

Nerve Injury-related Autoimmunity Activation Leads to Chronic Inflammation and Chronic Neuropathic Pain

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ABSTRACT

Background: Peripheral nerve injuries that provoke neuropathic pain are associated with chronic inflammation and nervous lesions. The authors hypothesized that chronic neuropathic pain might be caused by chronic inflammation resulting from a nervous autoimmune reaction triggered by nerve injury.

Methods: The authors observed chronic inflammation and neuropathic behaviors for up to 12 weeks after nerve injury in T lymphocyte-deficient nude mice and their heterozygous littermates. Lymphocyte proliferation and Schwann cell apoptosis were examined after coculture of each population with various neural tissues from normal rats and those with nerve injury.

Result: Nude mice recovered faster and exhibited less thermal hyperalgesia after nerve injury compared to their heterozygous littermates. A large number of IL-17⁺ cells indicative of lymphocyte activation were found in the injured sciatic nerve and spinal cord (L4-6) of heterozygous littermates, but far fewer of these populations were found in nude mice. *In vitro* lymphocyte proliferation was enhanced after coculture with nerve tissues from normal rats compared to nerve tissue-free phosphate-buffered saline controls. In particular, coculture with sciatic nerve tissue enhanced proliferation by 80%, dorsal root ganglion by 46%, and spinal cord by 14%. Moreover, neural tissues from rats with nerve injury markedly increased the lymphocyte proliferation compared

What We Already Know about This Topic

- Neuropathic pain is often difficult to treat because its pathophysiology remains obscure
- A growing body of evidence suggests immune cells and their mediators to be involved in the production/maintenance of neuropathic pain

What This Article Tells Us That Is New

- Mice lacking mature T cells showed diminished hypersensitivity and lymphocyte proliferation after nerve injury than did wild-type controls
- Treatment with neural tissue supernatant from rats with nerve injury increased lymphocyte proliferation compared to treatment with neural tissue from sham-operated rats
- This study suggests that chronic neuropathic pain might be caused by chronic inflammation resulting from a nervous autoimmune reaction triggered by nerve injury

to coculture with tissues from corresponding normal rats. Schwann cell apoptosis was triggered *in vitro* when cocultured with lymphocytes from neuropathic rats.

Conclusion: Our study suggests that chronic neuropathic pain might be caused by chronic inflammation resulting from a nervous autoimmune reaction triggered by nerve injury.

NEUROPATHIC pain is a global clinical problem affecting a number of people.¹⁻³ This clinical condition is difficult to treat because the underlying mechanisms are unclear. However, there is increasing evidence that immune cells and their mediators, both in the periphery and the central nervous system, contribute to the generation and maintenance of such persistent pain.⁴⁻⁸ Microglia, as part of the cellular immune response, is activated by peripheral nerve injury, and microglial inhibition alleviates the development of hyperalgesia and allodynia.^{9,10} Macrophages are recruited to the site of the injured nerve, and the inhibition or depletion of macrophages also attenuates neuropathic pain.^{11,12}

Lymphocytes have been shown to invade the nerve lesion site, dorsal root ganglia, and corresponding spinal cord in rat models of neuropathic pain.¹³⁻¹⁵ In most inflammatory events, lymphocytes play a protective role and inhibit immune responses.^{16,17} In our previous study, cyclosporine A, a potent inhibitor of lymphocyte activation, was found to reduce thermal hyperalgesia after nerve injury.^{18,19} Low-dose methotrexate, a known immune suppressor when given intrathecally, reduced peripheral nerve injury-evoked neuropathic pain behavior in rats.^{20,21} Moreover, congenitally

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athymic nude rats, which lack mature T cells, developed significantly less mechanical allodynia and thermal hyperalgesia after nerve injury when compared to their heterozygous littermates. However, passive transfer of proinflammatory cytokine-producing type 1 T cells into nude rats enhanced the recipients' pain hypersensitivity to a level similar to that of the heterozygous donor rats.¹⁵ Recently, Th17 cells, a subset of CD4⁺ T cells, have received considerable attention because both the interleukin (IL)-17A transcript and IL-17A⁺ T cells were detected in degenerating nerves²² and mechanical hyperalgesia was significantly reduced in IL-17 knockout mice.²³ Although Th17 cell is reported as participating in the pathogenesis of certain autoimmune diseases,^{24,25} it is unknown how lymphocyte activation is initiated and whether they cause neural tissue damage directly or indirectly. Wallerian degeneration after nerve injury is tightly linked to the development of both pain and other pathological changes.^{26,27} Recently, nerve biopsy of patients with trigeminal neuralgia revealed alterations in the peripheral myelin, including deformation, thickening, demyelination, and remyelination. Changes were also observed in the peripheral axons, including atrophy or hypertrophy, increases in neurofilaments, loss of myelin, and occasional sprouting.²⁸ It is possible that the lesion is induced by a chronic inflammation due to the interaction between the immune system and the peripheral nerves. Therefore, we hypothesized that inflammation resulting from a nervous autoimmune reaction triggered by the exposure of nerve tissues to the immune system during nerve injury might be one of the main mechanisms of acute neuropathic pain that eventually becomes chronic. First, we attempted to confirm whether a persistent inflammation was present for up to 12 weeks after nerve injury in T lymphocyte-deficient mice and the extent of lymphocyte activation. Second, we completed an *in vitro* experiment to test whether the exposure of lymphocytes *in vitro* to various neural tissues, taken from normal and neuropathic rats would alter lymphocyte proliferation. Third, we examined the interactions between lymphocytes from neuropathic rats and normal Schwann cells.

Materials and Methods

This study consists of three parts. Please see table 1 for details. Long-term observation of neuropathic pain behaviors in T lymphocyte-deficient mice and their heterozygous littermates.

Animals and Surgical Procedures

Male Balb/c-nu/nu nude mice (Laboratory Animal Center, Sichuan University, Chengdu, China), which lack mature T cells, and their heterozygous littermates (wild type), 6–8 weeks of age, weighing 25–30 g, were used in the following experiments. Animals were housed in a specific pathogen-free environment under controlled conditions (12-h light/dark cycle, room temperature 24°C, 70–80% relative humidity) with free access to food and water. Protocols were approved

by the Scientific and Ethics Committee of Sichuan University and adhered to the guidelines of the Committee for Research and Ethical Issues of the International Association for the Study of Pain. Mice were anesthetized with pentobarbitone sodium (10 mg/kg intraperitoneally for mice). Loose ligation of the sciatic nerve was carried out under aseptic conditions.²⁹ Briefly, the right sciatic nerve was exposed at the level of the proximal thigh and freed of connective tissue. Three loose ligations of the sciatic nerve were performed without any constriction of the nerve. Ligatures were tied loosely around the nerve with 5-0 silicon-treated silk sutures at a spacing of 1 mm to preserve epineural circulation. Control animals underwent a sham operation in which the sciatic nerve was exposed but not ligated. The incision was closed in layers with sutures. All animals received an injection of penicillin (10,000 U intraperitoneally) after surgery. Ten pairs of Balb/c-nu/nu nude mice and wild-type mice had nerve injury for the observation of thermal latencies and mechanical thresholds for up to 12 weeks, that is, up to postoperative day (POD) 84.

Nociceptive Testing Thermal Hyperalgesia.

Animals were adapted to the test environment for at least 30 min (until normal grooming behavior resumed) before any behavioral examinations. Thermal hyperalgesia was assessed according to the method proposed by Hargreaves³⁰ using a paw withdrawal test (7370-Plantar test, Ugo Basile, Comerio, Italy). Animals were placed in a transparent perspex box on a thin glass platform. An infrared light beam was used that was adjusted to the intensity at which normal mice reacted within approximately 6–10 s. The infrared beam stimulus was applied to the plantar surface of a hind paw. When the mouse withdrew its paw, the thermal stimulus was automatically switched off by a photocell, and an electronic timer recorded the withdrawal latency. This time was defined as the thermal latency and was accurate to within 0.1 s. To avoid tissue damage, trials were terminated after 20 s if the mouse had not withdrawn its hind paw. Five measurements were made on alternate paws at 3-min intervals, and the mean thermal latency for paw withdrawal was calculated. Left and right hind paws were measured in the same manner with a random order of presentation.

Mechanical Hyperalgesia

Mechanical hyperalgesia was assessed by placing each animal on an elevated wire grid and stimulating the plantar surface of the right hind paw using an electronic von Frey anesthesiometer (37400 - Plantar Von Frey, Ugo Basile). The device applied increasing pressure to the hind paw three times in 2-min intervals. The intensity of mechanical stimulation was increased until the hind paw was withdrawn, and a brisk withdrawal in response to the stimulus was considered as a positive reaction. The mechanical withdrawal threshold

Table 1. List of Experiments

Experiments	Type of Experiments	Animals Used	Examinations
Nude mice exp	<i>In vivo</i>	Nude mice and heterozygous littermates	Chronic neuropathic pain and chronic inflammation
Lymphocyte proliferation exp	<i>In vitro</i>	PBMCs and nervous supernatant from nerve injured and noninjured	Lymphocyte proliferation responses to normal nerve tissue and to those from nerve-injured rats
Schwann cell exp	<i>In vitro</i>	Schwann cells from newborn rats; lymphocytes from nerve injured and noninjured	Schwann cell apoptosis when directly or indirectly cocultured with lymphocytes from normal rats and from nerve-injured rats

exp = experiments; PBMCs = peripheral blood mononuclear cells.

was expressed as the force at which the mouse withdrew its paw. To avoid tissue damage, a cut-off threshold was set at 50g. Three responses were recorded per paw per session, and mechanical hyperalgesia was considered to be a significant reduction in mechanical withdrawal thresholds. The mechanical thresholds of both hind paws were tested separately in a random order.

Thermal latencies and mechanical thresholds were measured the day before the operation to derive baseline values and then at POD 3, 5, 14, 21, 28, 42, 56, 70, and 84.

Examination of Immune Responses in the Injured Nerve Tissues of T Lymphocyte-deficient Mice and Their Heterozygous Littermates

Nerve and Spinal Cord Harvesting and Processing. Twelve weeks after the nerve injury (POD 84) and after long-term observation of neuropathic pain behavior was complete, five nude mice and five of their heterozygous littermates were sacrificed with lethal injections of sodium pentobarbital (80 mg/kg intraperitoneally). Mice were perfused intracardially with 0.9% saline followed by fresh 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for tissue fixation. After the perfusion, a segment of injured sciatic nerve 5 mm in length and containing three ligatures were harvested. A corresponding segment of the sciatic nerve was also harvested from a normal mouse. The L4-6 spinal cord segments were also removed from all mice. All tissues were post-fixed in 4% paraformaldehyde for 12 h, transferred to 80% alcohol overnight, and then embedded in paraffin. All sections (5 μ m) were cut on a microtome and mounted on slides precoated with 2% 3-aminopropyl-triethoxysilane (ZSGB-BIO, 1:50). Tissue samples were also collected and processed as described above (from the second sentence to the sixth sentence in this paragraph) from another 10 pairs of nude mice and heterozygous littermates that had been subjected to nerve injury and sacrificed at 4 weeks (POD 28) and 8 weeks (POD 56) postinjury.

Immunohistochemical Staining of Immune Cells

Tissue sections were deparaffinized and rehydrated through graded concentrations of ethanol. To block non-specific staining, sections were treated with 5% normal

serum derived from the same host as the secondary antibody in 0.01 mol/l phosphate-buffered saline (PBS, pH 7.6). Sections were then incubated overnight at 4°C with one of the following primary antibodies: rabbit polyclonal anti-IL17 (sc-7927, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, 1:200) or mouse anti-ED-1 immunoglobulin G (Boster Biological Technology, Ltd., Wuhan, China, 1:100; macrophage marker in peripheral sciatic nerve and microglia marker in spinal cord, respectively). Tissue samples were then labeled with biotinylated secondary antibody (SPN-9001 SP kits, ZYMED, South San Francisco, CA, 1:300) at 37°C for 30 min followed by incubation with avidin-biotin peroxidase for 30 min (SPN-9001 SP kits, ZYMED). The biotin-avidin complex was detected with diaminobenzidine (DAB substrate kit, ZYMED), which produces a dark brown end product on catalysis of the biotin-avidin complex. Sections were finally dehydrated in graded ethanol solutions, cleared with xylene and cover slipped with gums.

Cell Counting

Cell profiles were viewed using an Olympus light microscope and analyzed with the Olympus Computer Assisted Stereological Toolbox system (Olympus Corporation, Tokyo, Japan). For each nerve section, the area of section in μ m² was measured, and then 80% of the section area was randomly sampled to count the number of macrophages and IL-17⁺ cells ($n = 5$ mice at each time-point, five sections per nerve specimen). For each spinal cord section, the total area of the dorsal horn ipsilateral to the injured sciatic nerve was measured, and 50% of this area was then randomly sampled to count the number of IL-17⁺ cells and microglia. The results were expressed as cells/mm². Four to six sections per specimen (from each mouse) were counted, and three to five animals were included at each time-point for calculation of mean cell densities in the spinal cord.

Lymphocyte Proliferation after In Vitro Exposure to Neural Tissue from Normal Rats or Nerve-injured Rats

Forty Sprague Dawley adult rats, weighing 250–300 g, were randomly divided into nerve injury group and sham-operated group. Rats were deeply anesthetized with

pentobarbitone sodium (40 mg/kg intraperitoneally). Loose ligation of the right sciatic nerve was carried out under aseptic conditions as described above (Please see Animals and surgical procedures section). Four loose ligations of the sciatic nerve were performed without any constriction of the nerve. Ligatures were tied loosely around the nerve with 5-0 silicon-treated silk sutures at a spacing of 1 mm to preserve the epineural circulation. In the sham-operated group, the sciatic nerve was exposed but not ligated. Thermal latency and mechanical threshold were measured the day before and up to 14 days after surgery (at POD 1, 3, 5, 7, and 14).

Rats were sacrificed on POD 14. Peripheral blood mononuclear cells from each rat were obtained by centrifugation at 1500 g/min for 20 min and resuspended in RPMI-1640 culture medium (supporting the growth of lymphocytes) supplemented with 10% fetal calf serum, L-glutamine, penicillin, and streptomycin. A segment of the right sciatic nerve (2 cm in length with the ligature in the middle in nerve-injured rats or the corresponding section in the sham-operated rats) and spinal cord segments (L4-L6) of each rat were collected, homogenized, and then resuspended in PBS. Samples were centrifuged, and the supernatants were harvested. Lymphocytes collected from each rat were divided equally into four wells. Supernatants and culture medium were mixed in 1:9 ratio and added to the lymphocytes from the same rat, then cultured at 37°C at a density of 5×10^6 cells/ml in a fully humidified atmosphere containing 5% CO₂. The same volume of PBS was added to the neural tissue supernatant-free controls. After lymphocytes were cultured for 68 h, MTT 50 µl/well was added. Lymphocyte proliferation was estimated at 72 h by absorbance (optical density value, OD value) with an automatic microplate reader at 570 nm (the mean value of the three replicates) after adding the neural tissue supernatant.

Apoptosis of Schwann Cells upon Coculture with Lymphocytes from Nerve-injured Rats

Schwann cells from the sciatic nerves of newborn Sprague Dawley rats (two days old) were dissociated and cultured as per the methods of Brookes, Fields, and Raff.³¹ Briefly, 20 neonatal rats were sacrificed with sodium pentobarbital and washed with 70% ethanol. Forty sciatic nerves were dissected, freed from blood vessels and fatty tissue, cut into small pieces, and digested for 10 min at 37°C in 10 ml Dulbecco's Modified Eagle's Medium (DMEM, Hyclone Laboratories Inc., South Logan, UT) containing 0.1% collagenase and 0.25% trypsin. To obtain a single cell suspension, the nerve pieces were gently triturated. The cell suspension was then plated at a density of 2×10^6 cells/ml in poly-L-lysine-coated 60 mm dishes with 5 ml DMEM containing 10% Fetal Bovine Serum Gold (PAA Laboratories Inc., Ontario, Canada; 1% glutamine, 100 U penicillin, and 100 µg/ml streptomycin). Fibroblast growth was reduced by the addition of 10 µM cytosine arabinoside

(Sigma, Saint Louis, MO) to the medium for four days. For the confirmation of Schwann cells, cells were plated onto poly-L-lysine-coated glass coverslips in 35-mm dishes and immunoassayed with S-100 antibody at a concentration of 1:500 (Beijing Biosynthesis Biotechnology Co. Ltd., Beijing, China).

Lymphocytes were extracted from the peripheral blood of rats 14 days after nerve injury or sham operation and then cocultured with Schwann cells by indirect or direct methods. For the indirect coculture, a trans-well (PET transparent Millipore Corporation, Billerica, MA) was used with lymphocytes in the upper chamber and Schwann cells in the lower chamber. The upper chamber had pore sizes of 1.0 µm, allowing cytokines released from lymphocytes to pass through the barrier and to interact with Schwann cells. For direct contact, lymphocytes were added directly to the Schwann cell culture at a concentration of 2×10^5 Schwann cells/ml and 2×10^6 lymphocytes/ml. Lymphocytes from one rat were divided into two equal parts, one of which was used for indirect coculture and one for direct coculture. A control Schwann cell culture without added lymphocytes was also prepared. After coculture for 24 h, apoptosis of Schwann cells was detected by flow cytometry for six groups of samples and by TUNEL staining for another six groups. Each group included six culture wells divided into two subgroups (one for nerve-injured rats and the other for sham-operated rats). Within each subgroup, one well contained an indirect coculture between Schwann cells and lymphocytes, another a direct coculture, and the third was a control in which there were Schwann cells but no lymphocytes. For flow cytometry analysis, Schwann cells were washed in PBS and resuspended in a binding buffer supplied with the Annexin V-PE/7-AAD apoptosis detection kit (Nanjing Kaiji Bioscience, Nanjing, China). Samples were incubated in the dark at room temperature for 15 min. Subsequently, apoptosis of Schwann cells was measured using flow cytometry (FACS Aria; BD Biosciences, San Jose, CA) followed by data analysis with FACSDiVa™ 6.0 software. For TUNEL staining, Schwann cells were cultured on the sterile cover slips of six wells, fixed with 4% paraformaldehyde, and stained according to the manufacturer's instructions for the TUNEL kit (Roche Applied Science, Mannheim, Germany). TUNEL-positive cells were viewed under a light microscope (DP70, Olympus Corporation) equipped with a digital camera (BX51, Olympus Corporation). The ratios of positively stained cells to total cells were calculated with the Computer Assisted Stereological Toolbox system (revision 0.9.5; 2000 Olympus Denmark A/S, Ballerup, Denmark). All examinations were carried out by blinded observers.

Statistical Analysis

Data were analyzed with the SPSS (Statistical Package for the Social Sciences) statistical package (SPSS 15.0, SPSS Inc., Chicago, IL). Two-way repeated measures

ANOVA with group/condition and time (Days postop) as a repeated measures factor, followed by the Tukey *post hoc* test, was used for the analysis of behavioral data. Cell counts of IL-17⁺ cell, microglia, and macrophages at different time-points in selected groups were compared with two-way ANOVA, followed by the Tukey *post hoc* test. Data on the apoptosis of Schwann cells derived from flow cytometry and TUNEL staining in selected groups were compared with one-way ANOVA, followed by the Tukey *post hoc* test. The extent of Schwann cell apoptosis between the direct group and indirect group was compared using the Student's *t*-test. Data are provided as the mean \pm SD. A *P* value less than 0.05 was regarded as statistically significant.

Results

Neuropathic Pain Behaviors Developed Differently between T Lymphocyte-deficient Mice and Their Heterozygous Littermates

Before nerve injury, no significant differences in thermal latency and mechanical thresholds were evident between nude mice and their heterozygous littermates. Thermal

latencies remained unchanged in both types of mice after sham operations compared with preoperative baseline values (fig. 1A). After injury to the sciatic nerve, thermal latencies in both nude mice and their heterozygous littermates showed a reduction on the injured side. The reduction for thermal latency ($P = 0.001$ for all by repeated-measures, two-way ANOVA) and mechanical threshold ($P = 0.011$ for all by repeated-measures, two-way ANOVA) was significant for time, group, and the interaction of time and group. Thermal latency in the heterozygous littermates was 5.68 ± 0.71 s on POD 3 and reached its minimum value on POD 7 (4.98 ± 0.73 s). These levels remained low and did not return to baseline by POD 84 (8.31 ± 0.61 s) (fig. 1B). In the nude mice, thermal latency began to decline on POD 3 (6.62 ± 0.61 s) and reached a minimum on POD 14 (6.61 ± 0.70 s) (fig. 1C), which was much higher than the minimum for the heterozygous littermates (fig. 1D). Furthermore, thermal latency in nude mice recovered to baseline values on POD 42. Figure 1 shows the time course of thermal latency changes in nude mice and their heterozygous littermates after nerve injury. Mechanical thresholds remained unchanged in both types of mice after sham operations compared with preoperative baseline values

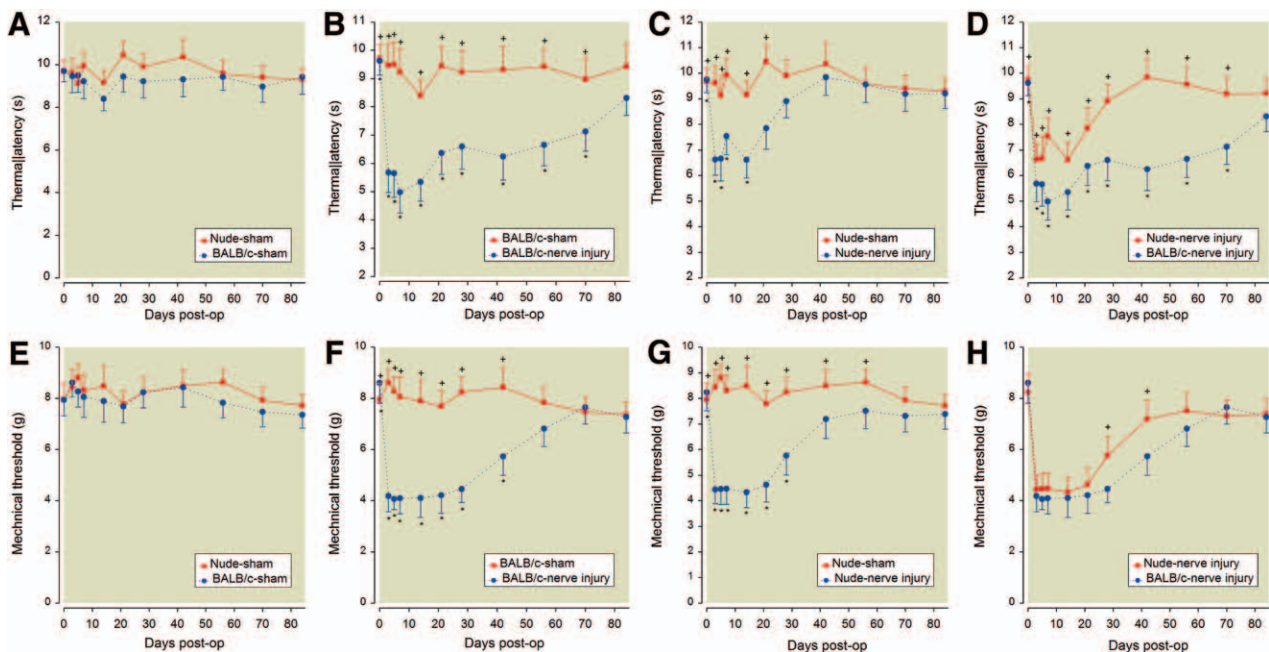


Fig. 1. Time course of hyperalgesia after nerve injury in nude mice and heterozygous littermates. Nerve injury was carried out on day 0, after measurement of baseline thermal latencies. Thermal latencies and mechanical thresholds remained stable after sham operation in both types of mice (A). After injury to the sciatic nerve, thermal latencies in both the nude mice and their heterozygous littermates showed a reduction on the injured side. Heterozygous littermates showed significant thermal hyperalgesia for up to 84 days after nerve injury (B, open symbols). Thermal latencies also decreased in nude mice after nerve injury, but did not decrease to the same level as in their heterozygous littermates, and their recovery was faster (C, D). Mechanical thresholds remained stable after sham operation in both types of mice (E). Heterozygous littermates showed significant mechanical hyperalgesia after nerve injury up to POD 56 (F). Nude mice developed mechanical hyperalgesia, but recovered faster after the nerve injury (G, H). Error bars represent SD ($n = 5$). Data were analyzed by two-way repeated measures ANOVA followed by the *post hoc* Tukey test. Asterisks indicate a significant difference from the baseline value on day 0 ($*P < 0.05$, Tukey). Plus symbols indicate a significant difference between two groups, at indicated time-points ($+P < 0.05$, Tukey). BALB = Wild Type BALB/c mouse; POD = postoperative day.

(fig. 1E). The time course of mechanical threshold values after nerve injury was slightly different from that of thermal latency. On the injury side, the mechanical thresholds of heterozygous littermates decreased and reached a minimum on POD 5 (4.06 ± 0.41 g). These values gradually recovered and returned to baseline value by POD 70 (fig. 1F). Mechanical threshold values of the nude mice declined to the same level as their heterozygous littermates on POD 3 (4.43 ± 0.55 g) but recovered faster, returning to baseline by POD 56 (fig. 1, G and H).

Immune Cell Infiltration in Nerve Tissue after Injury
Time Course of Lymphocyte Numbers in Peripheral Blood of Nude Mice. To confirm lymphocyte deficiency in nude mice, we measured the numbers of lymphocytes in the peripheral blood of nude and heterozygous mice at POD 28, 56, and 84 (fig. 2). The numbers of lymphocytes in the peripheral blood were 0.43 ± 0.01 ($\times 10^9$) in normal nude mice, and 0.85 ± 0.02 , 1.24 ± 0.03 , and 0.6 ± 0.09 ($\times 10^9$) on POD 28, 56, and 84, respectively. The numbers of lymphocytes in the peripheral blood were 1.2 ± 0.03 ($\times 10^9$) in normal heterozygous mice and 1.87 ± 0.04 , 3.96 ± 0.11 , and 1.48 ± 0.03 ($\times 10^9$) on POD 28, 56, and 84, respectively. The changes for lymphocyte numbers were significant for time, group, and the interaction of time and group ($P < 0.0001$, two-way ANOVA). The numbers of lymphocytes in the peripheral blood of nude mice were significantly lower than in the heterozygous mice either under normal conditions or after nerve injury.

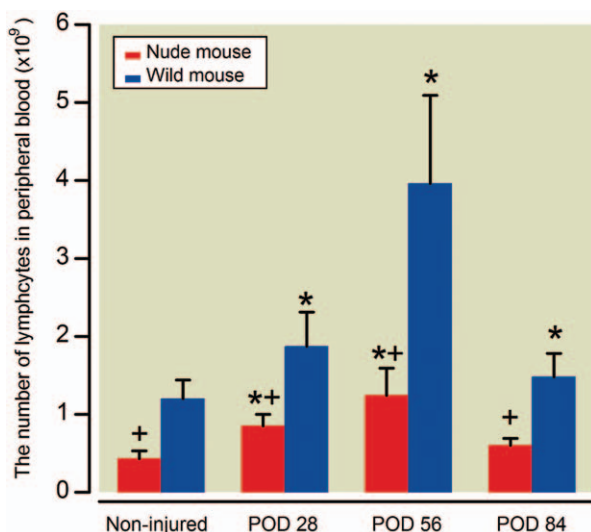


Fig. 2. Reduction in numbers of the lymphocyte of peripheral blood in nude mice. Numbers of lymphocytes in peripheral blood of nude mice were significantly lower than in the heterozygous mice. Error bars represent SD ($n = 5$). Data were analyzed by two-way ANOVA followed by the *post hoc* Tukey test. Asterisks indicate a significant difference from the baseline numbers of lymphocytes of peripheral blood in the nude mice ($*P < 0.05$, Tukey). Pluses indicate a significant difference between the two groups at indicated time-points ($+P < 0.05$, Tukey). POD = postoperative day.

IL-17⁺ Cell at the Sciatic Nerve Injury Site and L4-6 Lumbar Spinal Cord after Nerve Injury

IL-17⁺ cells were barely detectable in the noninjured sciatic nerves of both types of mice (fig. 3, A and B). Numbers of IL-17⁺ cell at the sciatic nerve injury site ($P = 0.013$, two-way ANOVA) and L4-6 lumbar spinal cord ($P = 0.003$, two-way ANOVA) were significantly increased in the injured nerve, by comparison with the noninjured nerve. The expression of IL-17⁺ cell in the sciatic nerve of the heterozygous littermates after nerve injury was $575 \pm 43/\text{mm}^2$, $107 \pm 11/\text{mm}^2$, and $39 \pm 13/\text{mm}^2$ on POD 28, 56, and 84, respectively (fig. 3, C–E). However, numbers of IL-17⁺ cell at the sciatic nerve injury site in nude mice were much lower than in the heterozygous littermates with $95 \pm 14/\text{mm}^2$, $42 \pm 12/\text{mm}^2$, and $14 \pm 9/\text{mm}^2$ on POD 28, 56, and 84, respectively (fig. 3, F–H). Unlike the sciatic nerve, which had almost no IL-17⁺ cells before nerve injury, there were IL-17⁺ cells in the noninjured spinal cords of both groups of mice (fig. 3, I and J), and these numbers increased dramatically in heterozygous littermates after sciatic nerve injury (fig. 3, K–M). However, the increase in IL-17⁺ cell was much less in the injured spinal cords of nude mice after nerve injury. For heterozygous littermates, the number of IL-17⁺ cell in the spinal cord increased to $506 \pm 31/\text{mm}^2$ on POD 28 (fig. 3K), subsequently dropped to $363 \pm 24/\text{mm}^2$ on POD 56 (fig. 3L), and increased to $117 \pm 8/\text{mm}^2$ on POD 84 (fig. 3M). In the nude mice, the IL-17⁺ cells in the spinal cord were $195 \pm 10/\text{mm}^2$, $82 \pm 7/\text{mm}^2$, and $78 \pm 11/\text{mm}^2$ on POD 28, 56, and 84, respectively (fig. 3, N–P). IL-17⁺ cells at the sciatic nerve injury site and L4-6 lumbar spinal cord are as shown in figure 3, Q and R after nerve injury.

Macrophage and Microglia Infiltration after Sciatic Nerve Injury

ED1⁺ profiles were barely detectable in the sciatic nerves of noninjured mice (fig. 4, A and B). Numbers of macrophages ($P < 0.0001$, two-way ANOVA) and microglia ($P = 0.001$, two-way ANOVA) were significantly increased in the injured nerve than the noninjured nerve. The expression of macrophages in the sciatic nerves of heterozygous littermates was dramatically increased after nerve injury to $557 \pm 37/\text{mm}^2$ on POD 28 (fig. 4C) followed by a decrease to $287 \pm 18/\text{mm}^2$ on POD 56 (fig. 4D) and $84 \pm 7/\text{mm}^2$ on POD 84 (fig. 4E). The numbers of macrophages in the sciatic nerves of nude mice after nerve injury were significantly lower than those in the heterozygous littermates at all time-points ($223 \pm 14/\text{mm}^2$, $80 \pm 8/\text{mm}^2$, and $37 \pm 4/\text{mm}^2$ on POD 28, 56, and 84, respectively; fig. 4, F–H). The mean numbers of microglia (fig. 4, I and J) was $58 \pm 5/\text{mm}^2$ in the noninjured spinal cords of heterozygous littermates and $56 \pm 5/\text{mm}^2$ in noninjured nude mice spinal cords. After sciatic nerve injury, the number of microglia in the spinal cords of heterozygous littermates increased to $323 \pm 17/\text{mm}^2$ on POD 28 (fig. 4K), $276 \pm 21/\text{mm}^2$ on POD 56 (fig. 4L), and 112 ± 13

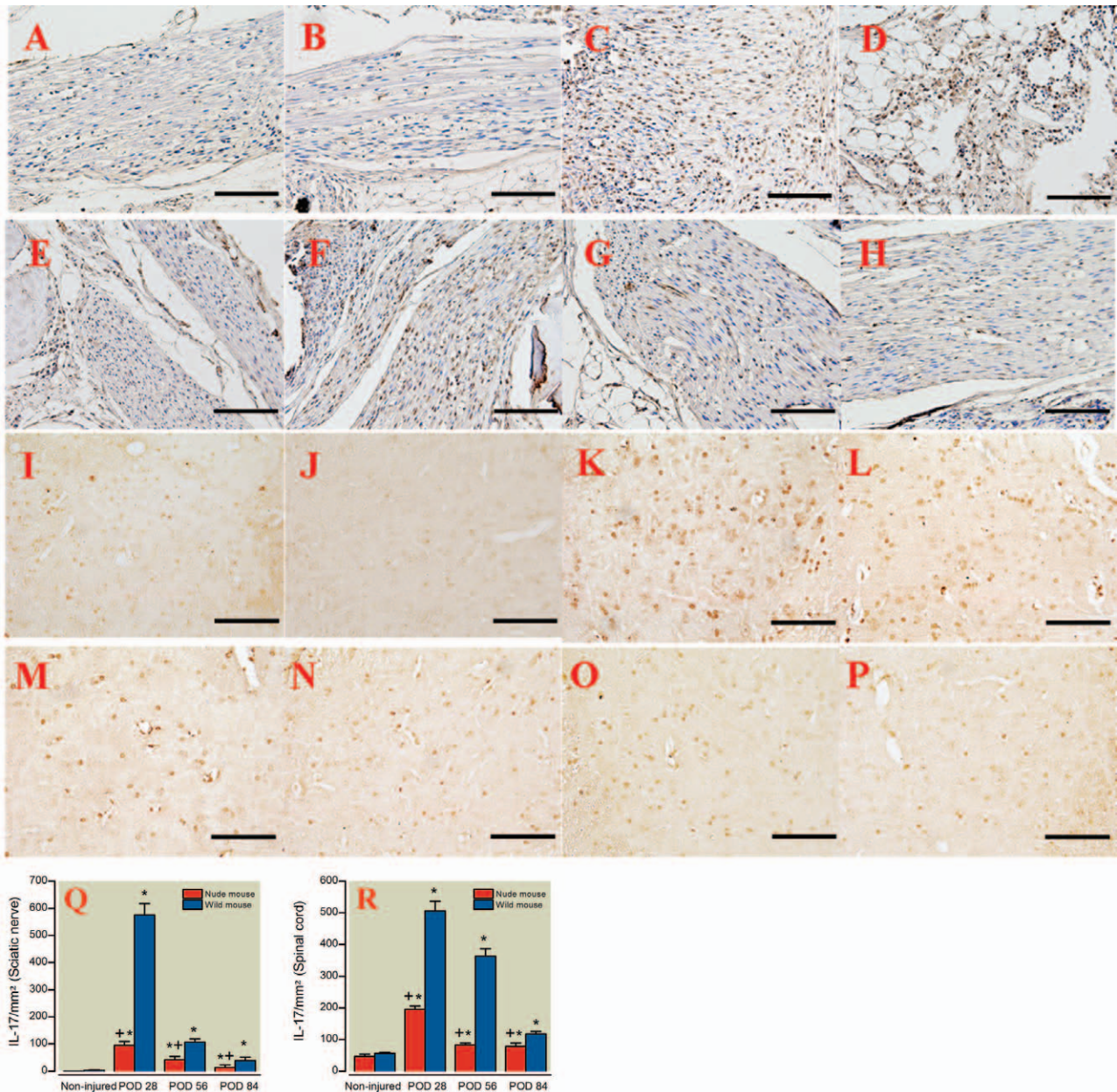


Fig. 3. Interleukin (IL)-17⁺ cell at injury site in the sciatic nerve and at L4-6 lumbar spinal cord after nerve injury. Representative photomicrographs of the injured region of the sciatic nerve region and the ipsilateral dorsal horn of L4-6 lumbar spinal cord. Almost no IL-17⁺ cell were seen in the noninjured sciatic nerve of heterozygous littermates and nude mice (A, B). After nerve injury, expression of IL-17⁺ cell in the sciatic nerve (C, D, E) of heterozygous littermates was dramatically increased on POD 28, and slightly increased on POD 56, and POD 84. There were far fewer IL-17⁺ cell in the sciatic nerve (F, G, H) of nude mice on POD 28, 56, and 84. Unlike the sciatic nerve, there were IL-17⁺ cell in the noninjured spinal cords of both groups of mice (I, J). Expression of IL-17⁺ cell in the spinal cord of heterozygous littermates (K, L, M) and nude mice (N, O, P), is shown on POD 28, 56, and 84. The numbers of expression of IL-17⁺ cell in spinal cord L4-6 and injured nerve were much fewer in nude mice on POD 28, 56, and 84 than in their heterozygous littermates (Q, R). Error bars represent SD (n = 5). Data were analyzed by two-way ANOVA followed by the *post hoc* Tukey test. Asterisks indicate a significant difference from the baseline value (**P* < 0.05, Tukey). Pluses indicate a significant difference between the two groups at the indicated time-points (+*P* < 0.05, Tukey; 40×, scale bar = 5 μm). POD = postoperative day.

/mm² on POD 84 (fig. 4M). Microglia densities in the lumbar spinal cords of the nude mice were $225 \pm 13/\text{mm}^2$, $170 \pm 14/\text{mm}^2$, and $77 \pm 11/\text{mm}^2$ on POD 28, 56, and 84, respectively (fig. 4, N–P). Numbers of macrophages

and microglia are as shown in figure 4, Q and R after nerve injury.

Lymphocyte proliferation was enhanced by neural tissue supernatant, depending on the source.

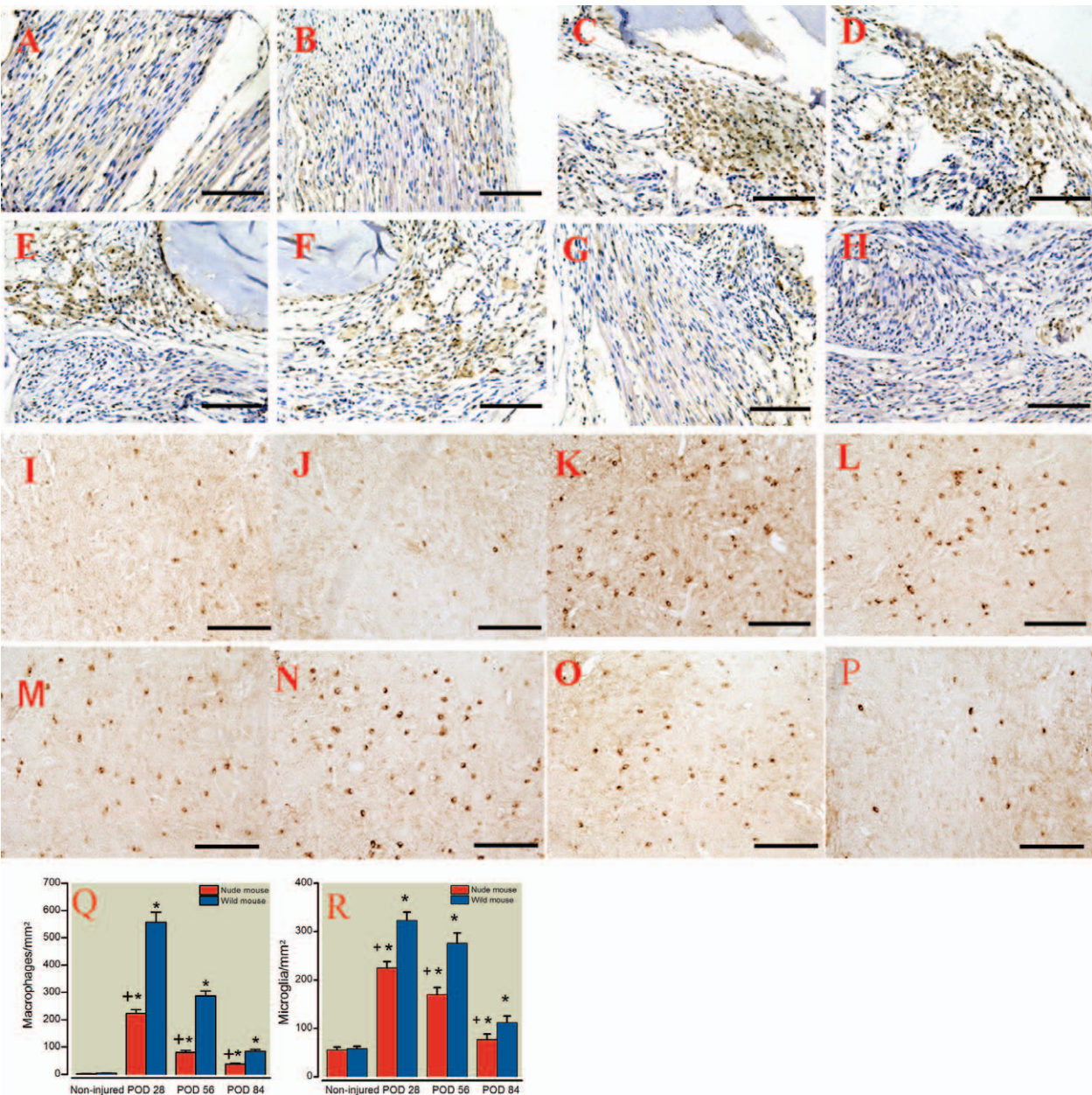


Fig. 4. Numbers of macrophages and microglia after nerve injury. Macrophages were barely detectable in the sciatic nerve of heterozygous littermates and nude mice before nerve injury (A, B). The number of macrophages in the sciatic nerve of heterozygous littermates after nerve injury is shown in C, D, and E at POD 28, 56, 84. There were significantly fewer macrophages at the nerve injury site in the nude mice on POD 28, 56, 84 (F, G, H) than in their heterozygous littermates. A basal level of ED1 positive profiles indicating microglia was expressed in the spinal cord of heterozygous littermates (I) and nude mice (J). Activation of microglia in the spinal cord of heterozygous littermates (K, L, M) and nude mice (N, O, P), is shown on POD 28, 56, and 84. The numbers of macrophages at the nerve injury site and microglia in spinal cord L4-6 were much fewer in nude mice on POD 28, 56, and 84 than in their heterozygous littermates (Q, R). Open bars represent the number of macrophages or microglia in nude mice; hatched bars represent the number of macrophages or microglia in heterozygous littermates. Error bars represent SD (n = 5). Data were analyzed by two-way ANOVA followed by the *post hoc* Tukey test. Asterisks indicate a significant difference from baseline values in normal mice (* $P < 0.05$, Tukey). Pluses indicate a significant difference between the two groups at indicated time-points (+ $P < 0.05$, Tukey). Representative photomicrographs of the injured region of the sciatic nerve and the ipsilateral dorsal horn of the L4-6 lumbar spinal cord are shown here (40 \times , scale bar = 50 μ m). POD = postoperative day.

Confirmation of Hyperalgesia in Rats with Nerve Injury

Before nerve injury, no significant differences in thermal latency or mechanical thresholds were evident between the

nerve injury and sham-operated groups. After injury to the sciatic nerve, thermal latencies in Sprague Dawley rats showed a significant reduction on the injured side.

Table 2. Optical Density Value (OD Value, 570 nm) of the Four Groups (mean \pm s, g, n = 20)

Group	Sham Group	Nerve Injury Group
Phosphate-buffered saline control	0.31 \pm 0.03	0.33 \pm 0.03
Sciatic nervous supernatant	0.57 \pm 0.05*	0.81 \pm 0.05*§
Dorsal root ganglion supernatant	0.46 \pm 0.06*†	0.62 \pm 0.10*†§
Spinal cord supernatant	0.36 \pm 0.04*†‡	0.45 \pm 0.07*†‡§

OD values measured at 570 nm in sham and nerve injury groups treated with various neural tissues.

* Significant difference from the control group; † significant difference from the sciatic nerve group; ‡ significant difference from the dorsal root ganglion group; § significant difference from their corresponding sham group (in each case, $P < 0.05$, Student's t -test).

Treatment with Neural Tissue Supernatant from Rats with Nerve Injury Increased Lymphocyte Proliferation Compared with Treatment with Neural Tissue from Sham-operated Rats

Lymphocyte proliferation was estimated using absorbance (OD value) derived from an automatic microplate reader at 570 nm. A higher OD value indicated greater proliferation. In the control group in which PBS was added to media instead of neural tissue supernatant, OD values measured at 72 h after addition of the neural tissue supernatant were 0.31 \pm 0.03 and 0.33 \pm 0.03 in the sham and nerve injury groups, respectively. OD values were significantly increased in the sham group by adding neural tissue supernatant. The greatest increase was produced by addition of supernatant from the sciatic nerve (OD increased by 80%) followed by dorsal root ganglion (46%) and spinal cord tissue, which produced the smallest increase (14%). Adding neural tissue supernatant from the rats with nerve

injury was more effective in increasing OD values than adding corresponding supernatants from the sham operated rats. Proliferation increases induced by supernatants from the respective tissues included the following: sciatic nerve, 42% (0.81 \pm 0.05); dorsal root ganglion, 34% (0.62 \pm 0.10); and spinal cord, 25% (0.62 \pm 0.10). Please see table 2 for details.

Survival or Apoptosis of Schwann Cells when Cocultured with Lymphocytes from Nerve-injured Rats

Schwann cell growth on poly-L-lysine with high proliferation rates (>90%) was confirmed by S-100 immunoassay and viewed using both phase-contrast (fig. 5A) and fluorescence microscopy (fig. 5B).

Schwann Cell Apoptosis Detected by Flow Cytometry

After 24 h coculture, Schwann cells were stained with Annexin V-PE/7-AAD and analyzed with FACS Aria flow cytometry to determine apoptosis rates, which were 3.9% \pm 0.84%, 30.1% \pm 4.50%, and 24.2% \pm 3.52%, in the control group (fig. 6A), nerve injury trans-well group (fig. 6B), and nerve injury direct contact group (fig. 6C), respectively. Apoptosis rates of Schwann cells in the sham trans-well group (fig. 6D) and sham direct contact group (fig. 6E) were 13.0% \pm 2.36% and 6.8% \pm 1.46%, respectively. Apoptosis rates of Schwann cells was higher *in vitro* when cocultured with lymphocytes from neuropathic rats ($P = 0.007$, one-way ANOVA) compared with the corresponding control group. Flow cytometry results (fig. 6F) showed a significant reduction in Schwann cell numbers in the nerve injury trans-well group ($P < 0.0001$, Tukey) and direct contact groups ($P = 0.001$, Tukey) compared with the corresponding sham groups. The differences in numbers of apoptotic Schwann cells in the sham trans-well group ($P = 0.012$, Tukey) and direct contact group ($P = 0.001$, Tukey) compared with the corresponding control groups were also statistically significant. However, the difference between apoptotic

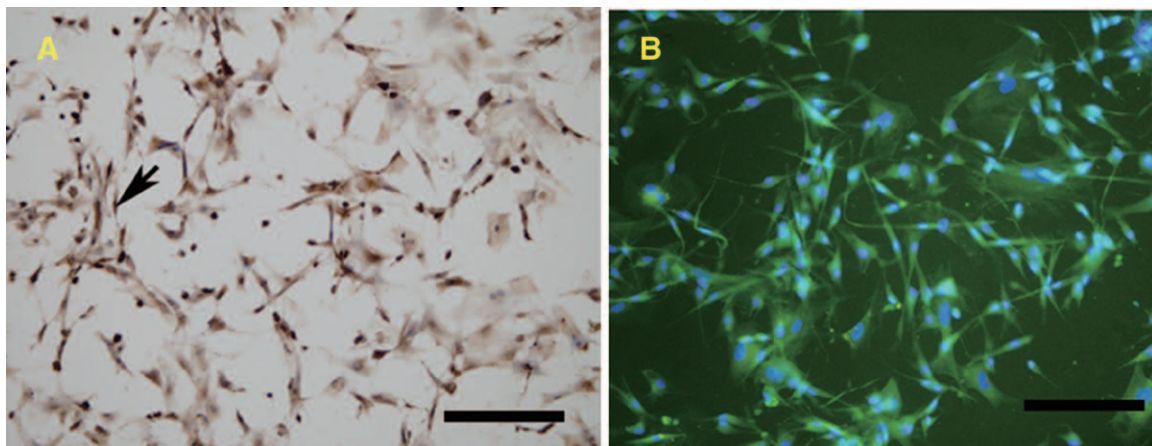


Fig. 5. Confirmation of Schwann cell identity with S-100 immunoassay. (A) Cultured Schwann cells labeled with S-100 antibody (phase-contrast microscopy). Arrow indicates Schwann cell. (B) Schwann cell cytoplasm and nuclei under fluorescence microscopy (scale bars = 50 μ m).

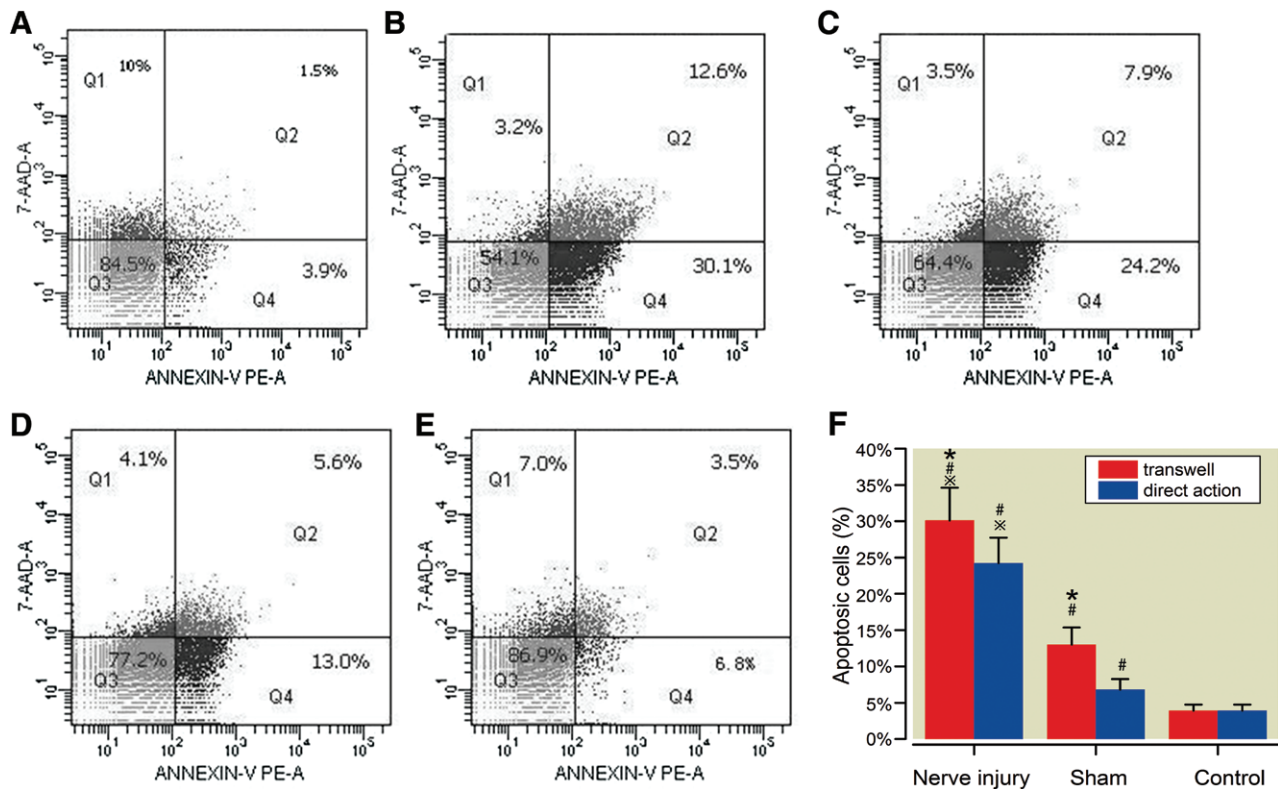


Fig. 6. Data from a representative flow cytometry analysis of Schwann cell apoptosis. The relative fluorescence intensity of Annexin V-PE/7-AAD is given in the dot plots. (A) control group; (B) nerve injury trans-well group; (C) nerve injury direct contact group; (D) sham trans-well group; (E) sham direct contact group. Q3: cells were negative for Annexin V-PE and 7-AAD, indicating that they were viable and not undergoing apoptosis; Q4: cells were Annexin V-PE positive and 7-AAD negative, indicating that they were in early stage apoptosis; Q2: cells were positive for both Annexin V-PE and 7-AAD, indicating that they were necrotic. (F) Histogram of percentages of apoptotic Schwann cells. Data are expressed as mean \pm SD ($n = 6$) and analyzed by one-way ANOVA followed by the *post hoc* Tukey test. # indicates a significant difference *versus* the control group ($\#P < 0.01$, Tukey). * indicates a significant difference *versus* the sham group ($*P < 0.01$, Tukey). * indicates the difference between trans-well group and direct contact group ($*P < 0.01$, Student's *t*-test).

Schwann cells in the trans-well group and those in the direct contact group was not significant.

Schwann Cell Apoptosis Detected by TUNEL Analysis

The effect of lymphocyte coculture on Schwann cell apoptosis was quantitatively evaluated using TUNEL staining (fig. 7). The percentages of apoptotic Schwann cells were $5.24 \pm 1.39\%$, $24.4 \pm 3.36\%$, and $20.9 \pm 1.48\%$ for the control group (fig. 7A), nerve injury trans-well group (fig. 7B), and nerve injury direct contact group (fig. 7C), respectively. Schwann cell apoptosis rates in the sham trans-well group and in the sham direct contact group were $10.8 \pm 1.74\%$ (fig. 7D) and $7.0 \pm 1.58\%$ (fig. 7E), respectively. Schwann cell apoptosis was higher *in vitro* when cocultured with lymphocytes from neuropathic rats ($P = 0.013$, one-way ANOVA) compared with the corresponding control group. The differences between the nerve injury trans-well group ($P < 0.0001$, Tukey) and the direct contact group ($P = 0.002$, Tukey) compared to their respective sham groups were statistically significant (fig. 7F). The differences between the sham trans-well group ($P = 0.011$, Tukey) and direct contact

group ($P = 0.003$, Tukey), and their respective control groups were also significant. However, the difference in apoptotic Schwann cells between the trans-well group and the direct contact group was not significant ($P = 0.67$, Student's *t*-test).

Discussion

The major findings of this study are as follows: (1) Peripheral nervous macrophage infiltration and central nervous microglia activation persisted up to 84 days after nerve injury. (2) Many IL-17⁺ cell were found in the injured sciatic nerves and spinal cords of heterozygous littermates, but far fewer were present in the nude mice. Moreover, the numbers of macrophages at the sciatic nerve injury site and of activated microglia in the L4-6 spinal cord were far fewer in the nude mice than in their heterozygous littermates. (3) Balb/c-nu/nude mice with T cell deficiency developed less thermal hyperalgesia and recovered faster after nerve injury than their heterozygous littermates. (4) The proliferation of lymphocytes was significantly enhanced when exposed to various neural tissue supernatants, particularly those from peripheral nerve and neural tissue harvested from neuropathic rats. (5)

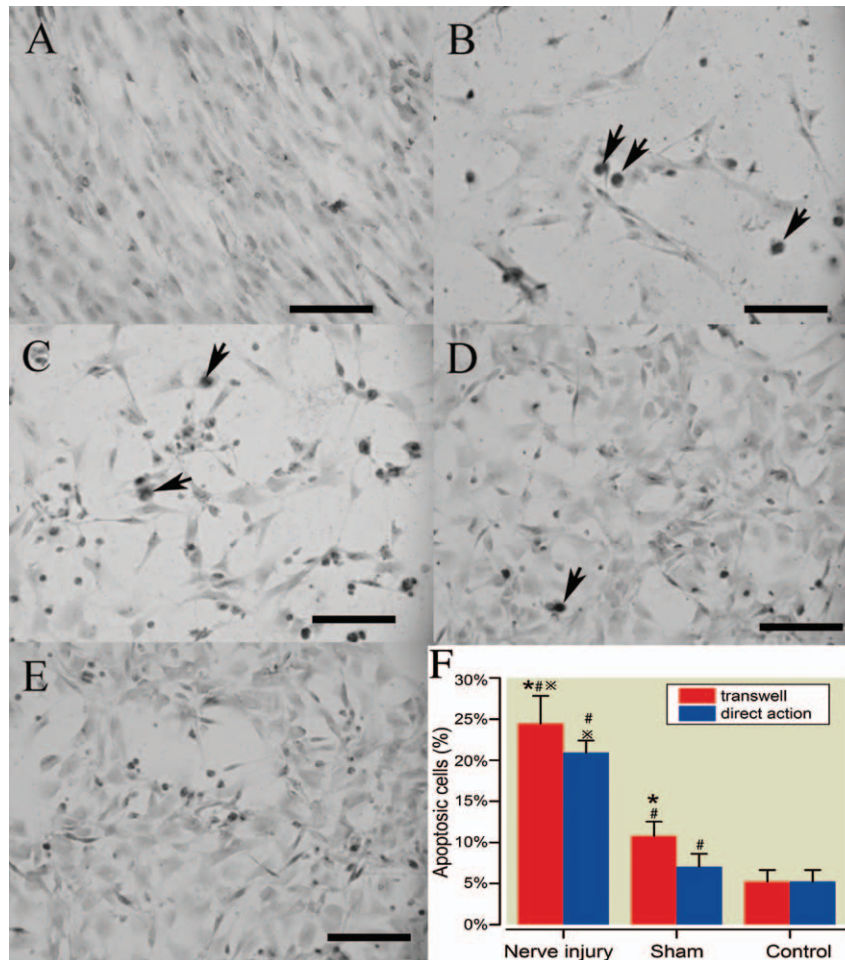


Fig. 7. Representative TUNEL analysis of Schwann cells after coculture with lymphocytes. (A) Schwann cells of the control group. (B) Schwann cells from the rats with nerve injury trans-well group. (C) Schwann cells from the rats with nerve injury direct contact group. (D) Schwann cells from the sham trans-well group. (E) Schwann cells from the sham direct contact group. (F) Histogram of the percentages of apoptotic Schwann cells. Arrows show apoptotic cells (40 \times , scale bars = 50 μ m). Data are expressed as mean \pm SD ($n = 6$) and analyzed by one-way ANOVA followed by the *post hoc* Tukey test. # indicates a significant difference versus the control group ($\#P < 0.01$, Tukey). * indicates a significant difference versus the sham group ($*P < 0.01$, Tukey). * indicates the difference between trans-well group and direct contact group ($*P < 0.01$, Student's *t*-test).

Schwann cell apoptosis was triggered *in vitro* when cocultured with lymphocytes from neuropathic rats.

In normal nerve or spinal cord tissue, few IL-17 $^{+}$ cell are present. Th17 cells are the principal source of IL-17. Kleinschultz *et al.* detected a transient 20-fold increase in IL-17A transcripts at POD 7 in mice with nerve injuries and confirmed IL-17A immunoreactivity to be located predominantly in the endoneurium at the same time-point.²⁴ In our behavioral study, we found that the lowest thermal latency occurred at approximately POD 7 in heterozygous littermates. This implies that Th17 may participate in the development of neuropathic pain. Our immunochemical data for IL-17 indicated that the number of IL-17 $^{+}$ cell on POD 28 was as high as $575 \pm 43/\text{mm}^2$ in the peripheral nerve and $506 \pm 31/\text{mm}^2$ in the spinal cord of heterozygous littermates, whereas these cells were barely detectable in the normal nerve and spinal cord. Similarly, on POD 28, the numbers of ED1 $^{+}$ macrophages were as high as $557 \pm 37/$

mm^2 , and microglia in the spinal cord were increased by sixfold compared with normal numbers in heterozygous littermates. All of these values remained higher than normal on POD 84, which strongly suggests that there is a chronic ongoing inflammation present both at the nerve injury site and at the corresponding level of the spinal cord after nerve injury. It is well known that tissue injury always causes acute inflammation that is essential for healing. However, why the peripheral tissue nerve injury causes such long-term chronic inflammation is not clear. We have confirmed that lymphocytes play a critical role in the process and promote the progression of acute inflammation to chronic inflammation because we have observed far less immune cell infiltration in T cell-deficient nude mice. Cao *et al.* found a significant leukocytic infiltration of predominantly CD4 $^{+}$, but not CD8 $^{+}$ T lymphocytes in the lumbar spinal cord that peaked at day 7 post a spinal nerve L5 transection neuropathic pain model (L5 Tx).³²

The mechanism of infiltration of T cells into the injured sciatic nerve and the corresponding section of spinal cord is unknown. This infiltration is probably secondary to inflammation caused by nerve injury, which is a nonspecific immune response. However, it is also possible that the infiltration of lymphocytes is caused by the exposure of normal or degenerated neural tissue to the immune system after breakdown of the blood–nerve barrier or blood–brain barrier resulting from nerve injury. Under normal circumstances, immune cells are separated from the nervous system by blood–nerve barrier or blood–brain barrier. Consequently, there are very few immune cells in normal neural tissues (figs. 3 and 4). Once nerve injury occurs, breakdown of the blood–nerve barrier or blood–brain barrier exposes the neural tissue to the immune system.³³ Part of our study was designed to investigate whether direct exposure of normal or injured neural tissue to lymphocytes would increase their proliferation *in vitro*. The results have shown that lymphocyte proliferation increased significantly when exposed to normal neural tissue compared with controls. However, the proliferation varied according to the type of neural tissue used. The proliferation was enhanced the most when exposed to the peripheral nerve tissue supernatant, followed by dorsal root ganglia and the spinal cord. This phenomenon suggests that peripheral neural tissue is more likely to cause an immune response than the central neural tissue when exposed to the immune system. Moreover, the exposure of lymphocytes to the nerve tissue supernatant harvested from neuropathic rats further increased their proliferation. Our results imply that injured peripheral nerve tissue may present as nonself antigens to the immune system, which might be the underlying mechanism of chronic inflammation after nerve injury. The evidence of chronic B lymphocyte activation and lupus-like autoantibody synthesis after spinal cord injury indicated that a systemic autoimmunity had been triggered.³⁴ The components that trigger immune response in the peripheral nerve tissue are unknown. Because Schwann cells are dominant in the peripheral neural tissue and the degeneration of Schwann cells is a common phenomenon after nerve injury, we suspect that Schwann cell proteins in neural tissue may present as a self-antigen after nerve injury. If this is true, active lymphocytes harvested from neuropathic rats may directly or indirectly attack Schwann cells.

Both flow cytometry and TUNEL staining demonstrated that coculture with nerve tissue significantly increased the apoptosis of Schwann cells. It is interesting to note that the increase of Schwann cell apoptosis was very similar between the direct contact group and indirect contact group, which may suggest that lymphocytes attack Schwann cells by releasing cytokines instead of direct cell-to-cell contact. Postherpetic neuralgia is one of the neuropathic pain conditions that follow varicella zoster virus infection. Most recently, one study found that varicella zoster virus antigen was localized almost exclusively in neurons, and large immune infiltrates consisted of noncytotoxic CD8⁺ T cells, with fewer numbers

of CD4⁺ T cells, B cells, natural killer cells, and macrophages. Interestingly, varicella zoster virus antigen-positive neurons did not express detectable major histocompatibility complex class I, nor did CD8⁺ T cells surround infected neurons, suggesting that mechanisms of immune control may not be dependent on direct contact.³⁵ However, the immune responses demonstrated in this study did not result from varicella zoster virus infected neurons but are most likely triggered by peripheral nerve injury. In the central nervous system, it is evident that injury induced by contusion would trigger systemic autoimmunity.³⁴ Lymphocytes respond to myelin proteins after spinal cord injury and may contribute to posttraumatic secondary degeneration.³⁶ Our study suggests that peripheral nerve injury may trigger an autoimmune response resulting in chronic inflammation that may contribute to chronic neuropathic pain. In our *in vivo* study, we confirmed that T-cell-deficient mice had reduced chronic inflammation, less thermal hyperalgesia, and faster recovery compared to heterozygous littermates. Other studies have demonstrated that CD4 knockout mice display significantly decreased mechanical hypersensitivity after day 7 of L5 spinal nerve transection injury, and the adoptive transfer of CD4⁺ leukocytes reverses this effect.³² The adoptive transfer of splenocytes and peripheral blood mononuclear cells from high pain donors to low pain recipients potentiates allodynia in a newly developed graded chronic constriction injury rat model.³⁷ The above evidence supports our hypothesis that chronic neuropathic pain might be caused by chronic inflammation resulting from a nervous autoimmune reaction triggered by nerve injury. The key proteins presenting as self-antigens require further investigation. The findings and the concepts raised here may have implications for the treatment of chronic neuropathic pain. In a previous study, we showed that cyclosporine A, a potent inhibitor of lymphocyte activation, reduced the development of thermal hyperalgesia after nerve injury. Methotrexate, a known immune suppressor administered intrathecally with a low dose from the start of injury, reduced neuropathic pain behavior in rats.^{20,21} Although immune suppressors are commonly used in chronic pain caused by arthritis, their efficacy and safety in neuropathic pain patients needs further investigation.

This study has a number of limitations. (1) We did not examine the Th17 cells, macrophages, and microglia within the first month of nerve injury. (2) We used mice for the first part of study, but rats for the remaining studies because mice were too small for either neural tissue harvesting or peripheral blood lymphocyte collection. Moreover, we exposed lymphocytes to the supernatant of nerve tissue that may contain many substances besides neural tissue components. (3) We also used Schwann cells from pups instead of adult mice to evaluate the interactions between lymphocytes and Schwann cells. Because the pup cells are still developing, their responses to injury are different from those of adult cells. However, fresh dissociated adult cells are difficult to culture. (4) If a pharmacological approaches applying

T lymphocytes growth inhibitor in our lymphocytes and Schwann cell coculture experiment, we may have confirmed that SC stay the same which could provide more knowledge on a likely dose effect of this inhibitor. We are considering verifying this in our further study.

Conclusion

There is a persistent inflammation at the site of injured nerve, corresponding dorsal root ganglia and spinal cord section after peripheral nerve injury. The interaction between the immune system and the peripheral nervous tissues underlies the mechanism of chronic inflammation. A nervous autoimmune reaction triggered by the exposure of nerve tissues to the immune system during nerve injury might be critical for developing persistent inflammation and resulting acute neuropathic pain that eventually becomes chronic.

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