Intravenous immunoglobulin preparations attenuate lysolecithin-induced peripheral demyelination in mice and comprise anti-large myelin protein zero antibody

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Abstract: Intravenous immunoglobulin (IVIg) has been used to treat inflammatory demyelinating diseases such as chronic inflammatory demyelinating polyneuropathy, Guillain–Barré syndrome, and multifocal motor neuropathy. Despite studies demonstrating the clinical effectiveness of IVIg, the mechanisms underlying its effects remain to be elucidated in detail. Herein, we examined the effects of IVIg on lysolecithin-induced demyelination of the sciatic nerve in a mouse model. Mice —administered with IVIg 1 and 3 days post-injection (dpi) of lysolecithin —exhibited a significantly decreased demyelination area at 7 dpi. Immunoblotting analysis using two different preparations revealed that IVIg reacted with a 36-kDa membrane glycoprotein in the sciatic nerve. Subsequent analyses of peptide absorption identified the protein as a myelin protein in the peripheral nervous system (PNS) known as large myelin protein zero (L-MPZ). Moreover, injected IVIg penetrated the demyelinating lesion, leading to deposition on L-MPZ in the myelin debris. These results indicate that IVIg may modulate PNS demyelination, possibly by binding to L-MPZ on myelin debris.

Keywords: intravenous immunoglobulin (IVIg), demyelination, peripheral nerve, neuropathy, lysophosphatidylcholine (lysolecithin), large myelin protein zero (L-MPZ)

1. Introduction

The myelin structure in the nervous system enables rapid propagation of nerve impulses along axons via saltatory conduction and the localization of axonal channels in specific regions.[1)–4)] Patients with inflammatory demyelinating diseases of the peripheral nervous system (PNS) exhibit various clinical symptoms including symmetric muscle weakness with or without sensory disturbances (*e.g.*, dysesthesia).[5),6)] Given its immunomodulatory and anti-inflammatory effects, high-dose intravenous immunoglobulin (IVIg) therapy has become the first-line treatment for inflammatory demyelinating diseases, including chronic inflammatory demyelinating polyneuropathy (CIDP), Guillain–Barré syndrome (GBS), and multifocal motor neuropathy (MMN).[7)] In addition to its demonstrated effects in human patients, IVIg treatment has been shown to attenuate aberrant clinical scores and improve conduction velocity in an experimental rodent model of autoimmune neuropathy.[8),9)]

The IVIg preparations produced by pharmaceutical companies represent a mixture of IgG from

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Non-standard abbreviation list: PNS: peripheral nervous system; IVIg: intravenous immunoglobulin; CIDP: chronic inflammatory demyelinating polyneuropathy; GBS: Guillain–Barré syndrome; MMN: multifocal motor neuropathy; dpi: day post-injection; P0/MPZ: myelin protein zero; L-MPZ: large myelin protein zero; MBP: myelin basic protein; DAPI: 4′,6-diamidino-2-phenylindole; NF200: neurofilament 200; PNGase F: peptide N-glycosidase F; SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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several thousand healthy donors and are manufactured by purifying and processing human IgG. As IVIg therapy is widely utilized in the treatment of inflammatory diseases,[10),11)] its anti-inflammatory effects in patients with demyelinating conditions can be expected; however, the therapeutic mechanisms by which IVIg counteracts demyelination have not been completely elucidated.

Lysophosphatidylcholine (lysolecithin) is commonly used to generate animal models of demyelination. Direct local injection of lysolecithin into the mouse sciatic nerve results in demyelination and inflammation at the injection site. Lysolecithin-induced demyelination transiently peaks 7 days post-injection (dpi), following which gradual remyelination and nerve recovery can be observed within approximately 3 weeks.[12)] In this study, we used lysolecithin to prepare a focal model of demyelinating neuropathy for investigating the effects of IVIg preparations.

Recent studies have identified a novel isoform of myelin protein zero (P0/MPZ) known as large myelin protein zero (L-MPZ), which plays key roles in the PNS.[13)] Compared with myelin P0, L-MPZ contains an additional 63 amino acids at the C-terminus, which are introduced via a translational read-through mechanism.[13)] Anti-L-MPZ antibodies are often detected in the serum of patients with CIDP,[13)] although the roles of these antibodies remain unknown.

In this study, to determine the putative mechanism of IVIg preparations on the demyelinating diseases, we investigated the effects of IVIg on the size of the sciatic nerve lesion in an experimental rodent model of demyelination induced using lysolecithin. In addition to its demonstrated effects on lesion size, our mechanistic results indicated that IVIg comprised an antibody against the 36-kDa L-MPZ in the PNS and co-deposited in the demyelinated lesion.

2. Materials and methods

2.1. Materials. The IVIg preparations used in the present study included Kenketu Glovenin®-I for intravenous injection (Glovenin®; lot numbers: N113FAN, N281FAN, and N433FAN, Nihon Pharmaceutical (Takeda Pharmaceutical Company Limited), Tokyo, Japan) and the sulfonated Kenketsu Venilon®-I IVIg preparation (Venilon®; lot number: SSV788C; KM Biologics, Kumamoto, Japan), both of which were gifted by the respective companies. Most of the experiments were carried out using Glovenin® (lot number: N281FAN). The other IVIg preparations were used for Western blot analysis to confirm that IVIg binds to the 36-kDa protein as the primary antibody.

Other reagents and chemicals of the highest purity were obtained from Fujifilm Wako Pure Chemical (Osaka, Japan), Sigma-Aldrich (St. Louis, MO), Nacalai Tesque (Kyoto, Japan), and Kanto Chemical (Tokyo, Japan).

2.2. Animals. Young adult male ICR mice (9- to 10-week-old, CLEA Japan, Tokyo, Japan), male Wistar rats (8-week-old, from Japan SLC, Hamamatsu, Japan), and male Crl: CD (SD) rats (15-week-old, Charles River Laboratories Japan, Kanagawa, Japan) were used in this study. All animals were maintained under specific pathogen-free conditions and were allowed free access to food and water. Animals were housed under a 12-h light/dark cycle at room temperature ($23 \pm 1\,^{\circ}C$) with constant humidity ($55 \pm 5\%$). All experimental procedures involving animals were approved by the Institutional Animal Use Committee at the Tokyo University of Pharmacy and Life Sciences (approval reference numbers: P14-14, P17-72, P18-80, P19-50, and P20-43).

2.3. Lysolecithin-induced demyelination model. To induce demyelination, we injected lysolecithin into the sciatic nerve under anesthesia. L-α-Lysophosphatidylcholine from egg yolk (Sigma-Aldrich) was dissolved to prepare 1% lysolecithin solution in Locke's solution [154 mM NaCl, 5.6 mM KCl, 2 mM $CaCl_2$, and 10 mM HEPES buffer, pH 7.4] containing with 0.05% Fast Green FCF (Sigma-Aldrich) for a coloration. ICR mice were anesthetized via intraperitoneal injection of sodium pentobarbital (50 mg/kg; Kyoritsu Seiyaku, Ibaraki, Japan) or a triple cocktail of medetomidine (0.3 mg/kg; Nippon Zenyaku Kogyo, Fukushima, Japan), midazolam (4.0 mg/kg; Maruishi Pharmaceutical, Osaka, Japan), and butorphanol (5.0 mg/kg; Meiji Animal Health, Kumamoto, Japan). Then, the femoral skin was incised to a length of approximately 10 mm, following which the sciatic nerve was carefully exposed. Thereafter, 1.0 µL of 1% lysolecithin solution was injected into the sciatic nerve at the point of trifurcation into the common peroneal, tibial, and sural branches. The injection was performed using a glass pipette (Warner Instruments, LLC, MA) or a 33G metal hub needle (Hamilton, Reno, NV) connected to a Hamilton microsyringe (700 series, Hamilton) under a stereomicroscope (Carl Zeiss, Oberkochen, Germany). The incision was closed using Aron Alpha jelly glue (Toagosei, Tokyo, Japan).

To evaluate the effect of IVIg on chemically induced demyelination, mice were divided into two experimental groups after lysolecithin-injection: saline-injection (control) and IVIg-injection groups. The IVIg was injected via an intravenous (20 mg/0.4 mL) and an intraperitoneal approach (10 mg/0.2 mL) at 1 and/or 3 dpi of lysolecithin, respectively. Control animals were administered the same volume of saline (Otsuka Pharmaceutical Factory, Tokushima, Japan) using the same procedures. These dosages were determined based on clinical evidence (400 mg/kg of human IgG for consecutive 5 days) to ameliorate muscle weakness in patients with CIDP.[14),15)]

At 7 or 14 dpi, mice were anesthetized as described above, and blood samples were collected from the heart. Then, the sciatic nerves were dissected to a length of approximately 15 mm around the injection site, following which they were immersed in 4% paraformaldehyde in 0.1 M phosphate buffer (PB; pH 7.4) overnight. The nerves were incubated in a series of 10–20% sucrose in 0.01 M phosphate-buffered saline (PBS; pH 7.4) and embedded in Tissue-Tek O.C.T. compound (Sakura Finetek, Tokyo, Japan). Sections (10 µm thickness) were prepared using a cryostat (CM1850; Leica Microsystems Wetzlar, Germany). The sections were placed on a Frontier glass slide (Matsunami, Osaka, Japan), dried at room temperature, and kept at −40 °C until use.

The blood samples were incubated at room temperature (20–24 °C) for 60 min and centrifuged at 1,500 × g at 24 °C for 15 min. The serum was stored at 4 °C until use.

2.4. Semi-quantification of the demyelinated areas. Multiple-fluorescence immunohistochemistry was performed as previously described[13)] with minor modifications. Longitudinal sciatic nerve sections were incubated in ice-cold methanol for 15 min and washed multiple times in PBS. The sections were then incubated with 10% normal goat serum (Fujifilm Wako Pure Chemical) in PBS containing 0.1% Triton X-100 (NGS) for 60 min at room temperature to block non-specific protein binding. Subsequently, the samples were incubated with rat anti-myelin basic protein (MBP; 1:200, a myelin marker), and rabbit anti-neurofilament 200 antibodies (NF200; 1:200, a neuronal marker) at 4 °C overnight. After thorough washing with PBS, the sections were incubated with Alexa Fluor 488-labeled goat anti-rat IgG (1:2,000) and Alexa Fluor 594-labeled goat anti-rabbit IgG (1:2,000) antibodies for 60 min in the dark. Finally, the sections were washed and enclosed using Vectashield HardSet Antifade Mounting Medium with 4′,6-diamidino-2-phenylindole (DAPI; H-1500; Vector Laboratories, Burlingame, CA) for nuclear staining. Information on all antibodies, including those used for Western blotting, is listed in Table 1.

Demyelination was examined in serial preparations of four sections at 100 µm intervals. The sections were automatically imaged in a tile-like fashion using an all-in-one BZ-X710 microscope (Keyence, Osaka, Japan) and a BZ-X analyzer (Keyence). Nerve and demyelinated regions were manually traced. The nerve area was manually outlined for the $NF200^{+}$ region, and the total nerve area (NA) was calculated by summing regional values for the four serial sections. The demyelinated region (MBP^{low} region) inside the nerve area was manually traced using an Axio Imager M1 (Carl Zeiss). The total demyelination area (DA) was calculated by summing regional values for the same four sections. The percentage of the total demyelination area (DA) calculated as a percentage using the following formula [1].

$$\mathrm{DA/NA} \times 100. \quad [1]$$

The procedures were performed by an investigator (A.K.) blinded to the experimental groups.

2.5. Quantification of human IgG levels in mouse serum by enzyme-linked immunosorbent assays (ELISA). The wells of a 96-well plate (Thermo Fisher Scientific) were coated with goat anti-human IgG (Fcγ fragment-specific) antibody (1:10,000; Jackson ImmunoResearch) in 0.1 M carbonate buffer (pH 9.65) overnight, followed blocking non-specific reactions with 2.5% bovine serum albumin (Sigma-Aldrich) at 37 °C. The diluted serum samples (1:50,000) were incubated for 60 min and washed repeatedly with PBS containing 0.05% Tween 20. They were then incubated with biotin-SP-conjugated $F(ab')_2$ fragment donkey anti-human IgG antibodies (1:5,000; Jackson ImmunoResearch). Subsequently, the wells were washed and incubated with HRP-conjugated streptavidin (1:5,000; Dako, Glostrup, Denmark). For visualization, the plate was incubated with *o*-phenylenediamine dihydrochloride (0.4 mg/mL, Sigma-Aldrich) in 0.05 M citrate buffer (pH 5.0) with 0.012% H_2O_2, following which 2.0 M HCl was added to stop the reaction. The absorbance of the reaction mixture at 492 nm was measured at a reference wavelength of 620 nm using a microplate reader (Varioskan Flash, Thermo Fisher Scientific).

Table 1. List of primary and secondary antibodies

Primary antibodies	Conjugations	Host	Companies		Catalog #	Application	Folds
human IgG (H+L)	Alexa Fluor 594	goat	Molecular Probes	Eugene, OR	A-11014	IHC	200
human IgG (Glovenin®)		human	Nihon Pharmaceutical	Tokyo, Japan	N/A	WB	100–500
human IgG (Venilon®)		human	KM Biologics	Kumamoto, Japan	N/A	WB	500
L-MPZ		rabbit	See Ref. 13 (Yamaguchi *et al.*, 2012)			IHC	250
						WB	120,000
MBP (Clone #: 12)		rat	Merck Millipore	Burlington, MA	MAB386	IHC	200
NF200		rabbit	Sigma-Aldrich	St. Louis, MO	N4142	IHC	200

Secondary antibodies	Conjugations	Host	Companies		Catalog #	Application	Folds
human IgG (H+L)	HRP	goat	Jackson ImmunoResearch	West Grove, PA	109-035-088	WB	10,000
rabbit IgG (H+L)	Alexa Fluor 488	goat	Biotium	Hayward, CA	20019-1	IHC	200
rabbit IgG (H+L)	Alexa Fluor 594	goat	Invitrogen	Waltham, MA	A-11012	IHC	2,000
rabbit IgG (H+L)	HRP	goat	Jackson ImmunoResearch	West Grove, PA	111-035-144	WB	10,000
rat IgG (H+L)	Alexa Fluor 488	goat	Molecular Probes	Eugene, OR	A-11006	IHC	200–2,000

L-MPZ: large myelin protein zero, MBP: myelin basic protein, NF200: neurofilament 200, IHC: immunohistochemistry, WB: Western blotting, HRP: horseradish peroxidase.

Serially diluted IVIg preparations were used as standard concentrations of human IgG.

2.6. Detection of human IgG in mouse sciatic nerves. The sections were incubated in methanol for 5 min at −20 °C, following which they were incubated in 10% NGS. Thereafter, the samples were incubated with Alexa Fluor 594-labeled goat anti-human IgG (1:200) antibody for 60 min in the dark. Finally, they were stained with DAPI (1:40,000; Molecular Probes, Eugene, OR) for 5 min and enclosed using Vectashield Vibrance Antifade mounting medium (H-1700; Vector Laboratories). The sections were observed in the aid of florescence microscope Axio Imager M1.

The sections were incubated in ice-cold methanol for 15 min, following which non-specific reaction was blocked with 10% NGS. Thereafter, the samples were incubated rat anti-MBP (1:200) overnight. They were then incubated with Alexa Fluor 488-labeled goat anti-rat IgG antibody (1:200), and also with Alexa Fluor 594-labeled goat anti-human IgG (1:200) antibody to detect IVIg. The sections were stained with DAPI and enclosed as described above. The sections were observed in the aid of confocal microscope FLUOVIEW FV1000-D (Olympus Corporation, Tokyo, Japan). All antibodies used are listed in Table 1.

2.7. Determination of IVIg antigen via Western blotting.

2.7.1. Sample collection and homogenization. Following anesthesia induced using an intraperitoneal injection of an anesthetic cocktail, several organs (brain, spinal cord, muscle, lymph node, sciatic nerve, thyroid, thymus, heart, lung, liver, spleen, pancreas, kidney, adrenal gland, and testis) were removed, snap-frozen in liquid nitrogen, and stored at −80 °C until homogenization. The frozen samples were powdered using a mortar or BioMasher II (Nippi, Tokyo, Japan) under liquid nitrogen freezing, following which they were homogenized using a homogenizer (DIAX 900; Heidolph, Schwabach, Germany) or BioMasher II in homogenization buffer [0.32 M sucrose, 5.0 mM Tris-HCl (pH 7.5), 2.0 mM EGTA, 0.75 µM aprotinin, 1.0 µM leupeptin, 1.0 µM pepstatin A, and 0.4 mM PMSF]. The homogenates were centrifuged at 500 × g for 10 min to remove chromosomal DNA, cell debris, and fibers. The supernatant was carefully collected and stored as whole homogenate fractions at −80 °C. The homoge-

nates were determined protein concentration with TaKaRa BCA Protein Assay Kit (TaKaRa, Shiga, Japan) and were applied to electrophoresis.

2.7.2. Fractionation of the sciatic nerve membrane and cytosol. Rat sciatic nerves were homogenized in homogenization buffer, following which they were centrifuged at 200,000 × g at 4 °C for 30 min (CP80α; Eppendorf Himac Technologies). The supernatants were used for the cytosolic fraction. The precipitates were resuspended in homogenization buffer and homogenized again to obtain a membrane-rich fraction. The resulting fractions were stored at −80 °C until electrophoresis.

2.7.3. Deglycosylation of the sciatic nerve membrane fraction. Deglycosylation of the sciatic nerve membrane fraction was performed using peptide N-glycosidase F (PNGaseF) purified from *Flavobacterium meningosepticum*, as previously described.[13] Briefly, the membrane fractions of the rat sciatic nerve were incubated in 0.5% sodium dodecyl sulfatepolyacrylamide (SDS) and 100 mM of 2-mercaptoethanol at 95 °C for 3 min. Next, 10% Triton X-100 was added to each sample to produce a final concentration of 2%, following which the sample was diluted to a protein concentration of 1 µg/µL using 0.1 M PB and 25 mM EDTA. The mixtures were then incubated with 0.25 U of PNGaseF purified from *Flavobacterium meningosepticum* (Roche Diagnostics, Rotkreuz, Switzerland)/30 µg of protein for 15 h at 30 °C and subjected to electrophoresis.

2.7.4. Electrophoresis and Western blotting. Sodium dodecyl sulfatepolyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting were performed as previously described,[16),17] with minor modifications. Briefly, the protein samples were mixed with sample buffer (62.5 mM Tris-HCl, pH 6.8, 5.0% sucrose, 2.0% SDS, 0.1 M DTT, containing with 0.002% bromophenol blue) and loaded onto 10.5% SDS-PAGE. Each sample was transferred to a PVDF membrane (pore size: 0.45 µm; Merck Millipore, Tokyo, Japan). The membranes were incubated with 5.0% skim milk (Becton, Dickinson and Company, Franklin Lakes, NJ) in 20 mM Tris-HCl (pH 7.5) containing 150 mM NaCl and 0.1% Tween 20 (5.0% skim milk in T-TBS) for 60 min at room temperature to block non-specific reactions. Thereafter, membranes were incubated with IVIg (Glovenin® or Venilon®) as the primary antibody (1:100–500) diluted in blocking buffer or T-TBS for 60 min, washed three times with T-TBS, and incubated with HRP-conjugated anti-human IgG antibodies in T-TBS for 30 min. After washing with T-TBS, immunoreactivity was detected using Amersham ECL (Cytiva, Tokyo, Japan). Immunoreactive signals were captured using an LAS-3000 (Fujifilm, Tokyo, Japan). The size markers were aligned with the positive bands. The analyzed membranes were stained with 0.1% amido black 10B to visualize loaded total protein. All antibodies used are listed in Table 1.

2.8. L-MPZ peptide absorption test. We previously reported that the immunoreactivity of L-MPZ, a 36-kDa myelin protein, can be absorbed using serum IgG obtained from patients with CIDP.[13] To determine the antigen involved in the present study, we examined the peptide absorption of the IVIg preparation using synthetic L-MPZ peptides.

The peptides used for the absorption test were synthesized by GenScript (Piscataway, NJ) as follows: L-MPZ$_{1–30}$ (RLAGRAGDRGLGVESAKGPKVMVIEMELRK), L-MPZ$_{25–63}$ (EMELRKDEQSPELRPAVKSPSRTSLKNALKNMMGLNSDK), L-MPZ$_{25–36}$ (EMELRKDEQSPE), L-MPZ$_{32–43}$ (EQSPELRPAVKS), L-MPZ$_{38–49}$ (RPAVKSPSRTSL), L-MPZ$_{45–56}$ (SRTSLKNALKNM), L-MPZ$_{52–63}$ (ALKNMMGLNSDK), and a non-related (NR) scramble amino acid peptide (GLPGNEGPPGQK). Appropriately diluted IVIg solutions were incubated overnight at 4 °C with/without one of the synthetic peptides in TBS. The mixtures of IVIg and peptide were diluted 10-fold with 0.3% skim milk in T-TBS. Anti-L-MPZ antibody from an immunized rabbit with L-MPZ$_{37–56}$ peptide[13] were also used as a positive control of 36-kDa protein.

Sciatic nerve homogenates were loaded onto SDS-PAGE and transferred to a PVDF membrane. After blocking with 0.3% skim milk, the membranes were incubated with a mixture of IVIg and peptide, following which detection was performed using HRP-labeled anti-human IgG antibody. Membranes were incubated with anti-L-MPZ antibody (1:120,000), followed by HRP-labeled anti-rabbit antibody (1:10,000), to confirm that the 36-kDa protein was L-MPZ. The analyzed membranes were stained with amido black as described above.

2.9. Observation of L-MPZ and human IgG on myelin debris. After incubation in methanol at −20 °C, the sections were blocked non-specific reaction with 10% NGS, and were incubated with rabbit anti-L-MPZ antibody (1:250). The sections were then visualized using Alexa Fluor 488-labeled goat anti-rabbit IgG (1:200) and Alexa Fluor 594-labeled goat anti-human IgG (1:200) antibodies. All

antibodies used are listed in Table 1.

2.10. Statistical analyses. All statistical analyses were performed using Prism 5 software (GraphPad Software, San Diego, CA). Data are expressed as the mean ± standard error (SE). Means were compared using Student's *t*-test, Welch's *t*-test, or the Kruskal-Wallis test, followed by Dunn's multiple comparison test. Differences were considered statistically significant at $P < 0.05$. The details of the statistical analyses performed in the individual studies are provided in the figure legends.

3. Results

3.1. IVIg treatment significantly decreased lesion size in mice with lysolecithin-induced demyelination. Using an experimental mouse model of demyelination, we examined the effect of IVIg preparations on demyelination in the mouse sciatic nerve. As shown in Fig. 1, at the peak stage of demyelination (7 dpi), MBP and NF200 co-immunofluorescence staining of longitudinal cryosections revealed demyelinated regions due to decreases in both MBP and NF200 immunoreactivity. These were distinguished from the surrounding normal-appearing regions at 7 dpi (Fig. 1B). The total area of demyelination in each sciatic nerve sample was calculated as the sum of the demyelinated areas in individual serial cryostat sections (100 µm apart) and was compared between the IVIg-treated and saline-treated (control) groups (Fig. 1C and 1D). The demyelinated areas in the IVIg-treated group were significantly smaller than those in the saline-treated group ($p = 0.025$), whereas the total nerve areas in the sections were similar between the groups (Fig. 1D and 1E). The percentage of the demyelination area was significantly decreased in the IVIg-treated group when compared with that in the control group ($p = 0.039$; Fig. 1F). These data suggest that IVIg treatment significantly mitigated lysolecithin-induced demyelination at 7 dpi.

3.2. IVIg treatment promoted human IgG deposition in demyelinated lesions in mouse peripheral nerves. Quantitative analyses of human IgG levels in the mouse serum at 7 and 14 dpi were performed using ELISA (Fig. 2A). Based on the official pharmaceutical documentation, the half-life of plasma IVIg in healthy humans is approximately 17.7 days.[14] The serum human IgG levels in the IVIg-treated group were 1.23 ± 0.12 and 0.67 ± 0.10 mg/mL at 7 and 14 dpi, respectively (Fig. 2B). Levels in the saline-treated group were undetectable at both time points (Fig. 2B).

The distribution of injected IVIg in the sciatic nerves of lysolecithin-treated mice at 7 dpi was examined via immunohistochemical analyses using an antibody against human IgG (Fig. 3). No positive signals for human IgG were detected in the saline-treated sciatic nerve (Fig. 3A, lysolecithin/saline/7 dpi). In IVIg-treated mice, human IgG immunoreactivities were observed few of weak signals in healthy nerves, whereas that was observed in epineurium. However, the nerves including demyelinated regions were obviously increased the intensity of human IgG immunoreactions (Fig. 3A, lysolecithin/IVIg/7 dpi). To confirm interaction with human IgG and myelin in the sciatic nerve, longitudinal nerve sections of mouse administrated lysolecithin and IVIg (Fig. 3B) were stained with MBP and human IgG. The human IgG-positive signals also co-localized with MBP-immunoreactions. Those were aggregated with round clumps, indicative of myelin debris in demyelinated lesions (Fig. 3C).

3.3. IVIg preparations bound to the 36-kDa protein in the PNS homogenates. As the distribution of human IgG immunoreactivities on the myelin debris in the demyelinated regions, we hypothesized that human IgG in IVIg preparations may bind to myelin proteins in the sciatic nerve. Western blotting was used to examine the binding partner of human IgG in the IVIg preparation. The IVIg interacted with a single 36-kDa in the sciatic nerve homogenates of mice and rats (Fig. 4A). Hence, rat sciatic nerve homogenates were used to identify the protein corresponding to the 36-kDa band in immunoblotting experiments. We then fractionated the whole nerve homogenate into cytosolic and membrane fractions and examined immunoblotting to determine which region of the protein reacted with the IVIg preparation. In the immunoblotting experiments, the 36-kDa band was detected in the whole nerve homogenate and the membrane fraction comprising myelin proteins, but not in the cytosolic fraction, suggesting a membrane protein (Fig. 4B).

Although the immunoblotting experiments described thus far were performed using a single lot of IVIg (Glovenin®, lot number N281FAN), we also aimed to determine whether findings could be generalized to other IVIg preparations. Immunoblotting of sciatic nerve homogenates were performed for three different lots of Glovenin® and an IVIg preparation from another company (Venilon®). All four IVIg preparations reacted with the 36-kDa proteins (Fig. 4C).

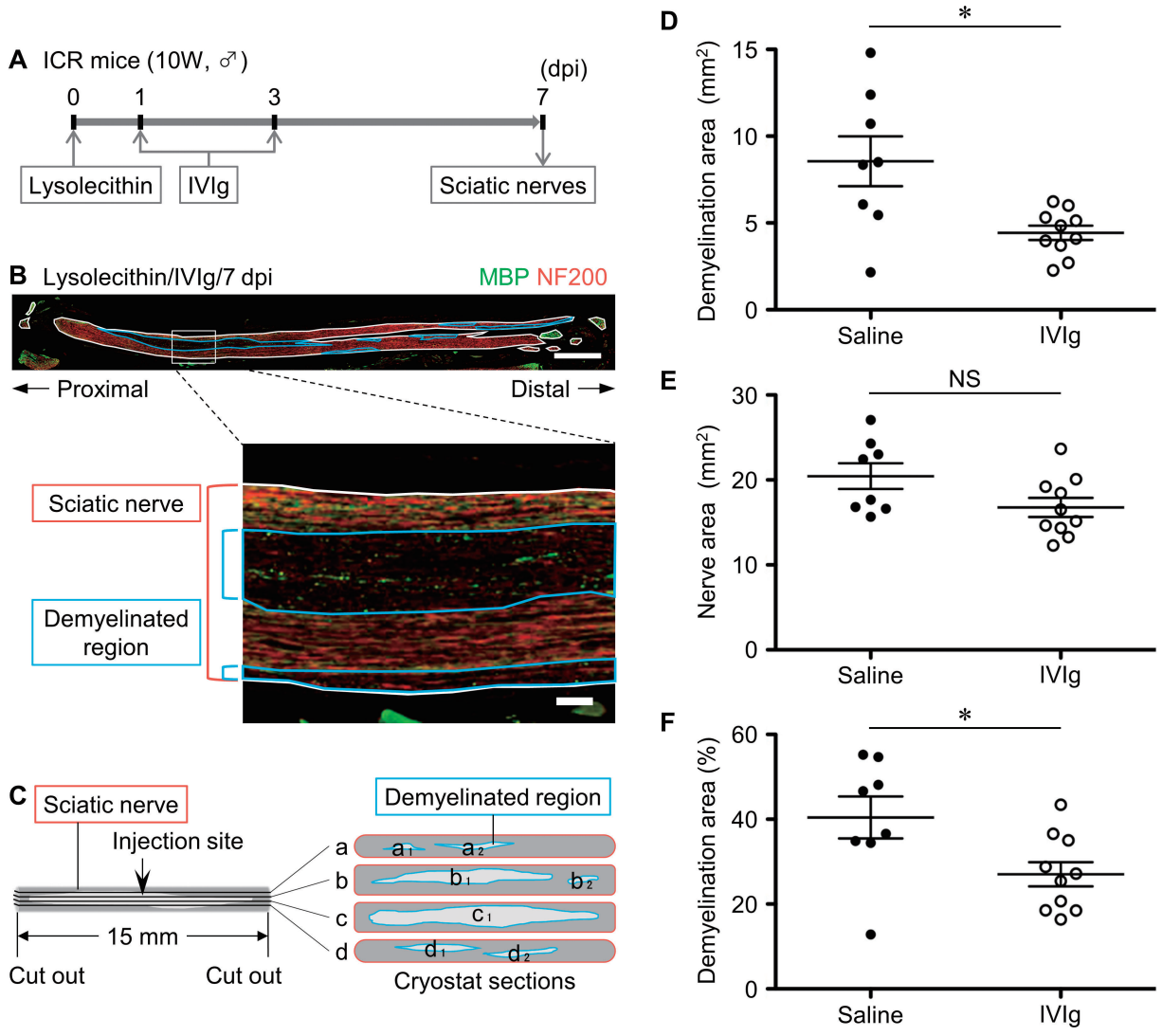

Fig. 1. Intravenous immunoglobulin (IVIg) treatment effectively reduces the size of the demyelinating lesion in mice with lysolecithin-induced demyelination. **(A)** Experimental timeline for lysolecithin-induced demyelination and IVIg treatment. A 1% lysolecithin (1.0 µL) was injected into the bilateral sciatic nerves in 10-week-old male ICR mice. The IVIg was injected with an intravenous and intraperitoneal at 1 and 3 days post-injection (dpi) of lysolecithin, respectively (n = 5). Control animals were administered the same volume of saline using the same procedures (n = 4). The sciatic nerves were removed at 7 dpi for evaluations of the demyelination area. **(B)** The demyelinated region was determined using longitudinal sections of the sciatic nerve subjected to immunofluorescence staining for myelin basic protein (MBP, green) and neurofilament 200 (NF200, red). White lines indicate the outline of the nerve, while blue lines indicate the demyelinated region. **(C)** The total nerve area (NA) was calculated as the sum of each nerve area (red-lined areas; a, b, c, and d) for four serial sections obtained at 100 µm intervals. The total demyelination area (DA) was calculated as the sum of each area within the blue lines (a_1, a_2, b_1, b_2, c_1, d_1, and d_2) in the same four sections. The percentage of the total demyelination area was calculated using the formula DA/NA × 100. **(D)** The demyelinated area was significantly smaller in IVIg-treated mice than in saline-treated mice. **(E)** There was no significant in the total nerve area between IVIg-treated and saline-treated mice. **(F)** The percentage of the total demyelination area was also significantly smaller in the IVIg-treated group than in the saline-treated group. *$P < 0.05$ (Welch's correction in D and unpaired *t*-test with Student's *t*-test in F); NS, not significant (Student's *t*-test in E). Data are presented as the mean ± standard error (SE). Scale bars: 1.0 mm and 100 µm.

Next, the tissue distribution of the 36-kDa protein was evaluated in homogenates of various organs. The 36-kDa protein band was exclusively detected in the sciatic nerve, suggesting PNS reactivity (Fig. 4D and Supplementary Fig. 1A). Moreover, when the membrane fraction in the sciatic

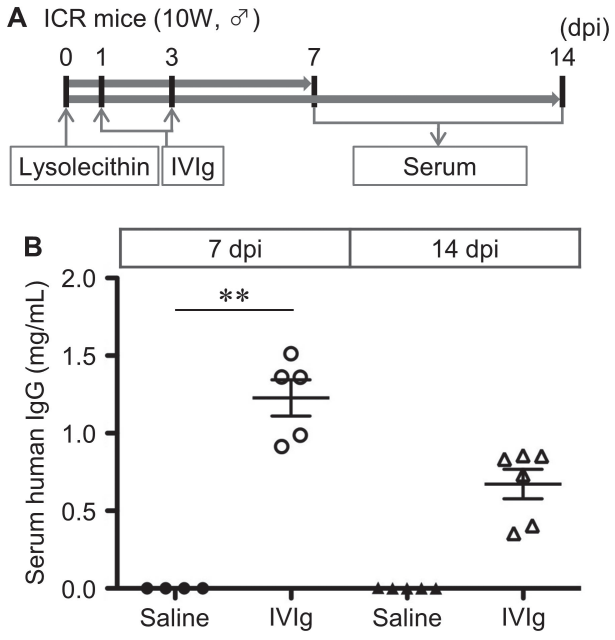

Fig. 2. In IVIg-treated mice, human IgG remained detectable in the mouse serum 14 dpi. **(A)** Experimental timeline for lysolecithin-induced demyelination and IVIg treatment. Lysolecithin (1.0 µL) was bilaterally injected into the sciatic nerves in 10-week-old male ICR mice. The IVIg was injected with an intravenous and intraperitoneal at 1 and 3 dpi, respectively. Control animals were administered the same volume of saline using the same procedures. Then, blood samples were obtained at 7 or 14 dpi to quantify serum levels of human IgG using enzyme-linked immunosorbent assays (ELISA). **(B)** Serum levels of human IgG were significantly higher in IVIg-treated mice (n = 5) than in saline-treated mice (n = 4) at 7 dpi. The same result was observed for IVIg-treated mice (n = 6) and saline-treated mice (n = 5) examined at 14 dpi. **$P < 0.05$ (Kruskal-Wallis test with Dunn's multiple comparison test). Data are presented as the mean ± SE.

nerve was deglycosylated with PNGaseF, the 36-kDa protein that reacted with IVIg shifted to a lower molecular weight (Fig. 4E). These results suggest that IVIg binds to a 36-kDa membrane glycoprotein of the sciatic nerve, likely in the PNS, and that the human IgG antibody in the IVIg preparation recognizes the peptide portion of this protein.

3.4. The 36-kDa PNS-specific protein recognized by human IgG in the IVIg preparation was identified as L-MPZ. We previously reported that the 36-kDa membrane-bound glycoprotein known as L-MPZ, which is found in the PNS, reacts with serum IgG obtained from patients with CIPD.[13] To determine whether the 36-kDa protein identified in the present study was L-MPZ, we examined the peptide absorption of IVIg using 7-different synthetic L-MPZ peptides and a non-related peptide (GLPGNEGPPGQK) (Fig. 5A). The reactivity of human IgG with the 36-kDa protein was markedly reduced upon pretreatment of the IVIg preparation with L-$MPZ_{25–63}$ and L-$MPZ_{45–56}$, but not upon pretreatment with other L-MPZ-specific or non-related control peptides (Fig. 5B and Supplementary Fig. 1B), indicating that the IVIg preparation contained an IgG antibody against L-MPZ. Because the 36-kDa protein was strongly detected in blotting experiments performed with the anti-L-MPZ antibody, the 36-kDa protein bound to IVIg was identified as L-MPZ.

The antigenic site was present within the peptide containing amino acids 45–56 in the L-MPZ-specific domain. L-$MPZ_{45–56}$ recognized by IVIg derived from healthy human donors has also been recognized by antibodies in the serum of patients with CIDP.[13] Furthermore, the amino acid sequence of the L-$MPZ_{45–56}$ region is conserved in humans, rats, and mice (Fig. 5A). Systemic administration of IVIg in the mouse demyelination model promoted the deposition of human IgG at demyelination sites (Fig. 3A), suggesting the involvement of a specific IgG antibody against L-MPZ in IVIg preparations.

3.5. IVIg bound to L-MPZ-positive myelin debris of the demyelinated regions. To confirm reproducibility the binding IVIg to L-MPZ *in vivo*, IVIg immunostaining with human IgG was performed in the mouse sciatic nerve sections. As expected, based on the results of Western blotting, the IVIg-immunoreactions were co-localized with L-MPZ-immunoreactions in the sciatic nerve lesions at 7 dpi (Fig. 6). Some of immunoreactivities were featured round clumps like MBP staining. These observations suggest that human IgG in IVIg is deposited in myelin debris, possibly through L-MPZ.

4. Discussion

The therapeutic effectiveness of IVIg has been clinically demonstrated in patients with inflammatory neuropathies including CIDP, GBS, and MMN.[10),11),18),19)] However, the mechanisms by which IVIg exerts it effects in the nervous system remain poorly understood. To our knowledge, the present study is the first to show that IVIg preparations contain IgG antibodies against a PNS myelin protein, L-MPZ. IVIg treatment significantly reduced the size of the demyelinated areas in the PNS in a mouse model of lysolecithin-induced chemical demyelination. Our results suggest that IVIg penetrates the demyelinating lesion and promotes the deposition of

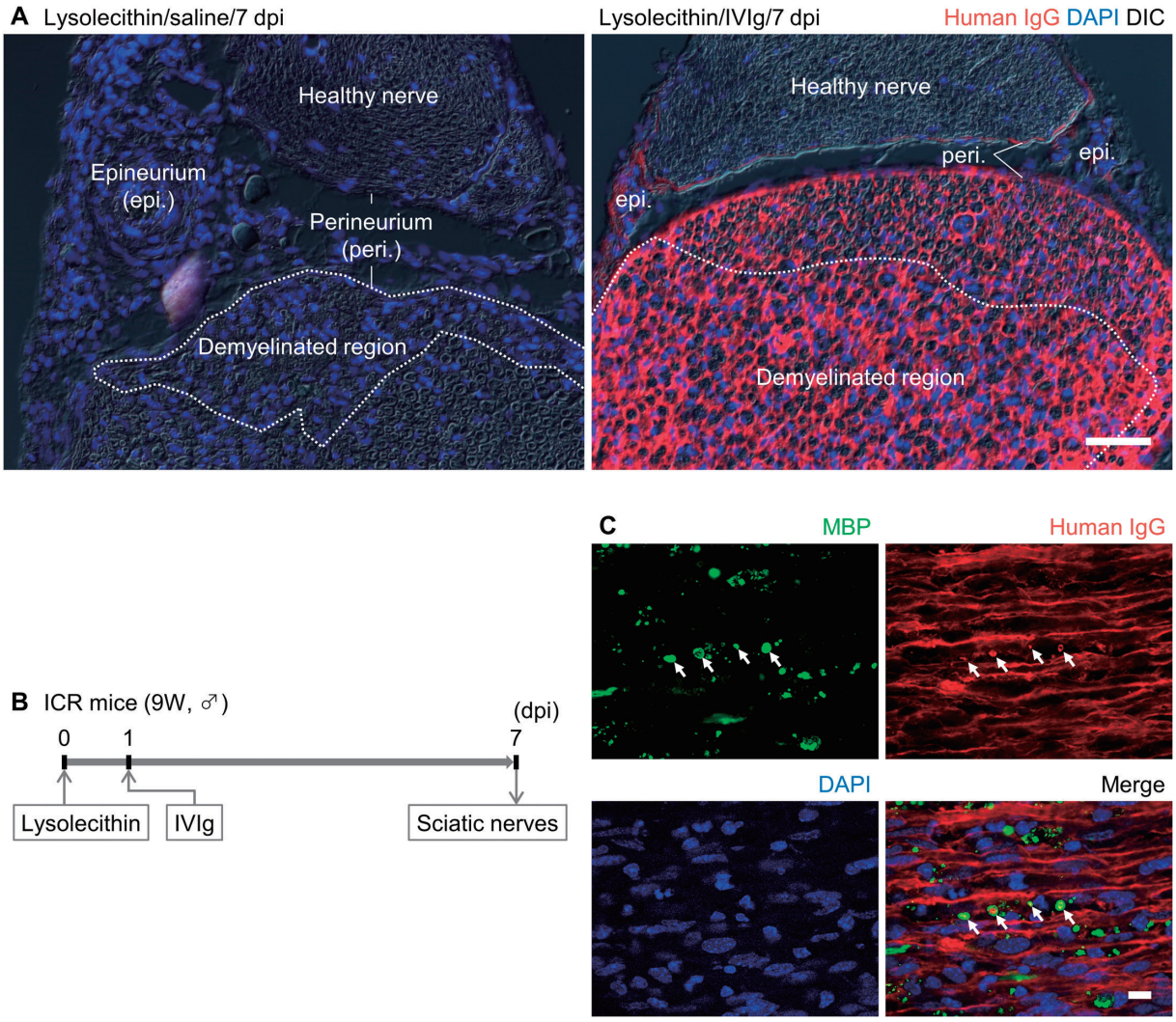

Fig. 3. IVIg treatment promotes deposition of human IgG at the demyelinated regions of mouse sciatic nerves, often appearing in areas with debris-like morphology. **(A)** Distribution of IVIg was evaluated using cross sections of the sciatic nerve (Fig. 1A) subjected to human IgG immunofluorescence staining. Human IgG-positive signals were not detected in the saline-treated mice (Lysolecithin/saline/7 dpi). The intensity of human IgG immunoreactions was greater in demyelinated regions within a dotted line, whereas weaker in healthy nerve and perineurium in the IVIg treated mice (Lysolecithin/IVIg/7 dpi). **(B)** Experimental timeline for lysolecithin-induced demyelination and IVIg treatment. To evaluate the distribution of injected human IgG in the demyelinated lesions, simplified IVIg injection protocol without intraperitoneal IVIg injection was used. Lysolecithin (1.0 µL) was injected into the left sciatic nerve in 9-week-old male ICR mice. Mice received an intravenous injection of either IVIg or saline at 1 dpi, following which the sciatic nerves were removed at 7 dpi for histological analysis. **(C)** IVIg staining, represented as human IgG (red), also overlapped with staining for MBP (green) and was often recognized as round clumps indicative of myelin debris (arrows) in the lysolecithin- and IVIg-treated mice at 7 dpi. Section was counterstained with DAPI (blue). Scale bars: 50 µm (A) and 10 µm (C).

human IgG at the damaged myelin by binding to L-MPZ, resulting in direct modulation of the demyelination process.

As IVIg preparations are based on a mixture of serum from more than 1,000 healthy donors, they contain various types of IgG antibodies that recognize diverse antigens. For example, antibodies against bacteria or viruses, anti-ideotype antibodies, and numerous antibodies against foreign and self-antigens—including those specific to cytokines, cell types, and adhesion molecules—have been reported to contribute to the effectiveness of IVIg treatment.[10),11)] In addition to these antibodies, the present results clearly demonstrated that anti-L-

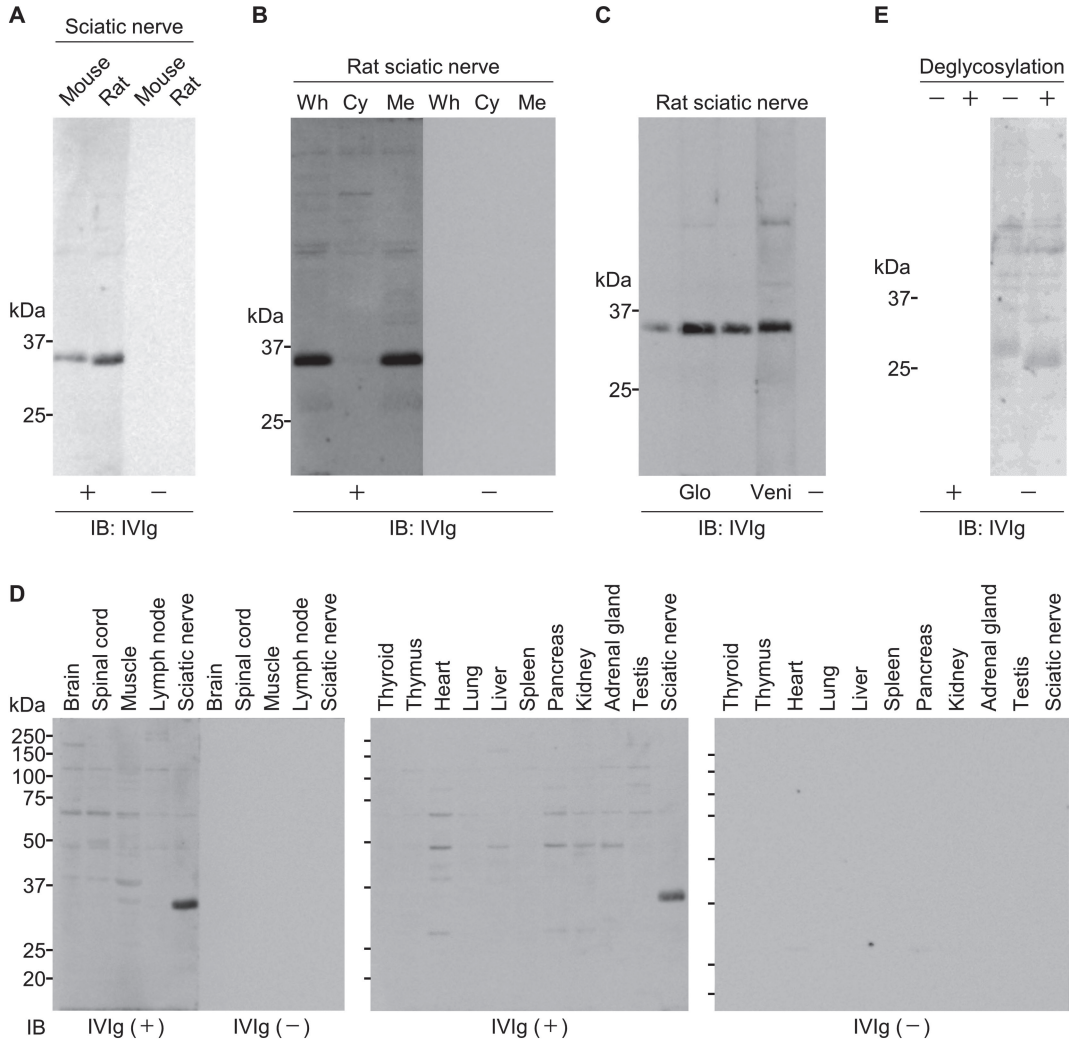

Fig. 4. IVIg preparations specifically react with the 36-kDa glycoprotein of the sciatic nerve membrane fraction, as demonstrated in Western blotting experiments. **(A)** Analysis of the IVIg-binding protein in the mouse and rat sciatic nerve homogenates (5 µg/lane) using Western blotting. IVIg was applied to the transferred membrane (+) as a primary antibody, following which the 36-kDa proteins were detected in both preparations. No signals were detected in the IVIg-free negative control membrane (−). **(B)** The 36-kDa protein was detected in whole homogenates (Wh; pre-fractionated sample) and membrane fractions (Me) of the sciatic nerve (5 µg/lane). However, it was not detected in the cytosolic fraction (Cy). **(C)** The 36-kDa protein in the sciatic nerve preparations (5 µg/lane) reacted with three different lot numbers (N113FAN, N433FAN, and N281FAN from left lane) of Glovenin® preparations (Glo) and with an IVIg preparation produced by another company (Venilon®; Veni). **(D)** The 36-kDa protein bound to IVIg was recognized only in the sciatic nerve. Protein samples for all organs were loaded at 5 µg/lane. **(E)** The 36-kDa protein bound with IVIg in the membrane fraction of the rat sciatic nerve shifted to a lower molecular weight after deglycosylation (+). IB: immunoblotting.

MPZ antibodies are present in multiple IVIg preparations with different modifications of IgG, including three Glovenin® products with different lot numbers (polyethylene glycol processing products) and a sulfonated Venilon® product (Fig. 4C). In our previous study,[13] not all serum samples from healthy individuals reacted to L-MPZ. Thus, the mechanism underlying the production of anti-L-MPZ antibodies in healthy donors remains unclear. However, since we previously demonstrated that some patients with CIDP were positive for L-MPZ antibodies,[13] we speculate that exposure of antigens in damaged nerves may promote antibody production. Given these results, future studies should aim to examine the proportion of anti-L-MPZ antibody-positive individuals among healthy donors in the Japanese population and other ethnic groups. Additionally, future research should focus on determining the

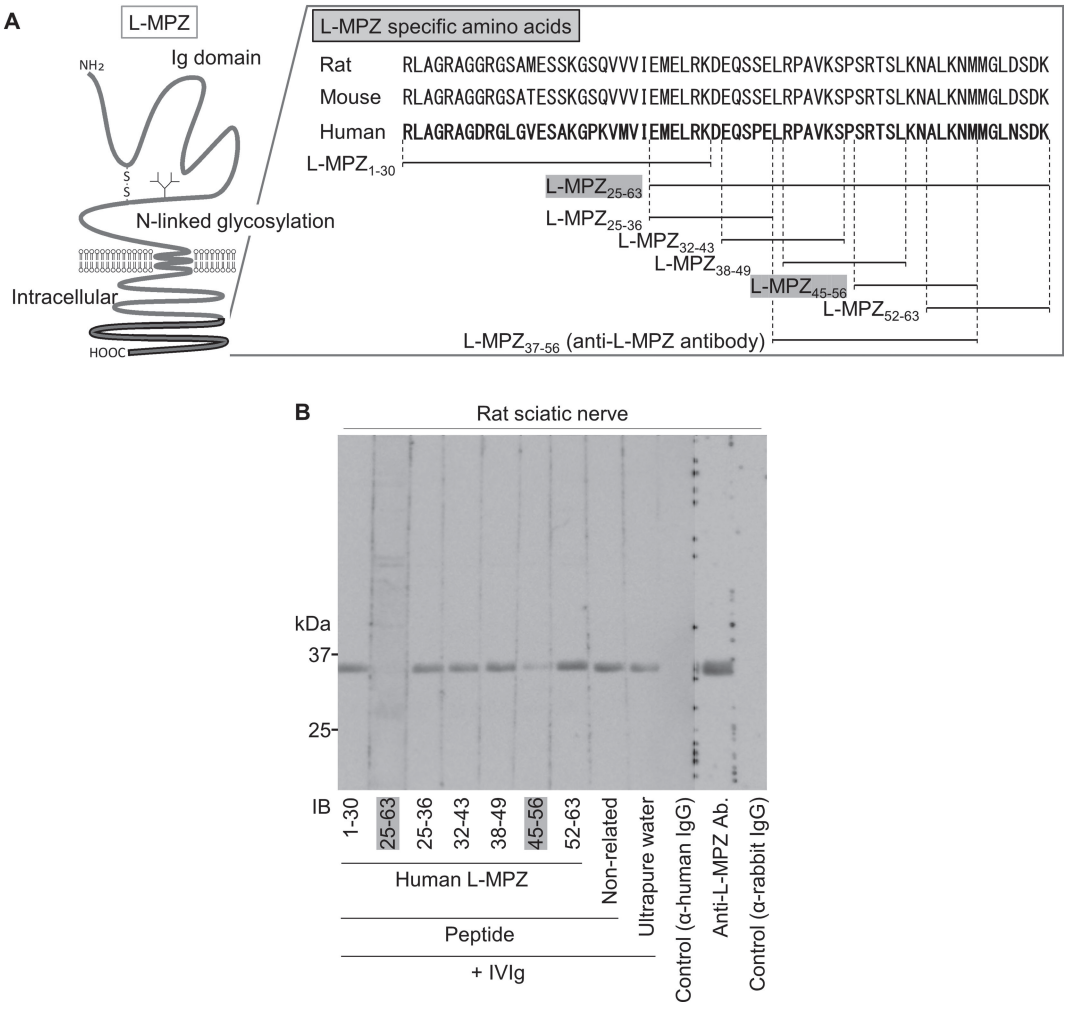

Fig. 5. IVIg contains human IgG antibodies against L-MPZ. **(A)** Illustration of L-MPZ and the specific amino acid sequences of the L-MPZ intracellular domain in rats, mice, and humans. In the present study, seven human L-MPZ peptides and a non-related scramble amino acid peptide (GLPGNEGPPGQK; non-related) were synthesized for use in a peptide absorption test. Anti-L-$MPZ_{37–56}$ antibody was used as a positive control. **(B)** Incubation of IVIg with L-$MPZ_{25–63}$ and L-$MPZ_{45–56}$ peptides resulted in little to no detection of the 36-kDa protein in sciatic nerve homogenates (5 µg/lane). Moreover, when the test was performed using the non-related peptide and the anti-L-MPZ antibody, the 36-kDa protein was detected. IB: immunoblotting.

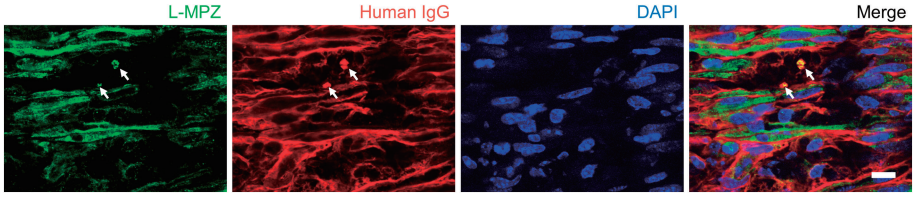

Fig. 6. IVIg co-localizes with the L-MPZ-positive myelin debris of the demyelinated regions. IVIg penetrating the sciatic nerve, represented as human IgG (red), overlapped with L-MPZ (green) immunoreactions in lysolecithin- and IVIg-treated mice at 7 dpi (arrows). Lysolecithin (1.0 µL) was injected into the left sciatic nerve in 9-week-old male ICR mice. Mice received an intravenous injection of IVIg at 1 dpi, following which the sciatic nerves were removed at 7 dpi for histological analysis. Section was counterstained with DAPI (blue). Scale bar: 10 µm.

correlation between anti-L-MPZ antibody levels and a history of nerve injury or other conditions.

Previously, we reported the presence of high-titer anti-L-MPZ antibodies in the serum of patients with CIDP.[13] In the present study, we demonstrated that the antigenic epitope of the anti-L-MPZ antibodies in IVIg preparations was located within the peptide L-MPZ$_{45–56}$, which consisted of the epitope recognized by the antibodies in patients with CIDP.[13] Although it is possible that some amount of the anti-L-MPZ antibodies found in the sera of patients originates from the IVIg treatment, high antibody titers in the patients' sera strongly suggest that these antibodies were primarily produced in the patients' bodies. We have suspected a pathogenic role of anti-L-MPZ antibodies in patients with CIDP given because certain types of anti-nerve protein antibodies have been associated with CIDP.[20] The presence of the anti-L-MPZ antibody in the serum itself might not cause neuropathy. Moreover, in healthy individuals, the anti-L-MPZ antibody may have limited access to the myelin antigen due to the presence of the blood–nerve barrier and the localization of its antigenic epitope in the cytoplasmic region. In contrast, under pathological conditions, anti-L-MPZ antibodies in IVIg preparations can easily penetrate demyelinating lesions through the damaged blood–nerve barrier, following which they can bind to the exposed epitope on myelin debris, which may help to attenuate peripheral demyelination. Currently, it remains unknown whether the role of anti-L-MPZ antibodies in patients is neuropathogenic or neuroprotective. However, IVIg-derived antibodies against the same L-MPZ peptide exerted a protective effect against demyelination in our mouse model. Furthermore, recent studies on autoimmune diseases have demonstrated that modifications of the N-linked glycan in the IgG Fc region, including sialylation, markedly alter the role of IgG from pro-inflammatory to anti-inflammatory.[21] Therefore, differential modifications of anti-L-MPZ antibodies between patients and healthy donors could lead to differences in function. Additional studies are required to clarify whether self-originating anti-L-MPZ antibodies in patients with CIDP play a distinct role from IVIg-derived antibodies.

In conclusion, the present results suggest that IVIg preparations comprise anti-L-MPZ antibodies. The administered human IgG was mainly distributed to demyelinating lesions, suppressing the exacerbation of lysolecithin-induced demyelination in the PNS. Thus, anti-L-MPZ antibodies in IVIg preparations may exert a therapeutic effect on peripheral demyelination.

Conflict of interest

The authors declare no conflicts of interest.

Data availability statement

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

Author contributions

Y.S. Methodology, Investigation, Writing – original draft, Preparation. A.H. Conceptualization, Methodology, Investigation, Writing – review and editing. A.K., A.I., D.Y., and R.S. Methodology, Investigation, Preparation. T.I., H.T., and Y.Y. Methodology. H.O. Writing – review and editing, Supervision. H.B. Conceptualization, Funding acquisition, Writing – review and editing, Supervision. All authors have read and approved the final version of the manuscript.

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Supplementary material

Supplementary material is available at https://doi.org/10.2183/pjab.99.004.

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