Polyamidoamine dendrimer-conjugated triamcinolone acetonide attenuates nerve injury-induced spinal cord microglia activation and mechanical allodynia

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Abstract

Background: Accumulating evidence on the causal role of spinal cord microglia activation in the development of neuropathic pain after peripheral nerve injury suggests that microglial activation inhibitors might be useful analgesics for neuropathic pain. Studies also have shown that polyamidoamine dendrimer may function as a drug delivery vehicle to microglia in the central nervous system. In this regard, we developed polyamidoamine dendrimer-conjugated triamcinolone acetonide, a previously identified microglial activation inhibitor, and tested its analgesic efficacy in a mouse peripheral nerve injury model.

Result: Polyamidoamine dendrimer was delivered selectively to spinal cord microglia upon intrathecal administration. Dendrimer-conjugated triamcinolone acetonide inhibited lipoteichoic acid-induced proinflammatory gene expression in primary glial cells. In addition, dendrimer-conjugated triamcinolone acetonide administration (intrathecal) inhibited peripheral nerve injury-induced spinal cord microglial activation and the expression of pain-related genes in the spinal cord, including Nox2, IL-1β, TNF-α, and IL-6. Dendrimer-conjugated triamcinolone acetonide administration right after nerve injury almost completely reversed peripheral nerve injury-induced mechanical allodynia for up to three days. Meanwhile, dendrimer-conjugated triamcinolone acetonide administration 1.5 days post injury significantly attenuated mechanical allodynia.

Conclusion: Our data demonstrate that dendrimer-conjugated triamcinolone acetonide inhibits spinal cord microglia activation and attenuates neuropathic pain after peripheral nerve injury, which has therapeutic implications for the treatment of neuropathic pain.

Keywords
Dendrimer-triamcinolone acetonide, neuropathic pain, peripheral nerve injury, spinal cord microglia

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Introduction

Studies in the past decade have clearly demonstrated that spinal cord microglia are important modulators of neuropathic pain.1 Upon peripheral nerve injury (PNI), microglia in the spinal cord dorsal horn become activated and express various pain-mediating molecules such as IL-1β, TNF-α, and BDNF. These pain mediators can induce central sensitization of pain at the spinal cord level by directly enhancing the excitability of pain-transmitting neurons or disinhibiting inhibitory synaptic transmission in the spinal cord pain circuits.2-4 These new findings suggest that spinal cord microglia...
activation is a potential therapeutic target, and that putative spinal cord microglia activation inhibitors might serve as novel analgesic drugs for neuropathic pain. In this regard, we have previously screened small molecules and identified triamcinolone acetonide (TA) as a potent microglia activation inhibitor in vitro.\textsuperscript{5} TA is a member of the corticosteroid family and is widely used as an anti-inflammatory drug.\textsuperscript{6} However, corticosteroid drugs including TA have limitations in clinical application due to their side effects. Concerns regarding neurotoxic and inflammatory side effects of corticosteroid treatments have been raised when considering its use in the nervous system.\textsuperscript{7–9} Literature shows that intrathecal (i.t.) TA administration can induce arachnoiditis and meningitis, which can occur from off-target side effects of corticosteroids.\textsuperscript{10,11} Therefore, it was reasoned that delivery of TA directly to microglia could minimize such complications.

Dendrimer is a synthetic nanoscale-sized polymer with a sphere-like shape. Given its biodegradable characteristics and globular protein-level size (\(\sim 3–10\) nm), its potential utility as a drug delivery vehicle has been widely investigated.\textsuperscript{12} Dendrimers derived from polyamidoamine (PAMAM) with a neutral hydroxyl terminal (PAMAM-OH dendrimer) have shown the highest biocompatibility.\textsuperscript{13,14} Recently, it was reported that neutral PAMAM dendrimers administered into the subarachnoid space were selectively localized within microglia, indicating a microglia-specific uptake of these nanomolecules.\textsuperscript{15} In support of these findings, fluocinolone acetoniode-conjugated PAMAM dendrimer was found to be selectively taken up by activated microglia in the retina.\textsuperscript{16} Based on these data, we hypothesized that TA can be selectively delivered to spinal cord microglia once conjugated to the PAMAM dendrimer, effectively blocking spinal cord microglia activation and neuropathic pain induction. In this study, we explored this hypothesis in a neuropathic pain mouse model with spinal nerve transection (SNT).

**Materials and methods**

**Animals**

All surgical and experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee at Seoul National University. The animal treatments were performed in accordance with the guidelines of the International Association for the Study of Pain. Experiments were carried out in male C57BL/6 mice aged 8–12 weeks. Mice were housed in plastic cages and were allowed to access food and water ad libitum. They were housed at a constant room temperature of \(23^\circ\text{C} \pm 2^\circ\text{C}\) and a 12-h dark/light cycle.

**Synthesis of dendrimer-conjugated TA and fluorescent dye-labeled dendrimers**

TA, gluatric anhydride (GA), N,N-dimethylacetamide (DMA), N,N-dimethylformamide (DMF), N,N-diisopropylethylaminde (DIPEA), triethylamine (TEA), PAMAM-OH G4 (ethylenediamine core), fluorescein isothiocyanate (FITC), and 5(6)-carboxy-X-rhodamine, and sephadex LH-20 (lipophilic sephadex) were purchased from Sigma (St. Louis, MO, USA).

N-Hydroxybenzotriazole (HOBT) and 2-(1H-betria-zole-1-yl)1,3,3-trtrimethyluronium (HBTU) were purchased from AnaSpec Inc. (San Jose, CA, USA).

A mixture of TA (50 mg, 0.115 mmol), five equivalents of GA (65.65 mg, 0.575 mmol), and 30 \(\mu\)l of TEA in anhydrous DMA/DMA 80:20 (v/v) was stirred for 48 h at \(37^\circ\text{C}\) in a nitrogen atmosphere. The crude product was purified with column chromatography by filtering through a silica gel using ethyl acetate:MeOH (99:1) as an eluent, yielding TA-glutarate.

For the synthesis of dendrimer-conjugated TA (D-TA), TA-glutarate (190 mg, 0.34 mmol), 32 equivalents of HOBT and HBTU, and 64 equivalents of DIPEA were dissolved in anhydrous DMF. The reaction mixture was stirred, followed by the addition of PAMAM G4-OH dendrimer (154 mg, 0.01 mmol). The final reaction mixture was stirred for 48 h at \(37^\circ\text{C}\) under nitrogen. The crude product was eluted into a sephadex LH-20 column (DMF only) to separate the unreacted excess GA-TA, HOBT, and HBTU. The reaction mixture was precipitated three times with cold ether to obtain the final product, dendrimer-TA (\(\sim 95\%\) yield). The conjugate was verified by \(^1\text{H}\) NMR spectra (Bruker DPX-400 NMR spectrometer, DMF-\(d_7\), \(\delta\) ppm) using \(\delta\) 0.91 (s, methyl protons of TA), 1.19 (s, \(-\text{CH}_3\) protons of TA), 1.38 (s, \(-\text{CH}_3\) protons of TA), 1.56 (s, \(-\text{CH}_3\) protons of TA), 2.31–2.83 (m, \(-\text{CH}_2\) protons of G4-OH, \(-\text{CH}_2\) protons of TA, and \(-\text{CH}_3\) protons of linker), 2.91–2.96 (m, \(-\text{CH}_2\) protons of G4-OH), 6.09 (s, aromatic protons of TA), 6.28–6.30 (d, \(J = 10.0\) Hz, aromatic protons of TA), 7.39–7.42 (d, \(J = 10.4\) Hz, aromatic protons of TA), and 8.08–8.20 (m, amide protons of G4-OH).

For the synthesis of rhodamine-labeled dendrimer (D-Rho), PAMAM (24 mg, 0.001 mmol) and 5(6)-carboxy-X-rhodamine (6.2 mg, 0.011 mmol) were dissolved in anhydrous DMF. A mixture of PAMAM, 8 equivalents of HOBT and HBTU, and 16 equivalents of DIPEA in anhydrous DMF. The reaction mixture was stirred for 48 h at room temperature. The crude product was eluted into a Sephadex\textsuperscript{\textregistered} LH-20 column (DMF only) to separate the unreacted excess rhodamine, HOBT, and HBTU. The product was precipitated three times with excess cold ether. The sample solution was dialyzed (MWCO 1000) against pure water for 4 h. After filtering through a 0.22-\(\mu\)m filter, the sample solution was freeze-dried.
All steps were performed in the absence of light. The FITC-labeled dendrimer (D-FITC) was synthesized using a similar procedure as that of D-Rho.

**Dynamic light scattering measurement of the dendrimer and D-TA**

Each material was diluted in distilled water in order to make the concentration of 1%. After each sample was sonicated in 30 min, dynamic light scattering (DLS) measurements were performed using Zetasizer Nano-ZS (green badge; ZEN3500, Malvern, Ltd, Malvern, UK) with a He–Ne ion laser at 633 nm.

**Zeta potential measurement**

The zeta-potential of the dendrimer and the D-TA was determined with laser-Doppler electrophoresis using Zetasizer Nano-ZS (green badge; ZEN3500, Malvern, Ltd) with a He–Ne ion laser at 633 nm and a detection angle of 173° at 37°C. Each material was diluted in distilled water and sonicated for 20 min. From the obtained electrophoretic mobility, the zeta-potentials of each material were calculated using the Smoluchowski equation: $\zeta = 4\pi\eta\nu/e$ in which $\eta$ is the viscosity of the solvent, $\nu$ is the electrophoretic mobility, and $e$ is the solvent dielectric constant.

**SNT-induced neuropathic pain model**

Mice were anaesthetized with an intraperitoneal (i.p.) injection of 50 mg/kg pentobarbital sodium, and PNI was induced by transecting the L5 spinal nerve as described previously. Briefly, a surgical incision was made to the skin, and the para-spinal muscles were partially removed, and the L5 spinal nerve was carefully transected. The wound was closed with surgical skin staples.

For the administration of D-Rho, D-FITC, or D-TA, mice were injected under pentobarbital sodium anesthesia (25 mg/kg). Using a 10-μl Hamilton syringe (Hamilton Company, Reno, NV, USA) with a 30-gauge, one-half-inch needle, D-TA (10 μl of 1 μg/μl in saline equivalent to 587.54 pmol), D-Rho (10 μl of 1.2 μg/μl), D-FITC (10 μl of 1.2 μg/μl), or saline alone was i.t. injected into each C57BL/6 mouse.

**Primary glial cell culture**

Primary mouse brain mixed glial cells were prepared from one-day-old C57BL/6 mice as previously described. Briefly, after being anesthetized, mice meninges were removed from the cerebral hemisphere, and the tissue was dissociated into a single-cell suspension by gentle repetitive pipetting. Cells were cultured in Dulbecco’s Modified Eagle’s medium supplemented with 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfo- neric acid, 10% fetal bovine serum (FBS), 2 mM L-glutamine, 1 × NEAA, and 1 × antibiotic/antimycotic in 75 cm² flasks at 37°C in a 5% CO₂ incubator, and the medium was changed every five days. To isolate microglia from the mixed glia cells, the flasks were tapped firmly 15–20 days after culturing. The floating cells were collected and plated on coated glass cover slips in a four-well plate with 2 × 10⁴ cells/well. After 30 min, the media was replaced with fresh media to eliminate unbound non-microglial cells and debris.

**Real-time RT-PCR**

Mice were deeply anesthetized with pentobarbital sodium and intra-cardially perfused with ice-cold saline. The whole spinal cord tissue from lumbar 4 to 5 was removed and mRNA was obtained. Real-time RT-PCR was performed using SYBR Green PCR Master Mix and ABI Prism 7500 sequences detection system (Applied Biosystems, Foster City, CA, USA) as described previously. The following PCR primer sequences were used: GAPDH forward, 5'-AGG TCA TCC CAG AGC TGA ACG-3'; GAPDH reverse, 5'-CAC CCT GTT GCT GTA GCC GTA-3'; Nox2 forward, 5'-GAC CCA GAT GCA GGA AAG GAA-3'; Nox2 reverse, 5'-TCA TGG TGC ACA GCA AAG TGA-3'; IL-1β forward, 5'-GTG CTG GAC CCA TAT GA-3'; IL-1β reverse, 5'-TTG TCG TTG CTT GGT TCT CC-3'; TNF-α forward, 5'-AGC AAA CCA CCA AGT GGA GGA-3'; TNF-α reverse, 5'-GCT GGC ACC ACT AGT TGG TTG-3'; IL-6 forward, 5'-CCA CGA TTT CCC AGC GAA CAT-3'; IL-6 reverse, 5'-TCC ATC CAG TTG CCT TCT TGG-3'. The mRNA level for each gene was normalized to the mRNA level of the GAPDH gene and represented as a fold induction. Fold induction was calculated using the 2^(-ΔΔCT) method, as previously described. All real-time RT-PCR experiments were performed at least three times, and the mean ± standard error of the mean (SEM) values were reported, unless otherwise noted.

**Flow cytometry analysis**

The mice were deeply anesthetized with pentobarbital sodium and intra-cardially perfused with ice-cold saline. The spinal cord tissue from lumbar 1 to sacral 3 was removed and homogenized mechanically to a single-cell suspension. Cells were washed with ice-cold PBS and 2% FBS and incubated with Fc Blocker™ (BD Bioscience, San Jose, CA, USA) for 10 min at 4°C prior to staining with CD11b-APC (Biolegend Inc., San Diego, CA, USA). A BD FACs Caliber flow
cytometer (BD Biosciences) was used to measure the CD11b+ microglia population and CD11b- non-microglia cells. Data were acquired and analyzed with the BD CellQuest™ system (BD Biosciences).

In vitro apoptosis assay and cell number counting

To examine apoptosis and necrosis, annexin V-FITC/PI (BioLegend Inc.) assays were used. Pure microglia (1 x 10⁶ cells/well) were seeded in six-well plates. Saline or D-TA (100 ng/ml) were treated 1 or 24 h. Then, cells were washed with PBS, and 100 µl of cold-PBS, 5 µl of FITC-annexin V, and 10 µl of propidium iodide were added in order. After 15 min at room temperature in the dark, cells were analyzed by Flow cytometer (FACSVerso, BD Biosciences). To count cell number, a 1-cm² square was marked on bottom of plate before seeding cells then images of cells in each condition were obtained by using microscopy (CKX41, Olympus Corporation, Tokyo, Japan). The number of cells was counted by ImageJ software.

Immunohistochemistry

Male mice were anesthetized with pentobarbital sodium (50 mg/kg, i.p.) and perfused intra-cardially with 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB, pH 7.4). The L4–L5 spinal cord was removed, post fixed in 4% PFA at 4°C overnight, and transferred to 30% sucrose in PBS for 48 h. Spinal cord sections (30-µm-thick) were prepared on gelatin-coated slide glass using a cryocut microtome. The sections were blocked in solution containing 5% normal donkey serum (Jackson ImmunoResearch, Bar Harbor, ME, USA), 2% BSA, and 0.1% Triton X-100 (Sigma) for 1 h at room temperature. Sections were then incubated overnight at 4°C with primary antibody for rabbit-anti-Iba1 (1:1000; Wako, Osaka, Japan), mouse-anti-GFAP (1:5000; Millipore, Billerica, MA, USA), or rabbit-anti-MAP2 (1:400; Millipore). After rinsing in 0.1 M PBS, the sections were incubated for 1 h at room temperature with a mixture of Cy3- or FITC-conjugated secondary antibodies (1:200; Jackson ImmunoResearch). The sections were mounted, and fluorescent images were obtained using a confocal microscope (LSM700; Carl Zeiss, Oberkochen, Germany).

Behavioral testing

All mice were allowed to acclimate to the testing apparatus and environment twice for 2 h before testing. Each mouse was placed alone in a Plexiglas cage on an elevated mesh in order to access the paws for observation. On each test day, mice were briefly habituated to the test environment for 30 min. Baseline thresholds were obtained two days before surgical treatment. Mechanical allodynia was assessed by measuring foot withdrawal thresholds in response to mechanical stimuli to the hind paw. All behavioral experiments were performed using the up-down method with von Frey filaments. Mechanical sensitivity was calculated using a mean threshold.

Statistical analysis

Statistical differences between the two groups were assessed using two-tailed Student’s t-tests. Differences between multiple groups were assessed using a one-way analysis of variance followed by Bonferroni corrected post hoc tests. All data were presented as mean ± SEM, and differences were considered significant if the p value was less than 0.05.

Results

Dendrimers specifically localize in spinal cord microglia

To test microglial uptake of neutral PAMAM dendrimer, we conjugated rhodamine to the terminal hydroxyl group of the PAMAM dendrimer (D-Rho) and treated them to primary mouse brain microglial cells. After 1 h of treatment, rhodamine fluorescence signals were detected in cell bodies morphologically identified as microglia (Figure 1(a)) indicating that dendrimers were efficiently transported to microglia. In some cells, the D-Rho signal showed a dot-like pattern (arrowheads) while in others the signal merged with the whole cell body (arrows), which was possibly due to the high concentration of fluorescent D-Rho taken up by primary microglia. To test microglia-specific delivery in vivo, D-Rho was i.t. injected into mouse spinal cord. Three days after injection, scattered rhodamine signals were detected in the lumbar spinal cord. These signals were merged with Iba1-positive-microglial cells (arrows) but not with GFAP-positive astrocytes or MAP2-positive neuronal cells (Figure 1(b)). To quantify microglia-specific uptake of dendrimer injected into the spinal cord in vivo, D-FITC was i.t. injected into the spinal cord, and then FITC-positive cells were analyzed in the spinal cord tissue by flow cytometry. Most of the FITC signals were detected mainly in CD11b+ microglia but not in CD11b- cells (Figure 1(c)). Among the CD11b+ microglia population, ~57% cells were FITC-positive, indicating that a single i.t. injection of dendrimer could be delivered to more than half of the spinal cord microglia (Figure 1(c)). Taken together, these data showed that neutral PAMAM dendrimer was delivered specifically and effectively to spinal cord microglia in vivo.
D-TA synthesis and its physiochemical properties

Upon confirming the microglia-specific delivery of the dendrimer, we synthesized D-TA following the synthesis procedure of D-fluocinolon acetonide. D-TA was synthesized using a two-step process with glutaric acid as a spacer, as shown in the synthetic scheme using a hydroxyl terminal Generation 4.0 PAMAM dendrimer (PAM-G4-OH) (Figure 2(a)). TA was conjugated to the PAMAM dendrimer with an ester bond so that TA could be easily released from the dendrimer nanomolecule upon microglial uptake. The average dendrimer and TA conjugated dendrimer diameter was determined by transmission electron microscopy (TEM) and Zetasizer (Figure 2(b) and (c)). Nanoparticle zeta potential was also analyzed. PAMAM dendrimer G4 showed a negative zeta potential of $-4.05 \pm 1.56$ mV due to its 64-hydroxyl group while the TA conjugated dendrimer zeta potential increased to $-1.74 \pm 0.28$ mV because its charge might be covered by TA (Figure 2(c)).

D-TA syntheses lipoteichoic acid-induced proinflammatory cytokine expression in glial cells

In our previous study, we found that TA inhibited lipoteichoic acid (LTA)-induced expression of proinflammatory genes (TNF-$\alpha$, IL-1$\beta$, and IL-6) in primary glial cells. To investigate whether dendrimer-conjugated TA also exerts anti-inflammatory effects on microglia, we treated toll-like receptor 2 (TLR2)-activated primary mouse mixed glial cells with D-TA, in which TLR2 was mainly expressed on microglia. Upon TLR2 stimulation with LTA in glial cells, the mRNA expression of IL-1$\beta$, TNF-$\alpha$, and IL-6 genes was upregulated 115-, 70- and 75-fold, respectively. These gene inductions, however, were dose-dependently inhibited by up to 90% by D-TA treatment (Figure 3(a) to (c)). In the meanwhile, D-TA treatment did not induce microglial cell death nor affect proliferation (Figure 3(d) and (e)). These data indicate that TA conjugated to dendrimer nanomolecules retains strong inhibitory effects on proinflammatory activation of glial cells.

Intrathecal administration of D-TA attenuates nerve injury-induced spinal cord microglia activation

To evaluate the inhibitory effects of D-TA on spinal cord microglia activation in nerve injury-induced neuropathic pain, D-TA was administered into the spinal cord immediately after L5 SNT. As measured with Iba1 immunostaining, spinal cord microglia in the ipsilateral dorsal horn (L4–L5) was morphologically activated at three
days after SNT (Figure 4(a) and (b)). However, this SNT-induced microglia activation was attenuated by 34% in D-TA or TA-treated mice compared to dendrimer-injected mice (Figure 4(a) and (b)). Pro-inflammatory gene inductions in the activated spinal cord microglia, such as IL-1β, TNF-α, and IL-6, have been shown to contribute to nerve injury-induced neuropathic pain.22,23 Similarly, microglial Nox2 expression after nerve injury is implicated in microglia activation and subsequent neuropathic pain.17 As reported, upon SNT, the transcripts of Nox2, IL-1β, TNF-α, and IL-6 were upregulated by 1.9-, 2.2-, 2.1-, and 4.7-fold, respectively, in L5 spinal cord tissue (Figure 4(c) to (f)). However, these pain-mediating gene inductions after SNT were reduced to 1.0-, 1.1-, 1.5-, and 2.0-fold, respectively, in D-TA-treated mice (Figure 4(c) to (f)). Taken together, these data demonstrate that D-TA attenuates nerve injury-induced spinal cord microglia activation in vivo.

**D-TA inhibits SNT-induced mechanical allodynia**

The inhibitory effects of D-TA on spinal cord microglia activation in vivo suggest a putative analgesic effect of D-TA. To test this possibility, we measured the susceptibility of D-TA-treated mice to nerve injury-induced pain hypersensitivity. After L5 SNT, wild-type mice showed increased sensitivity to mechanical stimuli measured by the von Frey test. The paw withdrawal threshold to mechanical stimuli decreased from 0.07 to 0.19 g at one-day post injury (dpi) (Figure 5(a)). The threshold remained below 0.1 g up to 7 dpi. In TA-treated mice, in which the same molar concentration of TA as D-TA was administrated, the paw withdrawal threshold was...
reduced to 0.31 g on 1 dpi and 0.24 g on 3 dpi, indicating a moderate reduction in mechanical allodynia. In the D-TA-treated mice, however, the paw withdrawal threshold was reduced to only 0.71 g on 1 dpi and 0.64 g on 3 dpi (Figure 5(a)) showing much stronger analgesic effects of D-TA compared to TA. The analgesic effect of a single injection of D-TA was maintained for up to three days, and the withdrawal threshold for the D-TA-treated mice returned to a level similar to vehicle-injected control mice at 7 dpi. To test the post-operative effects of D-TA, we injected D-TA on 1.5 dpi. A single post-operative injection of D-TA on 1.5 dpi significantly attenuated the mechanical hypersensitivity of SNT-injured mice at 4 dpi (Figure 5(b)). However, a post-operative D-TA injection on 4 dpi failed to induce any significant attenuation of mechanical hypersensitivity (Figure 5(c)). These data indicate that D-TA treatment at an early time point after SNT efficiently attenuate nerve injury-induced mechanical allodynia.

Discussion
In this study, newly synthesized D-TA was shown to specifically inhibit nerve injury-induced spinal cord microglia activation and neuropathic pain when administered intrathecally. TA is a synthetic glucocorticoid molecule and, accordingly, has potent anti-inflammatory effects. Glucocorticoid binding to glucocorticoid receptors (GR) can directly activate transcription of anti-inflammatory genes. In addition, it can inhibit the transcription of proinflammatory genes by inhibiting key transcription factors for proinflammatory gene expression such as NF-κB. Given its potent anti-inflammatory function, TA is widely used as an
anti-inflammatory drug to treat various inflammatory diseases including allergies, arthritis, and skin disorders.\textsuperscript{27,28} In clinical pain management, TA is being used via epidural injection to treat low back and neck pain.\textsuperscript{29,30} Intrathecal TA injection has also been tested as a treatment option for multiple sclerosis.\textsuperscript{31} However, direct i.t. injection of TA into the CNS is not recommended due to the risk of complications such as brain or spinal cord infarct\textsuperscript{32} or neurotoxicity.\textsuperscript{33,34} The exact molecular mechanisms of these unwanted effects are unclear. It has been suspected that TA tends to aggregate due to its hydrophobicity and can cause embolisms once
delivered to the vessel.\textsuperscript{35} It was also previously reported that high-dose corticosteroids had neurotoxic effects.\textsuperscript{36} Therefore, it is conceivable that delivery of TA specifically into microglia could efficiently modulate neuroinflammation, while minimizing its side effects.

Given this hypothesis, we synthesized a dendrimer-based nanomolecule, D-TA. Dendrimer is a biodegradable and biocompatible nanoscale molecule. Since its terminal active moieties can be conjugated with other small molecules, its potential as a drug delivery vehicle is currently being extensively explored. The characteristics of a dendrimer are largely dependent on its terminal moiety. A positively charged dendrimer due to lysine conjugation is used to deliver negative nucleic acids into negatively charged cell membranes.\textsuperscript{37} Meanwhile, hydroxyl group-containing neutral dendrimers are selectively taken up by microglia in the CNS, suggesting an efficient microglia-specific delivery vehicle.\textsuperscript{15} Indeed, the results of the present study confirm through immunohistochemistry and FACS analysis that i.t.-injected dendrimers are specifically located in the spinal cord microglia. Thus far, it is not clear why D-TA has microglia specificity. Among the cells in the CNS, microglia function as innate immune cells that are dedicated to the clearance of “foreign molecules” with their well-equipped phagocytic/endocytic machineries. Therefore, it is conceivable that dendrimer nanomolecules are specifically detected and cleared by microglia via their phagocytic machineries.

During the preparation of the manuscript, the synthesis of D-TA and its inhibitory effects on microglial activation were reported by another group.\textsuperscript{38} In their study, the inhibitory effects of D-TA on microglial cells were studied only in BV-2 cell lines. Our study confirms their results in primary glia. In our in vitro study to confirm the anti-inflammatory effects of D-TA, we used primary mouse mixed glial cells instead of pure microglia. We reasoned that the characteristics of microglia in mixed glia are more similar to those of resting microglia in vivo, since pure microglia in vitro tend to be spontaneously activated.\textsuperscript{39} Since TLR2 expression in pure astrocytes is minimal\textsuperscript{21}, the anti-inflammatory effects of D-TA on the TLR2-activated mixed glia observed in the present study are most likely due to its microglia-specific effects. In addition, the inhibitory effects of D-TA on microglia in the spinal cord were confirmed in vivo. Interestingly, D-TA was much more effective in decreasing mechanical allodynia compared to the same molar amount of TA in vivo. It is possible that the hydrophobic feature of free TA resulted in its aggregation upon injection, which rendered it less diffusible in the spinal cord. On the contrary, dendrimer-conjugated TA is more soluble and thusdiffuses more in the spinal cord, and does not experience aggregation, which might underlie its greater analgesic efficacy in vivo. It is also conceivable that TA that is directly injected into the spinal cord not only inhibits microglial activation but also affects other microglia-independent targets, thereby masking its microglia-dependent analgesic effects. Considering that i.t. TA injection could attenuate SNT-induced microglia activation at a similar level as D-TA injection (Figure 4(a) and (b)), we think this latter possibility is more likely. Thus, by driving TA specifically into microglia via a dendrimer, more potent analgesic effects were induced compared to those induced by free TA.

In SNT-injured mice, a single injection of D-TA maintained its analgesic effects for up to three days. The analgesic effect was also observed on 4 dpi when D-TA was injected post-operatively on 1.5 dpi, supporting its clinical application. However, when it was injected on 4 dpi, the analgesic effect was not as significant. These data indicate that D-TA inhibited the development, not the maintenance, of pain hypersensitivity. This is consistent with the hypothesis regarding the function of spinal cord microglia activation in nerve injury-induced neuropathic pain, in which microglia activation is believed to be involved in the induction but not the maintenance phase of neuropathic pain. Although debatable, some studies argue that DRG-infiltrating macrophages contribute to nerve injury-induced neuropathic pain.\textsuperscript{40} Considering that intrathecally injected drugs can be delivered to DRG, our study does not completely exclude the possibility that the putative inhibitory effects of D-TA on DRG-infiltrating macrophages may partly contribute to the analgesic effects seen. Still, the pivotal role of spinal cord microglia activation on nerve injury-induced neuropathic pain induction and the near-complete inhibitory effect of D-TA on such microglia activation suggest that D-TA confers its analgesic effect mainly by inhibiting spinal cord microglia activation. In addition to neuropathic pain, i.t. TA injections seem to have beneficial effects in progressive multiple sclerosis patients.\textsuperscript{41} Considering the more potent efficacy of D-TA compared to free TA in vivo, it is also important to test the efficacy of D-TA in MS.

In conclusion, a newly developed dendrimer-based nanomolecule D-TA was selectively delivered to spinal cord microglia upon i.t. injection. In addition, D-TA inhibits nerve injury-induced spinal cord microglia activation and mechanical allodynia, suggesting therapeutic implications of D-TA in the treatment of neuropathic pain.

Authors’ contributions
HK and BC contributed equally to this work. HK, JSP, JGC and SJL designed the study, analyzed the data, and wrote the manuscript. HL, HM, JHL, and SC performed the experiments and analyzed the data. All authors read and approved the final manuscript.
Declaration of Conflicting Interests
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