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Original Reports

Alendronate Attenuates Spinal Microglial Activation and Neuropathic Pain

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Abstract: **Many derivatives of bisphosphonates, which are inhibitors of bone resorption, have been developed as promising agents for painful pathologies in patients with bone resorption-related diseases. The mechanism for pain relief by bisphosphonates remains uncertain. Studies have reported that bisphosphonates could reduce central neurochemical changes involved in the generation and maintenance of bone cancer pain. In this study, we hypothesized that bisphosphonates would inhibit spinal microglial activation and prevent the development of hyperalgesia caused by peripheral tissue injury. We investigated the effects of alendronate (a nitrogen-containing bisphosphonate) on the development of neuropathic pain and its role in modulating microglial activation in vivo and in vitro. Intrathecal and intraperitoneal administration of alendronate relieved neuropathic pain behaviors induced by chronic constriction sciatic nerve injury. Alendronate also significantly attenuated spinal microglial activation and p38 mitogen-activated protein kinase (MAPK) phosphorylation without affecting astrocytes. In vitro, alendronate downregulated phosphorylated p38 and phosphorylated extracellular signal regulated kinase expression in lipopolysaccharide-stimulated primary microglia within 1 hour, and pretreatment with alendronate for 12 and 24 hours decreased the expression of inflammatory cytokines (tumor necrosis factor α, and interleukins 1β and 6). These findings indicate that alendronate could effectively relieve chronic constriction sciatic nerve injury–induced neuropathic pain by at least partially inhibiting the activation of spinal microglia and the p38 MAPK signaling pathway.**

Perspective: ***Alendronate could relieve neuropathic pain behaviors in animals by inhibiting the activation of spinal cord microglia and the p38 MAPK cell signaling pathway. Therapeutic applications of alendronate may be extended beyond bone metabolism–related disease.***

Key words: *Alendronate, chronic constriction injury, microglial activation, p38 mitogen-activated protein kinase, inflammatory cytokines.*

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Neuropathic pain is a debilitating disease often caused by peripheral nerve injury. It can result in an increased response to noxious stimuli (hyperalgesia) and stimuli that do not normally provoke pain (allodynia).[71] It is known that central sensitization is involved in development of chronic pain. Several lines of evidence have verified that glia play an important role in central hyperactive states as well as in the multiple alterations in dorsal horn neurons after nerve injury.[63-65]

Microglia, the immune and defense glia, respond very quickly to noxious stimulus and enter an activated state. Histological analysis shows proliferation and hypertrophy of these activated microglia in neuropathic pain.[11,13,15] These cells not only exert morphological changes but also exhibit some functional alterations when activated compared with when in a quiescent state. Activated microglia can release multiple proinflammatory cytokines, such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-6, which play an important role in hypersensitivity in neurons.[6,12,23] It is well known that development of tactile allodynia after nerve injury depends on p38 mitogen-activated protein kinase (MAPK), 1 of 4 subgroups of the MAPK family.[28,30,44,65] The activation of p38 MAPK is observed in activated microglia, but not in neurons nor in astrocytes in the dorsal horn after nerve injury.[65] Furthermore, phosphorylated p38 (p-p38) is known to regulate the synthesis of numerous proinflammatory cytokines via transcriptional regulation. This cascade is also known to play an essential role in the initiation of neuropathic pain as well as in long-term maintenance of central sensitization in the spinal cord. Thus, suppression of microglial activation and p-p38 activation in microglia might prevent the development of hyperalgesia caused by peripheral nerve injury.[20,35,47]

Alendronate (ALN) is one of the nitrogen-containing bisphosphonates and is commonly used to treat osteoporosis.[5,31,70] Several reports have shown that ALN treatment of bone metabolism disorders contributes to pain alleviation.[45,57] Therefore, it is important to elucidate how ALN potentially modulates pain control mechanisms and chronic pain development. Bisphosphonates are pyrophosphate analogues in which the oxygen bridge has been replaced by a carbon with various side chains phosphorus-carbon-phosphorus.[24] It prevents bone absorption by inducing apoptosis in osteoclasts or by inhibiting osteoclast activation.[42,46] Meanwhile, ALN can inhibit the bacteria-induced protein tyrosine phosphatase (PTP) activities, such as CD45, at very low 50% inhibiting concentration values.[54] CD45 is a novel molecular therapeutic target to inhibit MAPK activation in microglia.[74] We previously reported that CD45 expression was significantly induced in activated microglia in the lumbar spinal cord after intraplantar injection of formalin or in models of nerve injury.[15,36] Because osteoclasts and microglial cells are members of the monocyte phagocytic system and derived from hematopoietic stem cells,[1] ALN may also exert a similar effect on microglia. In our study, we hypothesized that ALN would attenuate activation of spinal microglia to modulate pain transmission by inhibiting CD45 and/or downstream cell signaling molecules such as MAPKs. In this study, we used an in vivo chronic constriction injury (CCI) model to investigate the effects of ALN on mechanical and thermal hypersensitivity and activation of glial cells. We also used an in vitro microglia culture to test whether ALN could directly inhibit microglial activation.

Methods

Animals and Treatment

Adult male Sprague Dawley rats weighing 280 to 300 g were used. The animal room was artificially lighted from 7:00 AM until 7:00 PM. The experimental protocol was approved by the Institutional Animal Care and Use Committee of Peking University. The surgical procedure was performed aseptically using pentobarbital (50 mg/kg, intraperitoneal) anesthesia. CCI rats underwent loose ligation of the common sciatic nerve according to the method of Bennett and Xie.[3] Sham rats received the same surgical procedure, except the nerve ligation was omitted.

Drug Delivery and Nociceptive Behavioral Testing

A PE10 intrathecal catheter was implanted in rats at the level of the lumbar enlargement approximately a week before CCI surgery according to the method described previously.[72] Rats exhibiting postoperative neurological deficits (eg, paralysis) or poor grooming (2 of 44) were excluded from the experiments. ALN was purchased from Sigma-Aldrich (St. Louis, MO) and was dissolved in sterilized .9% saline. ALN of different doses or sterilized .9% saline was injected intrathecally in a 10-μL volume followed by a 10-μL saline flush once a day from day 1 to day 7. Rats (n = 42) were randomly placed into 6 groups: sham with vehicle sterilized .9% saline (n = 7), sham with ALN (20 μg/kg, n = 7), CCI with vehicle (n = 7), CCI with ALN (20 μg/kg, n = 7), CCI with ALN (10 μg/kg, n = 7), and CCI with ALN (4 μg/kg, n = 7).

For the peritoneal injection, animals were randomly placed into 4 groups. ALN was dissolved in saline at concentrations of .05 mg/mL, .5 mg/mL, and 2.5 mg/mL and administered peritoneally as follows: CCI with vehicle (n = 8), CCI with ALN (.1 mg/kg, n = 8), CCI with ALN (1 mg/kg, n = 8), and CCI with ALN (5 mg/kg, n = 8). Treatment was initiated at the first day after CCI surgery and was terminated on day 7. The day of the CCI surgery was marked as day 0. Peritoneal administration of ALN occurred from day 1 to day 7.

Animals were habituated to the behavior test setting 2 days before beginning the experiment and allowed at least 20 minutes to acclimatize before the testing. The testing procedure for thermal hyperalgesia was performed according to a previously published method.[21] Temperature was set to have the baseline latency at approximately 10 to 12 seconds. The maximum time allowed was 20 seconds to prevent tissue damage. The mechanical allodynia test procedure was developed according to the report from Tal and Bennett.[60] A range of von Frey filaments were applied to the plantar surface of each hind paw, starting from the middle weighted filament (10 g). Each filament was tested 5 times, and the final gram weight of the filament was recorded if animals responded positively to the filament 1 to 2 times in the trial. The cutoff force was 20 g. The behavior testing operator was blinded to treatment procedures. All

behavior tests were conducted during the same time period.

Rotarod Test

Animals from the 4 groups (sham with saline; sham with ALN [20 μg/kg]; CCI with saline; CCI with ALN [20 μg/kg]) were tested at different progressively higher speeds on the Rotarod apparatus (Gary J. Brenner's laboratory; Massachusetts General Hospital, Harvard Medical School, Boston, Massachusetts) to measure their ability to balance and remain on the rotating rod according to the previously described method.[52] Animals were trained 3 times before the actual test. The total time that the animal was able to remain on the rod without falling off was recorded at day 7 after surgery. In all of the tests, the rod was cleaned with 70% ethanol before the next animal was tested. The equipment was programmed for an initial speed of 2 rpm and gradually increased to a final speed of 40 rpm over the course of 60 seconds. The result was mean amount of time the animal stayed on the rotating rod for the 3 trials, with a minimum of 30 minutes of rest between each trial.

Immunohistochemistry

Pilot studies established that ALN administered intrathecally at a dose of 20 μg/kg/d and peritoneally at a dose of 1 mg/kg/d for 7 days were the most effective dosages in improving hypersensitive withdrawal responses in rodents. Immunofluorescence staining was performed on spinal cord sections from day 3, day 7, and day 14 after CCI surgery (n = 4 at each time point). Rats were anesthetized with an overdose of pentobarbital sodium and killed using transcardiac perfusion (250 mL .1 M phosphate buffered saline, pH 7.4 followed by 300 mL ice-cold 4% paraformaldehyde in .1 M PBS, pH 7.4). After perfusion, the lumbar spinal cords (L4–L6) were removed, postfixed in 4% paraformaldehyde for 4 hours and then placed in a 30% sucrose solution in .1 M PBS overnight at 4°C. Thirty-micron thick tissue sections were cut transversely on a cryostat for free-floating immunohistochemical staining for Iba-1 (1:200; Wako Chemicals, Osaka, Japan), GFAP (1:400; NeoMarkers, Fremont, California), CD45 (1:200; AbD Serotec, Oxford, United Kingdom) and p-p38 (1:400, Cell Signaling Technology, Beverly, Massachusetts). All of the sections were blocked with 5% normal goat serum in .3% Triton X-100 for 1 hour at room temperature before being incubated for 48 hours at 4°C with primary antibody. The sections were then incubated for 90 minutes at room temperature with a corresponding FITC conjugated secondary antibody.

Western Blot Analysis

Rats were deeply anesthetized and decapitated. The spinal cord segments (L4–L6) ipsilateral to the treatment side were removed rapidly and homogenized in an ice-cold RIPA buffer (Cell Signaling Technology) supplemented with 1 mM phenylmethylsulfonyl fluoride, phosphatase inhibitor, and protease inhibitor cocktail (Sigma-Aldrich). The homogenate was centrifuged at 15,000*g* for 45 minutes at 4°C. The protein concentration of tissue lysates was determined with a BCA Protein Assay Kit (Pierce, Rockford, Illinois).

For in vitro cell protein analysis, cells were washed with ice-cold PBS 3 times and then cell cultures were lysed in ice-cold lysis buffer (150 mM NaCl, .1% [wt/vol] NP-40, 50 mM Tris (pH 8.0), .5% [wt/vol] sodium deoxycholate, 1% [wt/vol] sodium dedecyl sulfate, 1 mM DL-Dithiothreitol, .1 mM phenylmethylsulfonylfluoride and protease inhibitor cocktail [Roche, Sweden]). After treatment, cells on the culture plates were scraped off and the whole lysates were briefly centrifuged; supernatants were removed and saved for Western blot analysis. The Lowry protein assay was used to measure the total protein content.

The prepared proteins of appropriate aliquots were boiled and analyzed with a standard Western blot procedure. Briefly, proteins were electrophoresed on a 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis gel and were then electrophoretically transferred onto polyvinylidene fluoride membranes (Millipore, Bedford, Massachusetts). The polyvinylidene fluoride membranes were blocked with 5% (wt/vol) bovine serum albumin in tris-buffered saline and tween buffer (.1 M Tris-HCl [pH 8.0], .9% [wt/vol] NaCl and .1% [vol/vol] Tween-20), and the target proteins were probed with diluted primary antibodies to tubulin (1:1000; Santa Cruz Biotechnology, Santa Cruz, California), GAPDH (1:1000; Cell Signaling Technology), CD45 (1:200; BD Bioscience, San Diego, California), Iba-1 (1:800; WAKO Chemicals), GFAP (1:3000; Cell Signaling Technology), p-p38 (1:1000; Cell Signaling Technology), phosphorylated extracellular signal regulated kinase (ERK; 1:1000; Santa Cruz), and p-c-Jun N-terminal kinase (JNK; 1:1000; Cell Signaling Technology) overnight at 4°C. After wash, the membranes were then incubated with secondary antibody conjugated with horseradish peroxidase (1:2000; Santa Cruz) for 1 hour at room temperature. Finally, the enzymatic chemiluminescence Western blot detection kit (Thermo Fisher Scientific, Chelmsford, Massachusetts) was used to detect the expression of the target proteins. The density of immunoreactive bands were quantified using Quantity One analysis software (Bio-Rad, Hercules, California), normalized to the density of internal control and expressed as the fold of change compared with the control group.

In Vitro Study

Microglial Cultures and Identification

Primary cultures of cerebral cortex microglia were prepared from newborn (within 24 hours after birth) ICR mice.[16] Mice were sacrificed by decapitation. The cerebral cortices were removed and the meninges and blood vessels were cleansed in ice-cold modified Dulbecco Modified Eagle Medium under a dissecting microscope. After mechanical dissociation, the cell suspension was passed through a 70-mm nylon filter (Spectra/Mesh, Spectrum Medical Industries, Los Angeles, California). Ten percent (vol/vol) fetal bovine serum (Hyclone, Logan,

Table 1. The Sequences of Primers Used in Real-Time PCR

Primer	Forward	Reverse
TNF-α	GCACCACCATCAAGGACTC	TGAGACAGAGGCAACCTGAC
IL-1β	TCAGCACCTCACAAGCAGAG	GCCCATACTTAGGAAGACACG
IL-6	CGGAGAGGAGACTTCACAGAG	ATTTCCACGATTTCCCAGA
GAPDH	TCACCACCATGGAGAGGC	GCTAAGCAGTTGGTGGTGCA

Utah) was added to the medium containing the filtered cell suspension. These mixed cell suspensions were first seeded at 1×10^7/mL in 75 mm^2 flasks (Corning, New York, New York) coated with .25% poly-L-lysine (Sigma-Aldrich). Flasks were then incubated at 37°C, with 95% air/5% CO_2 (vol/vol) and 95% humidity. Culture medium was changed twice per week with Dulbecco Modified Eagle Medium containing 10% (vol/vol) fetal bovine serum. After 12 to 14 days, when the cells were stratified, the mixed glial culture was placed on a Forma Orbital Shaker (Thermo Fisher Scientific; 250 rpm, 37°C) for 4 hours. After agitation, the suspended cells were collected, pooled, centrifuged, and resuspended. Cells were seeded at 3×10^5/mL in 35 mm^2 tissue culture dishes (Corning) for identification testing and 12-well plates (Corning) for other analysis.

We used immunofluorescent staining to verify the purity of the microglial cultures. Cultures were washed 3 times with ice-cold PBS, fixed with 4% paraformaldehyde for 20 minutes and permeabilized with .3% Triton X-100 for 15 minutes. The cultures were then blocked with 3% BSA in PBS for 30 minutes at room temperature and incubated with primary antibody (anti-GFAP, 1:200; Cell Signaling Technology; anti-Iba-1, 1:200; WAKO Chemicals) at 4°C overnight. After washing 3 times with PBS, the cultures were incubated with secondary antibody conjugated with DyLight 488 and DyLight 594 (1:200; Invitrogen, Carlsbad, CA) for 1 hour at room temperature. Hoechst 33342 (2 μg/mL; Sigma-Aldrich) was used to stain nuclei. Finally, the cultures were observed with a confocal microscope (Olympus, Tokyo, Japan).

Drug Treatment and Cell Counting Kit-8 Measurement

Complete culture medium was removed and replaced with serum-free medium containing 100 ng/mL lipopolysaccharides (Sigma-Aldrich) with or without ALN (Sigma-Aldrich). ALN was stocked at a dose of .5 mg/mL and used at doses of 10 μM, 20 μM, 50 μM, or 100 μM. Cell counting kit-8 (CCK8) was measured to check whether the doses of ALN used in vitro would cause cytotoxicity. We collected

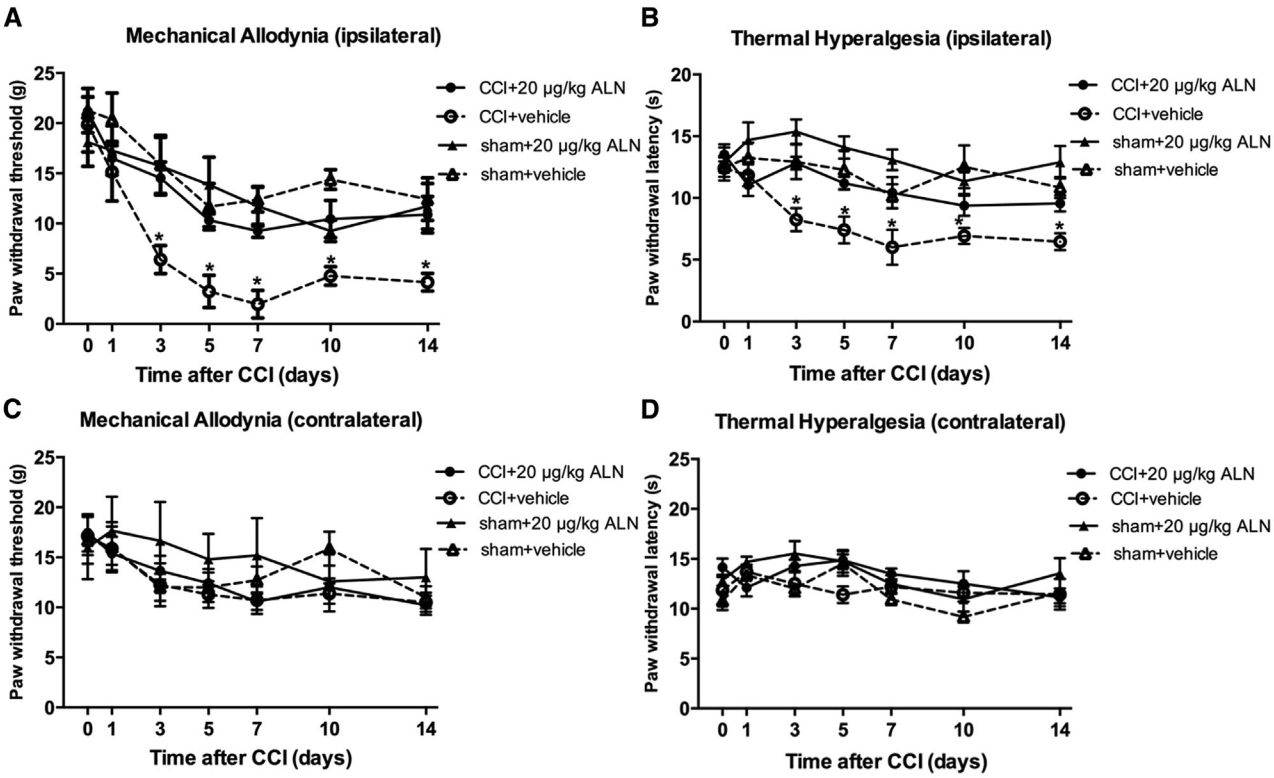

Figure 1. Effect of intrathecal ALN on CCI-induced pain behavior among different groups. CCI-induced mechanical allodynia and thermal hyperalgesia were prevented by intrathecal administration of ALN (20 μg/kg) from day 3 to day 14 compared with vehicle-treated CCI rats (**A** and **B**; *$P < .05$, n = 7). ALN did not change the mechanical threshold or thermal latency in sham-operated rats **(A** and **B)** and did not affect the contralateral sides (**C** and **D**; n = 7).

the culture medium from different treatment groups at different time points. Procedures were performed according to the instructions of the CCK8 kit (Sigma-Aldrich).

Real-Time Polymerase Chain Reaction for TNF-α, IL-1β, and IL-6 in Cultured Microglia

Gene expression of TNF-α, IL-1β, and IL-6 in primary microglia was measured with reverse transcription polymerase chain reaction (PCR). Total RNA from cultured cells given different treatments were extracted using Trizol reagent (Invitrogen) according to the manufacturer's instructions. Total RNA (1 μg) was used as a template for reverse transcription. cDNA and GoTaq PCR Master Mix (Promega, Madison, WI) were used to perform the real-time PCR according to the standard procedure from the ABI Prism 7500 real-time PCR system (Applied Biosystems, Foster City, CA). GAPDH was used as the internal control. The final data were normalized to each control group. Primers used were on the basis of the PubMed database (http://www.ncbi.nlm.nih.gov/pubmed). The sequences of primers used in real-time PCR are listed in Table 1.

Statistical Analysis

All data are presented as the mean ± standard error of the mean. Behavioral data between groups were compared using 2-way analysis of variance followed by Tukey post hoc tests. One-way analysis of variance followed by Bonferroni post hoc test was used for Western blot and real-time PCR results (multiple groups). These evaluations were performed using GraphPad Prism 5 for Windows (GraphPad Software, San Diego, California). The criterion for statistical significance was $P < .05$.

Results

Intrathecal Administration of ALN Improved Mechanical Allodynia and Thermal Hyperalgesia Induced by CCI Injury

The effects of intrathecal ALN on hypersensitive withdrawal responses were examined in 4 groups (n = 7 for each group). As illustrated in Fig 1, daily intrathecal administration of 20 μg/kg ALN for 7 days prevented the development of mechanical allodynia and thermal hyperalgesia compared with vehicle-treated CCI rats. A sharp decrease of mechanical withdrawal threshold (Fig 1A) and thermal withdrawal latency (Fig 1B) was observed in the groups of rats treated with CCI with vehicle from day 3 to day 14, which were prevented by administration of ALN. ALN alone did not change the withdrawal response threshold in sham-operated rats, nor did it affect the contralateral side of CCI rats (Figs 1C and 1D).

When 2 other doses of ALN (10 μg/kg and 4 μg/kg) were compared (Fig 2), a larger improvement of hypersensitive withdrawal responses in the higher dose group

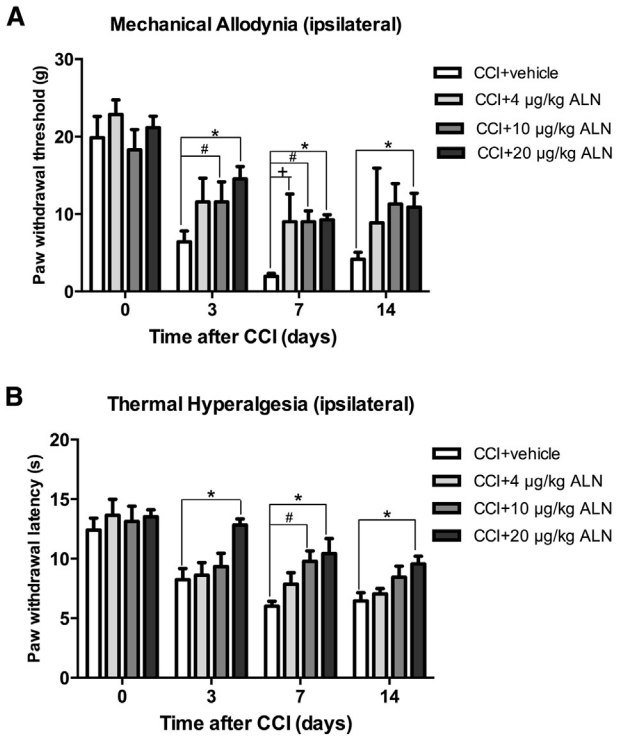

Figure 2. Effect of different doses of intrathecal ALN on CCI-induced pain behavior (**A** and **B**). Compared with the dose of 20 μg/kg ALN, 10 μg/kg ALN was less effective, and 4 μg/kg ALN treatment was only significantly different in mechanical allodynia at day 7 (+$P < .05$, CCI + 4 μg/kg ALN group vs CCI + vehicle group; #$P < .05$, CCI + 10 μg/kg ALN group vs CCI + vehicle group; *$P < .05$, CCI + 20 μg/kg group vs CCI + vehicle group; n = 7).

was observed. CCI-induced mechanical allodynia and thermal hyperalgesia was completely attenuated at the dose of 20 μg/kg from day 3 to day 14 (Fig 2). ALN was less effective at the dose of 10 μg/kg, and the dose of 4 μg/kg only showed a statistically effect on mechanical hypersensitivity on day 7 (Figs 2A and 2B).

No Significant Motor Function Impairment Was Observed After Intrathecal Administration of ALN

The dose of 20 μg/kg/d for 7 days in rodents was effective in improving hypersensitive withdrawal responses

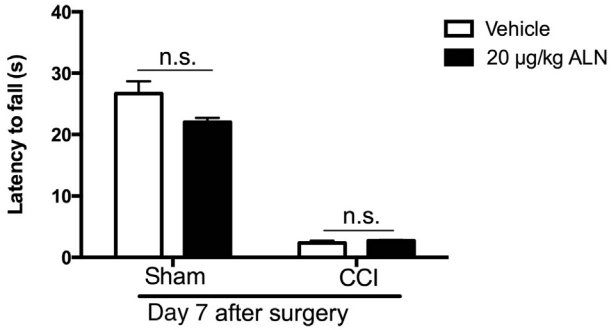

Figure 3. Rotarod test after sham or CCI surgery. No statistically significant difference was observed between the 20 μg/kg ALN-treated group and the vehicle group.

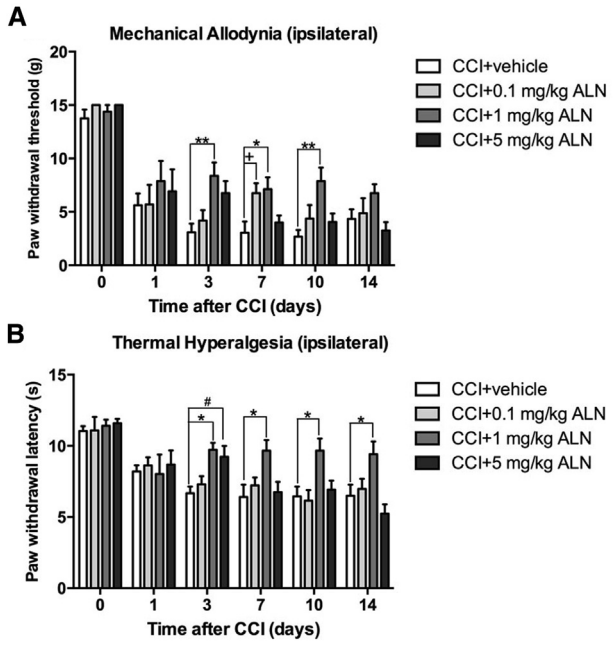

Figure 4. Effect of different doses of intraperitoneal ALN on CCI-induced pain behavior. Mechanical allodynia induced by CCI was significantly reduced by 1 mg/kg ALN from day 3 to day 10 and .1 mg/kg ALN was effective only on day 7 **(A)**. Thermal hyperalgesia was reversed by 1 mg/kg ALN treatment lasting from day 3 to day 14. Treatment with 5 mg/kg ALN caused a significant difference only on day 3 **(B)**. +$P < .05$, CCI + .1 mg/kg ALN group versus CCI + vehicle group; *$P < .05$, **$P < .01$, CCI + 1 mg/kg ALN group versus CCI + vehicle group; #$P < .05$, CCI + 5 mg/kg group versus CCI + vehicle group; n = 8.

and was safe, with no apparent side effects observed. To evaluate whether the increased withdrawal response threshold after intrathecal ALN administration was due to pain relief or motor function impairment, we used the Rotarod test to compare the riding time (latency to fall) between the ALN group and the vehicle group. As is shown in Fig 3, 7 days after CCI or sham operation, no statistically significant difference was shown between groups and indicating that intrathecal ALN did not impair motor function.

Systemic Injection of ALN Relieved Mechanical Allodynia and Thermal Hyperalgesia Induced by CCI

As shown in Fig 4, a sharp decrease in paw withdrawal threshold and latency appeared on day 1 after CCI surgery. However, there were no significant differences observed between different treatment groups. Consecutive peritoneal injection of 1 mg/kg ALN for 7 days reduced hypersensitive withdrawal responses in CCI rats compared with saline-treated CCI rats. The significant difference lasted from day 3 to day 10 for mechanical allodynia (Fig 4A), whereas for thermal hyperalgesia, it lasted to day 14 (Fig 4B). At a dose of .1 mg/kg ALN, a significantly higher mechanical threshold was observed only on day 7. However, the highest dose (5 mg/kg) of ALN was not effective. Neither mechanical threshold nor thermal latency in the contralateral side were affected in any different treatment group at any point in our study (data not shown).

ALN Did Not Change Protein Expression of CD45 in Spinal Microglia

In our previous study, we reported that CCI nerve injury induced CD45 expression in spinal microglia.[36] However, as illustrated in Fig 5, ALN in the current study did not decrease CD45 expression in the spinal cord, analyzed using immunostaining (Fig 5A) and Western blot analysis (Figs 5B and 5C) of the 20 μg/kg intrathecal injection dose of ALN, which significantly attenuated CCI-induced hypersensitive withdrawal responses.

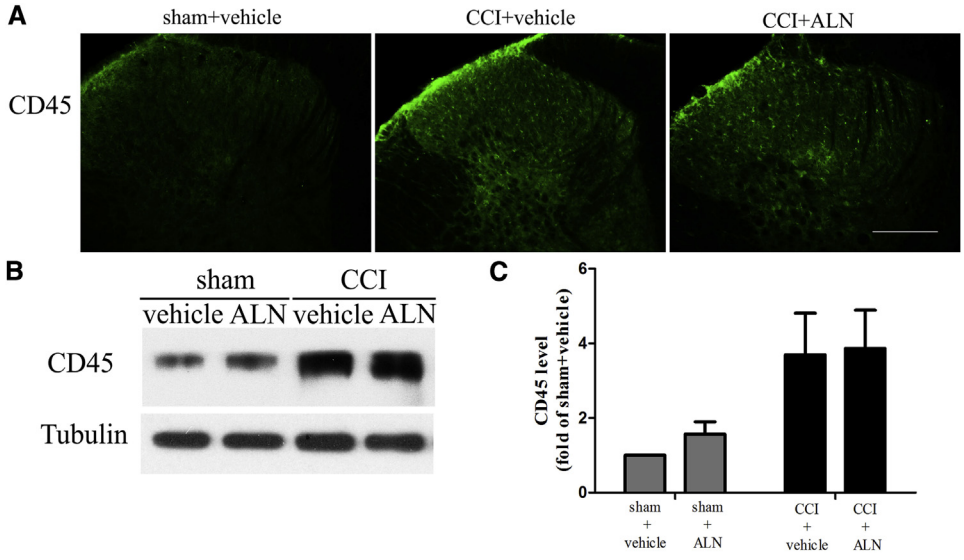

Figure 5. Effect of ALN treatment on CD45 expression in the lumbar spinal cord in CCI-injury rats. From the representative immunostaining pictures, ALN did not decrease the CCI-induced CD45 expression compared with the vehicle group (**A**, scale bar = 50 μm). Representative protein bands and quantification of Western blot analysis also showed no significant difference between ALN- and vehicle-treated group (**B** and **C**), n = 4.

ALN Attenuated CCI-Induced Activation in Spinal Microglia, But Not Astrocytes

CCI nerve injury resulted in an increase of Iba-1 immunoreactivity in the ipsilateral dorsal horn of the lumbar spinal cord (Fig 6A). Administration of ALN significantly reduced this Iba-1 expression in the ipsilateral dorsal horn (Figs 6A and 6B). Western blot semiquantitative analysis showed that ALN treatment significantly reduced the upregulation of Iba-1 induced by CCI, compared with vehicle (Figs 6C and 6D).

CCI nerve injury also could induce spinal astrocyte activation. Representative immunostaining results showed that GFAP (astrocyte marker) was slightly increased on day 7, and evident on day 14 after injury (Figs 7A and 7B), however, in the present study, we did not find a significant difference in GFAP protein level between the vehicle group and the ALN group on day 7 after injury (Figs 7C and 7D).

ALN Downregulated the Activation of the MAPK Signaling Pathway in the Spinal Dorsal Horn

The critical role of the MAPK family, including p38 kinase, ERK, and JNK has been well documented in various models of neuropathic pain. We also examined the effect of ALN on members of the MAPK family in CCI rats. Immunostaining and Western blot analysis showed that CCI surgery resulted in an increase in p-p38 expression,

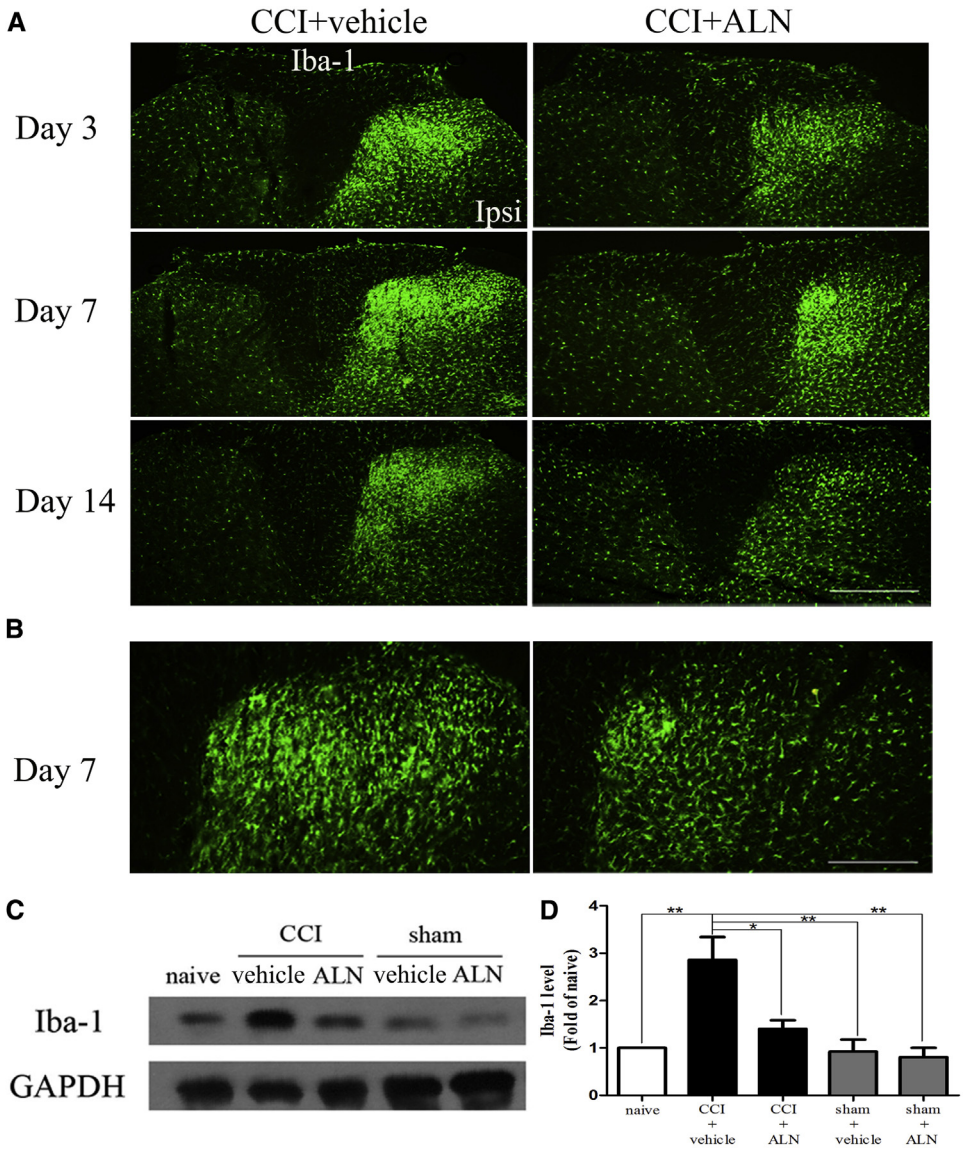

Figure 6. Effect of ALN treatment on microglial activation in the lumbar spinal cord in CCI-injury rats. Representative immunostaining pictures show that upregulation of Iba-1 expression was decreased by 1 mg/kg ALN treatment in the ipsilateral (Ipsi) dorsal horn on days 3, 7, and 14 (**A**, scale bar = 100 μm) with a higher magnification (×20) of the ipsilateral dorsal horn area of a day 7 sample (**B**, scale bar = 50 μm). Representative bands and quantification of Western blot analysis showed that ALN significantly suppressed the increased Iba-1 protein level on day 7 **(C** and **D)**. *P < .05, **P < .01, compared with CCI + vehicle group, n = 4.

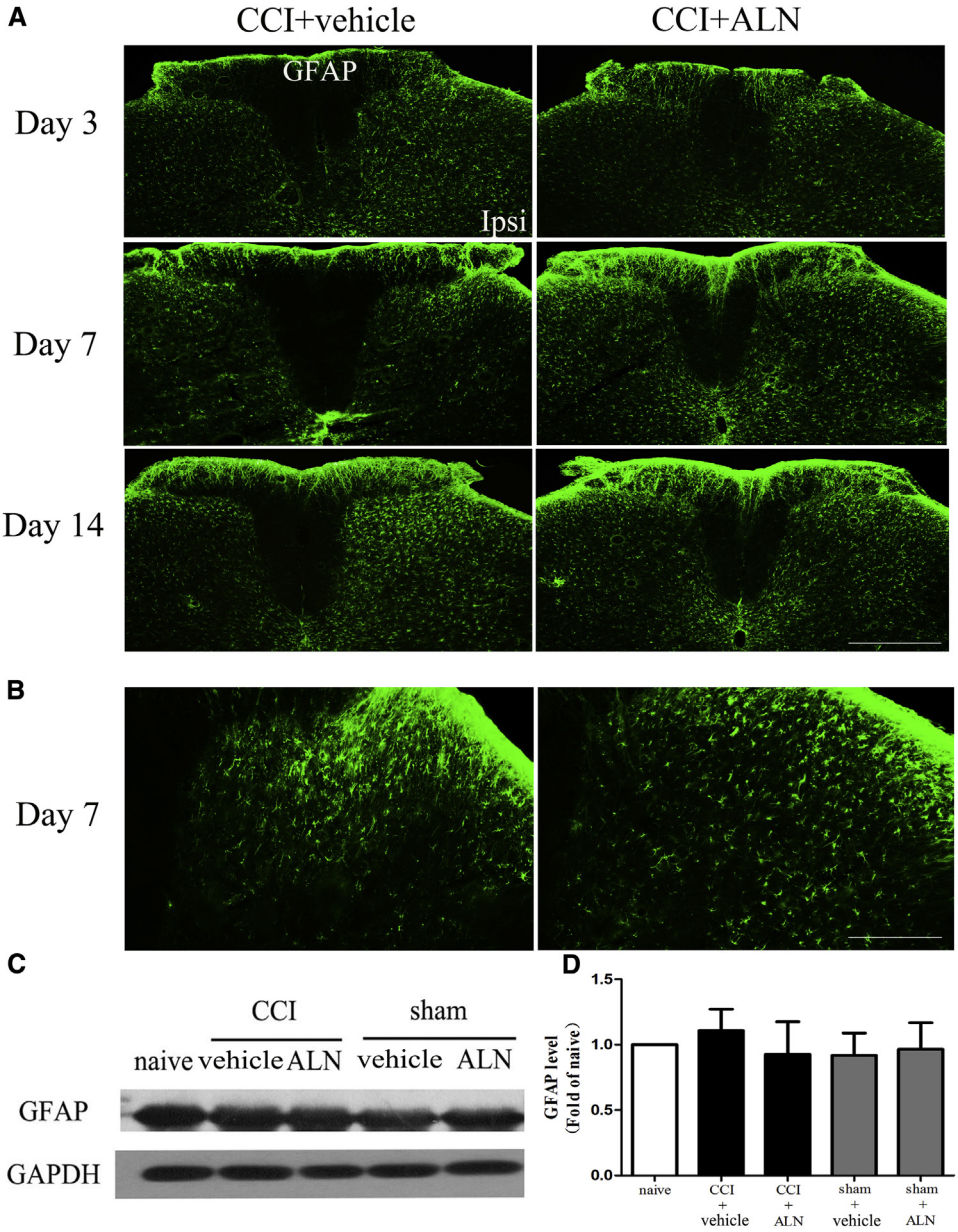

Figure 7. Effect of ALN treatment on astrocyte activation in the lumbar spinal cord in CCI-injury rats. CCI injury induced a slight increase of GFAP expression on day 14, but did not show a significant difference between vehicle- and ALN-treated groups in the ipsilateral dorsal horn on days 3, 7, and 14 (**A**, scale bar = 100 μm) with a higher magnification (×20) of the ipsilateral dorsal horn area of a day 7 sample (**B**, scale bar = 50 μm). Quantification of Western blot analysis also showed no significant difference by ALN treatment on day 7 compared with vehicle treated group **(C** and **D)**, n = 4.

which was significantly attenuated by ALN treatment (Figs 8A–8C). No significant differences in the expression of p-ERK or p-JNK were found with ALN treatment on day 7 (Figs 8D–8G).

Effects of Test of Doses of ALN on Primary Microglia Culture

Because our primary microglia were separated from mixed glia, we stained for the astrocyte marker GFAP and the microglia marker Iba-1. The results showed (Fig 9A) that there were almost no astrocytes in the shaken primary microglia culture. In the CCK8 experiments (Fig 9B), we did not observe a significant difference between different groups from 1 to 24 hours, indicating that the doses of ALN used in vitro had no cytotoxicity on primary microglia culture.

ALN Down-Regulated the Expression of p-P38 and p-ERK in LPS-Stimulated Primary Microglia at 1 Hour

We detected a time-dependent change of p-p38 and p-ERK expression after LPS treatment from 30 minutes to 24 hours. The peak time of phosphorylation was 1 hour after stimulation (data not shown). Here, we investigated the effects of different doses of ALN on the expression of p-p38 and p-ERK at the time point of 1 hour in primary microglia culture (Fig 10) and found that 20 μM ALN treatment significantly

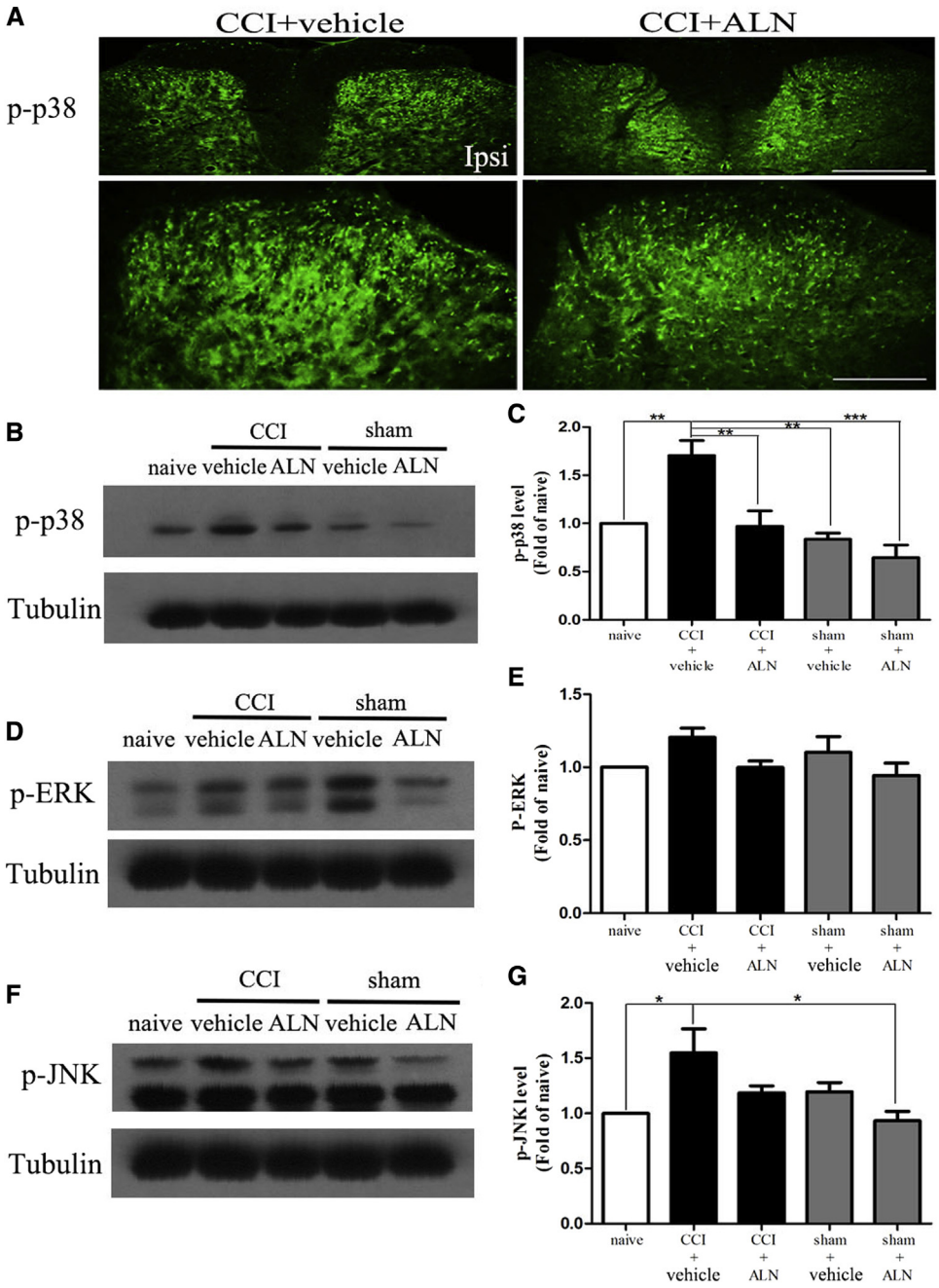

Figure 8. Effect of ALN treatment on p-p38, p-ERK, and p-JNK expression in the lumbar spinal cord on day 7 in CCI-injury rats. Representative immunostaining pictures show that p-p38 expression was decreased by 1 mg/kg ALN treatment in the ipsilateral dorsal horn (scale bar = 100 μm) with a higher magnification (×20) of the ipsilateral dorsal horn area below (scale bar = 50 μm) **(A)**. Representative bands and quantification of Western blot analysis showed that CCI-induced upregulated p-p38 expression was suppressed by ALN treatment with no effect among sham groups **(B** and **C)**, $^{**}P < .01$, $^{***}P < .001$, compared with CCI + vehicle group, n = 4. No statistically significant difference in p-ERK expression was found with ALN treatment **(D** and **E)**, n = 4. ALN lowered the level of the increased p-JNK expression, but did not show statistical significance **(F** and **G)**. $^{*}P < .05$, compared with CCI + vehicle group, n = 4.

downregulated the expression of p-p38 (Figs 10A and 10B). Although the level of p-ERK appeared to be downregulated dose-dependently with ALN treatment, no significant difference was found compared with the control (Fig 10C). However, the LPS group did not show a significant increase in p-p38 and p-ERK compared with the control group (serum-free treatment). We postulated that serum deprivation activated microglia,[33,37] and produced an effect ablating the LPS-induced increase, which we subsequently verified in another experiment (data not shown).

ALN Decreased the Expression of Inflammatory Cytokines (TNF-α, IL-1β, and IL-6) in Microglia Culture

We measured the expression of TNF-α, IL-1β, and IL-6 mRNA in primary microglia culture. At 12 hours (Figs 11A–11C), ALN treatment sharply decreased the

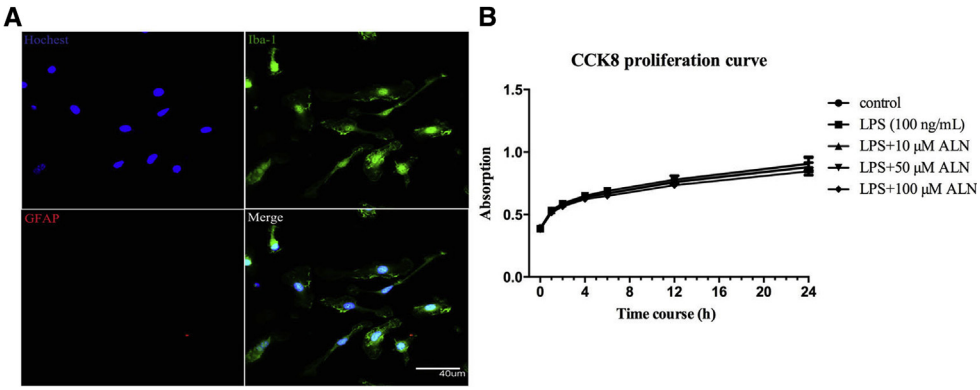

Figure 9. Identification of the purity of primary microglia culture and CCK8 test with different doses of ALN treatment. Triple staining with Hochest (blue), Iba-1 (green), and GFAP (red) showed little GFAP signal in the primary microglia culture **(A)**. CCK8 results show there was no significant difference between different doses of ALN treatment and control group at 24 hours **(B)**, n = 4.

expression of TNF-α and IL-6 in a dose-dependent way. The expression of IL-1β was also reduced, and significant differences were present in the 50-μM and 100-μM ALN treatment groups. At 24 hours (Figs 11D–11F), 50 μM and 100 μM ALN decreased the expression of TNF-α. A similar dose-dependent decrease with ALN treatment was observed in IL-1β. We did not find any significant differences in IL-6 mRNA expression among different groups at the 24-hour time point.

Discussion

The major findings of our present study were: 1) Intrathecal and peritoneal injection of ALN immediately after surgery relieved the hypersensitive withdrawal responses induced by CCI nerve injury; 2) CCI-induced microglial activation and phosphorylation of p38 in the spinal cord were attenuated by application of ALN; and 3) In vitro, ALN downregulated p-p38 expression within 1 hour and also decreased the expression of inflammatory cytokines (TNF-α, IL-1β, and IL-6) in LPS-stimulated primary microglia culture.

Many bisphosphonate derivatives are inhibitors of bone resorption. They are now also regarded as promising agents for painful pathologies such as Paget disease, tumoral osteolysis, tumoral hypercalcemia, osteoporosis, and rheumatoid arthritis.[8,14,18] The pain-alleviating mechanisms of bisphosphonates are currently unknown. One possible mechanism is related to their inhibition of osteoclasts, because clinical evidence indicates that bisphosphonates control bone pain in patients with bone resorption-related diseases.[27] ALN was reported to be able to attenuate the increase of the prohyperalgesic peptide dynorphin in the spinal cord in bone cancer pain.[55] The upregulation of dynorphin in the spinal cord was involved in the maintenance of chronic pain induced by peripheral nerve injury.[39,66,67] Sevcik et al[57] reported that ALN also attenuated

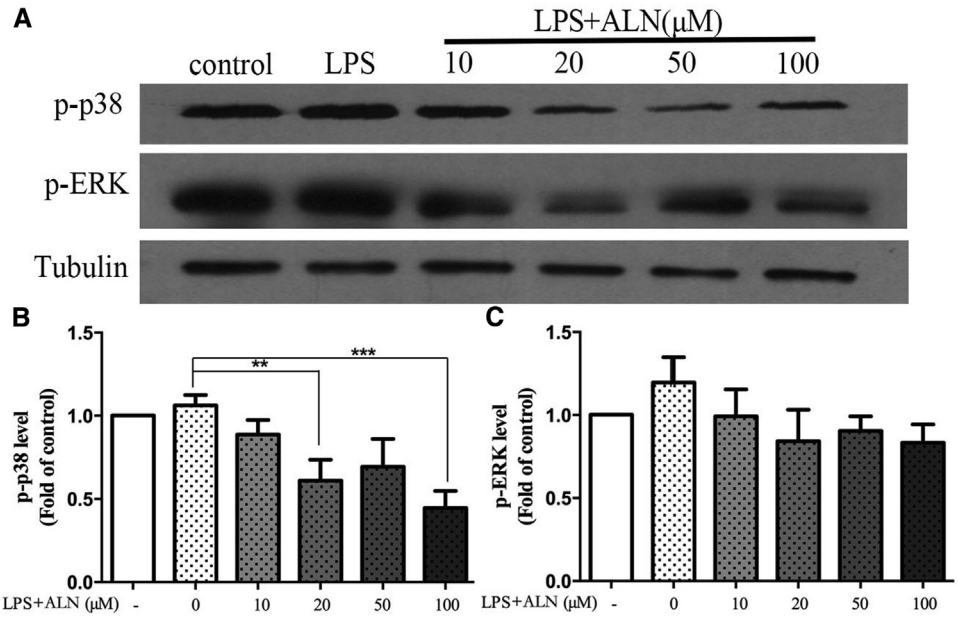

Figure 10. Effect of ALN treatment on p-p38 and p-ERK expression in activated primary microglia. Representative bands and quantification of Western blot analysis showed that ALN suppressed the level of p-p38 compared with the LPS-treated control group **(A** and **B)**, $^{**}P < .01$, $^{***}P < .001$, n = 4. The ALN treatment did not significantly influence p-ERK expression **(A** and **C)**, n = 5.

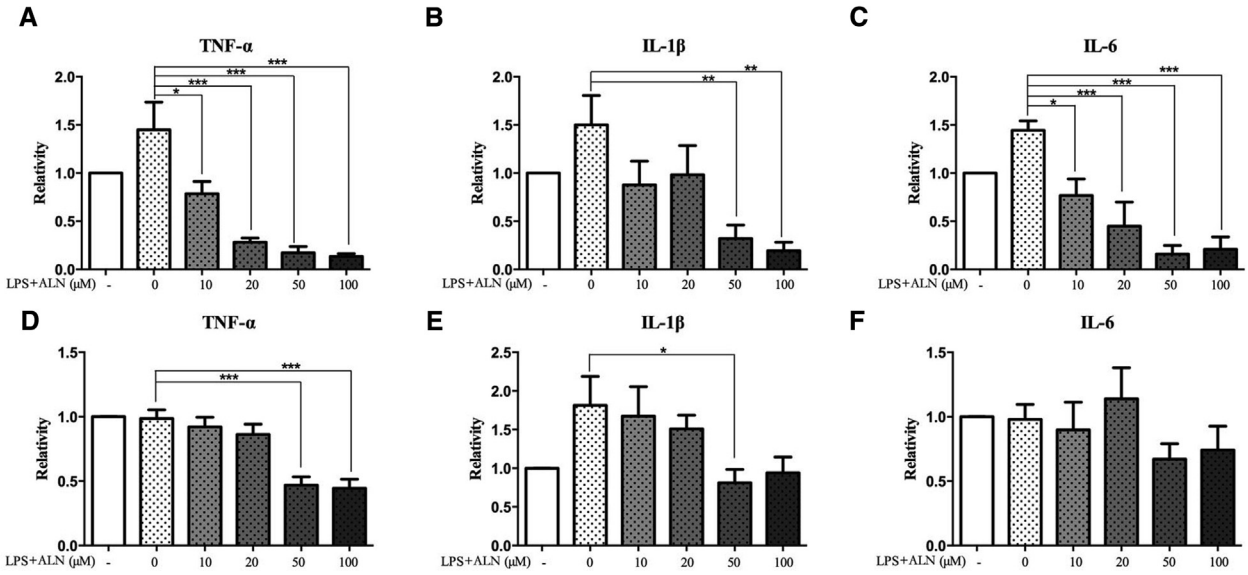

Figure 11. Effect of ALN treatment on inflammatory cytokine expression in activated primary microglia at 12 hours **(A–C)** and 24 hours **(D–F)**. *P < .05, **P < .01, ***P < .001, compared with LPS-treated control group, n = 5.

tumor-induced markers of peripheral and central sensitization such as activating transcription factor-3 (a marker of neuronal injury), galanin, and the astrocyte marker GFAP. These findings suggest that ALN not only attenuates tumor-induced activation and destruction of sensory fibers in the bone but also reduces the neurochemical changes in the peripheral and central nervous system, which may contribute to the generation and maintenance of bone cancer pain. In light of these previous reports, therapeutic applications of ALN may be extended beyond bone metabolism-related pain diseases. Bonabello et al[7] compared the antinociceptive action of 4 bisphosphonates (clodronate, ALN, pamidronate, and etidronate) with morphine and acetylsalicylic acid using the tail-flick and writhing tests. The results suggested that bisphosphonates played a central and peripheral antinociceptive role even in acute pain, a mechanism that was not related to an accelerated osteolytic response. Goicoechea et al[17] observed that ALN produced a dose- and time-dependent analgesic effect in mice in the acetic acid test without any effect on bone. In a small open-label placebo-controlled clinical study, bisphosphonates showed efficacy in the treatment of complex regional pain syndrome.[38] Bianchi et al reported the analgesic action of this bisphosphonate in a rat model of persistent inflammatory pain.[4] To our knowledge, the present study is the first to report that ALN significantly prevents development of CCI-induced mechanical/thermal hypersensitivity, after intrathecal and intraperitoneal administration. The highest dose (5 mg/kg) of ALN using systemic administration was not more effective than lower doses. The higher dose probably induced other effects, including influencing pain sensation. Although these behaviors are commonly used to measure pain behavior in animal studies, this type of hyper-reflexia is not a common component of clinical neuropathic pain in humans. Drugs that reduce hyper-reflexia in animals, such as analgesics, may have a poor track record.[32] Determining the effectiveness of ALN to reverse established mechanical/thermal hypersensitivity would be interesting.

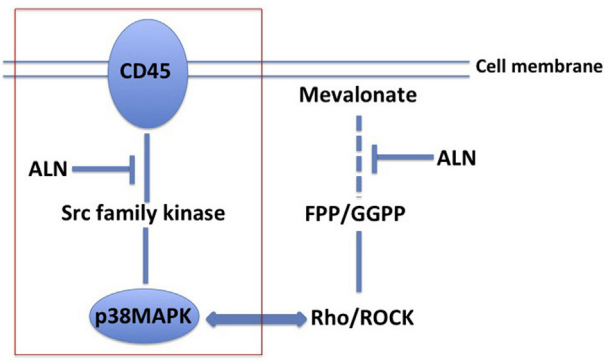

Figure 12. The possible sites of action for ALN to attenuate spinal microglial activation. Abbreviations: FPP, farnesyl pyrophosphate; GGPP, geranylgeranyl pyrophosphate.

In the present study, results of the Rotarod test indicate that ALN does not impair motor function. However, CCI impairs Rotarod performance. We loosely ligated the sciatic nerve, a nerve that controls motor function and sensing. The impairment of motor performance by CCI with marked reduced latencies to fall off the Rotarod has been reported.[2] Rats of neuropathic pain models are less active and could be considered examples of pain-related behavior depression.[25] Rotarod test behavior is designed to measure the ability to balance. ALN alleviated CCI-induced mechanical/thermal hypersensitivity but not Rotarod impairment, indicating neuronal control mechanisms for Rotarod test behavior is different from others such as the forced swimming test.[25]

Microglia are regarded as the immune cells in the central nervous system and are activated quickly in response to outside stimuli.[12,40,41] Upon activation, microglia release inflammatory cytokines, which play a critical

role in the development and maintenance of central sensitization and hyperalgesia by altering the sensitivity of the afferent neurons, thus modulating the transmission of painful stimuli to the central nervous system.[10,12,19,34,68,69] The present study showed that ALN significantly attenuated the activation of spinal cord microglia in vivo and decreased the expression of cytokines (TNF-α, IL-1β, and IL-6) in primary microglia culture. Our results indicate that ALN relieved neuropathic pain behaviors, at least in part, through inhibition of microglial activation in the spinal cord. We did not find a significant upregulation of GFAP or a difference between ALN treatment and vehicle in the study, which likely was because of the time point (7 days) being too early for astrocyte activation. In general, activation of astrocytes appears to proceed more slowly than that of microglia.[11,61] Actually we found GFAP upregulation by immunostaining, evident on day 14.

Studies on the mechanisms for bisphosphonates in osteoclast activity and bone resorption have suggested PTPs as a molecular target.[54] CD45 is a transmembrane PTP. We have previously reported that CD45 was induced in activated microglia in the lumbar region of the spinal cord after intraplantar injection of formalin or in a CCI model.[14,36] CD45, as an upstream molecule, can dephosphorylate different sites on Src family kinases and serve as a positive and negative regulator in a cell type- and context-dependent manner.[22,48] Src family kinases, one group of nonreceptor protein tyrosine kinases, were reported to be involved in many neurological disorders and in neuropathic pain states in which microglial activation played a role.[30,66] We also previously reported activation of the Src/p38 MAPK signaling cascade in spinal microglia, contributing to the persistent mechanical hyperalgesia evoked by formalin injection into the paw.[62] In the present study, in vivo and in vitro, ALN attenuated the phosphorylation of p38 in microglia, but did not reduce the expression of CD45. The result indicated that ALN might target some sites downstream of CD45, such as the Src/p38 MAPK signaling cascade, in spinal microglia as depicted in Fig 12.

Moreover, nitrogen-containing bisphosphonates have been shown to prevent the formation of farnesyl pyrophosphate and geranylgeranyl pyrophosphate through the inhibition of their synthases in the mevalonate pathway.[51] Preventing farnesyl pyrophosphate would induce the inhibition of small GTPase prenylation (Rho, Ras, Cdc). It would then prevent the prenylated small G-protein group from translocating to the cell membrane, thus blocking the downstream signals from passing.[50] Recently, the Rho/Rho kinase (ROCK) pathway has been shown to play important roles in the development and/or maintenance of chronic pain.[26,62] Intrathecal pretreatment with Y27632, a specific inhibitor of the Rho-associated kinase pathway, attenuated spinally administered mevalonate-induced hyperalgesia.[43] Some studies have implied a molecular link between the intracellular pathways of p38 MAPK and Rho/ROCK.[29,49,73] As an inhibitor of the enzyme in the mevalonate pathway, statins have been shown to regulate p38 MAPK through the inhibition of Rho activation in a variety of cell types and thus exert their pleiotropic effects through inactivation of the Rho-MAPK pathway.[53,56,59] It has been reported that statins could alleviate experimental nerve injury-induced neuropathic pain and abolish spinal cord glial activation and IL-1β expression.[58] Our previous work has elucidated that simvastatin alleviated formalin-induced nociceptive responses by inhibiting microglial RhoA and p38 MAPK activation.[9] From these findings, we postulated that the molecular mechanism of ALN inhibition of the Rho/ROCK signaling pathway might also contribute to the downregulation of p38 phosphorylation, thus attenuating the microglial activation through the Rho/p38 MAPK pathway (Fig 12).

Conclusions

In this study, we concluded that ALN, in addition to its already known functions, could relieve neuropathic pain behaviors through inhibiting the activation of spinal cord microglia and the p38 MAPK cell signaling pathway. Molecules upstream of Src/p38 MAPK and/or Rho/p38 MAPK pathways are potential molecular targets for ALN action and may be considered as novel targets for the microglial activation involved in central sensitization of chronic pain.

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