Intrathecal polymer-based interleukin-10^{*} gene delivery for neuropathic pain

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Research on communication between glia and neurons has increased in the past decade. The onset of neuropathic pain, a major clinical problem that is not resolved by available therapeutics, involves activation of spinal cord glia through the release of proinflammatory cytokines in acute animal models of neuropathic pain. Here, we demonstrate for the first time that the spinal action of the proinflammatory cytokine, interleukin 1 (IL-1) is involved in maintaining persistent (2 months) allodynia induced by chronic-constriction injury (CCI). The anti-inflammatory cytokine IL-10 can suppress proinflammatory cytokines and spinal cord glial amplification of pain. Given that IL-1 is a key mediator of neuropathic pain, developing a clinically viable means of long-term delivery of IL-10 to the spinal cord is desirable. High doses of intrathecal IL-10-gene therapy using naked plasmid DNA (free pDNA-IL-10) is effective, but the dose required limits its potential clinical utility. Here we show that intrathecal gene therapy for neuropathic pain is improved sufficiently using two, distinct synthetic polymers, poly(lactico-glycolic) and polyethylenimine, that substantially lower doses of pDNA-IL-10 are effective. In conclusion, synthetic polymers used as i.t. gene-delivery systems are well-tolerated and improve the long-duration efficacy of pDNA-IL-10 gene therapy.

Keywords: Glia, rats, allodynia, chronic constriction injury, neuropathic pain, polymer plasmid-DNA, gene therapy

INTRODUCTION

Microglia and astrocytes (glia) are important cell types that actively modulate neuronal function. Research in the past decade implicates glia in dorsal spinal cord as key players in the creation and maintenance of enhanced pain states such as neuropathic pain (DeLeo and Colburn, 1999; Watkins *et al.*, 2001; Ledeboer *et al.*, 2005). This recently recognized role of glia as powerful modulators of pain has major implications for drug development because neuropathic pain is resistant to currently available therapeutics that target neurons. Thus, new pharmacological approaches to control neuropathic pain are being developed that target spinal glial function (Watkins and Maier, 2003).

In response to either peripheral nerve inflammation or trauma that produces neuropathic pain, spinal cord glia become activated and release several neuroexcitatory

substances including the proinflammatory cytokines interleukin-1 (IL-1), tumor necrosis factor- α (TNF) and IL-6 (Watkins and Maier, 2004). Neurons and glia in the spinal cord contain receptors for these proinflammatory cytokines (Dame and Juul, 2000; Holmes *et al.*, 2004; Ohtori *et al.*, 2004). Proinflammatory cytokines, in turn, stimulate a cascade of events starting with the release of neuroexcitatory substances (Samad *et al.*, 2001; Viviani *et al.*, 2003; Samad *et al.*, 2004) that mediate enhanced pain (Marchand *et al.*, 2005; McMahon *et al.*, 2005; Tsuda *et al.*, 2005; De Leo *et al.*, 2006; Watkins *et al.*, 2006).

Although much evidence demonstrates that proinflammatory cytokines are crucial for the initiation and early phases (up to 2 weeks) of neuropathic pain in animal models (Watkins *et al.*, 2006), it is unknown whether neuropathic pain that has persisted for months also involves IL-1. Considering the proinflammatory cytokines, most evidence supports IL-1 as a crucial contributor to chronic neuropathological conditions (Allan *et al.*, 2005), in particular after neuropathic pain has persisted for 10 days (Milligan *et al.*,

^{*}This gene has a point mutation (F129S) as described in the Methods section.

2005a). Thus, in the studies described here we examine whether blocking the actions of IL-1 in spinal cord is effective at resolving neuropathic pain of prolonged duration (lasting several months). No other report has examined the role of IL-1 in a model of protracted neuropathic pain.

Given that proinflammatory cytokines are implicated strongly in neuropathic pain, previous research has explored a potential therapeutic role for anti-inflammatory cytokines in treating this condition. The production of proinflammatory cytokines by astrocytes and microglia is subject to targeted negative-feedback suppression by anti-inflammatory cytokines such as IL-10 (Moore *et al.*, 2001). Astrocytes and microglia express receptors for IL-10 whereas spinal cord neurons do not (Ledeboer, 2002; Mizuno *et al.*, 1994), which indicates that IL-10 might affect the proinflammatory functions of activated glia but have no direct effect on spinal neurons. Previously we have demonstrated that a bolus intrathecal (i.t.) injection of either IL-10 protein or i.t. viral vectors encoding IL-10 transiently reverses (from 2 hours to 2 weeks) 10-day, CCI-induced neuropathic pain (Milligan *et al.*, 2005a; Milligan *et al.*, 2005b). Furthermore, i.t. administration of free plasmid DNA (i.e. no viral vector) that encodes IL-10 (pDNA-IL-10) produces prolonged (~40 days) reversal in several neuropathic pain models in which early neuropathic pain is characterized to be mediated by IL-1 (Milligan *et al.*, 2005a; Milligan *et al.*, 2006; Ledeboer *et al.*, 2007). The dose required to achieve prolonged pain control in rats is high (100–200 µg per rat) (Sloane *et al.*, 2004) because of inefficient uptake of pDNA by cells (Sebestyen and Wolff, 1999; Kaneda, 2005). The high dose might limit the potential clinical application of pDNA-IL-10. Translating the therapy from laboratory animals to humans might require ~290 mg pDNA-IL-10 for a person of 81 kg (180 pounds). Thus, both the duration and dose of the gene therapy might limit clinical applicability.

Many non-viral gene-delivery materials, both synthetic and naturally occurring, are being developed to improve gene transfer for clinical utility (Huang *et al.*, 1999; Amiji, 2005). Compared to other materials that are being explored, synthetic polymers are promising because they are safer, more flexible in manipulating their chemistry, and easier to manufacture (Pack *et al.*, 2005). Several factors to be considered when designing a polymer for gene delivery include packaging and extracellular protection of pDNA, cellular uptake, endosomal escape and pDNA survival for nuclear transport. The polymers must also be non-immunogenic.

Polyethylenimine (PEI) is an effective gene-delivery polymer, with several reports demonstrating successful gene transfer to a variety of tissues (Huang *et al.*, 1999). PEI has a high percentage of positively charged nitrogen amines which complex readily with negatively charged pDNA. The success of PEI–pDNA complexes for gene delivery is thought to result from their efficient escape from the endosomal compartment. The underlying mechanism is based on a property of PEI that induces high ion influx into the endosome, which leads to swelling and, finally, rupture (Pack *et al.*, 2005). Indeed, several groups have examined PEI in combination with other polymer formulations (e.g. polyethylene glycol), resulting in highly efficient gene transfer (Pack *et al.*, 2005). Reports have shown that combining pDNA with cationic agents such as PEI leads to an improvement in gene expression while decreasing the

required dose by one-half to one-tenth (Meuli-Simmen *et al.*, 1999; Shi *et al.*, 2003). Moreover, PEI-complexed to pDNA in the CNS leads to minimal activation of glia (Boussif *et al.*, 1995; Abdallah *et al.*, 1996). Given these properties, we sought to determine whether the application of PEI to pDNA-IL-10 might provide a platform for further polymer development to improve the efficacy of pDNA-IL-10 gene delivery.

Other polymers such as poly(lactic-co-glycolic) (PLGA), which is a copolymer of lactic and glycolic acid, are approved by the US Food and Drug Administration and have a history of successful clinical and commercial applications when manufactured as microparticles for the slow release of peptides and proteins (Hedley, 2003). Microparticles prepared from PLGA take advantage of the body's innate immune defense system for gene uptake, which is one of the major advantages of using such polymers for gene delivery. This is particularly attractive for i.t. delivery because the meninges contain abundant, effective, phagocytic immune cells such as dendritic cells and macrophages (McMenamin *et al.*, 2003). These cell types readily phagocytose PLGA microparticles while remaining non-toxic to cells in the meninges (Ayhan *et al.*, 2002). Encapsulating pDNA within PLGA to create PLGA microparticles preserves the structural integrity and biological function of pDNA by protecting it from extracellular and intracellular degradative enzymes. Indeed, the inefficiency of free DNA delivery is caused mainly by degradation by extra- and intra-cellular enzymes (Sebestyen and Wolff, 1999; Kaneda, 2005). Several *in vivo* studies show that PLGA and its degradation products are safe and well tolerated (Visscher *et al.*, 1985). Intrathecal PLGA microparticles that release proteins are non-toxic acutely and after 35 days (Sendil *et al.*, 2003; Lagrace *et al.*, 2005a; Lagrace *et al.*, 2005b). However, the use of PLGA formulations in combination with spinal cord gene therapy to control long-term neuropathic pain has not been documented previously.

Chronic-constriction injury (CCI) is a model for examining novel approaches for controlling neuropathic pain because stable, prolonged, pain facilitation is observed for up to 3 months (Bennett and Xie, 1988; Milligan *et al.*, 2006). Previously, we have documented that both chemotherapeutic (paclitaxel) and CCI-induced neuropathic pain are reversed transiently by i.t. treatment with an IL-1-receptor antagonist (IL-1-ra) shortly after the onset of pain (Milligan *et al.*, 2005a; Ledeboer *et al.*, 2007). However, the early involvement of IL-1 does not indicate its long-term importance. Here, we have examined whether spinal IL-1 mediates CCI-induced neuropathic pain (light touch mechanical allodynia) of a long duration (2 months). This is important because although reports document that IL-10 reverses CCI-induced allodynia, which supports a general role for proinflammatory cytokines in long-duration neuropathic pain, a specific role for IL-1 has not been addressed to date. Given that IL-10 has effects in addition to inhibiting proinflammatory cytokines (e.g. inhibition of some chemokines and nitric oxide production), we wanted to examine whether the i.t. IL-10-mediated reversal of CCI-induced neuropathic pain of long duration observed previously, also involves inhibition of the effects of IL-1.

The involvement of proinflammatory cytokines in neuropathic pain has led to the exploration of anti-inflammatory cytokines such as IL-10 as therapeutic agents

for the control of chronic neuropathic pain (Watkins and Maier, 2004). Although endogenous anti-inflammatory factors such as IL-1-ra might be examined, IL-10 is the preferred candidate because it suppresses the actions of many proinflammatory factors including IL-1, TNF and IL-6 that mediate pathological pain (Watkins and Maier, 1999). More recently, we have reported that the chemotherapeutic, paclitaxel, creates long-duration allodynia and increases IL-1 and TNF in dorsal root ganglia (DRG) (Ledeboer *et al.*, 2007). In this study, pain reversal after i.t. treatment with pDNA-IL-10 is caused by IL-10-mediated decreases in IL-1 and TNF gene transcription. Gene-delivered IL-10 has been identified in spinal cerebrospinal fluid (CSF), lumbar spinal meninges and the related DRG at 2 and 18 days after i.t. IL-10 gene therapy (Milligan *et al.*, 2006; Ledeboer *et al.*, 2007). Additionally, dose-response analysis reveals that two sequential i.t. injections of pDNA-IL-10 leads to moderate–full reversal of pain (Sloane *et al.*, 2004; Milligan *et al.*, 2006; Ledeboer *et al.*, 2007), although the duration of pain reversal is dose dependent (Sloane *et al.*, manuscript in preparation). Here, we examined whether two synthetic polymers with different delivery mechanisms (PEI and PLGA) might decrease the amount of pDNA-IL-10 needed for i.t. IL-10 gene delivery. PEI tends to aggregate in aqueous solutions, which leads to decreased gene expression, and has moderate toxic effects in the body (Huang *et al.*, 1999; Sharma *et al.*, 2005), hence we characterized pDNA delivery from PLGA polymer microparticles further *in vitro* and *in vivo* for possible clinical utility. Specifically, we examined whether (a) the dose of untreated, i.t. free pDNA might be reduced substantially with minimal loss of therapeutic efficacy for neuropathic pain control, (b) the properties of PLGA *in vitro* and *in vivo* do not alter pDNA-IL-10 gene integrity, and (c) i.t. PLGA microencapsulated pDNA-IL-10 gene delivery causes long-term reversal of neuropathic pain in rats.

OBJECTIVES

Here, we have examined whether spinal IL-1 mediates CCI-induced neuropathic pain of long duration. IL-10 reverses CCI-induced allodynia, which supports a general role for proinflammatory cytokines in long-duration neuropathic pain. However, IL-10 has effects in addition to inhibiting proinflammatory cytokines, thus we have examined whether IL-10-mediated reversal of CCI-induced neuropathic pain of long-duration observed previously involves inhibition of IL-1 activity.

In addition, we examined whether using synthetic polymers (PEI and PLGA) might decrease the amount of pDNA-IL-10 needed for i.t. IL-10 gene delivery. Specifically, we examined whether (a) conjugation to polymer might reduce the dose of untreated, i.t. free pDNA needed for therapeutic efficacy, (b) PLGA-microencapsulation maintains pDNA-IL-10 integrity *in vitro* and *in vivo*, and (c) i.t. PLGA-microencapsulated pDNA-IL-10 gene delivery causes long-term reversal of neuropathic pain in rats.

METHODS

Animals

Pathogen-free, adult, male Sprague-Dawley rats were used in all experiments. Rats (350–375 g at the time of arrival; Harlan

Labs) were housed in temperature ($23 \pm 3^\circ\text{C}$) and light-controlled rooms (12:12 light:dark; lights on at 07.00 hr) with standard rodent chow and water available *ad libitum*. Behavioral testing was performed during the first 6 hours of the light cycle. All procedures were approved by the Institutional Animal Care and Use Committee at the University of Colorado at Boulder.

Surgery microinjections and drugs

CCI was performed under isoflurane anesthesia (1.5–2.0% vol. in oxygen) by loosely tying four chromic gut sutures around the sciatic nerve in the left hindleg at mid-thigh level, as described previously (Bennett and Xie, 1988). The sciatic nerves of sham-operated rats were identically exposed but not ligated.

Intrathecal (i.t.; sub-dural, peri-spinal) injections were used in all experiments and took 2–3 minutes to complete. An acute catheter-application method was employed under brief isoflurane anesthesia (5.0% vol. in oxygen), as described previously (Milligan *et al.*, 2005a), to inject compounds at the level of the lumbosacral enlargement. No abnormal motor behaviors were observed following injection.

The injection protocol in experiments described here are as follows. Two sequential, identical i.t. injections, 3-days apart, were used to examine the efficacy of: (1) 100 μg and 1 μg free pDNA-IL-10, because the 1 μg dose had not been used previously ($n = 3/\text{group}$); (2) PEI-treated pDNA-IL-10 (5 μg pDNA) ($n = 4\text{--}5/\text{group}$); and (3) PLGA-microencapsulated pDNA (PLGA-pDNA; 400 ng pDNA; $n = 4\text{--}6/\text{group}$). Single injections were used to compare pDNA efficacy following the double-injection procedures described above in experiments with (1) PEI-treated pDNA-IL-10 (5 μg pDNA; $n = 5/\text{group}$), and (2) pDNA-IL-10 (25 μg) co-infused with PLGA-microencapsulated pDNA-IL-10 (400 ng) versus PLGA-microencapsulated pDNA-IL-10 (400 ng) alone ($n = 3/\text{group}$).

Either endotoxin-free IL-1 receptor antagonist (IL-1ra; 100 μg μL^{-1} ; Amgen) or vehicle (Amgen) was injected ($n = 5\text{--}6/\text{group}$) once i.t. in 1 μL (Milligan *et al.*, 2001; Milligan *et al.*, 2003) followed by an 8 μL sterile-saline flush to ensure complete drug delivery.

Plasmid vectors

Plasmid vectors used in these studies were amplified as described previously (Milligan *et al.*, 2005b) and purified using an endotoxin-free Qiagen plasmid Giga purification kit. The transcriptional cassette of this plasmid, which has been described in detail previously (Milligan *et al.*, 2006), contains the cytomegalovirus enhancer/chicken β -actin promoter (CMV enhancer/CB-actin), which directs expression of either the rat IL-10 gene with a point mutation (F129S) and the SV40 polyadenylation signal region (pDNA-IL-10), or jellyfish green fluorescent protein (pDNA-GFP) used as a pDNA injection control.

Lipopolysaccharide (LPS), also known as endotoxin, is the cell membrane component of Gram-negative bacteria such as *E. coli* that is released into the liquid media during large-scale amplification of plasmid DNA. Co-purification of LPS with pDNA is possible using Qiagen plasmid purification

protocols that, typically, yield highly pure pDNA. Thus, the levels of LPS from each plasmid DNA purification process were determined by the photometric *Limulus* amoebocyte lysate assay according to the manufacturer's instructions (BioWhittaker Inc.). Random sampling of Qiagen-purified plasmids used in these studies revealed endotoxin levels of ~ 0.1 – 0.01 endotoxin units (EU) per μg DNA (0.050 – 0.005 ng LPS μg pDNA $^{-1}$).

All plasmid constructs were suspended in sterile Dulbecco's phosphate-buffered saline (DPBS, 0.1 μm pore-filtered, pH 7.2, #14190-144; Gibco) with 3% sucrose (DPBS-3%). The DPBS-3% vehicle was prepared using molecular biology grade D (+)-sucrose (β -D-Fructofuranosyl- α -D-glucopyranoside; Sigma-Aldrich) in DPBS, 0.2 μm sterile-filtered (pyrogen-free syringe filter unit, #25AS020AS, Life Science Products, Inc.) and stored in sterile, 50 ml conical tubes at 4°C until used.

After determining the concentration of pDNA using 260 nm adsorption, aliquots of ≥ 5 μg μl^{-1} and at a volume that allowed for two 100 μg pDNA injections, were stored at -20°C . The purity of the pDNA was determined by 260:280:320 nm adsorption. The DNA:RNA ratio (260:280) ranged from 1.95 to 2.0. Adsorption at 320 nm was examined for the presence of other impurities, which was minimal (optical density < 0.05).

Preparation of PEI-pDNA complexes

Plasmid DNA was subcloned, purified (as described above) and suspended at ≥ 5 μg μl^{-1} in DPBS-3% sucrose and stored at -80°C . Polyethylenimine (MW 25 kDa; Sigma-Aldrich) aqueous stock solution (0.1 M) was made in DPBS and stored in a sterile, 50 ml conical tube at room temperature until use. Thus, two solutions were prepared, (1) stock pDNA and (2) stock PEI, and stored appropriately before mixing. Procedures and calculations to complex PEI to pDNA were based on the following properties: 1 μl of 0.1 M PEI contains 100 nmol nitrogen amines and 1 μg of DNA contains 3 nmol phosphates (Boussif *et al.*, 1995; Abdallah *et al.*, 1996). The charge ratio of nitrogen amines contributed by PEI to phosphates contributed by DNA, previously reported to be non-toxic (Boussif *et al.*, 1995; Abdallah *et al.*, 1996), was examined at both 9:1 and 15:1 charge ratios (PEI:DNA). Based on pilot studies, no experimental behavioral differences were observed between ratios 9:1 and 15:1. Thus, we chose the 15:1 PEI:DNA charge ratio and a dose of pDNA shown previously to be within the effective range for enhanced gene expression after transfection (Boussif *et al.*, 1995) and i.t. injection (Shi *et al.*, 2003). The complex of PEI-pDNA solution was based on previously described methods (Boussif *et al.*, 1995) with the following changes. On the day of i.t. injection, stock pDNA was thawed on ice and both stock pDNA and stock PEI were diluted with DPBS-3% sucrose to an appropriate concentration to complete the final step. Thus, appropriate concentrations were such that the diluted PEI solution, added slowly and drop-wise to the diluted pDNA solution, resulted a final solution containing a complex of 5 μg pDNA/PEI with a 15:1 charge ratio in a final i.t. injection volume of 20 μl . The resultant PEI-pDNA (containing 5 μg pDNA per 20 μl i.t. injection) solution was inverted gently 5–6 times followed by a short incubation (10 min at room temperature) before i.t. injection. The control (equimolar PEI in DPBS-3% sucrose

with pDNA-GFP or without pDNA) was made fresh on the day of each i.t. injection ($n = 4$ – 5 /group).

Preparation of PLGA microparticles encapsulating pDNA

Microparticles were prepared as described previously (Tinsley-Brown *et al.*, 2000) using a double emulsion/solvent evaporation protocol. Briefly, pDNA was dissolved in deionized water at a concentration of 1 mg ml^{-1} . 300 mg of 50:50 PLGA copolymer (MW 75000) were dissolved in 2 ml of methylene chloride. The aqueous solution of pDNA (100 μl) was added to the polymer solution and the resulting solution sonicated for 10 sec to produce a homogeneous emulsion. Aqueous 1% polyvinyl alcohol (PVA, 25000 MW) solution (1 ml) was added drop-wise to the mixture and the emulsion sonicated for an additional 10 seconds. This double emulsion was poured into a beaker containing 100 ml of aqueous 0.3% PVA and stirred at high speed for 3 hours as the solvent evaporated and microparticles hardened. The microparticles were collected by centrifugation at 4000 rpm for 10 minutes and rinsed three times in deionized water. After the final rinse, the microparticles were resuspended in 5 ml deionized water, and rapidly frozen and lyophilized for > 24 hours.

Scanning electron microscopy was used to examine the morphology of microparticles, and size distribution was determined using a particle-size analyzer. Microparticles were 0.5 ± 0.3 μm diameter (data not shown), which is an appropriate size to be phagocytosed by immune cells such as macrophages and dendritic cells (Hedley, 2005; Meng and Butterfield, 2005). All microparticles were spherical and smooth. pDNA-loaded microparticles were slightly negative in charge (data not shown), indicated by Zeta potential measurements (the charge that develops at the interface between a solid surface and its liquid medium measured in millivolts (Brookhaven Instruments Corp.)). These characteristics are consistent with values reported in the literature (Hedley, 2005) and likely to be caused by the combined effect of pDNA and residual PVA associated with the surface (Sahoo, 2002). To assess loading of pDNA in microparticles, the polymer was dissolved with chloroform and pDNA extracted into tris-acetate-EDTA (TAE) buffer. pDNA concentration was determined by absorption at 260 nm. Microparticles used in the behavioral studies described below ($n = 4$ – 6 /group) were loaded with 1.0 ± 0.4 μg of pDNA mg^{-1} . PLGA-microparticle encapsulation (PLGA-pDNA-IL-10) efficiency was 74%. PLGA-pDNA-IL-10 was suspended at 20 mg ml^{-1} in DPBS immediately before i.t. injection. Each injection consisted of 400 ng pDNA encapsulated in 400 μg PLGA microparticles and delivered in 20 μl .

Release kinetics and stability of pDNA from PLGA microparticles

The release rate of an encapsulant (such as pDNA) from a microparticle can be controlled by changing the degradation kinetics of the polymer. Plasmid DNA-releasing microparticles were placed in phosphate buffered saline at 37°C . At regular time intervals, microparticles were pelleted by centrifugation, the PBS solution removed and microparticles re-suspended in fresh PBS. Content of pDNA in supernatants was assessed using a PicoGreen Assay (Invitrogen) according

to the manufacturer's instructions. Release kinetics were assessed from microparticles prepared with 75000 MW PLGA, and were similar to those reported in the literature (Tinsley-Brown *et al.*, 2000). Plasmid-DNA loading was $1.0 \pm 0.4 \mu\text{g mg}^{-1}$.

The stability of pDNA following the encapsulation protocol was examined by agarose gel electrophoresis. The PLGA microparticles were dissolved in chloroform and pDNA extracted into TAE buffer. To evaluate plasmid integrity, either naïve or extracted pDNA (200 ng) from PLGA microparticles was added to each well of a 1.0% agarose gel (Fisher Scientific), the gel was run at 75 V for 2.0 hours and imaged with UV trans-illumination at 305 nm.

IL-10 protein production in cell culture from pDNA released from PLGA microparticles

To verify that the IL-10 gene is biologically active following the pDNA-IL-10 encapsulation process, 2 μg of pDNA-IL-10 were extracted from microparticles, as described above. IL-10 gene expression was examined in human embryonic kidney 293 (HEK293) cells grown in Dulbecco's modified Eagle's medium (Gibco-BRL) supplemented with 5% fetal calf serum (Hyclone), 2 mM L-glutamine, 100 U ml^{-1} penicillin G sodium and 100 U ml^{-1} streptomycin sulfate in a 37°C humidified incubator with 5% CO_2 . Cells were transfected with 10 μg carrier pDNA (without the IL-10 gene) and 2 μg of either naïve, unprocessed carrier pDNA, pDNA-IL-10 or pDNA-IL-10 extracted from microparticles using a calcium phosphate transfection method (Sambrook *et al.*, 1989). Supernatants were collected and assayed in duplicate 48 hours post-transfection by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (R&D Systems).

Behavioral assessment of allodynia

Normally animals do not respond to non-noxious, light mechanical touch. However, in some cases after the development of neuropathy, normally non-noxious stimuli, such as light mechanical touch, elicit behavioral responses that are similar to those from noxious stimuli. This heightened sensitivity to light mechanical touch is referred to as allodynia. We and others have demonstrated previously that bilateral allodynia develops after neuropathy produced by CCI (Paulson *et al.*, 2000; Paulson *et al.*, 2002; Milligan *et al.*, 2005a; Milligan *et al.*, 2005b). Behavioral testing was performed within the sciatic and saphenous innervation area of the hind paws as described previously. Briefly, a series of calibrated Semmes-Weinstein monofilament fibers (von Frey hairs; Stoelting) were applied to the left and right hind paws to elicit paw-withdrawal responses (Milligan *et al.*, 2000). The range of monofilaments used in these experiments was 0.407–15.136 gm bending force. Assessments were made before (baseline; BL) and at days 3 and 10 after CCI surgery, and at indicated times after i.t. injections (see Figs). The behavioral response pattern, described in detail previously (Milligan *et al.*, 2000), was indicated by the number of non-withdrawal and withdrawal responses, which were used to calculate the 50% paw-withdrawal threshold (absolute threshold) by fitting a Gaussian integral psychometric function using a maximum-likelihood fitting method (Harvey, 1986). This

fitting method allows parametric statistical analyses. Data are presented as both the 50% paw withdrawal threshold (gm) and the $\log_{10}$ transformation of that value as $\log_{10}$ (milligrams $\times 10$). Because of the protracted nature of these experiments we used the fewest possible animals in each experimental condition to achieve meaningful statistical significance between groups. A pilot was conducted with experimental groups determined by the tester ($n = 1/\text{group}$ for experiments with PEI and PLGA) before setting up complete, prolonged 70-day experiments. During all other behavioral assessments for allodynia, the tester was blind with respect to the treatment groups. After each testing trial on each day, treatment groups were determined to avoid unnecessary, prolonged testing and discomfort to the animal. *A priori*, single-injection treatment groups were tested alongside double-injection treatment groups throughout the experimental period (to day 70 post-CCI) irrespective of the state of pain reversal.

CSF collection and analysis

Surgically naïve rats received two i.t. injections, 3-days apart, of a suspension of either PLGA-pDNA-IL-10 or PLGA-pDNA-GFP (as a control) (~ 400 ng pDNA/400 μg PLGA in 20 μl ; total dose ~ 800 ng pDNA). At 4 days after the initial i.t. injection rats were overdosed with sodium pentobarbital (Abbot Laboratories). Cervical ($n = 2\text{--}3/\text{group}$) and lumbosacral CSF ($n = 3/\text{group}$) was collected into 0.6 ml centrifuge tubes before sacrifice, as described previously (Milligan *et al.*, 2000; Milligan *et al.*, 2001), and frozen immediately in dry ice and stored at -80°C until analyzed by ELISA to detect IL-10 (R&D Systems). These initial studies required the entire CSF sample collected for IL-10 protein analysis.

Tissue preparation and microscopic examination of PLGA microparticles in spinal meninges

The spinal cord parenchyma is surrounded by the meninges, which includes the subarachnoid membrane and space. The pia and the subarachnoid space, which is filled with CSF, contains a variety of immune cells, including dendritic cells and macrophages (McMenamin *et al.*, 2003). To verify that microparticles are present within the meninges, PLGA-pDNA-IL-10 with rhodamine dextran (for visualization purposes) was injected into the subarachnoid space ($n = 4$). At 16 days after the initial injection, animals were sacrificed and the spinal cord with intact lumbar 4–6 DRG were dissected and fixed in a 4/30% paraformaldehyde/sucrose solution. Tissues were sectioned with a cryostat (30 μm). Tissue structure was assessed by co-staining cells with a nuclear counterstain (Sytox Green, Molecular Probes). The location of rhodamine dextran-containing microparticles was then examined by confocal microscopy using a Zeiss Pascal LSM microscope (Excitation/Emission: 543/560). Confocal images were acquired with 40 $\times$ Plan NeoFluor (1.3) oil-immersion objective. Sytox Green was excited with the 488 line of an argon laser and a 520 long-pass emission filter.

Data analysis

All statistical comparisons were computed using Statview 5.0.1 for Macintosh. Baseline measures for the von Frey were

analyzed by one-way ANOVA. Time-course measures for behavioral assessments were analyzed by repeated measures ANOVA followed by Fisher's protected least significant difference post hoc comparisons, where appropriate. Cervical and lumbosacral CSF IL-10 content was analyzed by two-way ANOVA, as appropriate, followed by Fisher's protected least significant difference post hoc comparisons. Statistical significance was defined as $P < 0.05$.

RESULTS

IL-1 mediates long-term CCI-induced mechanical allodynia

For the first time, we demonstrate that the spinal actions of IL-1 have a role in extending the duration of neuropathic pain. Hind-paw responses to low-threshold mechanical stimuli were assessed after a single i.t. injection of IL-1ra 8 weeks (56 days) after initiation of CCI. Hind-paw responses to the von Frey test were assessed at BL and after CCI (Fig. 1A,C). All animals showed a pronounced bilateral allodynia through 56 days. After von Frey testing on day 56 rats were administered i.t. IL-1ra (100 μ g) or control. This dose was based on previous reports of the efficacy of i.t. IL-1ra (Milligan *et al.*, 2001; Milligan *et al.*, 2003). Behavioral responses were reassessed every 30 minutes from 1 to 3 hours,

and at 24 hours after injection (Fig. 1B,D). Whereas i.t. vehicle did not alter allodynia, i.t. IL-1ra caused a pronounced reversal of allodynia that was maximal by 2.5 hours, with animals returning to allodynia by 24 hours. This brief reversal reflects the half-life of IL-1ra (Milligan *et al.*, 2005a). These data indicate that spinal IL-1 is important for the maintenance of long-duration neuropathic pain rather than being involved only in the initiation of neuropathic pain. Thus, spinal cord anti-inflammatory IL-10 gene therapy for long-term protein release is a feasible treatment strategy.

Intrathecal pDNA-rIL-10 complexed with PEI reverses CCI-induced mechanical allodynia

Earlier reports show that delivering free pDNA-rIL-10 reverses chemotherapeutic and CCI-induced neuropathic pain changes as measured by the von Frey test (Milligan 2006; Ledebor, 2007). We have reported that 50–200 μ g of i.t. pDNA-IL-10 causes pain reversal after CCI (Sloane *et al.*, 2005), although pain reversal following a dose of 50 μ g is of shorter duration (Sloane *et al.*, manuscript in preparation). Given previous observations show that complexing pDNA with cationic agents leads to substantially improved gene expression in the CNS (Meuli-Simmen *et al.*, 1999; Shi *et al.*, 2003), we examined the use of PEI as a delivery agent for

Fig. 1. IL-1 β is important for the maintenance of long-duration CCI-induced allodynia ($n = 5-6$ /group). (A,C) At baseline (BL), ipsilateral (A) and contralateral (C) threshold responses are similar ($F_{1,18} = 0.352$, $P > 0.56$). Bilateral mechanical allodynia was observed by day 3 after CCI ($F_{1,18} = 803.553$, $P > 0.0001$) and continued through day 56 after CCI in both the left and right hind paws ($n = 5-6$ /group) ($F_{7,126} = 0.059$, $P > 0.99$). (B,D) After chronic mechanical allodynia was maintained for 8 weeks, a single i.t. injection of IL-1ra (100 μ g) produced a clear bilateral reversal from allodynia ($F_{1,18} = 227.835$, $P < 0.0001$) with maximal reversal observed by 2.5 hours after i.t. IL-1ra ($P < 0.0001$). Allodynia in both hind paws returned fully by 24 hours ($P > 0.41$). This reversal by IL-1ra was transient, in keeping with the short half-life because the effect of time was revealed ($F_{5,90} = 30.072$, $P < 0.0001$). * indicates significance.

pDNA-IL-10. The use of PEI did not diminish the prolonged pain reversal associated with i.t. free pDNA-IL-10, which supports the proof-of-principle that polymer applications to improve spinal cord gene delivery are worthy of further investigation.

We examined the duration of reversal of allodynia after either one or two successive i.t. injections (3-day inter-injection interval) of PEI-complexed to pDNA-GFP (PEI-GFP; 5 μ g pDNA), pDNA-IL-10 (PEI-pDNA-IL-10; 5 μ g pDNA), or PEI alone (PEI-Control). Reliable reversal from allodynia was produced by two successive i.t. injections of PEI-pDNA-IL-10 (Fig. 2). Rats were assessed for BL responses to the von Frey test before either CCI or sham surgery and on days 3 and 10 post-surgery. Immediately after behavioral testing on day 10 rats received i.t. PEI-pDNA-IL-10 at a pDNA dose 1/10th that used in previous experiments. Behavioral assessment was conducted on days 11, 12, 13, followed by a second i.t. injection of PEI-IL-10 (total dose for both injections = 10 μ g). Behavioral assessment was conducted as indicated (Fig. 2). Compared to sham-operated control groups, CCI created allodynia by days 3 and 10 after surgery (Fig. 2A,B). Stable non-allodynic responses were

observed in PEI-Control sham-operated groups throughout the 54-days. In addition, i.t. PEI-Control treatment did not significantly alter CCI-induced allodynia. In contrast to the short-lived effects of a single injection of PEI-pDNA-IL-10, two successive i.t. injections of PEI-pDNA-rIL-10 led to prolonged reversal beginning on day 12 after CCI (Fig. 2A,B). A single injection of PEI-GFP in either sham or CCI control treatment did not alter normal and allodynic responses, respectively (data not shown). PEI-pDNA might be a feasible way to deliver pDNA-IL-10 for sustained reversal of allodynia (~40 days) at doses 10-fold lower. Under the conditions tested, two i.t. successive injections of PEI-pDNA-IL-10 are necessary for prolonged reversal, with one injection reversing pain for ~5 days. Although PEI is non-toxic to CNS tissues, it tends to aggregate in aqueous solutions under physiological conditions, which makes this compound not clinically viable for drug delivery (Pack *et al.*, 2005; Sharma *et al.*, 2005).

Release kinetics and stability of pDNA from PLGA microparticles

Total release of the pDNA is complete within ~60 days with 50% released in the first 25 days (Fig. 3A). These data support

Fig. 2. I.t. PEI-pDNA-IL-10 improves gene expression. 10-fold lower doses of pDNA-IL-10 are sufficient to reverse neuropathic pain ($n = 4-5/\text{group}$). Before CCI, no threshold differences were observed between groups ($F_{7,30} = 0.430$, $P > .87$). Responses of the hind paw ipsilateral (A) and contralateral (B) to the site of surgery were analyzed through 54 days after CCI surgery. The time of CCI or sham surgery and each i.t. injection are indicated by black arrows. (A,B) Robust bilateral allodynia was observed after CCI compared to BL values in the group designated for a single injection ($F_{2,16} = 47.398$, $P < 0.0001$) and in the group designated for two i.t. injections of PEI-pDNA-rIL-10 ($F_{5,22} = 44.178$, $P < 0.0001$; compared to sham-operated controls). A single injection of PEI-pDNA-IL-10 reversed bilateral allodynia transiently (significance indicated by *). Analyzing data between days 11–55 after surgery, bilateral allodynia after a single i.t. PEI-pDNA-rIL-10 (closed diamond = CCI with i.t. PEI alone, single injection) was reversed for ~7 days ($F_{15,120} = 7.743$, $P < 0.0001$) compared to CCI-treated PEI-Control (open diamond) or sham-treated PEI-control (open triangle; significance indicated by ** in key). Stable bilateral allodynia was observed for the remainder of the timecourse through day 55. In contrast, reliable reversal of persistent, CCI-induced mechanical allodynia after a first ($F_{5,22} = 12.276$, $P < 0.0001$; data analyzed between days 11–13 after CCI) and second i.t. injection of PEI-pDNA-IL-10 (each injection, 5 μ g pDNA-IL-10 complexed to PEI in 18–20 μ l) was observed through day 49 after CCI ($F_{5,22} = 103.803$, $P < 0.0001$; significance indicated by + in legend). Closed square = CCI with i.t. PEI-pDNA-IL-10, two injections, compared to CCI i.t. PEI-Control treatment of CCI (open diamond). Bilateral mechanical allodynia remained reversed in rats treated with i.t. PEI-pDNA-IL-10 through day 43 ($F_{2,44} = 21.921$, $P < 0.0001$).

Fig. 3. Plasmid DNA-IL-10 gene integrity is maintained *in vitro* and *in vivo* after PLGA microencapsulation. (A) Release of the total amount of pDNA-IL-10 from PLGA-microparticles is nearly complete within the first 60 days of a 73-day total release time course. PLGA co-polymer formulation is 50% lactide and 50% glycolide with a molecular weight of 75000. Plasmid DNA loading = 1.0 ± 0.4 $\mu\text{g}/\text{mg}$ PLGA ($P < 0.0001$). (B) pDNA-IL-10 remains largely intact following encapsulation in PLGA microparticles because supercoiled pDNA-IL-10 was observed by agarose gel electrophoresis. Some reduction in supercoiled pDNA is evident. Lane 1, naïve, unprocessed pDNA-IL-10; lane 2, molecular weight marker; lane 3, PLGA-microencapsulated, extracted pDNA-IL-10; M, multimers; R, relaxed; L, linear; and S, supercoiled. (C) PLGA-microencapsulation of pDNA-IL-10 results in robust gene expression, measured by IL-10 protein in the supernatant following transfection of HEK293 cells with pDNA. IL-10 was measured following transfection with unprocessed pDNA without the IL-10 gene (pDNA Control); unprocessed pDNA-IL-10 (pDNA-IL-10); and pDNA-IL-10 extracted from PLGA-microparticles (PLGA-pDNA-IL-10). Supernatants were collected 48 hours after transfection. pDNA-IL-10 extracted from PLGA-microparticles is biologically active, although cells produced less IL-10 after transfection with pDNA-IL-10 extracted from PLGA-microparticles than unprocessed pDNA-IL-10 ($F_{1,4} = 1874.305$, $*P < 0.0001$). (D) pDNA-IL-10 released from PLGA-microparticles *in vivo* is active because IL-10 protein is increased 4 days after i.t. injection of PLGA-pDNA-IL-10 (white bars) but not PLGA-pDNA-GFP (black bars) in the CSF ($F_{1,3} = 0.44.513$, $*P < 0.007$). PLGA-pDNA-GFP leads to a small increase in IL-10 concentration, which is greater around the lumbosacral injection site ($n = 3$) than in cervical ($n = 3$) spinal cord regions ($P > 0.18$). In contrast, PLGA-pDNA-IL-10 leads to robust gene expression in both the lumbosacral ($n = 3$) and cervical regions ($n = 2$). This might indicate the protective function of PLGA on pDNA-IL-10, which might remain bioactive for longer in the CSF.

the robust behavioral reversal observed in the first 25 days after i.t. injection, and indicate that the rate of pDNA release from PLGA-microparticles is crucial for the behavioral affects observed on CCI-induced allodynia. In addition, pDNA remains largely intact following encapsulation in PLGA microparticles and the pDNA extracted from the microparticles compares with native pDNA although there is evidence of a slight reduction in the percentage of supercoiled pDNA (Fig. 3B). Either exposure to solvents or sonication during the protocol is likely to cause this change in conformation, which is consistent with reports in the literature (Ando *et al.*, 1999).

Despite the procedures required for pDNA encapsulation, IL-10 protein was produced in cell culture from pDNA-IL-10 recovered following PLGA-microencapsulation procedures.

Production of IL-10 in cells that received pDNA-IL-10 extracted from microparticles was 27.2% of that achieved by cultures transfected with naïve pDNA-IL-10. This difference might be caused, in part, by reduced pDNA integrity independent of the content of supercoiled pDNA. Nevertheless, despite the decrease in protein production, the remaining functional pDNA extracted from the microparticle is capable of producing of IL-10 (Fig. 3C).

I.t. PLGA- pDNA-IL-10 causes elevated levels of IL-10 protein in CSF

To determine whether i.t. injection of PLGA-pDNA-IL-10 changes IL-10 protein content *in vivo*, animals received two i.t. injections spaced 3-days apart of a suspension of

microparticles (400 ng of pDNA/400 μ g PLGA in 20 μ l; 800 ng pDNA-IL-10 total dose). A dramatic increase in the release of IL-10 protein into the CSF was observed, with variability of transgene expression observed here and previously (Milligan *et al.*, 2006; Ledebor *et al.*, 2007). We believe this variability in gene expression at this early time point is caused by the meningeal environment in which the transgene is expressed at a time of enhanced immune cell flux. Currently, our group is examining the mechanisms that underlie transient and long-term gene expression in the meninges. The concentration of IL-10 was elevated significantly in the CSF of animals that received pDNA-IL-10 released from PLGA microparticles (Fig. 3D). This indicates that the small reduction in supercoiled content observed by gel electrophoresis, and the reduction in gene activation in cell culture, does not reduce the capacity of gene activation significantly *in vivo*. I.t. injection of microencapsulated pDNA-IL-10 induced nanogram quantities of IL-10 protein, which is $\sim$ 30-fold more than induced by i.t. injection of naked pDNA-IL-10 in a previous study (Milligan *et al.*, 2006). This indicates that PLGA microencapsulation of pDNA-IL-10 is an improvement over previous methods. We are now examining IL-10 transgene expression in CSF at multiple, longer timepoints (40, 60 and 90 days) after i.t. administration of a 2nd-generation PLGA-pDNA-IL-10.

Confocal microscopic examination of PLGA microparticles in spinal meninges

To examine the distribution and penetration into deeper parenchymal layers of PLGA-microparticles, surgically naïve animals were given two, sequential i.t. injections of PLGA-pDNA-IL-10 loaded with rhodamine-dextran, 3-days apart. The spinal cord surrounding the injection site examined two weeks after the second injection. Cellular nuclei were stained with Sytox Green and red-labeled microparticles were observed in contact with cellular nuclei only in the sub-arachnoid meningeal layer surrounding the spinal cord parenchyma (Fig. 4A,B; open white arrow), and in the lateral limit of the meningeal tissue, the meningeal angle (Zenker *et al.*, 1994), contacting the adjacent DRG (Fig. 4C). These findings are supported by our previous observations that the free plasmid-derived gene encoding IL-10 is activated in DRG 18 days after i.t. injection of -pDNA-IL-10 (Ledebor *et al.*, 2007). The distribution pattern of PLGA-pDNA-IL-10 reported here is ideal for the sustained delivery of pDNA-IL-10 because it is uniform throughout the meningeal layer and exposes all cells in close proximity to pDNA-releasing microparticles. Taken together, the tissue distribution of IL-10 protein and microparticles indicates that cells in the subarachnoid space internalize released pDNA-IL-10 and secrete IL-10 locally.

I.t. free pDNA-IL-10 is effective at 100 μ g but 1 μ g is ineffective in the absence of PLGA

These data replicate our earlier reports that two sequential i.t. injections of free pDNA-IL-10 (dose range, 50–200 μ g) are required for pronounced, long-lasting reversal of allodynia that lasts >40 days in rats with CCI (Fig. 5A,B) (Sloane *et al.*, 2005; Milligan *et al.*, 2006; Ledebor *et al.*, 2007). By contrast, two, sequential, i.t. injections of 1 μ g of free pDNA-IL-10 did

Fig. 4. PLGA-pDNA-IL-10 (rhodamine dextran, red) is evenly distributed in the meninges but not in the deeper parenchymal spinal cord layers ($n = 4$). (A) Morphological examination of a section of the spinal cord using a nuclear-specific stain (green). Confocal histological examination from the lumbar spinal cord injection site at lumbar segments $\sim$ 3–4, two weeks after i.t. injection of PLGA-pDNA-IL-10 (A). (B) Enlargement of the area in the white box in (A). PLGA microparticles (red) in the meninges (small arrow) appear to be associated closely with cellular nuclei (green nuclei, large arrow). This pattern is uniform throughout the meningeal layer from lumbosacral to cervical spinal cord. (C) Large clusters of PLGA microparticles are present in the meningeal angle at the lateral limit of the meninges leading to the DRG. Large and small clusters of microparticles (white arrows) contact cellular nuclei adjacent to spinal cord and rostral to DRG.

not reverse allodynia, either long-term or transiently. The 1 μ g dose of free pDNA-IL-10 was used to enable direct comparison of therapeutic efficacy after PLGA-microencapsulation procedures ($\sim$ 400 ng, as described below). Hind-paw responses were assessed at BL and at 3 and 10 days after CCI surgery. Potent allodynia was observed after CCI in all animals up to day 10 (Fig. 5A,B). After behavioral assessment on day 10, free pDNA-IL-10 (either 1 μ g or 100 μ g in 18–20 μ l) was injected i.t. and allodynia re-assessed 3 days later, followed by a second i.t. injection of free pDNA-IL-10 (either 1 μ g or 100 μ g). Response thresholds were re-assessed as indicated (Fig. 5A,B). Only 100 μ g per injection (200 μ g pDNA total following the combined injections) reversed allodynia. Thus, identifying a way to reduce the total dose of pDNA-IL-10 might be beneficial for clinical application (Pack *et al.*, 2005). We then examined whether i.t. pDNA-IL-10 (<1.0 μ g) microencapsulated in PLGA, a well-characterized synthetic polymer, might sustain the therapeutic effect of free pDNA-IL-10 observed here and previously (Sloane *et al.*, 2005; Milligan *et al.*, 2006; Ledebor *et al.*, 2007).

I.t. PLGA-microencapsulated pDNA-IL-10 (<1 μ g) reverses CCI-induced allodynia

These data show that PLGA-pDNA-IL-10 improves gene delivery and efficacy because a lower dose of the IL-10 gene was effective for sufficient gene expression to reverse neuropathic pain following CCI (Fig. 5C–F). At BL, no differences in threshold were observed between groups.

A Ipsilateral Hindpaw **B Contralateral Hindpaw** **C Ipsilateral Hindpaw** **D Contralateral Hindpaw** **E Ipsilateral Hindpaw** **F Contralateral Hindpaw**

Fig. 5. PLGA microencapsulation of pDNA-IL-10 improves gene efficacy in the spinal cord. Responses (von Frey test) of the ipsilateral (A,C,E) and contralateral (B,D,F) hindpaws to the site of surgery were analyzed through 41, 47 or 70 days after CCI surgery. (A,B) I.t. injection of 100 μ g but not 1 μ g free pDNA-IL-10 provides long-term reversal of CCI-induced allodynia ($n = 3$ /group). No group differences were observed at BL ($F_{3,8} = 0.237$, $P > 0.86$). All rats underwent CCI surgery (black arrow), which produced a strong allodynia at 3 and 10 days after surgery ($F_{2,16} = 164.110$, $P > 0.0001$). Each group received two injections, 3 days apart (black arrows) of either 100 μ g (closed symbols) or 1 μ g (open symbols) free pDNA-IL-10 in 18–20 μ l ($n = 3$ /group). Only 100 μ g produced a clear ipsilateral and contralateral reversal of allodynia ($F_{54,144} = 2.445$, $*P < 0.0001$); no change was observed after 1 μ g free pDNA-IL-10 ($F_{8,72} = 0.437$, $P > 0.97$). (C,D) Microencapsulated PLGA-pDNA-IL-10 substantially reduces the amount of pDNA-IL-10 required to produce reliable reversal of persistent allodynia ($n = 4$ –6/group) after two injections 3 days apart. At BL, no threshold differences were observed between groups ($F_{5,22} = 0.095$, $P > 0.97$). The day of surgery is indicated by black arrows. Bilateral allodynia was observed on days 3 and 10 after CCI compared to the sham-operated group ($F_{5,22} = 38.774$, $P < 0.0001$). Beginning on day 10, two i.t. injections (black arrows) were given (3 days apart) of either microencapsulated pDNA-IL-10 (each ~ 400 ng; 400 μ g microspheres suspended in 20 μ l PBS) or empty microspheres alone (Control microspheres; 400 μ g microspheres suspended in 20 μ l PBS). Sham-operated rats given control microspheres remained non-allodynic throughout the experiment (open squares; significance indicated by +). I.t. injection of control microspheres in CCI rats (closed squares) did not alter allodynia, whereas reversal of bilateral allodynia was observed through day 37 after injection of microencapsulated pDNA-IL-10 in CCI rats (closed circles) ($F_{5,22} = 31.090$, $P < 0.0001$). The magnitude of reversal was robust, with similar threshold values following sham-operated empty-microsphere treatment and microencapsulated pDNA-IL-10 between days 13 and 27 after surgery ($F_{3,12} = 1.436$, $P > 0.28$). (E,F) A single i.t. injection ($n = 3$ /group) of microencapsulated pDNA-IL-10 in combination with free pDNA-IL-10 (open squares) or microencapsulated pDNA-IL-10 alone (closed squares) in CCI treated rats only reversed bilateral allodynia for ~ 12 days ($F_{3,8} = 23.095$, $*P < 0.003$). No group differences were observed at BL ($F_{3,8} = 0.039$, $*P > 0.978$), or after allodynia developed by days 3 and 10 after CCI ($F_{2,16} = 276.243$, $*P > 0.0001$). Neither treatment revealed meaningful, long-term reversal of allodynia. Bilateral allodynia after day 24 post-CCI remained stable, with no significant differences ($F_{21,56} = 0.640$, $P > 0.86$) for the remainder of the timecourse.

However, allodynia was observed on days 3 and 10 after CCI surgery compared to sham-operated animals. Although normal threshold responses were observed in sham-operated animals treated with control, empty PLGA-microparticles (each 400 μ g in 20 μ l PBS), stable allodynia continued after control, empty microparticle in CCI-operated animals. Conversely, after two successive i.t. injections of PLGA-pDNA-IL-10, reversal of allodynia was observed through day 37. Importantly, 800 ng of pDNA-IL-10 in microparticles was required for prolonged pain control. This is substantially less than the range required in the free pDNA-IL-10 gene therapy studies described above and previously (Sloane *et al.*, 2005; Milligan *et al.*, 2006; Ledebor *et al.*, 2007). These results demonstrate the potential of PLGA-microparticles to reduce the amount of pDNA-IL-10 required for effective pain control.

Two identical i.t. injections were required for substantial reversal of allodynia, with a single injection of PLGA-pDNA-IL-10 attenuating allodynia for $\sim$ 4 days only (Fig. 5E,F). In an attempt to boost exposure and surface contact of cells surrounding the i.t. meningeal injection site with pDNA-IL-10, free pDNA-IL-10 (25 μ g) was co-infused with PLGA-pDNA-IL-10 (400 ng pDNA), so that a bolus of pDNA-IL-10 contacts meningeal cells immediately followed by a prolonged release with, presumably, greater cellular contact time. However, a single injection of this cocktail did not improve pain reversal substantially (Fig. 5E,F), which further supports the observation that two sequential i.t. injections are required for effective reversal of CCI-induced allodynia.

CONCLUSIONS

- Long-duration (2 month) CCI-induced neuropathic pain involves the spinal actions of the proinflammatory cytokine IL-1 because i.t. IL-1ra produced transient ($\sim$ 2.5 hour) anti-allodynia. Thus, IL-1 is important for the early phases of and for long-duration neuropathic pain, which substantiates the hypothesis that IL-1 might provide a therapeutic target for clinical pain control.
- The anti-inflammatory cytokine IL-10 has the potential to provide effective neuropathic pain control by suppression of IL-1 released long-term in response to peripheral nerve injury. However, driving transgene expression using free pDNA is inefficient because high doses of pDNA are required. PEI and PLGA reduce the amount of pDNA-IL-10 required by 10–100-fold. Nevertheless, a second injection of pDNA-IL-10 treated with either polymer was needed to achieve long-term pain reversal.
- The *in vitro* characteristics of PLGA microparticles indicate that $\sim$ 40% of the pDNA is released in the first 3 days, followed by a more gradual release. Anti-allodynia effects occur in parallel with changes in the bioavailability of pDNA-IL-10.
- PLGA microparticles are evenly distributed throughout the meninges, make cellular contact with cells in the meninges and adjacent to DRG after i.t. injection.
- I.t. injection of polymer-based, nonviral gene therapy to drive the release of the anti-inflammatory cytokine IL-10 is a viable option for the control of chronic neuropathic pain.

DISCUSSION

These experiments demonstrate that long-duration (2 month) CCI-induced neuropathic pain involves the spinal actions of the proinflammatory cytokine IL-1 because i.t. IL-1ra produced transient ($\sim$ 2.5 hour) anti-allodynia. Thus, IL-1 is important for the early phases of neuropathic pain (Milligan *et al.*, 2005a; Watkins *et al.*, 2006; De Leo *et al.*, 2006; Ledebor *et al.*, 2007) and for long-duration neuropathic pain. This further substantiates that hypothesis that IL-1 might be a viable therapeutic target for clinical pain control. As such, the anti-inflammatory cytokine IL-10 has the potential to provide effective neuropathic pain control by suppressing IL-1 released long-term in response to peripheral nerve injury. Indeed, i.t. free pDNA that encodes IL-10 (pDNA-IL-10) leads to significant pain reversal for a long duration (>30 days) and decreases in IL-1 and TNF mRNA expression (Ledebor *et al.*, 2007), while transgene-derived mRNA for IL-10 was increased substantially in lumbar meninges, lumbar 4–6 DRG and cauda equina either 2 or 18 days after i.t. injection of pDNA-IL-10 gene therapy (Milligan *et al.*, 2006; Ledebor *et al.*, 2007). However, driving transgene expression using free pDNA is inefficient and needs high doses of pDNA to achieve therapeutic efficacy. In light of this, we used two, well-characterized, synthetic polymers with different properties, PEI and PLGA, to produce long-term anti-allodynia in neuropathic rats with pDNA-IL-10 doses 10–100-fold less than required previously. However, a second injection of pDNA-IL-10 treated with either polymer was needed to achieve long-term pain reversal. This effect might reflect a cumulative response of phagocytic immune cells (resident and/or migrating to the injection site) to pDNA-IL-10 and/or pDNA-IL-10 combined with polymer such that phagocytic function increases following the initial exposure to either pDNA-IL-10 or PLGA-pDNA-IL-10. Ongoing studies are examining changes in IL-10 protein in the CSF after i.t. injection of PLGA-pDNA-IL-10 during long-duration (up to 90 days) reversal of pain. In addition, several proinflammatory factors, including IL-1 and other immune-cell signals, are being examined to further characterize the underlying mechanisms of PLGA-IL-10-mediated pain reversal (Soderquist *et al.*, in preparation).

Based on its established safety record in medical applications (Athanasίου *et al.*, 1996; Fournier *et al.*, 2003), PLGA has the potential for successful i.t. gene transfer. However, the clinical safety of PLGA in the spinal cord remains to be determined. The *in vitro* characteristics of PLGA microparticles used in this study indicate that a large amount of pDNA ($\sim$ 40%) is released in the first 3 days, followed by a more gradual release. This burst effect is consistent with previous studies (Tinsley-Brown *et al.*, 2000) and is attributed to large amounts of pDNA located on or near the surface of the microparticles. The initial anti-allodynia corresponds to the initial burst in release of encapsulated pDNA, whereas allodynia returns, in part, when is pDNA release negligible. These results show that anti-allodynia trends parallel changes in the availability of delivered pDNA-IL-10. Furthermore, some encapsulated pDNA-IL-10 remains intact, stable and biologically active *in vitro* and *in vivo*, because substantial increases in IL-10 protein were measured in culture medium and CSF after PLGA-microparticle encapsulation. Elevation of IL-10

protein in CSF after i.t. delivery of encapsulated pDNA-IL-10 supports and extends our previous findings that i.t. free pDNA-IL-10 increases IL-10 mRNA and protein around the injection site (Milligan *et al.*, 2006; Ledeboer *et al.*, 2007). In addition, confocal microscopic examination of the PLGA-microparticles, 2 weeks after i.t. injection revealed a consistent, evenly distributed pattern in the meninges surrounding the spinal cord and CSF and DRGs. Importantly, the microparticles appear to be in direct contact with cells resident in the meninges, which indicates that prolonged exposure of cells to PLGA-microencapsulated pDNA-IL-10 and to pDNA-IL-10 released from microparticles. Both of these mechanisms improve gene uptake. This is the first report to show that i.t. polymer-based, nonviral gene therapy to drive the release of the anti-inflammatory cytokine IL-10 is a viable option to control chronic neuropathic pain.

Although the mechanisms underlying neuropathic pain are understood poorly, attention to the role of glia in neuropathic pain has increased considerably. Factors released from activated spinal cord glia, such as the pro-inflammatory cytokine IL-1, increase responses of pain projection neurons to incoming signals from peripheral nerves (Watkins *et al.*, 2006). There is growing evidence that IL-1 released from activated spinal glial cells is crucial for the initiation and early phases of pain facilitation in several animal models of neuropathic pain. Our finding that IL-1 is important for mediating protracted neuropathic pain indicates that glia and proinflammatory cytokines are also crucial during long-term pain. Because glial IL-1 exerts an ongoing role in long-duration neuropathic pain, polymer-based drug therapies designed for long-term delivery to target activated glia in the spinal cord are potentially a powerful new approach for pain control.

Polymer-based gene-delivery methods given as either single or multiple injections in the periphery, brain or spinal cord parenchyma lead to transgene expression for up to 4 weeks, without activating glia in the CNS (Schwartz *et al.*, 1996; Brooks *et al.*, 1998; Meuli-Simmen *et al.*, 1999; Shi *et al.*, 2003; Tan *et al.*, 2001). A few studies have examined i.t. pDNA-based gene transfer for pain control (Yao *et al.*, 2002a; Yao *et al.*, 2002b; Milligan *et al.*, 2006; Ledeboer *et al.*, 2007). A single i.t. injection of either polymer-treated IL-2 pDNA or free pDNA-IL-10 reduced CCI-induced neuropathic pain transiently (for 3 days), prevented subcutaneous hind-paw carrageenan-induced pain enhancement, and led to mRNA expression and protein in the spinal cord (Yao *et al.*, 2002a; Yao *et al.*, 2002b; Milligan *et al.*, 2006). Other reports have demonstrated that multiple, rather than single, injections of nonviral-based vectors improve transgene expression (Shi *et al.*, 2003; Ito *et al.*, 2004; Luby *et al.*, 2004). Furthermore, it has been suggested that the interval between i.t. injections is crucial for gene expression and efficacy in the spinal cord (Shi *et al.*, 2003; Sloane *et al.*, 2005; Milligan *et al.*, 2006). Two, sequential, i.t. injections of PLGA-microencapsulated pDNA-IL-10 improve free pDNA gene delivery because lower doses (nanogram range) are sufficient to achieve long-term control of neuropathic pain.

I.t. gene therapy with pDNA might be effective because of the area of gene delivery. Previously, we and others have shown transgene expression in the meninges surrounding the injection site using viral vectors (Mannes *et al.*, 1998; Milligan

et al., 2005a; Milligan *et al.*, 2006). The transgene expression examined here using plasmid-based methods might result from macrophages and dendritic cells that readily phagocytose free pDNA and polymer-treated pDNA (Tabata and Ikada 1991; Stacey *et al.*, 1996; Meuli-Simmen *et al.*, 1999; Prior 2002; McMenamin *et al.*, 2003). Macrophages and dendritic cells in the meninges form an extensive, functional network (McMenamin *et al.*, 2003), are in constant contact with the CSF (Guseo, 1977) and express receptors that respond to DNA, such as scavenger receptors and toll-like receptors (TLRs). Unmethylated, cytosine-linked guanine dinucleotide (CpG) motifs, which are prevalent in the plasmid DNA that is used typically to deliver transgenes (Krieg, 2002), bind to and activate dendritic cells and macrophages by scavenger receptors and TLR-9 (Stacey *et al.*, 1996; Krieg, 2002; Latz *et al.*, 2004; McCoy *et al.*, 2004). Furthermore, unmethylated CpG motifs in pDNA activate immune cells including phagocytic macrophages and dendritic cells, and lead to the production of endogenous IL-10 (Donnelly *et al.*, 1999; Moore *et al.*, 2001; Yi *et al.*, 2002; Lammers *et al.*, 2003; Gordon, 2003). Consequently, IL-10 enhances the capacity for phagocytosis of immune cells (Goerdts and Orfanos, 1999). Together, these reports indicate that CpG motifs might cause endogenous production of IL-10 followed by enhanced phagocytosis of pDNA-delivered transgenes. In these experiments the transgene encodes IL-10. Thus, in the meningeal cellular environment, endogenous production of IL-10 increases in response to CpG-containing pDNA-IL-10. In addition, exogenous IL-10 is produced as a consequence of enhanced pDNA-IL-10 phagocytosis, which leads to further transgene expression. Our data show that nanogram amounts of pDNA-IL-10 are effective at resolving neuropathic pain after two injections. Enhanced phagocytosis might be an important mechanism for these therapeutic effects. This is especially noteworthy given that nuclear entry of only a few pDNA molecules is sufficient for gene expression (Sebestyen and Wolff, 1999) with cells in the meninges particularly permissive for transgene uptake.

An advantage of PLGA microparticles over other methods for delivery of free pDNA is that encapsulation within the polymer microparticle protects pDNA from physical and chemical damage, including degradation by nucleases present in tissues (Capan *et al.*, 1999). Generally, encapsulating a gene and releasing it from microparticles improves the bioavailability for cellular uptake and expression (Hedley, 2005). For example, following PLGA treatment, high levels of gene expression were observed in the lung 21 days after intranasal administration (Nagesh *et al.*, 2005), which is considerably longer than without PLGA treatment (detectable levels observed at ≤ 48 hours). Another study also shows the long-term delivery potential of PLGA microparticles, with DNA detected near the site of injection for up to 100 days after injection (Lunsford, 2000). Thus, PLGA microspheres are a good candidate for long-term drug-delivery because they offer excellent biocompatibility and protection of pDNA (Athanasίου *et al.*, 1996; Fournier *et al.*, 2003).

Key factors that impact the therapeutic effect of PLGA-delivered pDNA-IL-10 are: (1) the rate at which drug is released from the microparticles; (2) phagocytosis of released, free pDNA-IL-10; and (3) phagocytosis of PLGA-pDNA-IL-10. Our data indicate that each of these mechanisms might contribute to long-term pain reversal. After microparticles

are injected into the body they begin to degrade and undergo phagocytosis. PLGA-based materials degrade by hydrolysis to produce lactic acid and glycolic acid, which, in turn, leads to pDNA release. These degradation products are eliminated safely via the citric acid cycle. Ultimately, the released pDNA is transported to the nucleus and expressed (Sebestyen and Wolff, 1999). Long-term, sustained release is possible by either changing the ratio of polylactide to polyglycolide in the copolymer or varying the molecular weight of either of the components. Drug release from biodegradable microparticles involves a combination of diffusion through and erosion of the polymer. In general, the rate at which drug is released is increased by decreasing the molecular weight of the copolymer and by incorporating higher ratios of glycolic acid (Tinsley-Brown *et al.*, 2000). Thus, the amount of gene contact with cells in the meninges can be fine-tuned by altering the release-rate of PLGA. In this regard, it might be possible to treat chronic pain with a single injection by manipulating the rate of pDNA-IL-10 release from microparticles. For example, pDNA might be released in multiple phases following a single i.t. injection, with a large release of pDNA in the first 24 hours and again at 72 hours, followed by a slow, steady release over the next 60 days. This pattern of release would mimic the two discrete bolus pDNA injections given 3 days apart, as used here, and might improve therapeutic efficacy.

Released pDNA might be protected when associated with various polymer formulations (Pack *et al.*, 2005), including during PLGA degradation (Hedley, 2005). Sustained presentation of PLGA-released pDNA might improve the probability of pDNA phagocytosis. As microparticles degrade, pDNA is released slowly into the surrounding environment, contacting meningeal cells after i.t. injection. In turn, this might expose cells to low levels of pDNA over longer periods of time. This increase in cell-surface contact over time might increase the probability of cellular phagocytosis of pDNA.

Physicochemical properties of the microparticles (e.g. size, surface charge and hydrophilicity) (Tabata and Ikada, 1991; Prior 2002) influence the extent to which cells internalize microparticles by either phagocytosis (uptake that does not require a receptor-mediated process) (Medzhitov and Janeway, 1997), fluid-phase pinocytosis and receptor-mediated endocytosis. Particles in the low micrometer range, as used here, are internalized most efficiently (O'Hagan, 2001). Other studies indicate that particles with a positively charged surface are internalized more efficiently (Thiele, 2001; Thiele *et al.*, 2003). Once internalized, microparticles must escape from either the endosomal or phagosomal membrane to enter the cytoplasm. Currently, our group is examining a combination of the modifications described above to improve gene delivery and long-term pain control.

In summary, these studies indicate that IL-1 is important for ongoing neuropathic pain, which supports the concept that anti-inflammatory cytokines such as IL-10 are a therapeutic target in long-term clinical pain. The present study applied a clinically viable synthetic polymer (PLGA) to deliver pDNA-IL-10 to achieve long-term anti-allodynia in neuropathic rats. With the development of safe, commercially successful polymers such as PLGA, it might be possible to extend the success of our pDNA-IL-10 gene therapy to the application for pain control in humans.

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