

Prophylactic treatment with sulphonated immunoglobulin G attenuates development of mechanical allodynia-like response in mice with neuropathic pain

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ABSTRACT. Human immunoglobulin G (IgG) concentrates are immune-modulating, anti-inflammatory plasma-derived products. Clinical studies in recent years have suggested that IgG attenuates neuropathic pain. In this study, effects of sulphonated IgG on the development and maintenance of a mechanical allodynia-like response were examined in mice with neuropathic pain induced by a partial sciatic nerve ligation (PSL). When sulphonated IgG (400 or 1,000 mg/kg/day, i.p.) was administered for 5 days, from 1 day before surgery to post-operative day (POD) 3, the development of a mechanical allodynia-like response was attenuated. On the other hand, sulphonated IgG had little effect on the maintenance of a mechanical allodynia-like response when administered for 5 days, from POD 11 to POD 15, at which time a mechanical allodynia-like response had already been developed. To explore the mechanism of sulphonated IgG, the mRNA expression of inflammatory cytokines was evaluated in the injured sciatic nerve. Sulphonated IgG (1,000 mg/kg/day, i.p.) that was administered for 3 days, from 1 day before surgery to POD 1, significantly attenuated the up-regulation of tumor necrosis factor- α and monocyte chemoattractant protein-1 mRNAs on POD 1. These results suggest that prophylactic treatment with sulphonated IgG attenuates the development of mechanical allodynia-like response by inhibition of inflammatory cytokine expression in mice with PSL.

KEY WORDS: mechanical allodynia-like response, neuropathic pain, partial sciatic nerve ligation, sulphonated immunoglobulin G

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Neuropathic pain is currently treated with drugs, such as anti-depressants and anti-convulsants [18, 23]. Although these drugs have been shown to reduce neuropathic pain in clinical trials, they are not satisfactory for all patients and are often accompanied by adverse effects. New therapeutic strategies for neuropathic pain are therefore desired.

Human immunoglobulin G (IgG) concentrates are immune-modulating, anti-inflammatory plasma-derived products that can be applied either intravenously (intravenous immunoglobulin; IVIg) or subcutaneously (subcutaneous immunoglobulin; SCIG). IgG is widely used in the clinic for the treatment of severe infectious diseases, idiopathic thrombocytopenic purpura, kawasaki disease, sjögren's syndrome, guillain-barre syndrome, chronic inflammatory demyelinating polyradiculoneuropathy and churg-strauss syndrome [2, 7, 22, 25, 26]. In recent years, clinical studies have suggested that IgG attenuates neuropathic pain associated with diabetes mellitus, sjögren's syndrome and complex regional pain syndrome (CRPS) [12, 13]. For example, IVIg treatment reduces the pain score of patients with refractory CRPS [6] and improves neuropathic pain in patients with sjögren's syndrome [19]. Although, as mentioned above, clinical analgesic effects of IgG have been reported, there

has been no preclinical study that investigated the effects of IgG on neuropathic pain, and its mechanism has not yet been examined.

In this study, the effects of IgG (sulphonated human IgG) on a mechanical allodynia-like response, which is a representative symptom of neuropathic pain, were examined in mice with partial sciatic nerve ligation (PSL).

MATERIALS AND METHODS

Animals: Male ddY mice (16–19 g body weight) were obtained from Japan SLC Inc. (Hamamatsu, Japan). The animals were group-housed under a light-dark cycle (light on: 06:00–18:00) with free access to food and water. All animal experimental procedures were carried out according to a protocol approved by the Animal Care and Use Committee of Teijin Institute for Bio-Medical Research.

Partial sciatic nerve ligation: The partial sciatic nerve ligation (PSL) was made according to the method of Seltzer *et al.* [21] and Malmberg and Basbaum [15]. Briefly, the mice were anesthetized with isoflurane in oxygen during surgery. The right sciatic nerve was exposed and ligated with a 9-0 silk suture around approximately one-half of the nerve diameter under a light microscope. A sham operation was performed in the same manner, except for the sciatic nerve ligation.

Mechanical allodynia-like response: The paw withdrawal threshold (PWT) for mechanical stimulation was determined using calibrated von Frey hair (VFH) filaments (0.008, 0.02, 0.04, 0.07, 0.16, 0.4, 0.6, 1.0 and 1.4 g; Semmes-Weinstein Monofilaments, North Coast Medical Inc., San Jose, CA, U.S.A.) as described previously [32]. The mice were indi-

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vidually placed in suspended cages with wire mesh bottoms for at least 60 min. The plantar surface of the hind paw was stimulated with a VFH filament in ascending order. Each VFH filament was applied ten times until a withdrawal response was observed. Once the withdrawal response was observed, the paw was re-tested with the same VFH filament to confirm the response. The lowest amount of force required to elicit a response was recorded as the PWT (g). A mechanical allodynia-like response was defined when the animals responded to a VFH of 0.04 g or below, which was innocuous for normal or sham-operated mice. All the VFH measurements were performed in a blinded fashion.

Materials: IgG (sulphonated human IgG; venilon) was kindly supplied by the Chemo-Sero-Therapeutic Research Institute (KAKETSUKEN) (Kumamoto, Japan). In previous studies, we showed that sulphonated IgG is converted back into the non-sulphonated IgG both *in vitro* and *in vivo* [17] and that sulphonated IgG exhibits almost the same antibody activities against various antigens as those of the non-sulphonated IgG [16]. The sulphonated IgG was administered intraperitoneally daily at doses of 400 or 1,000 mg/kg/day (8 ml/kg or 20 ml/kg, respectively) according to previous studies [11, 14]. Physiological saline was used as a vehicle control (20 ml/kg). Although the human IgG used in this study was xenogenic to the host, no abnormal symptom, such as decreased body weight or body weight gain, was noted during the experiment (data not shown), suggesting that IgG antigenicity between different species was not likely to be a problem under this experimental condition. This finding is consistent with those of previous reports [3, 31].

Test protocol: To examine the effects of prophylactic treatment with IgG on PSL-induced mechanical allodynia-like response, 45 mice were used. The PWTs of these mice were tested 2 days before surgery to obtain baseline values, and the mice were randomized to 3 groups (n=15/group) in which they received vehicle or sulphonated IgG (400 or 1,000 mg/kg/day). PSL surgery was performed as described above. In the PSL surgery, 1 mouse in the sulphonated IgG 1,000 mg/kg/day group died from anesthesia overdose. Sulphonated IgG was administered for 5 days, from 1 day before surgery to post-operative day (POD) 3. The PWTs of these mice were examined on PODs 5, 10, 14, 21 and 28.

To examine the effect of therapeutic treatment with IgG on the PSL-induced mechanical allodynia-like response, 60 mice were used. The PWTs of these mice were tested on 1 day before surgery to obtain baseline values. PSL surgery was performed as described above. In the PSL surgery, 4 mice died from anesthesia overdose. On PODs 10 and 11, 47 PSL mice showed a reduction in the PWT to 0.04 g or a lower force, and 36 PSL mice were then selected. These 36 PSL mice were randomized to 3 groups (n=12/group) in which they received vehicle or sulphonated IgG (400 or 1,000 mg/kg/day). Sulphonated IgG was administered for 5 days, from POD 11 to POD 15. The PWTs of these mice were measured on PODs 18, 24, 30 and 36.

To explore the mechanism of the effect of IgG on the PSL-induced mechanical allodynia-like response, the mRNA expression of inflammatory cytokines in the injured

sciatic nerve on POD 1 and POD 5 was evaluated. For POD 1 sampling, 60 mice were used. The mice were divided into 3 groups (n=20/group); sham/vehicle, PSL/vehicle and PSL/sulphonated IgG (1,000 mg/kg/day). The mice were treated with vehicle or sulphonated IgG for 3 days, from 1 day before surgery to POD 1. The sciatic nerve segments (about 1-cm long) proximal to the ligatures were harvested on POD 1. The sciatic nerves from 2 mice were pooled and analyzed using quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR). For POD 5 sampling, 60 mice were divided into 3 groups (sham/vehicle, PSL/vehicle and PSL/sulphonated IgG 1,000 mg/kg/day, n=20/group) that were treated with vehicle or sulphonated IgG for 5 days, from 1 day before surgery to POD 3. The sciatic nerve was harvested on POD 5 and analyzed as for the POD 1 samples.

Quantitative real-time reverse transcription polymerase chain reaction: The qRT-PCR analyses were performed using an ABI Prism 7500 Sequence Detection System (Applied Biosystems, Foster City, CA, U.S.A.) and SYBR Green Mix (Applied Biosystems) according to the manufacturer's instructions. Briefly, total RNA was extracted from the sciatic nerve within 1 cm of the ligation site using the ISOGEN reagent (Nippon Gene, Toyama, Japan). First strand cDNA was synthesized from 1,000 ng of total RNA in a final volume of 12 μ l using the Omniscript RT Kit (Qiagen GmbH, Hilden, Germany), RNaseOUT Recombinant Ribonuclease Inhibitor (Invitrogen, Carlsbad, CA, U.S.A.) and Random Primer (Takara Bio Inc., Otsu, Japan). The cDNA was analyzed using qRT-PCR analysis. The reaction conditions were as follows: 50°C for 2 min and then 95°C for 10 min, followed by 40 cycles at 95°C for 15 sec (denaturation) and 60°C for 1 min (annealing and elongation). Messenger RNA (mRNA) expression for each sample was calculated by using the ΔC_T procedure ($\Delta C_T = C_T$ of the target mRNA - C_T of the control mRNA). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH, TaqMan[®] Rodent GAPDH Control Reagents, Thermo Fisher Scientific Inc., Waltham, MA, U.S.A.) was used as a reference control gene. The relative expression levels were calculated by $2^{-\Delta C_T}$. The primer sets used were as follows: tumor necrosis factor- α (TNF- α) [5], (5'-AAGCCTGTAGCCCACGTCGTA-3' and 3'-GGCACCCTAGTTGGTTGTCTTTG-5'), interleukin-1 β (IL-1 β) [27] (5'-TGTGAAATGCCACCTTTTGA-3' and 3'-AGCTTCTCCACAGCCACAAT-5'), monocyte chemotactic protein-1 (MCP-1) [27] (5'-AACTGCATCTGCCCTAAGGTCT-3' and 3'-GGAAAAGGTAGTGATGCATTAGC-5') and macrophage inflammatory protein-1 α (MIP-1 α) [10] (5'-TGCCCTTGCTGTTCTTCTCT-3' and 3'-CAGGCATTCAGTTCCAGGTC-5').

Statistical analysis: The data relating to PWT are expressed as the medians and the 1st and 3rd quartiles that indicate the range of median values. These data were subjected to a nonparametric-type Dunnett's test. The qRT-PCR data are expressed as means \pm SD and were subjected to a parametric-type or a nonparametric-type Tukey test. All data were statistically analyzed using SAS software for Windows, Release 9.2 (SAS Institute Inc., Cary, NC, U.S.A.). Differences with a *P* value of 0.05 were considered significant.

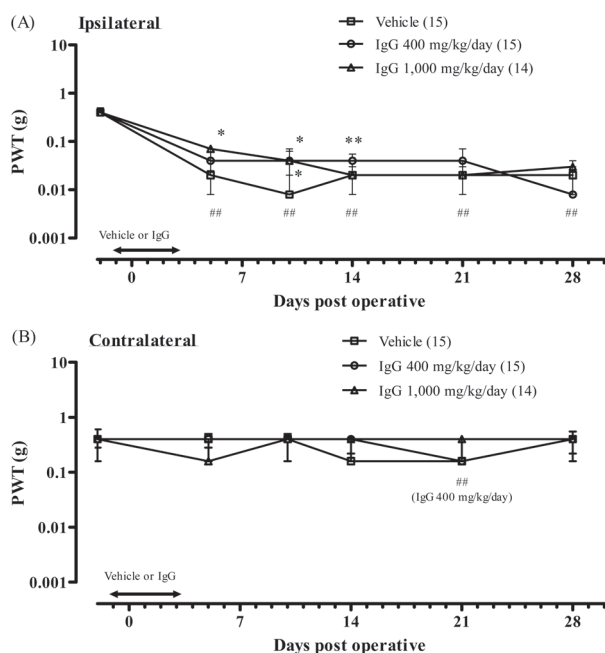


Fig. 1. Effects of sulphonated IgG on the development of a mechanical allodynia-like response. Vehicle or sulphonated IgG (400 or 1,000 mg/kg/day) was intraperitoneally administered to mice with PSL from 1 day before surgery to POD 3. The PWT was measured using a von Frey hair filament on the ipsilateral (A) and contralateral (B) paw of a mouse with PSL. In the ipsilateral paw, PWTs in the PSL/vehicle group were significantly decreased from POD 5 to POD 28 (## $P < 0.01$) compared with the PWTs before surgery, suggesting that a mechanical allodynia-like response had developed. Data are expressed as the median force (g) required to induce paw withdrawal in 14–15 animals per group (parenthesis indicates the number of animals used), and vertical bars represent 1st and 3rd quartiles. * $P < 0.05$, ** $P < 0.01$ vs. vehicle, and ## $P < 0.01$ vs. before surgery (Dunnett's test).

RESULTS

Effects of prophylactic treatment with sulphonated IgG on PSL-induced mechanical allodynia-like responses: In the paw ipsilateral to the PSL, the PWTs in mice with PSL were significantly decreased from POD 5 to POD 28 compared with the PWTs before surgery ($P < 0.01$). This result indicated that a mechanical allodynia-like response had developed on POD 5 and was sustained until the end of the study (POD 28). In PSL/sulphonated IgG groups, these decreases in PWTs were attenuated, and significant differences were noted between the PWTs of PSL/vehicle and PSL/sulphonated IgG (400 or 1,000 mg/kg/day) on PODs 5, 10 and 14 ($P < 0.05$ for all comparisons) (Fig. 1A). In the contralateral paw, sulphonated IgG did not affect the PWTs at either dose (Fig. 1B). Although a significant difference was noted between the PWTs before surgery and the PWTs in the PSL/sulphonated IgG 400 mg/kg/day group on POD 21, this difference was not considered to be related to sulphonated IgG treatment, because there was no significant difference between the

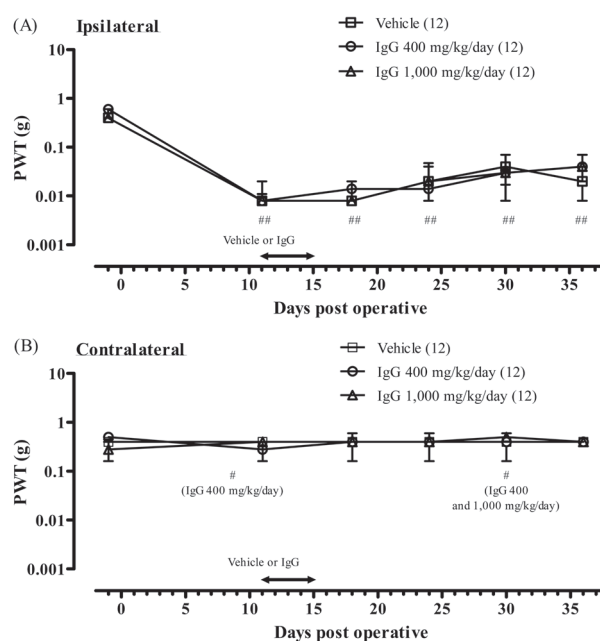


Fig. 2. Effects of sulphonated IgG on the maintenance of a mechanical allodynia-like response. Vehicle or sulphonated IgG (400 or 1,000 mg/kg/day) was intraperitoneally administered to mice with PSL from POD 11 to POD 15. The PWT was measured using a von Frey hair filament on the ipsilateral (A) and contralateral (B) paw of a mouse with PSL. In the ipsilateral paw, PWTs in the PSL/vehicle group were significantly decreased from POD 11 to POD 36 (## $P < 0.01$) compared with the PWTs before surgery, suggesting that a mechanical allodynia-like response had developed by POD 11. Data are expressed as the median force (g) required to induce paw withdrawal in 12 animals per group (parenthesis indicates the number of animals used), and vertical bars represent 1st and 3rd quartiles. # $P < 0.05$ and ## $P < 0.01$ vs. before surgery (Dunnett's test).

PWTs of the PSL/vehicle group and the PWTs of the PSL/sulphonated IgG 400 mg/kg/day group on POD 21.

Effects of therapeutic treatment with sulphonated IgG on PSL-induced mechanical allodynia-like responses: In the paw ipsilateral to the PSL, the PWTs in mice with PSL were significantly decreased from POD 11 to POD 36 compared with the PWTs before surgery ($P < 0.05$), suggesting that a mechanical allodynia-like response had developed by POD 11 (before sulphonated IgG treatment). When sulphonated IgG was administered from POD 11 to POD 15, it did not affect the PWTs at either dose (400 or 1,000 mg/kg/day) (Fig. 2A). In the contralateral paw, sulphonated IgG did not affect the PWTs at either dose (Fig. 2B). Although significant differences were noted between the PWTs before surgery and the PWTs in the PSL/sulphonated IgG group on POD 11 (400 mg/kg/day) and on POD 30 (400 or 1,000 mg/kg/day), these differences were not considered to be related to sulphonated IgG treatment. This is because significant differences between the PWTs of the PSL/vehicle and the PSL/sulphonated IgG groups were not observed at those time points.

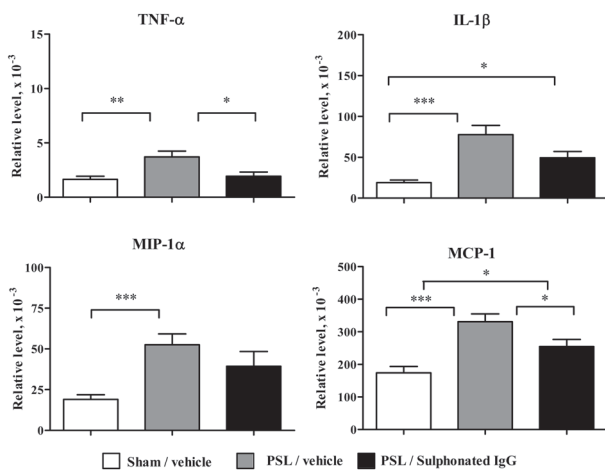


Fig. 3. Effects of sulphonated IgG on the expression of inflammatory cytokine mRNAs on POD 1. Vehicle or sulphonated IgG (1,000 mg/kg/day) was intraperitoneally administered to mice with PSL from 1 day before surgery to POD 1. The mice were sacrificed on POD 1, and total RNA was isolated from the sciatic nerves. The mRNA expression levels of TNF- α , IL-1 β , MIP-1 α and MCP-1 were measured using qRT-PCR. The amount of the target mRNA relative to the expression of GAPDH mRNA is expressed as the mean \pm SEM (n=10). * P <0.05, ** P <0.01 and *** P <0.001 (Tukey's test).

Effects of sulphonated IgG on the expression of inflammatory cytokines in mice with PSL: To explore the mechanism of the above sulphonated IgG effect, the mRNA expression of inflammatory cytokines (TNF- α , IL-1 β , MIP-1 α and MCP-1) in the injured sciatic nerve was examined using qRT-PCR. On POD 1, inflammatory cytokine mRNAs were significantly up-regulated in PSL/vehicle when compared with those of sham/vehicle. In the PSL/sulphonated IgG group, the up-regulation of TNF- α and MCP-1 mRNAs was significantly attenuated (P <0.05) (Fig. 3). On POD 5, TNF- α and MCP-1 mRNAs were still up-regulated, and pre-treatment with sulphonated IgG up to POD 3 showed a tendency to suppress the up-regulation of the MCP-1 mRNA ($P=0.0542$) (Fig. 4).

DISCUSSION

The present study showed that prophylactic treatment with IgG may attenuate the development of a mechanical allodynia-like response in mice with neuropathic pain. The reasons why we used a PSL model and detected a mechanical allodynia-like response were as follows; PSL model was widely used as a mouse model of neuropathic pain like chronic constriction injury (CCI) model, mechanical allodynia, an exaggerated sensory response to non-noxious stimuli, is one of the representative symptom in patients with neuropathic pain, detection of the mechanical allodynia-like response is a very popular assay performed in an animal model of neuropathic pain, and previous experiment conducted in our laboratory demonstrated that gabapentin, positive control drug, significantly suppressed mechanical

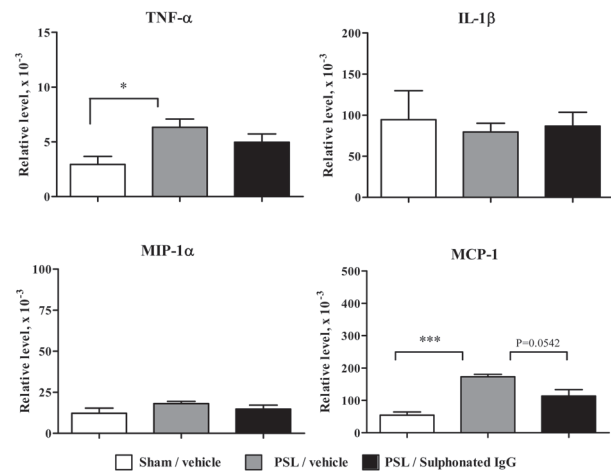


Fig. 4. Effects of sulphonated IgG on the expression of inflammatory cytokine mRNAs on POD 5. Vehicle or sulphonated IgG (1,000 mg/kg/day) was intraperitoneally administered to mice with PSL from 1 day before surgery to POD 3. The mice were sacrificed on POD 5, and total RNA was isolated from sciatic nerves. The mRNA expression levels of TNF- α , IL-1 β , MIP-1 α and MCP-1 were measured using qRT-PCR. The amount of the target mRNA relative to the expression of GAPDH mRNA is expressed as the mean \pm SEM (n=10). * P <0.05 and *** P <0.001 (Tukey's test).

allodynia-like response in this model.

In contrast to our findings, Sommer *et al.* reported that IgG did not show any effects on the development of a mechanical allodynia-like response in mice with CCI [24]. Although the exact reason for the discrepancy between our findings and theirs is unclear, it might be due to, at least in part, the different dose of IgG used in each study and/or the different IgG preparations. The sulphonated IgG used in this study was prepared from pooled human plasma from thousands of donors. It is known that IgG prepared from a large donor pool contains variable amounts of IgG dimer, whereas IgG isolated from the plasma of a single individual, which was used in the previous report, is essentially monomeric [28, 29]. Teeling *et al.* demonstrated that therapeutic effects of IgG preparations on the platelet counts of rats with immune thrombocytopenia depended on the amount of IgG dimers in the preparations [29].

In this study, sulphonated IgG significantly attenuated the up-regulation of TNF- α and MCP-1 mRNA in mice with PSL. Wagner [30] and Zelenka [33] showed that a single injection of TNF- α into a rat sciatic nerve decreased the mechanical withdrawal threshold for a week. Czeschik *et al.* reported that TNF- α increased voltage-gated sodium channels currents and potassium ion conductance in a non-voltage gated fashion in isolated rat DRG neurons [4] and leading to neuronal hyper-excitability. Abbadie *et al.* also demonstrated that an intraplantar injection of MCP-1 induced a mechanical allodynia-like response for more than 180 min in mice [1]. It is therefore postulated that the reduction in inflammatory cytokine expression induced by sulphonated IgG on POD 1 contributes to its attenuation of the development

of a mechanical allodynia-like response on POD 5 in mice with PSL. However, we cannot rule out any other factors to contribute the prophylactic effects of sulphonated IgG.

It is not yet known how IgG may attenuate cytokine expression in the injured sciatic nerve. It has been reported that IgG reduces TNF- α and IL-1 α production by human peripheral blood mononuclear cells in the presence of lipopolysaccharide [8]. Kishimoto *et al.* reported that treatment with sulphonated IgG decreased T-lymphocyte infiltration and serum cytokine levels (TNF- α , INF- γ and MIP-2), and attenuated the development of myocarditis induced by encephalomyocarditis virus in mice [11]. Therefore, an inhibitory effect of IgG on inflammatory cell infiltration into the injured sciatic nerve and/or on cytokine expression in macrophages might be considered as a mechanism by which IgG attenuates neuropathic pain.

In contrast to clinical studies, therapeutic treatment with sulphonated IgG has little effects on the maintenance of a mechanical allodynia-like response in mice with PSL. The differences of the relative contributions of inflammatory cytokines on the maintenance of neuropathic pain might cause the discrepant results. Although we have not examined the temporal profile of inflammatory cytokines, several studies have reported that cytokine mRNAs are immediately elevated in the injured sciatic nerve (from 12 hr to 7 days after nerve injury) and then gradually return to the control level by 7–14 days in mice with PSL [9, 10, 20]. The relative contribution of inflammatory cytokines on maintenance of mechanical allodynia-like response might be small in our experimental conditions. In the clinical studies, IgG treatment attenuates some types of neuropathic pain, whose pathophysiology may involve immune changes in the peripheral tissues and/or CNS, and the analgesic action of IgG in these conditions was thought to be the suppression of cytokine expression [25].

In summary, our results have provided the first preclinical evidence that prophylactic treatment with sulphonated IgG attenuates the development of a mechanical allodynia-like response via an inhibition of inflammatory cytokine expression in mice with PSL.

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