Inflammation Confers Dual Effects on Nociceptive Processing in Chronic Neuropathic Pain Model

Jin-Tarng Liou, M.D.,* Fu-Chao Liu, M.D., Ph.D.*, Chih-Chieh Mao, M.D., Ph.D.*, Ying-Shu Lai, M.Sc.,† Yuan-Ji Day, M.D., Ph.D.‡

ABSTRACT

Background: Although inflammation induces pain, immune cells also produce mediators that can effectively counteract it. To further elucidate the role of the immune response, we analyzed the relationship of pain behavior, several inflammatory signals, and opioid peptides using partial sciatic nerve ligation in mice at different levels of immunocompromise.

Methods: Sciatic nerves of C57BL/6C, nonobese diabetic (NOD), or nonobese diabetic–severe combined immune deficiency (NOD-SCID) mice were partially ligated. Responses to mechanical and radiant heat stimuli were observed. Inflammation was detected by immunohistochemistry and flow cytometry. Inflammatory cytokines and opioid peptides were analyzed using real-time polymerase chain reaction and enzyme-linked immunosorbent assay or immunostaining.

Results: Inflammation in immunocompromised mice was subordinate when compared with that seen in C57BL/6C mice. In addition, immunocompromised mice had less pain hypersensitivity at early stages. Whereas proinflammatory tumor necrosis factor-α (TNF-α), interleukin 1β (IL-1β), interleukin 6 (IL-6), and interferon-γ (IFN-γ), as well as antiinflammatory interleukin 1 receptor antagonist (IL-1Ra), interleukin 4 (IL-4), interleukin 10 (IL-10), and interleukin 13 (IL-13) cytokine expression and protein were increased in C57BL/6C mice, they were lower in immunocompromised mice. Although enkephalin, dynorphin, and β-endorphin messenger RNA expression also increased in C57BL/6C mice, peaking on day 14, this result was not observed in immunocompromised mice.

Conclusion: The contribution of inflammation to nerve injury is complex with biphasic modulation. During the early phase, a wide range of proinflammatory cytokines are released, leading to enhanced pain. In contrast, the analgesic effect of opioid peptides and antiinflammatory cytokines was more predominant in the later phases of injury, leading to attenuated pain responses.

EUROPATHIC pain is a common sequela resulting from multiple neural diseases and has been extensively studied.1,2 Growing evidence has demonstrated that the recruitment and infiltration of inflammatory cells, such as neutrophils and macrophages, which were activated during nerve injury,3–5 leads to microenvironmental changes that subsequently initiate hyperalgesia.6–7 In previous studies, the relationship between mediators released by inflammatory cells on their activation and the development of hyperalgesia in neuropathic pain has been identified.8–10 Proinflammatory cytokines, such as tumor necrosis factor-α (TNF-α), interleukin 1 (IL-1), and interleukin 6 (IL-6), have been shown to induce acute or short-term hyperalgesia10–12 and were implicated directly in chronic hyperalgesia and allodynia.12–14 In contrast, the antiinflammatory cytokines and opioid peptides released by inflammatory cells, which may reduce or eliminate behavioral hypersensitivity, were also present in neuropathic pain models.15,16 In these studies, the recruitment of circulating polymorphonuclear cells, monocytes, and lymphocytes that contain opioid peptides17,18 may induce the analgesic effects. These analgesic effects could be attenuated by anti-opioid peptide antibodies.19,20 Moreover, inhibiting the migration of opioid-containing leukocytes sig-

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* Assistant Professor, † Research Assistant, ‡ Associate Professor, Department of Anesthesiology, Transgenic and Molecular Immunogenetics Laboratory, Chang Gung Memorial Hospital and Graduate Institute of Clinical Medical Sciences, Chang Gung University, Linkou, Taiwan.

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Address correspondence to Dr. Day: Department of Anesthesiology, Chang Gung Memorial Hospital, 5 Fushing Road, Guishan Taoyuan, 333 Taiwan, Republic of China. yjday@adm.cgmh.org.tw. Information on purchasing reprints may be found at www.anesthesiology.org or on the masthead page at the beginning of this issue. ANESTHESIOLOGY’s articles are made freely accessible to all readers, for personal use only, 6 months from the cover date of the issue.

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What We Already Know about This Topic

• Nerve injury results in recruitment of immune cells that secrete products capable of enhancing and inhibiting pain and hypersensitivity.

What This Article Tells Us That Is New

• Using normal and immunocompromised mice with acute mechanical nerve injury, immune responses appear to exacerbate hypersensitivity shortly after injury, but reduce hypersensitivity later, reflecting functional changes in secreted products over time.

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nificantly augmented hyperalgesia.\textsuperscript{21} Taken together, it is not certain whether recruitment of leukocytes to the injured nerve tissue during the inflammatory process will augment or attenuate hyperalgesic effects. According to these reports, we hypothesized that inflammation may have multiple effects on the modulation of pain progression after neuroinflammation postinjury. To clarify this issue, we used wild-type C57BL/6C, nonobese diabetic (NOD), and nonobese diabetic–severe combined immune deficiency (NOD-SCID) mice—which all have different levels of immunocompromise—to examine the effect of inflammation on neuropathic pain. In the current study, we analyzed the profile of immune cell infiltrates as well as messenger RNA (mRNA) and proteins expression levels of various cytokines and opioid peptides in mice receiving partial sciatic nerve ligation (PSNL).

Materials and Methods

Animals and Preparation

Adult male C57BL/6C mice (15–20 g, aged 8–10 weeks) were purchased from BioLASCO Taiwan Co., Ltd. (Taipei, Taiwan). Adult male nonobese diabetic (NOD/ShiLt or NOD/Lt), and NOD-SCID mice (15–20 g, aged 8–10 weeks) were purchased from National Laboratory Animal Center, Taiwan. NOD/Lt mice develop insulinitis, a leukocytic infiltrate of the pancreatic islets. Approximately 40–60% of NOD/Lt males develop insulin-dependent diabetes mellitus by age 30–40 weeks. NOD mice also exhibit multiple aberrant immunophenotypes, including defects in antigen-presenting cells, immunoregulatory functions, regulation of the T lymphocyte repertoire, natural killer cell function, and cytokine secretion from macrophages, as well as lack of hemolytic C5 complement.\textsuperscript{22} Mice homozygous for the severe combined immune deficiency spontaneous mutation (Prkdc\textsuperscript{scid} or scid) are characterized by an absence of functional T and B cells, hypogammaglobulinemia, lymphocytopenia, and a normal hematopoietic microenvironment. In general, scid leakiness is high on the C57BL/6J and BALB/c genetic backgrounds, but lower on the NOD/Lt background. NOD.CB17-Prkdc\textsuperscript{scid}J (NOD-SCID) mice are characterized by an absence of functional T and B cells, hypogammaglobulinemia, lymphocytopenia, insulinitis (but no diabetes), and a normal hematopoietic microenvironment. In addition, they exhibit low natural killer cell activity, no hemolytic complement activity, defects in myeloid differentiation, and poor antigen-presenting cell functions as a result of the NOD genetic background.\textsuperscript{23,24}

All experimental mice were first housed in individually ventilated cages without food and water restrictions. They were monitored for blood glucose levels. Mice with diabetes, signs of wound infection, or dehiscence were excluded from study. Animal protocols were approved by the Institutional Animal Care and Use Committee at Chang Gung Memorial Hospital (Taipei, Taiwan). All mice were housed in the Chang Gung Memorial Hospital animal facility whereby the husbandries were followed by the approved 1996 guidelines from the National Institutes of Health§ and the International Association for the Study of Pain.\textsuperscript{11}

Surgical Procedures

Mice were anesthetized in a prone position using isoflurane, 1.5–2%, in 100% oxygen via a modified nose cone. Surgical procedures were modified from previous publications\textsuperscript{25,26} for rats and mice. In brief, a small incision was made at high thigh level and the right sciatic nerve was exposed. Half of the diameter of the nerve was tightly ligated with a 6-0 polydioxanone suture (Ethicon, Inc., New York, NY). A sham operation was also performed, exposing the right sciatic nerve without ligation.

Nociceptive Tests

In response to mechanical and radiant heat stimuli, the nociceptive baseline was measured as described in our previous study.\textsuperscript{27} In brief, animals were allowed to acclimate to their environment for at least 1 h before testing. Thermal and mechanical tests were performed on all animals in the same isolation room to minimize bias. One author (Y-S.L.) who was blinded to group assignments performed pain behavior testing. The first test was thermal stimulation in four animals of each group (n = 8) and mechanical stimulation in the other four animals of the same group. In addition, animals were placed in an elevated plastic cage at least 60 min before they were moved to other sites for testing. Mechanical withdrawal responses were determined with a calibrated electronic von Frey anesthesiometer (model 2290CE, IITC Life Science, Inc., Woodland Hills, CA). Each stimulus was applied from 0 g to the point at which a withdrawal response was observed, or until the cutoff value of 20 g was reached. These tests were repeated more than three times between withdrawal responses with a 5-min resting period between each test. The value of mean forces collected from these tests was considered the withdrawal threshold.

Withdrawal latencies to heat stimulation were evaluated with a focused radiant heat source and a tail flick analgesia meter (model 33; IITC Life Science, Inc.). In brief, the heat stimulus was applied from beneath a heat-tempered glass floor on the distal portion of the plantar aspect of the hind paw. The intensity of radiant heat was adjusted to achieve basal withdrawal latencies of 8–10 s. Cut-off value was set at 15 s. Thermal baseline values were obtained from animals before nerve injury and withdrawal latencies were measured to the nearest 0.1 s. These tests were repeated more than three times with 5–10 min resting periods between each trial. Tests results were then averaged to obtain the paw withdrawal latency. After PSNL, responses to mechanical stimuli and radiant heat were determined at 2–6 h as well as at 3, 7, and 14 days.


**Immunohistochemistry**

Animals (n = 6 per group) were anesthetized with isoflurane and the sciatic nerves were removed and fixed in 4% paraformaldehyde for 3–6 h. After dehydration, samples were embedded in paraffin and cut into 5-μm sections. They were then deparaffinized with xylene, rehydrated in graded ethanol and phosphate-buffered saline, and subjected to detection. Antigen retrieval was performed whereby slides were heated in sodium citrate buffer (10 mM sodium citrate, 0.05% Tween-20, pH 6.0) to a temperature of 95–100°C for 20–30 min. After several washes in phosphate-buffered saline, sections were pretreated with 0.3% H₂O₂ and 10% normal goat serum (Vector Laboratories, Burlingame, CA) for 1 h at room temperature. They were then incubated for 24–36 h at 4°C in a rat anti-mouse Ly-6G monoclonal antibody for neutrophils (1:500; BD Biosciences, San Diego, CA), rat anti-CD3 monoclonal antibody (1:500; AbD Serotec, Oxford, United Kingdom), and rabbit polyclonal lba1 antibody to macrophages (1:500; Biocare Medical, LLC, Concord, CA) diluted in phosphate-buffered saline containing 0.3% Triton X-100 and 3% normal goat serum. Subsequently, sections were incubated in biotinylated goat anti-rat or anti-rabbit immunoglobulin G secondary antibodies (1:2000; Vector Laboratories) using VECTASTAIN Elite ABC Kit (Vector Laboratories). Immunoprecipitates were developed with 0.05% diaminobenzidine in phosphate-buffered saline for 3–10 min. Negative control sections received the same treatment without the addition of primary antibody. Six to eight sections were randomly selected from each animal to be analyzed. Images were captured using a digital camera (DXM 1200 with Eclipse E800; Nikon Corporation, Tokyo, Japan).

To observe coexpression of opioid peptides with infiltrated inflammatory cells in the injured nerve, we incubated the sections with monoclonal rat anti-mouse CD45 receptor antibody—recognizing inflammatory cells (1:500; Novus Biologicals LLC, Littleton, CO), Ly6G, CD3, and anti-mouse Mac-2 for macrophages (1:100; eBioscience, Inc., San Diego, CA), as well as polyclonal goat antibody against β-endorphin (1:200; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), rabbit polyclonal to methionine enkephalin (1:400; Abcam, Cambridge, MA), or rabbit polyclonal to dynorphin A (1:50; Abcam) overnight at 4°C under gentle agitation. Sections were then incubated with the respective secondary antibodies (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA): donkey anti-goat conjugated with Rhodamine Red-X (1:100); goat anti-rabbit conjugated with Rhodamine Red-X (1:200); and goat anti-rat conjugated with fluorescein isothiocyanate (1:200). Thereafter, sections were washed and cover slips were mounted on glass slides with ProLong® antifade medium (Molecular Probes Inc., Eugene, OR). Nonspecific staining was determined by excluding primary antibodies. Images were captured using a fluorescence microscope operated by Case Data Manager Expo 4.5 software (DM 2500; Leica, Houston, TX).

**Cell Preparation and Flow Cytometry**

To obtain a single cell suspension for fluorescence-activated cell-staining analysis, animals (n = 6 per group) were anesthetized with isoflurane and sacrificed at 1, 3, 7, or 14 days after PSNL or sham surgery. Ligated portions of the sciatic nerve (approximately 1 cm long, including sites proximal and distal to PSNL) were collected. Single-cell suspensions were prepared as described in previous reports. In brief, samples were digested for 1 h at 37°C with 10 ml RPMI 1640 medium (Life Technologies, New York, NY) containing the following (Sigma-Aldrich, St Louis, MO): 30 mg collagenase, 10 mg hyaluronidase, and 0.5 ml HEPES 1 M. Digested samples were pressed through a 70-μm nylon filter (Becton, Dickinson, Franklin Lakes, NJ) to remove particles. Samples were stained with specific phycocerythrin conjugated rat anti-mouse monoclonal antibodies recognizing monocytes/macrophages (CD 11b), hamster anti-mouse T lymphocytes (CD3ε), or monoclonal antibody recognizing granulocytes (Ly6G) and, subsequently, with fluorescein isothiocyanate–conjugated monoclonal antibody recognizing IL-6 (1:200; eBioscience, Inc.). Staining specificity was verified by incubation of cell suspensions with appropriate isotype-matched control antibodies. We used Cytofix/Cytoperm Plus Kits (BD Biosciences) for intracellular cytokine staining according to manufacturer instructions. At least 30,000 fluorescence-activated cell-staining events were collected in each sample. Data were acquired and analyzed using flow cytometry (Cytomics FC500; Beckman Coulter, Inc., Fullerton, CA).

**Measurement of Cytokines Levels in Sciatic Nerves**

Different groups of animals (n = 6 per group) were sacrificed at 1, 3, 7, or 14 days after PSNL. Inflammatory mediators were measured by enzyme-linked immunosorbent assay. Ligated portions of the sciatic nerve were collected and analyzed simultaneously for 12 different cytokines, including IL-1β, interleukin 2 (IL-2), interleukin 4 (IL-4), IL-6, interleukin 10 (IL-10), interleukin 13 (IL-13), Interferon-γ (IFN-γ), and TNF-α, using the Multi-Analyte ELISAArray™ Kit System (Mix-N-Match; SABiosciences, Frederick, MD) according to manufacturer instructions. In addition, an interleukin 1 receptor antagonist (IL-1Ra) level was determined using enzyme-linked immunosorbent assay (R&D Systems Europe, Abdingdon, England, United Kingdom).

**Total RNA Isolation and Quantitative Real-time Polymerase Chain Reaction**

Fresh sciatic nerve specimens were harvested from C57BL/6G, NOD, and NOD-SCID mice at 1, 3, 7, or 14 days after PSNL (n = 4 per group). Samples were homogenized in 0.8 ml TRIZol® reagent (Invitrogen, Carlsbad, CA). Total RNA was isolated using the PureLink™ Micro-to-Midi Total RNA Purification System (Invitrogen) according to manufacturer instructions. Spectrophotometry was used to determine total RNA concentrations for each sample, which was then resuspended in diethyl pyrocarbonate–treated water.
Table 1. Sequences of Gene-specific Primers Used for Quantitative Real-time Polymerase Chain Reaction

<table>
<thead>
<tr>
<th>Gene (GeneBank Accession No.)</th>
<th>Primer</th>
<th>Sequence (5’ to 3’)</th>
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</thead>
<tbody>
<tr>
<td>β-actin (NM_007393)</td>
<td>Sense antisense</td>
<td>ACCCTAAGGCCAACCGTGAA ATGGCCTGAGGGAGACATAG</td>
</tr>
<tr>
<td>TNF-α (NM_013693)</td>
<td>Sense antisense</td>
<td>CCCCCTCATCGTGGCTATGAG AGCAAGGCTACAACCATCG</td>
</tr>
<tr>
<td>IL-1β (NM_008361)</td>
<td>Sense antisense</td>
<td>TTAGGGCAAGCAGGTATCAGTGGTCA CGTCAACACACACAGAGTGTGTT</td>
</tr>
<tr>
<td>IL-6 (NM_031168)</td>
<td>Sense antisense</td>
<td>TTGGCTTTGTTGGAGCTATG AGCAATTGCGATTCCGACACT</td>
</tr>
<tr>
<td>IFN-γ (NM_008337.1)</td>
<td>Sense antisense</td>
<td>TGGACGAGGACCTATGCGAGT CATTGGGAGGCTTAACAGAC</td>
</tr>
<tr>
<td>IL-1Ra (NM_031167)</td>
<td>Sense antisense</td>
<td>GATCATGTTGCTATGAGGCTT TGGACAGGCAGTCGTTCAAG</td>
</tr>
<tr>
<td>IL-4 (NM_021283)</td>
<td>Sense antisense</td>
<td>TGTACCAGGAGGCTAGTTCA AGGGCGTGAACGTTCAAG</td>
</tr>
<tr>
<td>IL-10 (NM_010548)</td>
<td>Sense antisense</td>
<td>ATCACAGGGGGCAGTACA TTCCGATAAGCTGGCAACCC</td>
</tr>
<tr>
<td>IL-13 (NM_008355)</td>
<td>Sense antisense</td>
<td>TCTGTGTAGGCGTGGATGAA CAGAATTGCCATTGCAAC</td>
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<tr>
<td>β-endorphin (NM_008895.3)</td>
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<td>GCCAGAAGCTCGCAGTAAGT GGTATGCGATTGCCAGT</td>
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<tr>
<td>Enkephalin (NM_001002927.2)</td>
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<td>TACACTGAGGCGTGGATGACAT GTTACAGAGGCTAGT</td>
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<tr>
<td>Dynorphin (NM_018863.3)</td>
<td>Sense antisense</td>
<td>GCCCTTCTGTCAGGTGGTCA GGGCGTGAACGTTCAAG</td>
</tr>
</tbody>
</table>

Primer sequences are shown in 5’ to 3’ orientation.

IFN-γ = interferon-γ; IL = interleukin; IL-1β = interleukin 1β; IL-1Ra = interleukin-1 receptor antagonist; TNF-α = tumor necrosis factor-α.

All RNA samples were pretreated with DNase I (Invitrogen) before complementary DNA synthesis to avoid genomic DNA contamination. Using 50 μM oligo(dT) primer and Superscript III First-Strand Synthesis SuperMix System (Invitrogen Life Technologies, Tokyo, Japan), 1 μg total RNA was reverse transcribed. Finally, complementary DNA was stored at −20°C until use. For polymerase chain reaction, specific primers for proinflammatory cytokines (TNF-α, IL-1β, IL-6, and IFN-γ), antiinflammatory cytokines (IL-1Ra, IL-4, IL-10, and IL-13) and opioid peptides (enkephalins, dynorphin, and β-endorphin) were designed and synthesized (table 1). Each reaction was performed with 10 μl master mix (DyNAme™, Flash Probe qPCR Kit; Finnzymes, Espoo, Finland), 1 μl mix containing two primers (10 μM each), and a template (0.2 μM) for each specific complementary DNA sample. Polymerase chain reactions were performed using the Chromo 4™ Four-Color Real-Time System (Bio-Rad Laboratories, Inc., Hercules, CA) according to manufacturer instructions. Each sample was measured in triplicate. Data sets were examined for integrity of linearization plot. All values were normalized relative to β-actin levels.

Use of Opioid Receptor Antagonists to Confirm Peripheral Opioid Distribution

To verify the distribution of peripheral opioids in early and later phases of neuropathic pain, the extent of thermal hyperalgesia was evaluated in C57BL/6JC and NOD-SCID mice (n = 8 per group) at 1 and 14 days after PSNL or sham surgery and reevaluated 30 min after subcutaneous injection of 5 μg opioid receptor antagonist naloxone methiodide or vehicle.

Statistical Analysis

Results were averaged for each group and values were expressed as mean ± SD. Mixed-design, two-way repeated-measures ANOVA was performed with group as a between-subjects factor and time after PSNL as a within-subjects factor. The Bonferroni adjustment was employed for multiple comparisons at individual time points between groups. One-way ANOVA was performed for comparison of individual group means. The Dunnett test was performed for multiple comparisons between experimental and control groups at different time points. Two-sample comparisons were made using a paired t test. When statistical evaluation was performed (Prism 5.0; GraphPad Software, San Diego, CA), for all tests, a P value of less than 0.05 was considered statistically significant.

Results

Reduced Pain Hypersensitivity. In NOD and NOD-SCID mice, reduced pain hypersensitivity was observed predominantly in the acute phase of PSNL injury (days 1–7). Mechanical and thermal pain hypersensitivities were measured in different groups of mice: one containing an intact immune function (C57BL/6), one with partial immune deficiency (NOD), and one with severe combined immune deficiency (NOD-SCID). When baseline values for mechanical and thermal pain hypersensitivities were obtained before PSNL, data showed no difference within groups. The baseline time of withdrawal for C57BL/6JC, NOD, and NOD-SCID mice, respectively, in response to thermal stimulus was 9.8 ± 0.6, 9.3 ± 0.8, and 9.7 ± 0.7 s, respectively; for baseline mechanical stimulus, response time was 5.8 ± 0.4, 6.6 ± 0.6, and 6.1 ± 0.8 g (P > 0.05 for all comparisons, two-way repeated-measures ANOVA; figs. 1A and B). However, compared with animals that had normal immune function (C57BL/6JC), immunocompromised mice (NOD and NOD-SCID) had significantly less thermal and mechanical pain hypersensitivities in the nerve-injured paw as seen in the
Acute phase (days 1–7) of PSNL injury. Thermal stimulus time needed to stimulate a withdrawal response at 1, 3, 7, and 14 days was, respectively, 3.4 ± 0.7, 3.7 ± 0.7, 4.4 ± 0.8, and 6.8 ± 1.0 for C57BL/6C; 5.3 ± 0.8, 5.4 ± 0.8, 6.5 ± 0.9, and 6.1 ± 0.5 for NOD; 6.9 ± 0.8, 7.9 ± 0.7, 7.5 ± 0.9, and 6.5 ± 0.9 for NOD-SCID. Significance levels were the same for C57BL/6C versus NOD and NOD-SCID, as well as NOD versus NOD-SCID at days 1 and 3, in addition to C57BL/6C versus NOD and NOD-SCID at day 7 (P < 0.05, two-way repeated-measures ANOVA; fig. 1A). In addition, these differences were more prominent in NOD-SCID mice. Similar results were observed with mechanical stimulation (g) and are shown in figure 1B (2.1 ± 0.3, 2.2 ± 0.3, 2.9 ± 0.4, and 3.5 ± 0.4 for C57BL/6C; 3.3 ± 0.5, 3.3 ± 0.5, 4.3 ± 0.5, and 3.9 ± 0.3 for NOD; 4.8 ± 0.5, 4.5 ± 0.5, 4.6 ± 0.6, and 4.0 ± 0.4 for NOD-SCID at days 1, 3, 7, and 14, respectively; C57BL/6C vs. NOD and NOD-SCID, NOD vs. NOD-SCID at days 1 and 3; C57BL/6C vs. NOD and NOD-SCID at day 7; P < 0.05; two-way repeated-measures ANOVA). Behavioral hypersensitivity was induced in all animals of our three PSNL groups (vs. control at different time points were P < 0.05; one-way ANOVA with Dunnett test). However, aging and repeated testing after surgery did not lower the threshold to stimulus-evoked response in the three respective sham groups. These data suggest that immune responses contribute to the development and maintenance of neuropathic pain.

**Less Inflammation and Cell Infiltration.** In the damaged nerves of NOD and NOD-SCID mice, less inflammation and cell infiltration was observed after PSNL. We carried out immunohistochemistry staining and flow cytometric analysis for immune cells (neutrophils, macrophages, and T cells) in sciatic nerves of the different groups of mice after PSNL. There was no significant cellular infiltration in the sham groups (data not shown). In fact, we found a pronounced infiltration of immune cells in the injured nerve as compared with the uninjured nerve (figs. 2, 3, and 4). Infiltrations were most prominent at the site of injury. Neutrophils were detected at the site of injury in C57BL/6C mice (intact immune function) at early stages (days 1–3), with neutrophil numbers peaking at day 3 postinjury and decreasing thereafter. Compared with intact immune function mice, immunocompromised mice (NOD/Lt and NOD-SCID) had significantly less neutrophil infiltration in the injured nerve. This difference was more prominent among NOD-SCID mice. Data are presented as mean ± SD (n = 6). *P < 0.05 versus C57BL/6C; #P < 0.05 versus NOD/Lt; two-way repeated-measures ANOVA with Bonferroni adjustment.
reached a peak on day 3 postinjury and decreased thereafter (1.6 ± 0.8, 8.9 ± 1.4, 9.4 ± 1.6, 2.5 ± 1.4, and 1.8 ± 1.2 \times 10^3 \text{Ly6G-positive cells/nerve} for C57BL/6C mice at days 0, 1, 3, 7, and 14, respectively; fig. 2). In contrast, macrophage and T-cell infiltration progressively increased and was most predominant at later postinjury stages (days 7–14), with macrophage numbers peaking at day 7 postinjury. Compared with intact immune function mice, immunocompromised mice (NOD/Lt and NOD-SCID) had significantly less macrophage infiltration in the injured nerve. This difference was more prominent among NOD-SCID mice. Data are presented as mean ± SD (n = 6). *P < 0.05 versus C57BL/6C, #P < 0.05 versus NOD/Lt; two-way repeated-measures ANOVA with Bonferroni adjustment.

Less Proinflammatory Cytokine Expression. After PSNL, less proinflammatory cytokine expression was observed in the damaged nerves of NOD and NOD-SCID mice. We analyzed proinflammatory cytokine (TNF-α, IL-1β, IL-6, and IFN-γ) mRNA expression in injured sciatic nerves collected from C57BL/6C, NOD, and NOD-SCID mice subjected to PSNL because they are well known to mediate cell-cell interplay and subsequent inflammatory responses (data not shown). These observed cytokine mRNA expres-

Fig. 3. Histopathologic features (A) and time course (B) of macrophage (iba1 or CD11b-positive) infiltration in the sciatic nerves of wild-type C57BL/6C, nonobese diabetic (NOD/Lt), and nonobese diabetic–severe combined immune deficiency (NOD-SCID) mice after partial sciatic nerve ligation (PSNL) versus sham surgery. Macrophages were detected at the site of injury in C57BL/6C mice (intact immune function) at later stages (days 7–14), with macrophage numbers peaking at day 7 postinjury. Compared with intact immune function mice, immunocompromised mice (NOD/Lt and NOD-SCID) had significantly less macrophage infiltration in the injured nerve. This difference was more prominent among NOD-SCID mice. Data are presented as mean ± SD (n = 6). *P < 0.05 versus C57BL/6C; two-way repeated-measures ANOVA with Bonferroni adjustment.

Fig. 4. Histopathologic features (A) and time course (B) of T-cell (CD3e-positive) infiltration in the sciatic nerves of wild-type C57BL/6C, nonobese diabetic (NOD/Lt), and nonobese diabetic–severe combined immune deficiency (NOD-SCID) mice after partial sciatic nerve ligation (PSNL) versus sham surgery. T cells were detected at the site of injury in C57BL/6C mice (intact immune function) at later stages (days 7–14). Compared with intact immune function mice, immunocompromised mice (NOD/Lt and NOD-SCID) had significantly less T-cell infiltration in the injured nerve. This difference was more prominent among NOD-SCID mice. Data are presented as mean ± SD (n = 6). *P < 0.05 versus C57BL/6C; two-way repeated-measures ANOVA with Bonferroni adjustment.
Less Antiinflammatory Cytokine Expression. NOD and NOD-SCID mice had less antiinflammatory cytokine expression in damaged nerves after PSNL. We also examined the expression patterns of several antiinflammatory cytokines (IL-1Ra, IL-4, IL-10, and IL-13) in injured sciatic nerves collected from C57BL/6/C, NOD, and NOD-SCID mice subjected to PSNL. Our results showed that there was a significant up-regulation of antiinflammatory cytokines in the C57BL/6/C mice subjected to PSNL. As shown in figure 7, IL-1Ra and IL-10 were significantly increased at the early phase (days 1–3). In contrast, IL-4 and IL-13 were detected most prominently in later phases (days 7–14). Furthermore, there was a significantly lower expression of IL-1Ra, IL-4, IL-10, and IL-13 observed in NOD and NOD-SCID mice compared with C57BL/6/C mice (C57BL/6/C vs. NOD and NOD-SCID at days 1, 3, 7, and 14 for IL-1Ra, IL-4, IL-10, and IL-13; P < 0.05, two-way repeated-measures ANOVA). This effect was more outstanding in NOD-SCID mice (NOD vs. NOD-SCID at day 1 for IL-1Ra; at days 1 and 3 for IL-4; and at days 7 and 14 for IL-13; P < 0.05, two-way repeated-measures ANOVA). Furthermore, there was a significantly lower basal expression of IL-4 in immunocompromised mice.

Less Peripheral Opioid Peptide Expression. Finally, less peripheral opioid peptide expression was observed in the damaged nerves of NOD and NOD-SCID mice after PSNL. We further analyzed mRNA expression levels of various opioid peptides (β-endorphin, enkephalin, and dynorphin) in injured sciatic nerves collected from C57BL/6/C, NOD, and NOD-SCID mice subjected to PSNL. The constitutive expression of each peptide was determined from mice that received a sham operation. No difference within groups was noted (data not showed). As shown in figure 8, increased expression of enkephalin, dynorphin, and β-endorphin was observed in C57BL/6/C mice after PSNL (baseline vs. days 1 and 14 for enkephalin, dynorphin, and β-endorphin; P < 0.05, one-way ANOVA). The expression of these peptides in the injured sciatic nerve was most prominent in the later phases in C57BL/6/C mice as shown in figures 8D, E, and F (day 1 vs. day 14 for enkephalin, dynorphin, and β-endorphin; P < 0.05, one-way ANOVA). In contrast, there was a significantly lower expression of enkephalin, dynorphin, and β-endorphin observed in NOD and NOD-SCID mice compared with C57BL/6/C mice (C57BL/6/C vs. NOD and NOD-SCID at days 1, 3, 7, and 14 for enkephalin, dynorphin, and β-endorphin; P < 0.05, two-way repeated-measures ANOVA). This effect was more prominent in NOD-SCID mice (NOD vs. NOD-SCID at days 7 and 14 for enkephalin and dynorphin; P < 0.05, two-way repeated-measures ANOVA; figs. 8A and C). These observations of opioid peptide mRNA expression could be further supported by immunostaining (fig. 9) that showed less antiinflammatory cytokine expression and less peripheral opioid peptide expression in the damaged nerves of NOD-SCID mice compared with NOD mice.
demonstrated opioid peptides synthesis by neutrophils and macrophages in the early phase and by mainly macrophages in the later phases (fig. 10).

Exacerbated Thermal Hyperalgesia. Among C57BL/6 mice, but not in NOD-SCID mice at the later phases, opioid receptor antagonists exacerbated thermal hyperalgesia. To verify the distribution of peripheral opioids in early and later phases of neuropathic pain, development of thermal hyperalgesia was evaluated in C57BL/6C and NOD-SCID mice (n = 8 per group) at days 1 and 14 after PSNL or sham surgery. To confirm the peripheral site of opioid receptor antagonist action, the opioid receptor antagonist (5 μg naloxone methiodide, the most effective dose) was applied subcutaneously near the surgical site as described in a previous investigation that used the same animal model.31 We found no significant difference in thermal hyperalgesia response between treated and untreated NOD-SCID mice at 1 and 14 days after PSNL (naloxone vs. vehicle at days 1 and 14 for NOD-SCID mice; P > 0.05, paired t tests). However, naloxone injection exacerbated thermal hyperalgesia in C57BL/6 mice in the later phases, but not in the early phase (naloxone vs. vehicle at day 14 for C57BL/6 mice; P < 0.05, paired t test; fig. 11).

Discussion

In the current study, we demonstrated that neuropathic allodynia and hyperalgesia were significantly reduced in both mildly and severely immunodeficient mice. The alleviation of neuropathic pain during the early postinjury phase may be attributed to reduced inflammatory responses observed in immunohistochemistry staining, flow cytometric studies, and cytokine expression profiles of tissue sections. Our results are in line with a previous report by Clatworthy et al.32 that used pharmacologic suppression of the inflammatory response at the injured sciatic nerve, which subsequently reduced hyperalgesia severity whereas enhancing inflammatory response augmented the hyperalgesia. Here we provide direct evidence using genetic models. Mice with mild or severe immunodeficiency harvested less inflammatory severity after...
Neuropathic Pain and Immune Deficiency

Fig. 7. Antiinflammatory cytokine protein levels in the sciatic nerves of wild-type C57BL/6C, nonobese diabetic (NOD/Lt), and nonobese diabetic–severe combined immune deficiency (NOD-SCID) mice after partial sciatic nerve ligation (PSNL). Antiinflammatory cytokine protein levels interleukin 1 receptor antagonist (IL-1Ra) (A), interleukin 4 (IL-4) (B), interleukin 10 (IL-10) (C), and interleukin 13 (IL-13) (D) were increased in C57BL/6C mice after PSNL. Baseline expression values of IL-4 in immunocompromised mice were significantly lower than those observed in C57BL/6C mice. Among immunocompromised mice, the production of these cytokines was attenuated after PSNL, most notably in NOD-SCID mice. Results are expressed as mean ± SD. For each data set, n = 6. *P < 0.05 versus C57BL/6C, #P < 0.05 versus NOD/Lt; two-way repeated-measures ANOVA with Bonferroni adjustment.

PSNL, consequently developing less sensitivity to mechanical and thermal stimuli. Moreover, the nociceptive alleviation observed in immunocompromised mice was predominant in the early phase, but not in later postinjury phases—correlating well with the stage of inflammation observed in C57BL/6C mice. Our results are further supported by a recent study7 where significantly less mechanical allodynia and thermal hyperalgesia developed in athymic nude rats compared with their heterozygous littermates after sciatic nerve injury. To eliminate the confounding effects of genetic background, C57BL/6C and NOD mice were deployed as general control and background control, respectively. It is noteworthy that rendered neuropathic pain thresholds and degrees of inflammation after PSNL were compatible with the severity of immune defects in an order of C57BL/6C (normal), NOD (defects in natural killer cell function), and NOD-SCID (complete immunodeficiency) mice. Although Gabra et al.33 found significant age-dependent hyperalgesia responses to heat stimuli in NOD mice, it is reasonable to assume that NOD mice may develop hyperalgesia due to diabetic neuropathy. However, the incidence of diabetes in NOD mice is low at an age of 8 weeks, 40% at 16 weeks, and 73% at 24 weeks. In our experiments, only NOD mice at an age of 8 weeks without diabetes were included in the study. In addition, there is a substantial difference between diabetes neuropathic pain and PSNL-related neuropathic pain. In the former, chronic hyperalgesia seems to be related to bradykinin B1 receptor; in the latter, prominent acute inflammation is observed during the first week after PSNL.

Based on observations from immunocompromised mice, it seems that the contribution of inflammation to neuropathic pain was predominantly observed in the early postinjury phases. As previously described, there is an infiltration and activation of immune cells in response to nerve damage, leading to the production and secretion of various inflammatory mediators10,34 that sensitize primary afferent neurons, leading to nociceptive hypersensitivity.2,34,35 Both neutrophil and macrophage infiltration have been suggested as promoting the initiation of hyperalgesia during nerve injury.3–5 Mediators released from infiltrated cells such as TNF, IL-1, and IL-6 have been shown to induce acute or short-term hyperalgesia10–12 and were implicated directly in neuropathic pain, chronic hyperalgesia, and allodynia.12–14 Our results also demonstrated the infiltration of the injured nerve tissue by neutrophils and macrophages in normal C57BL/6C mice after PSNL. Moreover, both the infiltration of neutrophils and macrophages and the release of proinflammatory cytokines were significantly lower in immunodeficient mice. Nonetheless, considerable hyperalgesia was still observed in immunodeficient mice subjected to PSNL. This discrepancy suggests that inflammation may not be the sole factor influencing neuropathic pain. Mechanisms other than inflammation may be involved in the process of injury-induced neuropathic pain. We further calculated the difference of each response time as the attribution of inflammation to the quantity of neuropathic pain, as there was no referenced data previously reported. It is noteworthy that neuropathic pain attributed to inflammation was 70% at 24 h postinjury and gradually decreased to 56% and 50% at days 3 and 7, respectively. However, though hyperalgesia was still observed, the influence of inflammation on the amount of neuropathic pain was a minimal, at 3% or of a negligible amount, as compared with NOD-SCID, NOD, and C57BL/6C mice at day 14 after PSNL. These results indicate that mechanisms other than those involved in injury-related immune response contribute to chronic hyperalgesia.

Our studies demonstrate that inflammation mobilizes leukocytes to the site of injury and augments behavioral hypersensitivity. This effect is in contrast to other studies that have shown an attenuation of behavioral hypersensitivity via the secretion of opioid peptides from recruited circulating leukocytes,17,18,29 such as polymorphonuclear cells, monocytes, and lymphocytes. These opioid peptides were identified as dynorphin, methionine enkephalin, and endorphin.17,15 Furthermore, the circulating opioid-containing leukocytes that mediate analgesia can be induced by several
exogenous stimulations. In fact, the analgesic effects of leukocyte-derived opioids were antagonized by naloxone or anti-opioid peptide antibodies. In addition, the prevention of leukocyte recruitment to the site of injury drastically augmented pain hypersensitivity. Here, we provide a more direct genetic rendered animal model to reexamine the effect of inflammation on neuropathic pain. Opioid peptide mRNA expression was minimal in injured nerve tissue at an

Fig. 8. Expressions of peripheral opioid peptide messenger RNA were decreased in immunocompromised mice after partial sciatic nerve ligation (PSNL). (A–C) Baseline expression values of enkephalins, dynorphin, and β-endorphin were not different within groups. Expression levels of these opioid peptides were much lower in immunocompromised nonobese diabetic (NOD/Lt), and nonobese diabetic–severe combined immune deficiency (NOD-SCID) mice than in wild-type C57BL/6C mice. Results are expressed as mean ± SD. For each data set, n = 4. *P < 0.05 versus C57BL/6C, #P < 0.05 versus NOD/Lt; two-way repeated-measures ANOVA with Bonferroni adjustment. (D–F) expression values of enkephalins, dynorphin, and β-endorphin were subsequently increased after PSNL in C57BL/6C and NOD/Lt mice. *P < 0.05 versus control (day 0), #P < versus day 1; one-way ANOVA with Dunnett test.

Fig. 9. Representative double-immunofluorescence images showing coexpression of enkephalin (Enk), β-endorphin (End), or dynorphin (Dyn) with CD45 receptors (CD45R) in immune cells at injured nerves of wild-type C57BL/6C, nonobese diabetic (NOD/Lt), and nonobese diabetic–severe combined immune deficiency (NOD-SCID) mice at days 3 and 14 after partial sciatic nerve ligation (PSNL).
early phase, peaking at day 14 after PSNL in wild-type C57BL/6C mice. Although only a few opioid-containing leukocytes were recruited in early stages of inflammation, they accumulated to peak numbers at late stages. Previously assumed leukocyte recruitment based on opioid peptide mRNA expression was the result of poorly defined opioid-containing leukocytes in earlier reports. However, our findings account for the markedly lower inflammatory states and behavioral hypersensitivity observed in NOD-SCID mice during the early postinjury stages as well as the differences observed in behavioral hypersensitivity between C57BL/6C and NOD-SCID mice that gradually diminished during the later stages. According to the time frame of opioid peptide mRNA expression and data from our immunohistochemical studies, we postulated that most of the opioid-containing leukocytes were macrophages, as T lymphocytes and neutrophils were low in number at later stages. Therefore, we hypothesized that neuropathic pain processes were dominated by inflammation in the acute stage of PSNL injury and that endogenous opioid peptide–mediated analgesia was masked because low amounts of opioid-containing leukocytes were recruited at an early stage. However, endogenous opioid peptide–mediated analgesia gradually accumulated as opioid-containing leukocytes were recruited, such as macrophages, at the late stage after PSNL. In addition, because inflammation subsided at the same time, this process led to less hyperalgesic sensitivity as observed in thermal and mechanical behavior analysis.

There is growing evidence that the balance of proinflammatory versus antiinflammatory cytokines secreted during inflammation determines whether an inflammatory response is elicited. Several antiinflammatory cytokines modulate immune and inflammatory events by inhibiting the production and action of proinflammatory cytokines, also reducing or eliminating pain hypersensitivity. It can be concluded from previous findings that antiinflammatory cytokines (e.g.,}

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**Fig. 10.** Representative double-immunofluorescence images showing coexpression of enkephalin (Enk) with neutrophils (Ly6G), macrophages (Mac 2), and T cells (CD3) at injured nerves of wild-type C57BL/6C mice at days 3 and 14 after partial sciatic nerve ligation (PSNL).

**Fig. 11.** Assessment of peripheral opioids distribution in early (A) and later (B) phases of neuropathic pain in C57BL/6C and nonobese diabetic–severe combined immune deficiency (NOD-SCID) mice. The opioid receptor antagonist (subcutaneous 5 μg naloxone methiodide) exacerbated thermal hyperalgesia at the later phases, after partial sciatic nerve ligation (PSNL) in wild-type C57BL/6C mice but not in NOD-SCID mice. Results are expressed as mean ± SD. For each data set, n = 8. *P < 0.05 for naloxone versus vehicle; paired t tests.
IL-4, IL-10, IL-13, and IL-1Ra) may produce analgesia by inhibiting several proinflammatory cytokines.38 We found a significantly lower expression of IL-1Ra, IL-4, IL-10, and IL-13 in immunocompromised (NOD and NOD-SCID) versus normal (C57BL/6C) mice. However, expression of proinflammatory cytokines was also decreased in immunocompromised mice, so the balance between proinflammatory and antiinflammatory cytokines was maintained.

In addition, Kraus et al.39 reported a different mechanism, IL-4–induced expression of μ- and δ-opioid receptors leading to analgesia through the endogenous opioid system. In contrast, Vale et al.40 reported that IL-4, IL-10, and IL-13 can cause analgesia independent of the endogenous opioid system. Furthermore, it is important to mention that several antiinflammatory cytokines can also act as proinflammatory cytokines during allergic responses.41,42 Thus, although evidence supports the role of antiinflammatory cytokines as direct or indirect mediators of chronic pain development and maintenance after nerve injury, further work is needed to clarify their role.

Our results demonstrate that inflammation may have a dual role in the modulation of behavioral hypersensitivity in the murine PSNL model used in the current study. Inflammation would facilitate the development of behavioral hypersensitivity in the early stage of nerve injury, and, with the accumulation of opioid-containing leukocytes and inflammatory cytokines, attenuate behavioral hypersensitivity in the late stage of injury. It is our hope that our current experiments, which relate the immune response to chronic neuropathy, will eventually lead to innovative possibilities for therapeutic intervention in neuropathic pain, based on targeting the inflammatory response in different stages.

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