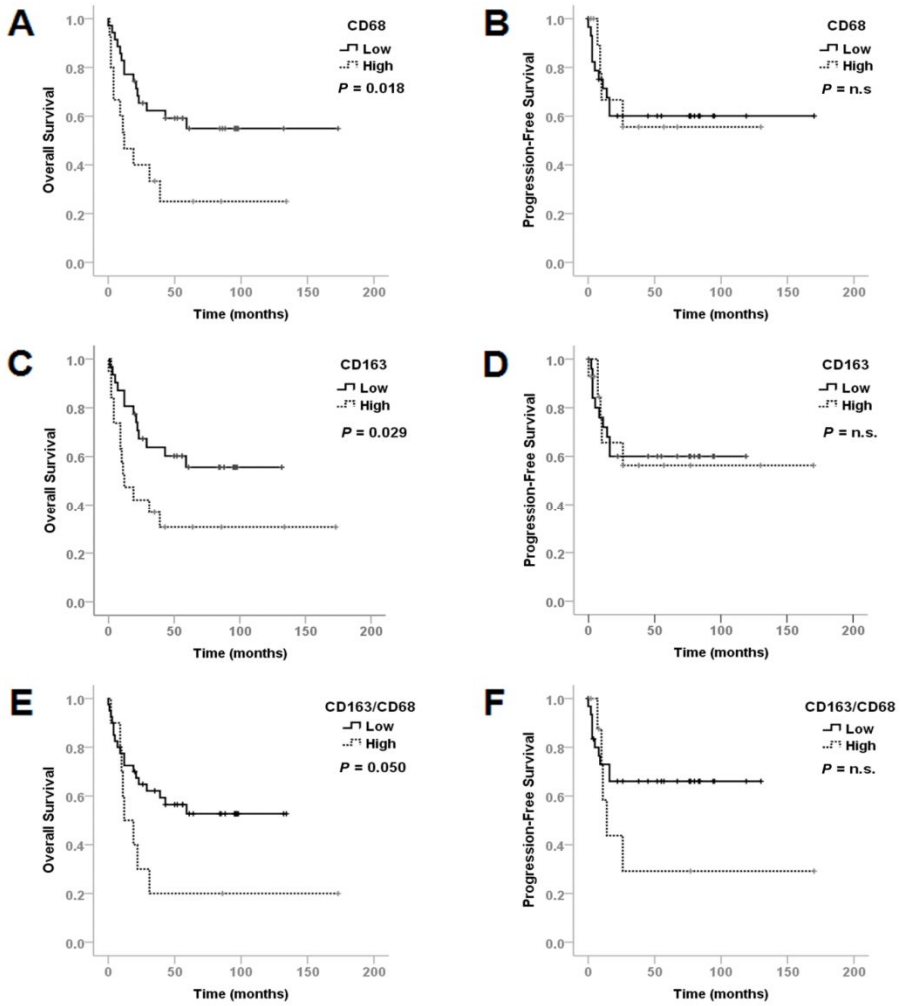

Figure 7-1. Survival analysis in systemic DLBCL patients treated with CHOP in the absence of Rituximab according to the number of CD68, CD163, and FOXP3 (+) cells, and the CD163/CD68 (+) cell ratio.

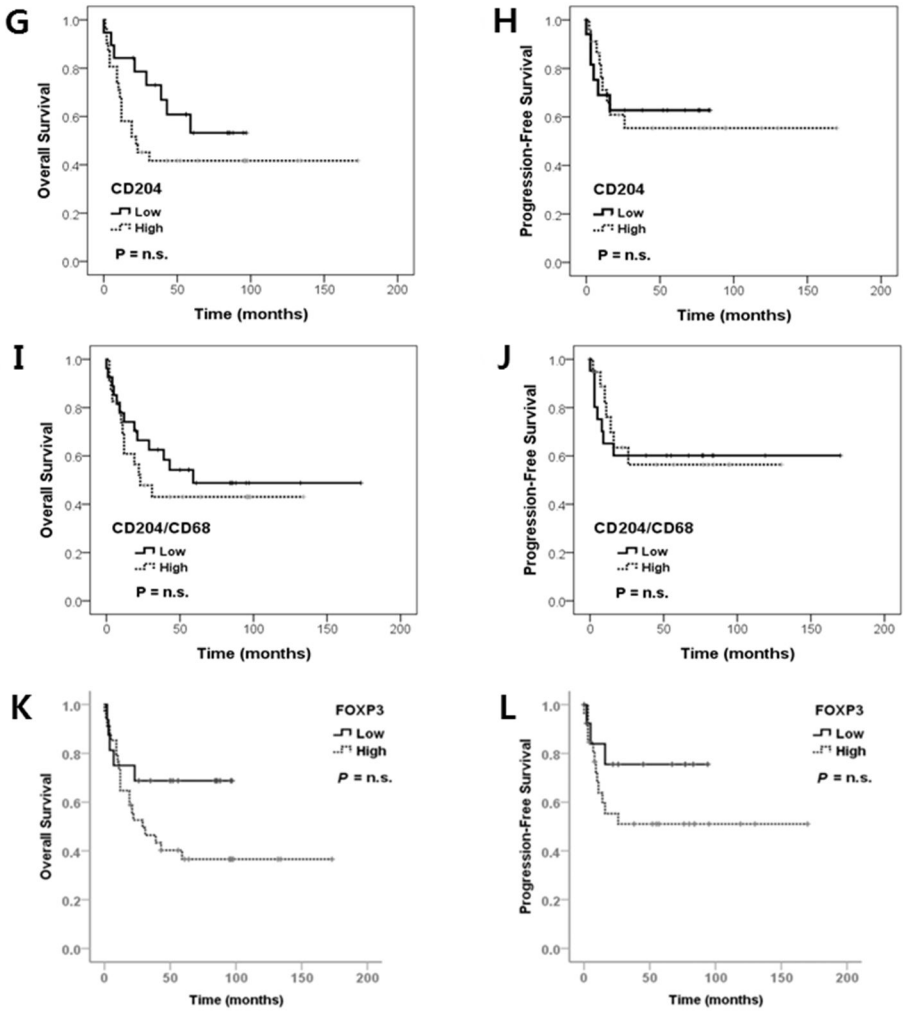

Figure 7-2. Survival analysis in systemic DLBCL patients treated with CHOP in the absence of Rituximab according to the number of CD68, CD163, and FOXP3 (+) cells, and the CD163/CD68 (+) cell ratio.

OS and PFS of systemic DLBCL patients treated with CHOP in the absence of rituximab were evaluated according to the number tumor-infiltrating of CD68 (+) cells (A, B), CD163 (+) cells (C, D), the ratio of CD163/CD68 (+) cells (E, F),

CD204 (+) cells (G, H), the ratio of CD204/CD68 (+) cells (I, J), and the number of FOXP3 (+) Tregs (K, L). Kaplan-Meier curves for OS (A, C, E, G, I, K) and PFS (B, D, F, H, J, L) are illustrated with the *P* values from the log-rank test.

국문 초록

원발성 중추신경 광범위큰B세포림프종에서 종양관련면역세포의 임상, 병리학적, 예후적 의미

종양미세환경은 종양 주위의 간질 세포, 여러 면역세포와 그 사이의 상호작용을 포함하며 일반적으로 종양의 생장을 촉진하고 항암제 저항성에 관여한다고 알려져 있다. 그러나 림프종의 종양미세환경은 다른 암과는 달리 림프종의 기원 세포에 따라 서로 다른 상호작용을 하기 때문에 다양한 양상을 보인다. 종양관련대식세포(또는 중추신경계의 소아교세포)와 조절 T세포는 종양미세환경의 주된 구성요소 중 하나이다. 대식세포는 일반적으로 M1과 M2의 아형으로 나뉘어지며 서로 반대의 역할을 하는 것으로 알려져 있다. 한편, 원발성 중추신경 광범위큰B세포림프종은 드문 악성림프종 중 하나로 광범위큰B세포림프종

중에서도 나쁜 예후를 보이는 특징이 있다. 중추신경계는 혈뇌장벽으로 인해 몸의 다른 부분과는 달리 면역학적으로 상이한 환경을 가지며, 이는 종양미세환경에서도 마찬가지이다. 최근 원발성 중추신경 림프종의 종양형성에 종양미세환경이 중요하게 작용한다는 몇몇 연구결과가 보고되고 있다. 그러나 종양미세환경에서 주된 작용을 하는 것으로 알려진 종양관련대식세포와 조절 T세포가 원발성 중추신경 광범위큰B세포림프종에 미치는 영향과 전신성 광범위큰B세포림프종과의 차이점에 대해서는 아직 밝혀진 것이 매우 적다. 따라서 본 연구에서는 원발성 중추신경 광범위큰B세포림프종과 전신성 광범위큰B세포림프종에서 종양관련대식세포와 M2 대식세포, 조절 T세포의 발현과 임상, 예후적 의미를 분석하고 비교하기로 하였다.

129례의 원발성 중추신경 광범위큰B세포림프종과 165례의 전신성 광범위큰B세포림프종의 포르말린 고정 파라핀 포매 종양조직을 대상으로 하여 CD68, CD163, CD204, FOXP3의 면역조직화학염색을 시행하였다.

염색된 유리 슬라이드를 가상현미경 파일로 스캔하고 자동 이미지 분석 프로그램을 이용해 양성세포의 개수를 세어 환자의 임상소견과 함께 통계 분석하였다.

종양관련대식세포와 M2 대식세포, 조절 T세포 모두 원발성 중추신경 광범위큰B세포림프종에서 전신성 광범위큰B세포림프종에 비해 통계적으로 유의한 수준으로 낮은 발현을 보였다. 그러나 M2 아형으로의 편향 정도를 나타내는 CD163/CD68의 비와 CD204/CD68의 비는 원발성 중추신경 광범위큰B세포림프종과 전신성 광범위큰B세포림프종에서 큰 차이를 보이지 않았다. 원발성 중추신경 광범위큰B세포림프종에서 CD163 양성세포의 수는 ECOG 활동도가 증가할수록 높아지는 양상이었다 (P=0.052). FOXP3 양성세포는 ECOG 활동도가 증가할수록 감소하였으며 (P=0.015), IELSG 지수와 노팅엄/바르셀로나 지수가 증가할수록 증가하였고(P=0.011, 0.010), 뇌척수액 단백질이 증가할수록 감소하는 경향을 보였다 (P=0.064). 전신성 광범위큰B세포림프종에서는 CD68,

CD163, CD204 양성 세포와 CD163/CD68의 비와 CD204/CD68의 비 모두 엡스타인바 바이러스 양성일 때 증가하는 양상을 보였다(*P*=0.005, 0.046, 0.028, 0.004, 0.007). CD68 양성세포는 60세 이하의 환자군에서 더 높게 나타났다 (*P*=0.015). 생존분석은 리툭시맵 치료를 받지 않은 100례의 원발성 중추신경 광범위큰B세포림프종을 대상으로 분석하였다. 높은 CD68 양성세포의 수는 전반적 생존율(overall survival)과 재발 없는 생존율 (progression free survival) 모두에서 더 좋은 예후를 보였다(*P*=0.014, 0.001). 반면에 높은 CD204 양성세포의 수는 전반적 생존율과 재발 없는 생존율 모두 나쁜 예후를 보였다(*P*=0.057, 0.012). 다변량분석에서 낮은 CD68과 높은 CD204 양성세포의 수는 모두 독립적인 나쁜 예후인자로 나타났다. R-CHOP 치료를 받은 109례의 전신성 광범위큰B세포림프종 에서 시행한 생존분석 결과 높은 CD68 양성세포의 수는 전반적 생존율 에서 좋은 예후인자로 나타났다(*P*=0.033). 반면에, 높은 CD163 양성 세포의 수와 높은 CD163/CD68의 비는 전반적

생존율(*P*=0.041, 0.003)과 재발 없는 생존율(*P*<0.001 and 0.002) 모두에서 통계적으로 의미 있는 나쁜 예후인자로 나타났다. 증가된 FOXP3 양성세포는 통계적으로 유의하지는 않았지만 좋은 예후를 보이는 경향이었다. 다변량분석에서 CD163/CD68의 비는 독립적인 나쁜 예후인자로 나타났다.

본 연구의 결과는 M2 대식세포가 원발성 중추신경 광범위큰B세포림프종과 전신성 광범위큰B세포림프종 모두에서 림프종 형성을 촉진하고 나쁜 경과를 가져오는 역할을 한다는 것을 보여준다. 반면에 CD68 양성 전체 종양관련대식세포는 원발성 중추신경 광범위큰B세포림프종과 리툭시맵 치료를 받은 전신성 광범위큰B세포림프종에서 종양형성을 억제하는 역할을 하고 좋은 임상 경과를 유도하는 역할을 할 수 있다. CD68 양성 전체 종양관련대식세포는 리툭시맵 치료를 받지 않은 전신성 광범위큰B세포림프종 환자군에서는 반대의 결과를 보여 나쁜 예후인자로 작용하였다. 따라서 이러한 종양관련대식세포의 분화에 대해 분석하는

것은 중추신경 광범위큰B세포림프종과 광범위큰 B세포림프종 환자의 예후를 예측 하는데 도움이 될 수 있을 것이다.

주요어: 종양미세환경, 종양관련대식세포, M2 대식세포, 원발성중추신경림프종, 광범위큰B세포림프종, 조절 T세포

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