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RESEARCH ARTICLE

Cannabinoid Receptor and N-acyl Phosphatidylethanolamine Phospholipase D—Evidence for Altered Expression in Multiple Sclerosis

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Keywords
blood–brain barrier, cannabinoid receptors, cerebral endothelial cell, multiple sclerosis, N-acyl phosphatidylethanolamine phospholipase D.

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Abstract

Cannabinoids have been shown to have a beneficial effect in both animal models of multiple sclerosis (MS) and human disease, although the mechanisms of action are unclear. We examined expression of the major cannabinoid receptors [(CBRs) cannabinoid receptor 1 (CB1) and cannabinoid receptor 2 (CB2)] and a key enzyme involved in synthesis of the endocannabinoid anandamide [N-acyl phosphatidylethanolamine phospholipase D (NAPE-PLD)] in autopsy brain samples from patients with MS. CB1 was expressed in neurons, injured axons, oligodendrocytes, macrophages/microglia, some astrocytes, endothelial cells, smooth muscle cells and pericytes. CB2 and NAPE-PLD were localized to cerebral endothelial cells, pericytes, smooth muscle cells, astrocytes and macrophages/microglia. NAPE-PLD immunoreactivity was also seen in neurons. Endothelial CB2 expression was greatest in chronic inactive plaques, and in areas was seen in segments of endothelium where the endothelial expression of adhesion molecules (VCAM-1 and ICAM-1) was focally undetectable, and was often expressed in areas of blood–brain barrier damage. Vascular density was increased in chronic active plaques and normal-appearing white matter compared with controls. These data support findings from animal models which suggest a role for the endocannabinoid system in the MS, particularly in the regulation of endothelial leukocyte adhesion and the cellular response to injury.

INTRODUCTION

Multiple sclerosis (MS) is an inflammatory disease of the central nervous system (CNS), characterized by breakdown of the blood–brain barrier (BBB) and episodes of demyelination, often associated with axonal damage and permanent neurological disability (10). The etiology of MS is not known, but genetic and environmental factors have been implicated with recent interest in the potential roles of Epstein–Barr virus (35) and vitamin D deficiency (38). An increasing emergence of new potential treatments for MS have centered on agents with anti-inflammatory and immunomodulatory activity (including beta-interferons and glatiramer acetate) as well as methods for reducing inflammatory traffic across the BBB (including natalizumab) (19).

Recent clinical trial studies have demonstrated the benefit of cannabinoids in alleviating MS-related symptoms (44, 45). Cannabinoids have also been shown to be beneficial in several animal models of MS (8, 36, 46). Cannabinoids have anti-inflammatory activity (29, 32) and neuroprotective activity (14) as well as promote oligodendrocyte survival (30), but the role of endocannabinoids in MS is uncertain. The major endocannabinoids, N-arachidonoylethanolamine [anandamide (AEA)] and 2-arachidonoylglycerol (2-AG), have been shown to exert neuroprotective activity, largely thought to be mediated through two specific receptors [cannabinoid receptor 1 (CB1) and cannabinoid receptor 2 (CB2)] (4), but relatively little information exists regarding the cellular distribution of CBRs and endocannabinoids in MS (5, 43). More specifically, if cannabinoids are involved in recovery after tissue injury, we might expect to observe different patterns of CBR and endocannabinoid activity at different stages of MS, and between areas of active inflammation compared with normal-appearing white matter (NAWM) and control tissue.

We therefore investigated the expression of both CB1 and CB2 receptors, together with N-acyl phosphatidylethanolamine phospholipase D (NAPE-PLD) (the major enzyme associated with synthesis of AEA) expression in a variety of MS lesions, and control autopsy brain tissue in order to explore possible mechanisms and sites of action of cannabinoids in MS. We observed novel alterations of expression of both CBRs and NAPE-PLD in cerebral endothelium at different stages of MS. We went on to investigate the association between endothelial integrity, CBR and adhesion molecule (VCAM-1 and ICAM-1) expression, angiogenesis, and provide evidence supporting anti-inflammatory and reparative roles for the endocannabinoid system in MS.

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MATERIALS AND METHODS

Human brain specimens

Sixteen brain specimens from patients with MS were kindly provided by the UK MS Society Brain Bank, London, UK, and the Department of Cellular and Anatomical Pathology, Derriford Hospital, Plymouth, UK. Thirteen control brains were obtained from the same sources. The patients with MS ranged in age from 32 to 72 years with a mean of 56 years. Patients in the control group were ranged in age from 25 to 87 years with a mean of 57 years. Patient information is summarized in Tables 1 and 2. None of the cases or controls was known to have received cannabinoid treatment. All tissue samples were obtained from cases where consent for use of tissue in research was given by the qualifying relative, and the study protocol was approved by the South West Devon Research Ethics Committee, UK.

Classification of MS plaques

To identify regions of demyelination in MS tissue samples, hematoxylin and eosin staining, luxol fast blue staining, and immunohistochemistry of the myelin basic protein (MBP) antigen were performed. Areas of demyelination were classified according to Trapp *et al* (41) into active plaques (APs) with abundant, evenly distributed inflammatory cells, many containing myelin debris; chronic active plaques (CAPs) with an enrichment of macrophages at the border of the lesion; and chronic inactive plaques (CIPs) that contained very few, predominantly perivascular, inflammatory cells. White matter showing no evidence of myelin loss and normal cellularity was classified as NAWM. Grey matter plaques were identified with immunocytochemistry, using an antibody to MBP, as areas of loss of staining largely or entirely confined to the cerebral cortex.

Immunohistochemistry

The protocol used was as described previously (5) with the following modifications. Human brain specimens were embedded in paraffin and cut in 4-μm sections, which were de-paraffinized and placed into a stainless-steel pressure cooker containing antigen retrieval solution (DakoCytomation Ltd., Ely, Cambridgeshire, UK; 1:10). After heating under pressure for 2 minutes, samples were removed and washed extensively in Tris-Buffered Saline with Tween 20 (TTBS). Endogenous peroxidase activity was blocked by incubation in 3% H_2O_2 at room temperature for 30 minutes. After several washes with TTBS, sections were incubated in 1% normal horse serum for 30 minutes at room temperature. Primary antibodies used were (i) rabbit polyclonal anti-CB1 (Affinity BioReagents, Golden, CO, USA; 1:400); (ii) rabbit polyclonal anti-CB2 (Affinity BioReagents; 1:600); (iii) rabbit polyclonal anti-NAPE-PLD (Sigma-Aldrich, Poole, Dorset, UK; 1:150); (iv) mouse monoclonal anti-MBP (Calbiochem, Nottingham, Nottinghamshire, UK; 1:6000); and (v) mouse monoclonal anti-CD34 (BioGenex Laboratories, San Ramon, CA, USA; 1:400). Primary antibodies were then added in blocking buffer and incubated with sections at 4°C overnight. Sections were washed with TTBS and incubated with biotinylated universal antibody (Vector Laboratories, Peterborough, Leicestershire, UK; 1:50) for 30 minutes at room temperature the

Table 1. Summary of clinical data on patients with MS. Abbreviations: F = female; M = male; MS = multiple sclerosis; SPMS = secondary progressive multiple sclerosis; RRMS = relapse remitting multiple sclerosis; NAWM = normal-appearing white matter; BP = bronchopneumonia; NK = not known.

Patient number	Gender/age (years)	Diagnosis	Duration of MS (years)	No. of different types of plaques			Immunosuppressive treatment	Cause of death	Postmortem interval (h)
				Active	Chronic active	Chronic inactive			
MS1	F/55	SPMS	35	1	1	8		BP, MS	36
MS2	F/35	SPMS	5	5	0	1		MS	9
MS3	F/51	SPMS	10	12	2	11		BP, MS	190
MS4	M/69	SPMS	25	0	0	3		BP, MS	12
MS5	M/32	RRMS	9	0	3	20	Y	MS	72
MS6	F/58	SPMS	41	0	0	7	Y	BP, MS	36
MS7	F/67	SPMS	31	0	0	4		BP, MS	11
MS8	M/58	SPMS	32	0	0	4		BP, MS	29
MS9	F/56	SPMS	23	0	2	5		BP, MS	42
MS10	F/54	SPMS	24	0	0	11		MS	18
MS11	M/56	SPMS	25	0	0	4		BP, MS	NK
MS12	F/72	SPMS	21	0	0	8		MS	23
MS13	M/70	SPMS	24	0	0	2		BP, MS	24
MS14	F/60	MS	21	0	0	4		Cardiac arrest	24
MS15	F/56	SPMS	27	0	0	3	Y	Breast cancer, MS	20
MS16	F/52	SPMS	18	0	0	4	Y	MS	90
Total number of plaques				18	8	99			

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Table 2. Summary of clinical data on patients in control group. Abbreviations: F = female; M = male; C = control; MDEM = multiphasic disseminated encephalomyelitis; MND = motor neuron disease; PSP = progressive supranuclear palsy; SUDEP = sudden unexpected death in epilepsy; CVA = cerebrovascular accident; MSA = multiple system atrophy; NHL = non-Hodgkin lymphoma; DAI = diffuse axonal injury; BP = bronchopneumonia; DIC = disseminated intravascular coagulation.

Patient number	Gender/age (years)	Diagnosis	Immunosuppressive treatment	Cause of death	Postmortem interval (h)
C1	F/31	MDEM	Y	MDEM	70
C2	F/87	MND		BP, MND	96
C3	F/63	Sarcoid		Sarcoid	48
C4	F/63	MSA		CVA	24
C5	F/76	CVA		CVA	72
C6	F/49	DAI		Head injury	70
C7	F/69	PSP		BP, PSP	80
C8	F/61	Hypoxia		DIC	74
C9	F/77	NHL		NHL	63
C10	M/71	Normal		Septicaemia	47
C11	M/31	Epilepsy		SUDEP	28
C12	F/25	Epilepsy		SUDEP	40
C13	M/39	Epilepsy		SUDEP	70

next day. Avidin-biotin complex (Vectastain® Universal Elite® ABC Kit, Vector Laboratories, Peterborough, Leicestershire, UK) and diaminobenzidine (DakoCytomation) were used to obtain a visible reaction product. Controls for immunohistochemistry included preincubation of the antibodies with the corresponding antigens (when available) and omission of the primary antibody. The examiners were blinded to the source of the specimen (MS vs. control). A Nikon eclipse 80i microscope (Nikon UK Ltd., Kingston Upon Thames, Surrey, UK) and a Nikon digital color camera (Nikon UK Ltd., Kingston Upon Thames, Surrey, UK) were used for the examination and photography of the slides, respectively.

Double immunofluorescence

Double immunostaining was performed on brain sections as described previously (5). After the antigen retrieval procedure, tissue sections were washed with TTBS before overnight incubation at 4°C with (i) rabbit polyclonal anti-CB1 (Affinity BioReagents; 1:50); (ii) rabbit polyclonal anti-CB2 (Affinity BioReagents; 1:100); and (iii) rabbit polyclonal anti-NAPE-PLD (Sigma-Aldrich; 1:10) and then with Alexa Fluor® 568 anti-rabbit antibody conjugate (Invitrogen, Paisley, UK; 1:200) in TTBS containing 1% BSA incubate for 1 h at room temperature, rendering red fluorescence. After several washes with TTBS, the monoclonal antibodies used for identification of the cell types and for investigation of cerebral endothelial CB2 functions were applied at 4°C overnight: (i) mouse anti-CD68 (DakoCytomation; 1:50); (ii) mouse anti-HLA-DR (DakoCytomation; 1:100); (iii) mouse anti-gilial fibrillary acidic protein (GFAP) (Sigma-Aldrich; 1:300); (iv) mouse anti-CD34 (BioGenex Laboratories; 1:10); (v) mouse anti-smooth muscle actin (SMA) (DakoCytomation; 1:100); (vi) goat anti-VCAM-1 (R&D System, Abingdon, Oxfordshire, UK; 1:200); (vii) goat anti-ICAM-1 (R&D System; 1:200); and (viii) mouse anti-ZO-1 (LifeSpan Biosciences, Seattle, WA, USA; 1:25). Followed by incubation with Alexa Fluor® 488 antigoat antibody conjugate (Invitrogen; 1:200) or Alex Fluor® 488 antimouse antibody conjugate (Invitrogen; 1:200) at room temperature for 1 h, rendering green fluorescence. CB2 and NAPE-PLD were also visualized by incubation with biotinylated antirabbit secondary antibody, followed by streptavidin–Cy3 conjugate (Sigma-Aldrich; 1:100), as described previously (5). Slides were mounted using 4′, 6-diamidino-2-phenylindole (DAPI; Molecular Probes, Paisley, UK) for 30 minutes and were afterward mounted onto glass slides with aqueous solution (Vectashield®, Vector Laboratories). A Nikon Eclipse 80i microscope and digital camera were used for observation and photography of the slides. Fluorescence signals were also detected using laser scanning with a Zeiss 510 nonlinear optic confocal scanning system (Carl Zeiss Ltd, Welwyn Garden City, Hertfordshire, UK). Controls included omitting either the primary or the secondary antibody, or preabsorbing the primary antibody.

Quantification of blood vessel density

To quantify blood vessel density, images of endothelial cells stained by immunoperoxidase labeling using an antibody to CD34 were acquired using a Leica DMRB microscope (Leica Microsystems, Milton Keynes, Buckinghamshire, UK) with attached DC500 digital camera. Images were captured from 5 to 10 continuous fields from each plaque, and from normal-appearing gray and white matter, using an ×20 objective, with the area of each field being 0.28 mm^2. The area of CD34-positive microvessels was measured using LUCIA G 4.81 (Laboratory Imaging, Prague, Czech Republic) image analysis software. Values were averaged and expressed as the mean endothelial area fraction for each lesion assessed. A total of 55 plaques were assessed (8 APs, 27 CIPs, 5 CAPs and 15 gray matter plaques) from 14 cases, with corresponding areas of normal-appearing gray and white matter. Nine control cases were also examined.

Quantification of CB2 expression in macrophage/microglia

Images of macrophage/microglia labeled for CB2 (red) and HLA-DR (green) were acquired using Nikon eclipse 80i fluores-

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cence microscope and digital camera with ×20 object lens. Macrophage/microglia was identified by a combination of HLA-DR and morphology. Five to 10 continuous fields were captured from each plaque and from corresponding NAWM and control white matter, the area of each field being 0.28 mm^2. The number of CB2-positive macrophage/microglia and the number of HLA-DR-positive macrophage/microglia in each picture were counted separately in a blinded fashion. The ratio of CB2-positive macrophage/microglia compared with the total number of HLA-DR-positive macrophage/microglia in each field was counted and expressed as the mean fraction. A total of 22 plaques were assessed (7APs, 5CAPs, 10CIPs) from eight cases, with corresponding area of NAWM; five control patients were examined.

Statistical analysis

Quantitative results were expressed as the Mean ± SD. One-way analysis of variance (ANOVA) was used to compare group differences; $P < 0.05$ is considered statistically significant. Tukey post hoc tests were applied to compare specific group differences if the ANOVA revealed a significant difference. All analyses were performed using the SPSS software package (version 17.0, SPSS Inc, Chicago, IL, USA).

RESULTS

CB1 expression in MS

CB1 expression in various cell types in MS was examined by immunohistochemistry and double immunofluorescence labeling. CB1 was seen in neurons, oligodendrocytes and perivascular macrophages/microglia in active lesions (data not shown). We also observed expression in injured axons both in APs and in controls (Figure 1F). CB1 was seen in GFAP-positive reactive astrocytes in APs (Figure 1C). CB1 immunoreactivity could be detected in cerebral blood vessels (endothelial cells, pericytes and smooth muscle cells) in NAWM (Figure 1A,Bi), in CIPs of MS patients and in the white matter of control cases (data not show). Low level of expression was found in intact endothelium in APs (Figure 1Bii) and CAPs, and higher level of CB1 staining was seen in smooth muscle cells and pericytes in APs (Figure 1Bii). In view of the weak and focal nature of CB1 staining in endothelium in MS, quantization was not undertaken.

CB2 expression in MS

CB2 immunoreactivity was detected in cerebral endothelial cells (Figure 1Di), pericytes, smooth muscle cells (Figure 1E), astrocytes and macrophages/microglia (data not show). Higher levels of CB2 staining were seen in the few intact endothelial cells in APs (Figure 1Dii); however, lower level of staining was found in disrupted endothelial cells (Figure 1Diii); in CIPs, higher level of CB2 staining was detected in intact endothelial cells (Figure 1Div). CB2 staining was also increased in pericytes and smooth muscle cells in APs, revealed by double labeling with SMA in Figure 1E.

In addition, endothelial CB2 expression level (CB2 positive endothelial area/total endothelial area) was highest in CIPs (32.12% ± 2.62%) and lowest in APs (7.25% ± 1.33%) compared with CAPs (19.08% ± 2.60%; $P < 0.01$; $n \geq 4$), NAWM (21.77% ± 1.75%; $P < 0.05$; $n \geq 4$), and controls (19.17% ± 2.74%; $P < 0.05$; $n \geq 4$) (Figure 1G).

Expression of CB2 in macrophages/microglia was analyzed with double immunostaining using morphology and HLA-DR as a marker for macrophage/microglia. As expected, our data showed abundant macrophage/microglia distribution in APs (675.25 ± 14.20/mm^2). A lower density of macrophage/microglia was seen in CIPs (133.64 ± 9.74/mm^2) than CAPs (287.18 ± 10.34/mm^2), and higher density was seen in NAWM (34.79 ± 3.74/mm^2) than control human brain (6.79 ± 0.28/mm^2) (Figure 2A). However, the proportion of CB2-positive macrophage/microglia varied in different types of MS plaques and was highest in APs (79.10% ± 1.52%; $P < 0.05$, $n \geq 4$) and CIPs (78.77% ± 1.74%; $P < 0.05$, $n \geq 4$) compared with other plaques and control white matter (Figure 2B).

NAPE-PLD expression in MS

Additionally, NAPE-PLD immunoreactivity was detected in a range of cells in different stages of MS, namely endothelial cells, pericytes, smooth muscle cells, reactive astrocytes, microglia and macrophages in active stages (Figure 3A), and in macrophages/microglia, endothelial cells, smooth muscle cells and pericytes in CAPs; high levels of NAPE-PLD expression were seen in endothelial cells, pericytes, smooth muscle cells and astrocytes in CIPs. Intense astrocytic NAPE-PLD immunoreactivity was seen in APs and CIPs, and was confirmed by double immunofluorescence staining with GFAP (Figure 3E). The expression of NAPE-PLD in macrophages, macrophage/microglia, astrocytes, endothelial cells, pericytes and smooth muscle cells was confirmed by double immunofluorescence staining with specific markers (Figure 3C–E,Fi–iii,G). NAPE-PLD was also expressed in neurons, with particularly strong expression in neurons showing evidence of acute ischemic/hypoxic injury (Figure 3B), and also in endothelial cells and perivascular muscle cells in control tissue (data not show).

Lower expression of NAPE-PLD was observed in disrupted endothelium in APs (Figure 3Fii), and higher expression was seen in intact endothelium in CIPs (Figure 3Fiii), compared with NAWM and the control group. Higher level of NAPE-PLD was also seen in the disrupted pericytes in the blood vessels in APs and was revealed by double staining with SMA in Figure 3G.

Further, endothelial NAPE-PLD expression was illustrated in average endothelial NAPE-PLD area fraction, which was lower in APs (12.81% ± 0.70%; $P < 0.05$; $n \geq 4$) and highest in CIPs (49.76% ± 5.17%; $P < 0.05$; $n \geq 4$) compared with the expression in CAPs (27.58% ± 0.74%), in NAWM (24.87% ± 1.37%), and in controls (24.36% ± 1.61%). There was no statistical significance between the other groups (Figure 3H).

Correlation of cerebral endothelial CB2 with adhesion molecule expression and BBB disruption

We examined the expression of adhesion molecules in different stages of MS, using labeling for VCAM-1 and ICAM-1, and observed a low level of VCAM-1 expression in inflammatory cells, endothelial cells, and astrocytes, either in the center of APs or in CIPs; the expression of ICAM-1 was low in the middle of APs but higher in some blood vessels in CIPs, while the expression level of

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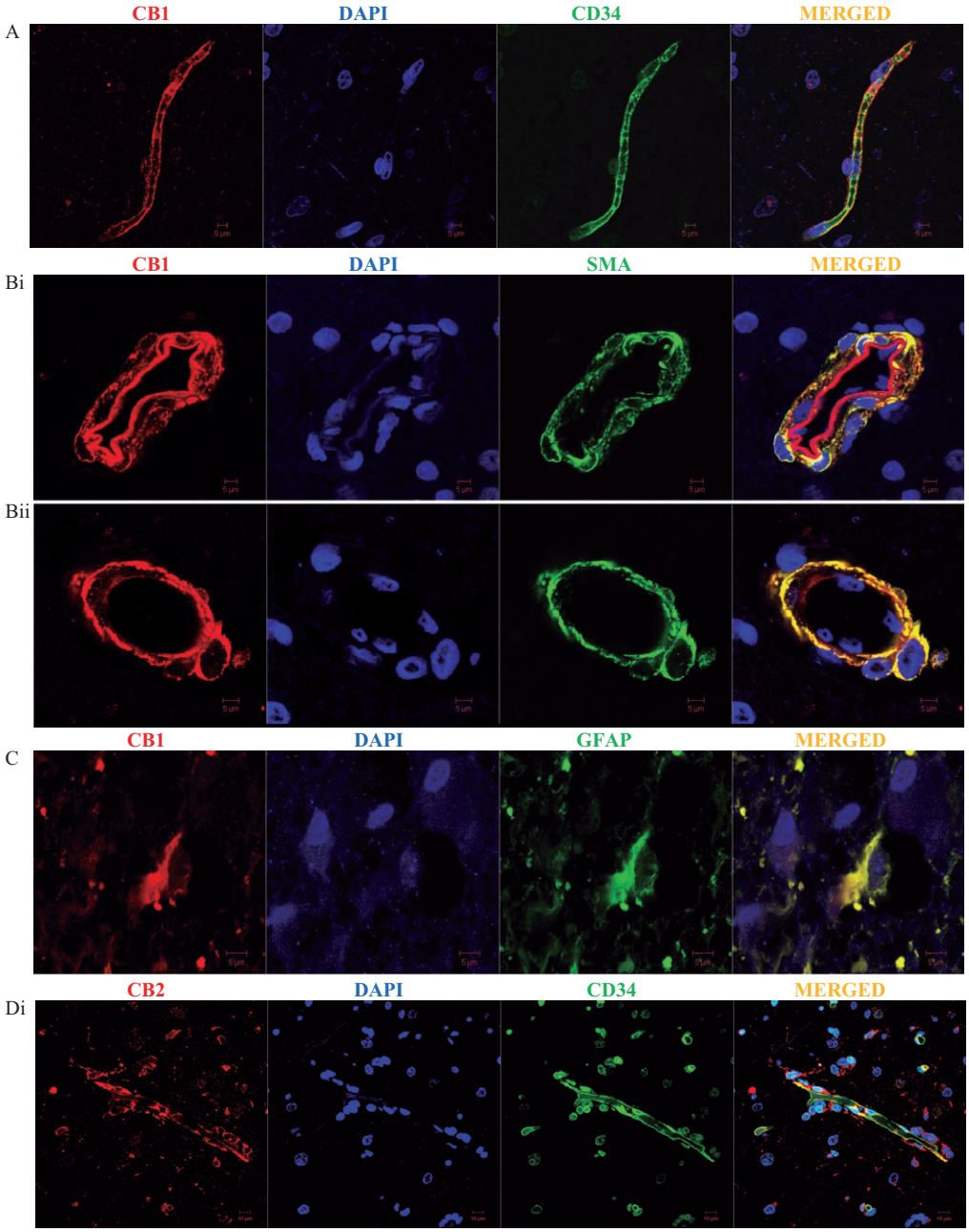

Figure 1. *Expression of cannabinoid receptor 1 (CB1) and cannabinoid receptor 2 (CB2) in multiple sclerosis (MS).* **A.** Double immunofluorescence labeling of a blood vessel in normal-appearing white matter (NAWM) of MS for CB1 (red), nuclei [blue; 4′, 6-diamidino-2-phenylindole (DAPI)] and CD34 (green); the overlapped area is shown in yellow. **Bi.** Immunofluorescence labeling of a blood vessel in NAWM for CB1 (red), nuclei (blue; DAPI) and smooth muscle actin (SMA) (green), the focal area of co-expression in smooth muscle is shown in yellow, strong endothelial expression is noted. **Bii.** Higher level of CB1 staining was seen in smooth muscle cells and pericytes in active plaques (APs), and lower level of staining was found in endothelial cells. **C.** CB1 receptor expressed in reactive astrocytes in APs by double immunofluorescence labeling of astrocytes for CB1 (red), nuclei (blue; DAPI) and marker GFAP (green), with areas of overlap (yellow). **Di–iv.** Double immunofluorescence staining for CB2 (red) with CD34 (green) in different stages of MS with overlapping expression seen as yellow; scale bars are 5 μm. (**Di**) CB2 staining was seen in endothelial cells in NAWM; (**Dii**) Higher level of CB2 staining was found in few intact endothelial cells in APs; (**Diii**) lower level of CB2 staining was seen in disrupted endothelial cells in APs; (**Div**) higher level of staining was also detected in endothelial cells (mainly expressed in luminal surface) in chronic inactive plaques (CIPs). **E.** Higher level of CB2 staining was found in smooth muscle cells in APs, and the endothelium shows extensive disruption. **F.** CB1 receptor was seen in injured axons in APs (arrows), (left upper; inset, the fluorescence staining of CB1 receptor in swollen axon in APs showing membrane localization). **G.** Quantification of endothelial CB2 expression in different stages of MS and controls (endothelial CB2 area/total endothelial area). AP group < all the other groups ($^*P < 0.05$, $n \geq 4$); CIP group > all the other groups ($^*P < 0.05$, $n \geq 4$); there is no statistically significant difference between the other groups ($P > 0.05$; $n \geq 4$).

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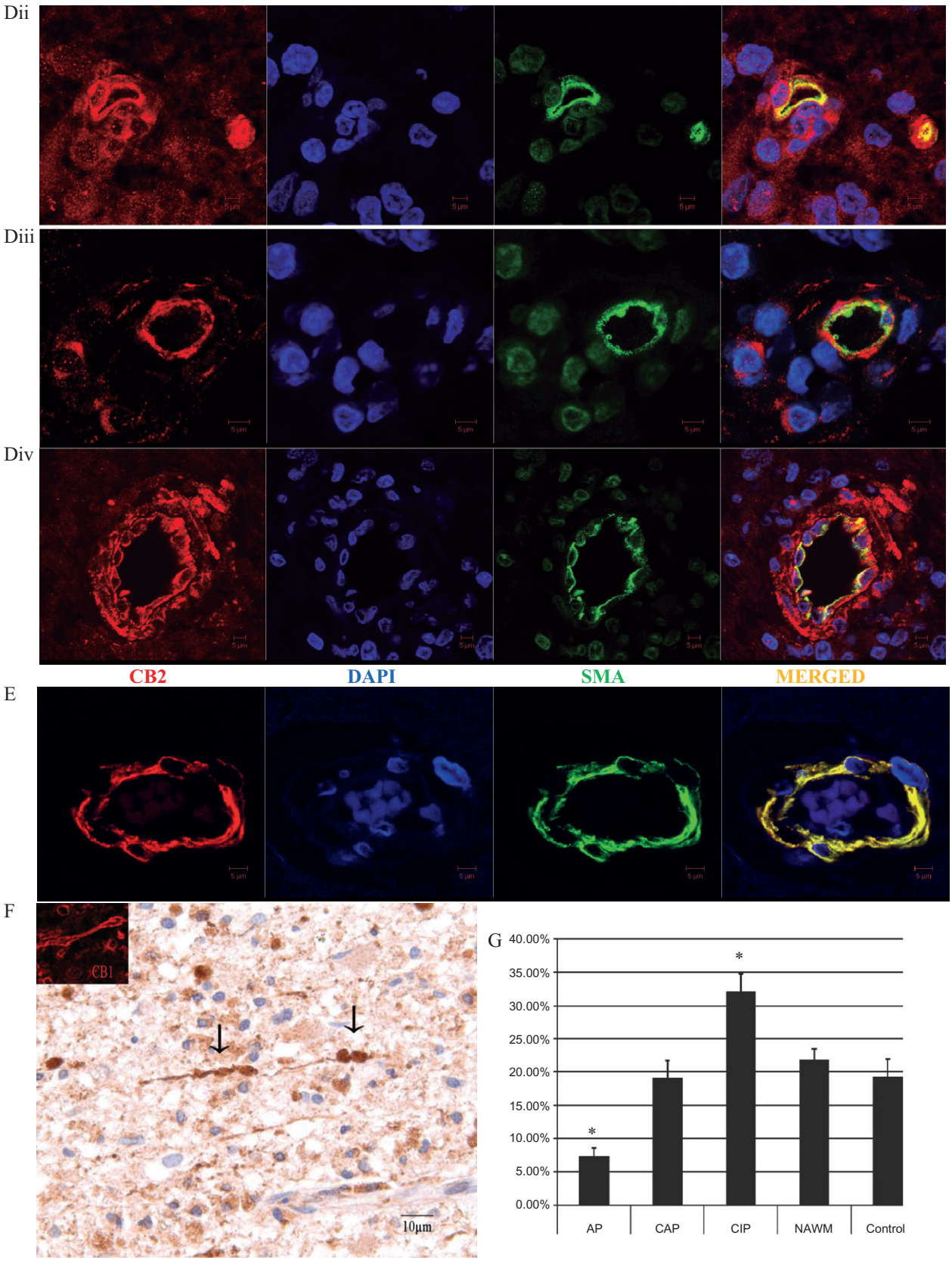

Figure 1. *Continued*

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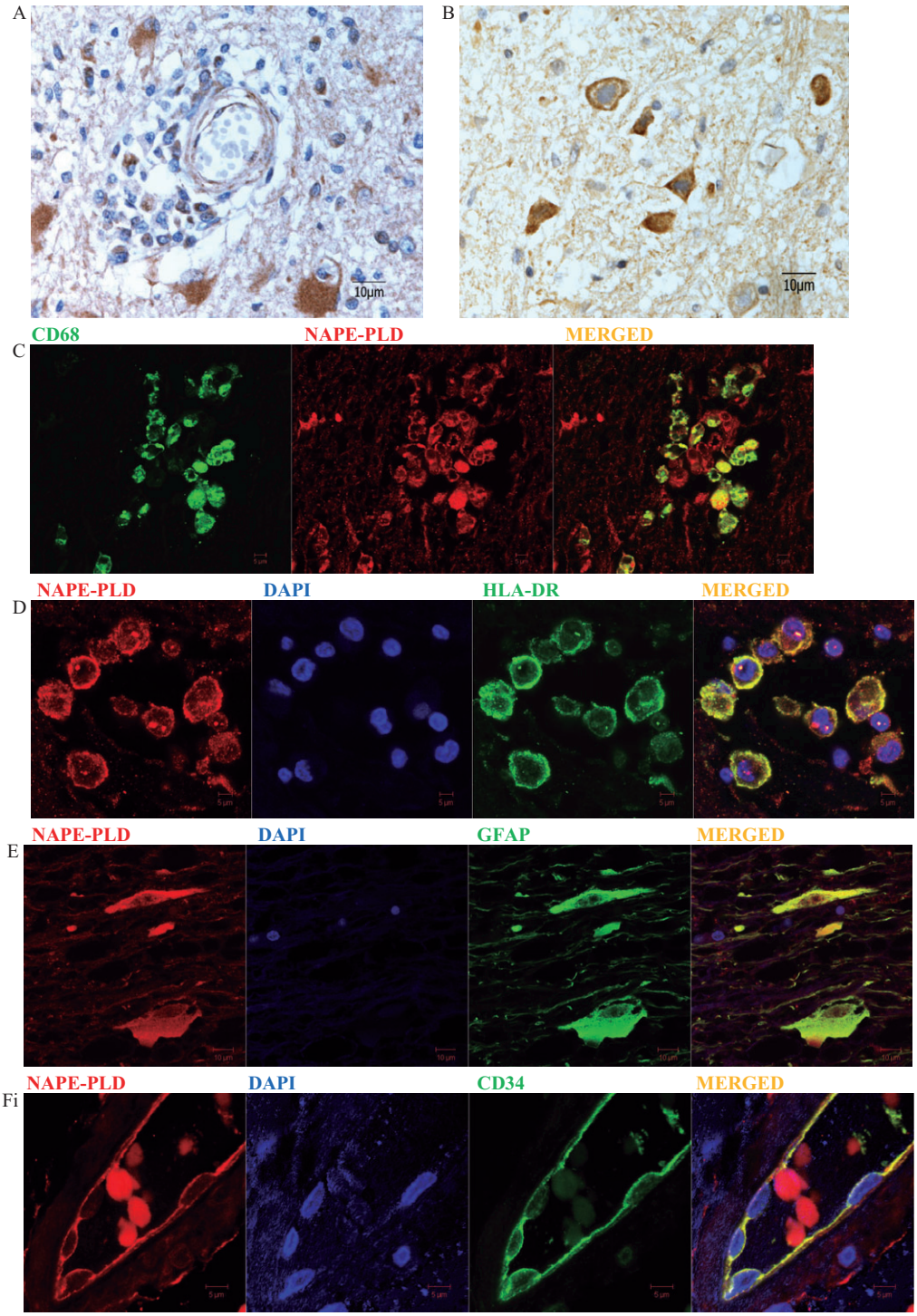

Figure 2. *Detection of N-acyl phosphatidylethanolamine phospholipase D (NAPE-PLD) in multiple sclerosis (MS).* Immunohistochemistry showing NAPE-PLD staining was seen in endothelial cells, smooth muscle cells, pericytes, reactive astrocytes, microglia and macrophages in active plaques (APs) (**A**) and neurons (**B**); scale bars are 10 μm. Double-labeling immunofluorescence confirming NAPE-PLD expression in perivascular lymphocytes and macrophages/microglia (**C,D**), reactive astrocytes (**E**), endothelium (**Fi**), and pericytes (**G**). (**Fii–iii**) Double immunofluorescence for NAPE-PLD (red) with CD34 (green) in different stages of MS with areas of coexpression in yellow; scale bars are 5 μm. Lower level of NAPE-PLD staining was seen in disrupted endothelial cells in APs (**Fii**) compared with higher level of staining was detected in intact endothelial cells in chronic inactive plaques (CIPs) (**Fiii**). (H) Quantification of endothelial NAPE-PLD expression in different stages of MS and controls (endothelial NAPE-PLD area/total endothelial area). AP < the other groups ($^*P < 0.05$; $n \geq 4$); CIP > the other groups ($^*P < 0.05$; $n \geq 4$). There is no statistically significant difference between the other groups.

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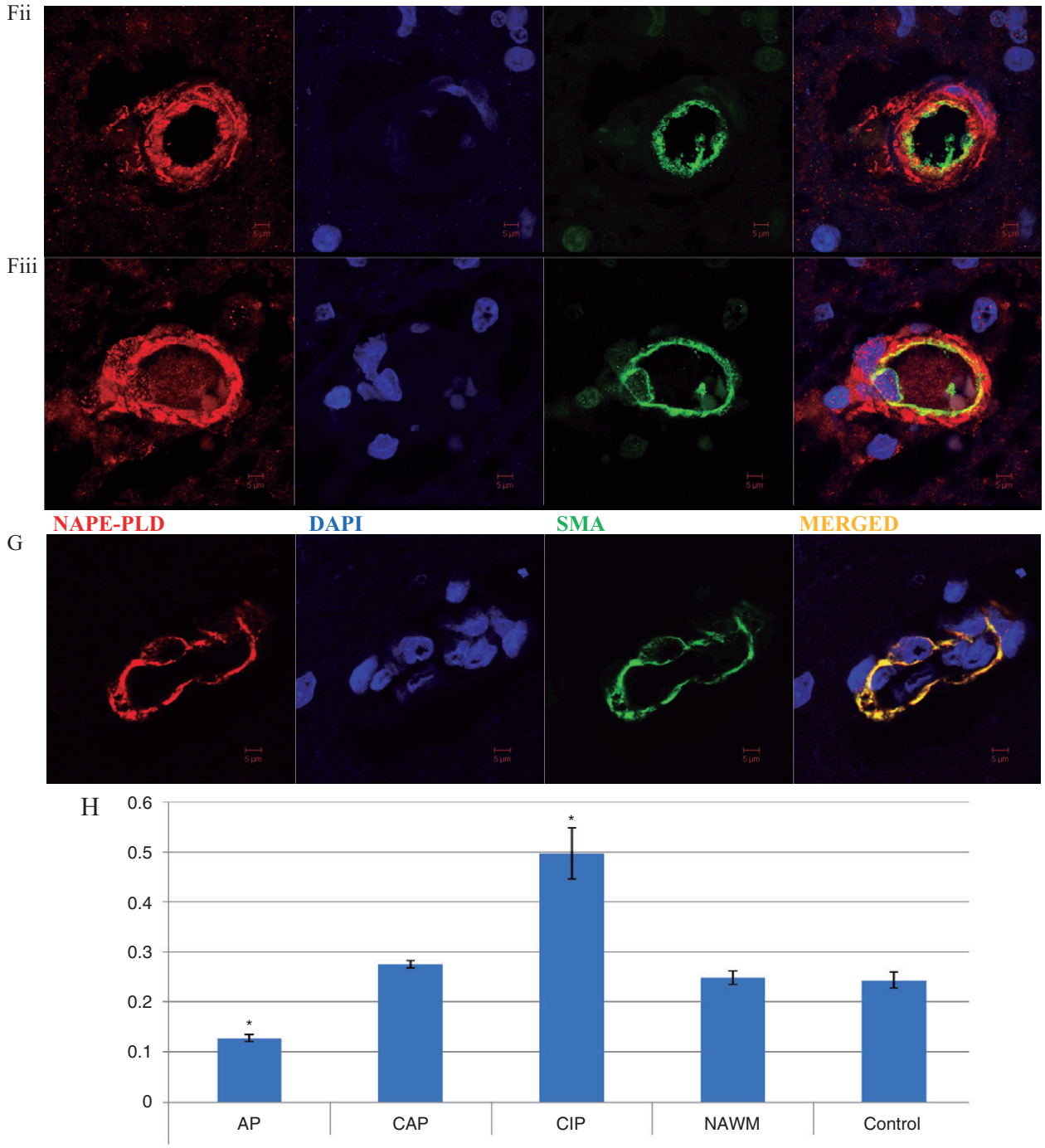

Figure 2. *Continued*

both adhesion molecules was high in the area around the lesions, mainly in inflammatory cells, endothelial cells and astrocytes. Further, double immunostaining of CB2 with adhesion molecules (VCAM-1 and ICAM-1) revealed that CB2 expression largely correlated with adhesion molecule expression, but in some blood vessels there were segments of alternate strong expression of CB2 and VCAM-1 (Figure 4A,B), and CB2 and ICAM-1 (Figure 4C,D) in APs and CIPs.

We then went on to investigate the relationship between BBB disruption and CB2 expression in various stages of MS. Single labeling of tight junction protein (ZO-1) revealed areas of BBB disruption, with discontinuous ZO-1 staining in all stages of MS, most obvious in APs. Double labeling of CB2 with ZO-1 demonstrated that increased vascular CB2 staining was seen in areas of BBB breakdown where ZO-1 staining was discontinuous (Figure 5A). Low levels of vascular CB2 expression were seen in

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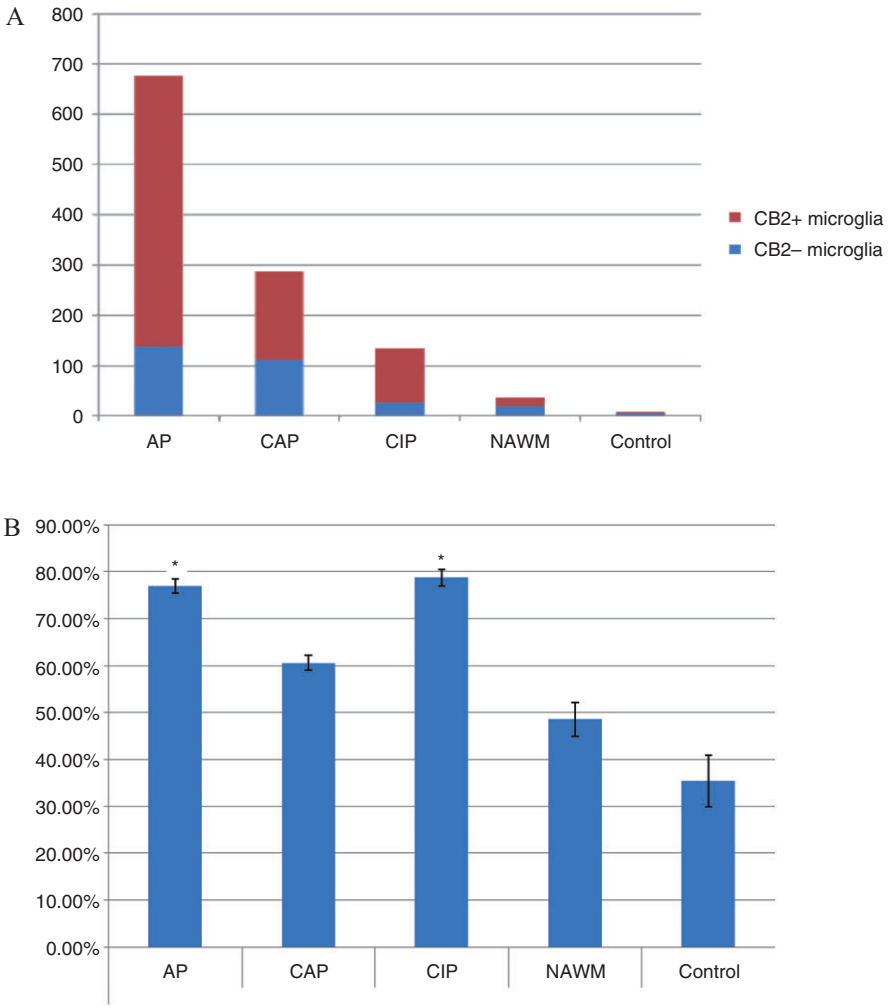

Figure 3. *Quantification of cannabinoid receptor 2 (CB2) expression in macrophage/microglia in multiple sclerosis (MS).* **A.** The number of CB2-positive macrophage/microglia and total macrophage/ microglia, per square millimeter, are shown for the different MS lesions. **B.** The proportion (%) of CB2-positive macrophage/microglia in different MS lesions and controls. The proportion of CB2-positive macrophage/ microglia is highest in chronic inactive plaques (CIPs) and active plaques (APs), and significantly higher than the other groups (* $P < 0.05$; $n \geq 4$).

areas of BBB with continuous ZO-1 staining both in NAWM (Figure 5B) and in white matter in control tissue.

We investigated whether vascular density was altered in different MS lesions by assessing endothelial area fraction using immunocytochemistry with an antibody to CD34. Endothelial area fraction in the different types of MS plaque in white matter is shown in Figure 6; we found increased expression in CAPs and NAWM but decreased expression in CIPs and APs, with statistical significance between CIPs and NAWM ($P < 0.05$; $n \geq 5$), APs and NAWM ($P < 0.05$; $n \geq 5$). Although the mean value of vascular density in gray matter was higher than that in white matter (0.0156 ± 0.0018 vs. 0.0076 ± 0.0065), it was not significantly altered in gray matter or white matter plaques compared with their respective adjacent normal-appearing tissues.

DISCUSSION

In the current study, strong CB1 expression in injured axons both in APs of MS and in control brains was noted. Previous studies have shown that CB1 is expressed abundantly in cortical neurons and axonal fibers, especially at presynaptic terminals in the hippocampus (17, 20) and in postsynaptic elements (39), and that activation of CB1 expressed on nerves may induce immunosuppression by driving the production of immunosuppressive molecules including glucocorticosteroids (3). Additionally, there is evidence that endocannabinoids (AEA and 2-AG) may be neuroprotective via presynaptic CB1 activation, leading to reduced excitotoxicity (23). Although the increased axonal expression of CB1 that we observed in APs may have functional significance, it could also simply reflect disturbed axonal transport in MS. We also observed CB1 expression in reactive astrocytes in MS. There is limited information about the function of CB1 in astrocytes, although they have been reported as mediating AEA-induced enhancement of IL-6 release (31) and may be involved in astrocytic cell death/survival decisions (16).

We have demonstrated both CB1 and CB2 expression in cerebral blood vessels (endothelial and smooth muscle cells) of MS brains and control tissue. Although previous studies of MS brain tissue did not note the presence of endothelial expression of CB2 in their publications (5, 43), our observations are consistent with the previous demonstration of both CBRs in cultured cerebrovascular endothelial cell lines (15, 25, 28) and CBRs in the blood vessels of rat brain (1, 2). In contrast to the relative absence of endothelial CB1 in MS patients, we found CB2 expressed in endothelium in almost all MS

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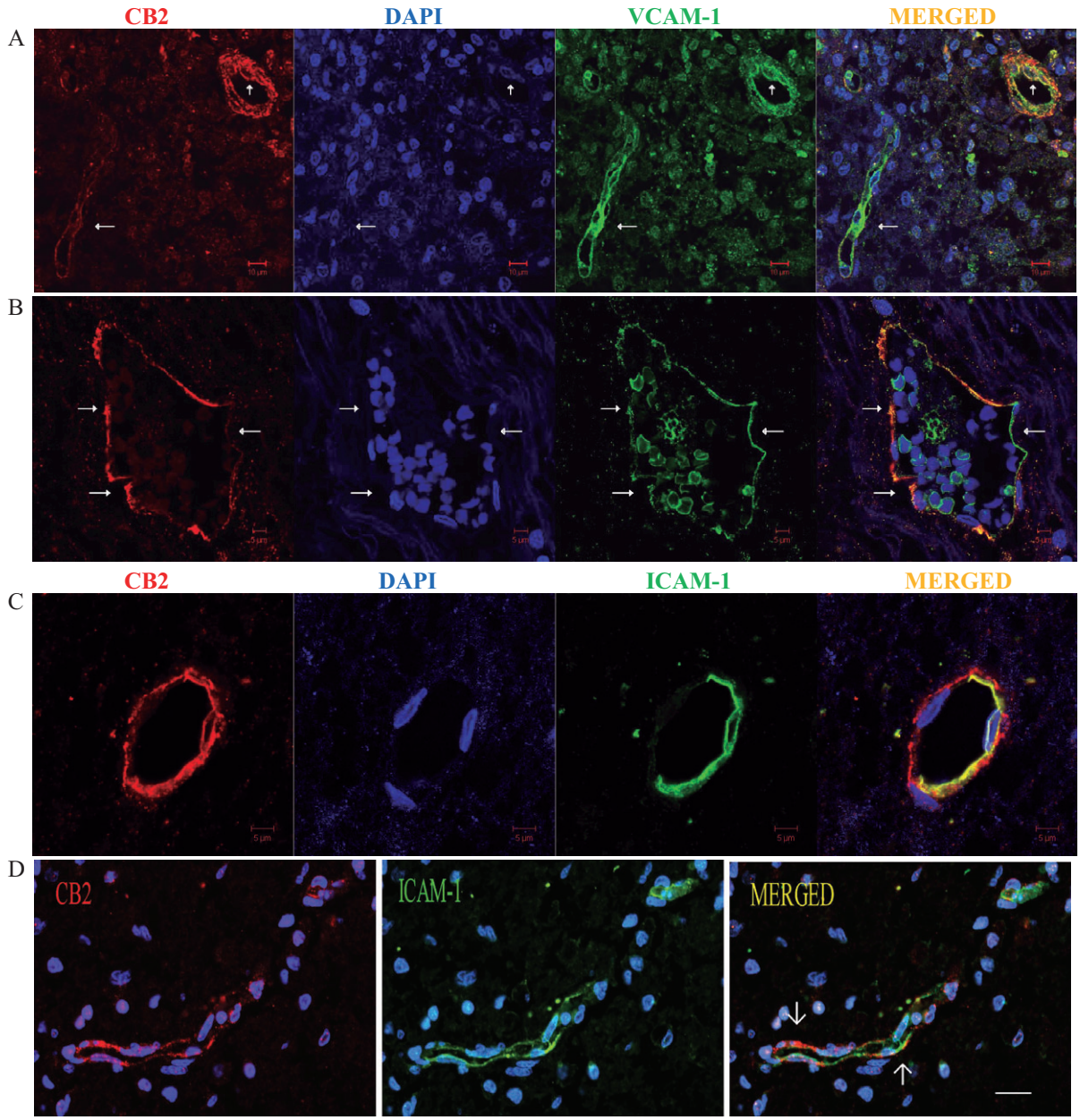

Figure 4. *Endothelial expression of cannabinoid 2 (CB2) receptor and adhesion molecules (VCAM-1/ICAM-1) in multiple sclerosis (MS).* **A.** Double immunostaining in active plaques (APs) showing high level of VCAM-1 expression in some blood vessels, in which the CB2 was undetectable (arrow←). In others, VCAM-1 was colocalized with the CB2 (arrow↑). **B.** Chronic inactive plaques (CIPs) showing areas of inversed endothelial expression of VCAM-1 and CB2 (arrows), and VCAM-1 expression in the inflammatory cells in the lumen of blood vessel. **C.** Blood vessel in CIPs showing undetectable ICAM-1 in one endothelial cell with upregulated CB2 expression in the abluminal side, and coexpression in another. **D.** Blood vessel in APs showing inversed expression of ICAM-1 and CB2 (arrows); the scale bar is 10 μm.

patients, with a variable expression pattern among different stages, which may suggest a role in MS pathology. We have also demonstrated NAPE-PLD expression in cerebral blood vessels; thus, the receptor and one of the enzymes important in AEA synthesis are expressed, suggesting a probable ligand. Together these findings are in keeping with a functional role of endothelial cannabinoid expression in MS; however, further work using *in vitro* cultures and animal models are required to investigate this further.

The significance of differential expression between CB1 and CB2 in MS endothelium is still unknown, although previous animal models of MS support a similar imbalanced expression of these receptors in the brain with evidence for reduced CB1 expression (6, 8, 9) and a 100-fold increase in CB2 mRNA expression (26).

The functional significance of endothelial expression of CBRs, especially CB2 and NAPE-PLD, is unclear, but the CBRs' presence does imply a role for endocannabinoid metabolism (25) and

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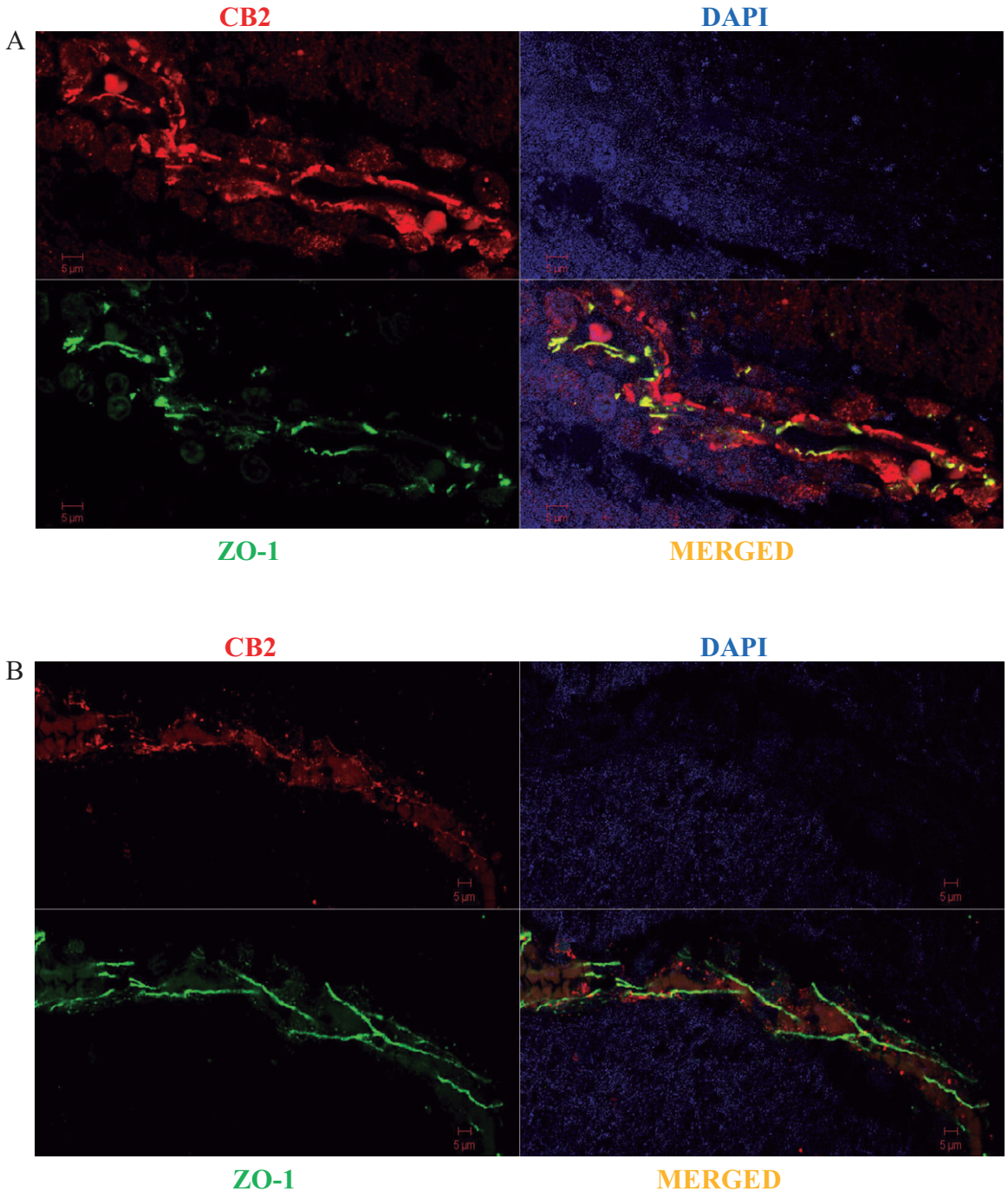

Figure 5. *Relationship between cannabinoid receptor 2 (CB2) expression and blood–brain barrier (BBB) integrity using ZO-1 staining in multiple sclerosis (MS).* **A.** Discontinuous ZO-1 expression (green) was associated with increased CB2 staining (red) in active plaques (APs). **B.** In normal-appearing white matter (NAWM), continuous ZO-1 staining (green) was associated with lower CB2 expression (red).

binding in vascular pathophysiology. It is of interest that endothelial CB2 and NAPE-PLD both appear at reduced levels in active disease but are elevated in CIPs. Tight junction abnormalities, which represent BBB disruption, were most frequently seen in active lesions and persist in inactive lesions of MS, which is consistent with previous descriptions (21, 22). We also observed that increased CB2 staining is associated with discontinuous ZO-1 expression in APs. When taken in conjunction with evidence from other models of brain injury that suggest that endocannabinoids may reduce BBB damage (27, 34), the current data are in keeping with a role for endothelial CB2 in the protection and/or repair of BBB injury in both acute and chronic disease.

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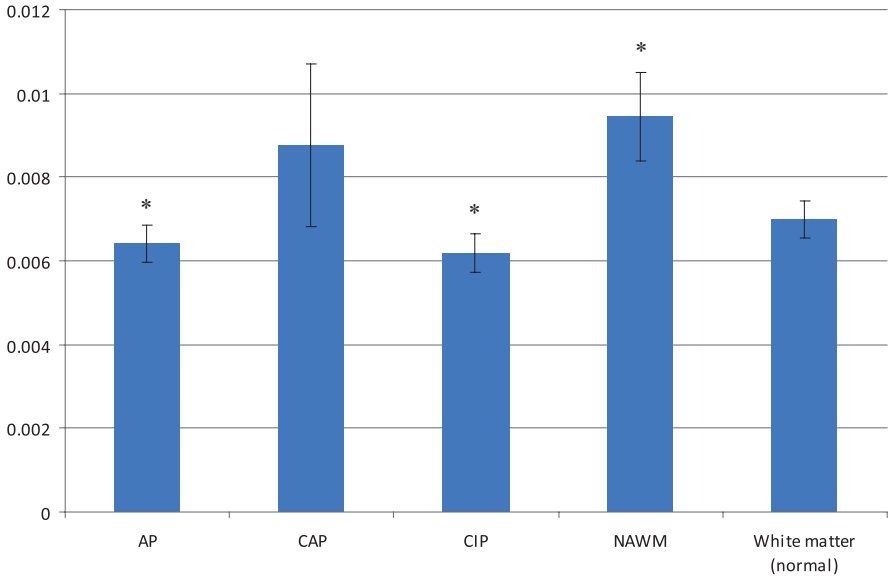

Figure 6. *Vascular density as measured by endothelial area fraction (CD34 positive area/total area of field) in different multiple sclerosis (MS) white matter lesions and control group.* The vascular density in the chronic inactive plaques (CIPs) and active plaques (APs) groups are less than that in the normal-appearing white matter (NAWM) group (*$P < 0.05$; $n \geq 5$). There is no statistical significance between the other groups ($P > 0.05$; $n \geq 5$).

Cannabinoids may also have a role in the inhibition of angiogenesis (7), and previous publications have suggested that there may be an increase in vascular proliferation toward the edge of MS lesions (18, 24). We have found convincing evidence for the reduced vascular density in CIPs, which is inversely associated with endothelial CB2 expression, in keeping with a possible role of endothelial CB2 in inhibiting angiogenesis, in the chronic repair stages of MS.

It has previously been shown that cannabinoid treatment in animal models of MS leads to reduced white cell rolling and adhesion in cerebral vasculature (32), associated with CB2 activation (46). A similar action for CB2 activation, with reduced leucocyte/endothelial adhesion and transmigration, has also been demonstrated in cultured human endothelial cells (37). However, in a viral model of MS, the role of endothelial CB2 in reducing endothelial adhesion molecule expression and perivascular inflammation (29) has been questioned. Therefore, we investigated the relationship between endothelial CB2 and vascular adhesion molecules, and found that in areas expression was nonoverlapping between these sets of molecules, especially in the active stages and chronic inactive stages of MS. This observation is in keeping with endothelial endocannabinoid activity having an anti-inflammatory role in MS. The specificity of this response in MS compared with other causes of CNS inflammation is unclear. However, findings in inflammatory controls (neurosarcoid and multiphasic disseminated encephalomyelitis) suggest that this response may not be specific in MS (data not show). High levels of NAPE-PLD expression in reactive astrocytes distributed closely around the blood vessels in APs and CIPs are also in keeping with a contribution by the cells to BBB protection and an anti-inflammatory action in MS (12, 42), although cannabinoids may act in a CBR-independent manner in astrocytes (12).

Neuronal expression of NAPE-PLD has also been noted in animal brains where it seems to have a regional distribution (11, 13, 40), and although we noted strong staining in a few hypoxic neurons, it is not clear whether this indicates a direct neuroprotective effect for endocannabinoids *in vivo* (23).

There is evidence that endocannabinoids including AEA have an anti-inflammatory effect by the inhibition of microglial function in MS (33). Abundant CB2-positive macrophages/microglia was seen in APs and CIPs, although the numbers of such cells were reduced in inactive plaques. Macrophage/microglia may therefore be an anti-inflammatory target for AEA action, both in the early inflammatory stage and also in the repair stages of MS, via a CB2 mediated mechanism.

In this study we report the differential expression of CBRs and NAPE-PLD in a range of cells, including glia, neurons, and blood vessels, with a variable expression pattern in different stages of MS pathology. The novel finding of prominent changes in endothelial expression supports data from animal models that suggest roles for endocannabinoid system in modulating leucocyte adhesion, BBB repair and possibly angiogenesis in MS. The specificity of these functions to MS remains unclear, but preliminary findings suggest that these mechanisms are likely to apply to other inflammatory diseases of the CNS. Although further work is required to clarify the role of the endocannabinoid system in MS, our data support the findings from animal models that provide a rationale for the modulation of cannabinoid expression in the treatment of MS.

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