Original Article

M2 Macrophage/Microglial Cells Induce Activation of Stat3 in Primary Central Nervous System Lymphoma

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Primary central nervous system lymphoma (PCNSL) is one of the most aggressive malignant lymphomas with a median survival of less than 20～40 months. Interest in signal transducer and activator of transcription 3 (Stat3) has increased during the past decade because Stat3 activation was found to contribute to tumor progression by inducing angiogenesis, immunosuppression, and metastasis. We previously demonstrated a significant correlation between Stat3 activation in tumor cells and infiltrating anti-inflammatory (M2) macrophages. Here, we focused on the phenotypes of infiltrating macrophages/microglial cells and Stat3 activation in PCNSL cells. The correlation of Stat3 activation or density of M2 macrophage infiltration with patient prognosis was also evaluated. We performed immunostaining for CD68, CD163, CD204, and pStat3 using paraffin-embedded PCNSL specimens obtained from 43 patients. CD163 and CD204 served as markers of the M2 phenotype. Dense infiltration of CD68$^+$ macrophages was found in all samples. High numbers of CD163$^+$ and CD204$^+$ M2 macrophages/microglial cells were observed in 29 and 25 cases, respectively. Stat3 activation in lymphoma cells was enhanced in the patients who showed denser infiltration of CD163$^+$ macrophages/microglial cells in tumor tissues. *In vitro* co-culture experiment to investigate cell-cell interactions between macrophages and lymphoma cells found that Stat3 in lymphoma cells was strongly activated by coculture with macrophages. Numbers of CD68$^+$, CD163$^+$, and CD204$^+$ tumor-associated macrophages/microglial cells (TAMs) and Stat3 activation in lymphoma cells were not correlated with prognosis. However, because Stat3 involvement in tumor development was demonstrated in several malignant tumors, our present finding that cell-cell interactions of M2 macrophage/microglial cells with lymphoma cells induced Stat3 activation may provide novel insights into PCNSL pathogenesis. 〔*J Clin Exp Hematopathol 51(2) : 93-99, 2011*〕

INTRODUCTION

Primary central nervous system lymphoma (PCNSL) is one of the most aggressive malignant lymphomas, with a recently reported median survival of less than 20～24 months.[1-4] Although the origin of PCNSL is still uncertain, most cases of PCNSL have been classified as diffuse large B-cell lymphomas.[5,6]

Activated signal transducer and activator of transcription 3 (Stat3) is one of the main molecules involved in tumor cell survival and is related to poor clinical prognosis of patients with many kinds of malignant tumors.[7,8] Activation of Stat3 in tumor cells is mediated by receptor-type tyrosine kinases including vascular endothelial growth factor receptor, platelet-derived growth factor receptor, epidermal growth factor, and Src. Stat3 induces anti-apoptotic proteins such as

Bcl-2, Bcl-XL, and survivin in tumor cells.[9] Stat3 also mediates tumor immune escape by inhibiting expression of Th1 immunostimulatory molecules and by promoting production of immunosuppressive factors such as interleukin (IL)-6, IL-10, and transforming growth factor *β*.[10,11] In patients with diffuse large B-cell lymphoma, higher Stat3 activation in lymphoma cells is a predictor of poor clinical prognosis.[12,13] In our previous work, we demonstrated that significant Stat3 activation in cancer cells was caused by stimulatory signals from activated macrophages during ovarian cancer progression.[14] Many cytokines including IL-6 and IL-10 were believed to be involved in Stat3 activation in cancer cells.[14] In view of these findings, we investigated the correlation of Stat3 activation in lymphoma cells, phenotypes of tumor-associated macrophages/microglial cells (TAMs), and clinical prognosis in patients with PCNSL.

With regard to malignant lymphomas, the importance of tumor-infiltrating macrophages for poor clinical prognosis was reported for Hodgkin lymphoma and follicular lymphoma, whereas no significant correlation was reported for diffuse large B-cell lymphoma.[15-17] Interest in the anti-inflammatory phenotype (M2) of TAM has increased in recent years because M2 macrophages are suggested to contribute to tumor progression by inducing angiogenesis, immunosuppression, and tumor cell invasion.[18,19] We previously reported the importance of the M2 anti-inflammatory phenotype of macrophages in several malignant tumors including glioma, intrahepatic cholangiocarcinoma, and angioimmunoblastic T-cell lymphoma.[20-22] In this study, we examined the activation of Stat3 and its correlation with the infiltration of M2 TAMs and clinical prognosis in patients with PCNSL.

MATERIALS AND METHODS

Patients

This study included 43 patients with PCNSL (23 males, 20 females; median age: 68 years, range: 53-84 years) for whom tumor tissues were available. Patients were uniformly treated with standard protocols at Kumamoto University Hospital, Nagano Red Cross Hospital, and Toyama Prefectural Central Hospital. Diagnosis was achieved by biopsy (40 patients) and by resection (3 patients). At least two pathologists reviewed the diagnostic slides according to the World Health Organization classification.[6] Informed written consent was obtained from all patients in accordance with protocols approved by the institutional review board of both hospitals. Tumor cells in all specimens were positive for B-cell markers such as CD20 and CD79a, and negative for T-cell markers (data not shown).

Immunohistochemistry

Paraffin-embedded tumor tissue samples were used for analysis of macrophage infiltration and Stat3 activation. CD163 and CD204 were utilized as markers for M2 macrophages. The following mouse or rabbit monoclonal antibodies were used: CD68 (PG-M1: DAKO, Glostrup, Denmark), CD163 (10D6: Novocastra, Newcastle, UK), CD204 (SRA-E5: TransGenic, Kumamoto, Japan), and pStat3 (Cell Signaling, Danvers, MA, USA). Infiltration of $CD68^+$, $CD163^+$, and $CD204^+$ cells was evaluated by two pathologists who were blinded to any information on the samples. At least three $40 \times$ high-power fields (HPFs) without necrosis and gliosis were evaluated, and classified into high group (> 21 cells per HPF) and low group (< 20 cells per HPF). Immunostaining of pStat3 was scored semiquantitatively using the following system : a score of 0 to 8 + corresponded to the sum of staining intensity (0 = negative, 1 = weak, 2 = intermediate, 3 = strong, and 4 = very strong) and the percentage of positively stained cells (0 = 0%, 1 = 1-25%, 2 = 26-50%, 3 = 51-75%, and 4 = > 76%), as described previously,[21] and the scores were averaged.

Cell culture

B-cell lymphoma cell lines (Raji, Daudi, and SLVL) were obtained from Cell Bank, RIKEN BioResource Center (Tsukuba, Japan), and tumor cell supernatants (TCSs) were prepared as described previously.[21] The mycoplasma test was performed using a polymerase chain reaction detection kit (Takara Bio Inc., Otsu, Japan). Human monocyte-derived macrophages were prepared from peripheral blood mononuclear cells (PBMCs) obtained from healthy volunteers as described previously.[21,23] Briefly, PBMCs were plated in a 6-well plate (0.5×10^6 cells/well) for 1 hr, and nonadherent cells were removed by gentle washing with phosphate-buffered saline. Adherent cells were cultured with granulocyte macrophage-colony stimulating factor (GM-CSF, 10 ng/mL ; Wako, Tokyo, Japan) for 5 days to induce differentiation to immature macrophages.

Cell-cell interactions

GM-CSF-primed human immature macrophages were treated with 50% of TCSs for 2 days then stimulated with 100 ng/mL lipopolysaccharide (LPS; Sigma, St. Louis, MO, USA) for 18 hr. IL-10 production was measured by means of an enzyme-linked immunosorbent assay (ELISA; PeproTech, Rocky Hill, NJ, USA) to determine the M2 polarization status. Induction of CD163 and CD204 in M2 macrophages was evaluated by means of cell-enzyme-linked immunosorbent assay and Western blot, respectively.[21,24] In the co-culture experiment, SLVL cells in 24-well plates were co-cultured

with TCS-stimulated M2 macrophages or interferon-γ-stimulated M1 macrophages using transwell chamber dishes (Nunc, Rochester, NY, USA) for 4 or 5 days as described previously.[14] Stat3 activation was measured by means of Western blot as described previously.[25]

Statistics

The *in vitro* data are expressed as means ± standard deviation (SD). The Mann-Whitney U-test was used for two-group comparisons. A value of $p < 0.05$ was considered statistically significant. Statistical analysis was performed using StatMate IV (ATOMS, Tokyo, Japan).

RESULTS

Many TAMs infiltrated into PCNSL tissues

Immunostaining revealed that many CD68$^+$ TAMs infiltrated into all PCNSL tissues. High numbers of CD163$^+$ and CD204$^+$ M2 TAMs were detected in 29 and 25 cases, respectively (Fig. 1). Twenty-two patients had high infiltration of M2 TAMs with positive staining for both CD163 and CD204.

Stat3 activation in PCNSL tissues

Stat3 activation was evaluated by pStat3 immunostaining, and scores were assigned as described in Materials and Methods. Stat3 activation was significantly higher in patients with high infiltration of CD163$^+$ TAMs, whereas no significant correlation between Stat3 activation and infiltration of
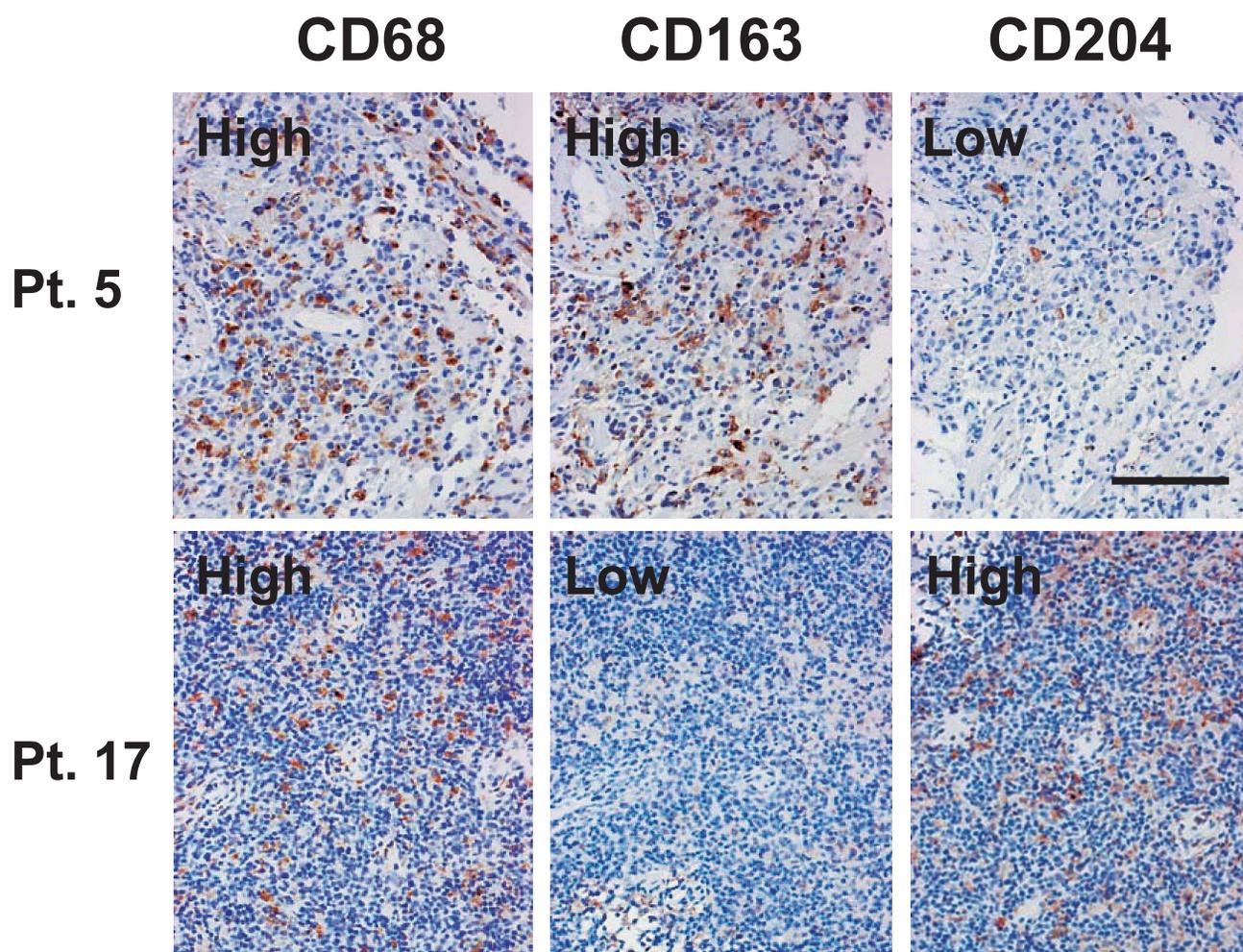

Fig. 1. Immunostaining of primary central nervous system lymphoma (PCNSL) tissues. Anti-CD68, anti-CD163, and anti-CD204 antibodies were used to detect pan-macrophages (CD68) and M-2-polarized macrophages (CD163 and CD204). Many CD68$^+$ macrophages were diffusely detected in all PCNSL tissues. The density of CD163$^+$ or CD204$^+$ cells was classified into two groups. *Scale bar* = 50 μm.

CD204$^+$ TAMs was found (Fig. 2).

Importance of cell-cell interactions between macrophages and lymphoma cells

Tumor-derived factors are known to induce polarization of macrophages to the M2 phenotype. Human macrophages were cultured with or without TCSs obtained from three lymphoma cell lines, and differentiation toward the M2 phenotype was evaluated. TCSs from all lymphoma cell lines enhanced LPS-stimulated IL-10 production (Fig. 3A). The expression of CD163 and CD204 was also increased by TCSs of all cell lines (Fig. 3B & 3C).

Next, a co-culture experiment was performed to investigate the importance of TAMs in the tumor microenvironment. After human macrophages were polarized to the M2 phenotype using TCS from SLVL lymphoma cells, the M2 macrophages and SLVL cells were co-cultured for 4 or 5 days. Co-culture induced strong activation of Stat3 in SLVL cells (Fig. 3D). However, less activation of Stat3 was detected in SLVL cells co-cultured with M1 macrophages (Fig. 3E).

Correlation with overall survival

We also examined the correlation between the phenotype of the TAMs and clinical prognosis. No significant correlation was found between the number of TAMs and overall survival of PCNSL patients (Fig. 4). Stat3 activation in lymphoma cells was also not significantly associated with overall survival of PCNSL patients (Fig. 4).

DISCUSSION

Stat3 activates various genes related to angiogenesis, cell survival, immunosuppression, and tumor invasion in many kinds of malignant tumors including malignant lymphoma.[7,8,12,13] In this study, we observed Stat3 activation in tumor cells of most PCNSL cases. We found that Stat3 activation in lymphoma cells was significantly higher in patients with high infiltration of CD163$^+$ TAMs. Our previous work on ovarian cancer demonstrated a similar relationship between Stat3 activation in tumor cells and density of M2 macrophage infiltration.[14] The findings that Stat3 activation in ovarian cancer cells was strongly up-regulated by co-culture with M2 macrophages and that this cell-cell interaction was partially inhibited by blocking antibodies against IL-6 and IL-10 indicated that various macrophage-derived factors may be involved in interactions between lymphoma cells and TAMs. To confirm the importance of cell-cell interactions in Stat3 activation in lymphoma cells, we used an *in vitro* co-culture experiment and indeed discovered that Stat3 activation in lymphoma cells was strongly induced by co-culture with M2 macrophages. Since cell-cell interactions between SLVL cells and macrophages were induced by indirect co-culture system using transwell dishes, unknown soluble factors might play an important role in cell-cell interac-
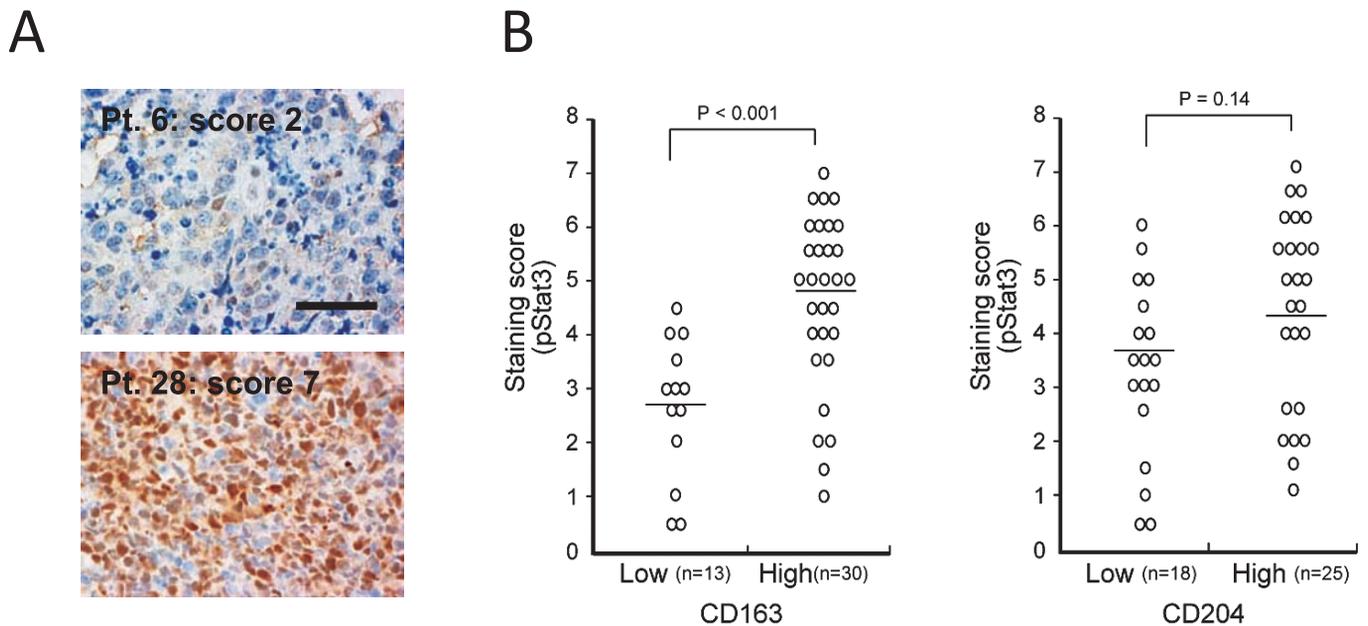

Fig. 2. Stat3 activation in lymphoma cells. (*1A*) pStat3 immunostaining was performed to evaluate Stat3 activation in lymphoma cells. Positive staining was detected in the nuclei of lymphoma cells. *Scale bar* = 50 μm. (*1B*) Correlation between macrophage infiltration and Stat3 activation in lymphoma cells was analyzed by means of the Mann-Whitney U test. Stat3 activation was increased in patients with a high level of infiltration of CD163$^+$ macrophages.
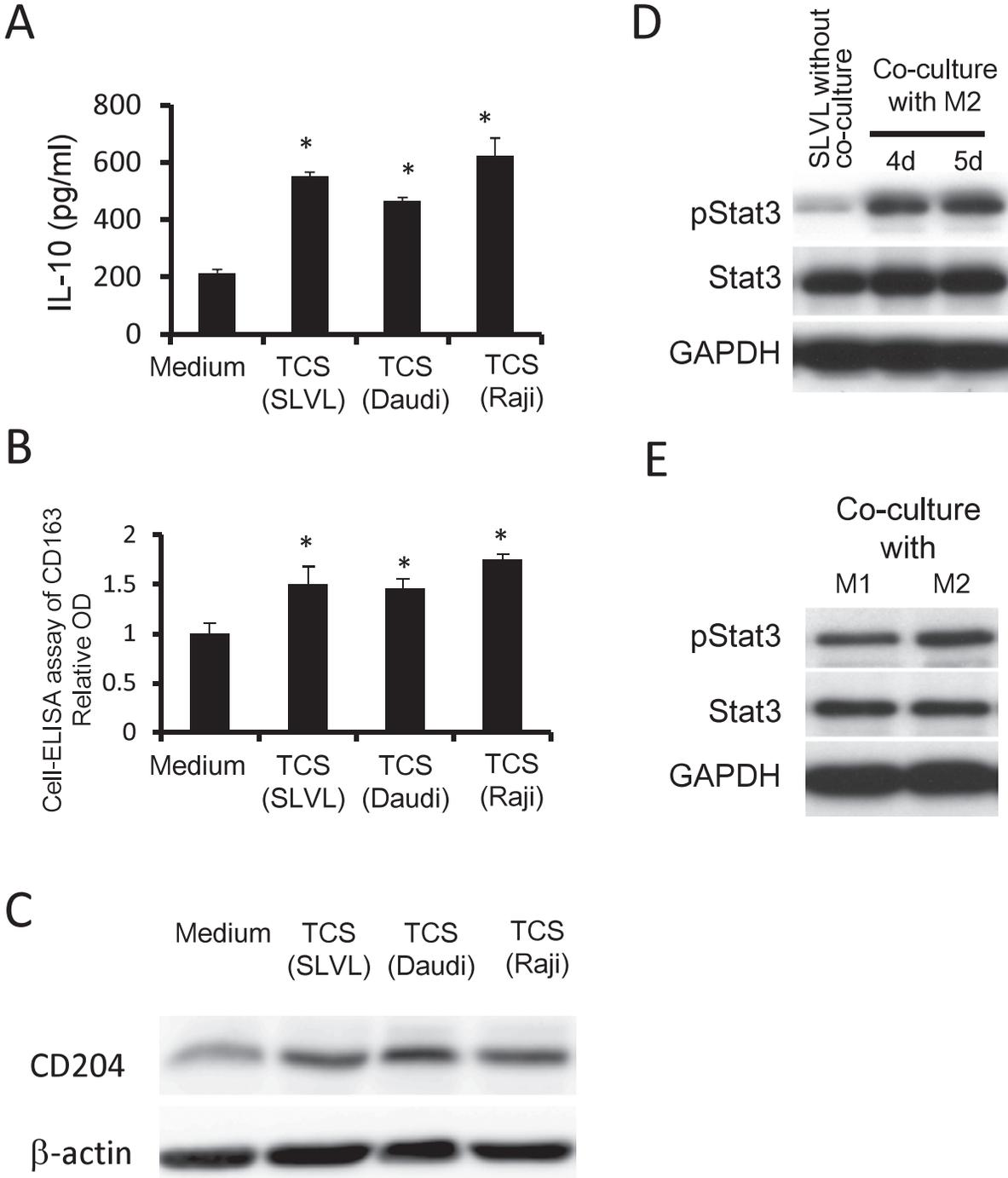
Fig. 3. Interactions between lymphoma cells and macrophages. (*3A*) Human monocyte-derived macrophages were cultured with tumor cell supernatants (TCSs). Following stimulation with lipopolysaccharide, interleukin-10 production was evaluated as an M2 activation marker. Interleukin-10 production was increased in TCS-stimulated macrophages. * : $p < 0.001$. (*3B*) Cell-enzyme-linked immunosorbent assay demonstrated the increased expression of CD163 in TCS-stimulated macrophages. * : $p < 0.001$. (*3C*) Western blot analysis showed the up-regulation of CD204 in TCS-stimulated macrophages. (*3D*) SLVL lymphoma cells were co-cultured with M2 macrophages differentiated via TCS. Stat3 activation in SLVL cells was significantly induced by co-culture. (*3E*) M2 macrophages induced stronger activation of Stat3 in co-cultured SLVL cells. The *in vitro* data represent two or three independent experiments.
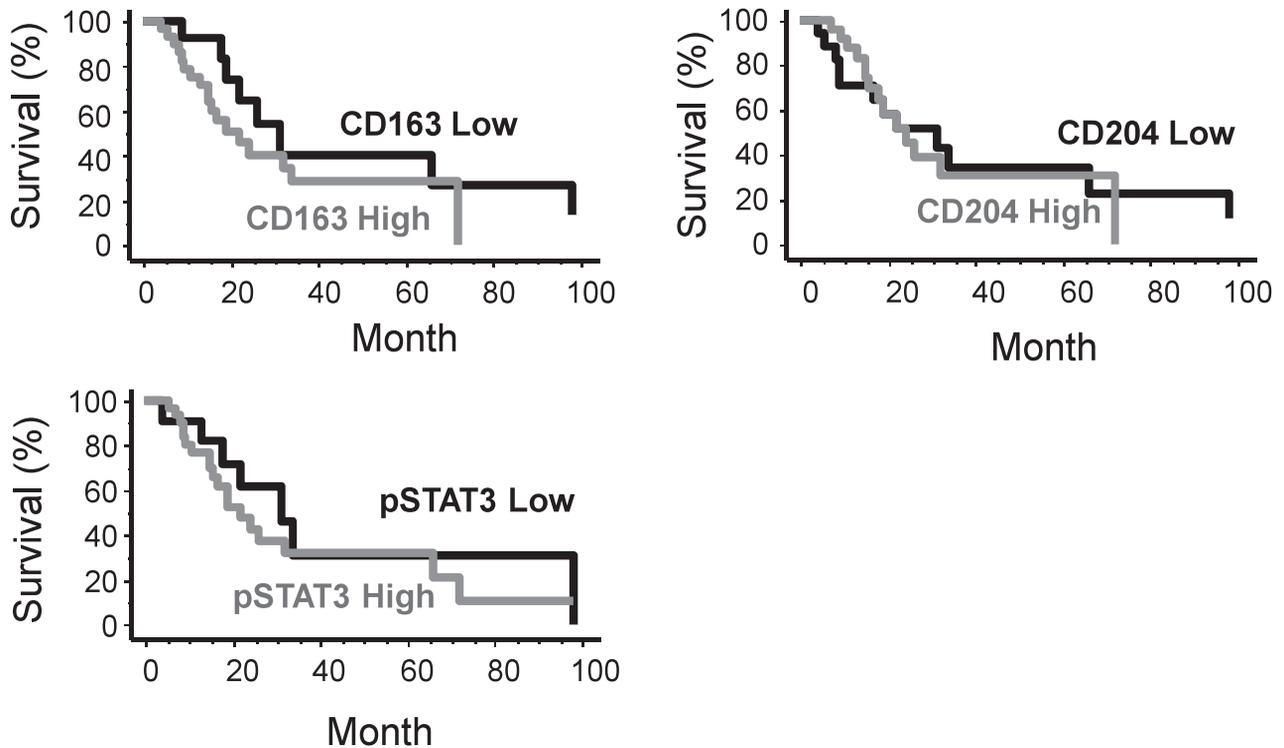
Fig. 4. Kaplan-Meier survival analysis of patients with high or low infiltration of CD163^{+} and CD204^{+} cells, and with high or low Stat3 activation in lymphoma cells. No significant correlation was shown between each molecule and overall survival.

tion.

An unexpected finding was the lack of significant association of Stat3 activation and clinical prognosis. Lam *et al.* demonstrated that not only Stat3 signaling but also nuclear factor-κB (NF-κB) signaling was activated in diffuse large B-cell lymphoma and that these signals contributed in a synergistic fashion to lymphoma cell proliferation.[26] Although we did not investigate NF-κB, both Stat3 and NF-κB may be involved in tumor progression and thus influence clinical prognosis. Further studies are necessary to determine the signaling molecules related to poor clinical prognosis in PCNSL.

In conclusion, we demonstrated here a close association of Stat3 activation in lymphoma cells with high infiltration of CD163^{+} TAMs, even though we found no significant correlation between TAMs and clinical prognosis of patients with PCNSL. Further studies are required to reveal detailed mechanisms of cell-cell interactions. However, because intracellular signaling via Stat3 is a main signaling pathway contributing to tumor development, our present findings may provide useful insights into the pathogenesis of PCNSL.

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