Spinal interleukin-10 produces antinociception in neuropathy through microglial β-endorphin expression, separated from antineuroinflammation

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1. Introduction

IL-10, one of the most important antiinflammatory and immunosuppressive cytokines, is involved in infectious and autoimmune diseases, like ulcerative colitis, Crohn’s disease, and multiple sclerosis (Fedorak et al., 2000; Fillatreau et al., 2002). In the central nervous system, IL-10 exhibits neuroprotection and antinociception in various rodent models (Ooboshi et al., 2005; Scholz and Woolf, 2007; Johnston et al., 2008). For example, intrathecal delivery of recombinant IL-10 blocked neuropathic pain induced by chronic constriction injury (Wagner et al., 1998), spared nerve injury (Wang et al., 2012b), or cancer chemotherapy with paclitaxel (Ledeboer et al., 2007). IL-10 was also effective in attenuating inflammatory pain induced by snake venom phospholipase A2 (Laughlin et al., 2000), chronic orofacial inflammation (Shimizu et al., 2009), acetic acid and zymosan (Vale et al., 2012). Interestingly, sustained IL-10 treatment did not induce antinociceptive tolerance in chronic pain states (Wagner et al., 1998; Milligan et al., 2006b; Dengler et al., 2014). Thus, to produce durable antinociception in chronic pain, IL-10 gene therapy using both intrathecal viral and non-viral delivery systems has been successfully employed in treating chronic pain in rodent models (Milligan et al., 2005a,b, 2006a,b; Soderquist et al., 2010). IL-10 could be a potential strategy to treat clinical pain, particularly chronic neuropathic pain, an unmet medical need.

Accumulated evidence indicated that spinal microglia played a crucial role in neuropathic pain, particularly at the initial stage through
induction of neuroinflammatory cytokines including tumor necrosis factor (TNF-α), IL-6, IL-1β, and brain-derived neurotrophic factor (BDNF) (Jim et al., 2003; Raghavendra et al., 2004; Wang et al., 2012a, 2014; Taves et al., 2013), which consequently sensitize the neurons in the spinal dorsal horn by altering the excitatory or inhibitory synaptic transmission to contribute to pain facilitation (Kawasaki et al., 2008; Milligan et al., 2012). IL-10 was able to disrupt expression of TNF-α, IL-1β, and IL-6 in microglia, by inducing antiinflammatory elements, like Bcl3 and Socs3 to subsequently inhibit NF-κβ-dependent signals (Moore et al., 2001; Ji et al., 2002; Milligan et al., 2012). To date, it is thus generally believed that IL-10 produced antinociception through inhibition of neuroinflammatory cytokine expression, in agreement with a well-accepted concept for the nonsteroidal antiinflammatory drugs (NSAIDs) used for the treatment of inflammatory pain (Vale et al., 2003; Milligan et al., 2005b; Zhou et al., 2008).

However, recent studies have weakened the role of microglia activation in the development of established neuropathic pain, evidenced by that expression of microglial proinflammatory cytokines (TNF-α, IL-1β and IL-6) and p38 phosphorylation in the central nervous system declined days or weeks after peripheral nerve injury (Schaifers et al., 2003; Lee et al., 2004; Wu et al., 2017a), and that the drugs targeting microglia failed to attenuate established neuropathic pain, although they could block expression of neuroinflammatory cytokines and prevent the initial of neuropathic pain. These drugs included the microglia activator minocycline (Gong et al., 2014c; Fan et al., 2016), microglia depletor clodronate liposome (Wang et al., 2018), p38 activation inhibitor SB203580 (Huang et al., 2016, 2017a; Wu et al., 2017a), IL-1 receptor antagonist (Sweitzer et al., 2001) and TNF-α inhibitor etanercept (Schaifers et al., 2003; Marchand et al., 2009).

On the other hand, microglia additionally exhibited expression of endogenous opioid peptides β-endorphin and dynorphin A, which produced antinociception in neuropathy via activation of neuronal μ- and κ-opioid receptors, respectively (Taves et al., 2013; Huang et al., 2016; Li et al., 2016; Huang et al., 2017a,b; Li et al., 2017). It has been implied that endogenous opioid peptides particularly β-endorphin was associated with the development of neuropathic pain (Backryd et al., 2014). IL-10 treatment was reported to upregulate the expression of the β-endorphin precursor gene POMC in arcuate nucleus in the brain (Nakata et al., 2016) and HL-60 cells (Awad et al., 2012). In addition, we recently demonstrated that IL-10 mediated the glucagon-like peptide-1 (GLP-1) receptor agonist exenatide-induced spinal microglial expression of β-endorphin and antinoiception in neuropathy (Wu et al., 2017b). Thus, this study aimed to test the hypothesis that IL-10 produces antinociception in neuropathy through spinal microglial expression of β-endorphin, rather than inhibition of the expression of neuroinflammatory cytokines.

2. Materials and methods

2.1. Chemicals and reagents

The β-endorphin neutralizing antibody and CTAP were purchased from Abcam (Cambridge, UK), and lipopolysaccharide (LPS), naloxone and 5′-guanidinonaltrindole (GNTI) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Recombinant rat IL-10, naltrindole and the STAT3 activation inhibitor NSC74859 were obtained from PeproTech (NJ, USA), Tocris (Bristol, UK) and Medchem Express (Princeton, NJ, USA), respectively, while the microglial inhibitor minocycline and specific microglial depletor clodronate liposome were purchased from Yuanye Biotech (Shanghai, China) and FormuMax Scientific (Sunnyvale, CA, USA), respectively. All the drugs were dissolved or diluted in normal saline.

2.2. Animals

Male and female Wistar rats used in this study were obtained from the Shanghai Experimental Animal Institute for Biological Sciences (Shanghai, China), including male and female one-day-old neonatal and male adult (8–10-week-old) rats. The adult rats were adopted in a 12-hour light/dark cycle with free access to food and water ad libitum. Three to five days were given for rats to acclimate to the laboratory environment prior to the experiments. The animal protocols were approved by the Animal Care and Welfare Committee of the Shanghai Jiao Tong University (Shanghai, China) and followed the regulatory animal care guidelines of the United State National Institute of Health (Bethesda, MD, USA).

2.3. Primary cell cultures

The spinal cords removed from the one day-old neonatal rats was minced and then digested in 0.05% trypsin. The dispersive cells were thereafter suspended in Dulbecco’s Modified Eagle’s medium (DMEM), supplemented with 10% (v/v) fetal bovine serum (FBS), penicillin (100 U/mL), and streptomycin (100 μg/mL). To collect neuronal cells, the medium after 1.5 h of incubation was changed to Neurobasal containing B27 supplement and 0.5 mM glutamine for further culture. All experiments were initiated 5–6 days after plating. The purity of neuronal cells was identified to be more than 85%, as determined by the neuronal nuclear antigen (NeuN) immunoreactivity (Gong et al., 2014c).

For glial cell cultures, suspended cells were seeded into 75-cm² tissue culture flasks (1 × 10⁶ cells/flask) that were pre-coated with poly-l-lysine (10 μg/mL), and cultured at 37 °C in a 5% carbon dioxide incubator. To prepare microglial cells, the flask after 8 days’ culture was shaken under 37 °C at 260 rpm for 2 h. The aliquots were transferred into new plates and unattached cells were removed by washing with serum-free DMEM. Harvested microglial cells exhibited a purity >95%, as determined by the Iba-1 immunoreactivity (Gong et al., 2014c; Wu et al., 2017a). To get astrocytes, the flasks after 11 days’ culture were also shaken for 2 h and then removed the aliquots and incubated with 10 mL of 0.05% trypsin-ethylenediamine tetraacetic acid (Invitrogen, Grand Island, NY, USA) in a cell incubator for 15 min to separate the oligodendrocytes. After neutralization of trypsin with 10 mL of the complete DMEM, the floating cell suspensions were discarded. A nearly intact layer of astrocytes in the bottom of the flask were then trypsinized and subcultured conventionally. Prepared astrocytes exhibited a purity >90%, as determined by the GFAP immunoreactivity (Gong et al., 2014c).

2.4. RNA isolation and quantitative reverse transcriptase-PCR (qRT-PCR)

The contralateral and ipsilateral spinal lumbar enlargements (L3-L5) were isolated from neuropathic rats and mechanically homogenized in Trizol (Invitrogen) on ice. Total RNA from the spinal homogenates and primary microglia were isolated using Trizol and reversely transcribed into cDNA using a ReverTraAce qPCR RT-kit (Toyobo, Osaka, Japan) according to the manufacturer’s instructions. Real-time quantitative PCR amplification was performed in a Mastercycler ep realplex (Eppendorf, Hamburg, Germany) using the RealmasterMix (SYBR Green I) (Toyobo). The primers were used as follows: 5′-CTATCAGCTTTGGGACATCTT-3′ and 5′-TGGCCTCTCGGAGGTCAAT-3′ (POMC exon2-3), 5′-AGGAAGTCTCCTCTCTGGA-3′ and 5′-TTCGTCGGGTTAGGATGATG-3′ (Bcl3, NM_001109422.1), 5′-AGGGCTCTTGATGGCGGA-3′ (Fan et al., 2015) and 5′-GGAAGGC AGTGTCACTCATTGTG-3′ (Fan et al., 2015). Real-time quantitative PCR was first identified to be specific using the melting curves and the relative
expression of each mRNA level was then calculated by using the \(2^{-\Delta\DeltaCT}\) method after normalizing Ct (cycle threshold) values with GAPDH Ct (Wu et al., 2017b,c).

2.5. Western blot

Protein supernatants were extracted from the homogenized spinal lumbar enlargements (L3–L5) and cultured microglia using the radio immunoprecipitation analysis buffer containing the protease inhibitor phenylmethylsulfonyl fluoride and phosphatase inhibitor cocktail A/B. The protein was denatured at 100 °C for 10 min and separated in 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and then transferred to a polyvinylidene fluoride membrane using the electrophoretic method. The membrane was then blocked in 5% skim milk dissolved in Tris-based saline (1× TBS) containing 0.1% Tween 20 (TBS-T) and then incubated with a primary antibody against phospho-STAT3 (Ser727) (1:1000, lot 49081, Cell Signaling Technology, Danvers, MA, USA) and GAPDH (1:5000, lot 6004-1-Ig, Protein Tech Group, Rosemont, IL, USA) at 4 °C overnight with slightly shaking. The Odyssey Infrared Imaging system (Li-Cor Biosciences, Lincoln, NE, USA) was used to detect the protein bands after one-hour incubation at 37 °C with the Dylight 680-conjugated anti-mouse IgG (1:10,000, lot 5470) and Dylight 800 conjugated anti-rabbit IgG (1:10,000, lot 5151) (Rockland Immunocorehemics, Gilbertsville, PA, USA). The protein band intensity was analyzed and quantified using the ImageJ software (NIH). The relative expression of each target protein was calculated after normalization to the GAPDH level. The experiments were independently repeated at least thrice (Gong et al., 2014c; Fan et al., 2016).

2.6. Immunofluorescence staining

β-Endorphin and microglia, astrocytes, or neurons in the spinal cord were doubly labeled with immunofluorescence and visualized under a TCS SP8 confocal microscope (Leica Microsystems, Wetzlar, Germany) as described previously (Huang et al., 2016). Briefly, rats were perfused intracardially with 100 mL normal saline, followed by 60 mL of 4% paraformaldehyde (w/v) in phosphate buffered saline (PBS) under pentobarbital anesthesia (40 mg/kg). Spinal lumbar enlargements (L3–L5) were isolated and fixed in 4% buffered paraformaldehyde for 12 h and dehydrated in gradient sucrose solutions (10–30%) at 4 °C. Tissues were entrapped in the OCT embedding agent (Leica Microsystems) and cut into 20-μm frozen sections. For the immunostaining of IL-10R and p-STAT3 in spinal microglia, primary cultured microglial cells were placed in 24-well plates (5 × 10⁴ cells/well) with poly-l-lysine-coated coverslips at the bottom and fixed in 4% PFA. The frozen sections and cell coverslips were incubated in 10% goat serum (v/v) and 0.5% Triton X-100 (v/v) in PBS for 1 h and then with the rat β-endorphin antibody (1:100; Phoenix Pharmaceuticals, Burlingame, CA, USA, lot G-022-33), IL-10 receptor-α antibody (1:25; Santa Cruz Biotechnology, lot SC-985), phospho-STAT3 (Ser727) antibody (1:200, from Cell Signaling Technology, Danvers, MA, USA, lot 49081) and other primary antibodies at 4 °C for additional 24 h. Spinal neuronal and glial cells were identified by the following biomarkers, e.g., Iba-1 (1:100; mouse monoclonal; Millipore, Darmstadt, Germany, lot MABN92) for microglia, GFAP (1:100; mouse polyclonal; Millipore, Darmstadt, Germany, lot MAB3402C3) for astrocytes, and NeuN (1:60; mouse polyclonal; Millipore, Darmstadt, Germany, lot MAB377) for neurons. The β-endorphin, IL-10 receptor-α and p-STAT3 staining was visualized with the Alexa-555-conjugated Goat anti-rabbit secondary antibody (1:200, Invitrogen, lot A-21428). The Alexa-488-conjugated goat anti-mouse secondary antibody (1:200, Invitrogen, lot A-11001) was used to detect the antibodies targeting the spinal cell biomarkers. DAPI staining was used to determine cell nuclei in cell coverslips. For quantification of β-endorphin-, Iba-1-, GFAP-, and NeuN-immunopositive cell density in the spinal cord, photomicrographs of the median three of the dorsal horn (laminas I–V) were taken under a confocal microscope with a 10× or 40× magnification. An investigator who blinded to the experiment groups measured the positively stained surface area using a computer-assisted image analysis program (ImageJ Software, NIH). The background fluorescence was excluded and only immunofluorescence intensity measurements from positive staining areas were included by low and high thresholds setup. For co-localization analysis, co-localization finder was used to generate merged-images in which co-localized pixels appeared as white. All the surface areas in each group were measured following the same configuration setup at the same time. The averaged percentage immunolabeled surface area was the fraction of the positive immunofluorescence surface area of the total measured picture area from three nonadjacent sections of each spinal cord (Huang et al., 2016; Wu et al., 2017b).

2.7. β-Endorphin measurements

For spinal homogenates, the contralateral and ipsilateral spinal lumbar enlargements were isolated from neuropathic rats 1 h after intrathecal injection of IL-10 and homogenized at 4000 rpm for 15 s with a homogenizer (Fluko Equipment) in 10 mM Tris-HCL (1 g tissue/5 mL) and centrifuged at 4000 rpm in 4 °C for 15 min, and the supernate was then collected for later protein measurement. The cultured primary microglia were stimulated with 30 ng/mL of IL-10 for 2 h, and then were placed in 24-well plates (1 × 10⁵ cells/well) and washed twice with 1 mL of warm DMEM containing 2 mg/mL of BSA and 15 mmol/L of N-(2-hydroxyethyl) piperazine-N’-2-ethanesulfonic acid. The total protein concentrations in the spinal homogenates and primary cells were measured using the standard bichinchoninic acid protein assay (Beyotime Institute of Biotechnology, Jiangsu, China), where the levels of β-endorphin were measured using enzyme-linked fluorescent immunomassay kits (Phoenix Pharmaceuticals, Burlingame, CA, USA). A fluorescent microplate reader (Thermo Labsystems, Vantaa, Finland) was used to measure the relative fluorescence values and the concentration of β-endorphin was calculated using a calibration curve performed at the same time. The assays were validated with the linear range of 1–100 pg/mL for β-endorphin. According to the manufacturer’s information, there was a cross-reactivity of the β-endorphin with α-endorphin (100%) and γ-endorphin (60%), but not with met-enkephalin (0%) or leu-enkephalin (0%).

2.8. RNA interference

The siRNAs targeting IL-10 receptor-α and the nonspecific oligonucleotides (oligos) were designed and synthesized by Shanghai GenePharma (Shanghai, China) to target the following sequences, i.e., POMC, 5′-CCUACUCUCAUGGCACUUUTT-3′ and 5′-AAGUGUCCCAUG GAGUAGGT T-3′; Bcl3, 5′-GCCUCAAGAAUGUCACAAAT-3′ and 5′-UUGUGACAGUUCUU AUGGCTT-3′; Socs3, 5′-CCUGGACUCAUG AGAAATTT-3′ and 5′-UUUCCUC AUAGGAGUCGAGTT-3′; and the non-specific oligos, 5′-UUUCGCAAGGUGUCGAGCTT-3′ and 5′-ACUGUA CACGUUGCAGGAATT-3′. The complex of siRNA and the Lipofectin DOTAP (1,2-di-oleyl-3-trimethylammonium-propane, from Avanti Polar Lipids, Alabaster, AL, USA) was prepared with a mass ratio of 1:8 following the manufacturer’s instruction. For in vitro transfection, primary microglial cells were seeded into 24-well/6-well plates. The siRNA-DOTAP complex was added into the plates with supplement of 300 μL/500 μl of basic DMEM to make the final siRNA concentration of 5 μg/mL and cells were then incubated for 5 h. After transfection, cells were further cultured for 24 h routinely. For the in vivo transfection, the siRNA-DOTAP complex was multiple daily intrathecally injected into rats for 6 days (Wu et al., 2017a).
2.9. Intrathecal catheterization and injection in rats

A catheter (PE-10: 0.28 mm inner diameter and 0.61 mm outer diameter, Clay Adams, Parsippany, NJ, USA) was administered into the rat lumbar level of the spinal cord under inhaled isoflurane anesthesia as described previously (Huang et al., 2012; Lu et al., 2012). Two days after recovery from anesthesia, the correct positioning of the catheter in the spinal cord was verified by administering 10 μL of 4% lidocaine. Rats that had no motor impairments such as paralysis and claudication following insertion of the intrathecal catheter and that developed immediate bilateral paralysis of the hindlimbs following intrathecal administration of lidocaine were used for the experiments. The exclusion rate was zero in our study. For intrathecal administration, 10 μL of the drug solution was administered in a 50-μL microinjector followed by a 15 μL saline flush.

2.10. Rat model of neuropathic pain, and assessments of mechanical allodynia and heat hyperalgesia

The left L5 and L6 spinal nerves of rats were carefully isolated and tightly ligated with 6-0 silk sutures under inhaled isoflurane anesthesia following the methodologies described previously (Kim and Chung, 1992). After nerve ligation, the lumbar fascia and skin were sutured by 4-0 absorbable polyglactin suture, and the rats were allowed to recover. The intrathecal catheterization was performed at the same time just before spinal nerve ligation. After recovery, only rats with significant unilateral allodynia to mechanical stimulation (hindlimb withdrawal thresholds in the operated side < 8 g) and with no major motor impairment were used for further studies. No rats were excluded due to motor impairment in our study. Neuropathic rats, starting the drug testing 2–3 weeks after spinal nerve ligation, were randomly assigned to experimental groups.

To evaluate mechanical allodynia, the animals were acclimatized for at least 30 min to the testing environment, namely a plexiglass box on a metal grid. An examiner who was blinded to the treatment groups performed the behavior testing using a 2290 CE electronic von Frey hair (IITC Life Science, Woodland Hills, CA, USA). The monofilament generated a force that ranged from 0.1 to 90 g. The withdrawal thresholds evoked in the contralateral and ipsilateral paws were determined by stimulation of the hindlimb while the rat stood on a metal grid. The increasing increments of force were applied to stimulate the footpad until the rat suddenly withdrew its hindlimb. The lowest force evoking a withdrawal response was considered the threshold, which was averaged from triplicate measurements at an approximately 1 min-interval (Wang et al., 2017).

To assess heat hyperalgesia, rats were adapted in a plexiglass box on an elevated glass surface for at least 30 min and a radiant heat source (at a low intensity of 45) was applied to stimulate the plantar medial surface of each hindpaw. The hindpaw withdrawal latency was determined by a 390G Plantar Test Analgesia Meter (IITC Life Science Inc.). The cut-off of 30 s was set to prevent skin damage. The paw withdrawal latency was defined as the period of time from the onset of radiant heat application to the withdrawal response. Each hindpaw was tested triply with a 5-min interval. The final result was calculated as the mean of the three repeated measurements (Wang et al., 2017).

2.11. Data evaluation and statistical analysis

The percentage of the maximal possible effect (% MPE) was calculated using the following formula: (post-drug threshold in ipsilateral hindlimb – baseline threshold in ipsilateral hindlimb)/(baseline threshold in contralateral hindlimb – baseline threshold in ipsilateral hindlimb) × 100 (Bowersox et al., 1996). The % MPE values near to 100 were regarded as normal mechanical thresholds (i.e., near contralateral thresholds), while values closed to 0 indicate mechanical allodynia. For analysis of the dose-response curves, the parameters, i.e., the minimum effect, maximum effect (Emax), half-effective dose (EC50), and Hill coefficient (n), were calculated by fitting nonlinear least-squares curves to the relation Y = a + bx, where x = [C]n/(EC50n + [C]n). The values of EC50 and b (Emax) were projected by yielding a minimum residual sum of squares of deviations from the theoretical curve (Wang and Pang, 1993).

Data were summarized as means ± standard error of the mean (SEM). The two-tailed and unpaired Student t-test and one-way or repeated measures two-way analysis of variance (ANOVA) were used to generate the statistical significance values using Prism (version 5.01, GraphPad Software, San Diego, CA, USA). The post-hoc Student-Newman-Keuls test was performed when the effect of the drug (dose) (for the one-way ANOVA, the factor was drug [dose]; for the two-way ANOVA, the factors were drug [dose], time and their interaction) was statistically significant. Probability values were two-tailed, and the statistical significance criterion P value was 0.05.

3. Results

3.1. Intrathecal IL-10 injection attenuated mechanical allodynia and thermal hyperalgesia in neuropathy

The antinociceptive effects of IL-10 on mechanical allodynia and thermal hyperalgesia were assessed in L5/L6 spinal nerve-ligated male and female rats 2–3 weeks following surgery. Saline (10 μL) or IL-10 (3, 10, 30, 100, or 300 ng) was intrathecally administrated to six groups of male neuropathic rats. The paw withdrawal thresholds and latencies were measured (with 5-min interval) at before, 0.5, 1, 2, and 4 h after intrathecal administration. As shown in Fig. 1A and B, spinal nerve-ligation dramatically induced mechanical allodynia and heat hypersensitivity in the ipsilateral paws, compared with the contralateral paws. Intrathecal saline injection did not change the mechanical withdrawal thresholds and heat withdrawal latencies in both contralateral and ipsilateral hindpaws over the 4-h period, whereas intrathecal IL-10 injection dose dependently increased the mechanical thresholds (F = 11.02, P < 0.0001, by repeated measures two-way ANOVA followed by the post-hoc Student-Newman-Keuls test) and withdrawal latencies in ipsilateral paws (F = 6.966, P < 0.0001, by repeated measures two-way ANOVA followed by the post-hoc Student-Newman-Keuls test), but had no effects on the paw withdrawal thresholds or latencies in the contralateral paws. The IL-10 antinociceptive effect was time-dependent, with a peak effect at 1 h after IL-10 injection. Dose-response analyses showed the ED50 and Emax values of IL-10 to inhibit mechanical allodynia were 40.8 ng (95% confidence limits: 15.6–107.2 ng) and 61.5% MPE, as calculated at one hour after injection (Fig. 1C). The ED50 and Emax values were 24 ng (95% confidence limits: 4.4–130.8 ng) and 100% MPE (Fig. 1D) in the thermal antihyperalgesic effect.

The antihyperactivity effect of IL-10 was also assessed in female rats. Three groups of female neuropathic rats received intrathecal injection of saline (10 μL) and IL-10 (30 and 100 ng), respectively. The hindpaw withdrawal thresholds and latencies were measured (with 5-min interval) at before, 0.5, 1, 2, and 4 h after intrathecal administration. As shown in Fig. 1E and F, intrathecal IL-10 administration dose dependently relieved mechanical allodynia (F = 9.38, P < 0.0001 by repeated measures two-way ANOVA followed by the post-hoc Student-Newman-Keuls test) and thermal hyperalgesia (F = 2.407, P = 0.02 by repeated measures two-way ANOVA followed by the post-hoc Student-Newman-Keuls test) in female rats, same as those in male rats.

3.2. IL-10 specifically stimulated spinal microglial β-endorphin expression

A previous study showed that IL-10 was able to induce β-endorphin expression in neutrophils (Awad et al., 2012). We assessed whether it is the case in cultured primary microglia obtained from the spinal cord. Expression of the β-endorphin precursor POMC mRNA in spinal
microglia 2 h after IL-10 treatment (1, 3, 10, 30, and 100 ng/mL) was dose-dependently upregulated \( (F = 4.8, P = 0.005, \text{by one-way ANOVA followed by the post-hoc Student-Newman-Keuls test; Fig. 2A}) \), with an EC\(_{50}\) value of 8.8 ng, but failed to change expression of prodynorphin mRNA (Fig. 2B), whereas POMC expression was not significantly changed within 2 h of IL-10 (30 ng/mL) treatment in primary astrocytes (Fig. 2C) or neurons (Fig. 2D).

The stimulatory effect of IL-10 on β-endorphin expression was further assessed in cultured primary microglia, astrocytes and neuronal cells by using a specific commercial fluorescent immunoassay kit. As shown in Fig. 2E, the baseline β-endorphin levels in the culture medium of microglia, astrocytes and neurons were 1.8 ± 0.4, 1.3 ± 0.6, and 2.2 ± 1.5 pg/mL, respectively. In parallel with POMC expression, IL-10 treatment (30 ng/mL) markedly increased β-endorphin level by 160% in microglia (\( F = 1.637, P = 0.002 \text{ by one-way ANOVA followed by the post-hoc Student-Newman-Keuls test} \)), but not in astrocytes or neurons.

To confirm our hypothesis that spinal IL-10 antinociception was mediated by microglial β-endorphin expression, we assessed spinal cell types that specifically promoted β-endorphin expression using single and double immunofluorescence labeling of β-endorphin and intracellular biomarkers of microglia (IBA-1), astrocytes (GFAP), or neurons (NeuN). There was no significant difference of the β-endorphin immunostaining between the contralateral and ipsilateral spinal dorsal horn. IL-10 treatment for 1 h markedly enhanced the β-endorphin immunostaining in both the contralateral and ipsilateral spinal dorsal horn (Fig. S1A and B) by 260% and 400%, respectively (\( F = 6.113, P < 0.05 \text{ by one-way ANOVA followed by the post-hoc Student-Newman-Keuls test, Fig. S1C} \)). In agreement with our previous study (Wang et al., 2018), the intensity of the IBA-1 staining in the ipsilateral dorsal horn was significantly higher than that in the contralateral dorsal horn. The treatment with IL-10 failed to change the IBA-1 staining in both contralateral and ipsilateral dorsal horns (Fig. S1D–F). In addition, there was no remarkable difference of the GFAP and NeuN staining between contralateral and ipsilateral dorsal horns, and IL-10 treatment failed to change the GFAP and NeuN staining in both sides (Fig. S1G–L).

In accordance with the previous studies (Wu et al., 2017a,b), β-endorphin was highly colocalized with IBA-1 in both the contralateral and ipsilateral dorsal horn under 10× and 40× magnifications (Fig. S1A–C). β-Endorphin was also colocalized with GFAP (Fig. S1G–I).
and NeuN (Fig. 3M–O), but with much lower density. Compared to the saline treatment, intrathecal IL-10 injection (100 ng) markedly increased level of β-endorphin with IBA-1 in both the contralateral and ipsilateral dorsal horn (Fig. 3D–F), but not with GFAP (Fig. 3J–L) or NeuN (Fig. 3P–R). Quantitatively, intrathecal IL-10 significantly increased the double immunofluorescence intensity of β-endorphin with IBA-1 by 200% and 300% in the contralateral and ipsilateral dorsal horn I–V under 10× magnification, respectively (F = 3.627, P = 0.0035 by one-way ANOVA followed by the post hoc Student-Newman-Keuls test; Fig. 3S), but not with GFAP (Fig. 3T) or NeuN (Fig. 3U).

3.3. IL-10 produced mechanical antiallodynia through spinal microglial β-endorphin expression

To explore the role of spinal microglial β-endorphin expression in IL-10-induced mechanical antiallodynia, we applied the β-endorphin neutralizing antibody into these experiments, i.e., three groups of neuropathic rats were first given with an intrathecal injection of 10 μL of blank serum (1:10 dilution) or anti-β-endorphin sera (1:10 dilution), followed by a second intrathecal injection of IL-10 (100 ng) or saline (10 μL) 30 min thereafter. Intrathecal IL-10 injection produced time-dependent mechanical antiallodynia in the ipsilateral hindpaws, whereas treatment with the β-endorphin neutralizing antibody did not significantly alter the baseline withdrawal responses in either hindpaws, but entirely blocked IL-10-induced mechanical antiallodynia in the ipsilateral hindpaws (F = 26.05, P < 0.0001, by repeated measures two-way ANOVA followed by the post hoc Student-Newman-Keuls test; Fig. 4A).

As β-endorphin is an endogenous ligand of μ-opioid receptors (Han, 2003), we further intervened IL-10 antinociception by using the selective opioid receptor antagonists. Two groups of neuropathic rats received an intrathecal injection of 10 μL of normal saline or the specific opioid receptor antagonist naloxone (20 μg), followed by a second intrathecal IL-10 injection (100 ng) 30 min thereafter. Intrathecal naloxone did not significantly change baseline paw withdrawal thresholds in ipsilateral hindpaws, but fully suppressed IL-10-induced mechanical antiallodynia (F = 27.59, P < 0.0001 by repeated-measures two-way ANOVA followed by the post-hoc Student-Newman-Keuls test; Fig. 4B).

Using the same protocol, we further assessed the subtypes of the opioid receptor that are responsible for IL-10 mechanical antiallodynia. We found that pretreatment (30 min earlier) with intrathecal the selective μ-opioid receptor antagonist CTAP (10 μg) (Steinmiller and Young, 2008) did not significantly alter withdrawal responses in either contralateral or ipsilateral hindpaws, but completely attenuated IL-10-induced mechanical antiallodynia (F = 8.153, P < 0.0001, by repeated measures two-way ANOVA followed by the post-hoc Student-Newman-Keuls test; Fig. 4C). In contrast, pretreatment with either intrathecal injection of the κ-opioid receptor antagonist GNTI (50 μg) (Jones and Portoghese, 2000) or δ-opioid receptor...
antagonist naltrindole (5 μg) (Granier et al., 2012) failed to reduce IL-10 mechanical antiallodynia. Furthermore, minocycline and clodronate liposome were applied to neuropathic rats. Minocycline was generally used for microglial inhibition (Tikka et al., 2001), but it also affected other cell types (Moller et al., 2016). Thus the specific microglial depletor clodronate liposome (Asai et al., 2015) was also employed in this study. We have recently tested specific depletory effect of clodronate liposome on spinal microglia (but not astrocytes or neurons) by using immunohistochemistry (Wang et al., 2018). The animals were intrathecally injected with normal saline (10 μL, 2 groups), minocycline (100 μg, 4 h earlier), or clodronate liposome (30 μg, 1 day earlier) followed by a second intrathecal administration of 100 ng of IL-10. The hindpaw withdrawal thresholds to the mechanical stimulus were assessed before and 1 h after IL-10 treatment. As shown in Fig. 5A, intrathecal IL-10 injection produced marked mechanical antiallodynia in ipsilateral hindpaws compared to the saline control. Although intrathecal injection of minocycline and clodronate liposome did not significantly alter baseline mechanical thresholds in both contralateral and ipsilateral hindpaws as reported earlier (Gong et al., 2014c; Fan et al., 2015; Huang et al., 2016), they entirely blocked IL-10-induced mechanical antiallodynia in the ipsilateral hindpaws (F = 4.873/2.68, P < 0.0001, by one-way ANOVA followed by the post-hoc Student-Newman-Keuls test).

Fig. 3. Specific stimulatory effects of interleukin 10 (IL-10), given intrathecally, on spinal microglial expression of β-endorphin expression in neuropathic rats induced by tight ligation of L5/L6 spinal nerves. Neuropathic rats received intrathecal injection of normal saline and IL-10. One hour later the rats were killed and the spinal lumbar enlargements (L3–L5) were obtained. β-Endorphin was double fluorescence immunolabeled with the microglial marker IBA-1 (A–C, J–L), astrocytic marker GFAP (D–F, M–O) and neuronal marker NeuN (G–I, Q–S) in the spinal cord and dorsal horn I–V laminate in both the contralateral and ipsilateral side. Scar bars: 750 μm for A, D, G, J, M and Q; 50 μm for B, C, E, F, H, I, K, L, N, O, R and S. Yellow double labeling of β-endorphin and biomarkers was indicated by arrows. The immunolabeled surface areas of β-endorphin/IBA-1 (S), β-endorphin/GFAP (T) and β-endorphin/NeuN (U) from the indicated spinal dorsal horn laminae I–V were quantified by using the ImageJ computer program. Data are means ± SEM (n = 5–6 per group). *P < 0.05 compared with the saline control group, by one-way ANOVA followed by the post-hoc Student-Newman-Keuls test.
Fig. 4. Blockade effects of intrathecal injection of the specific β-endorphin antiserum (A), naloxone (B), and selective opioid receptor subtype antagonists (C) on interleukin 10 (IL-10)-induced mechanical antiallodynia in L5/L6 spinal nerve ligation-induced neuropathic rats. The specific β-endorphin antiserum, non-selective opioid receptor antagonist naloxone, and selective μ-opioid receptor antagonists CTAP, κ-opioid receptor antagonist GNTI and δ-opioid receptor antagonist naltrindole were intrathecally administrated 0.5 h before spinal IL-10 treatment. The data are presented as means ± SEM (n = 6 per group). *P < 0.05 by two-way repeated measures ANOVA followed by the post-hoc Student-Newman-Keuls test.

Fig. 5. Inhibitory effects of the microglial inhibitor minocycline and specific microglial depletor clodronate liposome, given intrathecally, on spinal interleukin 10 (IL-10) mechanical antinociception (A), and spinal expression of β-endorphin protein (B), POMC mRNA (C), TNF-α mRNA (D), IL-1β mRNA (E) and IL-6 mRNA (F) in neuropathic rats induced by spinal nerve ligation. Minocycline or clodronate liposome was intrathecally injected 4 h or 1 day prior to IL-10 treatment. A single dose of IL-10 was injected intrathecally and mechanical nociceptive behaviors were quantified before and 1 h after injection. Spinal lumbar enlargements were immediately isolated after the completion of the behavioral assessment. The expressions of β-endorphin protein and POMC mRNA, TNF-α mRNA, IL-1β mRNA, and IL-6 mRNA were determined by using the specific fluorescent immunoassay kit and real-time quantitative PCR, respectively. Data are means ± SEM (n = 5–6 per group) *,#P < 0.05 compared with the saline control group or contralateral side and IL-10 treatment group, respectively, by one-way ANOVA followed by the post-hoc Student–Newman–Keuls test.
ANOVAs followed by the post-hoc Student-Newman-Keuls test).

The spinal lumbar enlargements were obtained immediately after the completion of the behavioral assessment and β-endorphin level was assessed using the commercial fluorescent immunoassay kit and qRT-PCR. The baseline β-endorphin level in the spinal homogenates was 39.9 ± 5.6 pg/mg, which was increased by 40% after intrathecal IL-10 injection. Inhibition or depletion of microglial cells by minocycline (Fan et al., 2015; Huang et al., 2016) or clodronate liposome (Tsunekawa et al., 2017) did not significantly affect the baseline β-endorphin expression in the spinal cord or brain. However, minocycline or clodronate liposome dramatically suppressed IL-10-induced β-endorphin overexpression (F = 3.978/2.418, P < 0.027/0.0316, by one-way ANOVA followed by the post-hoc Student-Newman-Keuls test; Fig. 5B).

Moreover, intrathecal IL-10 injection also significantly upregulated POMC mRNA in both contralateral and ipsilateral spinal cords by 90% and 80%, respectively, which were entirely blocked by pretreatment with minocycline (F = 1.705/4.047, P < 0.05 by one-way ANOVA followed by the post-hoc Student-Newman-Keuls test; Fig. 5C) and clodronate liposome (F = 6.13/8.213, P < 0.05 by one-way ANOVA followed by the post-hoc Student-Newman-Keuls test; Fig. 5C).

In addition, spinal nerve ligation dramatically elevated TNF-α, IL-1β and IL-6 expression in the ipsilateral spinal cords by 170% (F = 38.57, P < 0.05; Fig. 5D), 270% (F = 523.6, P < 0.05; Fig. 5E), and 190% (F = 101.9, P < 0.05 by one-way ANOVA followed by the post-hoc Student-Newman-Keuls test; Fig. 5F), respectively, compared to the contralateral spinal cords. Intrathecal IL-10 injection fully inhibited spinal nerve ligation-elevated TNF-α (F = 1.497, P < 0.05), IL-1β (F = 65.54, P < 0.05) and IL-6 (F = 5.92, P < 0.05 by one-way ANOVA followed by the post-hoc Student-Newman-Keuls test) in the ipsilateral spinal cords, although it did not alter the baseline expression of neuroinflammatory cytokines in the contralateral spinal cord (Fig. 5D–F). However, IL-10-inhibited expression of cytokines was not significantly affected by intrathecal minocycline or clodronate liposome injection, as each of them alone entirely blocked spinal nerve ligation-induced overexpression of neuroinflammatory cytokines (Kotter et al., 2001; Fan et al., 2015; Huang et al., 2016).

3.4. IL-10 stimulated spinal microglial β-endorphin but inhibited neuroinflammatory cytokine expression through STAT3 activation

We first confirmed the expression of IL-10R and p-STAT3 in spinal microglial cells by double immunostaining. Specific microglial marker IBA-1 was co-labeled with IL-10R and p-STAT3, respectively, in spinal microglial cells. Both IL-10R and p-STAT3 were colocalized with IBA-1, with a double immunopositive rate of about 100% (Fig. 6A), suggesting that all of spinal microglial cells expressed both IL-10R and p-STAT3.

We then explored the relationship between STAT3 activation and IL-10-stimulated β-endorphin expression since IL-10’s functions are mainly mediated by IL-10 receptors and their downstream transcription factor STAT3 (Weber-Nordt et al., 1996). It is known that IL-10 did not change the expression of total STAT3 in various cells including microglia (Strle et al., 2002; Qin et al., 2006), thus we detected phosphorylated STAT3 only in the current study. In primarily cultured microglial cells, IL-10 (30 ng/mL) incubation for 30 min markedly increased STAT3 phosphorylation by 120% (F = 1.765, P < 0.05 by two-tailed and paired Student t-test; Fig. 6B). We then added the STAT3 activation inhibitor NSC74859 (10, 30, and 100 μM) in the primary microglia cultures 1 h before IL-10 treatment. IL-10 treatment (30 ng/mL) for 2 h markedly increased level of POMC mRNA, whereas NSC74859 did not significantly alter the baseline POMC, but completely inhibited IL-10-induced POMC overexpression (F = 5.042, P < 0.05 by one-way ANOVA followed by the post-hoc Student-Newman-Keuls test; Fig. 6C).

We further confirmed the causality between STAT3 activation and IL-10-inhibited expression of neuroinflammatory cytokines. NSC74859 (100 μM) and IL-10 (30 ng/mL) were incubated with primary microglia 1 h and 0.5 h before LPS (10 ng/mL) stimulation. As shown in Fig. 6D–F, compared with control, LPS stimulation for 2 h dramatically increased level of TNF-α, IL-1β, and IL-6 mRNA by 10-F (F = 79.23, P < 0.05), 77-F (F = 5912, P < 0.05), and 31-fold (F = 18.83, P < 0.05), which were significantly suppressed by pre-incubation with IL-10 by 82% (F = 949, P < 0.05), 72% (F = 104.9, P < 0.05), and 58% (F = 15.34, P < 0.05), respectively. Moreover, NSC74859 pre-treatment did not significantly alter the baseline expression of neuroinflammatory cytokines, but almost fully reversed IL-10-suppressed expression of TNF-α (F = 1001, P < 0.05), IL-1β (F = 29.05, P < 0.05) and IL-6 (F = 36.7, P < 0.05 by one-way ANOVA followed by the post-hoc Student-Newman-Keuls test).

To further confirm the causal role of STAT3 in IL-10-produced mechanical antiallodynia, we measured IL-10-altered spinal STAT3 phosphorylation in neuropathic rats using Western blot. As shown in Fig. 7A, the phosphorylated STAT3 level in the ipsilateral spinal cord was comparable with that of the contralateral spinal cord, consistent with a previous study (Dominguez et al., 2008). Intrathecal IL-10 injection for 1 h significantly increased the phosphorylated STAT3 level by 120% and 130% in the contralateral (F = 6.305, P < 0.05) and ipsilateral side (F = 222.8, P < 0.05 by one-way ANOVA followed by the post-hoc Student-Newman-Keuls test), respectively. We then assessed the blockage effect of NSC74859 on IL-10 mechanical antiallodynia in neuropathic rats. NSC74859 did not change the baseline withdrawal thresholds in either contralateral or ipsilateral hindpaws, but it completely blocked IL-10 mechanical antiallodynia in the ipsilateral hindpaws (F = 15.87, P < 0.05, by repeated measures two-way ANOVA followed by the post-hoc Student-Newman-Keuls test; Fig. 7B).

3.5. IL-10 induced microglial β-endorphin expression via a non-Bcl3 and non-Socs3 mechanism

Bcl3 and Socs3 were main elements responsible for IL-10 to suppress antiinflammatory cytokine expressions (Sabat et al., 2010). To explore whether IL-10 stimulated β-endorphin expression indirectly through the Bcl3 and Socs3 signaling, and whether POMC indirectly participated in IL-10-suppressed expressions of neuroinflammatory cytokines via Bcl3 and Socs3 induction, three pairs of siRNA targeting Bcl3, Socs3, and POMC were applied in the primary microglial culture and neuropathic rats, respectively. We found that siRNA/Bcl3-3, siRNA/Socs3-3, and siRNA/POMC-2 were the best and exhibited 70%, 75% and 62% of silence efficacy, respectively, compared to the nonspecific oligonucleotide controls (Supplementary Fig. S1). Further as shown in Fig. 8A–C, IL-10 but not LPS treatment markedly increased expressions of Bcl3 by 110% (F = 22.79, P < 0.05), Socs3 by 60% (F = 11.11, P < 0.05), and POMC by 90% (F = 3.285, P < 0.05). Pre-transfection with siRNA/Bcl3, siRNA/Socs3 and siRNA/POMC separately blocked IL-10-stimulated expression of Bcl3 (F = 9.133, P < 0.05), Socs3 (F = 3.231, P < 0.05) and POMC (F = 5.329, P < 0.05 by one-way ANOVA followed by the post-hoc Student-Newman-Keuls test). Furthermore, as shown in Fig. 8D–F, LPS treatment remarkably increased expressions of TNF-α by 500%, IL-1β by 170%, and IL-6 by 390%, which were significantly (44–55%) suppressed by IL-10 treatment. Bcl3 silence fully reversed IL-10-suppressed expression of TNF-α (F = 7.124, P < 0.05) and IL-1β (F = 3.525, P < 0.05) although not IL-6, whereas Socs3 silence totally restored IL-10-suppressed expression of IL-1β (F = 3.448, P < 0.05) and IL-6 (F = 2.139, P < 0.05 by one-way ANOVA followed by the post-hoc Student-Newman-Keuls test) although not TNF-α. In contrast, silence of either Bcl3 or Socs3 did not affect IL-10-stimulated POMC expression (Fig. 8C), while POMC silence did not affect IL-10-inhibited expression of TNF-α, IL-1β, and IL-6.

To identify the separate relationship between IL-10-induced anti-neuroinflammation and mechanical antiallodynia, five groups of neuropathic rats received multiple daily intrathecal injections of non-specific oligonucleotides (5 μg/day, the first two groups), siRNA/Bcl3 (5 μg/day), siRNA/Socs3 (5 μg/day) and siRNA/POMC (5 μg/day) for
The withdrawal thresholds to mechanical stimuli were measured daily in both contralateral and ipsilateral paws prior to each siRNA injection during the observation period. As shown in Fig. 9A, multiple daily intrathecal injections of siRNA/POMC time dependently decreased baseline withdrawal threshold in the contralateral paws by 42% on the 6th day during the injections (F = 19.41, P < 0.05, by repeated-measures two-way ANOVA followed by the post hoc Student-Newman-Keuls test) compared with the nonspecific oligonucleotide control, but did not significantly alter the reduced withdrawal responses in the ipsilateral paws presumably due to the ceiling effect. On the seventh day, a single dose of normal saline (10 μL, the first group) or IL-10 (100 ng, the other four groups) was intrathecally injected into rats and the paw withdrawal thresholds were tested 1 h after injection. Intrathecal IL-10 injection in non-specific oligonucleotide control rats produced a significant mechanical antiallodynia in the ipsilateral hindpaws, which was completely blocked by pretreatment with siRNA/
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POMC (F = 5.928, P < 0.05 by one-way ANOVA followed by the post-hoc Student–Newman–Keuls test; Fig. 9B). In contrast, IL-10 mechanical antiallodynia was not obviously affected by multiple-daily intrathecal injections of siRNA/Bcl3 and siRNA/Socs3.

The spinal lumbar enlargements were obtained once the behavioral assessment was completed and expressions of Bcl3, Socs3, POMC, TNF-α, IL-1β, and IL-6 were assessed by qRT-PCR. As exhibited in Fig. 9C–E, intrathecal injection of IL-10 in the nonspecific oligonucleotide-pre-treated rats markedly increased level of Bcl3 (F = 26.98, P < 0.05), Socs3 (F = 12.97, P < 0.05) and POMC (F = 8.487, P < 0.05) mRNA, which were specifically and completely attenuated by intrathecal injection of each respective siRNA (F = 2.241/12.69/3.15, P < 0.05 by one-way ANOVA followed by the post-hoc Student–Newman–Keuls test). Furthermore, compared with the contralateral spinal cord, peripheral nerve injury in the ipsilateral spinal cord markedly stimulated the expressions of TNF-α by 140% (F = 6.333, P < 0.05), IL-1β by 140% (F = 17.61, P < 0.05), and IL-6 by 890% (F = 13.79, P < 0.05), which were fully inhibited by intrathecal IL-10 injection (F = 7.504/5.081/2540 P < 0.05 by one-way ANOVA followed by the post-hoc Student–Newman–Keuls test). In addition, treatment with intrathecal siRNA/Bcl3, siRNA/Socs3, or siRNA/POMC did not significantly alter the baseline expression of TNF-α, IL-1β, or IL-6 in the contralateral spinal cord. But same as the cultured primary microglia study, intrathecal siRNA/Bcl3 injection remarkably reversed IL-10-inhibited TNF-α (F = 9.916, P < 0.05) and IL-1β (F = 2.851, P < 0.05) expression, whereas intrathecal siRNA/Socs3 injection markedly restored IL-10-inhibited expression of IL-6 (F = 7.413, P < 0.05) and IL-6 (F = 3726, P < 0.05) by one-way ANOVA followed by the post-hoc Student–Newman–Keuls test). In contrast, intrathecal siRNA/POMC injection did not significantly affect levels of all three neuroinflammatory cytokines (Fig. 9F–H).

4. Discussion

The current study revealed that spinal IL-10 produced mechanical antiallodynia in neuropathy was through microglial β-endorphin expression, which is supported by the following in vitro and in vivo experiments. 1). Intrathecal IL-10 injection significantly induced β-endorphin mRNA and protein in both contralateral and ipsilateral spinal cords of neuropathy rats. The direct stimulatory effect of IL-10 on β-endorphin expression was confirmed in spinal microglial cells (but not in neurons or astrocytes) by using double fluorescence immunostaining technique. IL-10-upregulated expression of POMC (but not prodynorphin) mRNA and β-endorphin protein in cultured primary microglia, but not astrocytes or neuronal cells; 2). Intrathecal injection of the specific β-endorphin neutralizing antibody, specific opioid receptor antagonist naltrexone, or selective μ-opioid receptor antagonist CTAP totally blocked spinal IL-10-induced mechanical antiallodynia in neuropathic rats. The latter results conflicted with a previous study that systemic naloxone failed to reverse intraperitoneal IL-10-induced antinociception in the hotplate test (Vale et al., 2003), although the reason for this discrepancy is unclear; 3). Intrathecal injection of the microglial inhibitor minocycline (Tikka et al., 2001) and specific microglia depletor clodronate liposome (Asai et al., 2015) fully blocked spinal IL-10-induced mechanical antiallodynia and spinal overexpression of β-endorphin in neuropathic rats. On the other hand, long-term application of β-endorphin induced progressive antinociceptive tolerance (Bhargava, 1981; Fan et al., 2016), whereas IL-10, though through expression of β-endorphin demonstrated in our current study, failed to induce antinociceptive tolerance in a variety of studies (Wagner et al., 1998; Milligan et al., 2006b; Dengler et al., 2014). In addition, the GLP-1 receptor agonists exenatide and shanzhisi methylester produced antinociception through spinal β-endorphin expression, but did not induce analgesia tolerance (Gong et al., 2014b; Gong et al., 2014c; FansP
et al., 2016). The reason for the discrepancy is not completely known, but may be due to that although IL-10-stimulated β-endorphin level is sufficient to mediate antinociception, but may not be highly enough to induce antinociceptive tolerance, as sufficiently high concentrations and durations are required for morphine and β-endorphin to induce antinociceptive tolerance. Indeed, IL-10, exenatide and shanzhiside methylester stimulated β-endorphin expression at a much lower level of approximately 20–60 pg/mg in the spinal cord, which was in sharp contrast to that (approximately 4.7 ng/mg) obtained following intrathecal injection of exogenous β-endorphin at a dose of 1 μg to induce antinociceptive tolerance (Gong et al., 2014c; Fan et al., 2016).

Our current study characterized IL-10 antihypersensitivity in neuropathic rats induced by tight ligation of L5/L6 spinal nerves. Patients experienced peripheral nerve lesions usually exaggerated responses to light touch (mechanical allodynia) and temperature stimuli (thermal hyperalgesia) (LaMotte et al., 1982; Wahren and Torebjork, 1992). Thus both sensory mechanical and thermal tests were employed in this study, although the affective pain tests such as the place aversion test are also needed to perform later on. Intrathecal IL-10 injection did not change the baseline nociceptive responses in the contralateral hindpaws, but dose dependently attenuated mechanical allodynia and thermal hyperalgesia in the ipsilateral paws in both male and female rats, with ED_{50} values of 41 and 24 ng, and E_{max} values of 62% MPE and 100% MPE in male rats. These results indicate that IL-10, independent of gender difference, has a specific blockade effect on pain hypersensitivity but not on acute reflex nociception and exhibit greater sensitivity to thermal hyperalgesia than mechanical allodynia. The differential activity on pain hypersensitivity and acute reflex nociception is supported by the previous findings in which aconitines (Fan et al., 2016; Li et al., 2016) and GLP-1 receptor agonist exenatide and shanzhiside methylester (Gong et al., 2014c; Fan et al., 2016), through stimulation of spinal dynorphin A or β-endorphin, produced antinociception in ipsilateral paws but not contralateral paws in neuropathic rats. The reasons for the inability of IL-10 on acute nociception in the contralateral paws is not completely understood, as it stimulated β-endorphin expression by the same degree from both contralateral and ipsilateral spinal cords of neuropathic rats. However, the observation might be due to that contralateral paws with no central sensitization involved in neuropathic rats have lower sensitivity to exogenous and endogenous opioids. Indeed, morphine was more effective in reducing mechanical withdrawal thresholds in ipsilateral paws than contralateral paws in neuropathic and bone cancer pain rats (Huang et al., 2012; Gong et al., 2014a).

The pivotal role of STAT3 in IL-10-induced antiinflammation was demonstrated in STAT3-deficient mice (Takeda et al., 1999) and other studies (Williams et al., 2004; El Kasmi et al., 2006). Patients with hyper-IgE syndrome, harboring a dominant negative STAT3 mutation, showed a defective IL-10 inhibition of LPS-induced overexpression of...
TNF-α (Minegishi et al., 2007). Indeed, IL-10 treatment in our current study markedly induced STAT3 phosphorylation in primary cultures of microglia. Pretreatment with the specific STAT3 inhibitor NSC74859 almost completely reversed IL-10-suppressed expression of TNF-α, IL-1β, and IL-6. In addition, NSC74859 totally blocked IL-10-stimulated β-endorphin expression, consistent with the previous findings that STAT3 was an effective inducer of POMC expression in COS-7 cell lines (Scully and Rosenfeld, 2002; Bates et al., 2003). Furthermore, intrathecal IL-10 injection into neuropathic rats markedly increased phosphorylated-STAT3 levels in both contralateral and ipsilateral spinal cords, and pretreatment with intrathecal NSC74859 fully blocked spinal IL-10-induced mechanical antiallodynia, mediated by the β-endorphin expression. Our data revealed that STAT3 phosphorylation mediated IL-10-stimulated β-endorphin expression in microglia, in parallel to its inhibition of expression of neuroinflammatory cytokines.

De novo protein synthesis induced by STAT3 activation was essential for the pleiotropic functions of IL-10 (Sabat et al., 2010). Bcl2 expression was responsible for IL-10 antiapoptosis (Levy and Brouet, 1994; Cohen et al., 1997), whereas DUSP1 expression was responsible for IL-10 antiapoptosis (Levy and Brouet, 2005). IL-10-induced antiapoptosis responses in macrophages and monocytes were associated with a variety of STAT3-inducible antiinflammatory elements to subsequently suppress NF-κB activity. These antiinflammatory elements included Socs3 (Berlato et al., 2002), Bcl3 (Kuvata et al., 2003), Abln3 (Weaver et al., 2007), Etv3 (El Kasmi et al., 2007), DUSP1 (Hammer et al., 2005), and Ihbs (Hirota et al., 2005). In this study, Bcl3- or Socs3-deficient microglial cells showed a defective IL-10-mediated suppression of LPS-induced overexpression of TNF-α and IL-1β or IL-1β and IL-6 presumably due to reduction of NF-κB activation, consistent with the results in macrophages and monocytes (Williams et al., 2004). However, Bcl3 and Socs3 silence did not significantly affect IL-10-stimulated POMC expression in microglia. Similarly, POMC silence only blocked IL-10-stimulated POMC expression, but had no effects on reversing IL-10 inhibition of LPS-stimulated TNF-α, IL-1β, or IL-6 expression. In agreement with the cultured primary microglial cells, multiple daily intrathecal transfection with siRNA/Bcl3 and siRNA/Socs3, but not siRNA/POMC, nearly completely reversed IL-10-induced inhibition of spinal expression of TNF-α, IL-1β, and IL-6 but not mechanical antiallodynia. In contrast, siRNA/POMC, but not siRNA/Bcl3 or siRNA/Socs3, totally blocked IL-10-increased spinal expression of POMC and mechanical antiallodynia. These results suggested that IL-10/STAT3 transcriptionally specifically bound to the promoters of POMC, Bcl3 and Socs3 in parallel to induce expression of β-endorphin and antiinflammatory elements, and that the expressed β-endorphin did not interfere the IKKs activity, leading to a completely separation between IL-10-induced antinociception mediated by the β-endorphin expression and antineuroinflammation mediated by Bcl3 and Socs3 and subsequent inhibition of expression of neuroinflammatory cytokines (Fig. 10). The separation of antinociception and neuroinflammation is also supported by the previous and current findings that minocycline and clodronate liposome did not affect mechanical allodynia in established neuropathy although they completely ameliorated overexpression of neuroinflammatory cytokines in the ipsilateral spinal cords (Rutkowski et al., 2006; Kotter et al., 2001; Fan et al., 2015; Huang et al., 2016), and that the IL-1 receptor antagonist (Swietzer et al., 2001) and TNF-α inhibitor etanercept (Schafer et al., 2003; Marchand et al., 2009) failed to reverse established neuropathic pain.

There are several features of the spinal microglial IL-10/β-endorphin pathway to produce analgesia or hypoalgesia in the body, i.e., 1. The IL-10/β-endorphin pathway is an efficacious endogenous system in controlling pain transmission and transduction. The notion is supported by the facts that multiple daily injections of siRNA/IL-10 receptor-α (Wu et al., 2017b) and siRNA/POMC (present study) produced marked (approximately 40% reduction) mechanical allodynia in the contralateral hindpaws of the neuropathic rats. The latter results was consistent with a previous study in which POMC-deficient mice were even more sensitive to stress-related analgesia (Rubinstein et al., 1996), although they were in conflict with that the single intrathecal injection of the β-endorphin neutralizing antibody had no inhibitory effect on the mechanical thresholds in either contralateral or ipsilateral hindpaws. The reason for the conflicting finding is not clear, but may be due to that POMC was also the precursor of other bioactive peptides including adrenocorticotropic, melanotropins and lipotropins, in addition to endorphins (Smith and Funder, 1988; 2). The IL-10/β-endorphin pathway is activated regardless of whether microglia is in activation or rest states, as we demonstrated that, in contrast to the expression of neuroinflammatory cytokines, IL-10 stimulated β-endorphin expression by nearly the same degree in both contralateral and ipsilateral spinal cords from neuropathic rats. This characterization is supported by the previous findings that the GLP-1 receptor agonists, acotinoids, and cyamandelone A stimulated microglia to express β-endorphin and dynorphin A by the same degrees in primary cultures of microglia in the presence and absence of LPS, and in both contralateral and ipsilateral spinal cords of neuropathic rats (Gong et al., 2014c; Fan et al., 2015; Huang et al., 2016, 2017a; Li et al., 2016); and 3. The IL-10/β-endorphin...
pathway is likely implicated with many physiologically- and pharmacologically-involved hypoalgesic and analgesic conditions. Indeed, the GLP-1 receptor activation has been demonstrated to produce anti-
nociception in a variety of rodent models of pain hypersensitivity (Gong et al., 2014c; Fan et al., 2015) and the autocrine microbial IL-10/β-
endorphin pathway mediated its antinociception (Wu et al., 2017b). In addition, the up-regulation of the IL-10 and/or β-endorphin expression has been reported to mediate hypoalgesia in infants (McKelvev et al., 2015) and physical activity on analgesia (Goldfarb and Jamurtas, 1997; Gleeson, 2007). The pathway may also be involved in therapies for the treatment of chronic pain in the human or in experimental animals, such as the placebo effect on pain (Levine et al., 1978), acnepruritic or spinal cord injury-mediated analgesia (Zijlstra et al., 2003; Lin and Chen, 2008), and application of analgesics including the GPR40 agonists (Nakamoto et al., 2013), shanzhiside methyl ester (Zeng et al., 2015; Fan et al., 2016) and cyanidine A (Huang et al., 2016).

Acknowledgements

This study was supported in part by grants from the National Natural Science Foundation of China (#81374000 and #81673403) and the Shanghai Industrial Translational Project (#15401901300).

Author contributions

Conceived and designed the experiments: YXW and HYW; performed the experiments: HYW, KFM, XQT, AU, EA, HL and XYL; analyzed the data: HYW and YXW; and prepared the paper: YXW and HYW.

Conflict of interest statements

The authors declare that there are no competing interests in this work.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.bbi.2018.06.015.

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