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ORIGINAL COMMUNICATION

A controlled inflammation and a regulatory immune system are associated with more favorable prognosis of progressive multifocal leukoencephalopathy

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Abstract

Objective In the present study, we analyzed the inflammatory profiles of brain tissues obtained from patients with progressive multifocal leukoencephalopathy (PML) due to John Cunningham (JC) virus infection to identify potential prognostic factors.

Methods The study included seven patients (two men, five women) who had been pathologically diagnosed with PML, and all of whom were HIV negative. Fixed brain samples were analyzed via hematoxylin and eosin (HE) staining and Klüver–Barrera (KB) staining. We then performed immunohistochemistry (IHC) specific to JC virus capsid proteins (VP1 and VP2/3) and lymphocyte surface markers (CD4, CD8, CD138, and PD-1).

Results The mean age at onset was 53.4, while the mean duration until biopsy/autopsy was 4.7 months. Four patients were included in the good prognosis (GP) group, while three were included in the poor prognosis (PP) group. Pathological analysis revealed a significantly larger number of CD4-positive T-cell infiltrations ($P = .029$) in the GP group, along with a preserved CD4:CD8 ratio. Larger numbers of CD138-positive plasma cells were also observed in the GP group ($P = .029$) than in the PP group. Linear regression analyses revealed a significant association between the numbers of CD138-positive plasma cells and PD-1-positive cells ($R^2 = 0.80$).

Conclusions Viral loads in the cerebrospinal fluid, a controlled inflammatory response mediated by CD4- and CD8-positive T cells, and plasma cells are associated with PML prognosis. Our findings further indicate that regulatory plasma cells may regulate inflammatory T-cell activity via a PD-1/PD-L1 immuno-checkpoint pathway, thereby protecting the uninfected brain from excessive immune-mediated damage during an active JC virus infection.

Keywords Progressive multifocal leukoencephalopathy · CD4:CD8 ratio · Regulatory plasma cell · IRIS · JC virus · PML

Introduction

Progressive multifocal leukoencephalopathy (PML) is currently an untreatable, fatal demyelinating disease induced by the opportunistic John Cunningham (JC) polyomavirus infection to the central nervous system (CNS). Previously, PML was primarily diagnosed among patients with human

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immunodeficiency virus (HIV) or hematologic malignancy [32]; however, the number of cases has increased due to the use of natalizumab, rituximab, and other immunomodulatory drugs for multiple sclerosis and other autoimmune diseases [3].

Although host immunological inflammatory reactions were thought to rarely occur in the brains of patients with classic PML [17], detailed histopathological analysis revealed the presence of CD3-positive lymphocyte infiltration even in MRI-negative patients with PML; however, infiltration was relatively milder than that in MRI-positive patients. On the other hand, following treatments such as highly active anti-retroviral therapy (HAART) for HIV infection, reconstitution of the immune system may occur, during which severe inflammatory reactions could destroy uninfected brain parenchyma. Such conditions have collectively been referred to as inflammatory PML [19] or PML–immune reconstitution inflammatory syndrome (PML–IRIS) [37]. In most fatal PML–IRIS cases among HIV-positive patients, overwhelming CD8-positive T-cell proliferation has been observed in the brain upon autopsy. Severe inflammation is often accompanied by edema or mass effects, suggesting that catastrophic damage has spread to the uninfected parenchyma. Fatal PML–IRIS, which refers to an excessive T-cell response during immune system reconstitution following a JC virus infection, can result in catastrophic brain destruction spreading to the uninfected parenchyma, in turn leading to a poor prognosis. Although these abnormalities, which can be evaluated using contrast enhancement on T1-weighted magnetic resonance imaging (MRI) [31, 34], are associated with poor prognosis; however, this is not the case for patients with natalizumab-associated PML, in which approximately half of the patients with contrast-enhanced lesions on MRI exhibit a relatively favorable prognosis [10].

Although the precise distinctions between favorable and unfavorable inflammatory reactions remain to be clarified, we recently hypothesized that the preservation of the CD4:CD8 T-cell ratio reflects a controlled inflammatory response—a factor that may be associated with a more favorable prognosis [30]. The previous studies have suggested that the immunological circumstances typically differ between patients with PML exhibiting a preserved CD4:CD8 ratio and those with fatal PML–IRIS showing destruction by CD8-positive T cells [17, 24]. Therefore, cooperation between anti-JC virus activity and a well-controlled immune reaction is crucial in protecting the uninfected brain parenchyma, as the JC virus is cleared from the CNS. Hence, one major factor influencing PML prognosis is an insufficient immune response to the JC virus infection, which is often observed in immunocompromised patients leading to widespread of the JC virus in the CNS. Another critical factor is a lethal uncontrolled immune reaction (mostly associated with CD8-positive T cells), leading to the catastrophic destruction of uninfected neural cells.

In this study, we analyzed the inflammatory profile of brain tissues obtained from patients with PML to identify potential prognostic factors among patients with good prognosis (GP) or poor prognosis (PP). Our findings indicated that in addition to facilitating T-cell responses and the preservation of the CD4:CD8 ratio, regulatory plasma cells act in cooperation to fight against the active JC virus, protecting the uninfected brain parenchyma from immune-mediated damages via the PD-1/PD-L1 immune-checkpoint system.

Methods

The present study included seven patients that had been pathologically diagnosed with PML [4] at Tokyo Medical and Dental University Hospital and the following co-operative institutions between September 2005 and November 2015: Tokyo Medical University Hospital, Shinshu University Hospital, and Tokyo Metropolitan Bokutoh Hospital. We defined GP as a group of patients whose conditions improved clinically and they recovered, such that they were discharged from the hospital. PP was defined as a group of patients who died even after they received standard PML therapies, such as mefloquine, mirtazapine, and administration of immunosuppressive agents were tapered or discontinued. The three patients of the PP group underwent an autopsy, while the four patients of the GP group underwent brain biopsy (Table 1). All patients were HIV negative.

The brains tissues were fixed in 10% buffered formalin and paraffin-embedded, following which the tissues were sectioned and subjected to hematoxylin and eosin (HE) staining and Klüver-Barrera (KB) staining. We then performed immunohistochemistry (IHC) specific to agnoprotein and JC virus capsid proteins (VP1 and VP2/3) [33], followed by in situ hybridization (ISH). Antibodies specific to the JC virus capsid protein VP1 and agnoprotein were kindly provided by Dr. Nagashima (Hokkaido University). Detailed information about these antibodies have been previously described elsewhere [26]. Anti-VP2/3 antibody was originally generated by immunizing a rabbit with the C-terminal sequence 'RKEGPRASSKTSYKR' [32].

To characterize brain inflammation in each patient, fixed brain samples were immunohistochemically analyzed using anti-CD4 (Nichirei, Tokyo, Japan), anti-CD8 (Nichirei, Tokyo, Japan), anti-CD138 (clone B-A38, Thermo Fisher Scientific, Tokyo, Japan), anti-CD68 (Clone PG-M1, Dako, Glostrup, Denmark), and anti-PD-1 antibodies (clone EH12.2H7, LifeSpan Biosciences, Seattle, WA, USA).

The relationship among inflammation markers was evaluated using Mann–Whitney *U* tests and Pearson correlation

Table 1 Demographic feature of patients with progressive multifocal leukoencephalopathy

	Good prognosis group			Poor prognosis group			
Patient no.	1	2	3	4	5	6	7
Age/sex	52/M	38/F	32/F	72/F	70/F	57/F	53/M
Underlying disease	Follicular lymphoma	AIHA	SLE	Dermatomyositis	MCTD	SLE	Diabetes
Months between onset and biopsy/autopsy	2	4	5	2	6	2	9
Symptoms	Cognitive impairment, Gerstmann syndrome, right hemispatial agnosia	Cognitive impairment, right hemiparesis	Motor aphasia, right hemiparesis	Cognitive impairment	Cognitive impairment, Gerstmann syndrome, right hemiparesis	Cognitive impairment	Dysarthria, left hemiparesis, 6th cranial nerve palsy
Treatment	Mefloquine, risperidone, cytarabine	Mefloquine, mirtazapine, PSL tapering	Mefloquine, mirtazapine, PSL tapering	Mefloquine	Risperidone, PSL tapering	PSL tapering	PSL tapering
Outcome	Improvement	Improvement	Improvement	Improvement	Death	Death	Death
Cerebrospinal fluid JC viral load (copies/ml)							
At baseline	Not detected	4000	Not detected	NA	18,000	≧50	10,000
Evolution	NA	Not detected	NA	NA	75,000	NA	NA
Inflammatory cells (median, IQR)							
CD4 + cells (/LPF)	73, 38	56, 45	47.5, 32.8	161, 73	0, 0	1.5, 4.8	16, 19
CD8 + cells (/LPF)	93, 45	50, 23	61, 65.5	54, 35	6, 4	19, 29.5	64, 8
CD138 + cells (/LPF)	9, 12	84, 46	18, 9	313, 188	0, 0	0, 3.8	8, 10
CD4/CD8	0.98, 0.31	1.12, 0.55	0.97, 0.56	3.6, 2.0	0, 0	0.11, 0.23	0.25, 0.25
PD-1 (/LPF)	11, 4	52, 57	28.5, 25.5	97, 36	1, 1	2.5, 6	44, 37
PD-1/CD4 + CD8	0.084, 0.048	0.49, 0.38	0.32, 0.34	0.53, 0.33	0.14, 0.17	0.17, 0.31	0.59, 0.29

Data are described as medians and interquartile ranges

AIHA autoimmune hemolytic anemia; *PSL* prednisolone; *SLE* systemic lupus erythematosus; *MCTD* mixed connective tissue disease; *NA* not available; *LPF* low power field

coefficient, and a contingency was analyzed with Fisher's exact test. All analyses were performed using Prism (GraphPad Software, La Jolla, USA). Data are reported as medians and interquartile ranges. P values $< .05$ were considered to be statistically significant.

Standard protocol approvals, registrations, and patient consent

All patients or patient families provided written informed consent. Our protocol was conducted in accordance with ethical requirements and was approved by the Institutional Ethics Committee of Tokyo Medical and Dental University. The study was performed in accordance with the tenets of the 2013 Declaration of Helsinki.

Data availability statement

The data that support the findings of this study are available from the corresponding author (NS) upon reasonable request.

Results

The mean age at onset of PML was 53.4 (range 32–70 years), and the mean duration from PML onset to the date of biopsy/autopsy was 4.3 months (range 2–9 months). There was no significant difference in age, gender, and underlying diseases between the GP and PP groups. Although JC virus in the cerebrospinal fluid (CSF) at baseline was detected only 1 of 4 patients in the GP group, all patients exhibited positive and apparently larger number of JC viral load in the CSF in the PP group; however, the difference did not reach statistical significance. ($P = .14$; Table 1). All patients in the GP group were treated with mefloquine (Table 1).

Pathological examination demonstrated that the inflammatory reactions occurred more frequently in patients in the GP group than in patients in the PP group (Fig. 1a–d). Infiltration of lymphocytes and plasma cells was more apparent at various levels in patients in the GP group (Fig. 1a, b) than

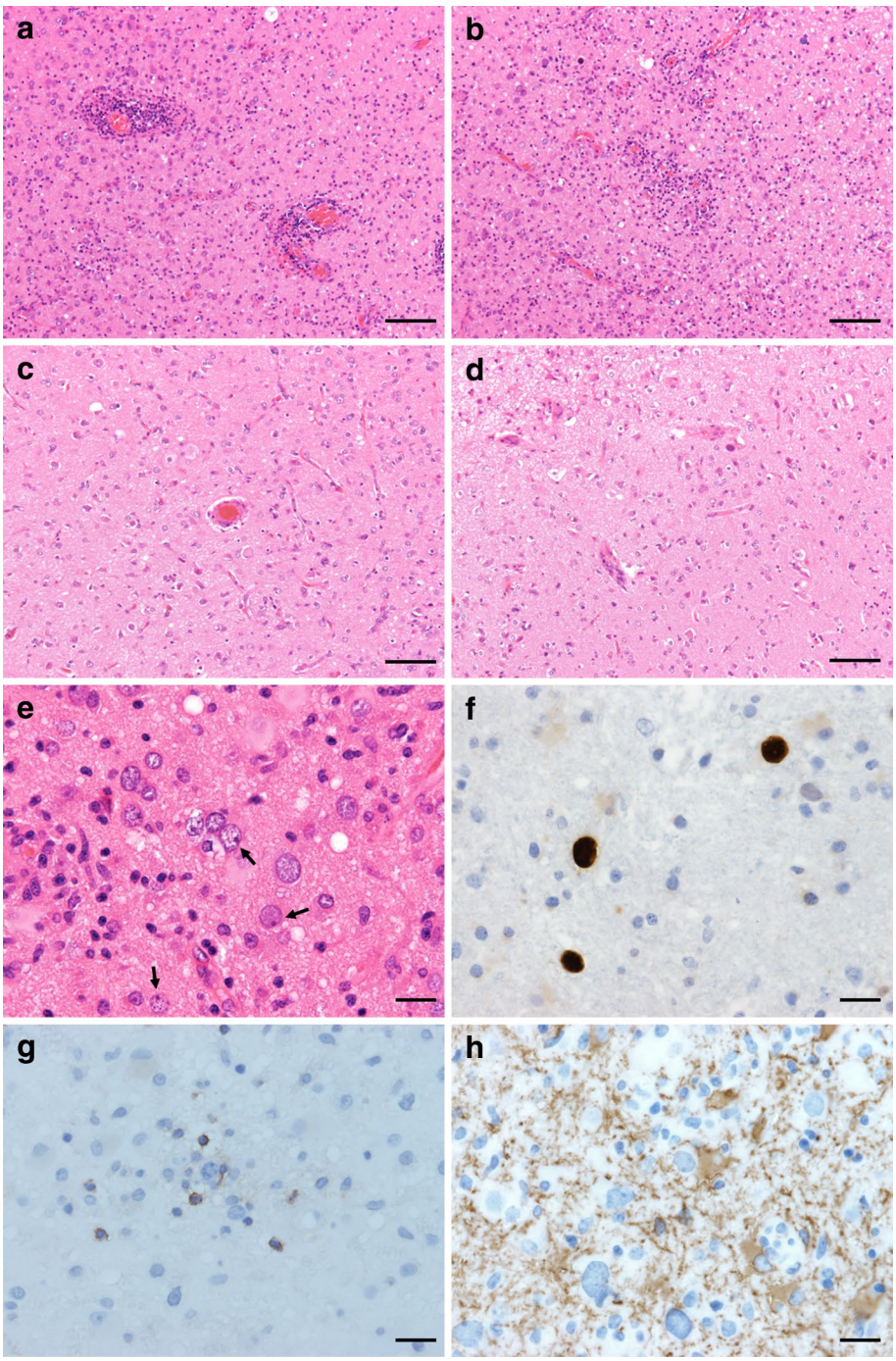

Fig. 1 Pathological findings of demyelinating lesions in patients with PML. Hematoxylin and eosin staining of brain sections from patients in the good prognosis (GP) and poor prognosis (PP) groups. A greater number of inflammatory cells were observed in the GP group (**a, b**) than in the PP group (**c, d**). In addition, in the GP group, inflammatory cells were clustered in the perivascular area (**a, b**), and there were a number of oligodendroglia-like cells with enlarged dot-shaped inclusions (**e**; arrows). Immunohistochemistry specific to the JC virus capsid proteins VP2/3 revealed a JC virus infection in multiple cells (**g**), which were negative for anti-CD45 (**g**) and anti-glial fibrillary acidic protein (GFAP) antibodies (**h**). Scale bar = 100 µm (**a**–**d**), 20 µm (**e**–**h**). *PML* progressive multifocal leukoencephalopathy; *JC virus* John Cunningham virus

patients in the PP group (Fig. 1c, d). In patients in the GP group, blood vessels proliferated in the area surrounding the demyelinating lesions, and lymphoid cells tended to be clustered in the perivascular spaces. In addition, there were a number of oligodendroglia-like cells with enlarged nuclei; however, amphophilic intranuclear viral inclusions were not clearly observed following HE staining. Instead, many cells exhibited nucleoli-like punctate structures, suggesting dot-shaped inclusions (Fig. 1e). Typical JC virus-infected cells with full inclusions were relatively small in number in the GP group. The ratios of dot-shaped inclusions to the total numbers of inclusions were 0.94 (0.83–1.0) and 0.30 (0.22–0.86) in the GP and PP groups, respectively ($P = .20$). IHC specific to the JC virus capsid proteins VP1 and VP2/VP3 was used to observe JC virus-infected cells (Fig. 1f). JC virus-infected cells with swollen nuclei were likely oligodendrocytes, because they were negative for CD45 (Fig. 1g) and glial fibrillary acidic protein (GFAP) (Fig. 1h). ISH revealed JC virus infection to those oligodendrocytes in both groups. In the PP group, JC virus-positive cells were typically observed at the periphery of demyelinated lesions.

We then performed IHC for lymphocyte surface markers, which revealed that most inflammatory cells in the GP group were T cells (CD4, CD8) or plasma cells (CD138), although a commensurate amount of PD-1 positive cells were observed. The distribution of these inflammatory cells corresponded well with that of JC virus-infected cell distributions. Figure 2 presents a comparison of CD4, CD8, CD138, and PD-1 expressions for infiltrating lymphocytes between the GP (Fig. 2a, b, e, f) and PP (Fig. 2c, d, g, h) groups.

Comparison of the numbers of lymphocytes expressing CD4, CD8, and CD138, and PD-1 between the GP and PP groups revealed a significantly larger number of CD4-positive T cells in the GP group (62.8, 47.8–137.9 vs. 2.3, 0–18.3; $P = .029$; Figs. 2a, c, 3a). Although a relatively larger number of CD8-positive T cells were also observed in the GP group, the difference did not reach statistical significance (54.6, 47.2–76.4 vs. 21.5, 5.3–66.0; $P = .20$; Figs. 2b, d, 3b). The average ratio of CD4:CD8 in the GP group was significantly higher than that in the PP group (0.95, 0.92–3.6 vs. 0.12, 0–0.27; $P = .029$; Fig. 3c). The number of CD138-positive plasma cells was also higher in the GP group than in the PP group (46.5, 9.84–222.9 vs. 1.25, 0–6.7; $P = .029$; Figs. 2e, g, 3d). The ratios of PD-1-positive cells to CD138-positive plasma cells tended to be stable, and a linear regression of those values yielded R^2 of 0.80 (Figs. 2e–h, 3d–f), indicating that the expression of PD-1 receptors on T-cell surfaces was induced via regulation of CD138-positive plasma cells.

Previously, the JC virus gene was analyzed and VP1 loop-specific polymorphisms were found to be associated with a favorable prognosis [13]. We tried to analyze the JC virus gene in the patients in the present study, but 3 of 4 patients in the GP group were negative for CSF JC virus, and JC virus gene analysis of the remaining patient in the GP group failed because of a low level of virus load.

Discussion

In the present study, we compared the inflammatory profiles of brain tissues obtained from patients with good and poor prognoses following PML. Our findings indicated that viral loads in the CSF, a controlled inflammatory response mediated mainly by CD4- and CD8-positive T cells, and plasma cells are associated with PML prognosis.

To the best of our knowledge, a low JC virus load in the CSF, VP1 loop-specific polymorphisms, a high CD4-positive T-cell count in the blood, and the presence of JC virus-specific cytotoxic CD8-positive T cells in the blood have been established as good prognostic factors among patients with PML [2, 5, 12, 15, 22, 35]. Some researchers have reported that the mean JC virus load in the CSF at baseline is associated with PML prognosis and that significantly smaller JC virus loads can be observed in patients with slowly progressing PML or stable neurological symptoms, relative to those observed in patients with classical PML [5, 14]. These findings are in accordance with our data obtained from the GP group of the present study, suggesting that some factors associated with the JC virus load should be observed in the CSF (e.g., viral genotype, administration of immune-mediated drugs, host immune surveillance system, etc.).

Studies involving long-term follow-up of patients with an HIV infection demonstrated that CD4-positive T-cell deficits represent a significant risk factor and critical prognostic factor for PML. Indeed, patients with a CD4-positive T-cell nadir of < 200 cells/μl are at a high risk of developing PML with a poor prognosis [27]. In addition to CD4-positive T cells, CD8-positive T cells are critical for survival and recovery from PML. PML cases in associated with HIV and other immunocompromised conditions have been linked to deficiencies in CD4- and/or CD8-positive T cells [27]. Efficient recognition of the JC virus by CD4-positive T cells is necessary for the complementary support of CD8-positive T cells in fighting against a JC virus infection of the CNS. The previous studies have indicated that cytolytic activity mediated by JC virus-specific CD8-positive T cells is correlated with recovery from PML [27]. Additional studies have demonstrated that the ratio of CD4:CD8 lymphocytes is significantly associated with the JC virus index during natalizumab treatment [9]. Lower ratios of CD4:CD8 lymphocytes indicate lower immune surveillance over JC virus activity; these lower ratios are typically observed in patients with multiple sclerosis treated with natalizumab, thus potentially leading to pathogenic PML variants. Gheuens et al.

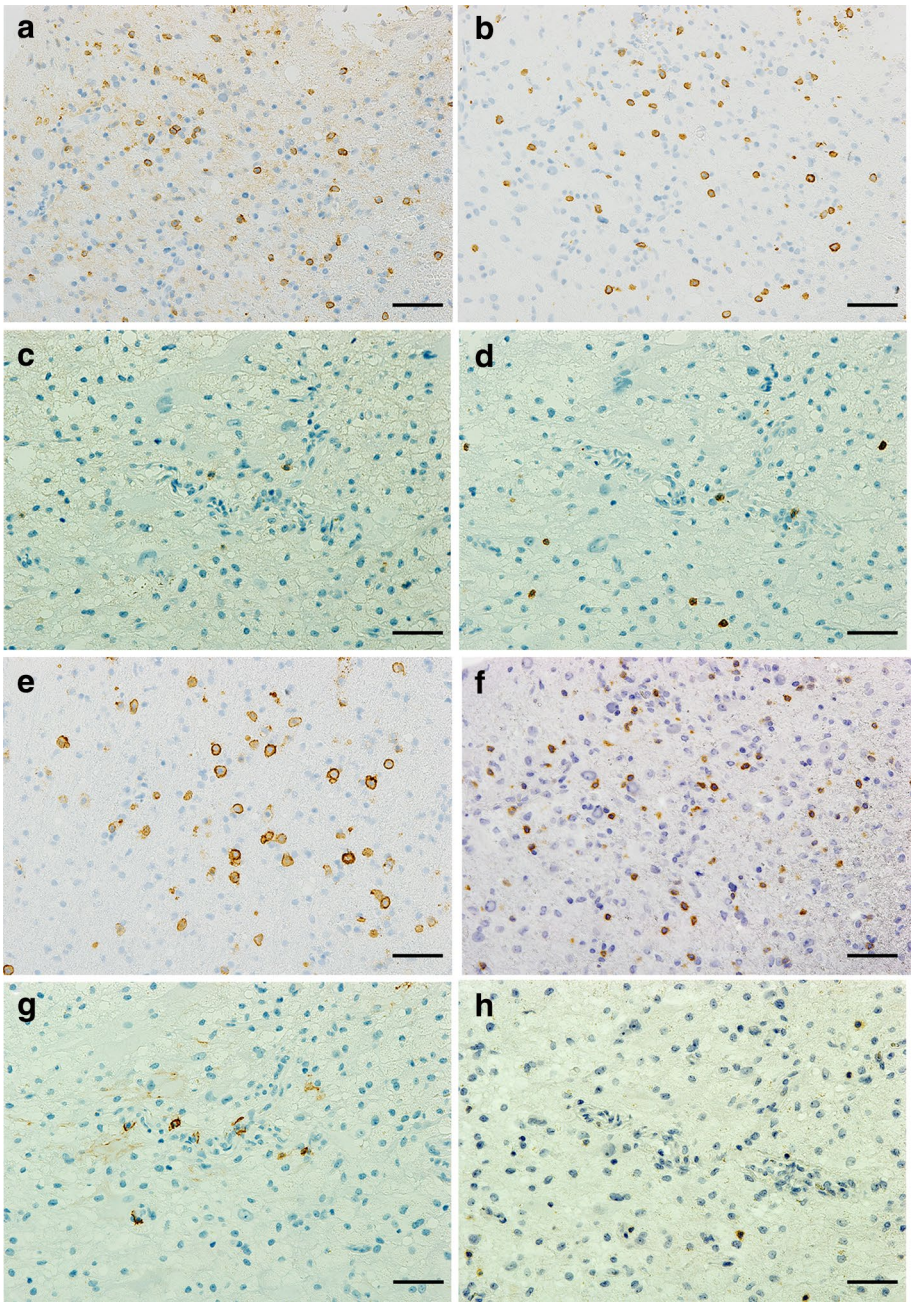

Fig. 2 Comparison of immunocytochemical lymphocyte markers between the good prognosis and poor prognosis groups. Representative immunohistochemical staining for lymphocytic surface markers and immune-checkpoint markers. The number of CD4-positive cells was significantly greater in the good prognosis (GP) group (**a**) than in the poor prognosis (PP) group (**c**). However, no significant differences in the number of CD8-positive cells were noted between the groups (**b, d**). CD138-positive (**c, e**) and PD-1-positive (**f, h**) cells were also more abundant in the GP group (**e, f**) than in the PP group (**g, h**). The pattern of CD4 (**a**) and CD8 (**b**) expressions in the GP group indicated a balanced infiltration of CD4- and CD8-positive T lymphocytes. Scale bar = 50 μm

verified the roles of both CD4- and CD8-positive T cells in patients successfully treated for JC viral infection, reporting that JC virus-specific CD8-positive T cells are critical for disease containment and survival among patients with PML (100% vs. 27.3%) and that a 4.8-fold greater response of CD4-positive T cells was observed in PML survivors than in PML progressors [16].

No previous studies have clarified the mechanism underlying the host immune system against virus infection to prevent such excessive T-cell responses. Notably, in the present study, we observed a significantly larger amount of CD138-positive plasma cells in the GP group than in the PP group. Furthermore, the GP group exhibited preservation of the CD4:CD8 ratio. In the GP group, the inflammatory lymphocytes did not spread excessively beyond the infected brain parenchyma, and there was no extensive damage to the uninfected brain parenchyma. Our result indicated that excessive inflammation was suppressed by PD-1 expressing on the surface of the inflammatory lymphocytes. We further observed a strong correlation between CD138-positive cells and PD-1 expression on the surface of lymphocytes (Fig. 3f)—a finding supported by the previous observations that CD138-positive plasma cells represent a distinct population of regulatory B cells that not only secrete antibodies, but also

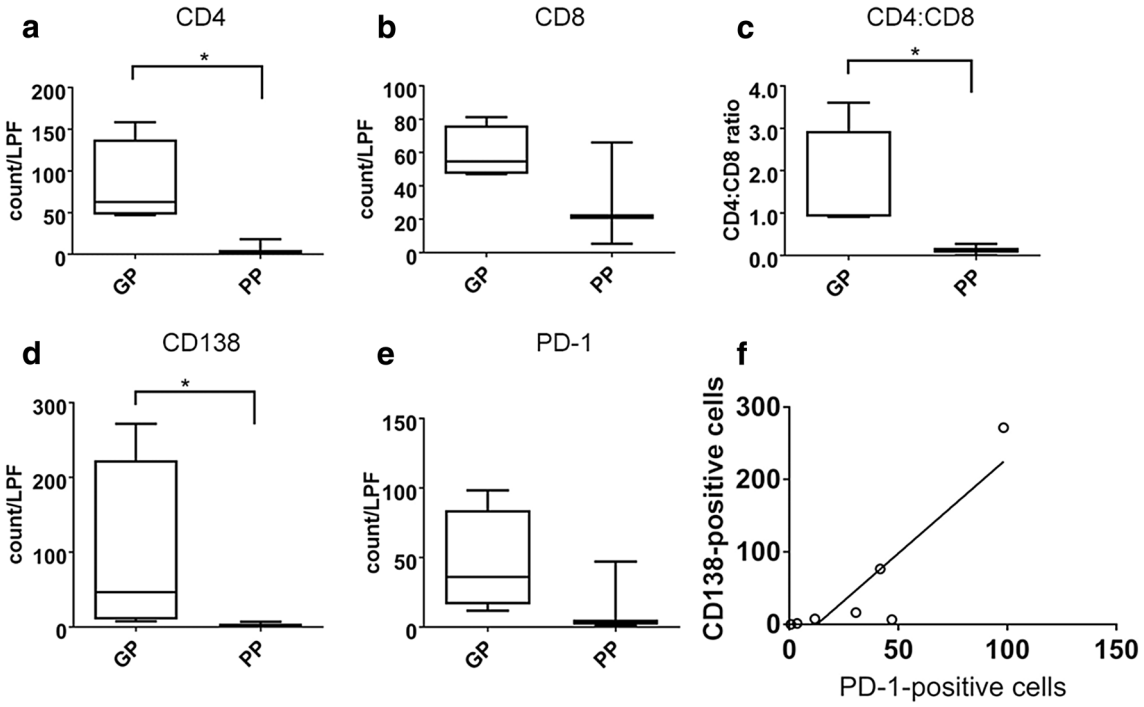

Fig. 3 Box-and-whisker plots of CD-positive cells and linear regression between CD138- and PD-1-positive cells. Significantly greater numbers of CD4-positive (**a**) and a relatively larger number of CD8-positive cells were observed in the GP group than in the PP group (**b**). The average ratio of CD4:CD8 was higher in the GP group than in the PP group (**c**). A larger number of CD138-positive plasma cells were observed in the GP group (**d**), and a relatively larger number of PD-1-positive cells were observed in the GP group than in the PP group (**e**). There was a strong correlation between the numbers of PD-1- and CD138-positive cells (F). *GP* good prognosis; *PP* poor prognosis

suppress T-cell activity via interleukin 10 (IL-10), IL-35, and PD-L1 [23]. Additional reports support the notion that CD138-positive plasma cells contribute to the suppression of excessive inflammation and prevent PML–IRIS. In one such report involving a patient with fingolimod-associated PML with mild IRIS, a large number of CD79a-positive cells were observed at the active inflammatory lesion in the brain [25]. Further studies have demonstrated that B-cell depletion disrupts CD4- and CD8-positive T-cell homeostasis [21] and that B cells produce inhibitory cytokines, thereby promoting a regulatory T-cell response and regulating the inflammatory responses [36].

The lymphocyte surface marker PD-1 emerges on the exterior of viral-specific CD4-positive or CD8-positive T cells induced to become 'exhausted', meaning that they eventually fail to produce effective cytokines upon antigen stimulation [38]. The PD-1 inhibitory pathway may regulate immune-mediated damage during a persistent infection by turning off the virus-specific T cells. Indeed, the previous studies have indicated that PD-1 plays an important role for in the inhibitory regulation of T-cell exhaustion during chronic viral infection [1]. This inhibitory pathway is critical for the appropriate regulation of immune-mediated reactions, which must occur according to the strength of the inflammatory response. To prevent tissue damage during persistent infection, this mechanism must suppress the destructive hyperactivity of the virus-specific T cells [1], which prevent lethal PML–IRIS.

Although all patients in the GP group were treated with mefloquine (Table 1), no patient in the PP group was. The efficacy of mefloquine remains controversial [6, 11, 20]. Interestingly, mefloquine has been reported to induce anti-inflammatory effects such as lymphocyte proliferation or IL-2 production [8, 29]. Although further studies are required to better understand the effects of mefloquine, our results indicate that mefloquine may be helpful, or at least non-harmful, among patients with a JC virus infection of the CNS. A few effective therapeutic candidates via immune response modulators have been reported, such as interferon-alpha, IL-2, and IL-7 [7, 18, 28], which were expected to modify the delicate balance of host immunity to fight the JC virus. The present study revealed that a considerable number of inflammatory cells around the demyelinating lesions were involved in the PD-1/PD-L1 immuno-checkpoint pathway, and this indicates that immune-checkpoint inhibitors may be new candidates for treating PML.

It is important to describe the factors that may influence the conditions that predispose a patient to PML. Here, we

compared the patients' medications before the onset of PML, especially for the immunosuppressive agents (Supplementary Fig. 1). Both the patients in the GP and PP group were treated with immunosuppressive agents except for patient 7 in the PP group. In the GP group, patient 2 was not administered immunosuppressive agents. In the other patients, high-dose prednisolone was started within 3 months and they did not receive prolonged immunosuppressive medications. Conversely, in the PP group, immunosuppressive agents were administered for more than half a year, or strong immunosuppressive therapies were repeated. Patient 7 in the PP group was treated for diabetes for 6 years. Although the number of patients in both groups was small, we observed that prolonged immunosuppressive medications or repeated immunosuppressive therapy seemed to put them at the risk of PML; this is supported by the fact that the number of lymphocytes was higher in the GP group than in the PP group (975.3, 325–1802 vs. 362.5, 135–590). Another possible factor that increased the risk of PML was the age of patients, because the mean age of the patients in the PP group was relatively higher than that in the GP group, but was not statistically significant (57, 53–70 vs. 45, 33.5–67, respectively; $p = .4$).

The present study was limited in that we performed ICH analyses for a limited number of CD series, including dendritic cell markers, which have been reported to induce a potent JC virus-specific cytotoxic T-lymphocyte response. We also investigated a limited number of immune-checkpoint candidates using a limited number of brain sections. Furthermore, we were unable to obtain brain sections from patients with PML–IRIS, or to determine the cells expressing PD-1, the effect of corticosteroids on PD-1 expression in each patient, or the pattern of PD-L1 expression.

In conclusion, the results of the present study demonstrated that viral loads in the CSF, a controlled inflammatory response mediated mainly by CD4- and CD8-positive T cells, and plasma cells are associated with PML prognosis. Our findings further indicate that plasma cells may regulate inflammatory T-cell activity via a PD-1/PD-L1 immunocheckpoint pathway, thereby protecting the uninfected brain from excessive immune-mediated damage during active JC virus infection.

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Author contributions NS: study concept and design, acquisition, analysis and interpretation of data, statistical analysis, and drafting/revising the manuscript; YN: acquisition of data and drafting/revising the manuscript; YS-H: acquisition of data and drafting/revising the manuscript; SM: acquisition of data and drafting/revising the manuscript; YS: acquisition of data and drafting/revising the manuscript; HA: acquisition of data and drafting/revising the manuscript; TT: drafting/revising the manuscript; TY: drafting/revising the manuscript.

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Compliance with ethical standards

Conflicts of interest The authors have NO affiliations with or involvement in any organization or entity with any financial interest, or non-financial interest in the subject matter or materials discussed in this manuscript.

Ethical standard The protocol followed ethical requirements and was approved by the Institutional Ethics Committee of Tokyo Medical and Dental University. This study was performed in accordance with the ethical standards laid down by the 2013 Declaration of Helsinki.

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