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RESEARCH ARTICLE

CNS inflammation after natalizumab therapy for multiple sclerosis: A retrospective histopathological and CSF cohort study

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Abstract

Natalizumab, a recombinant humanized monoclonal antibody directed against the α4 subunit of the integrins α4ß1 and α4ß7, has been approved for the treatment of active relapsing-remitting MS. Although natalizumab is a highly beneficial drug that effectively reduces the risk of sustained disability progression and the rate of clinical relapses, some patients do not respond to it, and some are at higher risk of developing progressive multifocal leukoencephalopathy (PML). The histopathological effects after natalizumab therapy are still unknown. We, therefore, performed a detailed histological characterization of the CNS inflammatory cell infiltrate of 24 brain specimens from natalizumab treated patients, consisting of 20 biopsies and 4 autopsies and 21 MS controls. To complement the analysis, immune cells in blood and cerebrospinal fluid (CSF) of 30 natalizumab-treated patients and 42 MS controls were quantified by flow cytometry. Inflammatory infiltrates within lesions were mainly composed of T cells and macrophages, some B cells, plasma cells, and dendritic cells. There was no significant difference in the numbers of T cells or macrophages and microglial cells in lesions of natalizumab-treated patients as compared to controls. A shift towards cytotoxic T cells of a memory phenotype was observed in the CSF. Plasma cells were significantly increased in active demyelinating lesions of natalizumab-treated patients, but no correlation to clinical disability was observed. Dendritic cells within lesions were found to be reduced with longer ongoing therapy duration. Our findings suggest that natalizumab does not completely prevent immune cells from entering the CNS and is associated with an accumulation of plasma cells, the pathogenic and clinical significance of which is not known. As B cells are considered to serve as a reservoir of the JC virus, the observed plasma cell accumulation and reduction in dendritic cells in the CNS of natalizumab-treated patients may potentially play a role in PML development.

KEYWORDS
multiple sclerosis, natalizumab, plasma cells, progressive multifocal leukoencephalopathy (PML), tysabri

1 | INTRODUCTION

Natalizumab, a recombinant humanized monoclonal antibody directed against the α4 subunit of the integrins α4ß1 (VLA4) and α4ß7, has been approved for the treatment of active relapsing-remitting multiple sclerosis (MS) based on the AFFIRM and SENTINEL phase 3 clinical trials (1, 2). It hinders the transmigration of immune cells into the CNS by blocking the interaction between VLA4, expressed by all leucocytes except neutrophils, and endothelial cells (3).

Analysis of cerebrospinal fluid (CSF) after natalizumab therapy demonstrated its beneficial therapeutic effects: the number of white blood cells (WBC) were found to be decreased (3, 4). Moreover, previous studies have shown increased lymphocyte numbers, predominantly of the B cell lineage, in the peripheral blood after natalizumab therapy (5, 6).

However, there is very limited data on the histopathological changes of the CNS parenchyma after natalizumab therapy. To our knowledge only one histological study has been published, an analysis of a single patient with confounding pathology who developed progressive multifocal leukoencephalopathy (PML) due to natalizumab therapy (7). Therefore, the aim of this study was to provide a detailed histopathological characterization of CNS inflammatory cell infiltration in natalizumab-treated MS patients. For this purpose, tissue samples from 24 natalizumab-treated MS patients were analyzed and the data were compared to disease duration-matched MS controls. To complement the analysis, immune cells in blood and CSF of 30 natalizumab-treated patients were quantified by flow cytometry.

2 | MATERIALS AND METHODS

2.1 | Patients

The present study included tissue samples from MS patients who had been treated with natalizumab (labeled as MS + Ntz) and afterward had either undergone brain biopsy (n = 20) or whose tissue samples had been collected at autopsy (n = 4). In the four autopsy cases, the cause of death was a fulminant MS disease course in two patients, cardiac infarction in one and recurrent infections in the other. Brain biopsies were taken for diagnostic reasons, e.g. to rule out progressive multifocal leukoencephalopathy (PML), a known severe side effect of natalizumab treatment, or due to clinical deterioration after stopping natalizumab therapy. Tissue in all patients showed inflammatory demyelinating lesions consistent with MS. Patients with PML and neuromyelitis optica spectrum diseases (NMOSD) were excluded from the study. Testing for JCV, the virus that causes PML, was done by immunohistochemistry (SV40 T Ag (Ab-2); clone: PAb416; Merck Millipore, Massachusetts, USA) and/or *in situ* hybridization. Of the 24 natalizumab-treated patients, 13 responded to the therapy (labeled as resp.), while 4 patients did not (labeled as non-resp.). Non-response was defined as presenting with relapses and/or new lesion formation on magnetic resonance imaging (MRI) during treatment with natalizumab. In 7 patients, no information on therapy response was available. Controls (labeled as MS) included 21 MS patients with no prior natalizumab therapy, and a disease duration comparable to natalizumab treated patients (n = 11 biopsies and 10 autopsies). Demographic and clinical characteristics of the study participants and MS controls are outlined in Table 1. For blood and CSF analyses, we included 30 natalizumab-treated MS patients and 42 MS controls (Table 1). Lumbar puncture was performed for diagnostic reasons, e.g. to rule out PML.

2.2 | Histology and immunohistochemistry

Biopsy and autopsy samples were fixated in formalin and embedded in paraffin. One µm-thick slices were stained with hematoxylin and eosin (H&E), Luxol fast blue/periodic acid Schiff (LFB/PAS) and Bielschowsky silver impregnation. Inflammatory cell infiltrates were detected by immunohistochemistry with an avidin-biotin technique using antibodies specific for CD3 (marker for T cells; clone: CD3-12; AbD Serotec), CD4 (marker for T helper cells; clone: SP35; Zytomed Systems GmbH, Berlin, Germany), CD8 (marker for cytotoxic T cells; clone: L26; Dako DakoCytomation Glostrup, Denmark), CD20 (marker for B cells; clone: L26; Dako), CD138 (marker for plasma cells; clone: MI15; Dako), IL-10 (marker for the anti-inflammatory cytokine interleukin 10; polyclonal; Bioss, Woburn, Massachusetts, USA), CD209 (marker for dendritic cells; clone: 120612; R&D Systems, Minneapolis, MN, USA), MCAM (marker for Th17 cells; clone: EPR3208; Merck Millipore) and KiM1P (marker for macrophages and microglia; clone: KiM1P, University Kiel, Germany). Double fluorescence immunohistochemistry to show MCAM+ T cells was performed using the same primary antibodies and using tyramid amplification for CD3 (Alexa Fluor 555 Tyramid SuperBoost Kit; Invitrogen GmbH, Karlsruhe, Germany). Macrophages and microglia were distinguished according to their morphology. Demyelinating lesion activity was determined using antibodies against

TABLE 1 Demographic and clinical characteristics of natalizumab-treated MS patients and controls

	Biopsies								Autopsies			Blood/CSF		
	Total biopsies		Active demyelinating biopsy lesions			Inactive demyelinated biopsy lesions			Inactive demyelinated autopsy lesions					
	MS + Ntz (n = 20)	MS (n = 11)	MS + Ntz[a] (n = 17)	MS (n = 7)	*p* value	MS + Ntz[a] (n = 5)	MS (n = 4)	*p* value	MS + Ntz[b] (n = 4)	MS (n = 10)	*p* value	MS + Ntz (n = 30)	MS (n = 42)	*p* value
Demographic characteristics														
Age, years, median (range)	38.5 (23–54)	32(14–52)	40 (29–52)	32(14–52)	0.1921	44 (23–54)	33 (28–44)	0.4606	41 (32–62)	47 (28–71)	0.6354	42 (29–61)	37.5 (18–64)	0.2761
Female, no. (%)	14 (70)	9 (81.8)	12 (70.1)	5 (71.4)		3 (60.0)	4 (100.0)		3 (75.0)	4 (40.0)		20 (66.7)	28 (66.7)	
Clinical characteristics														
Disease duration, years, median (range)	10 (1.5–23)	7 (5–14)	10 (1.5–23)	7 (5–10)	0.1801	6 (1.5–9)	8 (5–14)	0.3429	10.5 (6–20)	10.5 (2.5–23)	0.7761	11.9 (4–24)	0.9 (0–13)	<0.001
RR, no. (%)	15 (75)	6 (54.5)	13 (76.5)	3 (42.9)		4 (80.0)	3 (75.0)		3 (75.0)	0		30 (100)	36 (85.7)	
SP, no. (%)	5 (25)	5 (45.5)	4 (23.5)	4 (57.1)		1 (20.0)	1 (25.0)		1 (25.0)	2 (20.0)		0 (0)	1 (2.4)	
PP, no. (%)	0	0	0	0		0	0		0	4 (40.0)		0 (0)	1 (2.4)	
unknown, no. (%)	0	0	0	0		0	0		0	4 (40.0)		0 (0)	4 (9.5)	
Ntz treatment characteristics														
Therapy duration, months, median (range)	28.5 (1–106)		33 (1–106)			16 (1.5–53)			15 (5–51)			62 (25–117)		
Time after ntz. discontinuation and bx/ax/lumbar puncture, days, median (range)	185 (21–1848)		180.5 (21–617)			259 (22–1848)			188.5 (20–198)			0 (0)		
Previous treatment														
Mitoxantrone	1	5	1	3		1	2		0	0		0	0	
Interferon beta	0	6	0	4		0	2		1	0		19	0	
Glatiramer acetate	2	3	1	2		1	1		1	0		6	0	
Fingolimod	4	0	4	0		0	0		0	0		1	0	
DMF	2	0	1	0		1	0		0	0		0	0	
None	5	1	4	1		1	0		3	0		4	42	
Steroids[c]	11	2	10	2		2	0		1	0		0	10	
PLEX[c]	6	2	6	1		1	1		0	0		0	0	

Abbreviations: ax, autopsy; bx, biopsy; DMF, dimethyl fumarate; Ntz, natalizumab; PLEX, plasma exchange; PP, primary progressive; RR, relapsing-remitting; SP, secondary progressive.

[a]Patients may have more than one demyelinating activity.

[b]Number of lesions analyzed = 1–5.

[c]Relapse therapy within 6 weeks before biopsy/autopsy/lumbar puncture.

myelin basic protein (MBP; polyclonal; Dako) and proteolipid protein (PLP; clone: plpc1; AbD Serotec, Oxford, UK) according to Brück et al. (8) and Kuhlmann et al. (9). Myelin protein degradation products, including the major myelin proteins MBP and PLP, can be detected in the cytoplasm of macrophages during active demyelination, while inactive demyelinated MS lesions show no incorporation of these myelin proteins. Inactive demyelinated biopsy lesions typically still show a substantial number of macrophages, whereas inactive demyelinated autopsy lesions are in general older and present lower macrophage numbers. We cannot exclude with certainty that these biopsies are not representative of the disease process as a whole, but our experience from autopsy studies indicates that these biopsies are typical for the pathological process in general. Histological sections were captured using a digital camera (DP71; Olympus Europa GmbH, Hamburg, Germany) mounted on a light microscope (BX51; Olympus Europa GmbH). Inflammatory cells were quantified at 400x magnification in at least 10 visual fields using an ocular counting grid and are shown as cells/mm^2.

2.3 | Immune cell phenotyping by fluorescence-activated cell sorting (FACS)

Peripheral blood mononuclear cells (PBMCs) were prepared by Ficoll-Hypaque (Biochrom, Berlin, Germany) density centrifugation. CSF samples were centrifuged at 250 g for 10 min at 4°C within 20 min after collection, and the cell pellet was immediately processed by flow cytometry. Cell surface staining was performed using fluorescence labeled anti-CD3, anti-CD4, anti-CD8, anti-CD19, anti-CD45RA, anti-CD45RO and anti-CD138 antibodies (BD Biosciences, Heidelberg, Germany) according to the manufacturer´s instructions. Negative controls included directly labeled isotype-matched irrelevant antibodies (BD Biosciences). After the staining procedure, cells were evaluated by flow cytometry. Cells were measured on a LSR-Fortessa (BD Biosciences) and evaluated by FACS-Diva Software (BD Bioscience).

2.4 | Statistical analysis

Statistics were calculated using the software GraphPad Prism 5.01 and 6.01. The differences between two groups were analyzed using the Mann-Whitney *U* test. Group differences of immune cell distribution in the blood versus the CSF were verified by the Wilcoxon matched pairs test. Correlation analyses were done with the Spearman *r* test. All data are given as median. A value of $p < 0.05$ was considered significant and is shown by one asterisk. Two asterisks, three asterisks and four asterisks indicate significances of $p < 0.01$, $p < 0.001$ and $p < 0.0001$, respectively.

3 | RESULTS

3.1 | Pathological, demographic, and clinical characteristics

A total of 24 brain tissue samples taken after natalizumab therapy were available for analysis. Lesion staging showed 17 active demyelinating biopsy areas, 5 inactive demyelinated biopsy areas (2 biopsy specimens contained active demyelinating and inactive demyelinated areas) and 4 inactive demyelinated autopsy lesions of natalizumab-treated MS patients. The disease duration ranged from 1.5 to 23 years (median: 10 years; Table 1). 18 of these patients showed a relapsing-remitting and 6 patients a secondary progressive disease course. The median age at biopsy and autopsy was 38.5 and 41 years, respectively, and more than two-thirds of patients were women. The natalizumab medication was heterogeneous in regard to the number of natalizumab infusions given before biopsy/autopsy, varying between 1 and 101 infusions. In addition, one patient received an oral anti-α4 integrin inhibitor (firategrast) instead of natalizumab infusions. Furthermore, the interval between the last natalizumab infusion and biopsy/death varied between 20 days and more than 5 years. 11 MS biopsies and 10 autopsies with no prior natalizumab therapy were used as controls (Table 1). Lesion staging showed 7 active demyelinating biopsy lesions, 4 inactive demyelinated biopsy lesions, and 10 inactive demyelinated autopsy lesions. The median disease duration at biopsy or autopsy was 7 and 10.5 years, respectively. More patients treated with natalizumab received high-dose corticosteroids (HDCS) and/or plasma exchange before biopsy/autopsy (Table 1). For blood and CSF analyses we included 20 female and 10 male natalizumab-treated MS patients with a median number of 62 natalizumab infusions, ranging between 25 and 117 infusions, and compared them to 42 MS controls (28 female, 14 male; Table 1). The median age of the natalizumab and MS control cohort was 42 years and 37.5 years, respectively. MS controls received HDCS more often than natalizumab-treated patients (Table 1).

3.2 | CNS T cell inflammation despite natalizumab therapy

T cells were observed in active demyelinating lesions (Figure 1A; MS + Ntz median: 57.6 cells/mm^2, MS median: 81.6 cells/mm^2) as well as in inactive demyelinated lesions (data not shown) of natalizumab-treated patients, and showed no significant difference as compared to controls. In addition, dissection of the T cell population into CD4$^+$ T helper and CD8$^+$ cytotoxic T cells revealed no significant differences (Figure S1).

After discontinuing natalizumab treatment, a reduction in free CD49d receptor binding sites on PBMCs was described for a further 3½ months as compared to

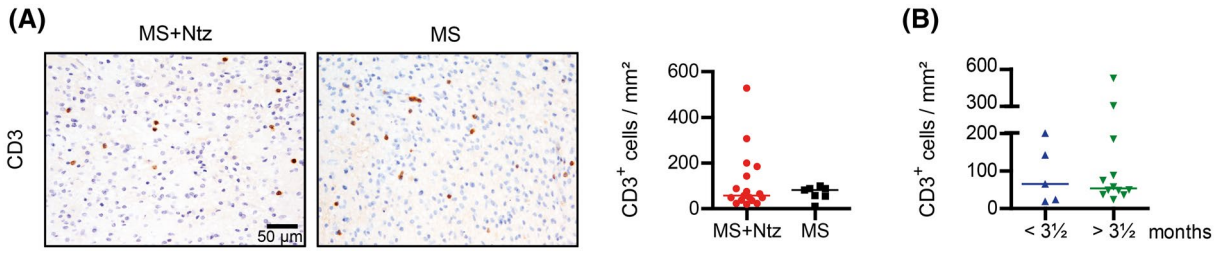

FIGURE 1 T cells are present in MS brain parenchyma despite natalizumab therapy. T cell infiltration was assessed by immunohistochemical staining for CD3 in active demyelinating white matter biopsy lesions. (A) T cell numbers in natalizumab-treated MS patients were compared to MS controls (representative sections, left; quantitative comparison of groups given as median, MS + Ntz: n = 17, MS: n = 7, right; Mann-Whitney *U* test). (B) T cell numbers with expected natalizumab activity (<3½ months = treatment interruption of less than 3½ months) were compared to T cell numbers where no more natalizumab activity was expected (>3½ months = treatment interruption of more than 3½ months; quantitative comparison of groups given as median, <3½ months: n = 5, >3½ months: n = 12; Mann-Whitney *U* test)

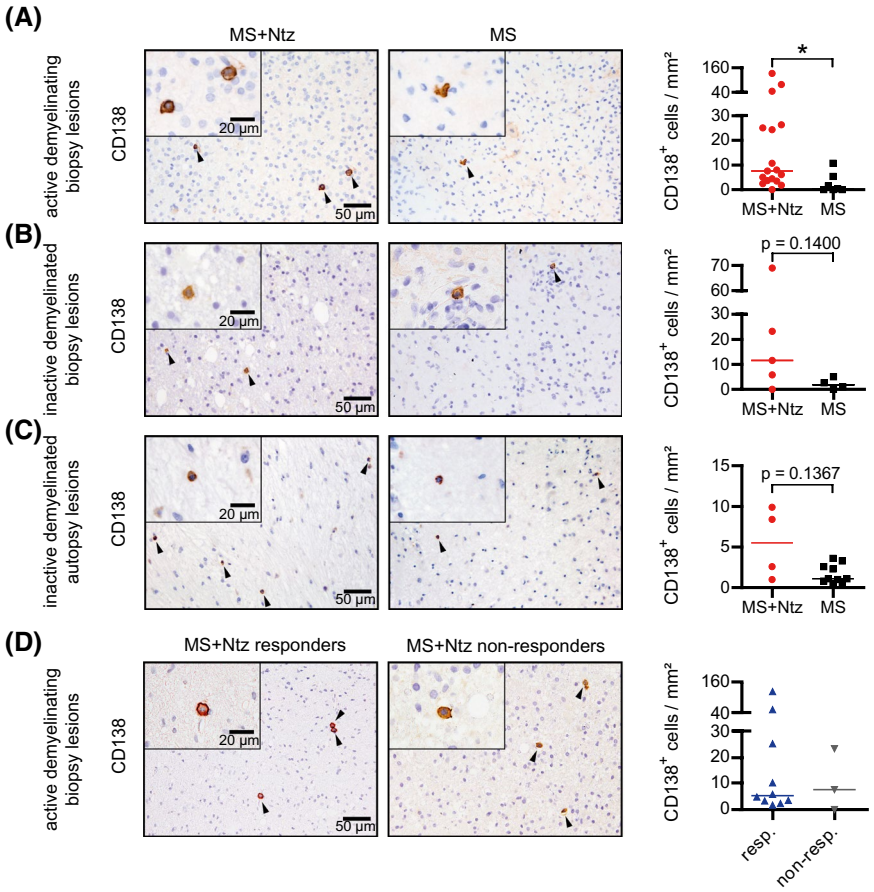

FIGURE 2 Increased plasma cell numbers in MS brain parenchyma after natalizumab therapy. Plasma cell infiltration was assessed by immunohistochemical staining for CD138 (A) in active demyelinating white matter biopsy lesions and in inactive demyelinated white matter (B) biopsy lesions and (C) autopsy lesions (representative sections, black arrowheads indicate individual cells, left; quantitative comparison of groups given as median, MS + Ntz: (A) n = 17 (B) n = 5 (C) n = 4, MS: (A) n = 7 (B) n = 4 (C) n = 10, right). (D) Plasma cell infiltration in patients with active demyelinating white matter biopsy lesions which responded to natalizumab therapy (resp.) were compared to non-responding patients (non-resp.) (representative sections, black arrowheads indicate individual cells, left; quantitative comparison of groups given as median, MS + Ntz resp.: n = 10, MS + Ntz non-resp.: n = 3; (A–C) Mann-Whitney *U* test)

levels before the first injection.(10) To determine whether natalizumab activity has an influence on CNS T cell inflammation, T cell numbers in active demyelinating biopsies until 3½ months after the last infusion were compared to T cell numbers with treatment discontinuation longer than 3½ months. No difference in T cell numbers was found when comparing these two groups (Figure 1B; interval between last natalizumab infusion and biopsy or autopsy <3½ months median: 65.6 cells/mm^2, >3½ months median: 53.7 cells/mm^2).

In experimental autoimmune encephalomyelitis (EAE), an animal model of MS, it was shown that Th17 cells are able to enter the CNS independently of VLA4, which is blocked by natalizumab, suggesting migration via the choroid plexus into the brain parenchyma in a LFA1/ICAM1 dependent manner (11). Along the same lines, natalizumab has been observed to promote differential routes into human CNS by involving PSGL-1 rolling and MCAM-adhesion of TH17 cells (12). To investigate whether natalizumab treatment effects Th17 cell migration, we quantified $MCAM^+$ cells, a proposed marker for Th17 cells (13–15) in the periventricular parenchyma of natalizumab-treated MS patients. We observed single Th17 cells in natalizumab-treated MS patients with no significant difference compared to MS controls (Figure S2).

Taken together, our results suggest that T cell infiltration into the brain parenchyma may occur despite natalizumab therapy.

3.3 | Plasma cell accumulation in CNS lesions after natalizumab therapy

Quantification of plasma cells in the CNS of natalizumab-treated MS patients revealed significantly increased numbers in active demyelinating lesions compared to MS controls (Figure 2A; MS + Ntz median: 7.6 cells/mm^2, MS median: 0.4 cells/mm^2, $p = 0.0156$). Also, higher numbers of plasma cells were found in inactive demyelinated biopsy and autopsy lesions (Figure 2B,C) as compared to MS controls, but these differences showed no statistical significance (Figure 2B; MS + Ntz median: 11.6 cells/mm^2, MS median: 1.9 cells/mm^2, $p = 0.14$; Figure 2C; MS + Ntz median: 5.5 cells/mm^2, MS median: 1.1 cells/mm^2, $p = 0.1367$). In a small number of patients (n = 4 patients with 5 lesion areas), tissue samples were stained for IL-10 and revealed IL-10-positive plasma cells ranging between 7.8% and 68.6% of all plasma cells (Figure S3). By dividing the natalizumab-treated MS patients with active demyelinating lesion activity according to therapy responders (resp.) and non-responders (non-resp.), no obvious difference in plasma cell numbers was evident (Figure 2D), although statistical analyses were not performed due to low numbers of non-responders. As it is known that plasma cell numbers increase with longer disease duration (16), a disease duration-related effect was ruled out by correlation analysis (Figure S4A). Moreover, plasma cell numbers were also not dependent on age (Figure S4B), suggesting a treatment-related effect by natalizumab. Single B cells were observed in natalizumab-treated MS patients with no significant difference to MS controls (data not shown).

3.4 | Reduction in dendritic cells in the CNS with longer natalizumab therapy

Dendritic cells play an important role in initiation and regulation of immune processes in infections.

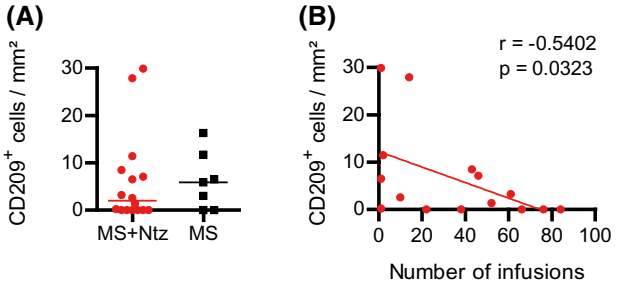

FIGURE 3 Decreased dendritic cell numbers with longer ongoing natalizumab therapy. (A + B) Dendritic cell infiltration was assessed by immunohistochemical staining for CD209 in active demyelinating white matter biopsy lesions and (B) correlated to the number of natalizumab infusions [(A) quantitative comparison of groups given as median, MS + Ntz: n = 16, MS: n = 7; Mann-Whitney *U* test; (B) n = 15; Spearman *r* test]

Natalizumab treatment is associated with the occurrence of the opportunistic infection PML, and we were thus interested in whether dendritic cells were reduced after natalizumab treatment. CD209-positive cells in the CNS of natalizumab-treated patients showed a typical dendritic cell morphology and quantification of dendritic cells revealed no significant difference in active demyelinating lesions (Figure 3A; MS + Ntz median: 2.0 cells/mm^2, MS median: 5.9 cells/mm^2) and in inactive demyelinated lesions (data not shown) as compared to MS controls. No correlation was found between the number of dendritic cells and the disease duration (Spearman r 0.219; $p = 0.94$). However, correlation analysis showed a decline in dendritic cells with an increasing number of natalizumab infusions (Figure 3B; Spearman r −0.5402, $p = 0.0323$), indicating an impaired immune surveillance with longer ongoing natalizumab therapy.

3.5 | No impact on macrophage and microglia numbers in CNS after natalizumab therapy

Natalizumab therapy did not alter macrophage and microglia numbers in active demyelinating white matter biopsy lesions (Figure 4).

We next analyzed the CSF cell numbers as well as the immune cell composition in the blood and CSF of natalizumab-treated patients and MS controls.

3.6 | Reduction in the absolute number of inflammatory cells in the CSF of natalizumab-treated patients, except for memory cytotoxic T cells, B cells, plasmablasts, and plasma cells

Natalizumab therapy is known to reduce the absolute number of inflammatory cells in the CSF (3), and this could also be seen in our cohort (Figure 5A). However, further cell subset analyses revealed that CD8+ cytotoxic T cells and specifically CD8+CD45RO+ memory cytotoxic T cells as well as B cells, plasmablast, and plasma

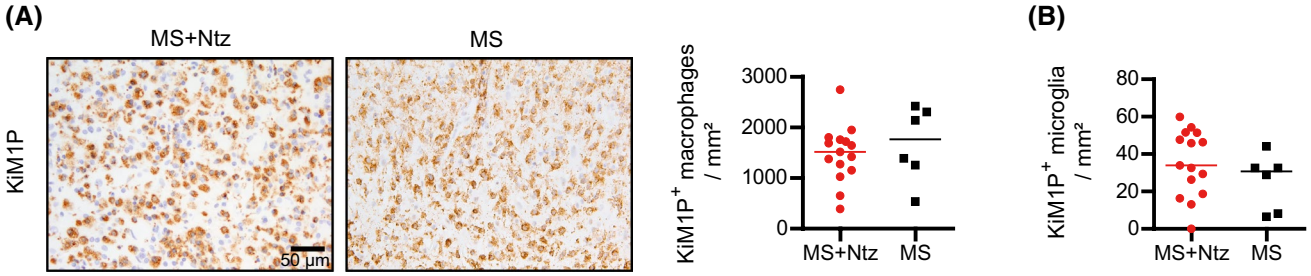

FIGURE 4 Natalizumab has no effect on macrophage and microglia numbers in MS brain parenchyma. (A + B) Macrophages and microglia were stained for KiM1P and distinguished according to their morphology in active demyelinating white matter biopsy lesions [(A) representative sections, left; quantitative comparison of groups given as median, (A) macrophages and (B) microglia; MS + Ntz: n = 15, MS: n = 6, right; (A + B) Mann-Whitney *U* test]

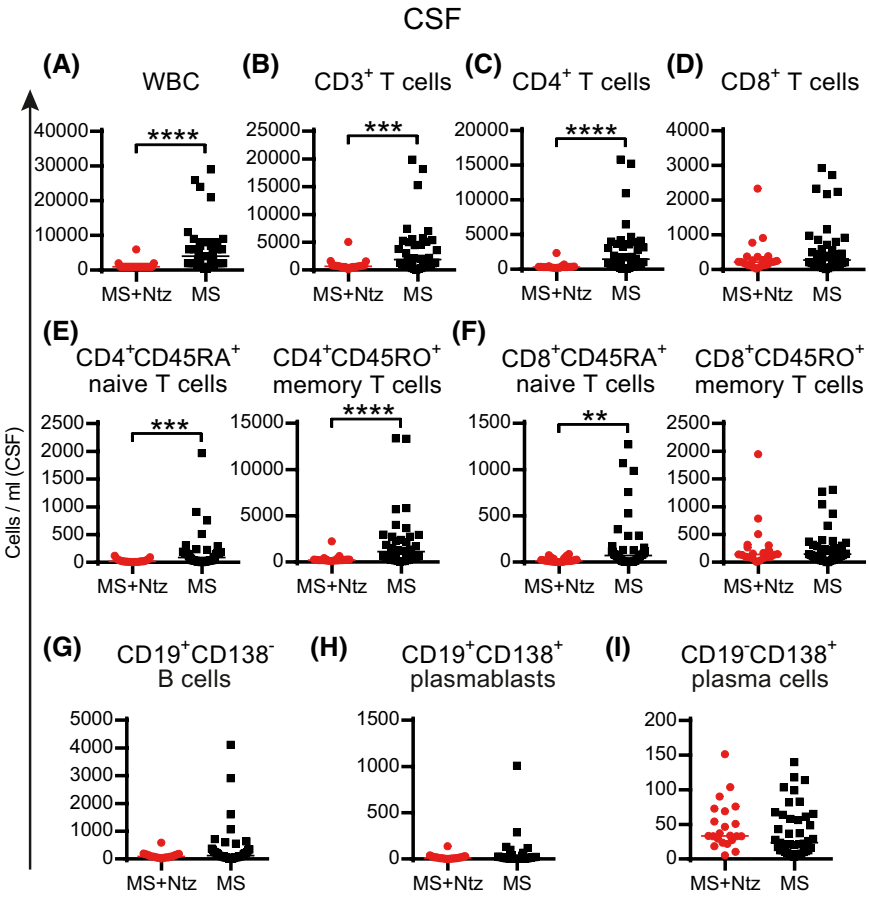

FIGURE 5 Reduced absolute number of inflammatory cells in the CSF of natalizumab-treated patients, except for memory cytotoxic T cells, B cells, plasmablast and plasma cells. In the CSF, absolute WBC numbers (A) were quantified after lumbar puncture. The absolute numbers of CD3+ T cells (B), CD4+ T cells (C), CD8+ cytotoxic T cells (D), CD4+CD45RA+ naive and CD4+CD45RO+ memory T cells (E), CD8+CD45RA+ naive and CD8+CD45RO+ memory T cells (F), CD19+CD138− B cells (G), CD19+CD138+ plasmablasts (H) and CD19−CD138+ plasma cells (I) were determined by flow cytometry based on total absolute WBC numbers (quantitative comparison of groups given as median, MS + Ntz: (A, B, D, F left, G, H, I) n = 22, (C, E, F right) n = 21; MS: (A–I) n = 41; (A–I) Mann-Whitney *U* test)

cells were not reduced in natalizumab-treated patients versus MS controls, indicating that these cell subsets were not affected by the treatment (Figure 5B–I).

We then focused in detail on the percentages of inflammatory cell subsets in the blood as well as CSF.

3.7 | Increased percentages of memory cytotoxic T cells in CSF of natalizumab-treated patients

We first focused on T cell inflammation and analyzed the percentage of different T cell subsets of total PBMCs; the results are given as frequencies. Although no changes in T cell subsets were found in peripheral blood after natalizumab treatment compared to controls (Figure S5A–E), within the CSF the frequencies of T cells increased (Figure 6A). Detailed characterization of T cells showed that the frequencies of $CD8^+$ cytotoxic T cells increased, whereas the frequency of $CD4^+$ T cells decreased (Figure 6B,C). The increase in $CD8^+$ cytotoxic T cells could be attributed to an increase in the frequency of memory cytotoxic T cells and a decrease in the frequency of naïve cytotoxic T cells, while no difference in $CD4^+$ naïve and memory T cells was found (Figure 6D,E).

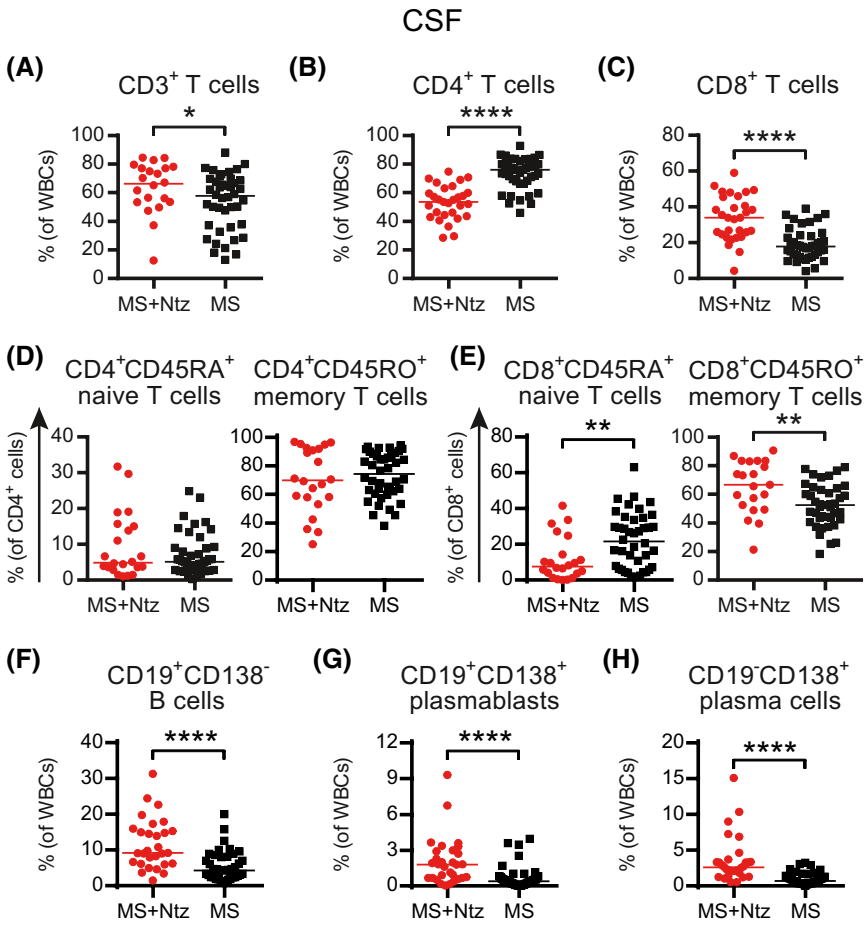

FIGURE 6 Increased percentage of memory cytotoxic T cells, B cells, plasmablasts and plasma cells in the CSF of MS patients after natalizumab therapy. In the CSF, percentages of CD3+ T cells (A), CD4+ T cells (B), CD8+ cytotoxic T cells (C), CD4+CD45RA+ naive and CD4+CD45RO+ memory T cells (D), CD8+CD45RA+ naive and CD8+CD45RO+ memory T cells (E), CD19+CD138− B cells (F), CD19+CD138+ plasmablasts (G) and CD19−CD138+ plasma cells (H) were analyzed by flow cytometry (quantitative comparison of groups given as median, MS + Ntz: (A, D, E left) n = 22, (B, F) n = 29, (C, G, H) n = 30, (E right) n = 21; MS: (A–D, E left, F, H) n = 42, (E right, G) n = 41; (A–H) Mann-Whitney *U* test)

3.8 | Increased percentages of B cells, plasmablasts, and plasma cells in CSF after natalizumab therapy

Next, we analyzed B lineages cells and observed increased frequencies of B cells in the blood, but no effects on plasmablasts and plasma cells were found (Figure S5F–H). In the CSF, the immune cell quantification revealed increased frequencies of B cells, plasmablasts and plasma cells (Figure 6F–H).

3.9 | Increased percentages of memory cytoxic T cells, B cells, plasmablasts, and plasma cells independent of disease duration

Natalizumab-treated patients had a significantly longer disease duration than MS controls ($p < 0.001$, Table 1). Thus, to exclude that our findings on immune cell infiltrates are related to a longer disease duration, we performed correlation analyses. The number of CD3+ T cells, CD8+ T cells, CD8+ memory T cells (CD8+CD45RO+), B cells, plasmablasts (CD19+CD138+), and plasma cells (CD19−CD138+), which were increased in the natalizumab-treated group as compared to untreated controls, did not increase with disease duration in either the natalizumab-treated or the untreated cohort (Table S1). These findings support the hypothesis of a natalizumab-related treatment effect.

4 | DISCUSSION

Natalizumab has been approved for the treatment of active relapsing-remitting MS. Although natalizumab is a highly beneficial drug that effectively reduces the risk of sustained disability progression and the rate of clinical relapses (1, 2), some patients do not respond to the therapy (17, 18) and natalizumab-treated patients are at higher risk of developing PML (2, 19, 20). The histopathological effects after natalizumab therapy are still unknown, which prompted us to perform a detailed histopathological characterization of the CNS inflammatory cell infiltrate in MS lesions. To complement the analysis, immune cells in blood and CSF were quantified by flow cytometry. We observed in our histological analyses that inflammatory infiltrates were present in natalizumab-treated patients and these were mainly composed of T cells and macrophages as well as some B cells and plasma cells. T cell numbers were not significantly reduced as compared to non-treated controls. Moreover, T cell numbers were also not lower

when the interval between the last natalizumab infusion and the biopsy or autopsy was short, indicating that T cells may enter the CNS despite natalizumab therapy.

CSF analyses revealed a reduction in the absolute number of inflammatory cells, with the exception of memory cytotoxic T cells, B cells, plasmablast, and plasma cells, indicating that these cells are not hindered at accessing the CSF compartment. Analysis of the percentages of cell subsets indicated a shift toward increased cytotoxic T cells and reduced T helper cells, as well as a higher proportion of memory $CD8^+$ cytotoxic T cells in natalizumab-treated patients as compared to controls. Our data are consistent with a previous study showing decreased $CD4^+/CD8^+$ ratios in the CSF of natalizumab-treated MS patients (4). Although an effective prevention of T cell migration into the CNS after blockage of VLA-4 receptors has been shown in animal models of MS (21–23), our results may indicate that natalizumab can only hinder a certain proportion of T cells from entering the human CNS. One explanation could be that natalizumab does not lead to a complete blockage of the VLA-4 receptors on blood cells with the monthly dosing interval (24). Along the same lines, α4 integrin expression varies throughout the T cell populations, with the highest expression on memory $CD8^+$ T cells (24), the T cell population we found not to be reduced in CSF after natalizumab treatment. Less likely is that cytotoxic T cells are preexisting and invaded before natalizumab therapy was started.

Our findings are of critical relevance, as there is substantial evidence implicating $CD8^+$ T cells in the pathogenesis of MS (25–27). Moreover, previous studies have also explored the importance of Th17 cells in MS pathogenesis (13, 28). In EAE it has been shown that Th17 cells are able to enter the CNS independently of VLA-4, suggesting migration via the choroid plexus into the brain parenchyma in a LFA1/ICAM1-dependent manner (11). Our analysis revealed single Th17 cells in natalizumab-treated MS patients with no clear difference to MS controls, a finding which is compatible with the concept of a VLA-4 independent migration into the CNS. However, quantification was limited due to the low number of patients with periventricular lesions.

Several lines of evidence also indicate essential roles for B cells in the pathogenesis of MS, which is broadly supported by the beneficial effect of B cell depleting therapies (29–32). Recently, we could show therapeutic efficacy in a B cell-dependent mouse model of MS upon therapy with a natalizumab analogon (anti-alpha 4 integrin antibody injections) (21). In the present study, quantification of PBMC subsets in blood revealed higher B cell frequencies in natalizumab-treated MS patients, which is in line with previous reports suggesting an increased release of B cells from the bone marrow (5, 6) and an impaired homing of mature B cell subsets into secondary lymphoid organs (33). Moreover, analysis of the circulating B cell pool revealed an increased activation and a progressed differentiation of B cells. This indicates a direct stimulating effect that may be mediated by the bidirectional signaling effect upon binding of natalizumab to VLA-4 (34). A higher proportion of B cells, plasmablasts, and plasma cells were found in the CSF of natalizumab-treated MS patients as compared to MS controls. It should be kept in mind, however, that the absolute cell numbers of B lineage cells were not increased. However, plasma cell numbers were significantly increased in active demyelinating lesions of natalizumab-treated MS patients. Of note, this effect was independent of the disease duration, as increased plasma cell numbers are observed in MS patients with longer disease duration (35–37). Thus, results indicate a plasma cell accumulation associated with natalizumab therapy.

What may be the reason for this plasma cell accumulation? Increased VLA-4 expression has been described on B cells as compared to T cells (24) and VLA-4 expression was even higher on plasmablasts and plasma cells (38). By modeling the migratory capacity of PBMCs in vitro, B cells have been shown to migrate more efficiently than T cells across human brain-derived endothelial cells (HBEC) (39). Moreover, natalizumab apparently does not reduce free VLA-4 binding sites to the same extent on B cells as compared to T cells (24), suggesting both the increased number of activated B cells in the blood and insufficient blockage of VLA-4 receptors as a possible cause for the plasma cell accumulation in the CNS. As it has been hypothesized that long-lived plasma cells do not migrate but rather remain in niches containing survival factors (40), it is likely that freshly migrated B cells locally persist and transform into plasmablasts and plasma cells. In the CNS of MS patients activated astrocytes express high levels of BAFF, which is a known survival factor for B cells and plasma cells, and thus may contribute to a local B cell/plasma cell-fostering environment (41, 42).

The pathological, pathogenic, and clinical relevance of the observed plasma cell accumulation upon natalizumab therapy remains unknown. Although high B cell and plasmablast percentages in the CSF after natalizumab treatment are associated with ongoing disease activity (43), we could not find an association between increased plasma cell numbers and a natalizumab treatment failure. Treatment concepts in MS and NMOSD with atacicept (TACI-Ig), which reduces circulating B cells and plasma cells by the neutralization of BAFF and APRIL, were stopped due to disease exacerbations (44, 45), raising the question as to which role plasma cells play in disease activity. Growing evidence indicates regulatory properties for plasma cells during neuroinflammation. Machado-Santos et al observed IL-10-producing plasma cells in MS lesions (46). Moreover, Rojas et al demonstrated in an animal model of MS that gut-derived IgA-producing plasma cell precursors may migrate into the CNS and ameliorate clinical severity in

an IL-10-dependent manner (47). We could also find a fraction of IL-10-positive plasma cells in a small number of analyzed patients.

Natalizumab patients are at higher risk of developing PML (2, 19, 20), which is an opportunistic infection of the CNS caused by the JC virus. Although the precise cause for this increased risk of PML is not completely understood, the impaired immune surveillance of the CNS is thought to play a critical role. Dendritic cells are of major importance during infection as they are involved in the effective pathogen defense, which is mediated through recognition of infectious agents, procession, and presentation of antigenic peptides via MHC class receptors to T cells. Del Pilar Martin et al. described a reduction in the number of antigen-presenting dendritic cells and the expression of MHC class II in cerebral perivascular spaces of a single natalizumab-treated MS patient with concomitant PML (7). Our findings are in line with this observation, showing decreased $CD209^+$ dendritic cell numbers in active demyelinating lesions with longer ongoing therapy duration. The present data also correspond well with the observation of an increased risk of PML in patients who are seropositive for the anti-JC virus and who were treated with natalizumab for more than one year (48).

B cells may also play a critical role in PML development, as they can be latently infected with JCV. The activation of transcriptional factors then leads to a viral transactivation (49) during the maturation of $CD19^+CD10^+$ B cell progenitor cells, which are released upon natalizumab administration (5). B lineage cells that enter the CNS may function as a Trojan horse for the JC virus, allowing the virus to enter the CNS. Our observed plasma cell accumulation may serve as a reservoir of the JC virus and act as a vector for its dissemination in the CNS. Taken together, our findings of increased plasma cell numbers, in synergy with an impaired immune surveillance due to a decrease in dendritic cells with longer ongoing therapy duration, could have critical consequences for PML development.

The limitations of our study include the heterogeneity of the natalizumab-treated patient cohort, a limitation immanent to retrospective histological studies. We consider the varying intervals between the last natalizumab infusion and biopsy/autopsy to be the most important limitation, yet our study contains to date the most comprehensive collection of CNS specimens obtained after natalizumab treatment. In the biopsy and autopsy cohorts, we noticed a higher number of natalizumab-treated MS patients who received HDCS and/or plasma exchange as compared to the control group. As these therapies are thought to reduce inflammation in lesions, this imbalance supports our findings that T cells enter the CNS despite the natalizumab therapy, and that natalizumab treatment is associated with higher numbers of plasma cells within lesions. In the CSF cohort, 10 MS controls but none of the natalizumab-treated patients were treated with HDCS. We excluded any effect of the HDCS on CSF cell subsets by exclusion of the HDCS-treated patients (data not shown). The only change we could observe was that the percentage of total CD3+ T cells was no longer significantly higher in natalizumab-treated patients compared to MS controls, a change that does not influence our main findings. Furthermore, the biopsies were taken for diagnostic reasons, implicating clinical worsening; a potential bias towards natalizumab non-responders could therefore be argued. However, retrospective clinical evaluation showed that most of the patients were natalizumab treatment responders. Finally, the number of disease duration-matched MS controls was limited, as MS biopsies are typically taken early during the disease course, but natalizumab treated patients had a median disease duration of 10 years.

In conclusion, although natalizumab is known to be an effective drug for MS treatment, it does not completely prevent immune cells from entering the CNS. Plasma cell numbers were even increased in MS lesions after natalizumab therapy as compared to controls, a finding with a yet unknown pathogenic and clinical significance. Since B cells may function as a reservoir of the JC virus, the higher proportion of B lineage cells in the CSF, the accumulation of plasma cells in brain parenchyma as well as a reduced immune surveillance caused by the reduction of dendritic cells in the CNS of natalizumab-treated patients may foster PML development.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHOR CONTRIBUTIONS

D.H., I.M., W.B., and H.L. analyzed the biopsy and autopsy material. K.A. analyzed the PBMC and CSF samples. D.H. prepared the figures. I.M. supervised the research. D.H. and I.M. wrote the manuscript. K.A., L.S., H.L., T.Z., and W.B. participated in reviewing and editing the manuscript.

DATA AVAILABILITY STATEMENT

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

ETHICAL APPROVAL

The study was approved by the ethical review committee of the University Medical Center Göttingen

(approval number 19-9-10) and the ethical review board of the Technical University Dresden (approval number EK348092014).

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

Supplementary Material

FIGURE S1 $CD4^+$ T cells and cytotoxic T cells in natalizumab-treated MS patients. (A) $CD4^+$ T helper and (B) $CD8^+$ cytotoxic T cell infiltration was assessed by immunohistochemical staining in active demyelinating white matter biopsy lesions and (C) the $CD4^+/CD8^+$ ratio was calculated (data given as median; MS + Ntz: n = 15, MS: n = 7; (A–C) Mann-Whitney *U* test)

FIGURE S2 Th17 cell infiltration in MS brain parenchyma in periventricular regions with natalizumab therapy. (A-C) To show MCAM+ T cells, double fluorescence immunohistochemistry was performed using tyramid amplification for CD3; white triangles = endothelial cells ($CD3^-MCAM^+$), white arrow = Th17 cells ($CD3^+MCAM^+$), grey arrow = CD3+ T cells ($CD3^+MCAM^-$). (D) Th17 cell infiltration was assessed by immunohistochemical staining for MCAM in periventricular active demyelinating white matter biopsy lesions and in periventricular inactive demyelinated white matter autopsy lesions; (quantitative comparison of groups given as median, MS + Ntz: n = 4, MS: n = 8; Mann-Whitney *U* test)

FIGURE S3 A fraction of plasma cells are positive for the anti-inflammatory cytokine IL-10. IL-10 expression in plasma cells was assessed by immunohistochemical staining for IL-10 and CD138 in active demyelinating white matter biopsy lesions and in inactive demyelinated white matter biopsy lesions in a small number of natalizumab-treated MS patients, MS + Ntz (active): n = 3, MS + Ntz (inactive): n = 2

FIGURE S4 Plasma cell numbers are not increased due to longer disease duration or higher age. Plasma cell infiltration was assessed by immunohistochemical staining for CD138 in active demyelinating white matter biopsy lesions and correlated to (A) disease duration (n = 16) and (B) age (n = 17); (A,B) Spearman *r* test

FIGURE S5. Increased percentage of B cells in the blood of MS patients after natalizumab therapy. Percentage of PBMCs consist of $CD3^+$ T cells (A), $CD4^+$ T cells (B), $CD8^+$ cytotoxic T cells (C), $CD4^+CD45RA^+$ naive and $CD4^+CD45RO^+$ memory T cells (D), $CD8^+CD45RA^+$ naive and $CD8^+CD45RO^+$ memory T cells (E), $CD19^+CD138^-$ B cells (F), $CD19^+CD138^+$ plasmablasts (G) and $CD19^-CD138^+$ plasma cells (H) were analyzed by flow cytometry (quantitative comparison of groups given as median, MS+Ntz: (A, D right) n = 20, (B, C, F, H) n = 29, (D left, E right) n = 21, (E left) n = 18, (G) n = 30; MS: (A, B, C, D left, F, G, H) n = 42, (D right) n = 41, (E left) n = 38, (E right) n = 39; (A–H) Mann-Whitney *U* test).

TABLE S1 Correlation analysis of immune cell infiltrates in the CSF with disease duration

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