

Interleukin-17 Contributes to Neuroinflammation and Neuropathic Pain Following Peripheral Nerve Injury in Mice

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Abstract: Cytokines, essential mediators of inflammatory and immune responses, play an important role in the pathophysiological processes associated with neuropathic pain following peripheral nerve injury. Recently, a novel proinflammatory cytokine, the interleukin (IL)-17, was found to orchestrate inflammatory responses in a wide range of inflammatory and autoimmune diseases of the nervous system. Here, we investigated the role of IL-17 in mediating neuroinflammation and pain hypersensitivity using the neuropathic pain model of partial ligation of the sciatic nerve in mice. Compared to wild-type, IL-17 knockout (KO) mice displayed significantly decreased mechanical pain hypersensitivity as well as decreased infiltration of T cells and macrophages to the injured sciatic nerves and the L3-L5 dorsal root ganglia and decreased activation of microglia and astrocytes in the L3-5 dorsal and ventral horns of the spinal cord. Further, intraplantar and intraneural injection of recombinant IL-17 into the hind paw and the sciatic nerve, respectively, induced both mechanical allodynia and thermal hyperalgesia, whereas intrathecal injection produced thermal hyperalgesia. IL-17 administration was associated with a significant increase in the numbers of infiltrating neutrophils and activated dendritic cells in the injected hind paws and infiltrating neutrophils in the injected sciatic nerves. Taken together, our results demonstrate that IL-17 contributes to the regulation of immune cell infiltration and glial activation after peripheral nerve injury and the ensuing neuropathic pain. **Perspective:** *IL-17 is an important regulator of immune responses and is involved in inducing and mediating proinflammatory reactions. Using IL-17 KO mice, we have demonstrated that IL-17 contributes to neuroinflammatory responses and pain hypersensitivity following neuropathic injury. This work identifies IL-17 as a potential therapeutic target in neuropathic pain.*

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Key words: *Proinflammatory cytokines, interleukin-17, nerve injury, hyperalgesia, allodynia.*

Neuropathic pain is a chronic pain condition arising as direct consequence of a lesion or disease affecting the somatosensory system.¹⁴ Such pain can be caused by a primary lesion or dysfunction to either the peripheral or the central nervous system, is relatively common, and severely debilitating.⁶⁵ Common symptoms include the appearance of abnormal sensory signs such as increased sensitivity to painful stimuli (hyperalgesia), pain sensitivity to normally innocuous stimuli (allodynia), and ongoing spontaneous pain.³ There is growing evidence that the functional interaction

between the immune system and the nervous system plays crucial roles in the mechanisms underlying neuropathic pain.³⁸ Previous research has demonstrated that following nerve damage, a cascade of inflammatory response is initiated. The complement system is activated,^{15,30} a variety of inflammatory cells are recruited to the site of nerve injury,⁸ to the dorsal root ganglia (DRGs),¹⁷ and to the spinal dorsal horn⁵⁴ and resident satellite cells,¹⁶ and immune-like glial cells (astrocytes and microglia) are activated.^{6,37} These events facilitate an upregulation of cytokines, chemokines and adhesion molecules, and subsequent production and secretion of various algogenic mediators, which contribute to the generation and maintenance of neuropathic pain.^{38,62}

Dysregulation of cytokines has been implicated in a variety of painful neurological diseases and in animal models of neuropathic pain. For example, differential expression of blood cytokines has been demonstrated in patients with complex regional pain syndrome having higher levels of tumor necrosis factor (TNF) and interleukin (IL)-2, and lower levels of IL-4 and IL-10.⁵⁸ In mice,

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2 The Journal of Pain

a rapid and marked upregulation of TNF, IL-1 β , and IL-10 has been observed as early as 1 hour after chronic constriction injury in injured sciatic nerves and DRGs.⁵⁹ Numerous experimental studies provide evidence that proinflammatory cytokines facilitate neuropathic pain.⁵³ Exogenous administration of TNF and IL-1 β elicits pain and hyperalgesia when applied either intraplantarly⁹ or intraneurally,⁶⁹ whereas blocking TNF and IL-1 β alleviates neuropathic pain.⁴⁹ Furthermore, anti-inflammatory cytokines such as IL-4 and IL-10 suppress genes that code for IL-1, TNF, and chemokines. Application of IL-10 using a single dose at the site of a chronic constriction injury⁶¹ or using intrathecal gene therapy with viruses or plasmid DNA vectors encoding IL-10³⁶ reduces or abolishes neuropathic pain. The effects of cytokines on pain can be either direct receptor-mediated actions on afferent fibers or indirect effects involving further mediators, such as inducing the expression of agents (eg, prostaglandin E₂, PGE₂) that themselves sensitize nociceptors.⁵³ Anti-cytokine agents are currently under trials for the treatment of neuropathic pain.

IL-17 (IL-17A, CTLA-8) is the founding member of a novel family of cytokines (IL-17A-F); it shares no sequence homology with other known mammalian proteins and has potent proinflammatory properties.⁶⁴ The main cellular sources for IL-17 are CD4 effector T cells from the T helper (Th)17 lineage,¹¹ although many other cellular sources such as CD8 T cells,⁵⁷ neutrophils,¹¹ and eosinophils⁴⁰ have been identified. Interestingly, recent studies have shown that cells of the central nervous system also express IL-17. For example, astrocytes and oligodendrocytes express IL-17 mRNA,^{29,57} and microglia produce IL-17 protein.²⁰ The best studied IL-17 receptor to date, IL-17R (or IL-17RA), binds both IL-17A and IL-17F, although IL-17A binds with more than tenfold higher affinity. Expression of IL-17RA appears to be ubiquitous on haematopoietic cells as well as many nonimmune cell types such as fibroblasts, endothelial cells, and epithelial cells.⁶⁶ Gene expression studies have demonstrated that signaling through IL-17RA promotes the expression of numerous genes relevant to the recruitment of innate immune cells to sites of infection or tissue damage.⁶⁷

Indeed, the involvement of the proinflammatory cytokine IL-17 in a number of inflammatory disorders, including multiple sclerosis,⁵⁷ arthritis,³³ psoriasis,²³ and cerebral ischemia²⁹ has been established. Recently, IL-17 positive T cells have been demonstrated in the endoneurium of injured sciatic nerve 7 days following chronic constriction injury.²¹ However, little research has been done on the involvement of IL-17 in neuropathic pain. In the present study, we set out to investigate the role of IL-17 in the development of behavioral pain hypersensitivity and neuroinflammatory responses induced by nerve injury using the partial ligation of the sciatic nerve (PSNL) model in mice.³⁴

Methods

Animals

Experiments were carried out in male C57BL/6J inbred mice (7–8 weeks old; Animal Resource Centre, Perth, WA,

Interleukin-17 Contributes to Neuropathic Pain

Australia) and in IL-17 knockout (KO) mice (IL-17 $^{-/-}$) on C57BL/6J background. Breeding pairs for IL-17 KO mice were kindly obtained from Prof Yoichiro Iwakura (Center for Experimental Medicine, The Institute of Medical Science, The University of Tokyo, Japan) and bred in the animal facility of the Animal Resource Centre, Perth, WA, Australia. All mice were group-housed with food and water ad libitum and maintained on a 12:12 hour light/dark cycle. Housing was kept at a constant room temperature and humidity level, and the well-being of the animals was monitored daily. All animal experiments were approved by the Animal Care and Ethics Committee of the University of New South Wales, Australia, and all efforts were made to minimize pain and discomfort in laboratory animals and their suffering.

Partial Ligation of the Sciatic Nerve

A unilateral partial ligation of the sciatic nerve was performed according to the method described previously.³⁴ Briefly, mice were anaesthetized with halothane in 1:1 mixture of O₂ and N₂O and the left sciatic nerve exposed at the level proximal to the thigh and freed of adhering tissue. A partial ligation of the sciatic nerve was made by tightly ligating the medial dorsal 1/3 to 1/2 of the nerve's diameter using 9-0 silk. In sham-operated mice, used as controls, the left sciatic nerve was exposed, but not ligated. Muscle layers were closed with 5-0 silk sutures and the skin wounds closed with skin staples.

Injection of IL-17

C57BL/6J mice were anaesthetized with halothane in 1:1 mixture of O₂ and N₂O. Injection of recombinant mouse IL-17 (ProSpec-Tany TechnoGene Ltd, Rehovot, Israel) or vehicle (saline, control) took place as follows. For intraplantar injection, 10 μ l of 5 or 25 ng IL-17 or vehicle was injected into the plantar aspect of the left hind paw. For intraneural injection, the left sciatic nerve was exposed at midhigh level and 2 μ l of 10, 25, or 50 ng IL-17 or vehicle was injected into the nerve in 2 spots, 1 μ l each, using a pulled micropipette. For intrathecal injection, a small cut was made in the skin and the muscles to expose the vertebrae at L5-6 level of spinal segments. Then, a pulled micropipette was inserted between the vertebrae until a brisk tail flick observed and 25 or 50 ng per 4 μ l IL-17 or vehicle was slowly injected into the subdural space. In the cases of intraneural and intrathecal injections, the incisions were closed in layers by suturing.

Behavior Testing

Mice were habituated to the behavioral testing apparatus for 30 minutes to 1 hour prior to data collection and the testing environment was kept quiet and well controlled. Behavioral tests carried out before (baseline) and at time-points 1, 3, 5, and 24 hours, and in some cases 48 hours, after IL-17 injection, or every several days after partial ligation of the sciatic nerve.

Mechanical allodynia was assessed by placing mice in enclosures, with an elevated wire grid and stimulating the midplantar surface of the hind paws using the tip

Kim and Moalem-Taylor

of an electronic von Frey anesthesiometer (IITC Inc, Woodland Hills, LA) until the mouse withdrew its paw in response to the mechanical stimulus. The device automatically recorded and displayed the force (in grams) that elicited a withdrawal response. The threshold was recorded and repeated 3 times with an interval of at least 3 minutes between trials on the same paw and the mean was calculated.

Thermal hyperalgesia was measured by exposing the midplantar surface of the hind paws to a beam of radiant heat through a transparent perspex surface, using a plantar analgesia meter for paw stimulation (Ugo Basile, Comerio, Italy). The paw withdrawal latency to heat stimulus was measured automatically and recorded, as the time from onset of the thermal stimulus to withdrawal response. Each paw was tested 3 times, and the mean withdrawal latency was calculated. The interval between trials on the same paw was at least 5 minutes. The cut-off latency was set at 20 seconds to avoid tissue damage in the case of failure to remove paw.

Immunohistochemistry

The mice were deeply anaesthetized with .1mL Letharbip and intracardially perfused with .9% saline containing heparin, followed by 4% paraformaldehyde in .1 M phosphate buffer (pH 7.4) for fixation. Sciatic nerves, L3-L5 DRGs, and spinal cord tissues were harvested from nerve-injured or sham-operated mice. The wide part of the plantar hind paws was cut to the bone ground and removed from mice injected intraplantarly with IL-17 or vehicle, and sciatic nerves were excised from intraneurally IL-17- or vehicle-injected mice. All tissue was postfixed overnight in 4% paraformaldehyde and then cryoprotected in 30% sucrose overnight. Using a cryostat, the sciatic nerves and DRGs were sectioned longitudinally at a thickness of 10 μ m, spinal cords were sectioned transversely at a thickness of 20 μ m, and hind paw tissues were sectioned longitudinally at a thickness of 10 μ m.

Sciatic nerve and DRG sections from nerve-injured mice were stained for T cells and macrophages. Spinal cord sections from nerve-injured mice were stained for microglia and astrocytes. Sciatic nerve and hind-paw sections from IL-17- or vehicle-injected mice were stained for T cells, macrophages, neutrophils, and dendritic cells. The sections were fixed for 10 minutes at room temperature with absolute ethanol and washed twice in distilled water and once in phosphate-buffered saline (PBS) containing .05% Tween-20. Sections stained for T-cells were then incubated in acetone for 3 minutes followed by 3 washes in PBS containing .05% Tween-20. All sections were blocked for 1 hour at 37°C with blocking solution (PBS containing 5% donkey or goat serum, .2% Tween-20; and for astrocyte and neutrophil staining also .3% triton X-100) followed by 1 hour incubation at room temperature with the primary antibody diluted in PBS containing 5% bovine serum albumin. Primary antibodies used are: Monoclonal purified rat anti-mouse CD3 molecular complex antibody (1:100; BD Biosciences Pharmingen, San Diego, USA) for the detection of infiltrating T cells; F4/80 (1:100; Santa Cruz Biotechnology,

Inc, Santa Cruz, CA) for the detection of macrophages; rabbit anti-ionized calcium binding adaptor molecule 1 (IBA1, 1:2000; Wako Pure Chemical Industries Ltd, Osaka, Japan) for the detection of microglia; rabbit anti-glial fibrillary acidic protein (GFAP, 1:1000; Dako Cytomation, Copenhagen, Denmark) for the detection of astrocytes; monoclonal rat (clone 7/4) anti-mouse neutrophil (1:2500; Abcam, Cambridge, UK); and hamster anti-mouse CD11c for the detection of dendritic cells (1:250; AbD Serotec, Oxford, UK). The sections were then washed 4 times with PBS containing .05% Tween-20 and incubated with the appropriate secondary antibody for 1 hour at room temperature. Secondary antibodies used are: Cy2-conjugated donkey anti-rat, Cy3-conjugated donkey anti-rabbit, Dylight 488 goat anti-Armenian hamster IgG (Jackson ImmunoResearch, West Grove, PA), and Alexa Flour 488 goat anti-rabbit (Invitrogen, Victoria, Australia). The sections were then washed 4 times with PBS containing .05% Tween-20. In some cases, sciatic nerve sections from nerve-injured mice were treated for 8 minutes with .5% Sudan Black B (Sigma) in 70% ethanol to eliminate possible autofluorescence. Then, the sections were washed and mounted with fluorescent mounting medium (DakoCytomation) to inhibit quenching of fluorescence, before being cover-slipped.

Image Analysis

Sections were viewed on an Olympus fluorescence microscope. Images were captured using an Olympus DP70 camera and DP Controller software (Olympus, Tokyo, Japan) and were taken from 3 to 4 sections in each animal. The sections were identified under the microscope and sciatic nerve images from nerve-injured mice were taken at $\times 40$ objective at the site of injury (identified by the suture) and regions distal and proximal to injury site on nonoverlapping fields of view. DRG images were taken at $\times 40$ objective on regions containing cell bodies of the DRG and spinal cord images were taken at $\times 40$ objective in consistent areas of the spinal cord dorsal and ventral horns (Fig 4C), corresponding to the sciatic nerve territories.¹⁷ Sciatic nerve and hind paw images from IL-17- and vehicle-injected mice were taken either at $\times 20$ or $\times 40$ objective around the site of injection. In each photograph, immunoreactivity was evaluated using NIH ImageJ software (NIH, Bethesda, USA) in 2 ways: 1) for T-cell, neutrophil and dendritic-cell immunoreactivity, which contained clear cellular staining, single stained cells were counted manually using the cell counter plug-in; and 2) for macrophage, microglia and astrocyte immunoreactivity where individual cells were difficult to demarcate, thresholding was used to detect labeled structures and the % areal fraction covered by stained structures determined.

Statistical Analyses

All data are expressed as means \pm SEM. Figures and statistical analyses were made with GraphPad Prism software package version 5.0 (GraphPad Prism, La Jolla, CA). Immunohistochemistry data were analysed

4 The Journal of Pain

using 2-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison post-test, or unpaired Student *t* test, as appropriate. Behavioral data were analyzed using repeated measures (rm) 2-way ANOVA followed by Bonferroni's multiple comparison post-test for each time-point. A probability of .05 or less was considered statistically significant.

Results

Reduced Mechanical Allodynia in IL-17 KO Mice

To investigate the role of IL-17 in the production of pain hypersensitivity following neuropathic injury, we compared pain behaviors of IL-17 KO and C57BL/6J (wild-type control) mice following peripheral nerve injury. We measured the sensitivity of the mice to mechanical and thermal stimuli in both the injured ipsilateral and uninjured contralateral hind paws before (baseline values) and up to 2 weeks after surgery. Baseline values of both mechanical and thermal pain sensitivity were not different between the C57BL/6J mice and the IL-17 KO (Figs 1A and 1B). Following PSNL, all mice developed pain hypersensitivity in the paw ipsilateral to PSNL compared with either the contralateral side or baseline values before surgery. A marked decrease in paw withdrawal threshold to mechanical stimuli was observed in the ipsilateral hind paw of the C57BL/6J mice and a smaller decrease in the ipsilateral hind paw of the IL-17 KO mice (Fig 1A). Paw withdrawal thresholds in the ipsilateral hind paws of C57BL/6J mice were significantly lower than that of IL-17 KO mice between days

Interleukin-17 Contributes to Neuropathic Pain 5 to 12 after PSNL (Fig 1A; $P < .05-.001$; 2-way ANOVA, followed by Bonferroni post-test).

A significant decrease in paw withdrawal latencies to thermal stimuli was observed in the ipsilateral hind paws of both the C57BL/6J mice and IL-17 KO mice. Paw withdrawal latencies were not significantly different between the groups (Fig 1B; $P > .05$; 2-way ANOVA, followed by Bonferroni post-test).

Neuroinflammatory Responses Are Reduced in IL-17 KO Mice

Because IL-17 is a potent proinflammatory cytokine, a deficiency in IL-17 is expected to alter activation of immune cells and infiltration of inflammatory cells in the nervous tissue following nerve injury. Thus, we compared the inflammatory responses in sciatic nerves, L3-5 DRGs, and spinal cord segments between C57BL/6J and IL-17 KO mice using immunohistochemistry for T cells, macrophages, microglia, and astrocytes on day 7 following PSNL.

We found almost no labeled T cells in uninjured sciatic nerves from sham-operated mice, but a significant infiltration of T cells into the injured sciatic nerve, with most cells accumulating around the injury site and fewer in regions distal and proximal to the site of injury (Fig 2A). T-cell numbers were significantly lower at the injury site and distal to injury in IL-17 KO mice than in C57BL/6J mice (Fig 2A; $P < .01-.001$; 2-way ANOVA, followed by Bonferroni post-test). In DRGs, a minimal number of T cells was detected in uninjured DRGs from sham-operated mice. However, T-cell numbers increased considerably in L3, L4, and L5 DRG following PSNL, and

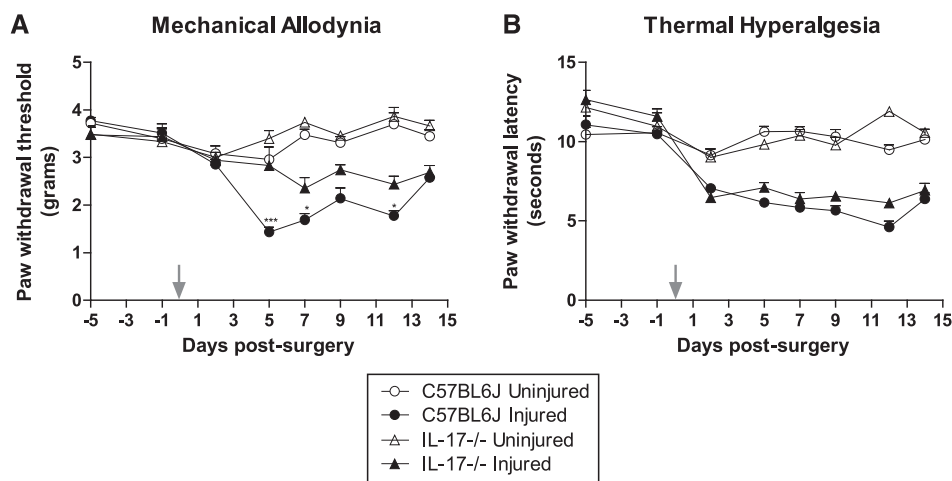


Figure 1. Neuropathic pain behaviors induced by unilateral partial ligation of the sciatic nerve (PSNL) in C57BL/6J mice in comparison to IL-17 KO mice. Behavioral responses for mechanical allodynia (A) and thermal hyperalgesia (B) at the injured (ipsilateral) and uninjured (contralateral) hind paws were measured before surgery (baseline) and after PSNL (day 0; ↓). Behavioral results are presented as paw withdrawal mechanical thresholds and thermal latencies of the injured and the uninjured side. A decrease in values represents pain hypersensitivity to the stimulus. (A) Paw withdrawal threshold to mechanical stimuli (mean \pm SEM, $n = 6$ per group) in the ipsilateral hind-paws of C57BL/6J mice (filled circles) was significantly lower than in the ipsilateral hind-paws of IL-17 KO mice (filled triangles) between days 5 to 12 after PSNL ($*P < .05$, $***P < .001$, 2-way ANOVA, followed by Bonferroni post-test). No significant changes were observed in the contralateral hind paws of C57BL/6J mice (open circles) and IL-17 KO mice (open triangles). (B) Paw withdrawal latency to thermal stimuli (mean \pm SEM, $n = 6$ per group) in the ipsilateral hind-paws of C57BL/6J mice (filled circles) was slightly lower than in the ipsilateral hind paws of IL-17 KO mice (filled triangles); however, there was no significant difference between the groups, with both showing a marked decrease in withdrawal latency from day 2 onwards, through up to day 14 following PSNL. No significant changes were observed in the contralateral hind paws of C57BL/6J mice (open circles) and IL-17 KO mice (open triangles) (2-way ANOVA, followed by Bonferroni post-test).

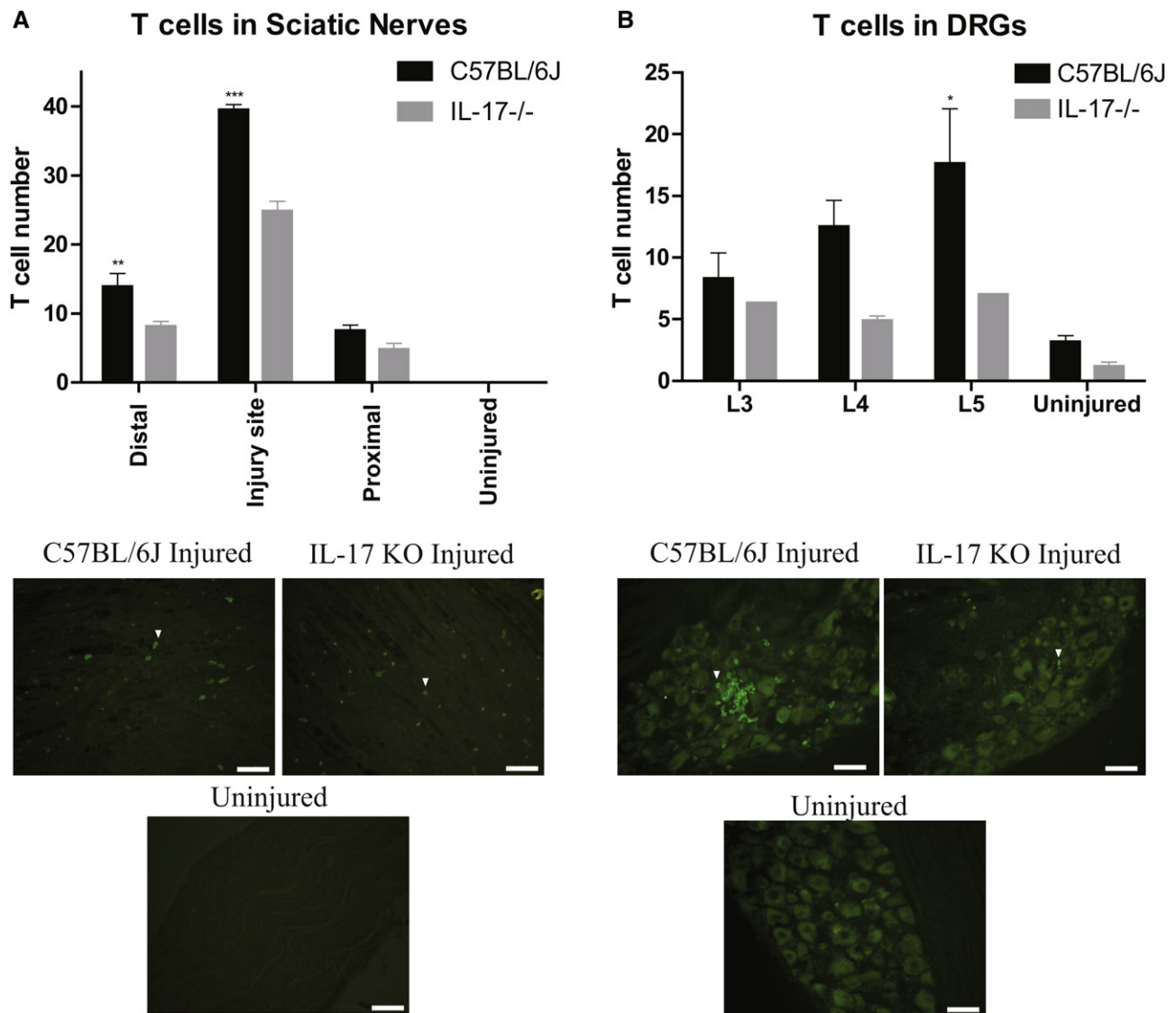


Figure 2. Reduced T-cell infiltration into the injured sciatic nerves and DRGs in IL-17 KO mice. The number of T cells was determined 1 week after PSNL in injured sciatic nerves (distal to, proximal to, and at injury site) and L3-L5 DRGs and in uninjured nerves and DRGs of sham-operated mice. (A) T-cell numbers in the sciatic nerves (mean \pm SEM, $n = 3$ per group) notably increased at the injury site, proximal to, and distal to injury as compared to uninjured nerves in both C57BL/6J mice and IL-17 KO mice following PSNL. However, the number of T cells was significantly lower in IL-17KO than in C57BL/6J mice at the site of injury and distal to nerve injury ($**P < .01$, $***P < .001$, 2-way ANOVA, followed by Bonferroni post-test). Representative photomicrographs (below graph) illustrate T-cell immunoreactivity in sections distal to injury site (CD3, green) within the sciatic nerve of C57BL/6J mice and IL-17 KO mice. Arrowheads indicate the circular T-cells present after PSNL. Scale bar = 100 μ m. (B) T-cell numbers (mean \pm SEM, $n = 3-6$ per group) in L3-5 ipsilateral DRGs were considerably greater after PSNL than in sham-operated mice in both C57BL/6J mice and IL-17 KO mice. However, the number of T cells in the DRGs was lower in IL-17KO than in C57BL/6J mice and was significantly lower in the L5 DRG ($*P < .05$, 2-way ANOVA, followed by Bonferroni post-test). Representative photomicrographs (below graph) illustrate T-cell immunoreactivity in DRG sections (CD3, green) of C57BL/6J mice and IL-17 KO mice. Arrowheads indicate the circular T-cells present after PSNL. Scale bar = 100 μ m.

were significantly lower in L5 DRG of IL-17 KO mice than in L5 DRG of C57BL/6J mice (Fig 2B; $P < .05$; 2-way ANOVA, followed by Bonferroni post-test).

Few macrophages were observed in uninjured sciatic nerves. Macrophage cell density was significantly increased in all areas of the injured sciatic nerve following PSNL. However, compared to C57BL/6J mice, IL-17 KO mice had significantly less macrophage immunoreactivity at the site of injury as well as proximal and distal to the injury site in the injured nerve (Fig 3A; $P < .05-.001$; 2-way ANOVA, followed by Bonferroni post-test). In addition, macrophage cell density in L3-5 DRG from the

injured side was significantly lower in IL-17 KO mice than in C57BL/6J mice (Fig 3B; $P < .05-.01$; 2-way ANOVA, followed by Bonferroni post-test).

In the spinal cord, IBA1 immunoreactivity (microglia) and GFAP immunoreactivity (astrocytes) were detected in the contralateral side. In the ipsilateral side, both microglia (Fig 4A) and astrocytes (Fig 4B) were markedly upregulated around regions of termination of sciatic primary afferents in the dorsal and ventral horns (Fig 4C). However, microglia and astrocyte cell density was significantly lower in the ipsilateral dorsal and ventral horns of the spinal cord in IL-17 KO

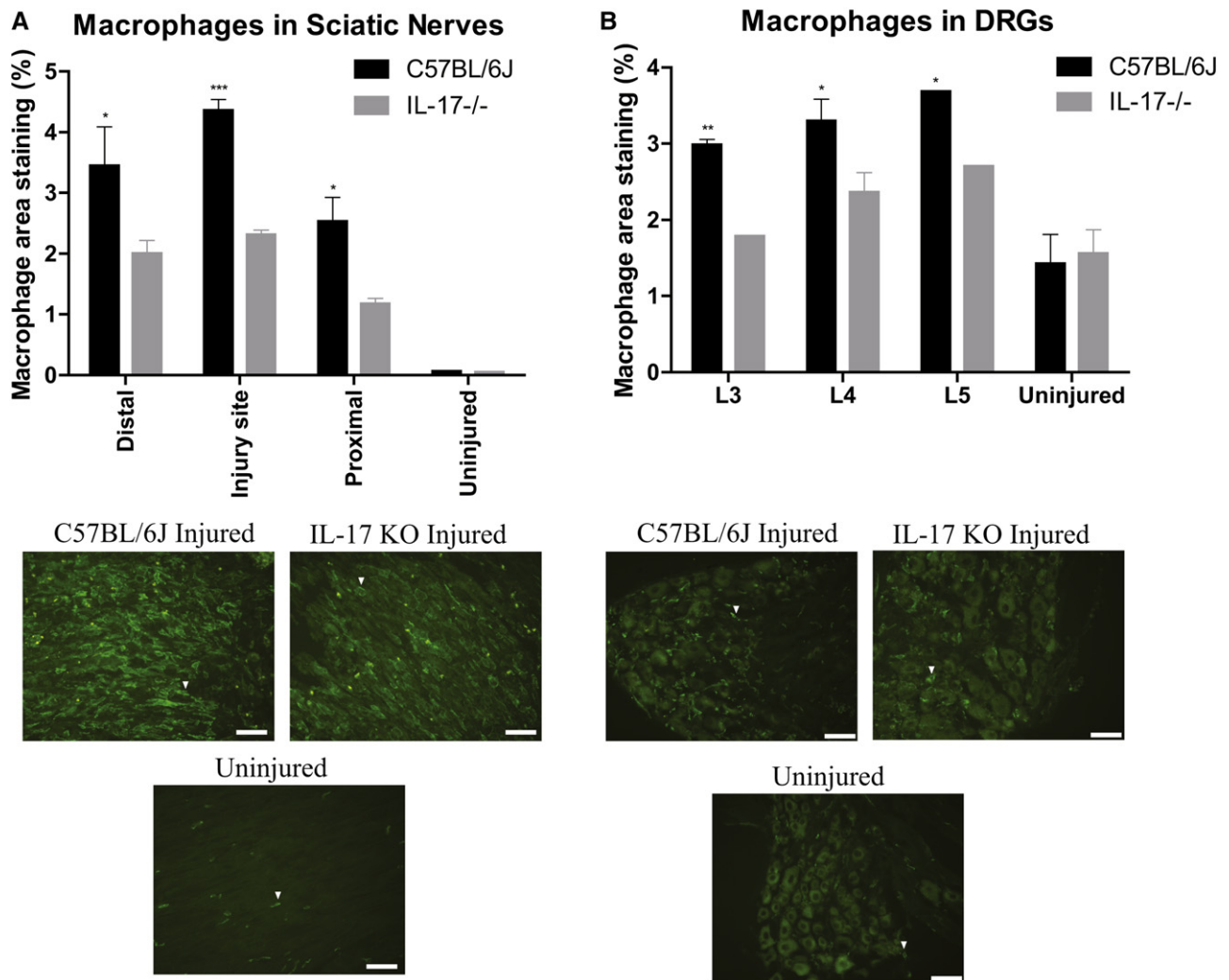


Figure 3. Macrophage presence in the injured sciatic nerve and DRGs is significantly reduced in IL-17 KO mice. The level of macrophage immunoreactivity was determined 1 week after PSNL in injured (distal to, proximal to, and at injury site) sciatic nerves and L3-L5 DRGs and in uninjured sciatic nerves and DRGs from sham-operated mice. **(A)** Percentage of macrophage area staining (mean \pm SEM, $n = 3$ per group) at each site in injured nerves was considerably increased in both C57BL/6J mice and IL-17 KO mice following PSNL, as compared to sham control mice. However, the level of macrophage area staining was significantly lower at the site of injury and in the proximal and distal parts in IL-17 KO mice than in C57BL/6J mice ($*P < .05$, $***P < .001$, 2-way ANOVA, followed by Bonferroni post-test). Representative photomicrographs (below graph) illustrate immunoreactivity to a marker of macrophages (F4/80, green) in C57BL/6J mice and IL-17 KO mice. Arrowheads show macrophage staining. Scale bar = 100 μ m. **(B)** Macrophage numbers in the ipsilateral L3-L5 DRGs (mean \pm SEM, $n = 3$ per group) of nerve-injured mice were higher in both C57BL/6J mice and IL-17 KO mice than in sham controls. However, the level of macrophage area staining in L3-L5 DRGs was significantly lower in IL-17 KO mice than in C57BL/6J mice ($*P < .05$, $**P < .01$, 2-way ANOVA, followed by Bonferroni post-test). Representative photomicrographs (below graph) illustrate macrophage immunoreactivity (F4/80, green) in C57BL/6J mice and IL-17 KO mice. Arrowheads show macrophage staining. Scale bar = 100 μ m.

mice as compared to C57BL/6J mice (Figs 4A and 4B; $P < .01$ –.001; 2-way ANOVA, followed by Bonferroni post-test).

Administration of IL-17 Induces Pain Hypersensitivity in Normal Mice

Next, we assessed pain responses in naïve mice injected with recombinant IL-17. We evaluated the effects of IL-17 on the development of mechanical and thermal pain sensitivity using: 1) injection of IL-17 into hindpaw (intraplantar); 2) injection of IL-17 into normal sciatic nerve (intraneural); and 3) injection of IL-17 into normal spinal cord (intrathecal injection via lumbar puncture). Pain be-

haviors were measured 48 to 24 hours before injection (baseline) and 1 to 48 hours after injection.

Following intraplantar injection of IL-17, mice showed an exaggerated response to mechanical (Fig 5A) and thermal (Fig 5B) stimuli. For both doses of IL-17 (5 ng and 25 ng), a significant decrease in paw withdrawal threshold and latency (Figs 5A and 5B; $P < .001$; rm-ANOVA, followed by Bonferroni post-test) was already observed within the first hour following injection and was maintained for a 24-hour period before returning to normal baseline values within 48 hours. Control mice injected with saline showed minimal change in pain sensitivity throughout the 48-hour time period. There was no difference between 5 ng and 25 ng doses in paw

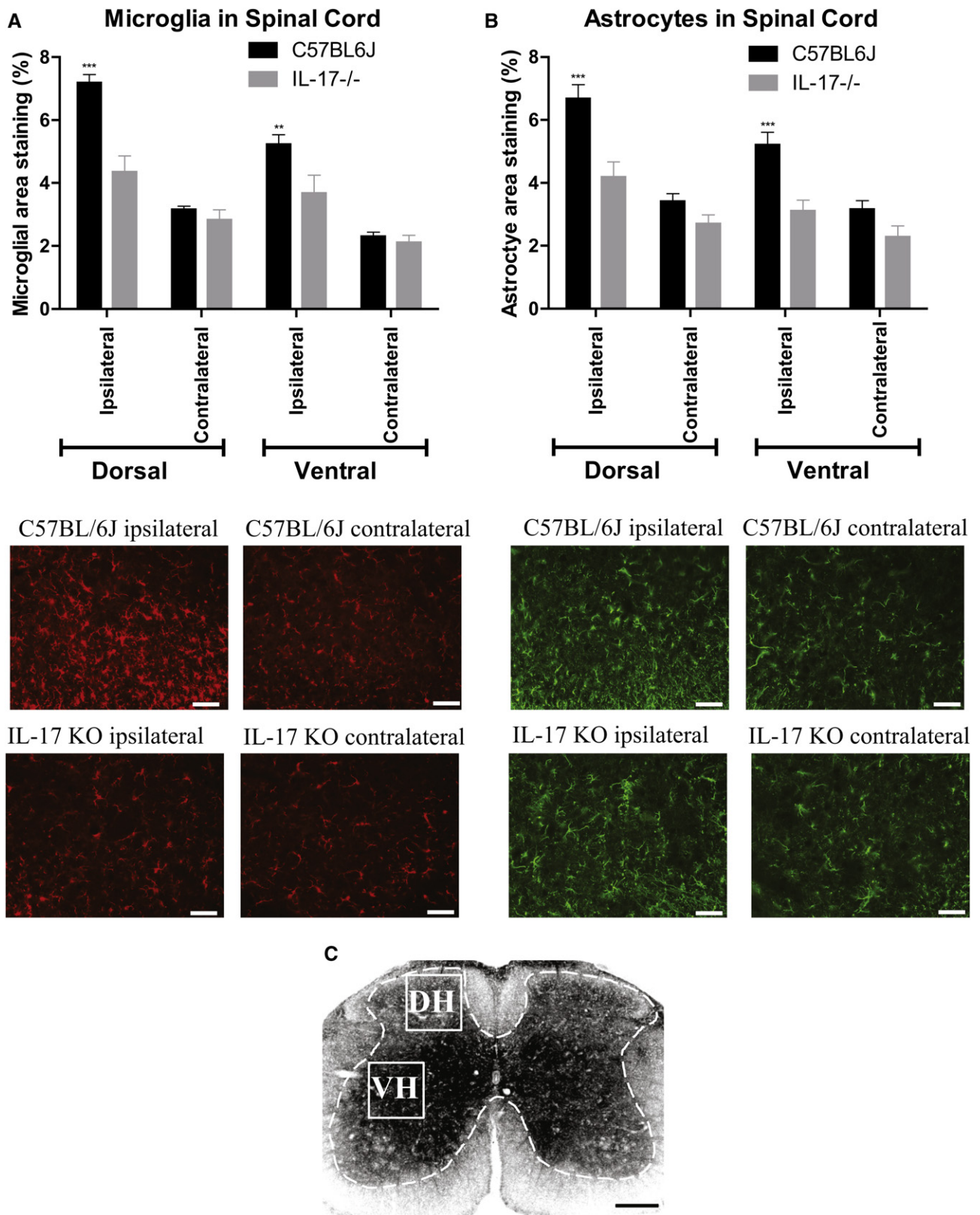


Figure 4. Decreased activation of microglia and astrocytes in spinal cord of nerve-injured IL-17 KO mice. Peripheral nerve injury induced microglial activation at the ipsilateral side dorsal horn and ventral horn evidenced by an increase in the level of IBA1 immunoreactivity (A, mean \pm SEM, n = 7 per group) and astrocyte activation at the ipsilateral side dorsal horn and ventral horn evidenced by an increase in the level of GFAP immunoreactivity (B, mean \pm SEM, n = 7 per group). IL-17 KO mice had significantly reduced microglia (A) and astrocyte (B) activation than C57BL/6J mice in both dorsal horn and ventral horn of the spinal cord (** P < .01, *** P < .001, 2-way ANOVA, followed by Bonferroni post-test). Representative photomicrographs (below graphs) illustrate microglia immunoreactivity (A; IBA1, red) and astrocyte immunoreactivity (B; GFAP, green) in C57BL/6J mice and IL-17 KO mice. Scale bar = 100 μ m. (C) The regions illustrated are the areas analyzed, as indicated by boxes labeled dorsal horn (DH) and ventral horn (VH), and were determined according to known sciatic nerve territories in the dorsal and ventral horns of the spinal cord. Scale bar = 100 μ m.

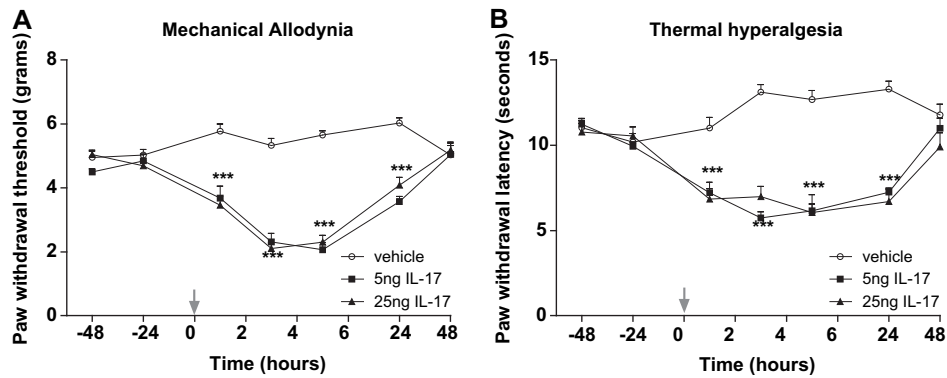


Figure 5. Intraplantar IL-17 administration induces mechanical and thermal pain hypersensitivities. Behavioral responses for mechanical allodynia (A) and thermal hyperalgesia (B) at the injected hind paws were measured before (baseline) and after injection of IL-17 (↓) and compared to control mice injected with vehicle (saline). A decrease in paw withdrawal thresholds to mechanical stimuli and paw withdrawal latency to thermal stimuli represents mechanical and thermal pain hypersensitivity, respectively. Paw withdrawal threshold (A) and paw withdrawal latency (B) (mean \pm SEM, $n = 6$ per group) in mice injected with 5 ng IL-17 (filled squares) and 25 ng (filled triangles) were significantly lower 1 to 24 hours following intraplantar IL-17 injection than in mice injected with vehicle (open circles) and gradually returned to near normal values in 48 hours (** $P < .001$, 2-way ANOVA, followed by Bonferroni post-test).

withdrawal threshold and latency, suggesting that these doses have already reached the maximal effect of IL-17 on behavioral outcomes.

Following intraneural injection of IL-17 into the sciatic nerve, mice developed an increased sensitivity to mechanical and thermal stimuli in a dose-dependent manner (Fig 6). No significant differences were observed in paw withdrawal thresholds (Fig 6A) and latencies (Fig 6B) between mice injected with 10 ng IL-17 and control mice injected with saline ($P > .05$; rm-ANOVA, followed by Bonferroni post-test). Injection of 25 ng IL-17 had no effect on paw withdrawal thresholds in response to mechanical stimuli (Fig 6C); however, significant reduction in paw withdrawal latencies to thermal stimuli was observed between 1 and 5 hours following injection, with values returning to normal latencies at 24 hours (Fig 6D; $P < .05-.001$; rm-ANOVA, followed by Bonferroni post-test). Mice injected with 50 ng IL-17 exhibited increased sensitivity to both mechanical and thermal stimuli (Figs 6E and 6F). A significant decrease in paw withdrawal thresholds to mechanical stimuli was observed in IL-17-injected mice from 1 hour, and up to 24 hours, following injection (Fig 6E; $P < .05-.001$; rm-ANOVA, followed by Bonferroni post-test). A significant decrease in paw withdrawal latencies to thermal stimuli was observed in IL-17-injected mice at 1, 3, and 24 hours postinjection (Fig 6F; $P < .05-.001$; rm-ANOVA, followed by Bonferroni post-test).

Intrathecal administration of IL-17 resulted in increased sensitivity to thermal stimuli, but not mechanical stimuli (Fig 7). There were no significant differences in paw withdrawal threshold to mechanical stimuli between mice injected with either 25 or 50 ng IL-17 and control mice injected with saline (Figs 7A and 7C; $P > .05$; rm-ANOVA, followed by Bonferroni post-test). However, intrathecal injection of both IL-17 doses (25 and 50 ng) resulted in a significant reduction in paw withdrawal latency to thermal stimuli from 1 to 24 hours postinjection

(Figs 7B and 7D; $P < .05-.001$; rm-ANOVA, followed by Bonferroni post-test).

To assess whether IL-17 injection is associated with increased infiltration and activation of inflammatory cells, we analyzed immunohistochemically the presence of T cells, macrophages, neutrophils, and dendritic cells at the site of application 3 to 4 hours following injections of IL-17 intraplantarly (25 ng) and intraneurally (50 ng), which were most potent at inducing hyperalgesia and allodynia (Figs 5 and 6). We found minimal or almost no labeled T cells and macrophages in the hind paws, or the sciatic nerves, 3 to 4 hours after intraplantar, or intraneural injection, respectively. Whereas T cells and macrophages are known to recruit to the site of injury/inflammation within days following trauma, neutrophils are one of the first responders of inflammatory cells to migrate toward a site of inflammation (within minutes) by chemotaxis. Vehicle (saline) injection by itself induced a considerable accumulation of neutrophils at the site of injection (ipsilateral hind paws and sciatic nerves). However, compared with vehicle injection, IL-17 injection into the hind paw or the sciatic nerve resulted in a significant increase in the number of infiltrating neutrophils (Table 1, $P < .05$, $P < .0001$, respectively; Student t test). There were no significant differences in the numbers of neutrophils in the contralateral un-injected side (Table 1; $P > .05$). Dendritic cells are present in small quantities in some tissues, such as the skin and the peripheral nerve,⁴⁸ and function as antigen-presenting cells to initiate the adaptive immune response. Vehicle injection by itself induced some accumulation of dendritic cells at the site of sciatic nerve injection (ipsilateral sciatic nerves). Compared with vehicle administration, intraplantar, but not intraneural, injection of IL-17 resulted in a significant increase in the number of dendritic cells in the injected hind-paws (Table 1, $P < .0001$; Student t test). There were no significant differences in the numbers of dendritic cells in the contralateral un-injected side (Table 1, $P > .05$).

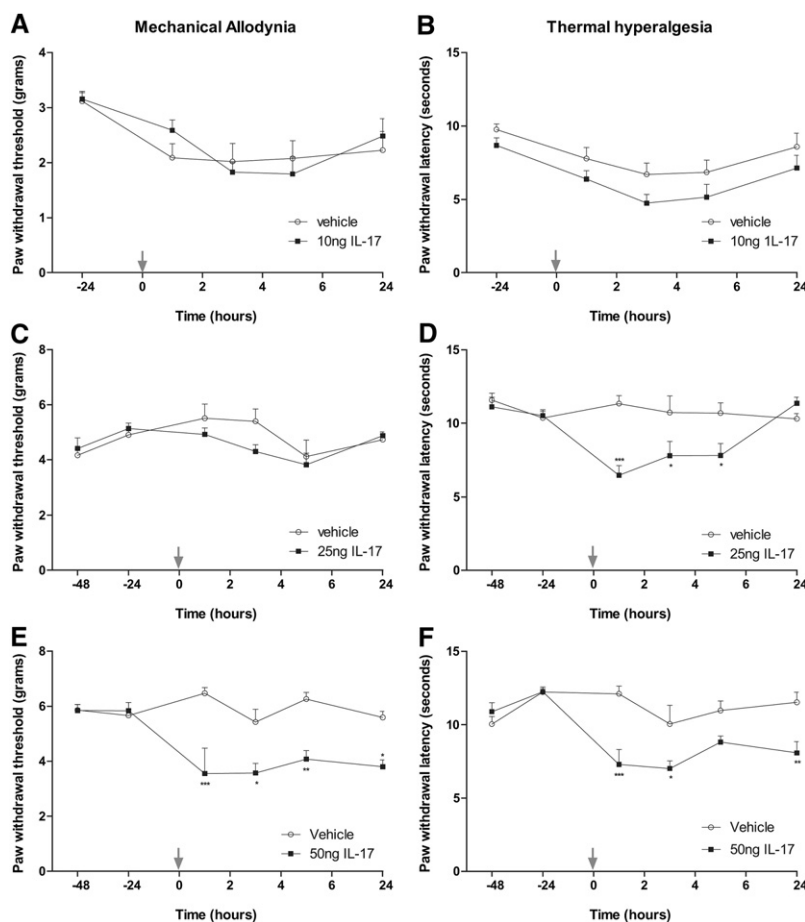


Figure 6. Intraneural IL-17 administration induces mechanical and thermal pain hypersensitivities. Behavioral responses (mean \pm SEM, $n = 6$ per group) for mechanical allodynia (A, C, E) and thermal hyperalgesia (B, D, F) at the hind paws were measured before (baseline) and after sciatic nerve injection of IL-17 (\downarrow) and compared to control mice injected with vehicle (saline). A decrease in paw withdrawal thresholds to mechanical stimuli and paw withdrawal latency to thermal stimuli represents mechanical and thermal pain hypersensitivity, respectively. No difference was observed in paw withdrawal threshold between mice injected with 10 ng (A) and 25 ng (C) IL-17 (filled squares) and mice injected with vehicle (open circles) (2-way ANOVA, followed by Bonferroni post-test). However, intraneural injection of 50 ng IL-17 (E) induced a significant decrease in paw withdrawal threshold 1 to 24 hours following injection ($*P < .05$, $**P < .01$, $***P < .001$, 2-way ANOVA, followed by Bonferroni post-test). There was no significant difference in paw withdrawal latencies between mice injected with 10 ng (B) (filled squares) and mice injected with vehicle (open circles). However, paw withdrawal latencies were significantly lower in mice injected with 25 ng (D) and 50 ng (F) IL-17 (filled squares) in comparison to those injected with vehicle (open circles) between 1 to 5 hours following injection, as well as in 24 hours for 50 ng IL-17 injection ($*P < .05$, $**P < .01$, $***P < .001$, 2-way ANOVA, followed by Bonferroni post-test).

Discussion

Strong evidence is emerging that inflammatory cells and glia, the immune cells of the nervous system, play a key role in the pathogenesis of pain hypersensitivity following peripheral nerve injury. A variety of immune cells including neutrophils, mast cells, macrophages, T cells, microglia, and astrocytes have been implicated in neuropathic pain.³⁸ These cells contribute to pathological pain by release of proinflammatory cytokines. IL-17 is a key proinflammatory cytokine that is primarily secreted from T cells and is now considered as the defining cytokine of a recently discovered new subset of T helper cells, Th17.⁶³ A number of studies indicate the involvement of IL-17 and Th17 cells in a wide range of inflammatory diseases including autoimmune diseases of the nervous system, such as multiple sclerosis⁵⁷ and the animal model experimental autoimmune encephalomyelitis (EAE).^{22,25,45} Additionally, recent studies have demonstrated that T cells in the injured peripheral

nerve and in the dorsal spinal cord contribute to neuropathic pain^{4,7,39} and T-cell infiltration after peripheral nerve injury was found to be associated with IL-17 expression.²¹ In the present study, we investigated the effects of IL-17 on neuropathic pain behaviors. We demonstrate that IL-17 deficiency significantly attenuates mechanical, but not thermal, pain hypersensitivity and significantly reduces neuroinflammatory responses following peripheral nerve injury. Furthermore, local injection of IL-17 increases pain sensitivity in naïve animals, as well as promotes the recruitment of immune cells. Our finding of IL-17 involvement in the development of neuropathic pain is consistent with a recent study showing that IL-17 mediates articular mechanical hypernociception in a model of antigen-induced arthritis.⁴⁷

The specific mechanisms underlying the reduced mechanical allodynia in IL-17 KO mice (Fig 1) are unknown. However, it is reasonable to assume that IL-17 deficiency results in an altered local environment with different

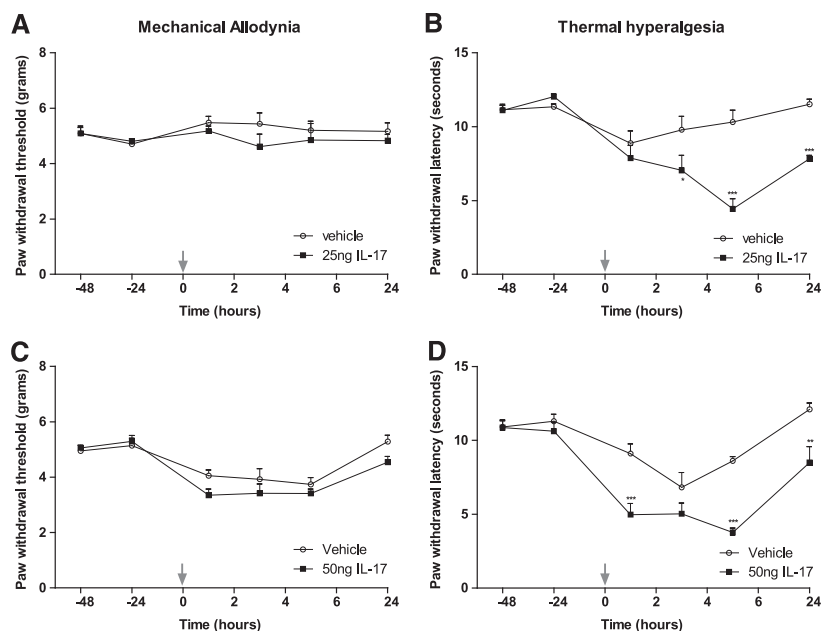


Figure 7. Intrathecal IL-17 administration induces thermal pain hypersensitivity. Behavioral responses (mean \pm SEM, $n = 6$ per group) for mechanical allodynia (A, C) and thermal hyperalgesia (B, D) at the hind paws were measured before (baseline) and after intrathecal injection of IL-17 (I) and compared to control mice injected with vehicle (saline). A decrease in paw withdrawal thresholds to mechanical stimuli and paw withdrawal latency to thermal stimuli represents mechanical and thermal pain hypersensitivity, respectively. There was no significant difference in paw withdrawal threshold between mice injected with 25 ng (A) and 50 ng (C) IL-17 (filled squares) and mice injected with vehicle (open circles). However, paw withdrawal latency in mice injected with 25 ng (B) and 50 ng (D) IL-17 (filled squares) were significantly lower than in vehicle-injected mice (open circles) 1 to 24 hours following intrathecal IL-17 injection ($*P < .05$, $**P < .01$, $***P < .001$, 2-way ANOVA, followed by Bonferroni post-test).

cellular and chemical milieu in the injured nervous system. Some of the gene products most strongly regulated by IL-17 are matrix metalloproteases, acute phase proteins, IL-6, and chemokine ligands, particularly CXC chemokines that function to recruit neutrophils. Accordingly, IL-17 has potent actions to mobilize, recruit, and activate neutrophils.^{11,68} Neutrophils have been implicated in the development of hyperalgesia caused by allergen provocation²⁶ and by peripheral nerve injury.^{41,46} In addition, IL-17 can induce the expression of diverse proinflammatory cytokines, and chemokines from a large variety of cells including fibroblasts, epithelial and endothelial cells, osteoblasts, and monocytes/macrophages.⁶⁴ For example, IL-17 increases PGE2 production via upregulation of cyclooxygenase-2 and increases nitric oxide by stimulating inducible nitric oxide synthase.²⁸ PGE2 and nitric oxide are important inflammatory mediators that contribute to pain hypersensitivity in neuropathic pain.³⁸ Interestingly, some actions of IL-17 are potentiated by other inflammatory cytokines, particularly TNF and IL-1 β .^{64,67} Several studies have shown that IL-17 and TNF synergistically increase production of IL-6, chemokines, beta-defensins, and cyclooxygenase-2 from fibroblasts and osteoblasts.^{19,50} This functional cooperation might be central in neuropathic pain conditions where a significant role for TNF and IL-1 β is well established.

The finding that IL-17 KO mice displayed a significant decrease in mechanical allodynia, but did not display a significant decrease in thermal hyperalgesia following nerve injury, indicates that PSNL-induced neuropathic pain is only partially modulated by IL-17. Indeed, there

has been a growing acknowledgment that mechanical allodynia and thermal hyperalgesia are mechanistically distinct symptoms mediated via different afferent fiber input and may involve different neural pathways including spinal or supraspinal circuits.^{2,44,52} Studies have found that nerve injury-induced mechanical allodynia is mediated by myelinated A-fibers, and thermal hyperalgesia is mediated by heat-nociceptive C-fibers.^{44,52} Thermal hyperalgesia caused by nerve injury involves both spinal and supraspinal circuits, whereas tactile allodynia depends on a supraspinal loop.² Upregulation of the transient receptor potential ion channel TRPV1 in undamaged primary sensory neurons has been implicated in nerve injury-induced heat hyperalgesia.^{18,43} Phosphorylation of p38 mitogen-activated protein kinase in the spinal dorsal horn and the gracile nucleus⁵⁵ and Toll-like receptor 3-mediated activation of spinal glial cells⁴² have been shown to play a crucial role in the development of tactile allodynia, but not thermal hyperalgesia, following peripheral nerve injury. Thus, it is possible that IL-17 does not contribute to the mechanisms of thermal hyperalgesia caused by nerve injury.

Recruitment and activation of T cells, macrophages, microglia and astrocytes have been strongly implicated in the development of neuropathic pain.³⁸ IL-17 influences the recruitment of leukocytes and plays a pivotal role in inflammatory processes. Indeed, our results show that IL-17 deficiency results in a significant decrease in the numbers of T cells (Fig 2) and macrophages (Fig 3) in the injured sciatic nerves and the DRGs following nerve injury. Depletion of macrophages³² and lack of functional T cells^{4,39} have been

Table 1. Neutrophil and dendritic cell counts following local administration of IL-17 or vehicle in the hind paw and sciatic nerve

TREATMENT	HINDPAW			SCIATIC NERVE			
	IL-17 INJECTION	VEHICLE INJECTION	P VALUE	IL-17 INJECTION	VEHICLE INJECTION	P VALUE	
Neutrophils/.5 mm ² ± SEM	Ipsilateral	65.5 ± 10.9	38.2 ± 5.7	<.05*	152.6 ± 4.6	111.5 ± 6.3	<.0001†
	Contralateral	2.9 ± .7	3.7 ± 1.3	ns	0 ± 0	0 ± 0	ns
Dendritic cells/.5 mm ² ± SEM	Ipsilateral	10.4 ± 1.4	1.1 ± .5	<.0001†	30.9 ± 2.9	26.9 ± 4.4	ns
	Contralateral	3.8 ± .7	2.5 ± .6	ns	8.2 ± 1.9	4.9 ± 1.4	ns

NOTE. Neutrophils and dendritic cells were analyzed immunohistochemically in hind-paw sections obtained 3 to 4 hours after intraplantar injection of IL-17 (25 ng) or vehicle, and in sciatic nerve sections obtained 3 to 4 hours after intraneural injection of IL-17 (50 ng) or vehicle. Data are presented as means per .5 mm² ± SEM; n = 3 to 4 mice per group. The numbers of neutrophils and dendritic cells were significantly greater in the ipsilateral hind paws of mice injected intraplantarly with IL-17 than in mice injected with vehicle (**P* < .05, †*P* < .0001, Student *t* test). The numbers of neutrophils, but not dendritic cells, were significantly greater in the ipsilateral sciatic nerves of mice injected intraneurally with IL-17 than in mice injected with vehicle (***P* < .0001, Student *t* test). There were no significant differences in any of the cell counts in the contralateral side. (ns = not significant; *P* > .05, Student *t* test).

shown to attenuate neuropathic pain in animal models. IL-17 could also act directly on glial cells in the central nervous system. Several lines of evidence support the notion that IL-17 affects the functions of glial cells. Using *in vitro* assays, IL-17 was found to be produced by microglia in response to IL-23 and IL-1 β , and treatment of microglia cell culture with IL-17 upregulated the microglial production of IL-6, macrophage inflammatory protein-2, nitric oxide, adhesion molecules, and neurotrophic factors.²⁰ Studies in ischemic brain tissue have demonstrated that levels of IL-17 were elevated in the ischemic hemispheres of human brain and in rats with permanent middle cerebral artery occlusion, and that IL-17 was expressed by neuroglial cells in the ischemic lesion region.²⁹ IL-17 was recently found to play a pivotal role in the evolution of brain infarction and the accompanying neurological deficits.⁵¹ Expression of IL-17 mRNA and protein was also demonstrated in astrocytes and oligodendrocytes located in the active areas of multiple sclerosis lesions.⁵⁷ Furthermore, expression of functional IL-17 receptor A was shown in astrocytes and microglia and was upregulated in the central nervous system of mice with experimental autoimmune encephalomyelitis.¹⁰ This study also demonstrated that treatment of glial cells with IL-17 induced biological responses in these cells, including significant upregulation of monocyte chemoattractant protein (MCP)-1, MCP-5, macrophage inflammatory protein (MIP)-2, and KC chemokine secretion through constitutively expressed IL-17RA.¹⁰ Our results clearly demonstrate that injury to the sciatic nerve induced activation of microglia and astrocytes in the ipsilateral dorsal and ventral horns of the spinal cord and that IL-17 deficiency significantly reduced this glial cell activation (Fig 4). Spinal microglial and astrocyte activation has been previously shown in both the dorsal and ventral horns following peripheral nerve injury^{17,70} and was suggested to be triggered by MCP-1 induction in DRG neurons and their central terminals in the superficial dorsal horn and in motor neurons in the ipsilateral ventral horn.⁷⁰ Peripheral nerve injury could potentially induce IL-17 production by glial cells, which could directly target the same cells expressing IL-17RA to become activated and release proinflammatory cytokines and chemokines. Upregulation of inflammatory

mediators including nitric oxide, IL-6,²⁰ and MCP-1¹⁰ by glial cells exposed to IL-17 could have contributed to the neuropathic pain behaviors in wild-type mice and may partially explain the reduced mechanical pain hypersensitivity in the IL-17 KO mice. In fact, nitric oxide,³⁵ IL-6,²⁷ and MCP-1¹³ enhance central sensitization and play a role in the development of neuropathic pain.

Supporting a role of IL-17 in neuropathic pain, injections of recombinant IL-17 into naïve animals produced short-term pain hypersensitivity. In accordance with the proinflammatory actions of IL-17, we found that intraplantar injection of low dose of IL-17 (5 ng) already induces significant pain hypersensitivity from 1 hour following the injection (Fig 5). IL-17 regulates inflammatory responses by inducing the production of other proinflammatory cytokines and chemokines, thereby promoting the recruitment of monocytes and neutrophils.¹ For example, it can stimulate a variety of cells to produce C-X-C chemokines, IL-8, IL-6, granulocyte-colony-stimulating factor, MCP-1, PGE2, nitric oxide, and numerous matrix-metalloproteinases.^{12,24,56,60} Some of these mediators have been shown to contribute to neuropathic pain.³⁸ We also found that intraplantar injection of IL-17 resulted in a significant increase in the numbers of infiltrating neutrophils and activated dendritic cells at the region of the hind paw where injection took place (Table 1). It is logical to assume that these cells released proinflammatory mediators, and hence contributed to the immediate pain hypersensitivity. Intraneural injection of IL-17 produced time- and dose-dependent hypernociception, with only high doses (25 and 50 ng) showing effects (Fig 6), and pain hypersensitivity was less pronounced compared to the pain responses after intraplantar injection. The existence of a blood-nerve barrier around the sciatic nerve may limit the infiltration of inflammatory cells under normal conditions, but might be compromised following peripheral nerve injury.³¹ We found that intraneural injection of IL-17 resulted in a significant increase in the numbers of infiltrating neutrophils (Table 1), which could have contributed to the observed pain hypersensitivity. Endoneurial accumulation of neutrophils at the site of sciatic nerve injury has been shown to play a role in the early development of hyperalgesia and depletion of neutrophils at the

12 The Journal of Pain

time of nerve injury significantly attenuated neuropathic pain.⁴⁶ Intrathecal administration of high doses of IL-17 produced only thermal hypernociception, but not mechanical hypernociception (Fig 7). This is in contrast to our results of IL-17 KO mice displaying significantly reduced mechanical allodynia, but not thermal hyperalgesia, suggesting different mechanisms mediating the pain hypersensitivity in normal animals and following sciatic nerve injury. Additionally, as mentioned previously, abnormal sensory responses to thermal and mechanical stimuli differ mechanistically involving different fiber types and neural pathways.^{2,44,52} Because cytokines normally have a short half-life in vivo, the pain hypersensitivity observed hours after IL-17 injections might be due to indirect effects. However, the exact mechanisms involved in this IL-17-mediated hypernociception are yet to be clarified.

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Interleukin-17 Contributes to Neuropathic Pain

In conclusion, we demonstrate here that IL-17-mediated inflammatory responses in the injured sciatic nerve, DRGs, and spinal cord contribute to neuropathic pain processing after nerve injury and that direct administration of IL-17 produces pain hypersensitivity. Clearly, IL-17 is not the sole factor responsible for neuropathic pain because, although IL-17 KO mice have reduced mechanical allodynia, they still exhibit thermal hyperalgesia following nerve injury. However, cross-communication between IL-17 and other cytokine signaling systems is most likely involved in the pathogenesis of neuropathic pain, as has been demonstrated in other inflammatory diseases.^{5,28} The molecular and cellular characterization of IL-17 in the nervous system following peripheral nerve injury and the effects of pharmacologically suppressing IL-17 bioactivity on neuropathic pain warrant further studies.

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Interleukin-17 Contributes to Neuropathic Pain
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