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Immune cell distribution and immunoglobulin levels change following sciatic nerve injury in a rat model

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ABSTRACT

Objective(s): To investigate the systemic and local immune status of two surgical rat models of sciatic nerve injury, a crushed sciatic nerve, and a sciatic nerve transection

Materials and Methods: Twenty-four adult male Sprague-Dawley rats were randomly divided into three groups: sham-operation (control group), sciatic nerve crush, and sciatic nerve transaction. Sciatic nerve surgery was performed. The percentage of $CD4^+$ cells and the $CD4^+/CD8$+ratio were determined by flow cytometry. Serum IgM and IgG levels were analyzed by ELISA. T-cells (CD3) and macrophages (CD68) in sciatic nerve tissue sections were identified through immunohistochemistry.

Results: Compared to sham-operated controls, in rats that underwent nerve injury, the percentage of $CD4^+$ cells and the $CD4^+/CD8^+$ ratio in the peripheral blood were significantly decreased 7 days after surgery, serum IgM levels were increased 14 days after surgery, and serum IgG levels were increased 21 days after surgery. There were a large number of $CD3^+$ cells and a small number of $CD68^+$ cells in sciatic nerve tissue sections 21 days after surgery, indicating T-cell and macrophage activation and infiltration. Local IgG deposition was also detected at the nerve injury site 21 days after surgery.

Conclusion: Rat humoral and cellular immune status changed following sciatic nerve injury, particularly with regard to the cellular immune response at the nerve injury site.

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Introduction

Peripheral nerve injury is an under-appreciated clinical problem, even though it is more common than injury to the central nervous system. Indeed, repair and regeneration of peripherally injured nerves is an extremely complex process (1), which includes adhesion, the extracellular matrix, regulation of neurotrophic factors, formation and extension of the growth cone, synthesis and release of neurotransmitters, and neuron remodeling. In addition, inflammation, inflammatory cells and their products, and major histocompatibility complex class II (MHC II) antigens have a very important role in the degeneration and regeneration of axons (2, 3). As such, the success of peripheral neuroregeneration after trauma or disease is limited, even when compared to neuroregeneration after damage to the central nervous system (4).

Evidence suggests that neuro-immune interacttions take place in response to disease and trauma in the nervous system (5). Following peripheral nerve injury, a series of immunological responses occurs (3). Both neuroprotective and neurodestructive effects of the immune system have been described, although how these contradictory effects are regulated has yet to be determined (6). Importantly, the immune response after peripheral nerve injury may cause secondary damage to neurons and inhibit their repair and regeneration. Some studies show that peripheral nerve damage stimulates the innate immune response and the production of a variety of cytokines and inflammatory mediators, which induce non-specific nerve damage (7-9). Elucidating the crucial immune elements that regulate peripheral nerve injury may inform the development of novel therapeutic strategies that suppress immune reactions, promote nerve repair and regeneration, and restore normal function following nerve injury.

To further understand the role of the humoral and cellular immune response in regulating peripheral nerve damage, we used rat models of sciatic

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nerve injury. We focused on characterizing $CD4^+$ cells, the $CD4^+/CD8^+$ ratio, and local immune-globulin (Ig) G and IgM levels in the peripheral blood and cellular infiltrates at the nerve injury site. The percentage of $CD4^+$ cells and the $CD4^+/CD8^+$ ratio in the peripheral blood are good indicators of overall immune status (10). The local expression of CD3 and CD68, and the deposition of immunoglobulin following sciatic nerve injury provide evidence of interactions between the immune response during nerve regeneration.

Materials and Methods

Animals

This study was approved by the Committee on the Ethics of Animal Experiments of the Peking University People's Hospital, China. All surgery was performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering. Twenty-four adult male Sprague-Dawley rats (mean body weight, 275 g; range, 250–300 g) from the Animal Center of the Military Academy of Medical Sciences were used in this study. The rats were maintained under standard pathogen-free laboratory conditions on a 12 hr light/dark cycle with free access to pellet food and water. Rat care and all experimental procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals and approved by the Chinese National Committee for the Use of Experimental Animals for Medical Purposes, Beijing Branch.

Sciatic nerve surgery

The rats were randomly divided into three groups: sham-operation (control group) (n= 8), sciatic nerve crush (n= 8), and sciatic nerve transection (n= 8). The rats were anesthetized with sodium pentobarbital (30 mg/kg) by intraperitoneal injection. All of the surgical procedures were performed under aseptic conditions. The sciatic nerves of the rats in the sham-operated control group were exposed, but no deliberate injury was performed. In the sciatic nerve crush group, the left sciatic nerve of each rat was clamped for 1 min using pincers with a 2 mm width (11). The sciatic nerve transection in the third treatment group was performed by severing the left sciatic nerve with a scalpel (12). The stump of the nerve was sutured with 3 to 4 stitches of 9-0 silk thread by neurorrhaphy.

Percentage of $CD4^+$ cells and $CD4^+/CD8^+$ ratio in peripheral blood

Blood was collected through the orbital venous plexus of each rat at 7, 14, and 21 days post-surgery. Peripheral blood samples were obtained from individual rats, and the percentage of $CD4^+$ cells and the $CD4^+/CD8^+$ ratio were determined by flow cytometry using a FACS Calibur instrument, according to the manufacturer's instructions (BD Biosciences). Peripheral blood samples were stained with a combination of allophycocyanin (APC)-anti-CD3, fluorescein isothiocyanate (FITC)-anti-CD4, and phycoerythrin (PE)-anti-CD8 (eBioscience, USA).

Enzyme-linked immunosorbent assay (ELISA) analysis of serum antibodies

The concentrations of serum antigen-specific IgG and IgM in individual animals were analyzed by ELISA, according to the manufacturer's instructions (Bethyl, USA). Individual serum samples at 1:100-1:500 dilutions were tested in triplicate and incubated at 37 ˚C for 1 hr. Subsequently, the bound antibodies were incubated with horseradish peroxidase (HRP)-conjugated goat anti-rat IgG (1:100) or HRP-conjugated goat anti-rat IgM (1:100) (Bethyl, USA) at 37 ˚C for 1 hr. After washing, the bound HRP-conjugated secondary antibodies were detected with tetramethylbenzidine substrate. The reaction was stopped by adding 50 µl/well of 1 M H_2SO_4, and the optical density was measured at 450 nm.

Immunohistochemistry

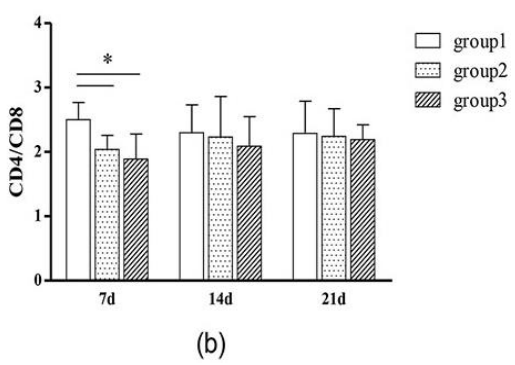
The rats were euthanized 21 days after surgery. The left sciatic nerve was dissected, fixed in 4% paraformaldehyde for 48 hr, conventionally dehydrated, cleared, embedded in paraffin, and sectioned into 4 µm sections. The sections were dewaxed and incubated in 3% H_2O_2 at room temperature for 10 min to inactivate endogenous peroxidase. After washing 3 times, the sections were microwaved for 10 min in 0.01 M citrate buffer (pH= 6.0) and washed 3 times.

Primary antibodies were diluted with phosphate-buffered saline (PBS) and 5% goat serum. CD3 and CD68 (1:500, Abcam, USA) and IgG (1:200, PLlabs, USA) antibodies were added in sequential treatments, according to the manufacturer's instructions. After overnight incubation and PBS washing, a secondary antibody conjugated with biotin was added. The sections were incubated with HRP-conjugated streptavidin at room temperature for 30 min. The antigens were visualized with 3,3'-diaminobenzidine. The sections were washed with double-distilled water, air-dried, and fixed with neutral balsam.

Sections were observed under a Leica DM6000B microscope (Leica, Germany), and the images were processed with IPP6.0 software. T-cells (CD3) and macrophages (CD68) were quantified on 4 sec/rat (n= 8 rats/group) at the region with cell infiltration or at the sciatic nerve injury site.

Statistical analysis

The experimental data are expressed as mean±standard error of the mean (SEM). Statistically significant differences between the groups were determined by one-way analysis of variance



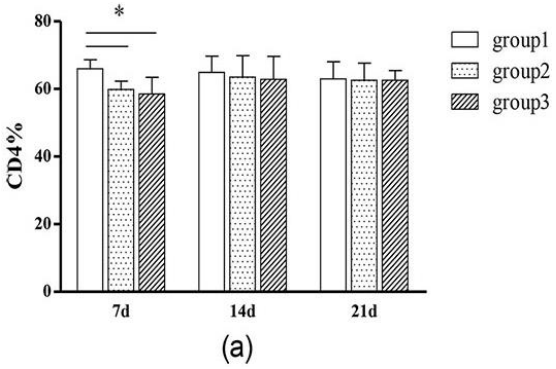


Figure 1. Percentage of CD4+ cells and the CD4+/CD8+ ratio in peripheral blood at 7 days, 14 days, and 21 days after surgery. The percentage of CD4+ cells in the blood (A) and the CD4+/CD8+ ratios (B) are shown. Group 1- sham-operated control (open bars); Group 2- sciatic nerve crush injury (dotted bars); and Group 3- sciatic nerve transection (striped bars). Error bars represent the SEM of three independent experiments. Asterisks (*) indicate statistically significant differences compared with Group 1. Statistically significant decreases in the CD4+ cell percentage and the CD4+/CD8+ ratio were observed 7 days post-surgery, but not at 14 days or 21 days post-surgery in groups 2 and 3 compared to group 1

(ANOVA) followed by Tukey's *post hoc* multiple comparison test. $P<0.05$ was considered statistically significant. Statistical analyses were performed using SPSS version 19.0 (SPSS, Chicago, IL, USA).

Results

Percentage of CD4+ cells and CD4+/ CD8+ ratio in the peripheral blood

At 7 days post-surgery, there were 60.84±2.49% CD4+ cells in the peripheral blood of rats that underwent sciatic nerve crush injury and 58.5±3.92% CD4+ cells in rats that received sciatic nerve transection. These were significantly lower than the percentage of CD4+ cells in the peripheral blood of the sham-operated control group (65.94±2.66%, $P<0.05$). The ratio of CD4+/CD8+ in the peripheral blood was also significantly lower in the rats that underwent a sciatic nerve crush injury (2.04±0.22) or sciatic nerve transection (1.97±0.39) compared to the sham-operated control group (2.5±0.27) ($P< 0.05$). At 14- and 21 days post-surgery, there were no statistically significant differences between the sciatic nerve injury groups and sham-operated controls in the percentage of CD4+ cells or the CD4+/CD8+ ratio in the peripheral blood ($P>0.05$) (Figure 1).

Serum levels of IgG and IgM

At 7 days post-surgery, there were no statistically significant differences in serum IgG or IgM levels between the sciatic nerve crush, sciatic nerve transection, and sham-operated control groups. At 14 days post-surgery, serum IgM levels were significantly increased in the rats that underwent sciatic nerve injury compared to the sham-operated control group ($P<0.05$); there were no significant

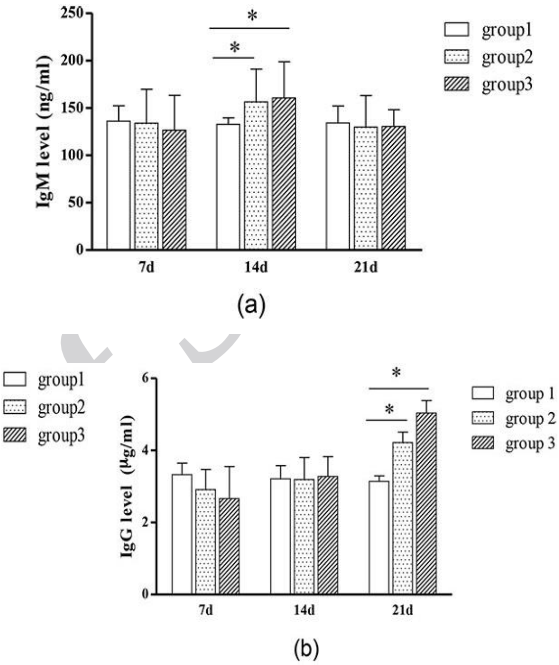


Figure 2. Serum IgM and IgG levels at 7 days, 14 days, and 21 days after surgery. Group 1- sham-operated control (open bars); Group 2- sciatic nerve crush injury (dotted bars); and Group 3- sciatic nerve transection (striped bars). Error bars represent the SEM of three independent experiments. Asterisks (*) indicate statistically significant differences compared with Group 1. Serum IgM was significantly elevated at 14 days, but not 7 days or 21 days post-surgery in Groups 2 and 3 compared to group 1. Serum IgG was significantly elevated 21 days post-surgery in Groups 2 and 3 compared to Group 1

differences in serum IgG levels. At 21 days post-surgery, serum IgM levels had decreased in the rats with sciatic nerve injury, and there were no statistically significant differences between the groups. In contrast, serum IgG levels were significantly increased in the rats with nerve injury; both the sciatic nerve crush and the sciatic nerve transection groups had significantly higher serum IgG levels compared to the sham-operated control group ($P<0.05$; Figure 2).

Hematoxylin and eosin (H&E) staining

At 21 days post-surgery, sciatic nerve tissue sections were collected from each group and stained

Table 1. Number of CD3+ or CD68+ immune cells in the sciatic nerve in each group

Group	Sham	Group 2	Group 3
CD3	0.00 ± 0.00	85.25 ± 15.17[a]	112.75 ± 10.90[a]
CD68	0.00 ± 0.00	16.48 ± 4.55[a]	27.22 ± 3.56[a]

Data are expressed as mean±SD of each group (n=8 per group) from three separate experiments
[a] $P < 0.01$ vs. sham group
Group 2- sciatic nerve crush
Group 3- sciatic nerve transection

with H&E. In the sham-operated control group, medullated fibers appeared to be arranged normally, and there was no inflammatory cell infiltration (Figure 3, group 1). In the rats with sciatic nerve injury, the numbers of medullated fibers were reduced, axons were inflamed and disarranged, and there were a large number of infiltrated lymphocytic cells. In addition, a portion of the nerve fibers had disintegrated and was engulfed by phagocytes (Figure 3, group 2, group 3).

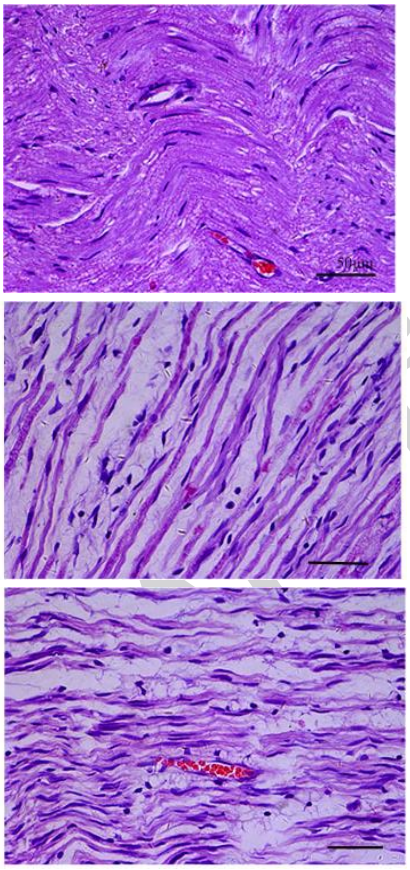


Figure 3. Histological analysis of the rat sciatic nerve 21 days after surgery. Representative examples of hematoxylin and eosin staining of the sciatic nerve 21 days after surgery in rats from Group 1- sham-operated control (*left panel*); Group 2- sciatic nerve crush injury (*middle panel*); and Group 3- sciatic nerve transection (*right panel*). Group 1 showed well-organized myelin sheets, round axons, and an absence of mononuclear infiltrates. Groups 2 and 3 showed several areas of edema, degraded myelin sheets, and several infiltrated mononuclear cells (arrows)

Immunohistochemical staining

At 21 days post-surgery, immunohistochemical staining of sciatic nerve tissue sections revealed a large number of CD3+ cells and a small number of CD68+ cells in rats with sciatic nerve injury (Figure 4). In contrast, CD3+ or CD68+ cells were not detected in the sham-operated control group. There were significant differences in the number of CD3+ and CD68+ cells in infiltrates between the rats with sciatic nerve injury and the sham-operated control group ($P<0.01$; Table 1). Although the numbers of CD3+ and CD68+ cells in the rats that underwent sciatic nerve transection were higher compared with rats with sciatic nerve crush injury, this difference was not statistically significant ($P>0.05$). These results indicate that a large number of lymphocytic cells had been recruited locally at the sciatic nerve injury site.

Qualitative analysis of immunoglobulin deposition

At 21 days post-surgery, IgG deposition (Figure 5, *group 1*) was not detected in sciatic nerve tissue sections in the sham-operated control group. However, there was sporadic IgG deposition along the nerve fiber bundle in rats from the sciatic nerve crush group and a moderate amount of IgG deposition along the nerve fiber bundle in rats with sciatic nerve transection (Figure 5, group 2, group 3).

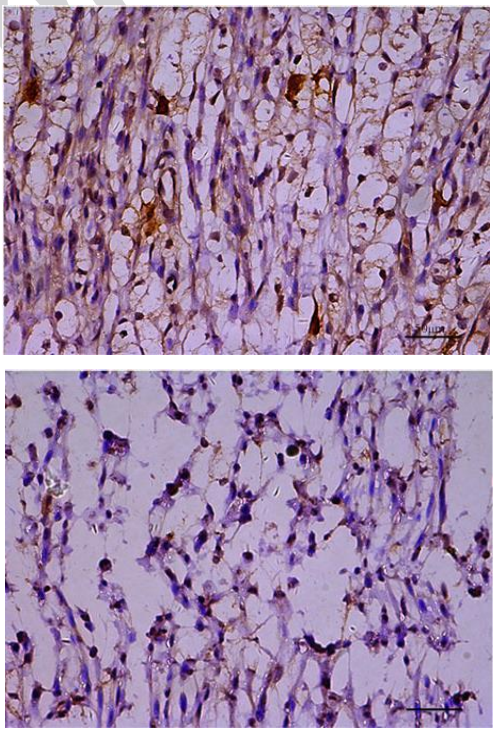


Figure 4. CD68 and CD3 expression in the sciatic nerve 21 days after surgery. Representative example of CD68 (A) and CD3 (B) immunohistochemical staining in the sciatic nerve from a rat that had undergone sciatic nerve crush injury. Macrophage (CD68) and T-cell (CD3) quantification at the sciatic nerve injury site or the region with cell infiltration was performed on 4 sections/rat (n=8 rats/group) and processed using the IPP6.0 software. Arrows indicate positive cells

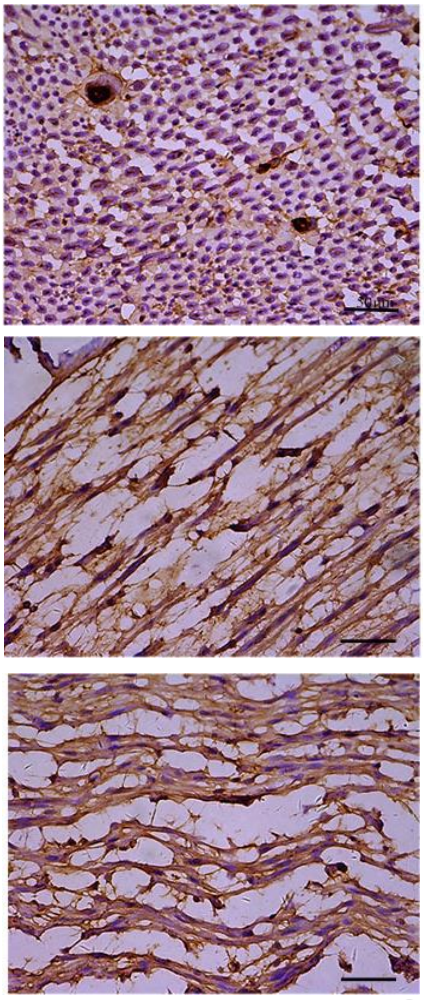


Figure 5. IgG deposition in the rat sciatic nerve 21 days after surgery. Representative examples of IgG immunohistochemical staining of the sciatic nerve 21 days after surgery in rats from Group 1- sham-operated control (*left panel*); Group 2- sciatic nerve crush injury group (*middle panel*); and Group 3- sciatic nerve transection (*right panel*). IgG was not observed in Group 1. Different degrees of IgG deposition were observed in rats from Group 2 and 3. Arrows indicate deposited IgG

Discussion

In this study, we used two rat models of sciatic nerve injury to investigate the humoral and cellular immune response following peripheral nerve damage. In both the sciatic nerve crush and sciatic nerve transection rat models we observed systemic and local changes in immune status after nerve injury and found that humoral and cellular immune responses were altered. Seven days after nerve injury, the percentage of $CD4^+$ cells and the $CD4^+/CD8^+$ ratio in the peripheral blood were significantly lower in rats with nerve damage compared with the sham-operated control rats. These findings indicate that rats have a low immune status after nerve injury, which may result in an underactive and poor performing immune system. This is consistent with the period of immunosuppression seen in patients after trauma (13). The period of low immune status in the rats was transient and T cell levels returned to normal 14 days after nerve injury.

It is well known that IgG and IgM are the major immunoglobulins in blood serum (14, 15). IgG is a product of the initial response to infection and secondary immune response and memory and therefore has important immune effects. IgM can activate complement more effectively than IgG, and serum levels of IgM begin to elevate when the humoral immune response is initiated. In our rat models of sciatic nerve injury, we observed significantly increased levels of serum IgM 14 days after nerve injury, which normalized by day 21. Levels of serum IgG increased only after 21 days; the delay may be because the generation of IgG in the blood requires antigen uptake, processing, and presenting. The systemic immune response seen in our rat models was likely the result of a breakdown in the blood-nerve barrier following nerve injury and the subsequent release of nerve antigens into the blood circulation, which stimulates immune cells and generates specific antibodies (16). We also observed IgG deposition locally at the sciatic nerve injury site, IgG deposition was not observed in the sham-operated controls. This further substantiates the suggestion that specific antibodies act on nerve cells after their release into the circulation to propagate an immune response.

In this study, we hypothesized that differences in the degree of nerve damage might be reflected by the cellular immune response at the nerve injury site. However, we found no significant difference in the percentage of $CD4^+$ cells, the $CD4^+/CD8^+$ ratio, and serum IgG and IgM levels in rats that had undergone sciatic nerve crush injury and sciatic nerve transection. This may be due to the acute nature of the injuries. A study of chronic lumbar disc herniation patients revealed significant differences in peripheral blood counts of $CD4^+$ cells and the $CD4^+/CD8^+$ ratio in patients stratified according to the degree of lower back pain (17). In contrast, CD3+ and CD68+ infiltration was less in the rats that had undergone sciatic nerve crush injury compared to the rats with a sustained sciatic nerve transection. CD3 is required for T cell activation and CD63 is expressed on the surface of monocytes/macrophages. Macrophages are able to take up, process, and present antigens to T lymphocytes. Macrophages also express MHC II antigens, which have a crucial role in the regulation of the immune response after peripheral nerve injury (18). The enhanced expression of MHC II antigen can cause the synthesis and deposition of immunoglobulin and immune cell infiltration, which has an inhibitory effect on nerve regeneration.

The present study showed that Wallerian degeneration of nerve fibers occurred after peripheral nerve injury. Wallerian degeneration refers to the sequence of events that occurs following nerve transection. Wallerian-like degeneration refers to similar events that occur in damaged axons after blunt or crush injury. Secondary damage to the neurons caused by the immune response after peripheral nerve injury can aggravate Wallerian degeneration and inhibit the repair and regeneration of peripheral nerves (19, 20).

Conclusion

In the present study, we found that during the 21 days following sciatic nerve injury in rats, there were changes in both the humoral and cellular immune systems, especially the cellular immune response that locally affects the injured nerve. Alterations in the humoral and cellular immune systems varied during the 21 days post-injury, likely reflecting different stages of activation. The significance of such changes in terms of the rate and efficacy of peripheral nerve recovery remains unclear. Furthermore, how signals of these changes in immune system status are relayed to the nervous system and translated requires further investigation. Additional study is needed to understand the mechanism of these processes to provide an adequate theoretical basis for the clinical application of immune system modulating drugs following peripheral nerve injury.

Conflict of Interest

We declare that we have no conflict of interest.

Acknowledgment

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