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Microglia and Immune cells interactions in multiple sclerosis cognitive impairment: a *postmortem* study

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Abstract

Multiple Sclerosis (MS), a neuroinflammatory disease of the central nervous system, is one of the commonest causes of non-traumatic disability among young adults. Impaired cognition arises as an impactful symptom affecting more than 50% of the patients and with substantial impact on social, economic, and individual wellbeing. Despite the lack of therapeutic strategies, many efforts have been made to understand the mechanisms behind cognitive impairment in MS patients. Here, we aimed to investigate whether microglia-derived synaptic elimination and immune interactions are exacerbated in MS patients with impaired cognition when compared to non-demented controls (NDC) and cognitively preserved MS patients, that may clarify the role of immune cell interplay in MS cognitive deficits. *Postmortem* hippocampal samples were obtained from NDCs and MS patients. Sixteen MS patients were categorized based on their cognitive status: preserved cognition (MSCP) and impaired cognition (MSCI). Immunohistochemistry studies were conducted to explore the density of microglia, their role in synaptic engulfment, and their interaction with $CD8^+$ immune cells in the context of cognitive impairment in MS. In high synaptic density hippocampal regions, MSCI patients exhibited a massive presence of microglia cells actively engulfing both excitatory and inhibitory synapses, accompanied by morphological alterations. Additionally, there was an increased expression of the complement protein C1q particularly localized at inhibitory synapses within microglia cells, suggesting a preferential engulfment of complement-tagged inhibitory synapses in MSCI patients. Furthermore, in hippocampal lesions of MSCI patients, we detected a significant infiltration of microglia and CD8 T cells that may be contributing to the smouldering MS and cognitive deterioration. These findings demonstrate that cognitive deficits occurring in MS are associated with microglia engulfment of C1q-tagged inhibitory synapses, which may be driven by direct or indirect stimulation from CD8+ T cells.

Keywords Cognitive impairment, CD8 T cells, Microglia, Multiple sclerosis, Synaptic pruning

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Background

The autoimmune and neurodegenerative disorder, multiple sclerosis (MS), is the leading cause of non-traumatic disability among young adults [1]. Epidemiological data shows a continuously increasing incidence and prevalence of MS affecting nowadays 2.8 million people worldwide [2]. Despite the inflammatory mechanisms occurring in MS pathology, the formation of focal demyelinated plaques within the central nervous system (CNS) is a key disease hallmark [3]. Consequently, irreversible pathological changes result in the appearance of symptoms, particularly affecting motor and cognitive function [1].

Over a century ago, Charcot JM first described a "marked enfeeblement of the memory" in people diagnosed with MS, a symptom that was then overlooked for a long time [4]. Currently, cognitive impairment is recognized as a main clinical symptom affecting over 50% of MS patients throughout disease phenotypes [5]. Specifically, cognitive decline is more prevalent in advanced phases as secondary progressive MS (SPMS, 50–70%), and less prevalent in the earliest stages namely in clinically and/or radiologically isolated syndrome (20–25%) and in relapsing-remitting MS (RRMS, 30–45%) [6, 7]. In-depth, MS-associated cognitive deficits involve multiple cognitive domains, commonly linked to slow information processing speed and episodic memory impairment, where the hippocampus is the main affected brain region [8]. However, additional difficulties might be also found in MS patients mostly associated with specific impairments in some executive behavior, being correlated with alterations in the prefrontal cortex and related networks [9]. These brain alterations are usually detected using neuropsychological assessments and through imaging techniques [10]. Recent advances using magnetic resonance imaging (MRI) have revealed correlations between cognitive functions and brain pathology in MS. Cognitively impaired MS patients show whole-brain and parenchyma atrophy [11, 12], an increased number of cortical lesions [13], and the involvement of grey matter structures such as the hippocampus [14]. *Postmortem* studies highlight the impact of hippocampal degeneration in MS patients [15], together with extensive demyelination [16], synaptic changes [17, 18] and the involvement of microglia in neuroinflammatory processes [16, 19].

The precise mechanisms underlying cognitive impairment in MS are poorly understood, however, some studies pointed to the link between immune system activation, inflammation, and synaptic degradation in critical brain structures, including the hippocampus [20]. Recent reports have shown that proteins of the classical complement system (complement component 1q or C1q, and component complement 3 or C3) are critical mediators of synaptic refinement and plasticity [21]. Wernerburg and colleagues demonstrated that C3-tagged synaptic material is engulfed by microglia in the retinogeniculate system in the in vivo MS models and in the visual thalamus of MS patients [22]. Additional studies also support the role of the C1q-C3 complement system in synaptic alterations particularly within the hippocampus of MS patients [23]. Interestingly, later studies comparing patients with and without impairment of cognitive function have shown higher amounts of C1q in specific hippocampal regions in those with cognitive deficits [18].

Lacking current therapeutic strategies for cognitive impairment in MS, it becomes a priority to understand its underlying mechanisms to improve patients' quality of life. Therefore, in this *postmortem* study, we shed light on the implications of microglia in synapse elimination and immune interactions in the hippocampus of MS patients diagnosed with impaired cognition and compare them with those with preserved cognition and non-demented controls.

Materials and methods

Hippocampal tissue collection

Postmortem hippocampi of 8 non-demented controls (NDC) and 16 MS donors were obtained from the Netherlands Brain Bank (NBB; Amsterdam, the Netherlands). Approval was granted from the Independent Review Board of Amsterdam, UMC, registered with the US Office of Human Research Protection. Informed consent was obtained by the NBB for brain autopsy and for the use of material and clinical data for research purposes following institutional and national ethical guidelines. Specimens were fixed in 10% buffered formalin and processed for embedding in paraffin. NDC and MS donors were matched for age (NDC: 74.1±8.4, mean±SD; MS: 62.1±10.9 years, mean±SD) and *postmortem* delay (PMD) (NDC: 8.5±1.1, hours, mean±SD; MS: 9.2±1.2, hours, mean±SD). MS paraffin-embedded hippocampi included 1 donor with RR, 3 donors with PP, 3 donors with SP, and 9 with a progressive disease of undetermined type (PP/SP). NDC were chosen excluding patients with CNS inflammatory diseases. Detailed clinicopathological data are provided in Table 1.

Cognitive function

Reviewing the clinical data of all cases, high-quality hippocampal samples were selected: 8 MS donors cognitively preserved (MSCP) and 8 MS donors cognitively impaired (MSCI). MS cases were also age- (MSCP: 64.6±9.9 years, mean±SD; MSCI: 59.6±11.8, mean±SD) and PMD-matched (MSCP: 9.2, ± 1.2, hours, mean±SD; MSCI: 8.5±1.1, hours, mean±SD). Data provided by the NBB included the clinical dementia rating (CDR) for all donors, which is a 5-point scale used to characterize cognitive and functional performance: 0 – no cognitive

Table 1 Clinical data of non-demented controls and multiple sclerosis donors

Case	Sex	Age, yr	PMD, h	MS Type	Cognitive Impairment	CDR score	COD
2005-068	M	56	09:15	-	-	0	Myocardial infarction
2011-049	F	83	04:40	-	-	0	Ileus with pancreatic cancer
2015-027	F	76	04:45	-	-	0	Adenocarcinoma
2017-016	M	72	04:20	-	-	0	Endocarditis
2017-093	M	82	05:45	-	-	0	Euthanasia
2017 – 131	F	71	06:15	-	-	0	Multi-organ failure
2018-005	F	76	05:30	-	-	0	Respiratory insufficiency
2018-072	M	77	04:50	-	-	0	Euthanasia
2012-008	F	66	10:45	SP	-	0	Pulmonary hypertension
2012 – 113	M	59	10:45	PP	-	0	Euthanasia
2014-001	M	57	10:15	SP/PP	-	0	Sepsis by urinary tract infection
2014-071	F	60	09:25	SP/PP	-	0	Euthanasia
2016-050	F	52	08:40	SP/PP	-	0	Euthanasia
2017-044	F	77	08:20	SP/PP	-	0	Respiratory insufficiency
2017-083	F	81	07:20	RR	-	0	Anorexia
2018-044	M	65	09:30	SP/PP	-	0	Euthanasia
2015-022	M	82	08:05	SP/PP	+	1	Pneumonia
2015-082	F	47	08:35	SP	+	1	Aspiration pneumonia
2016 – 104	F	49	08:30	PP	+	1	Euthanasia
2013-015	M	56	09:35	PP	+	2	End of stage of MS
2017 – 100	M	66	08:30	SP	+	2	Pneumonia and/or heart failure
2019-011	M	63	10:00	SP/PP	+	2	Aspiration pneumonia and sepsis
2015-073	F	49	09:45	SP/PP	+	3	Euthanasia
2016-030	F	65	06:25	SP/PP	+	3	Euthanasia

CDR clinical dementia rating, *COD* cause of death, *F* female, *M* male, *MS* multiple sclerosis, *PMD postmortem* delay, *PP* primary progressive, RR remitting relapsing, *SP* secondary progressive

impairment; 0.5 – questionable cognitive impairment; 1 – mild cognitive impairment; 2 – moderate cognitive impairment; and 3 – severe cognitive impairment. The CDR score is used to characterize domains of cognitive and functional performance considering 6-items: memory, orientation, judgement and problem solving, community affairs, home and hobbies, and personal care. Knowing that hippocampal damage impairs functions related to memory, orientation and problem solving, the categorization was performed based on alterations of those functions. Indeed, NDC and MSCP patients had degrees of CDR score equal to zero, whereas MSCI patients exhibited highest scores on memory and orientation, which was reflected in CDR scores higher than one (Table 1).

Immunohistochemistry

Paraffin sections were deparaffinized in xylene for 20 minutes and rehydrated through a series of ethanol baths (100% for 20 minutes, 95% for 20 minutes, and 70% for 10 minutes) followed by H_2O (10 minutes). Then, sections were pre-treated with microwave antigen retrieval for 15 minutes in citrate buffer (10 mM, pH=6.0). Sections were permeabilized with 0.25% Triton X-100 (Sigma-Aldrich) in 1x PBS for 10 minutes and blocked with 5% bovine serum albumin (Sigma-Aldrich), 5% fetal bovine serum (Gibco) and 0.1% Triton X-100 in 1x PBS for 1 hour at room temperature. Selected primary antibodies (see Additional File 1) were diluted in blocking solution and incubated overnight at 4ºC. The next day, slices were washed three times with 1x PBS for 10 minutes each and incubated with respective secondary antibody (see Additional File 2) diluted in blocking solution for 2 hours at room temperature. After washing, sections were incubated with 2'-6'-diamidino-2-phenylindole (DAPI, 1:1000 in 1x PBS) for 5 min to visualize the nuclei and mounted in Fluoromount-G (Southern Biotech).

Image acquisition was carried out using a Leica DMi8-CS inverted microscope with Leica LasX software (Leica Application Suite X; RRID: SCR_013673). Samples were acquired at 40x and 63x magnification with high-resolution digital images (16 Gb, 1024×1024 pixels). For each immunostaining, a total number of images (5–7 images x 24 donors) for each subfield, including myelinated areas, demyelinated lesions, and *Cornu Ammonis* (CA)1-CA3 fields, were captured. Myelinated areas were identified with positive myelin basic protein (MBP) staining, while demyelinated lesions were classified by the absence of MBP and an increased DAPI staining. The CA1-CA3 regions were recognized by the dense neuronal fields. 18-20x z-stacks were taken per slice to reduce variation in image acquisition and analysis.

Quantification and image analysis

Quantitative analysis was performed on the region of interest (ROI) using the open-source FIJI ImageJ (v.2.3.0; RRID: SCR_002285) or AIVIA (v.12, Leica Microsystems Inc, Bellevue WA, USA). Using FIJI ImageJ, the number of ionized calcium-binding adaptor molecule 1 positive ($Iba1^+$) cells and $CD8^+$ T cells was calculated by manual counting in merged z-stacks. For the quantification in the lesions, the area measurement function was used and the $Iba1^+$ or $CD8^+$ cells were expressed as the number of cells per mm^2 in each ROI. For the colocalization, ROIs were created for each $Iba1^+$ cell present in high synaptic regions and single-color channels (C1q, vGat, and vGlut) were subjected to thresholding that captures the specific staining. The area of colocalization was performed across three different z-stacks. The results are the sum of each z-stack and further average between the different images taken from each individual region. For assessing microglia morphology and synaptic vesicle engulfment, AIVIA software with a specific plugin (3D Cell Analysis – Meshes) was used. 3D reconstructs were created for each $Iba1^+$ cell and synaptic vesicles. Then, microglia properties were automatically calculated by AIVIA (sphericity, surface area, volume, total number of vesicles, and the number of vesicles contained in cells) and further exported for analysis.

Statistical analysis

Data analyses were performed using GraphPad Prism Software (v. 8.0.0, RRID: SCR_002798; GraphPad Software Inc, San Diego, CA, USA). The type of variability of distributions was assessed by the Shapiro-Wilk normality test. Non-normally distributed data were analyzed with the non-parametric Mann-Whitney test for two groups comparison or with the non-parametric Kruskal-Wallis test followed by Dunn's correction for multiple comparisons. Normally distributed data were analyzed using one-way ANOVA followed by post-hoc analysis with Tukey's multiple comparisons test. Statistical significance was ranked $^*p<0.05$, $^{**}p<0.01$ and $^{***}p<0.001$.

Results

Cognitively impaired MS patients have microglia alterations in hippocampal demyelinated lesions

Hippocampal pathological changes are known to contribute to the emergence of cognitive impairment in MS, however, the precise molecular mechanisms remain unknown [10]. Notably, the formation of demyelinating lesions is a key hallmark of MS, with microglia standing as the main players. One study suggests a potential link between early memory impairment and microglia activation in the in vivo model of MS [24], but further research is needed. Therefore, to understand the importance of microglia in cognitive processes, we quantified the density of $Iba1^+$ cells in hippocampal lesions and lesion-free myelinated areas.

Demyelinated areas, known as lesions, were delineated by the absence of myelin (MBP, in red) staining and the presence of cell nuclei (DAPI, in blue) infiltration as identified in Fig. 1a. Surprisingly, lesions were detected in all experimental groups, even in NDC, that may reveal age-associated myelin loss with no association of symptoms in these individuals. Additionally, MSCI patients exhibited a significant increase in lesion area when compared to NDC (Fig. 1b; $^*p<0.05$ vs. NDC), and curiously a higher density of microglia at the lesion rim suggesting the presence of smouldering MS lesions (Additional File 3) [25, 26]. Regarding $Iba1^+$ microglia cells, we observed a trend toward increased density in MS donors (Fig. 1c), with MSCI patients exhibiting higher numbers of infiltrating $Iba1^+$ microglia cells within hippocampal lesions when compared to NDC and MSCP (Fig. 1d; $^*p<0.05$ vs. NDC; $\#p<0.05$ vs. MSCP). Morphometric analysis of microglia cells in hippocampal lesions revealed no differences in cell membrane sphericity (Fig. 1e). Nevertheless, we detected a decrease in cell volume in MSCP when compared to NDC (Fig. 1f-g; $^*p<0.05$ vs. NDC), while MSCI patients had a significant increase in both cell surface area and volume (Fig. 1f-g; $\#\#p<0.01$ vs. MSCP). In lesion-free areas, we detected a variety of myelin patterns and no changes in the density of microglia cells (Additional File 4a-b).

These data shows microglia alterations in hippocampal lesion areas of MSCI patients emphasizing their possible role in accelerating cognitive deficits.

C1q preferentially tag inhibitory synapses in hippocampal subfields of MS patients with cognitive decline

Multiple studies have highlighted the role of synaptic alterations across hippocampal regions in MS-cognitive impairment [17]. Moreover, components of the classical pathway of the complement system, particularly C1q, have recently emerged as key mediators of synaptic elimination throughout development [27] and in neurodegenerative disorders [23]. Thus, we quantified the density of excitatory (vesicular glutamate transporter 1 or vGlut1) and inhibitory (vesicular GABA transporter or vGat) synapses, together with the expression of the complement protein, C1q, in the high synapse density CA subfields and dentate gyrus of *postmortem* hippocampus.

Quantification of the synapse density revealed a gain of excitatory $vGlut1^+$ synaptic vesicles in MSCI patients when compared to MSCP (Fig. 2a, $\#p<0.05$ vs. MSCP), while no changes were observed in the number of inhibitory $vGat^+$ synaptic vesicles (Fig. 2b). In addition, a notable increase in the percentage of C1q was observed in MSCI patients compared to NDC (Fig. 2c, $^*p<0.05$ vs. NDC). Double staining for vGlut1 (Additional File 5a)

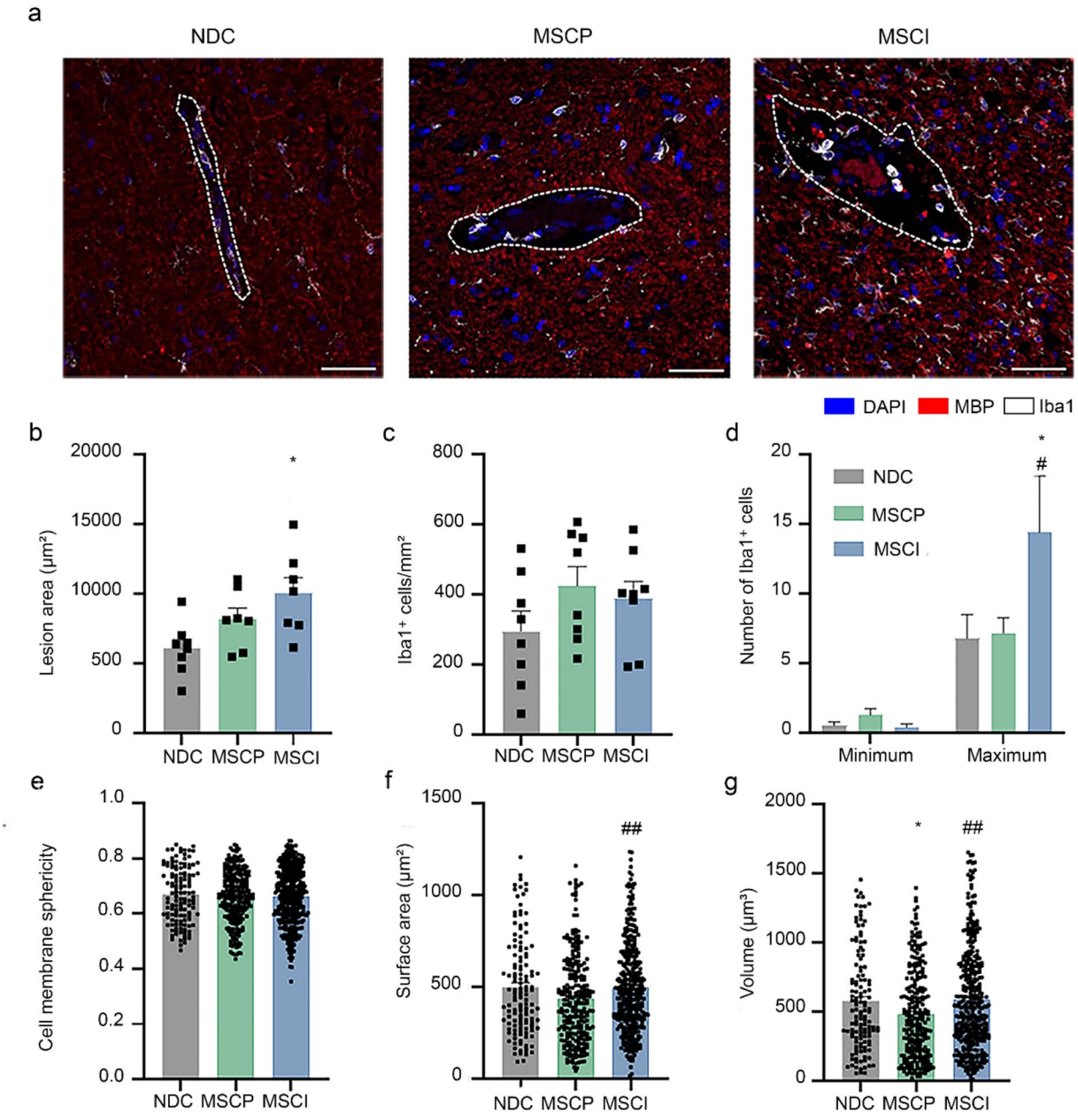

Fig. 1 Massive infiltration of microglia in hippocampal plaques in cognitively impaired MS patients. **(a)** Representative immunohistochemical staining for myelin (MBP, in red) and microglia cells (Iba1, in white) and staining for cell nuclei (DAPI, blue) of *postmortem* hippocampal slices of non-demented donors (NDC) and multiple sclerosis donors diagnosed with preserved (MSCP) and impaired cognition (MSCI). White dash lines indicate the areas of demyelination. Scale bars: 50 µm. Graph bars stand for the quantification of **(b)** lesion areas in μm^2, the manual counting of **(c)** Iba1$^+$ cells normalized to the lesion area and **(d)** the minimum and maximum number of Iba1$^+$ cells able to infiltrate lesion areas ($N = 8$ for NDC, MSCP and MSCI). Microglia morphometric analysis, as **(e)** cell membrane sphericity, **(f)** surface area and **(g)** volume, were automatically calculated by AIVIA software. Each data point represents a different Iba1$^+$ cell detected ($N = 137$ for NDC, $N = 278$ for MSCP, and $N = 435$ for MSCI). *$p < 0.05$ vs. NDC; # $p < 0.05$, and ##$p < 0.01$ vs. MSCP

or vGat (Fig. 2c) with C1q revealed no changes in excitatory synapses tagging (Fig. 2d), but a preferential tagging of C1q for vGat$^+$ inhibitory synapses in MSCI patients (Fig. 2e, #$p < 0.05$ vs. MSCP).

Therefore, we confirmed findings from previous studies showing the exacerbated presence of C1q in MSCI patients [18]. Most attractively, for the first time, we specifically showed signs of C1q preferential tag for vGat$^+$

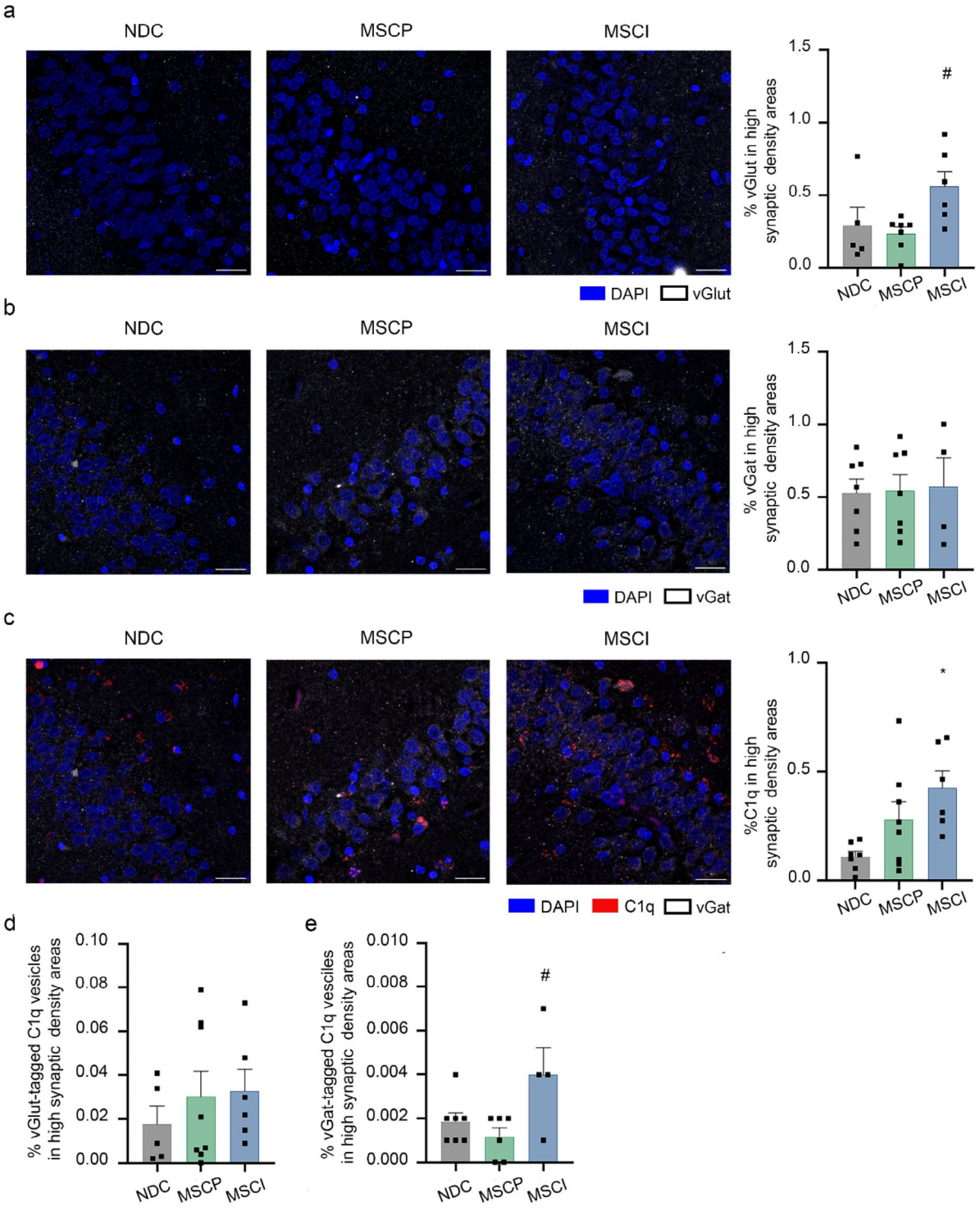

Fig. 2 C1q is increased and preferentially tag vGat synapses in cognitively impaired MS patients. Representative immunohistochemical staining and respective quantification of synaptic partners, **(a)** vGlut (in white) and **(b)** vGat (in white), and the complement protein **(c)** C1q (in red) in high synaptic fields found in *postmortem* hippocampal tissues from non-demented donors (NDC) and multiple sclerosis donors diagnosed with preserved (MSCP) and impaired cognition (MSCI). Scale bar: 25 µm. Graph bars represent the percentage of colocalization between each synaptic partner, **(d)** vGlut 1 and **(e)** vGat, and the complement protein, C1q. Each sample is represented by the average of seven images analyzed in the specific regions of interest ($N = 8$ for NDC, MSCP and MSCI). Staining for cell nuclei (DAPI, blue) was performed in all samples. *$p < 0.05$ vs. NDC, and # $p < 0.05$ vs. MSCP

inhibitory synapses in the MS hippocampus of cognitively impaired patients.

Cognitively impaired MS patients have C1q-tagged inhibitory synaptic elements that localize within microglia in hippocampal subfields

Many authors have proposed that synaptic loss observed in MS could be a consequence of aberrant synaptic elimination – synaptic pruning – where microglial cells act as crucial players [28]. Complement-tagged synapses are eliminated via phagocytosis by microglia throughout the lifespan [27] and in neurodegenerative conditions [22], but no studies have established this association in the context of cognitive impairment in MS. So, we next assessed microglia morphology and their ability to prune tagged excitatory and inhibitory synapses throughout hippocampal subfields identified by areas of increased nuclei density (Fig. 3a).

Interestingly, with a higher magnitude than what we have observed in hippocampal lesions, there was a significant increase in the number of Iba1$^+$ cells in the hippocampal high synaptic density area of MSCI patients (Fig. 3b; $*p<0.05$ vs. NDC; $\#p<0.05$ vs. MSCP), together with increased cell surface area and volume when compared to MSCP (Fig. 3c-d; $\#p<0.05$ vs. MSCP). Furthermore, 3D reconstruction of each Iba1$^+$ cell with vGlut1 or vGat (Fig. 4a) showed a basal synaptic vesicle engulfment in NDC and MSCP hippocampus reflecting an ongoing surveillance of microglia, but a significant increase in the percentages of vGlut1$^+$ (Fig. 4b; $*p<0.05$ vs. NDC; $\#p<0.05$ vs. MSCP) and vGat$^+$ (Fig. 4c; $*p<0.05$ vs. NDC) synaptic particles engulfed by Iba1$^+$ cells in MSCI patients. Furthermore, we detected increased C1q levels in Iba1$^+$ cells (Fig. 4d-e; $*p<0.05$ vs. NDC; $p=0.05$ vs. MSCP), together with a preferential engulfment of

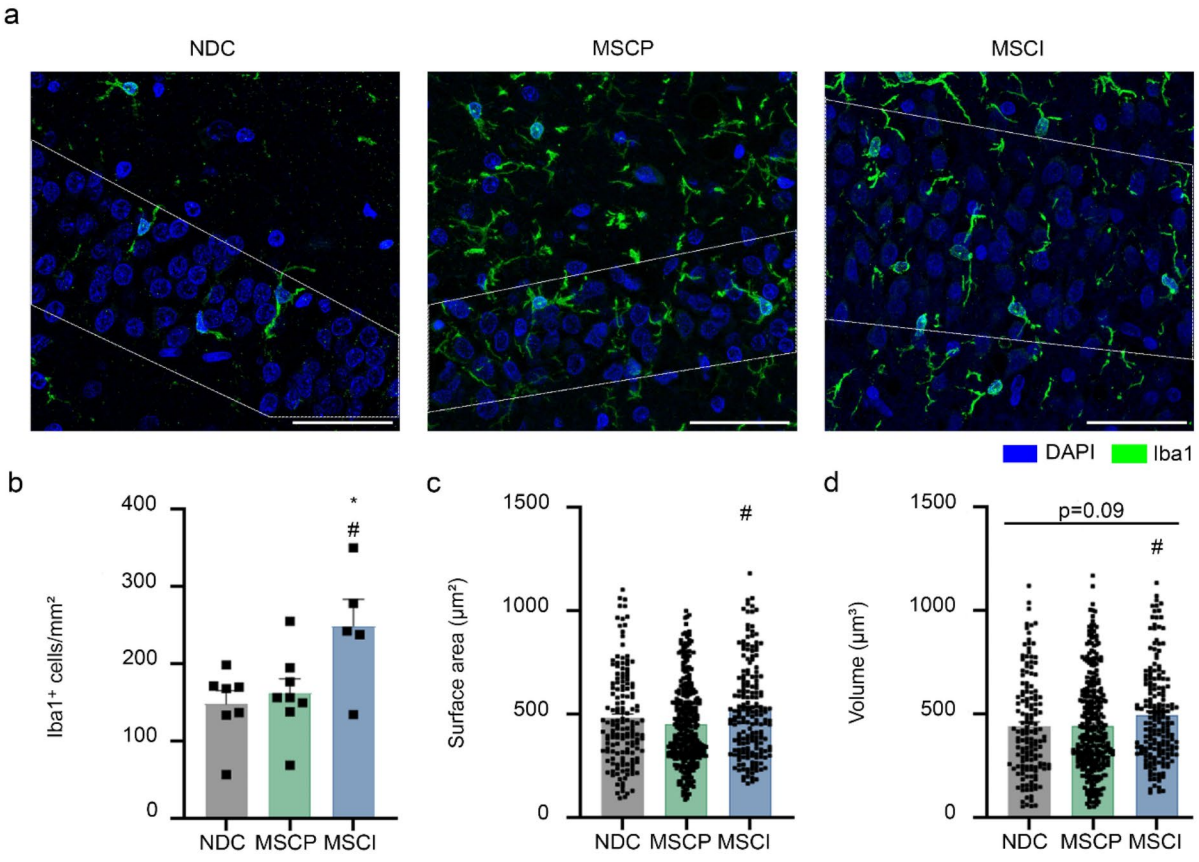

Fig. 3 Microglia is present in high synaptic hippocampal region of cognitively impaired MS patients. **(a)** Representative immunohistochemical staining of the CA1-CA3 regions of *postmortem* hippocampal slices of non-demented donors (NDC) and multiple sclerosis donors diagnosed with preserved (MSCP) and impaired cognition (MSCI). White dash lines indicate the region of interest (ROI) detected by the higher density of nuclei staining (in blue, DAPI). Scale bars: 50 µm. Graph bars stand for the quantification of **(b)** the manual counting of Iba1$^+$ cells normalized to the CA1-CA3 regions of *postmortem* hippocampal area ($N=8$ for NDC, MSCP and MSCI). Microglia morphometric analysis, as **(c)** cell membrane surface area and **(d)** volume, were automatically calculated by AIVIA software. Each data point represents a different Iba1$^+$ cell detected ($N=161$ for NDC, $N=358$ for MSCP, and $N=209$ for MSCI). $*p<0.05$ vs. NDC; $\#p<0.05$ vs. MSCP

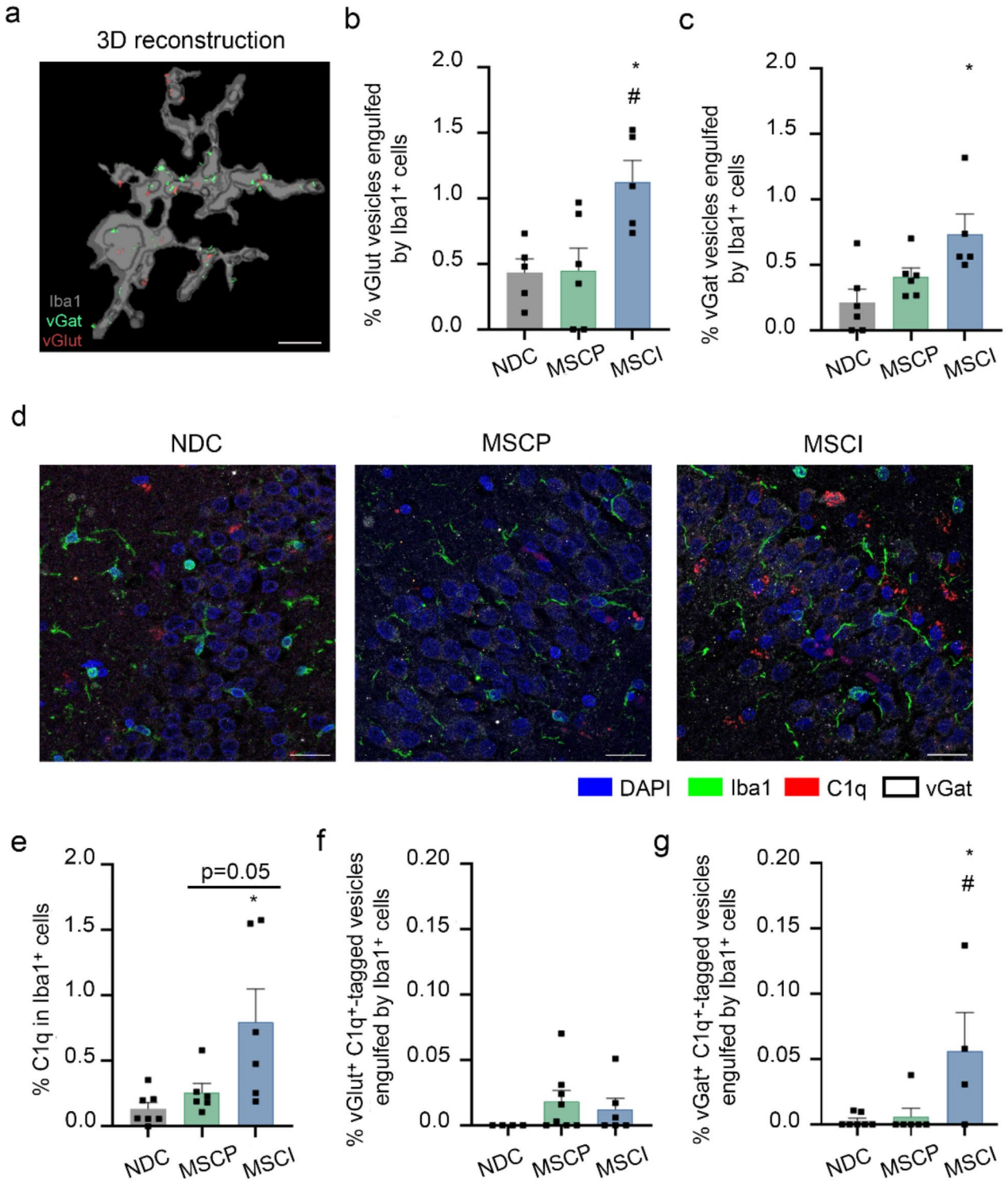

Fig. 4 (See legend on next page.)

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Fig. 4 Microglia engulf inhibitory synaptic elements in hippocampal subfields of cognitively impaired MS patients. **(a)** 3D rendering of a Iba1$^+$ microglia cell (in grey) engulfing vGat$^+$ (in green) and vGlut1$^+$ (in red) synaptic vesicles. Graph bars represent the percentage of **(b)** vGlut and **(c)** vGat synaptic partners engulfed by Iba1$^+$ cells ($N=8$ for NDC, MSCP and MSCI). **(d)** Representative immunohistochemical staining of microglia (Iba1, in green), the complement protein (C1q, in red) and the synaptic partner (vGat, in white), and staining for cell nuclei (DAPI, blue) in the CA1-CA3 regions of hippocampal slices of non-demented donors (NDC) and multiple sclerosis donors diagnosed with preserved (MSCP) and impaired cognition (MSCI). Graph bars represent the percentage of area stained for **(e)** C1q in Iba1$^+$ cells, and the percentage of colocalization between the synaptic partners, **(f)** vGlut and **(g)** vGat, with C1q in Iba1$^+$ cells. All Iba1$^+$ cells were delineated and considered ROIs in the high synaptic regions. The analyses were performed in three different z-stacks followed by the sum of three measurements in each image. Graph bars represent the average of seven images for each donor ($N=8$ for NDC, MSCP and MSCI). *$p<0.05$ vs. NDC; #$p<0.05$ vs. MSCP

C1q-tagged vGat$^+$ synaptic vesicles in MSCI patients (Fig. 4f-g; *$p<0.05$ vs. NDC; #$p<0.05$ vs. MSCP).

These results clearly emphasize the role of microglia in synaptic clearance in MS, particularly in cognitively impaired patients, with C1q playing a crucial role in tagging inhibitory synapses.

Microglia interacts with CD8 T cells throughout MS hippocampal lesions

Microglia actively surveil the CNS establishing contact with neurons and immune cells [29]. Studies have demonstrated that CD8 T cells-interferon signaling impacts on microglia function promoting aberrant synaptic elimination, therefore contributing to cognitive decline in a viral-induced in vivo model [30]. Here, we aimed to correlate the presence of CD8 T cells with microglia, particularly in hippocampal demyelinated lesion areas.

We detected a massive increase in the number of CD8$^+$ T cells in hippocampal demyelinated areas of MSCI patients when compared to NDC and MSCP (Fig. 5a-b; **$p<0.01$ vs. NDC; ##$p<0.01$ vs. MSCP). When we assessed the possible interaction of these cells with Iba1$^+$ cells, we observed that more than 30% of the CD8$^+$ T cells were in close adherence to Iba1$^+$ cells in MSCI patients when compared to MSCP (Fig. 5c-d and Additional File 6; *$p<0.05$ vs. MSCP).

These results highlight the potential interplay between CD8$^+$ T cells and activated microglia that, directly or indirectly, may contribute to MS-cognitive impairment.

Discussion

Cognitive impairment is gaining recognition as a significant debilitating symptom since it affects more than 50% of the MS patients directly impacting their quality of life [10]. Without effective therapeutic strategies, several efforts are being made to clearly understand the mechanisms underlying cognitive impairment. While some studies highlighted the role of microglia in eliminating synapses via complement system, particularly in the retinogeniculate system of the in vivo MS model [22], others showed a direct correlation between the immune system and microglia in cognitive outcomes [30]. In this study, we aimed to unravel the role of microglia in synaptic pruning and immune cell interplay in MS-associated cognitive impairment.

Through MRI techniques, many authors correlated cognitive impairment in MS to structural brain changes. Studies have shown higher lesion volumes and increased number of cortical lesions, together with whole-brain atrophy in MSCI patients [14]. Some studies also highlight the involvement of prefrontal cortex in MS patients diagnosed with deficits in memory and in executive functions. More specifically, the right and left superior frontal lobes are described as the regions more susceptible to atrophic changes being correlated to defective cognitive performances [31]. Additionally, some authors also showed that, due to the existence of numerous connections as the thalamic-hippocampal-prefrontal, microstructural damages in one of the circuitry regions can affect cognitive and executive function particularly even before functional impairment is evident [32, 33]. Nevertheless, hippocampal atrophy is still considered one of the best predictors of cognitive impairment, where some clinical manifestations as memory deficits are partially related to the involvement of this brain structure [14, 15]. Knowing this, for this study, the NBB provided neuropsychological information guaranteeing the use of hippocampal samples from patients diagnosed with preserved – MSCP – or impaired – MSCI – cognition. We found increased areas of *postmortem* hippocampal demyelinated lesions in MSCI patients, together with a substantial infiltration of microglia cells with altered morphology linking their activation with cognitive deficits. The presence of microglial activation might correlate with the presence of smouldering plaques which are highly prevalent among patients with progressive MS [34], which is the most prevalent disease type in our MS patients' samples. Moreover, these chronic active plaques, mostly evident throughout the hippocampus of MSCI patients, explain the impact of these lesions in the disruption of neuronal connections and pathways necessary for proper cognitive function [35].

Pathological lesions alone are insufficient to explain MS-associated cognitive impairment. Indeed, molecular hippocampal changes in MS, including progressive synaptic alterations, have been implicated in the mechanisms underlying cognition [17, 18, 23]. Intriguingly, our results show an imbalance between excitatory and inhibitory systems revealing an increased vGlut1$^+$ along with unchanged vGat$^+$ in hippocampal regions of MSCI

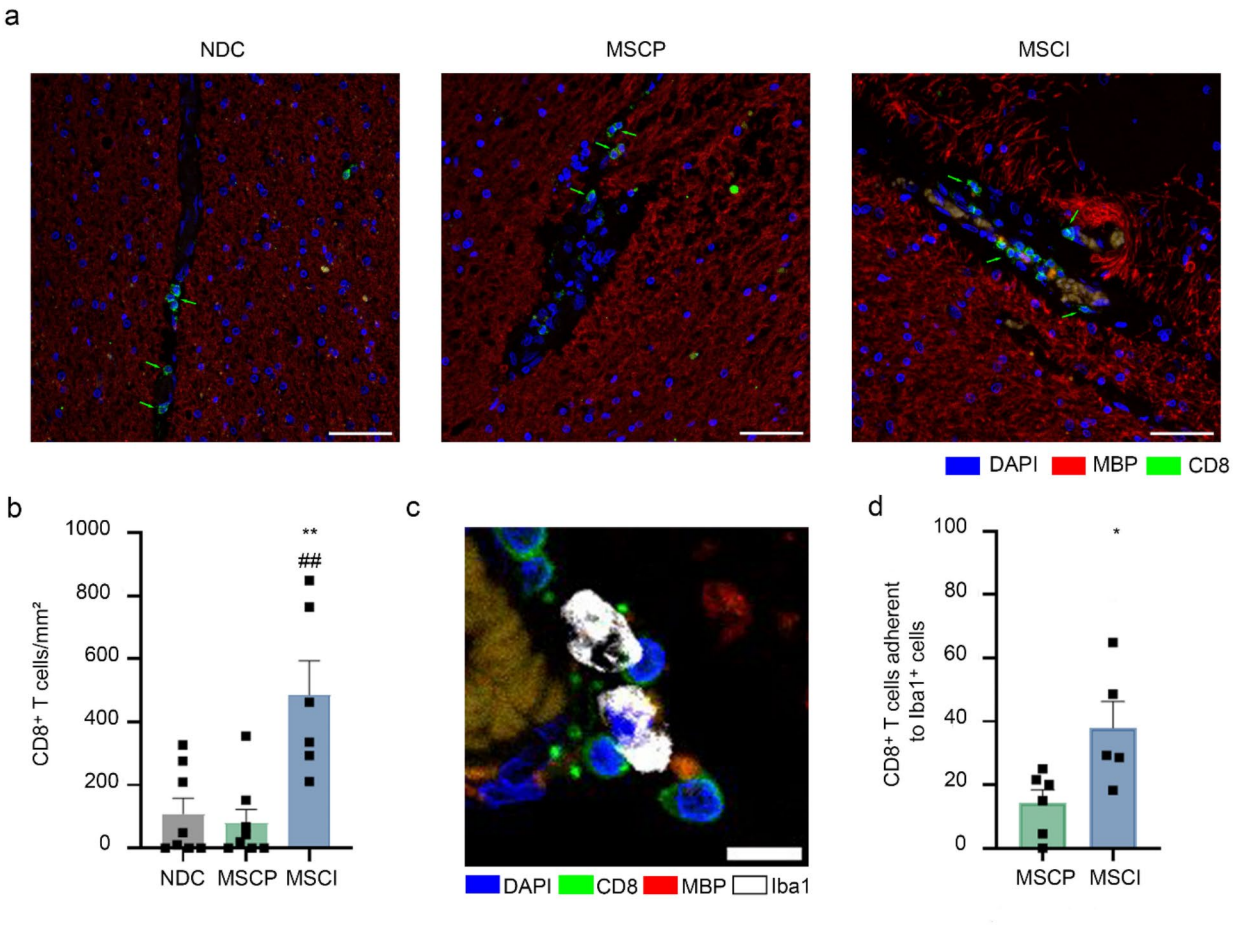

Fig. 5 Increased number of CD8$^+$ T cells in MS patients with impaired cognition. **(a)** Representative immunohistochemical staining for myelin (MBP, in red) and CD8 + T cells (in green) and staining for cell nuclei (DAPI, blue) in demyelinated areas of *postmortem* hippocampal slices of non-demented donors (NDC) and multiple sclerosis donors diagnosed with preserved (MSCP) and impaired cognition (MSCI). Scale bar: 100 µm. **(b)** Graph bars represent the quantification of the number of CD8$^+$ T cells normalized to the lesion area. **(c)** Representative close-up of the immunohistochemical staining of CD8 T cells (in green) and microglia (Iba1, in grey) interactions. Scale bar: 10 µm. **(d)** Graph bars the number of CD8$^+$ T cells adherent to Iba1$^+$ cells ($N = 8$ for NDC, MSCP and MSCI). *$p < 0.05$, and **$p < 0.01$ vs. NDC; ##$p < 0.01$ vs. MSCP

patients when compared to preserved ones. Although many authors have reported a significant loss of glutamatergic synapses [17], others have demonstrated a strong immunoreactivity of glutamatergic markers such as vGlut1 and Homer1 throughout intrahippocampal regions in both MS patients and demyelinating mouse models [18]. The presence of the glutamatergic vesicular marker vGlut1 may represent the accumulation of vesicles at sites of injured axons particularly in MSCI patients, as already shown in progressive MS patients [36]. Additionally, this enhanced expression of vesicular glutamate transporters was already proved to be sufficient to cause excitotoxicity leading to neurodegeneration in a Drosophila model [37], emphasizing the possible role of vGlut1 in hippocampal pathology. Nevertheless, the impact of changes in the glutamate neurotransmitter system in MS-associated cognitive impairment needs to be further addressed.

As main players in MS pathology, microglia reactivity has been described in the hippocampus and output tracts (e.g. fimbria) of both in vivo MS models and in MS patients [19, 38]. Going in line, we found a massive presence of microglia cells alongside increased synaptic engulfment in the hippocampus of MSCI patients corroborating the role of microglia in disease symptomatology. Together with synaptic changes and microglial reactivity, recent studies also demonstrated signs of immune system activation along with areas of active inflammation in hippocampal tissue of MS patients [18, 19, 23]. Indeed, some authors report that synapse loss occurs via complement system tagging being phagocytosed by microglia in the retinogeniculate system of in vivo models of demyelination and in the visual thalamus

of MS patients [22]. Others show that C1q is deposited particularly in the hippocampal CA2-3 regions at synapses that localize within microglial processes and lysosomes [18, 23]. In cognitively impaired MS patients, we showed for the first time that C1q was selectively enriched only at vGat$^+$ synaptic vesicles and further phagocytosed by microglial processes suggesting a specific elimination pathway for inhibitory synapses. The question that arises is what mechanisms determine the vulnerability of synapses to C1q targeting in MS cognitive impairment. In the context of other neurodegenerative disorders such as Alzheimer's disease and epilepsy, studies already indicate that microglia preferentially prune inhibitory synapses, whereas astrocytes may contain excitatory synaptic material revealing an unexpected division of labor [39, 40]. Similarly, this specificity was also observed in non-diseased models where $GABA_B$ receptors are expressed in a subset of microglia enabling the pruning during development, which in turn might contribute to the engulfment of inhibitory synapses in a disease context [41].

Given the immune nature of MS, focal demyelination is often associated with inflammatory infiltrates such as microglia and T lymphocytes. CD8 T cells outnumber CD4 T cells in the blood, and in the cerebrospinal fluid of MS patients [42], as well as in multiple lesions, particularly the cortical and smouldering plaques [25, 43]. In our study, we found a significant infiltration of CD8$^+$ T cells in hippocampal demyelinated lesions of MSCI patients corroborating their role in both MS pathology and symptomology. Additionally, we observed interactions between CD8$^+$ T cells and microglia contributing to compartmentalized inflammation – a characteristic of smouldering and silent progressive MS [43]. Interestingly, these ongoing processes might contribute to the disruption of neural networks in MS patients, ultimately leading to changes in cognitive performances [26, 43]. However, whether hippocampal microglia activation and consequent aberrant synaptic pruning results directly or indirectly from infiltrating CD8 T cells in the context of MS-cognitive impairment remains unclear. Nevertheless, recent studies highlighted the role of CD8 T cells-microglia interactions as drivers of cognitive impairment in a viral-induced in vivo model. Indeed, Vasek et al. showed that long-term memory impairment after a viral infection was due to complement-mediated elimination of synapses by microglia [44]. Later studies revealed that microglia-mediated synaptic elimination was promoted by a specific subset of CD8 T cells, thus causing neurological and cognitive deficits [30].

Conclusions

Although extremely valuable, the use of *postmortem* tissue and the restricted tissue sampling confined to a specific brain region might represent a limitation of the present study as it offers an endpoint of a complex pathological cascade and limit the ability to generalize findings to other brain areas. Indeed, microglia plays a crucial role in both demyelinated hippocampal lesions and throughout intrahippocampal regions. In these regions, microglia are responsible for the aberrant elimination of synapses, mainly the inhibitory synapses tagged by C1q. Our findings also demonstrate an interplay between CD8 T cells and microglia in hippocampal lesions of MSCI patients, emphasizing an involvement of this axis. Thus, this study represents a step forward on the understanding of the biological mechanisms involved in cognitive impairment in MS paving the way for the development of new therapeutic strategies.

Supplementary Information

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Supplementary Material 1

Supplementary Material 2

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Author contributions

AF, CB and AA contributed to the conception and design of the present study. CB and AA performed the immunohistochemistry studies. CB contributed to the acquisition and analysis of data. CB and AF contributed to drafting the text and preparing the figures. CB, AA and AF reviewed and approved the final draft of the manuscript.

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Data availability

No datasets were generated or analysed during the current study.

Declarations

Ethics approvals

All procedures performed using human postmortem samples were approved by the Independent Review Board of Amsterdam, UMC, registered with the US Office of Human Research Protection.

Consent for publication

Not applicable.

Competing interests
The authors declare no competing interests.

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