

RESEARCH PAPER

Increased IP-10 production by blood–nerve barrier in multifocal acquired demyelinating sensory and motor neuropathy and multifocal motor neuropathy

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ABSTRACT

Objective Dysfunction of the blood–nerve barrier (BNB) plays important roles in chronic inflammatory demyelinating polyneuropathy (CIDP) and multifocal motor neuropathy (MMN). The aim of the present study was to identify the candidate cytokines/chemokines that cause the breakdown of the BNB using sera from patients with CIDP and MMN.

Methods We determined the levels of 27 cytokines and chemokines in human peripheral nerve microvascular endothelial cells (PnMECs) after exposure to sera obtained from patients with CIDP variants (typical CIDP and multifocal acquired demyelinating sensory and motor neuropathy [MADSAM]), MMN and amyotrophic lateral sclerosis (ALS), and healthy controls (HC), using a multiplexed fluorescent bead-based immunoassay system.

Results The induced protein (IP)10 level in the cells in both the MADSAM and MMN groups was markedly increased in comparison with the typical CIDP, ALS and HC groups. The other cytokines, including granulocyte colony-stimulating factor, vascular endothelial growth factor (VEGF) and interleukin-7, were also significantly upregulated in the MADSAM group. The increase of IP-10 produced by PnMECs was correlated with the presence of conduction block in both the MADSAM and MMN groups.

Conclusion The autocrine secretion of IP-10 induced by patient sera in PnMECs was markedly upregulated in both the MADSAM and MMN groups. The overproduction of IP-10 by PnMECs leads to the focal breakdown of the BNB and may help to mediate the transfer of pathogenic T cells across the BNB, thereby resulting in the appearance of conduction block in electrophysiological studies of patients with MADSAM and MMN.

sensory and motor neuropathy [MADSAM]).¹ Patients with t-CIDP show relatively uniform manifestations characterised by symmetric motor-dominant polyneuropathy.² In contrast, MADSAM neuropathy is clinically characterised by multiple mononeuropathy, which is involved in multifocal nerve conduction block (CB) on nerve conduction studies.^{3–4} The difference in these clinical phenotypes suggests the possibility of a different immunopathogenesis.⁵

Multifocal motor neuropathy (MMN) is an acquired motor neuropathy that is characterised electrophysiologically by the presence of multifocal persistent CBs on motor nerves but not on sensory nerves.⁶ The disease is considered to have an immunological basis, mostly based on clinical improvement after immune therapy including high-dose intravenous immunoglobulin (IVIg) treatment. Basically, MADSAM can be distinguished from MMN by the presence of overt sensory involvement and responsiveness to steroid therapy, although MMN and MADSAM share a similar electrophysiological feature, the presence of CB.^{6,7}

The breakdown of the blood–nerve barrier (BNB) is an important step in the pathogenesis of CIDP and MMN.^{8,9} Using our established human in vitro BNB model, we previously demonstrated that the pattern and severity of the BNB breakdown induced by sera differ among t-CIDP, MADSAM and MMN.^{10,11} However, little is known about the importance of the BNB breakdown induced by cellular immunity in patients with CIDP and MMN. In our present study, we compared the levels of 27 cytokines/chemokines produced by the endothelial cells comprising the BNB after exposure to sera obtained from patients with t-CIDP, MADSAM and MMN, and healthy controls (HCs).

INTRODUCTION

Chronic inflammatory demyelinating polyneuropathy (CIDP) is an immune-mediating neuropathy characterised by electrophysiological and pathological evidence of peripheral nerve demyelination. The European Federation of Neurological Societies and the Peripheral Nerve Society (EFNS/PNS) classified CIDP into two clinical subtypes, which show different features: typical CIDP (t-CIDP) and atypical CIDP (eg, multifocal acquired demyelinating

MATERIALS AND METHODS

Sera

Written informed consent was obtained from each participant. As previously described, sera were collected from a total of 22 patients with CIDP (t-CIDP, n=12; MADSAM, n=10) who met the clinical criteria for CIDP based on the 2010 EFNS/PNS guideline.^{1,10} Sera were also obtained from 11 patients with MMN who fulfilled the diagnostic criteria for possible MMN based on the guidelines reported by the EFNS/PNS in 2010,¹² and 9 patients



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with definite amyotrophic lateral sclerosis (ALS) (diagnosed by the El Escorial criteria) were included as disease controls.¹³ Sera from 10 healthy individuals were used as HCs. All serum samples were immediately stored at -80°C until the analysis and were inactivated at 56°C for 30 min immediately before the analysis.

The clinical data of all patients with CIDP/MMN were analysed. The clinical course in all patients with CIDP/MMN showed a stepwise or continuous worsening without treatment. The cerebrospinal fluid (CSF) protein and CSF/serum albumin ratio (Q Albumin) were collected from CSF samples from 25 patients with CIDP. The total Medical Research Council (MRC) scale values in four muscle groups (weaker side of the deltoid, wrist extensor, iliopsoas and tibialis anterior muscles) were collected for clinical evaluation. All 25 patients with CIDP received corticosteroid or IVIg treatment, and all 11 patients with MMN received IVIg treatment. Treatment was considered to be effective when the MRC score improved after treatment. Nerve conduction studies were performed using a standard electromyography machine (Neuropack M1, Nihon Kohden, Tokyo, Japan; Viking 4, Nicolet Biomedical Japan, Tokyo, Japan). Motor nerve studies of the median, ulnar and tibial nerves, including F wave analyses, were performed. A partial motor CB was defined as a $>50\%$ reduction in the compound muscle action potentials (CMAP) between the stimulus sites in accordance with the EFNS/PNS guideline.^{1 10 12}

Cell culture and treatment

Immortalised human BNB-comprising endothelial cells, named 'FH-BNB cells', were described previously.¹⁴ The cells were cultured in a medium containing 10% serum from the patients or HCs in a CO_2 incubator at 37°C . The total proteins were extracted from the cells 1 day after the conditioned media were completely removed.

Multiplexed fluorescent bead-based immunoassays

The concentrations of 27 cytokines/chemokines in equal amounts of protein ($22.5\ \mu\text{g}$) from each sample obtained from cells after exposure to sera were analysed using Bio-Plex Human 27-Plex Cytokine Panels and a Bio-Plex Cytokine Reagent Kit (Bio-Rad, Hercules, California) in accordance with the manufacturer's instructions. The levels of interleukin (IL)-1 β , IL-2, IL-4, IL-5, IL-6, IL-7, CXCL8/IL-8, IL-9, IL-10, IL-12 (p70), IL-13, IL-15, IL-17, IL-1 receptor antagonist (IL-1ra), VEGF, granulocyte colony-stimulating factor (G-CSF), platelet-derived growth factor (PDGF)-11, fibroblast growth factor (FGF)-2, granulocyte-macrophage colony-stimulating factor (GM-CSF), tumour necrosis factor (TNF)- α , interferon (IFN)- γ , CCL2/macrophage chemoattractant protein-1 (MCP-1), CCL3/macrophage inflammatory protein (MIP)-1 α , CCL4/MIP-1 β , and CCL5/regulated on activation, CCL11/eotaxin, CXCL10/IFN-inducible protein of 10 kDa (IP-10) and normal T cell expressed and secreted (RANTES) were measured in this study. The concentrations of cytokines/chemokines were calculated by referencing a standard curve for each set of molecules derived from the various concentrations of the standard assays. The approximate lower limits of quantification (assay sensitivity) were as follows: IFN- γ , 6.4 pg/mL; IL-1 β , 0.6 pg/mL; IL-1ra, 5.5 pg/mL; IL-2, 1.6 pg/mL; IL-4, 0.7 pg/mL; IL-5, 0.6 pg/mL; IL-6, 2.6 pg/mL; IL-7, 1.1 pg/mL; IL-8, 1.0 pg/mL; IL-9, 2.5 pg/mL; IL-10, 0.3 pg/mL; IL-12, 3.5 pg/mL; IL-13, 0.7 pg/mL; IL-15, 2.4 pg/mL; IL-17, 3.3 pg/mL; IL-1ra, 5.5 pg/mL; VEGF, 3.1 pg/mL; G-CSF, 1.7 pg/mL; PDGF-BB, 2.9 pg/mL; FGF-2, 1.9 pg/mL; GM-CSF, 2.2 pg/mL; TNF- α , 6.0 pg/mL; IFN- γ , 6.4 pg/mL; MCP-1, 1.1 pg/mL; MIP-1 α , 1.6

pg/mL; CCL4/MIP-1 β , 2.4 pg/mL; eotaxin, 2.5 pg/mL; IP-10, 6.1 pg/mL; and RANTES, 1.8 pg/mL (Bio-Rad). The specificity of this assay has not been reported; however, it is considered to be almost the same as the double-sandwich ELISA.

Immunocytochemistry

FH-BNB cells were cultured in a conditioned medium containing 10% serum from patients (2 MADSAM, 2 t-CIDP, 2 MMN and 2 ALS)/healthy volunteers (2 individuals) or 5 U/mL IFN- γ (Thermo Fisher Scientific; Waltham, Massachusetts, USA). After 24 hours of incubation, cells were fixed with 4% paraformaldehyde, washed, then permeabilised with 0.3% Triton X. After blocking overnight in 5% goat serum in phosphate buffered saline (PBS), primary antibodies (IP-10 from R&D; Minneapolis, Minnesota, USA) were added (2 hours, room temperature), then mouse-specific Alexa Fluor secondary antibodies (1:400). Images were captured on an LSM 510 META confocal microscope.

Data analysis

Statistical analyses were performed using the Prism V7 software program (GraphPad Software). A one-way analysis of variance (ANOVA) between individual groups was performed using Bonferroni correction or Kruskal-Wallis test (two sides) of 27 cytokine and chemokines. All values are expressed as mean \pm SEM. P values of <0.001 (Bonferroni correction) were considered to indicate statistical significance. The unpaired Mann-Whitney U (single comparison) or one-way ANOVA (multiple comparisons) was used (two sides) for analyses of the correlation between clinical/electrophysiological parameters and the IP-10. Pearson's correlation coefficients were also used to test associations. Fisher's exact probability test was used to assess differences between groups. Two-sided p values of <0.05 were considered to indicate statistical significance.

RESULTS

Clinical characteristics

The clinical profiles of patients with t-CIDP, MADSAM and MMN are described in [table 1](#). The mean total MRC score in the four muscle groups in patients with t-CIDP was significantly lower in comparison with patients with MADSAM. When sera were collected, none of the CIDP and 1 of 11 patients with MMN were receiving IVIg treatment. The mean CSF protein concentrations in patients with t-CIDP were higher than those in patients with MADSAM and MMN. The mean Q Albumin level in the t-CIDP group was higher than that in the MADSAM group. A nerve conduction study of the median nerve revealed that the patients with t-CIDP had a more prolonged average motor nerve distal latency and a greater slowing of the mean motor nerve conduction than patients with MADSAM and MMN. CB was more frequently observed in patients with MADSAM than in patients with t-CIDP or MMN. The CMAPs of the groups did not differ to a statistically significant extent.

The overproduction of IP-10 by FH-BNB cells in the MADSAM and MMN groups

The concentrations of 27 cytokines/chemokines were analysed in protein samples obtained from the FH-BNB cells after exposure to sera from patients with t-CIDP (t-CIDP group), MADSAM (MADSAM group) and MMN (MMN group) and healthy individuals (HC group). Eight of the 27 cytokine/chemokines (IL-2, IL-10, IL-15, IL-17, GM-CSF, MCP-1, MIP-1b and IFN- γ) were not detected in this assay. [Table 1](#) shows the profiles of the other 19 cytokines/chemokines that were detected in the FH-BNB

Table 1 Clinical profiles of t-CIDP, MADSAM and MMN

	t-CIDP (n=12)	MADSAM (n=10)	MMN (n=11)	ALS (n=9)	HC (n=12)	P values
Clinical profile						
Age (year)	53 (±25)	56 (±12)	42 (±19)	70 (±10)	35 (±6)	NS
Male:Female	9:3	8:2	7:4	5:4	6:6	NS
Disease duration† (year)	4.2 (±6.9)	4.4 (±5.5)	4.2 (±5.0)	1.9 (±1.6)	NA	NS
Total MRC score‡	15.7 (±2.6)	18.3 (±1.5)	ND	NA	NA	<0.05*
Treatment when collecting sera§	0% (0/12)	0% (0/10)	9% (1/11)	NA	NA	NS
Response to treatment¶	67% (8/12)	80% (8/10)	82% (9/11)	NA	NA	NS
Response to IVIg	44% (4/9)	88% (7/8)	82% (9/11)	NA	NA	NS
CSF protein (mg/dL)	95.3 (±34.7)	55.8 (±31.4)	37.0 (±18.2)	NA	NA	<0.05*, <0.01**
CSF Q Albumin	0.028 (±0.047)	0.009 (±0.006)	ND	NA	NA	<0.01*
Motor conduction study (median nerve)						
Distal latency (ms)	9.0 (±5.9)	4.7 (±1.0)	3.8 (±1.0)	NA	NA	<0.05*, <0.01**
CV (m/s)	30.2 (±12.6)	42.3 (±8.3)	51.5 (±13.4)	NA	NA	<0.001**
CMAP (mV)	4.6 (±3.4)	6.1 (±3.5)	4.8 (±3.3)	NA	NA	NS
CB††	58% (7/12)	100% (10/10)	45% (5/11)	NA	NA	<0.05*, <0.01***

*t-CIDP vs MADSAM, **t-CIDP vs MMN, ***MADSAM vs MMN. Data are expressed as mean (±SD) or % (number).

†Disease duration when collecting the samples.

‡Total MRC score (deltoid + wrist extensor + iliopsoas + tibialis anterior).

§IVIg or steroid treatment when collecting the samples.

¶Response to treatment (IVIg or steroid).

††Conduction blocks (median nerve, ulnar nerve, radial nerve or tibial nerve).

ALS, amyotrophic lateral sclerosis; CB, conduction block; CMAP, compound muscle action potential; CSF, cerebrospinal fluid; CV, conduction velocity; HC, healthy control; IVIg, intravenous immunoglobulin; MADSAM, multifocal acquired demyelinating sensory and motor neuropathy; MMN, multifocal motor neuropathy; MRC, Medical Research Council; NA, not applicable; ND, not determined; t-CIDP, typical chronic inflammatory demyelinating polyradiculoneuropathy.

cells. A comparison of the levels of 15 cytokines/chemokines revealed no statistically significant differences between the groups.

The levels of four proteins (IL-7, VEGF, G-CSF and IP-10) produced by FH-BNB cells in the MADSAM group were significantly increased in comparison with the other groups (table 2). The IP-10 protein level in both the MADSAM and MMN groups was significantly increased in comparison with that in both the t-CIDP and HC groups (table 2). Figure 1 shows that the levels of four cytokines/chemokines in MADSAM and/or MMN group were significantly increased in comparison with the other groups (figure 1A–D). Out of these four cytokines/chemokines, the amount of IP-10 produced by FH-BNB cells (range, 96.3–225 pg/mL) was markedly increased in all of the samples from the MADSAM group (figure 1D), and was above the upper levels recorded in the HC (51.0 pg/mL), ALS (63.2 pg/mL) and t-CIDP (60.3 pg/mL) groups. In contrast, the level of IP-10 produced by FH-BNB cells was also significantly increased in the MMN group (range, 50–165 pg/mL); however, in a few MMN group samples, the levels of this chemokine was below the upper levels observed in the HC (51.0 pg/mL), ALS (63.2 pg/mL) and t-CIDP (60.3 pg/mL) groups.

Overall, the values of the other three cytokines/chemokines in the MADSAM group (IL-7 (range, 10.2–20.7 pg/mL), VEGF (range, 111–187 pg/mL) and G-CSF (range, 474–1453 pg/mL)) were also significantly increased in comparison with the HC, ALS and t-CIDP groups (figure 1A–C); however, the levels of these cytokines remained within the normal ranges in most of the samples in the MADSAM group.

Immunocytochemical analyses showed that the IP-10 expression in FH-BNB cells was increased after exposure to sera from patients with MADSAM and MMN, in comparison with after incubation with sera from patients with t-CIDP and ALS and HCs (figure 2).

Correlations between the clinical findings and the IP-10 levels in patients with CIDP and MMN

We next examined the correlations between the clinical findings and the IP-10 levels in FH-BNBs after exposure to sera from patients with CIDP/MMN. A higher IP-10 level in BNB was associated with the presence of CB in patients with CIDP or MMN (figure 3). The IP-10 level was not significantly associated with the disease duration from onset, MRC score, response to immunotherapy (including IVIg), the amount of CSF protein, the Q Albumin or the distal latency/velocity/CMAP amplitude in the median nerve.

DISCUSSION

Previous studies have demonstrated the levels of various cytokines/chemokines, including TNF- α , IL-8, IL-17, CXCL-9, CCL-3 and IP-10, were increased in the serum/CSF of patients with t-CIDP. These studies focused on the key roles of these cytokines/chemokines in the pathogenesis of CIDP.^{15–19} In contrast, there have been few studies on the cytokine/chemokine profiles in patients with MMN; these studies have shown that the serum levels of IL-1 Ra, IL-2, G-CSF and TNF- α in patients with MMN were higher than those in the controls.^{20–22} However, when serum/CSF sample from patients was analysed, it was difficult to discern whether these cytokines/chemokines were increased as important pathogenic molecules or whether they were merely non-specifically upregulated as a byproduct of inflammation. In the present study, we therefore analysed the cytokine/chemokine profiles produced by FH-BNB cells as a result of the cellular response after exposure to sera from patients with t-CIDP, MADSAM, MMN and ALS and HCs in order to identify the pathogenic cytokines/chemokines responsible for the malfunction of the BNB. We observed that the higher levels of IL-7, VEGF, G-CSF and IP-10 after exposure to sera from patients

Table 2 Summary of cytokine and chemokine levels secreted by PnMECs after exposure to a patient's sera

	HC (n=12)	ALS (n=9)	t-CIDP (n=12)	MADSAM (n=10)	MMN (n=11)	P values (Bonferroni correction)
	Mean (SD)	Mean (SD)	Mean (SD)	Mean (SD)	Mean (SD)	
IL-7	10.9 (1.2)	10.7 (1.2)	10.0 (1.8)	16.2 (2.9)	13.2 (5.4)	<0.001 (MADSAM vs t-CIDP)
VEGF	119 (14)	116 (15)	103 (29)	178 (41)	133 (38)	<0.001 (MADSAM vs HC, t-CIDP, MMN)
G-CSF	491 (91)	511 (90)	458 (106)	778 (261)	559 (171)	<0.001 (MADSAM vs ALS)
IP-10	44.2 (4.6)	51.7 (6.5)	48.7 (5.8)	149 (34)	92.1 (41.1)	<0.001 (MADSAM vs HC, ALS, t-CIDP, MMN) <0.001 (MMN vs HC, t-CIDP)
IL-4	0.41 (0.44)	0.99 (0.41)	1.12 (0.53)	0.41 (1.28)	1.27 (1.73)	NS
IL-9	3.88 (2.04)	4.74 (1.39)	3.17 (1.76)	6.89 (2.80)	5.72 (2.36)	NS
IL-12	0 (0)	0.69 (2.09)	2.42 (3.02)	4.39 (3.60)	4.45 (3.93)	NS
PDGF-BB	86.4 (20.0)	82.4 (38.5)	133 (43)	150 (71)	168 (108)	NS
TNF- α	20.7 (71.8)	87.5 (118)	133 (125)	68.1 (144)	130 (268)	NS
IL-1 β	0	0	0	0.32 (0.66)	0.29 (0.66)	NS
IL-5	0.43 (0.63)	1.07 (0.62)	0.78 (0.70)	1.15 (0.90)	1.05 (1.17)	NS
IL-6	0 (0)	0.17 (0.52)	0.83 (1.13)	0.46 (0.75)	0.79 (1.43)	NS
IL-8	4.85 (1.24)	6.70 (1.56)	6.43 (2.15)	5.98 (3.20)	7.43 (5.17)	NS
IL-13	1.88 (0.21)	2.15 (0.30)	2.15 (0.37)	2.34 (0.48)	2.18 (0.73)	NS
IL-1ra	0 (0)	1.89 (5.68)	3.73 (8.87)	12.8 (40.4)	10.7 (20.7)	NS
FGF-2	243 (56)	253 (70)	256 (39)	212 (44)	300 (233)	NS
Eotaxin	3.65 (1.73)	5.23 (1.87)	3.96 (1.85)	7.06 (4.49)	4.35 (3.33)	NS
RANTES	272 (82)	249 (93)	228 (136)	207 (121)	249 (110)	NS
MIP-1a	0 (0)	0.06 (0.18)	0.06 (0.14)	0.30 (0.95)	0.22 (0.56)	NS

Undetermined cytokines: IL-2, IL-10, IL-15, IL-17, GM-CSF, MCP-1, MIP-1b, IFN- γ .

ALS, amyotrophic lateral sclerosis; FGF, fibroblast growth factor; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; HC, healthy controls; IFN- γ , interferon- γ ; IL, interleukin; IL-1ra, IL-1 receptor antagonist; IP-10, inducible protein of 10 kDa; MADSAM, multifocal acquired demyelinating sensory and motor neuropathy; MCP-1, macrophage chemoattractant protein-1; MIP, macrophage inflammatory protein; MMN, multifocal motor neuropathy; PDGF, platelet-derived growth factor; PnMECS, peripheral nerve microvascular endothelial cells; RANTES, CCL4/MIP-1 β and CCL5/regulated on activation, normal T cell expressed and secreted; t-CIDP, typical chronic inflammatory demyelinating polyneuropathy; TNF- α , tumour necrosis factor- α ; VEGF, vascular endothelial growth factor

with MADSAM were significantly higher than after exposure to serum from patients with t-CIDP and ALS and HCs. Among these cytokines/chemokines, we identified that the level of IP-10 produced by the cells was markedly increased after incubation with sera from patients with MADSAM and MMN, suggesting that IP-10 may contribute to the pathogenesis of MADSAM and MMN. Furthermore, we found a significant correlation between the IP-10 produced by the cells after exposure to sera and the presence of CB in nerve conduction studies in both patients with CIDP and MMN.

Some reports revealed that the concentration of IP-10 was increased in the CSF samples from patients with t-CIDP.^{15–17} CXCR3, a chemokine receptor for IP-10, is known as a specific chemokine receptor for type 1 helper T cells (Th1 cells).^{23–25} Importantly, in sural nerve biopsy samples from patients with t-CIDP, the greatest number of CXCR3-positive T lymphocyte in the invading T cells was observed,²⁶ suggesting that endothelial IP-10 can facilitate the trafficking of activated Th1 cells expressing the CXCR3 receptor across the BNB.²⁶ Our present results suggest that the humoral factors present in the MADSAM and MMN sera markedly increase the production of IP-10 by FH-BNB cells via an autocrine mechanism, and that their transfer of CXCR3-positive T lymphocyte to lesion sites across the BNB can be mediated.

MADSAM and MMN share a common electrophysiological feature: multiple CBs in the peripheral nerve trunk.^{6–7} The focal breakdown of the BNB and the passage of inflammatory cells across the BNB at the site of CB(s) were considered to be associated with the pathogenesis of MADSAM and

MMN.^{5,27} The overproduction of IP-10 by FH-BNB cells may induce the migration of T cells across the BNB, and eventually lead to the development of inflammatory CB in MADSAM and MMN. On the other hand, recent studies have emphasised the importance of functional CB in MMN.^{28–30} The largest series of motor nerve biopsies from the site of CB in MMN showed multifocal degeneration and a loss of nerve fibre without inflammation or lymphocyte infiltration.³¹ Electrophysiological studies of patients with MMN revealed the presence of axonal membrane hyperpolarisation due to the overactivation of Na⁺/K⁺ATPase at the node of Ranvier around the sites of CB, resulting in a decrease in the persistent Na⁺ current.^{29,30} The association between inflammatory and functional CBs is still elusive; however, we hypothesise that focal inflammation and oedema induced by autocrine IP-10 secretion around BNB may trigger functional CB in MMN.

IgG4 antibodies against proteins of the paranodal junction were recently found in 2–10% of patients with CIDP who presented the t-CIDP and distal acquired demyelinating symmetric polyneuropathy (DADS) phenotypes, and the concept of autoimmune nodoparanodopathy is now proposed.^{32–33} NF155 on the terminal myelin loop and contactin 1 on the axonal side are major targets of these antibodies.^{34–35} Some clinical features, including a poor response to IVIg, are different from those in CIDP.³⁵ Myelin loop detachment and increased periaxonal space are observed at the site of paranode in the sural nerve biopsy, suggesting that nerve injury due to the dismantling of the paranode, which is induced by antibodies, may be the primary pathogenic

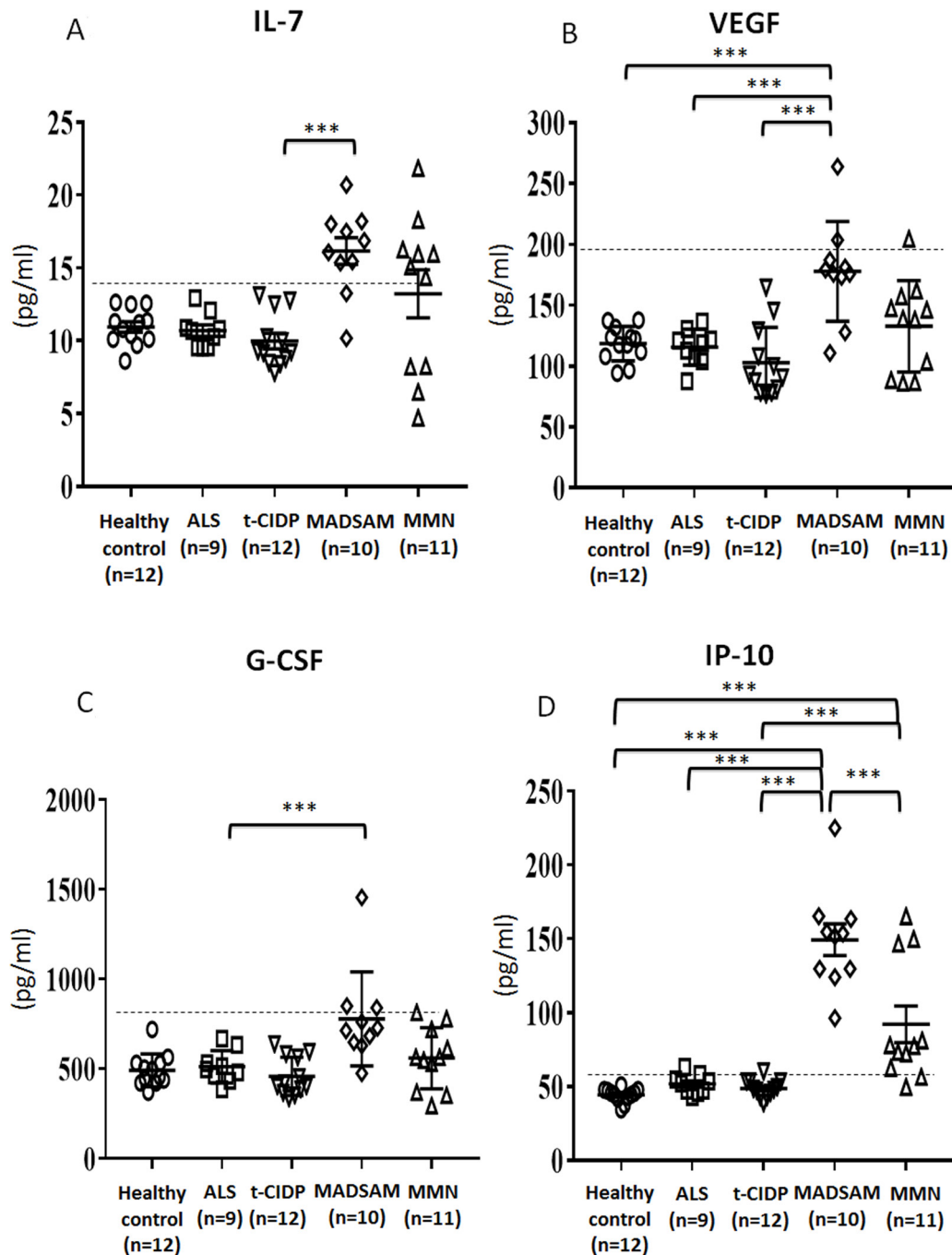


Figure 1 The upregulation of cytokines/chemokines in the MADSAM group. The concentrations of cytokines/chemokines including IL-7 (A), VEGF (B), G-CSF (C) and IP-10 (D) in protein samples obtained from FH-BNB cells after exposure to sera from patients with t-CIDP (t-CIDP group), MADSAM (MADSAM group), MMN (MMN group) and ALS (ALS group) and healthy volunteers (healthy control group). These four cytokines/chemokines were significantly increased in the MADSAM group compared with the t-CIDP, ALS and healthy control groups ($***p < 0.001$, one-way analysis of variance followed by Bonferroni multiple comparison test). Of these four cytokines/chemokines, the IP-10 levels produced by FH-BNB cells in the MADSAM and MMN groups were markedly increased in comparison with the ALS and healthy control groups. The dashed lines indicate the upper or lower limits of the cytokine levels in the healthy control group (mean \pm 3 SD). Healthy control, cells exposed to sera from healthy controls; t-CIDP, cells exposed to sera from patients with t-CIDP; MADSAM, cells exposed to sera from patients with MADSAM; MMN, cells exposed to sera from patients with MMN; ALS, cells exposed to sera from patients with ALS; healthy control, cells exposed to sera from healthy controls. ALS, amyotrophic lateral sclerosis; FH-BNB cells, immortalized human BNB-comprising endothelial cells; G-CSF, granulocyte colony-stimulating factor; VEGF, vascular endothelial growth factor; IL-7, interleukin-7; IP-10, inducible protein of 10 kDa; MADSAM, multifocal acquired demyelinating sensory and motor neuropathy; MMN, multifocal motor neuropathy; t-CIDP, typical chronic inflammatory demyelinating polyneuropathy.

mechanism of this disease, rather than segmental demyelination and inflammation. In this study, we did not detect NF155 antibodies or contactin-1 antibodies in patients with

CIDP/MMN.³⁶ A further analysis will be needed to clarify the pathological mechanism through which these antibodies affect the BNB function in patients with CIDP.

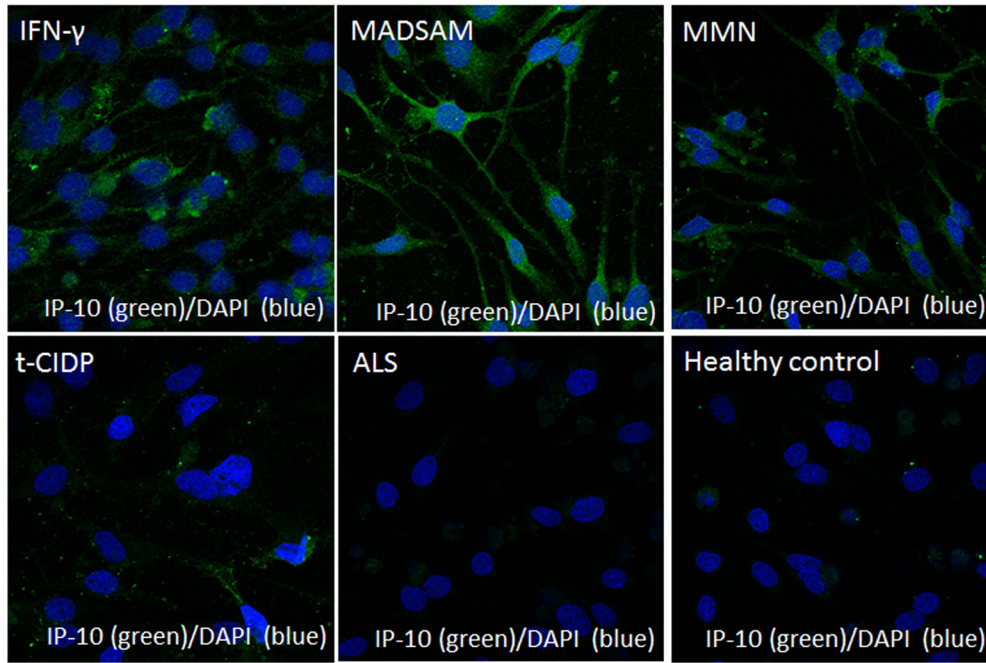


Figure 2 The increase of IP-10 in the MADSAM group using immunohistochemistry. The IP-10 expression in FH-BNB cells was assessed by immunohistochemistry and was found to be increased after exposure to sera from patients with MADSAM and MMN, and incubation with IFN- γ , in comparison with after exposure to sera from patients with t-CIDP and healthy controls. IFN- γ , cells exposed to 5 U/mL IFN- γ ; healthy control, cells exposed to sera from healthy controls; t-CIDP, cells exposed to sera from patients with t-CIDP; MADSAM, cells exposed to sera from patients with MADSAM; MMN, cells exposed to sera from patients with MMN; ALS, cells exposed to sera from patients with ALS. ALS, amyotrophic lateral sclerosis; IFN- γ , interferon- γ ; IP-10, inducible protein of 10 kDa; MADSAM, multifocal acquired demyelinating sensory and motor neuropathy; MMN, multifocal motor neuropathy; FH-BNB cells, immortalized human BNB-comprising endothelial cells; t-CIDP, typical chronic inflammatory demyelinating polyneuropathy; DAPI, 4',6-diamidino-2-phenylindole

The serum VEGF levels are elevated in a host of neuropathies, including polyneuropathy, organomegaly, endocrinopathy, M-protein and skin change (POEMS) syndrome, CIDP, neuropathies associated with monoclonal gammopathy of undetermined significance and Guillain-Barré syndrome.³⁷ The serum VEGF

concentration is clinically useful for monitoring disease activity in POEMS syndrome³⁸; however, the role of serum VEGF in other immune-mediated neuropathies is still unclear. VEGF is a key regulator of barrier permeability in the blood-brain barrier (BBB) and BNB. In this study, the secretion of VEGF secreted by

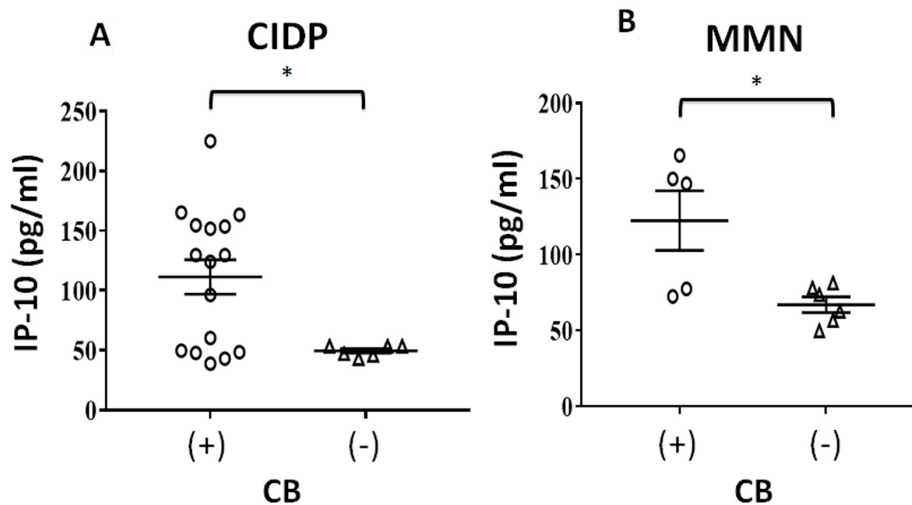


Figure 3 The correlation between IP-10 in FH-BNB cells after sera exposure and the clinical parameters of patients with CIDP or MMN. A nerve conduction study revealed that a higher IP-10 level was highly associated with the presence of conduction blocks in CIDP (A) and MMN (B). CIDP, cells exposed to sera from patients with CIDP, including t-CIDP or MADSAM; MMN, cells exposed to sera from patients with MMN. Statistical significance was assessed by the unpaired Mann-Whitney U (* $p < 0.05$). CB, conduction block; CIDP, chronic inflammatory demyelinating polyneuropathy; FH-BNB cells, immortalised human BNB-comprising endothelial cells; IP-10, inducible protein of 10 kDa; MADSAM, multifocal acquired demyelinating sensory and motor neuropathy; MMN, multifocal motor neuropathy, t-CIDP, typical CIDP.

the BNB in the MADSAM group was significantly increased in comparison with the HC, ALS and t-CIDP groups. We previously reported the role of VEGF secretion by BNB-endothelial cells on the increase in BNB permeability in patients with MMN,¹¹ although the present study did not show any significant differences in the VEGF levels of patients with MMN and controls as a group. Further retrospective and prospective studies will be needed to understand the role of the VEGF and IP-10 produced by BNB-endothelial cells as a clinical biomarker of the disease activity in CIDP and MMN.

In conclusion, our present study showed that the markedly overproduction of IP-10 by the BNB was induced by humoral factors in the sera from MADSAM and MMN. Our data suggest the potential application of neutralising anti-IP-10 antibodies in the treatment of MADSAM and MMN. Further investigations using the dynamic flow-based in vitro BNB model will be required to clarify the association between the IP-10 produced by the BNB and the migration of leucocytes, and to better understand the pathogenesis of MADSAM and MMN.

Contributors FS and TK were responsible for conception and design of the study. FS performed the experiments, analysed and interpreted the data, and wrote the manuscript. MO, HN, YS, TM, RS and YT performed the experiments and analysed the data. FS, MO, SS, MB, SM, AM and NM were responsible for collecting sample and clinical data from patients. SK, RK and TK edited the manuscript.

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Competing interests None declared.

Patient consent Not required.

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