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Characterization of and Cellular Mechanisms Underlying Spinal Non-viral Interleukin-10 Gene Therapy Formulated with D-mannose for Treatment of Peripheral Neuropathic Pain

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CHARACTERIZATION OF AND CELLULAR MECHANISMS UNDERLYING SPINAL NON-VIRAL INTERLEUKIN-10 GENE THERAPY FORMULATED WITH D-MANNOSE FOR TREATMENT OF PERIPHERAL NEUROPATHIC PAIN

by

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DISSERTATION

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DEDICATION

The pursuit of knowledge is an adventure like none other. My journey could not have been possible without the love and support of my family. My parents, Elizabeth Trickey and Richard Glassman, have always taught me to be adventurous, inquisitive, and perseverant. My brother, Andrew Trickey-Glassman, is a life-long best friend who has always helped me see the lighter side of things, especially when they seemed most dark. My husband, Philip Vanderwall, has truly been my rock. His unconditional love, support, goofiness, love of cooking, sense of adventure, and limitless understanding have helped get me through this enormous undertaking.

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CHARACTERIZATION OF AND CELLULAR MECHANISMS UNDERLYING SPINAL NON-VIRAL INTERLEUKIN-10 GENE THERAPY FORMULATED WITH D-MANNOSE FOR TREATMENT OF NEUROPATHIC PAIN

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ABSTRACT

Anti-inflammatory intrathecal non-viral interleukin-10 (IL-10) gene therapy provides enduring relief of chronic pain in numerous animal pain models. Co-administration of the mannose receptor (MR) ligand D-mannose (DM) improves non-viral IL-10 gene therapeutic efficacy, but questions remain regarding which pain-relevant tissues are critical for non-viral transgene expression resulting in long-lasting pain relief. Additionally, the role of endogenous IL-10 in non-viral IL-10 therapeutic effects is completely unknown.

Chapter I is an Introduction to the studies detailed in this dissertation, providing a historical perspective of the field of neuroinflammation and a framework upon which neuroimmune processes intersect with mechanisms underlying pathological pain. The work presented in Chapter II investigates *in vivo* the mechanisms that underlie pain relief following intrathecal non-viral IL-10 gene therapy formulated with DM for treatment of peripheral neuropathic pain. Naked plasmid DNA encoding the IL-10 transgene (pDNA-IL-10) was co-injected intrathecally with DM in both IL-10 wildtype (WT) and IL-10

deficient (IL-10 KO) neuropathic mice. We show that DM/pDNA-IL-10 is efficacious in both backgrounds, indicating that endogenous IL-10 is not required for efficacy of DM/pDNA-IL-10 therapy. We next demonstrate the biodistribution of the IL-10 transgene, with key expression in the ipsilateral dorsal root ganglia (DRG) of pain-relieved mice that induces local anti-inflammatory effects. This drives further anti-inflammatory changes at the level of the lumbar spinal cord, which are mirrored by decreased expression of glial activation markers. We further demonstrate that MR activation itself provides transient pain relief in IL-10 KO mice. This supports an IL-10-independent anti-inflammatory mechanism by which MR-mediated actions modulate pain signaling.

The work presented in Chapter III utilizes *in vitro* primary cell culture techniques to explore the cellular mechanisms by which MR activation mediates IL-10 independent anti-inflammation. This approach is also applied to examine and the cellular conditions (basal vs. inflammatory stimulation) required for DM-mediated transgene adjuvant effects. These preliminary studies indicate that MR activation decreases nitric oxide (NO) production in an IL-10-independent manner. We also present evidence that the timing of inflammation and the relative expression levels of MR dramatically impact the efficacy of MR-mediated anti-inflammatory-like conditions.

Chapter IV discusses pain as a chronic inflammatory disease and explores the versatility of DM/pDNA-IL-10 gene therapy in the treatment of chronic pain of different etiologies. It also indicates that MR activation by DM has utility beyond the realm of non-viral gene therapy and puts forth that MR should be explored as a therapeutic target in its own right for treatment of chronic pain.

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CHAPTER I

Introduction to Pain and the Neuroimmune Interface

1. Overview

Since the time of the ancient Greeks pain has been considered a cardinal sign of inflammation. However, modern science has been slow to appreciate that pain and the immune system are interrelated. There has been growing recognition since the 1970s that the nervous and immune systems play intertwining roles in behavior and physiology (Watkins and Maier, 2005). It was first noted that sick or injured animals often display a broad array of 'sickness responses'(Hart, 1988). Characteristic physiological changes include fever and increased sleep, as well as changes to activity of the hypothalamic-pituitary axis and the sympathetic branch of the autonomic nervous system. These biologic changes are paired with behavioral stereotypes including decreases in ingestion of food and water, motility, and social/sexual behavior. Intriguingly, sickness responses also include sickness-induced hyperalgesia, a pathological pain state when pain from noxious stimuli is enhanced (Maier et al., 1992; Watkins and Maier, 2000).

These alterations all require changes in brain functioning, suggestive of an avenue of communication from the periphery to the brain to specifically relay that something serious (e.g. injury or infection) has happened (Watkins and Maier, 2005). It has since been demonstrated that pro-inflammatory cytokines produced by peripheral immune cells, especially interleukin-1β (IL-1β), IL-6, and tumor necrosis factor-α (TNF-α), provide this critical signal to the central nervous system (CNS) (Milligan and Watkins, 2009; Mika et

al., 2013; Grace et al., 2014; Ji et al., 2016). Additionally, glia (astrocytes and microglia) and neurons (both CNS and peripheral nervous system [PNS]) express a variety of cytokine receptors like the IL-1β receptor (IL-1βR) and the TNF-α receptor (TNF-αR) (Milligan and Watkins, 2009). Glia are also known to respond to important chemokines such as chemokine C-C motif ligand 2 (CCL2) (Grace et al., 2014). Not only can glia respond to peripherally derived pro-inflammatory mediators, but consequent glial activation results in further production of pro-inflammatory mediators. Importantly, these centrally derived glial cytokines are both necessary and sufficient for the production of sickness responses (Rothwell and Luheshi, 1994; Watkins and Maier, 2005).

This introduction to the topic of pain and neuroimmunology will discuss key aspects of neuronal-mediated pain signaling under acute and chronic conditions following a brief overview of normal pain processing. Major findings will be discussed addressing why pathological pain is now considered a product of neuroimmune dysfunction involving neurons, glia, and peripheral immune cells. Finally, the reader will be introduced to potential avenues for improvements in therapeutic approaches for pain control using methods that target neuroimmune glial cells.

2. Chronic Pain is a Global Health Problem

Pain lasting greater than three months is considered chronic. Worldwide, chronic pain is estimated to afflict more than 1.5 billion people, and in 2013 pain was ranked in a global top ten list of causes for years lived with disability (YLD) (Yaqub, 2015). A different survey in Europe found that 60% of respondents had experienced chronic pain from two to

15 years, with nearly a fifth of respondents reporting pain suffering for ≥20 years (Breivik et al., 2006). In the United States alone, chronic pain exacts an estimated $560-635 billion per year in costs for treatment and loss of productivity and, for many patients, adequate pain control remains out of reach (Institute of Medicine Committee on Advancing Pain Research and Education, 2011). Neurons are clearly critical in pain signaling, and many pharmaceutical agents are designed to control pain through limiting neuronal activity. Unfortunately, such medications offer insufficient analgesia. Furthermore, as is the case with opioids like morphine, they can be associated with the rapid onset of tolerance and a high potential for abuse.

3. Function and Anatomy of Physiological Pain

3.1 Pain vs. Nociception

Physiological pain is an evolutionarily advantageous sensory warning system and can be understood as the conscious awareness of nociceptive information (Nash, 2005; Tracey and Mantyh, 2007). Nociception is the 'pre-conscious' process by which potentially harmful stimuli, such as intense thermal, mechanical, or chemical insults, are detected by a subpopulation of peripheral neurons called nociceptors (Basbaum et al., 2009). Primary nociceptors that innervate the body have peripheral sensory receptors in target tissues (e.g. skin) and house their cell bodies in the dorsal root ganglia (DRG) found at each level of the vertebral column (Basbaum et al., 2009). Importantly, these nociceptors synapse with second order neurons in the spinal cord dorsal horn (Tracey and Mantyh, 2007; Watkins et

al., 2007b; Basbaum et al., 2009). For example, pricking one's forefinger should activate nociceptors traveling in the median nerve to the dorsal horn within the cervical spinal cord (Standring, 2016a). The dorsal horn of the spinal cord is a CNS locale with a complex assortment of pain transmitting neurons, interneurons, and non-neuronal glial cells. Unlike nociceptors found in the rest of the body, those traveling within cranial nerves (e.g. the trigeminal nerve) communicate not with second order neurons in the dorsal horn, but rather with second order neurons found in brainstem nuclei (Standring, 2016b).

3.2 Nociceptor Subtypes and Pain Projection Pathways

There are two main types of nociception-specific neurons, those with thin unmyelinated axons (C fibers) or medium diameter lightly myelinated axons (Aδ fibers) (Watkins et al., 2001a; Basbaum et al., 2009; Milligan and Watkins, 2009). Generally, Aδ fibers mediate acute well-localized 'fast' pain, whereas C fibers are characterized by slow conduction velocity and 'slow' pain (Basbaum et al., 2009; Milligan and Watkins, 2009). These nociceptor subtypes are distinct from the large diameter heavily myelinated Aβ fibers that are critical for relaying non-noxious information (e.g. mechanical stimulation such as light touch). Within the dorsal horn, sufficiently noxious nociceptive inputs can initiate protective spinally-mediated withdrawal reflexes designed to remove oneself from immediate harm (e.g. withdrawing one's fingertips from a hot pan) (Schouenborg and Sjolund, 1983). Nociceptive information is also processed by higher order structures in the brainstem and brain, leading to perception of pain, promotion of learning, and fostering of healing behaviors (Tracey and Mantyh, 2007; Basbaum et al., 2009). Information about

noxious stimuli can ascend within the spinal cord to the thalamus via the contralateral spinothalamic tract (STT) or to the medulla and midbrain through the spinoreticular and spinomesencephalic tracts (Tracey and Mantyh, 2007). The most common cerebral regions activated during acute pain experiences are the primary and secondary somatosensory, insular, anterior cingulate, and prefrontal cortices (Tracey and Mantyh, 2007).

Descending modulation of nociceptive signaling (particularly that which alters activity in the spinal cord dorsal horn) can be utilized to either facilitate or inhibit pain (Fields and Basbaum, 1978; Porreca et al., 2002). Many of the higher order structures mentioned to be activated by acute pain also play roles in spinal mechanisms of pain modulation, including the frontal lobe, anterior cingulate cortex (ACC), insula, amygdala, hypothalamus, periaqueductal gray (PAG), nucleus cuneiformis (NCF), and rostral ventromedial medulla (RVM) (Porreca et al., 2002; Tracey and Mantyh, 2007). There is also interest in understanding how cognitive processes like attention can influence brainstem activity, leading to changes in spinal nociceptive processing (Tracey and Mantyh, 2007).

3.3 Acute Pain Neurotransmitter Signaling

During acute pain transmission, peripheral nociceptor activation leads to glutamatergic signaling in the spinal cord dorsal horn, which in turn leads to excitatory postsynaptic currents (EPSCs) in second order pain projection neurons (Basbaum et al., 2009). Under ‘normal pain’ conditions, glutamatergic signaling is mediated by postsynaptic AMPA receptors. Though NMDA receptors are present, they are inhibited by the presence of a

magnesium ion ‘plug’ (Basbaum et al., 2009). Summation of subthreshold EPSCs mediated by AMPA activation eventually causes action potential firing of the second order pain projection neurons, allowing for transmission to higher order structures in the brain (Basbaum et al., 2009).

4. Neuronal Mechanisms of Pathological Pain

4.1 Pathological Pain

‘Normal’ physiological pain correlates with acute or potential tissue damage. Unfortunately, damage to the CNS or PNS by disease, injury, or infection can lead to pathological pain. That is, pain that is now maladaptive, extending beyond the window of usefulness (Grace et al., 2014). Such pathological pain can be observed in a variety of conditions including inflammatory pain such as that following joint injury (e.g. arthritis), cancer pain, neuropathic pain following peripheral nerve damage (e.g. trauma, diabetic neuropathy, or chemotherapeutic side effects), or CNS damage (e.g. stroke, trauma, or HIV infection) (Milligan and Watkins, 2009; Grace et al., 2014). In such cases, pain resulting from normally nociceptive stimuli, such as heat, may now be exaggerated (hyperalgesia) (Milligan and Watkins, 2009). Pain may even occur in response to non-nociceptive stimuli (allodynia) such as light touch, which is referred to as mechanical allodynia (Milligan and Watkins, 2009).

4.2 Peripheral and Central Sensitization

Incremental changes in neuronal activity are required to elicit pathological pain. One such change in neuronal signaling is called peripheral sensitization in which primary nociceptors become overly responsive as result of exposure to pro-inflammatory mediators released by local immune cells at the site of tissue injury or infection (Basbaum et al., 2009). One mechanism facilitating heightened exposure is known as neurogenic inflammation. Neurogenic inflammation is possible, in part, because nociceptive activation of C fibers not only leads to orthodromic signaling to the spinal cord, but also to antidromic signaling back to the periphery as a result of axonal or dorsal root reflexes (Chiu et al., 2012). This causes peripheral nociceptor terminal release of neuropeptides like substance P (Sub P), calcitonin gene-related peptide (CGRP), neurokinin A (NKA), NKB, vasoactive intestinal protein (VIP), and neuropeptide Y (NPY) (Chiu et al., 2012). These neuropeptides lead to local increases in blood flow, vascular permeability, and immune cell accumulation (Chiu et al., 2012). Trafficking immune cells release a host of pro-inflammatory mediators for which sensory neurons express receptors, such as IL-1βR and TNF-αR (Scholz and Woolf, 2007). Activation of these receptors on nearby C fibers and Aδ fibers leads to enhanced membrane excitability and sensitization to subsequent stimulation (Scholz and Woolf, 2007; Littlejohn, 2015).

Peripheral nerve sensitization can in turn lead to enhanced nociceptor release of excitatory neurotransmitters at their terminal synapses within the spinal cord dorsal horn. Key spinal pain neurotransmitters include glutamate, Sub P, CGRP, and adenosine triphosphate (ATP) (Grace et al., 2014). Unlike during acute pain conditions, the enhanced

excitatory signaling enables sufficient postsynaptic depolarization for removal of the once inhibitory magnesium ion from the NMDA receptor pore. This results in increased cation (e.g. Ca2+) influx and strengthened synaptic connections between primary nociceptors and secondary pain transmission neurons in the dorsal horn of the spinal cord (Latremoliere and Woolf, 2009). This type of neuronal plasticity is known as central sensitization and can be observed clinically as hyperalgesia, allodynia, and the expansion of receptive fields that enables input from non-injured tissue to produce pain (secondary hyperalgesia) (Basbaum et al., 2009; Latremoliere and Woolf, 2009). While the NMDA receptor-mediated mechanism of central sensitization is the best understood, alternatives have been proposed. For example, peripheral nerve injury could induce phenotypic changes in Aβ fibers, leading to ectopic expression of CGRP and Sub P (neuropeptides associated with nociceptive C- and Aδ- fibers) (Nitzan-Luques et al., 2011).

4.3 Traditional Neuron-Centric Pain Control Therapeutics and Their Shortcomings

To combat pathological pain, clinicians have traditionally relied on medications that target neuronal activity. Common treatments include opioid and non-opioid analgesics including anti-depressants (e.g. tricyclic anti-depressants [TCAs] or serotonin norepinephrine reuptake inhibitors [SNRIs]) and anticonvulsants (e.g. gabapentin or pregabalin) (Dworkin et al., 2007; Colloca et al., 2017). For most patients, initial treatment for neuropathic pain involves anti-depressants or anticonvulsants, but are almost inevitably followed by opioid use, the second-line option (Dworkin et al., 2007). Opioids such as morphine mimic endogenous endorphins by acting on opioid receptors, such as the μ, κ, and δ subtypes,

found on sensory neurons within the brain and spinal cord (Hutchinson et al., 2011; Jamison and Mao, 2015). Activation of μ opioid receptors on ascending pain projection neurons decreases neuronal firing, thereby limiting the perception of pain (Hutchinson et al., 2011). Though opioids are known to produce powerful analgesia, they are also associated with rapid development of tolerance, paradoxical hyperalgesia, and potential for opioid use disorder (Song and Zhao, 2001; Raghavendra et al., 2002; Milligan and Watkins, 2009; Hutchinson et al., 2011; Grace et al., 2014). Current neuron-centric treatments offer insufficient pain control, but a growing body of evidence suggests that neuroimmune glial cells can serve as novel targets for development of future chronic pain control therapeutics.

5. Glial Pro-inflammatory Activation is Both Necessary and Sufficient for Pathological Pain

5.1 Spinal glial activation: more than simple homeostasis

A great deal of work has begun to focus on astrocytes and microglia and their roles in pain dysregulation (Watkins et al., 2001a). Astrocytes arise from neuroepithelium and are found throughout the brain and spinal cord (Rowitch and Kriegstein, 2010), outnumbering neurons and accounting for 40-50% of all CNS glia (Lee et al., 2000). In contrast, microglia possess a mesodermal hematopoietic origin and constitute only 5-10% of all glia and are considered the resident mononuclear phagocytes of the CNS (Watkins et al., 2007a; Rowitch and Kriegstein, 2010). Previously, astrocytes and microglia were thought to serve purely homeostatic and surveillance functions, but it has become clear that they are far

more than “housekeepers.” For instance, following infection or injury of the CNS, both cell types can assume ‘activated’ phenotypes, experiencing changes in morphology (e.g. switch from highly ramified to amoeboid appearance) as well as upregulation of cell-type specific activation markers like astrocytic glial fibrillary acidic protein (GFAP) and microglial complement receptor 3 (CR3; CD11b) (Watkins et al., 2001b; Milligan and Watkins, 2009). Astrocytes and microglia also release increased levels of pro-inflammatory mediators including cytokines, chemokines, ATP, excitatory amino acids (EAAs), and nitric oxide (NO) (Watkins et al., 2007a). Importantly, spinal glia are closely associated with neural synapses and are known to express many of the same neurotransmitter receptors as neighboring neurons, making them well-positioned to modulate neural communication or to become reactive in response to abnormal or heightened neuronal signaling (Watkins et al., 2001b; De Leo et al., 2006; Scholz and Woolf, 2007; Milligan and Watkins, 2009).

Oligodendrocytes are another important glial cell type found throughout the CNS, though their potential role in pain dysregulation remains poorly understood. Oligodendrocytes are derived from neuroepithelium and are critical for CNS myelination (Rowitch and Kriegstein, 2010). They are capable of producing pro-inflammatory cytokines and chemokines, and a recent study by Zarpelon *et al.* suggests that pro-inflammatory IL-33 produced by oligodendrocytes may be important for neuropathic pain following peripheral nerve injury in mice (Zarpelon et al., 2016). Overall, the relationship between oligodendrocytes and pain has been understudied and is worth further investigation.

5.2 Initial evidence for glial activation following peripheral nerve damage

Interestingly, glia increase their expression of activation markers following peripheral nerve damage (Watkins and Maier, 2005). Garrison *et al.* provided the first anatomical evidence that spinal glia may play a role in pain modulation through the observation that astrocytes of the lumbar spinal cord demonstrate signs of activation (e.g. changes in morphology and increased GFAP expression) following chronic constriction injury (CCI) of the rat sciatic nerve (Garrison et al., 1991). Though prior work had identified CNS glial activation following peripheral nerve damage, Garrison *et al.* were the first to connect glial activation with a functional outcome (Garrison et al., 1994). They found that administration of an NMDA receptor antagonist (MK-801) known to alleviate neuropathic pain (clinically and in animal models) leads to a dramatic decrease in the level of glial activation markers in the spinal cord dorsal horn normally observed following sciatic nerve damage (Garrison et al., 1994).

5.3 Glial activation observed in a variety of animal models of neuropathic pain

Glial activation and the notion that glia are potent contributors to exaggerated pain states is further supported by the observation that pain in animal models can be prevented or reversed by application of agents that inhibit glial activation or production of pro-inflammatory mediators. Meller *et al.* showed that following intraplantar injection of zymosan (an inflammatory component of yeast cell walls) and subsequent hyperalgesia, intrathecal (i.t.; delivery into subarachnoid cerebrospinal fluid) injection of fluorocitrate (a

selective metabolic inhibitor of astrocytes) results in pain relief (Meller et al., 1994). Later, Watkins *et al*. demonstrated that i.t. fluorocitrate, IL-1 receptor antagonist (IL-1Ra), or semapimod (CNI-1493; inhibitor of both NO synthesis and p38 MAP Kinase activation) all block hyperalgesia caused by subcutaneous formalin injection (Watkins et al., 1997). Milligan *et al.* then established that i.t. fluorocitrate, IL-1Ra, and TNF binding protein (TNFbp) all prevent pain induced by i.t. HIV-1 envelope glycoprotein gp120, a model of spinal glial inflammation (Milligan et al., 2001). Additionally, Sweitzer *et al.* found that propentofylline (a methylxanthine derivative found to attenuate astrocyte activation) could both prevent and reverse mechanical allodynia (pathological sensitivity to light touch, as noted above) in a rodent L5 spinal nerve transection model of neuropathic pain (Sweitzer et al., 1999). Importantly, microglial activation was further implicated in neuropathic pain by Ledeboer *et al.* who showed that i.t. minocycline (a selective inhibitor of microglial activation) attenuates mechanical allodynia following either acute or persistent sciatic inflammatory neuropathy (SIN) or i.t. HIV-1 gp120 (Ledeboer et al., 2005).

Since then, the observation that glial activation and resultant production of pro-inflammatory mediators are both necessary and sufficient for neuropathic pain has been shown in a wide variety of animal models. Spinal cord glial activation is observed in virtually every animal model of enhanced pain, and this pain can be reversed or prevented through inhibition of glial activation or nullification of pro-inflammatory actions (Watkins et al., 2001a; Watkins and Maier, 2003; Marchand et al., 2005). It is worth noting that recent work in pre-clinical animal models have begun to reveal a role for leukocyte (e.g. macrophages and T-cells) infiltration of the spinal cord following peripheral nerve damage

in mechanisms of neuropathic pain (Ji et al., 2016). The role of the peripheral immune system in chronic pain will be examined in greater detail in Chapters III and IV.

5.4 Opioids: Their newly recognized role in glial pro-inflammatory actions

Opioid analgesics are associated with a variety of unwanted side effects. Some of these can be partly explained by neuronal mechanisms, but glial activation is now recognized as a major contributor. Opioid receptors are stereoselective and become activated only through binding with [-]-isomers (Hutchinson et al., 2011). Interestingly, classical opioid receptors are not the only avenue for opioid-induced effects. For instance, glia express a variety of receptors designed to recognize pathogen associated molecular patterns (PAMPs) and endogenously derived markers of insult such as danger associated molecular patterns (DAMPs) (Grace et al., 2014; Ji et al., 2016). Key among these are toll-like receptors (TLRs). TLR4 is a well characterized pathogen recognition receptor (PRR) known for its ability to become activated by the PAMP lipopolysaccharide (LPS; endotoxin; cell wall component of gram negative bacteria) and DAMPs such as heat shock proteins (HSPs) or high mobility group box 1 (HMGB1) protein (Agalave et al., 2014; Grace et al., 2014). Intriguingly, Hutchinson *et al*. demonstrated that both [+]- and [-]-opioid isomers can activate TLR4 through binding its co-receptor, myeloid differentiation factor 2 (MD2) (Hutchinson et al., 2007). Prior to this, it was known that morphine administration leads to dramatic increases in spinal levels of mRNA for pro-inflammatory cytokines, with consequent increases in observed cytokine release (Watkins et al., 2007a). Additionally, co-administration of morphine with glial inhibitors is known to abolish the development

of opioid tolerance (Song and Zhao, 2001). Interestingly, spinal pro-inflammatory cytokines in neuropathic pain have been shown to produce a state of "naïve morphine tolerance" in which activated glia overreact to morphine compared to glia from sham treated controls, thereby preventing the anti-nociceptive effects of acute morphine (Raghavendra et al., 2002; Raghavendra et al., 2003). Opioids are just one example for which a clear understanding for neuroimmune function will be key in improving pain control approaches.

6. Neuroimmune Targets for Improved Pain Control

It has become clear that glial activation is a major reason why widely used neuron-centric therapeutics, like opioids, fail to provide adequate pain control for a majority of patients. Thus far the development of neuroimmune modulators for pain control has targeted three areas: 1) direct inhibition of neuroimmune pro-inflammatory signaling, 2) stimulation of anti-inflammatory pathways, and 3) pro-resolution lipid mediators (Grace et al., 2014).

6.1 Direct inhibition of neuroimmune pro-inflammatory signaling

Pro-inflammatory cytokine actions can be targeted at several different levels including cellular activation, production of pro-inflammatory mediators, and interaction with specific receptors. Fluorocitrate is a well-known astrocytic activation inhibitor shown to be highly effective at blocking neuropathic pain in animal models (Meller et al., 1994; Milligan and Watkins, 2009). Unfortunately it has a narrow dose range with heightened neurotoxic risk

at higher doses, making it less than ideal for human application (Romero-Sandoval et al., 2008). Propentofylline is another glial activation modulator that specifically inhibits phosphodiesterases (Sweitzer and De Leo, 2011). Minocycline, a second generation tetracycline, is a blood-brain-barrier (BBB) permeable activation inhibitor of mononuclear phagocytes like microglia (Mika, 2008). Ibudilast (formerly AV411, now MN-166) administration results in decreased microglial activation by inhibiting both phosphodiesterase function and TLR4 signaling (Ledeboer et al., 2007a; Johnson et al., 2014). Other TLR4 antagonists that have shown promise as pain control therapeutics alone or as adjuvants to opioids include [+]-naloxone and [+]-naltrexone (Hutchinson et al., 2008). Interestingly, some pain control medications thought to solely target neurons have been found to modulate glial pro-inflammatory functions, such as the anti-depressant amitriptyline, a TCA that also inhibits TLR4 activation through binding to the MD2 co-receptor (Hutchinson et al., 2010; Grace et al., 2014). Cytokine antagonists like the biologic agents anakinra (recombinant human IL-1 receptor antagonist; IL-1RA) and etanercept (recombinant human soluble TNF receptor) have also shown beneficial effects in animal models of neuropathic pain (Milligan and Watkins, 2009). Unfortunately host development of neutralizing antibodies, compromised host defenses against infection, or BBB impermeability make these biologic agents less than ideal for neuropathic pain control (Fleischmann et al., 2006; Milligan and Watkins, 2009; Rider et al., 2016).

6.2 Stimulation of Anti-inflammatory pathways

Gene therapeutic delivery of anti-inflammatory cytokines offer unique advantages over small molecule approaches. For instance, the vector can be applied directly to the relevant portions of the nociceptive pathway (e.g. level of the spinal cord) (Milligan and Watkins, 2009). Anti-inflammatory IL-2, IL-4, and IL-10 gene therapies have all been demonstrated efficacious in the reversal of neuropathic pain in a variety of animal models (Goss et al., 2007; Milligan and Watkins, 2009). Transgene expression and pain reversal is associated with decreased phosphorylation of the pro-inflammatory transcription factor p38-MAPK and decreased production of pro-inflammatory mediators like TNF-α, IL-1β, and prostaglandin E2 (PGE2) (Grace et al., 2014). There is also a dramatic decrease in the expression of glial activation markers like GFAP (Grace et al., 2014).

Both viral and non-viral methods are effective in treating rat models of chronic neuropathic pain. Non-viral gene transfer methods, such as i.t. injection of naked plasmid DNA (pDNA) provide several advantages over viral methods (e.g. problems with immunogenicity or insertional mutagenesis) (Glover et al., 2005). Gene transfer adjuvants, such as the recently reported D-mannose, have been shown to greatly enhance the enduring efficacy of non-viral IL-10 gene therapy, reversing established neuropathic pain for greater than 80 days in rats (Dengler et al., 2014). Work reported in this dissertation addresses important questions that had long remained unanswered regarding the identities of the tissues and cell types responsible for IL-10 transgene uptake and pain reversal, which are now detailed in Chapter II.

6.3 Pro-resolution Lipid Mediators

An emerging area of chronic pain research focuses on pro-resolution lipid mediators (PRLMs) such as resolvins, protectins, and lipoxins (Grace et al., 2014; Ji et al., 2014). PRLMs act to restore normal sensitivity through re-establishment of neuroimmune communication. Resolvins were originally identified from inflammatory exudates associated with the resolution of inflammation in rodents and humans(Ji et al., 2014). Resolvins and protectins are derived from dietary essential fats such as the omega-3 polyunsaturated fatty acids docohexaenoic acid (DHA) and eicopentaenoic acid (EPA), while lipoxins are derived from arachidonic acid (Ji et al., 2014). PRLMs have been found to signal through G protein-coupled receptors (GPCRs) found on both glia, neurons, and peripheral immune cells, and have great promise for neuropathic pain treatment based on data from experiments showing pre-surgical prevention of neuropathic pain associated with nerve damage in mice (Ji et al., 2014).

7. Conclusions

In this introductory chapter, the differences between acute and pathologic pain were briefly described. Traditional mechanisms of pain processing from a purely neuronal perspective were examined, followed by current clinical approaches to the treatment of chronic pain and the insufficient pain control of neuron-centric therapeutics. A succinct history of the realization that glial activation and production of pro-inflammatory mediators are critical for the onset and maintenance of chronic pain was detailed, supporting that glia should be

targeted in the development of novel pain control therapeutics. Though it has been nearly 25 years since glia were first recognized as central actors in pain pathology, there is still much to be learned about the neuroimmune interface in this field of psychoneuroimmunology.

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CHAPTER II: PREFACE

While considerable progress has been made towards understanding the anti-inflammatory actions on neuroimmune dynamics of pathological pain, questions remain regarding the underlying mechanisms of anti-inflammatory intrathecal non-viral IL-10 gene therapy. The focus of this second chapter is to elucidate the role of endogenous IL-10 in enduring pain relief mediated by intrathecal non-viral IL-10 gene therapy for treatment of neuropathic pain.

Though a significant body of work demonstrates the efficacy of this therapeutic approach in a variety of animal pain models, these studies were performed in the presence of endogenous IL-10. Consequently, it remained unclear whether endogenous IL-10 is required for any component or phase of intrathecal non-viral IL-10 gene therapy. Further, prior work utilized an IL-10 transgene that possessed a non-silent point mutation that could interfere with IL-10 homodimerization, a preliminary requirement for activation of the IL-10 receptor. Thus, it was previously unknown whether the exogenous transgene's mutant protein product requires heterodimerization with wildtype (e.g. endogenous) IL-10 for sufficient receptor interaction leading to downstream anti-inflammatory signaling. Additionally, due to the presence of endogenous IL-10, prior works could not unambiguously indicate in which pain-relevant anatomic regions IL-10 transgene uptake and expression drives relief of peripheral neuropathic pain.

This chapter also examines the role of IL-10 in D-mannose-mediated improvement of non-viral IL-10 gene therapy. A prior report demonstrated that D-mannose enhances therapeutic efficacy (i.e. transgene uptake and expression with consequent pain relief) if

co-injected with non-viral plasmid DNA encoding the IL-10 transgene. Additionally, a single intrathecal dose of D-mannose alone provides transient pain relief in neuropathic wildtype rats. Whether IL-10 is required for these desirable effects is further explored here.

CHAPTER II

Effects of spinal non-viral interleukin-10 gene therapy formulated with D-mannose in neuropathic interleukin-10 deficient mice: behavioral characterization, mRNA and protein analysis in pain relevant tissues.

Abstract

Studies show that spinal (intrathecal; i.t.) interleukin-10 (IL-10) gene therapy reverses neuropathic pain in animal models, and co-administration with the mannose receptor (MR; CD206) ligand D-mannose (DM) greatly improves therapeutic efficacy. However, the actions of endogenous IL-10 may be required for enduring pain control observed following i.t. IL-10 gene therapy, potentially narrowing the application of this non-viral transgene delivery approach. Here, we show that i.t. application of naked plasmid DNA expressing the IL-10 transgene co-injected with DM (DM/pDNA-IL-10) for the treatment of peripheral neuropathic pain in IL-10 deficient (IL-10 KO) mice results in a profound and prolonged bilateral pain suppression. Neuropathic pain is induced by unilateral sciatic chronic constriction injury (CCI), and while enduring relief of light touch sensitivity (mechanical allodynia) in both wild type (WT) and IL-10 KO mice was observed following DM/pDNA-IL-10 co-therapy, transient reversal from allodynia was observed following i.t. DM alone. In stably pain-relieved IL-10 KO mice given DM/pDNA-IL-10, mRNA for the IL-10 transgene is detected in the cauda equina and ipsilateral dorsal root ganglia (DRG), but not the lumbar spinal cord. Further, DM/pDNA-IL-10 application increases anti-inflammatory TGF-β1 and decreases pro-inflammatory TNF mRNA in the ipsilateral DRG compared to allodynic controls. Additionally, DM/pDNA-IL-10 treated mice exhibit decreased spinal pro-inflammatory mRNA expression for TNF, CCL2 (MCP-1), and for

the microglial-specific marker TMEM119. Similarly, DM/pDNA-IL-10 treatment decreases immunoreactivity for the astrocyte activation marker GFAP in lumbar spinal cord dorsal horn. Despite transient reversal and early return to allodynia in DM-treated mice, lumbar spinal cord revealed elevated TNF, CCL2 and TMEM119 mRNA levels. Both MR (CD206) and IL-10 receptor mRNAs are increased in the DRG following CCI manipulation independent of injection treatment, suggesting that pathological conditions stimulate upregulation and availability of relevant receptors in critical anatomical regions required for the therapeutic actions of the DM/pDNA-IL-10 co-therapy. Taken together, the current report demonstrates that non-viral DM/pDNA-IL-10 gene therapy does not require endogenous IL-10 for enduring relief of peripheral neuropathic pain and does not require direct contact with the spinal cord dorsal horn for robust and enduring relief of neuropathic pain. Spinal non-viral DM/pDNA-IL-10 co-therapy may offer a framework for the development of non-viral gene therapeutic approaches for other diseases of the central nervous system.

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1. Introduction

Non-viral transgene delivery is one of the least efficient methods of gene transfer for therapeutic applications (Glover et al., 2005), but due to its improved safety profile and reduced cost burden, it has been pursued for the treatment of diseases of the central nervous system (CNS) (Jayant et al., 2016). While non-opioid treatments for the control of chronic neuropathic pain are limited, one promising avenue is the application of spinal non-viral interleukin-10 (IL-10) gene therapy, an approach previously demonstrated to provide enduring pain relief in a variety of animal models (Milligan et al., 2006b; Milligan et al., 2006a; Ledeboer et al., 2007b; Sloane et al., 2009b; Soderquist et al., 2009; Soderquist et al., 2010b; Milligan et al., 2012; Dengler et al., 2014; Grace et al., 2017).

IL-10 is a powerful anti-inflammatory cytokine that pleiotropically inhibits the actions of many pro-inflammatory factors by mechanisms that include the destabilization of mRNA transcripts for the pro-inflammatory cytokines tumor necrosis factor (TNF) and interleukin-1β (IL-1β) (Moore et al., 2001; Lobo-Silva et al., 2016). Following peripheral nerve injury, a brief compensatory upregulation in IL-10 protein production is followed by decreased IL-10 expression below baseline levels in pain-relevant anatomic locations (Jancalek et al., 2010; Jancalek et al., 2011; Khan et al., 2015). Studies show that spinal non-viral IL-10 gene delivery in neuropathic animals produces pain relief through elevated spinal IL-10 production with corresponding reduction of pro-inflammatory mediators of pathological pain (Ledeboer et al., 2007b; Sloane et al., 2009a; Soderquist et al., 2010a; Dengler et al., 2014). However, whether endogenous IL-10 is required for the long-lasting pain relief observed following i.t. spinal non-viral IL-10 gene therapy remains unknown.

Additionally, the anatomical regions in the pain pathway necessary for IL-10 transgene expression that leads to pain relief are still unclear.

While a single large dose of naked plasmid DNA encoding the IL-10 transgene (pDNA-IL-10; 100μg) or repeated doses (100μg followed by ≥25μg within 3-72hr) result in transient or enduring pain relief, respectively (Milligan et al., 2006a; Ledeboer et al., 2007b; Sloane et al., 2009b), the doses used render these approaches clinically unfeasible. A novel gene delivery formulation, whereby a single co-injection of as little as 1μg of naked pDNA-IL-10 with the immune cell adjuvant D-mannose (DM), a known mannose receptor-specific (MR; CD206) ligand, greatly improves the efficacy of spinal non-viral IL-10 gene therapy in rats, allowing for stable long lasting pain relief following a single i.t. injection (Dengler et al., 2014). The MR is expressed by subpopulations of macrophages and dendritic cells, as well as by microvascular endothelial cells (Taylor et al., 2005). In the CNS, the MR is expressed by astrocytes, microglia, and some neurons (Burudi et al., 1999; Burudi and Regnier-Vigouroux, 2001), and in the PNS by Schwann cells (Baetas-da-Cruz et al., 2009). Increased MR expression is often associated with anti-inflammatory macrophages (Gordon, 2003). Macrophages and other trafficking lymphocytes (i.e. T cells), along with non-leukocytic resident cell types such as satellite glia, are present within the DRG following sciatic nerve injury and likely contribute to neuropathy (Hu et al., 2007; Hanani, 2015). Notably, MR expression is present on leukocytes (Martinez-Pomares, 2012), and MR-activation itself leads to anti-inflammatory signaling as well as transient pain relief (Dengler et al., 2014). However, the transcriptional regulation of critical pro- and anti-inflammatory cytokines and chemokines in the pain pathway following DM-mediated pDNA-IL-10 co-therapy is not known.

In the current report, we applied spinal non-viral DM/pDNA-IL-10 co-therapy to neuropathic wild type (WT) mice and IL-10 deficient (IL-10 KO) mice. Sciatic nerve chronic constriction injury (CCI) was induced, an established mouse model of peripheral neuropathy, resulting in reliable pathological sensitivity to light touch known as allodynia (Bennett and Xie, 1988; Colleoni and Sacerdote, 2010; Jaggi et al., 2011b). Both central and peripheral nervous tissues associated with the pain pathway were analyzed for IL-10 transgene mRNA, as well as transcriptional regulation of pro- vs. anti- inflammatory cytokine and chemokine mRNA and protein. The findings reported here support that DM acting as an immune adjuvant for improved spinal non-viral pDNA-IL-10 gene transfer provides a new strategy for gene therapeutics to treat chronic pain, with the potential for application to other chronic CNS diseases.

2. Materials and Methods

2.1 Animals

All experiments were performed using adult male mice (8-12 weeks of age). C57BL/6J (WT; RRID: IMSR_JAX:000664) or B6.129P2-Il10^{tm1Cgn}/J (IL-10 KO; RRID: IMSR_JAX:002251) mice were purchased from Jackson Laboratories or bred in-house from breeders also purchased from Jackson Laboratories (Bar Harbor, ME, USA). Mice were maintained in specific-pathogen free conditions confirmed negative for detection of *Helicobacter spp.* Prior to handling, all animals were acclimated to the mouse colony at the University of New Mexico (UNM) Health Sciences Center Animal Facility for a

minimum of 7 days. Animals were housed in groups of 3-5 at 23˚ ± 2˚C in light controlled rooms (12:12 light:dark; lights on at 6:00am) and fed standard rodent chow and water *ad libitum*. All procedures were approved by the Institutional Care and Use Committee (IACUC) of the UNM Health Sciences Center, conducted in accordance to the NIH Guidelines for the Care and Use of Laboratory Animals, and closely adhered to recommendations from the International Association for the Study of Pain for the use of animals in research.

2.2 Animal Model of Peripheral Neuropathy

A modification of the sciatic nerve chronic constriction injury (CCI) model developed by Bennett and Xie (Bennett and Xie, 1988) was used for application in the mouse (Costa et al., 2008; Martucci et al., 2008; Liu et al., 2017) and briefly described here. Under isoflurane anesthesia (induction at 3.0 followed by 2.0-2.5 vol.% in oxygen, 2.0L/min), the lower back and dorsal left thigh were shaved and then cleaned with diluted Bacti-Stat AE (EcoLab Health Care Division, Mississauga, Ontario, Canada), followed by water, and lastly swabbed with 70% EtOH that was allowed to air dry before proceeding. Using aseptic procedures, the left sciatic nerve was carefully isolated by gentle blunt dissection through the fascia between the *gluteus superficialis* and *biceps femoris* muscles. The exposed sciatic nerve was snuggly ligated with three segments of sterile 4-0 chromic gut suture (Ethicon; Cat#:635H) proximal to the nerve's trifurcation and without pinching of the nerve. To allow enhanced malleability of the thick suture material, thereby reducing the risk of unintended damage, segments of chromic gut material were briefly soaked in a

bath of isotonic sterile saline *(Hospira; Cat#:NDC 0404-4888-10)* prior to application. Additionally, great attention was paid to keeping the sciatic nerve moist via regular irrigations with isotonic sterile saline. Sham surgery was identical to the CCI surgery but without nerve ligation. The overlying muscle was sutured closed with one 3-0 sterile silk suture (Ethicon; Cat#:K572H). The overlying skin was closed using two Reflex^{TM} wound clips (Kent Scientific Corp.; Cat#:INS750344). Full recovery from anesthesia was observed within 10-15 minutes following surgery. At this time, mice that had undergone CCI showed minor ventroflexion of the ipsilateral hindpaw while Sham mice revealed no postural abnormalities. Animal body weights were monitored prior to and following surgery. One day after surgery, animals were monitored for wound condition, hindpaw health, and general activity level. If autotomy was present at any point during the experiments, mice were immediately euthanized (less than 1.0% of all mice).

2.3 Behavioral Assessment of Mechanical Allodynia

Hindpaw threshold responses to innocuous light mechanical touch were assessed in mice by adopting principles from the von Frey fiber test. The von Frey behavioral testing approach used in this report blends components of previously described von Frey behavioral assessment in rodents (Chaplan et al., 1994; Sommer and Schafers, 1998; Milligan et al., 2000; Bonin et al., 2014). Following habituation to the testing environment (30-45 minutes per day, 4 days), baseline (BL) responses were assessed as follows. A series of calibrated monofilaments were applied randomly to the plantar surface of left and right hindpaws for a maximum of 3.0s per application. A $\geq$30s interval was required between

applications to the same mouse. The log stiffness of the nine monofilaments used is defined as $\log_{10}$ (grams x 10,000) with the following range of filaments having log stiffness values (the value in grams is given in parentheses) of 2.36 (0.022g), 2.44 (0.028g), 2.83 (0.068g), 3.22 (0.166g), 3.61 (0.407g), 3.84 (0.692g), 4.08 (1.202g), 4.17 (1.479g), and 4.31 (2.042g). The first monofilament applied was always 3.22 (0.166g). If a positive response (i.e. lifting, licking, or shaking of the paw) was observed, then the next weaker hair was applied. In contrast, a negative response indicated that the next stronger monofilament be applied to that hindpaw in the subsequent round. This testing sequence progressed for a total of six applications per hindpaw. Testing was stopped prematurely if a positive response was observed following application of the weakest filament (2.36). This testing paradigm requires far fewer hair applications (a maximum of 6 times) to a given paw as compared to previous methods, thereby curbing artifacts caused by overstimulation. The total numbers of positive responses and negative responses at each of the tested monofilaments were used to calculate the absolute (50%) paw withdrawal threshold via the computer program PsychoFit (http://psych.colorado.edu/~lharvey; RRID: SCR_015381), as previously described (Milligan et al., 2000; Dengler et al., 2013). This software fits a Gaussian integral psychometric function to the observed withdrawal rates for each of the tested von Frey hairs using a maximum-likelihood fitting method (Milligan et al., 2000). The computed log stiffness threshold values were then used for subsequent statistical analyses, but graphical representations present data as stimulus intensity in grams. A considerable benefit of this new method of assessment and analysis is that all hairs tested, (i.e. the entire pattern of responses) collectively contribute to calculating the stimulus threshold, whereas prior approaches in mouse heavily depend on the observed response to

the final hair applied (Sommer and Schafers, 1998; Bonin et al., 2014). All behavioral hindpaw-response assessments were performed within the first four hours of the light cycle to minimize physiological influences regulated by circadian rhythms.

Behavioral assessment was conducted at BL prior to and after surgery. Timepoints for behavioral assessment on Days post-surgery were carefully chosen to avoid disruption of behavioral responses that may result from frequent testing. For the long-duration timecourse study, behavioral assessment was conducted at BL, Days 3 and 5 post-surgery, and Days post-injection 1, 3, 5, 7, 10, 14, 17, 22, 26, 30, 35, and 40. In the short-duration studies that terminated prior to tissue collection for mRNA, protein, and immunohistochemical analysis, behavioral assessment occurred at BL, Days 3 and 5 post-surgery, and Days post-injection 1, 2, 3, 4, 5, 6, 7, 10, and 12. For immunohistochemical analysis, an additional control group of IL-10 KO CCI mice (N = 4) received no injection, and was behaviorally verified at BL, and Days post-surgery 3, 5, 7, 10, and 17. Pilot studies (data not shown) were conducted using this approach to validate hindpaw response thresholds demonstrated in previous reports (Sommer and Schafers, 1998; Shimoyama et al., 2002).

2.4 Preparation of Plasmid DNA

The plasmid vector pDNA-IL-10 (also called pTR2-CB-mIL10^{F129S}) used in these studies (**Fig. 1C)** is the mouse IL-10 equivalent of a rat IL-10 plasmid that was fully described previously and is derived from an adeno-associated virus-2 (AAV-2) expression cassette (Milligan et al., 2005b; Milligan et al., 2006b). It consists of a 5.9 kilobase circular plasmid

DNA (pDNA) containing a transcriptional cassette consisting of a hybridized cytomegalovirus enhancer/chicken beta-actin promoter (CMV enh/CB pro) driving expression of the mouse Il10 gene containing a point mutation (mIL-10^{F129S}), and a simian virus 40 (SV40) polyadenylation signal (SV40 poly(A)). The transcription cassette is flanked by 149 bp inverted terminal repeat (ITR) sequences. The plasmid backbone contains an ampicillin resistance (Amp^r) gene, as well as components unique to the original AAV-2 expression cassette such as an intervening sequence (IVS; intron). The control plasmid (pDNA-Ctrl) is an analogous plasmid cassette that instead drives expression of enhanced jellyfish green fluorescent protein (eGFP) (Milligan et al., 2006b). Plasmids were amplified in SURE2 Supercompetent Cells (Agilent Technologies; Cat#:200152) because the ITR elements are often deleted in conventional E. coli strains (Milligan et al., 2006b) resulting in reduced transgene expression. Plasmid DNA was isolated using an EndoFree Plasmid Giga Kit (Qiagen; Cat#:12391) according to manufacturer's instructions. Purified endotoxin-free plasmids were resuspended in sterile Dulbecco's PBS (1x) (Gibco; Cat#:14190-144) with 3% sucrose, aliquoted, and stored at -20˚C.

2.5 Intrathecal Injections

Intrathecal (i.t.) injections were acutely administered under isoflurane anesthesia following behavioral assessment on Day 5 post-surgery and conducted as previously described (Hylden and Wilcox, 1980) with modification as indicated here. Injector units were constructed as follows. First, a needle adaptor was created by inserting an intact sterile 27G x 0.5in needle (PrecisionGlide, Becton Dickinson & Co.; Cat#:305109) into one end of a

30cm long segment of PE20 polyethylene tubing (Becton Dickinson & Co; Cat#:427406). At the opposite end, a second 27G needle with the needle hub removed was inserted such that the blunt end was inserted inside the tubing while leaving the beveled sharpened end available for lumbar puncture. These needle adaptors were placed in a sterile dry place until the time of use. At the time of injection, sterile isotonic saline (Hospira; Cat#: NDC 0404-4888-10) was used to fill the line as well as to check for leakage. Sterile isotonic saline was also used to fill a sterilized 50μL gastight calibrated syringe (Hamilton Co.; Cat#:CAL80901) which was then connected to the needle adaptor via the intact needle hub. Excess saline was expelled from the syringe leaving saline only within the tubing. The line was then loaded with the following four components in this order: (1) a 1μL air bubble is drawn into the tubing followed by (2) 3μg of plasmid DNA (in 7.5μL), (3) a second 1μL air bubble, and finally (4) either 25μg D-mannose (Sigma-Aldrich; Cat#:M6020) dissolved in 3μL of sterile isotonic saline or sterile isotonic sterile saline alone.

Under isoflurane anesthesia (induction at 2.5% followed by 1.5% volume in oxygen at 2.0 liters per minute), the mid- to lower-back was shaven and swabbed with 70% ethanol. The beveled sharpened end of the 27G needle of the injector unit was inserted percutaneously between lumbar vertebrae 5 and 6 (L5-L6). A tail flick was considered indicative of a successful i.t. puncture. Next, the i.t. injection bolus was slowly administered over the course of 30s. The total time required for each injection was ~3 minutes, excluding anesthesia induction. Following injection, all mice resumed motor activity consistent with that observed prior to i.t. injection.

2.6 Tissue Collection and Total RNA Isolation

Following behavioral assessment on Day 12 post-injection (Day 17 post-surgery), mice were deeply anesthetized (≥10min, 5% volume in oxygen at 2 liters per minute) followed by transcardial perfusion with ice-cold 0.1M phosphate buffered saline (pH = 7.40). The body was then placed on a frozen gel refrigerant pack (Glacier Ice, Pelton Shepherd Industries) previously maintained on dry ice. Rapid laminectomy followed by tissues dissection in the following order: lumbar spinal cord (L3-L6), contralateral lumbar (L3-L5) dorsal root ganglia (DRG), ipsilateral L3-5 DRG, cauda equina, and lastly ipsilateral sciatic nerve (~1cm). Samples were placed in DNase/RNase-free 1.5mL centrifuge tubes (VWR International; Cat#:47747-362), quickly frozen on dry ice, and then stored at -80˚C for future analysis.

Total RNA was extracted from ipsilateral DRG, contralateral DRG, lumbar spinal cord, and cauda equina using the miRNeasy Mini Kit (Qiagen; Cat#:217004) per manufacturer's instructions. Homogenizations were performed using a motorized VWR Disposable Pellet Mixer and cordless motor pestle system (VWR International; Cat#:47747-3). For ipsilateral sciatic nerves, special care was taken to process samples so that they could be analyzed for both RNA and protein. To accomplish this, frozen tissues were placed in 100µL chilled 1x phosphate buffered saline (10x PBS diluted to 1x with DNase/RNase free water; Sigma-Aldrich; Cat#: P7059) and quickly chopped with scissors, and then briefly homogenized with the motorized pestle system. Within 1.5min from initial chopping, 40% of the PBS-suspended homogenate was transferred to chilled Qiazol Lysis Reagent (Qiagen) and further homogenized. Total RNA was extracted using an miRNeasy

Micro Kit (Qiagen; Cat#:217084) per manufacturer's instructions but with single additional RPE and 80% EtOH wash steps in order to remove excess salts introduced by PBS. The remaining 60% of the PBS-suspended homogenate was pelleted at full speed (20,627 x g) for 1.5 minutes, the PBS aspirated, 35µL of protease inhibitor solution (MesoScale Discovery) added, frozen on dry ice, and finally stored at -80˚C for future protein analysis (see section 2.7).

RNA concentration and quality was assayed by NanoDrop (Thermo Scientific). RNA samples were then diluted to a standardized RNA concentration for the following tissues: ipsilateral DRG (30ng/µL), contralateral DRG (30ng/µL), cauda equina (100ng/µL), and ipsilateral sciatic nerve (50ng/µL), and lumbar spinal cord (100ng/µL). Total RNA reversed transcribed to cDNA per sample was 1,400ng for lumbar spinal cord, 1,000ng for cauda equina, and 525ng for ipsilateral sciatic nerve. For DRG, which had low total RNA yields due to the typically small DRG tissue volumes, 390ng of total RNA were reverse transcribed for contralateral DRG, while 165ng and 390ng were used in two separate rounds of cDNA for ipsilateral DRG. Reverse transcription was performed for DRG, lumbar spinal cord, and sciatic nerve using a SuperScript™ IV VILO™ cDNA Synthesis Kit (Invitrogen; Cat#:11754250) per manufacturer's instructions but required further optimization by extending the 42˚C-incubation step to 2hr in order to improve cDNA yields from small samples such as the DRG. Reverse transcription was performed for cauda equina using a SuperScript IV First-Strand Synthesis System (Invitrogen; Cat#:18091050) per manufacturer's instructions.

2.7 mRNA Analysis by Quantitative Real-Time PCR

Levels of mRNA expression were measured and analyzed as previously described (Mellios et al., 2014). The following dilution factors (indicated in parentheses) were applied to cDNA samples for assessment of transcripts of interest in given tissues: ipsilateral DRG (undiluted), contralateral DRG (undiluted), lumbar spinal cord (1:4), cauda equina (1:6), ipsilateral sciatic nerve (1:2.5). The following cDNA dilutions were used for assessment of the normalizer, mouse 18S rRNA: ipsilateral and contralateral DRG (1:40), lumbar spinal cord and cauda equina (1:200), ipsilateral sciatic nerve (1:100). Levels of mRNAs, as well as "Normalizer" 18S rRNA (*Rn18s*, Taqman Assay ID#: Mm03928990_g1), were assayed in triplicate via quantitative real-time PCR (qRT-PCR) with Taqman Gene Expression Assays (ThermoFisher Scientific). All selected mouse gene expression assays were identified by the manufacturer to be "best coverage" assays, unless otherwise noted, and to exclude detection of genomic DNA. mRNA levels were analyzed with the formula $C = 2\text{^}CT^{Normalizer}/2\text{^}CT^{Target}$, as previously described (Livak and Schmittgen, 2001; Schmittgen and Livak, 2008; Mellios et al., 2014).

The IL-10 Taqman Gene Expression Assay was selected to target sequences that span exons 1 and 2 (*Il10*; Taqman Assay ID#: Mm00439614_m1) in order to ensure that IL-10 transcripts detected in IL-10 KO mice truly represent exogenous transgene, versus the possible endogenous null mutant transcript. IL-10 KO mice possess a null copy of IL-10 in which a 500bp fragment of exon 1 (codons 5-55) has been replaced by a 24bp linker sequence, a *neo* expression cassette, and a termination codon (Kuhn et al., 1993).

Therefore, any potential endogenous null IL-10 transcripts are predicted to be unrecognizable by the IL-10 Taqman Gene Expression Assay.

Because mannose receptor activation by DM is sufficient to improve non-viral IL-10 gene therapy (Dengler et al., 2014), mannose receptor gene expression was assayed (*Mrc1*; Taqman Assay ID#: Mm01329362_m1). Additionally, IL-10 transgene expression is known to limit the actions of many pro-inflammatory mediators (Moore et al., 2001; Milligan et al., 2012). Consequently, transcripts for the following pain-relevant pro-inflammatory cytokines were assessed: interleukin-1β (IL-1β, *Il1b*, Taqman Assay ID#: Mm00434228_m1) and tumor necrosis factor (TNF, *Tnf*, Taqman Assay ID#: Mm00443258_m1).

To address whether IL-10 deficient knockout mice possess compensatory changes in other anti-inflammatory pathways, the anti-inflammatory cytokine transforming growth factor β1 (TGF-β1, *Tgfb1*, Taqman Assay ID#: Mm01178820_m1) was evaluated. To investigate basal and post-CCI expression levels of the IL-10 receptor in IL-10 KO mice, transcript levels for IL-10R alpha (IL-10Rα, a.k.a. IL-10 R1; *Il10ra*, Taqman Assay ID#: Mm00434151_m1) were examined.

To assess whether actions of transgenic IL-10 may modulate microglia in the lumbar spinal cord, we tested the newly identified microglial specific marker (Bennett et al., 2016) transmembrane protein 119 (TMEM119, *Tmem119*, Taqman Assay ID#: Mm00525305_m1). The “best coverage” option was not available for the TMEM119 Taqman gene expression assay.

2.8 Multiplex Determination of Cytokine and Chemokine Expression

Ipsilateral sciatic nerve homogenates (see section 2.6) previously stored in a buffer with protease inhibitors (MesoScale Discovery) were kept on ice, further homogenized using a motorized homogenization system (VWR International; Cat#:47747-3), and subsequently sonicated. Tissue samples were centrifuged at 4,200 x g at 4°C for 10min to pellet cellular debris. Cellular lysates were collected from the supernatant and protein concentrations were determined by Quickstart™ Bradford Protein Assay (BioRad; Cat#:500-0201). Sciatic nerve protein expression levels were then determined using a V-PLEX™ immunoassay (MesoScale Discovery; detailed below) panel for quantification of the following cytokines and chemokines: TNF, IL-1β, IFN-γ, IL-6, CXCL1 (a.k.a. KC/GRO), and IL-12p70. All V-PLEX™ immunoassays were conducted according to manufacturer's instructions.

The V-PLEX™ immunoassay is well validated for quantifying protein from small CNS tissue samples (Maxwell et al., 2015; Robinson et al., 2016; Noor et al., 2017). The methods are briefly described here. V-PLEX™ immunoassays apply electrochemiluminescence technology to precisely measure protein concentrations of multiple protein targets simultaneously with high sensitivity and reproducibility. Tissue lysates from experimental tissue samples, or calibrator (provided by kit), were loaded onto a 'multi-spot' plate. Each plate-well is pre-coated with antigen-specific 'capture' antibodies on independent spatially well-defined 'spots' that are in turn connected to a working electrode surface. Following incubation with protein lysates, immobilized proteins are next recognized by SULFO-TAG™-conjugated antigen-specific 'detection'

antibodies. A Quickplex SQ120 Imager (MesoScale Discovery) was used to detect signal in each well in the plate via application of an electrical current to the plate electrodes and subsequent measurement of light intensity emitted by SULFO-TAG labeling. Where possible, 35µg total protein was loaded per well in duplicate, but due to limitations in tissue availability singlets were also accepted. For some samples, especially those from Sham mice, less than 35µg total protein was available, therefore all assay outputs were normalized by dividing the measured concentration for a specific analyte (e.g. 0.12pg TNF) for a given well by the total protein loaded to that same well, yielding units of (pg Analyte)/(µg Protein). In the two conditions, CCI+DM/pDNA-IL-10 and CCI+DM/pDNA-Ctrl, pairs of "low-protein" samples were combined once per condition to make a single N with a protein load of 35µg, yielding N = 6 and N = 8, respectively. The V-PLEX™ immunoassay system has high content validity and inter-assay variations less than 12% in our laboratory.

2.9 Tissue Preparation for Immunohistochemistry

Lumbar spinal cord tissue was collected from Naïve IL-10 KO and WT mice (N = 3 mice/group), and from CCI-treated IL-10 KO mice (N = 3-4 mice/group) following behavioral assessment on Day 12 after intrathecal injection (Day 17 post-surgery). Samples were processed similarly as previously described (Dengler et al., 2014). Briefly, a lethal dose of pentobarbital (Fatal-Plus Solution, Vortech Pharmaceuticals, LTD.) was administered by intraperitoneal injection. Mice then underwent transcardial perfusion with 0.1M phosphate buffered solution (PBS; pH = 7.40) for 4min at 5mL/min, followed by

room temperature 4% paraformaldehyde (PFA; pH = 7.40) (Sigma-Aldrich; Cat#:P6148) for 4min at 5mL/min, and finally ice cold 4% PFA for 4min at 5mL/min. Entire intact spinal vertebral columns (cervical 2 to coccygeal vertebrae) were collected and post-fixed 24hr in 4% PFA at 4˚C. Columns were then washed 24hr in 0.1M PBS at 4˚C and decalcified 4 weeks in 1.5L water containing 10% ethylenediaminetetraacetic acid (EDTA; Cat#:M101; VWR International) with 0.01% sodium azide (Sigma-Aldrich; Cat#:S2002) and 0.5% PFA with gentle consistent agitation atop a stir plate at room temperature. The method was used to allow examination for intact meninges and visualization of the subarachnoid matrix. The decalcification solution was changed every 5-7 days. Lumbar 3-6 (L3-L6) spinal vertebral column segments were paraffin processed according to previously described standard methods (Wilkerson et al., 2012b) and later sliced on a microtome, with 7μm tissue sections mounted on VWR VistaVision™ HistoBond® Adhesive Slides (VWR International; Cat#:16004) and stored in slide boxes at room temperature.

2.10 Immunohistochemistry

To investigate potential changes in lumbar dorsal horn spinal astrocytic activation following intrathecal non-viral IL-10 gene therapy, we analyzed the expression of the astrocyte marker glial fibrillary acidic protein (GFAP) in L4-L5 spinal segments, as previously described (Wilkerson et al., 2012b; Noor et al., 2017). Briefly, randomly selected paraffin-processed L4-L5 spinal cord tissue sections underwent deparaffinization in Hemo-De (Scientific Safety Solvents; Cat#: HD-150A) followed by rehydration via

descending alcohols to 0.1M PBS (pH = 7.40). Antigen retrieval was applied by placing tissue slices in Nuclear Decloaker (Tris-based buffer (pH = 9.50); BioCare Medical; Cat#: CB911M) and heating the samples in a conventional rice cooker (15min, ~94˚C). After sufficient cooling, blocking was performed using 5% normal donkey serum (Jackson ImmunoResearch Labs; Cat#: 017-000-121, RRID: AB_2337258) (2hr, room temperature), followed by overnight primary antibody incubation at 4˚C with 1:1,000 chicken anti-mouse GFAP (Abcam, Cat#: ab4674, RRID: AB_304558). The next day tissues were washed 4 x 3min in 0.1M PBS (pH = 7.40) followed by a 2hr incubation at room temperature with 1:200 donkey anti-chicken Alexafluor488-conjugated secondary antibody (Jackson ImmunoResearch Labs; Cat#: 703-545-155, RRID: AB_2340375). Slices were washed 4 x 3min in 0.1M PBS and then stained with 4',6-Diamidino-2-phenylindole dihydrochloride (DAPI) (Sigma-Aldrich; Cat#:D9542) before coverslipping. All incubation steps were performed using a humidity chamber.

2.11 Microscope Spectral Imaging for Immunofluorescent Quantification

Image acquisition for spectral analysis was performed using the Nuance Multispectral Imaging System (PerkinElmer Inc.; RRID:SCR_015382) (Mansfield, 2014), as described previously (Wilkerson et al., 2012b; Dengler et al., 2014; Noor et al., 2017). Briefly, lumbar spinal cord dorsal horn images were obtained using a 20X objective with a Nikon TE-200 U inverted fluorescence microscope. Flat-field correction was applied in order to remove artifacts, including uneven field illumination, to produce a uniform illumination during image acquisition. Images of single-labeled control slides (one each for AF488 and

DAPI) and a label-free (autofluorescence) slide were used to create a 'spectral library'. Pure signals for each fluorophore were then computed by separating a known spectral profile (autofluorescence) from a 'mixed' spectrum profile (single labeled + autofluorescence). This allowed for the un-mixing of multi-labeled slides to obtain composite images containing only the labels of interest. Composite images were then used for further analysis using Slidebook 6 software (see Section 2.11).

2.12 Slidebook software image analysis

Composite images were analyzed using Slidebook 6 software (Intelligent Imaging Innovations; RRID: SCR_014300). To eliminate signals originating from artifact, an experimenter created a 'threshold mask' designed to limit the lowest-level fluorescent emissions in such a way that the final image file replicated that which was visible when the specimen was viewed through the microscope eyepiece, as described previously (Dengler et al., 2014). Additionally, an individualized 'area mask' was created for each image such that only the spinal cord dorsal horn gray matter was included in analyses, thereby excluding surrounding white matter and peri-spinal blank space. The location of nuclei, as indicated by the DAPI channel image, for each slice was used to more accurately approximate the true borders of the dorsal horn when making the 'area mask'. These masks were further refined to create a 'final analysis mask' for each slice that included only signal above the predetermined lower threshold (defined by the 'threshold mask') within the outlined region. 'Fluorescence Intensity' was calculated using the equation Fluorescence Intensity = (Sum Intensity)/(Area), where 'Sum Intensity' (the total signal measured by the

'final analysis mask,') is divided by 'Area' (the total area of the dorsal horn as indicated by the original 'area mask,' in microns squared [μm^2]). The average Fluorescence Intensity of four slices (2 slice pairs taken ~140μm apart, mounted on a single slide) from one slide per animal was calculated to determine the value for each respective animal. Data from individual animals were analyzed as indicated in section 2.12, below.

2.13 Experimental Design and Statistical Analysis

To determine the efficacy of the DM/pDNA-IL-10 formulation in mice and to test whether endogenous IL-10 is required for long-lasting pain reversal, a complete timecourse of non-viral IL-10 efficacy for controlling allodynia was assessed in IL-10 KO mice compared to WT mice (N = 6-8 mice/group). In this long-duration timecourse, animals were followed behaviorally until Day 45 post-surgery (Day 40 post-injection), a timepoint by which pilot experiments (data not shown) indicated both IL-10 KO and WT mice reveal complete spontaneous reversal from CCI-induced allodynia. No tissues were collected.

All subsequent behavioral studies were performed using only IL-10 KO mice in order to (1) replicate initial observations, (2) to better understand the biodistribution of IL-10 transgene mRNA, (3) to examine the underlying mechanisms of long-lasting pain reversal independent of endogenous IL-10, and (4) to further explore the role of D-mannose in improved efficacy of non-viral transgene uptake and expression.

Behavioral results from the long-duration characterization were used to design a truncated timecourse such that tissues could be collected at a time when therapeutically treated neuropathic IL-10 KO mice had achieved stable reversal from allodynia (Day 12

post-injection; Day 17 post-surgery). In the first replication study (behavioral N = 6-12 mice/group), the following conditions were manipulated: surgery (Sham vs. CCI), plasmid DNA (pDNA-IL-10 vs. pDNA-Control), and adjuvant (DM vs. Saline). Collected tissues were analyzed by qRT-PCR (N = 5-8 mice/group). For ipsilateral sciatic nerve, samples from each animal were split for use in both qRT-PCR (Section 2.6) mRNA analysis and V-PLEX^{TM} Immunoassay protein expression analysis (Section 2.7), and assessed typical injury-associated pro-inflammatory changes following peripheral nerve damage (Okamoto et al., 2001; Kleinschnitz et al., 2006; Uceyler et al., 2007). All corresponding tissues were collected from naïve WT (N = 3) and IL-10 KO (N = 2) mice to serve as IL-10 expression positive and negative controls, respectively, and to identify potential baseline transcriptional differences that may result from developmental IL-10 deficiency.

In a third study designed to replicate behavioral non-viral IL-10 efficacy for controlling allodynia, tissues were collected to assess changes in astrocytic activation markers at the level of the lumbar spinal cord. IL-10 KO mice given CCI-surgery were behaviorally verified and followed through Day 12 post-injection (N = 4 mice/group). On Day 12 post-injection (Day 17-post-surgery), the entire spinal cord enclosed within the vertebral column was collected from each mouse for subsequent immunohistochemical analysis. Decalcification and paraffin-embedding procedures were followed as described above. Prior work examining immunoreactive markers for spinal cord astrocytes following decalcification and paraffin-embedding procedures demonstrated through power analysis (Noor et al., 2017) and previously documented reports using similar methods (Wilkerson et al., 2012a; Wilkerson et al., 2012b; Noor et al., 2017) that N = 3 spinal cords per experimental condition was sufficient to yield reliable group differences. Therefore, to

minimize unnecessary duplication, animal numbers were restricted to N = 3-4 per experimental condition.

All baseline behavioral data of hindpaw threshold responses were analyzed by One-way analysis of variance (ANOVA) using GraphPad PRISM version 7.02 (GraphPad Software Inc.; RRID:SCR_002798) to ensure no group differences were present at baseline, as well as to confirm complete spontaneous reversal from allodynia on Day 40 post-injection. Two-way repeated measures (RM) ANOVA using SPSS (IBM; RRID: SCR_002865) was performed for all other behavioral timepoint analyses. The assumption of sphericity for Two-way RM ANOVAs was assessed using Mauchly's Test of Spericity ($\alpha = 0.05$). If the assumption of sphericity was violated ($P < 0.05$), to protect against Type I errors, the reported degrees of freedom and p-values were adjusted using the conservative Greenhouse-Geisser correction. Data from microscope-acquired images reflecting immunoreactivity, relative mRNA transcript levels from qRT-PCR, and protein V-PLEX^{TM} immunoassays were analyzed using One-way ANOVA with GraphPad PRISM. To control the type I error rate during all multiple comparisons, Fisher's LSD test (reported with adjusted P values) was applied for *post hoc* examination of possible group differences selected *a priori*. The threshold for statistical significance for all sets of multiple comparisons was set *a priori* to $\alpha = 0.05$. All data are presented as the Mean $\pm$ Standard Error of the Mean (SEM). For mRNA and protein analyses, within-group outliers where tested for by Grubbs' Test using the GraphPad QuickCalc Outlier Calculator (https://graphpad.com/quickcalcs/grubbs1/) with $\alpha = 0.05$.

3. Results

3.1 Intrathecal non-viral IL-10 gene therapy provides stable long-lasting relief of allodynia in mice, and is efficacious in the absence of endogenous IL-10.

Previous investigations (Milligan et al., 2006b; Milligan et al., 2006a; Ledeboer et al., 2007b; Sloane et al., 2009b; Soderquist et al., 2010b; Dengler et al., 2014) of non-viral IL-10 gene therapy were conducted in rodent models that were capable of expressing endogenous IL-10. Consequently, these studies were not able to unambiguously demonstrate whether enduring non-viral therapeutic efficacy relies on the actions of endogenous IL-10. To address this possibility, long-duration efficacy of i.t. non-viral IL-10 gene therapy was examined in IL-10 deficient mice, that is, IL-10 KO mice. In addition, D-mannose has previously been shown to improve the therapeutic efficacy of i.t. non-viral IL-10 gene delivery in rats for the treatment of allodynia (Dengler et al., 2014). However, the possibility that DM induces endogenous IL-10, acting as an immune adjuvant to enhance phagocytosis of pDNA-IL-10, suggests that endogenous IL-10 may be necessary for enduring pDNA-IL-10 efficacy. Therefore, the therapeutic efficacy DM/pDNA-IL-10 gene therapy in IL-10 KO vs. WT mice was examined in the current report.

Light mechanical touch assessed at BL revealed similar levels of hindpaw sensory threshold responses between all groups (**Fig. 1A-D**), which demonstrates that basal IL-10 is not required to maintain normal healthy sensory responses. Following surgery, CCI-treated WT and IL-10 KO mice develop clear bilateral allodynia by Day 3-5 post-surgery. All groups received an i.t. injection following behavioral assessment on Day 5 post-surgery

(**Fig 1A-B**). By Day 2 post-injection, both IL-10 KO and WT CCI-treated mice given DM/pDNA-IL-10 reveal similar bilateral reversal of allodynia compared to CCI-treated mice given Saline/pDNA-IL-10, suggesting that possible additional actions of endogenous IL-10 in WT mice are not outwardly observable following gene therapy. Both WT and IL-10 KO CCI control groups given Saline/pDNA-Ctrl demonstrate spontaneous reversal of allodynia, with hindpaw responses similar to those observed in Sham-treated mice, on Day 35 post-injection (Day 40 post-surgery). However, both WT and IL-10 KO mice treated with CCI+DM/pDNA-IL-10 exhibit long-lasting and stable pain relief, never returning to allodynia.

Lastly, it is additionally important to note that the non-silent point mutation present in the mouse IL-10 gene (IL-10^{F129S}) used in the current study and previously characterized in rat (Milligan et al., 2006b; Milligan et al., 2006a; Ledeboer et al., 2007b; Sloane et al., 2009b; Soderquist et al., 2010b; Dengler et al., 2014) supports predicted IL-10 protein-receptor interaction. That is, IL-10 protein homodimerizes prior to binding to and activating the IL-10 receptor (Moore et al., 2001). The current report demonstrates that the IL-10^{F129S} product does not require interaction with endogenous wild type IL-10 protein to achieve stable and enduring IL-10 transgene efficacy. This is the first demonstration that both non-viral IL-10 gene therapy and DM, the non-viral gene therapy adjuvant, are efficacious in mice and do not require endogenous IL-10.

3.2 The combination of DM/pDNA-IL-10 is necessary for long-lasting relief of allodynia

To better understand the effective relationship between pDNA-IL-10 and DM to induce long-lasting pain relief, i.t. DM/pDNA-IL-10 co-therapy was compared to various control conditions in neuropathic IL-10 KO mice (**Fig. 2A-B**). Threshold responses at BL were similar between all groups for both the ipsilateral and contralateral hindpaws, and following surgery, Sham-treated mice maintain normal levels of light touch sensitivity, replicating that observed in **Fig. 1**. CCI-treated mice exhibit bilateral allodynia by Day 3 post-surgery, and CCI+Saline/pDNA-Ctrl-treated mice maintain stable allodynia through Day 12 post-injection. Surprisingly, CCI mice given DM with control plasmid DNA (lacking IL-10 transgene) exhibited delayed onset bilateral reversal from allodynia beginning on Day 3 post-injection. However, reversal of allodynia was transient, with increased hindpaw light touch sensitivity occurring by Day 6 post-injection and full allodynia by Day 12 post-injection. The data presented here support that DM-mediated relief of pathological light touch sensitivity is independent of IL-10. Overall, these data reveal that it is the combination of pDNA-IL-10 plus DM that is required for long-lasting relief of allodynia.

3.3 I.t. non-viral IL-10 transgene mRNA is expressed in lumbar dorsal root ganglia

To investigate the IL-10 expression patterns that underlie i.t. non-viral IL-10 transgene-mediated pain relief in the absence of endogenous IL-10, nervous tissues were collected from behaviorally verified IL-10 KO mice (See section 3.2) on Day 12 post-injection (Day

17 post-surgery). This "midway" timepoint was chosen for tissue analysis because CCI+DM/pDNA-IL-10 mice are stably reversed from allodynia compared to all other CCI-treated groups (**Fig. 2A-B**). IL-10 transgene expression was examined in relevant "pain pathway" tissues, including ipsilateral and contralateral lumbar (L3-5) DRG, cauda equina, lumbar spinal cord, and ipsilateral sciatic nerve. Of note, the i.t. injections were performed such that pDNA was deposited into the spinal subarachnoid area at the level of the cauda equina (acute transcutaneous puncture between vertebral L5 and L6 interspinous processes). In IL-10 KO Naïve (no surgery and no injection) mice, IL-10 mRNA was undetectable in ipsilateral and contralateral DRG, but was present in samples from WT Naïve mice serving as positive controls (**Fig2C-D**). In ipsilateral DRG (**Fig. 2C**), IL-10 transgene mRNA was significantly elevated in pain relieved IL-10 KO+CCI+DM/pDNA-IL-10 mice compared to chronically allodynic CCI+DM/pDNA-Ctrl controls and un-injured Sham controls. In contralateral DRG (**Fig. 2D**), IL-10 transgene expression was not significantly different across treatment groups. However, an *a priori* comparison revealed that contralateral DRG from CCI-treated mice given DM co-treated with the IL-10 transgene contained significantly greater IL-10 mRNA levels than DRG from CCI-treated mice given DM with the control plasmid ($P < 0.05$) or Sham-treated mice with DM and the control plasmid ($P < 0.05$). Similar findings in the cauda equina (**Fig. 2E**) revealed IL-10 transgene was significantly increased in pain-relieved CCI-treated mice given DM plus the IL-10 transgene compared to either CCI-treated mice given DM plus the control plasmid or Sham-treated mice given DM plus the control plasmid. While IL-10 mRNA levels were not significantly different between WT Naïve and IL-10 KO Naïve cauda equina samples, a strong trend for positive expression in WT animals was observed ($P =$

0.06). Surprisingly, IL-10 transgene mRNA was not detectable in lumbar spinal cord (**Fig. 2F**) of any experimental condition, though it was readily detectable from spinal cords of WT Naïve positive controls. As expected, IL-10 mRNA levels were not detected in ipsilateral sciatic nerve in any treatment condition, nor were IL-10 mRNA levels detected in the uninjured WT Naïve tissue (data not shown; WT Naïve vs. IL-10 KO Naïve, $P = 0.226$). Similarly, though IL-10 protein was detected in ipsilateral sciatic nerve tissue of WT Naïves (**Fig. 2G**), it was not observed in IL-10 KO sciatic nerve tissues from any treatment condition. These data demonstrate that long-duration relief of allodynia is likely mediated by long-term survival of the IL-10 plasmid and corresponding protein expression in the DRG. Additionally, non-viral gene therapeutic modulation of the lumbar DRG is sufficient to relieve bilateral light touch sensitivity following unilateral peripheral nerve damage.

3.4 IL-10 transgene mediates pain relief through anti-inflammatory changes in ipsilateral lumbar DRG.

To further characterize the cytokine environment in the presence of elevated IL-10 transgene mRNA levels, the mRNA expression levels of pro- and anti-inflammatory cytokines in the ipsilateral and contralateral lumbar (L3-L5) DRG were assessed in tissues collected on Day 12 post-injection (See section 3.3). Interestingly, significantly greater mRNA levels for the pro-inflammatory TNF (*Tnf*) were observed in ipsilateral DRG of IL-10 KO Naïves compared to WT Naïves (**Fig. 3A**), but not in contralateral DRG (**Fig. 3B**). In addition, while TNF mRNA transcript levels were elevated in ipsilateral DRG following

CCI, significantly less TNF mRNA expression was observed in pain-relieved mice treated with DM plus the IL-10 transgene compared to allodynic CCI-treated mice given Saline plus the IL-10 transgene (**Fig. 3A**). No differences in TNF mRNA levels were detected in un-injured contralateral DRG (**Fig. 3B**). Expression of mRNA for the pro-inflammatory cytokine IL-1β (*Il1b*) in ipsilateral DRG was similar across all conditions and was not different between WT Naïve and KO Naïve mice (**Fig. 3E**).

Given existing evidence that the anti-inflammatory cytokine TGF-β1 relieves neuropathic pain in animal models and is increased by IL-10, the current study examined potential changes in DRG TGF-β1 (*Tgfb1*) mRNA levels as a consequence of DM/pDNA-IL-10 gene therapy. TGF-β1 mRNA expression is significantly elevated in ipsilateral DRG (**Fig. 3C**) of only pain-relieved CCI+DM/pDNA-IL-10 mice. Additionally, allodynic CCI+Saline/pDNA-IL-10 mice have significantly less TGF-β1 mRNA expression compared to both non-injured Sham controls and CCI+DM/pDNA-IL-10 mice. In contrast, no significant changes in TGF-β1 mRNA levels are observed in the contralateral DRG (**Fig. 3D**).

Several striking aspects of the behavioral response to the DM plus pDNA-IL-10 co-therapy are: 1) the dramatic improvement of enduring pain control over the individual treatment of DM or pDNA-IL-10, and 2) the complete, albeit transient, allodynic reversal following DM treatment alone (no IL-10 transgene). These results support that the mannose receptor (MR; CD206) is not simply promoting phagocytosis of surrounding material, but rather MR-mediated signaling is able to induce significant changes in the DRG microenvironment. Therefore, to confirm that the DRG environment is amenable to DM treatment strategies, mRNA levels for the mannose receptor (*Mrc1*) were assessed. In

addition, alterations in IL-10 receptor (IL-10R) expression could exist in IL-10 KO mice thereby underlying unforeseen behavioral adaptations. Thus, comparisons of mRNA levels for IL-10Rα (a.k.a. IL-10R1; *Il10ra*), the IL-10R component responsible for binding IL-10, between WT and IL-10 KO Naïve mice, and between various gene therapy treatment groups were assessed in the ipsilateral lumbar DRG (**Fig. 3F-G**). While there were no significant differences for MR or IL-10Rα mRNA levels between WT and IL-10 KO Naïves, mRNA expression levels for both IL-10Rα and MR were significantly elevated in the ipsilateral DRG following peripheral nerve injury (i.e. CCI). Surprisingly, though stably pain-relieved CCI+DM/pDNA-IL-10 mice had elevations in MR mRNA expression compared to Sham controls, their levels were significantly less than those observed in allodynic CCI+Saline/pDNA-IL-10 controls. Similarly, MR mRNA levels were also decreased in mice that had undergone transient pain reversal following DM treatment (CCI+DM/pDNA-IL-10). Together, these findings support that non-viral IL-10 transgene therapy acts at the level of the ipsilateral lumbar DRG to promote an anti-inflammatory environment and consequent relief of allodynia. Given that MR mRNA expression is elevated following CCI, these data further suggest that an initial pro-inflammatory environment may be beneficial for the "transgene adjuvant" effects of DM.

3.5 Transcriptional and protein characterization of the CCI-damaged sciatic nerve: i.t. non-viral gene therapy relieves pain in IL-10 KO mice despite an ongoing pro-inflammatory environment at the level of the sciatic nerve

Ipsilateral sciatic nerve collected on Day 12 post-injection (See section 3.3) was analyzed for typical injury-associated pro-inflammatory transcriptional changes following peripheral nerve damage (**Fig. 4**). There were no significant differences observed between WT Naïve and IL-10 KO Naïve mice for any mRNA transcript examined ($P > 0.05$). The transcript levels for the key pro-inflammatory cytokines TNF and IL-1β (**Fig. 4A-B**), and the pro-inflammatory chemokine CCL2 (*Ccl2*) (**Fig. 4C**) were significantly elevated in all IL-10 KO CCI conditions compared to Shams. To further assess typical immune cellular responses to sciatic nerve injury, markers for macrophages and T cells were examined. mRNA levels for the macrophage marker CD11b (*Itgam*) were significantly elevated compared to Sham-treated mice (**Fig. 4D**). Additionally, mRNA transcript levels for the general T cell marker CD3e (*Cd3e*) were elevated in CCI mice compared to Shams (**Fig. 4E**). mRNA transcript levels for the anti-inflammatory cytokine TGF-β1 were examined (**Fig. 4F**) as a possible compensatory cytokine in the absence of endogenous IL-10. *Tgfb1* transcript expression was significantly elevated in all CCI conditions following injury.

While mRNA analysis provides strong evidence for the presence of key factors, their protein products could be quickly degraded resulting in little to no physiological impact. Consequently, complementary protein analysis provides a balanced approach to characterizing the damaged sciatic nerve. Here, multiplex protein analysis of injury-associated pro-inflammatory chemokines and cytokines in the sciatic nerve ipsilateral to

the CCI surgery were examined in samples from the same animals previously assessed for injury-induced changes in mRNA (**Fig. 4G-L**). While no significant differences between WT Naïve and IL-10 KO Naïve nerves for any protein target examined were observed (data not shown; $P > 0.05$), not surprisingly, pro-inflammatory TNF protein levels were elevated in all CCI conditions (**Fig. 4G**). Additionally, the detected protein levels for the pro-inflammatory cytokines IL-1β, IFN-γ, and IL-6 were significantly elevated in both allodynic CCI+Saline/pDNA-IL-10 mice and non-allodynic CCI+DM/pDNA-IL-10 mice compared to Sham controls (**Fig. 4H-J**). CXCL1, a neutrophil chemoattractant molecule, was elevated in all CCI conditions compared to Sham controls (**Fig. 4K**). No differences in bioactive IL-12p70, a pro-inflammatory cytokine downstream of CpG-activated TLR-9, were observed (**Fig. 4L**).

Together, these mRNA and protein observations support that all IL-10 KO CCI animals experienced similar local responses to peripheral nerve damage regardless of i.t. gene therapy manipulation. Furthermore, the pain-suppressive actions of intrathecally delivered DM and IL-10 transgene co-therapy appear to occur in the DRG in IL-10 KO mice, despite a potent pro-inflammatory milieu at the level of the sciatic nerve.

3.6 I.t. non-viral IL-10 gene therapy alters lumbar cytokines and microglial activation.

To assess the spinal impact of DRG-mediated IL-10 transgene expression, mRNA levels were assessed in lumbar spinal cord tissue collected on Day 12 post-injection (See section 3.3). Levels of mRNA for the pro-inflammatory cytokine TNF (*Tnf*) (**Fig. 5A**) and the pro-inflammatory chemokine CCL2 (*Ccl2*) (**Fig. 5C**) were significantly elevated in allodynic

mice given non-IL-10 control gene therapy, while spinal cords from pain-relieved IL-10 gene therapy-treated mice (CCI+DM/pDNA-IL-10) revealed a significant decrease in TNF and CCL2 mRNA compared to Sham-treated controls. Additionally, significant decreases for both targets were observed in pain-relieved mice compared to allodynic CCI+Saline/pDNA-IL-10 controls. (**Fig. 5B**) Surprisingly, no significant differences in pro-inflammatory cytokine IL-1β (*Il1b*) mRNA transcript levels were observed between any condition.

Interestingly, though mRNA levels for the anti-inflammatory cytokine TGF-β1 (*Tgfb1*) were found to be elevated only in ipsilateral DRG from pain-relieved DM/pDNA-IL-10 mice (**Fig. 3D**), no significant differences in *Tgfb1* levels were observed in the lumbar spinal cord for any condition (**Fig. 5D**).

Spinal microglial activation is a critical component of pathologic pain (Hughes et al., 2009) but has not yet been examined following naked non-viral IL-10 gene therapy. A previously described novel transmembrane protein 119 (TMEM119) identifies solely microglia (Bennett et al., 2016). Though TMEM119 has not been previously identified as a marker of microglial activation, we chose to examine changes in TMEM119 mRNA expression to assess whether spinal non-viral IL-10 gene therapy reduces pathological microglial activation. Examination of TMEM119 (*Tmem119*) mRNA (**Fig. 5E**) revealed spinal cords from allodynic CCI-treated mice given control gene therapy treatment (CCI+DM/Ctrl or CCI+Saline/IL-10) have significantly elevated *Tmem119* transcript levels compared to Sham controls. However, Tmem119 mRNA expression is decreased in pain-relieved (CCI+DM/pDNA-IL-10) mice compared to allodynic CCI+Saline/pDNA-IL-10 controls. This evidence suggests that changes in expression of TMEM119 may serve

as a useful marker of both pathologic microglial activation and a biomarker of therapeutic efficacy. Furthermore, these data support that non-viral DM/pDNA-IL-10 gene therapeutic actions at the level of the DRG lead to decreased microglial activation at the level of the lumbar spinal cord.

Together, these data support that the therapeutic actions of IL-10 transgene at the level of the ipsilateral DRG lead to decreased lumbar spinal microglial activation in parallel with decreased spinal production of classic pain-relevant pro-inflammatory cytokines and chemokines.

3.7 I.t. non-viral IL-10 gene therapy decreases astrocyte activation in the dorsal horn of the lumbar spinal cord.

While lumbar spinal glial activation has been extensively examined in a variety of animal models (Garrison et al., 1991; Meller et al., 1994; Watkins et al., 1997; Colburn et al., 1999; Sweitzer et al., 1999; Hashizume et al., 2000; Milligan et al., 2001; Watkins and Maier, 2002), the underlying glial mechanisms at the level of the lumbar spinal cord following naked non-viral IL-10 gene therapy were previously unknown. It has not yet been characterized whether subarachnoid IL-10 gene therapy that alleviates allodynia also reduces L3-L5 dorsal horn astrocyte activation. Therefore, the current study aimed to examine whether subarachnoid IL-10 gene therapy that alleviates allodynia in behaviorally verified mice also reduces L3-L5 dorsal horn astrocyte activation as indicated by immunoreactive levels of glial fibrillary acidic protein (GFAP). GFAP is an astrocytic protein that increases in expression as astrocyte activation increases. At BL prior to surgery

and i.t. injection, all mice revealed similar responses to light mechanical touch (**Fig. 6A-B**). CCI-treated IL-10 KO mice develop clear bilateral allodynia by Day 3 post-surgery. All groups received an i.t. injection following behavioral assessment on Day 5 post-surgery. On Day 2 post-injection, mice given DM/pDNA-IL-10 reveal bilateral reversal of allodynia compared to CCI mice treated with various control transgene injections. As before, IL-10 KO mice treated with CCI+DM/pDNA-IL-10 exhibit stable pain relief for the remainder of the timecourse. Mice given i.t. DM/Saline or i.t. DM/pDNA-Ctrl reveal transient pain reversal beginning on Day 3 post-injection, and returning to allodynia beginning on Day 6 post-injection. The additional control group of un-injected IL-10 KO CCI mice (N = 4) was behaviorally verified and revealed bilateral allodynia on Day 17 post-surgery with the following stimulus intensity thresholds (Mean $\pm$ SEM) 0.032g $\pm$ 0.004g and 0.048g $\pm$ 0.009g for the left and right hindpaws, respectively (data not shown).

Following behavioral assessment on Day 12 post-injection, lumbar spinal tissues were collected and assessed for GFAP immunoreactivity (IR) by immunohistochemistry. Representative photomicrographs (**Fig. 6C-J**) of ipsilateral lumbar dorsal horn are shown. As expected, GFAP IR is present at low levels in Naïve conditions, but notably elevated in most CCI conditions. Importantly, GFAP IR for pain relieved CCI+DM/pDNA-IL-10 mice reveals GFAP IR levels similar to those observed for Naïve mice. Analysis of (**Fig. 6K**) ipsilateral and (**Fig. 6L**) contralateral (contralateral lumbar dorsal horn images not shown) lumbar dorsal horn GFAP IR quantification show no significant difference between WT and IL-10 KO Naïves. However, significantly more GFAP fluorescence intensity is observed in the ipsilateral dorsal horn following CCI surgery, with no significant difference between CCI alone, CCI+Saline, and CCI+DM/Saline groups. Curiously, the greatest

levels of GFAP IR in the spinal cord were observed bilaterally in control therapy treated CCI-mice (DM/pDNA-Ctrl and Saline/pDNA-IL-10), which are also significantly greater than those observed in pain-relieved DM plus IL-10 gene co-therapy treated mice (CCI+DM/pDNA-IL-10).

Several key comparisons, were predicted, *a priori,* to be significantly different. Unpaired t-test revealed differences between CCI+DM/pDNA-IL10 vs. CCI alone ($P < 0.001$) and CCI+Saline ($P < 0.05$). Conversely, GFAP IR levels for CCI+DM/pDNA-IL-10 mice are strikingly similar (not significantly different) to Naïve conditions. The heightened GFAP IR observed in DM/pDNA-Ctrl and Saline/pDNA-IL-10 treated mice supports the possibility that the IL-10 transgene does not influence GFAP IR. Rather, it is hypothesized that pDNA itself (either lacking or encoding IL-10) at the DRG creates additional pro-inflammatory signaling downstream of TLR-9 activation via unmethylated CpGs present in the plasmid vector (Hughes et al., 2009). Indeed, pDNA treatment leads to increased production of pro-inflammatory mediators (Hughes et al., 2009). Thus, elevated DRG pro-inflammatory drive results in elevated spinal GFAP. Though pain-relieved DM/pDNA-IL-10 treated mice were also exposed to unmethylated CpGs, these mice additionally received D-mannose. That is, MR activation, in conjunction with IL-10 transgene expression, increases TGF-β1 mRNA transcription in the DRG resulting in greater anti-inflammatory drive from the DRG with a consequent reduction of the pro-inflammatory drive to the spinal cord. Thus, reduced spinal astrocyte activation following DM/pDNA-IL-10 co-therapy is a predicted result despite the presence of CpG DNA.

Therefore, these data support that i.t. non-viral gene therapy leading to DRG expression of IL-10 transgene modulates pro-inflammatory factors in the lumbar spinal cord dorsal horn, supporting an anti-inflammatory spinal milieu.

4. Discussion

While i.t. non-viral IL-10 gene therapy is efficacious for the treatment of chronic pain in several rat models of peripheral neuropathy (Milligan et al., 2006b; Milligan et al., 2006a; Ledeboer et al., 2007b; Sloane et al., 2009b; Sloane et al., 2009a; Milligan et al., 2012; Dengler et al., 2014), the current data provide new insight into underlying mechanisms by which a unique non-viral vector formulation results in robust and enduring pain control. Here, the unique combination of delivering D-mannose (DM) with non-viral plasmid DNA encoding the IL-10 transgene (DM/pDNA-IL-10) unambiguously demonstrates that endogenous IL-10 is not required for enduring and profound pain reversal following DM/pDNA-IL-10 co-therapy. Additionally, the biodistribution of transgene IL-10 mRNA expression following i.t. DM/pDNA-IL-10 co-therapy in IL-10 deficient mice is robustly observed in the ipsilateral DRG and cauda equina, but not in the lumbar spinal cord. The corresponding pain-associated pro-inflammatory cytokine TNF mRNA expression is decreased in pain-relieved mice given DM/pDNA-IL-10 co-therapy compared to allodynic controls. In contrast, the anti-inflammatory cytokine TGF-β1 mRNA expression is increased in the DRG of gene therapy-treated pain-reversed animals. Robust mRNA changes observed in the DRG of neuropathic mice prompted examination of mRNA levels for the mannose receptor (MR) and the IL-10 receptor (IL-10Rα), both of which are

significantly upregulated following peripheral nerve damage, supporting their availability and action for DM/pDNA-IL-10 co-therapy in treatment of pathological conditions.

IL-10 transgene mRNA expression is predominantly present in the DRG and not in the lumbar spinal cord, while dramatic pain-related cytokine changes are observed in the lumbar spinal cord. Specifically, mRNA levels for TNF and the pro-inflammatory chemokine CCL2, but not IL-1β or TGF-β1, are upregulated in spinal cords of allodynic CCI mice. In contrast, pain-relieved mice given DM/pDNA-IL-10 co-therapy have spinal levels of TNF and CCL2 mRNA no different from uninjured Sham controls. Upon examination of the lumbar spinal mRNA expression for the microglial-specific transmembrane protein 119 (TMEM119), previously reported to upregulate upon increased microglial activity (i.e. proliferation) (Bennett et al., 2016), the activation state of microglia is significantly reduced in DM/pDNA-IL-10-treated pain-reversed mice. Similarly, immunohistochemical analysis for changes in astrocyte activation in the dorsal horn of the spinal cord reveal a profound reduction in GFAP immunoreactivity in DM/pDNA-IL-10 treated pain-reversed mice. Importantly, cytokine mRNA and protein levels from the injured mouse sciatic nerve reveal equivalently strong upregulation of pro-inflammatory cytokines, as well as general monocyte and T cell involvement regardless of injection formulation. Thus, these data support that i.t. non-viral DM/pDNA-IL-10 co-therapy provides stable and long-lasting relief from peripheral neuropathic pain through anti-inflammatory actions at the level of the DRG and consequent anti-inflammatory actions at the level of the lumbar spinal cord.

4.1 Endogenous IL-10 is not required for efficacy of therapeutic mouse IL-10 gene containing a known non-silent point mutation: mIL-10^{F129S}

We show for the first time that the known point mutation present in the mouse IL-10 (mIL-10^{F129S}) transgene expressed by the pDNA-IL-10 used in these studies (Fig 1E) does not require endogenous IL-10 to exert IL-10 action. IL-10 monomers associate as homodimers prior to binding with the interleukin-10 receptor via IL-10Rα (a.k.a. IL-10R1) (Moore et al., 2001). Though this point mutation lies outside the IL-10 dimer's known receptor binding region, it may still interfere with IL-10 homodimerization resulting in the possibility that the IL-10^{F129S} protein could require wildtype IL-10 for adequate dimerization, receptor binding, and downstream anti-inflammatory function. However, preliminary *in vitro* work using macrophages and B cells suggest IL-10^{F129S} may result in greater anti-inflammatory actions than wildtype IL-10 alone (Sloane et al., 2009b). While it is currently unknown whether pDNA-IL-10^{F129S} lacks the requirement for dimerization, the findings in the current report demonstrate two critical issues: that 1) wildtype IL-10/IL-10^{F129S} heterodimerization is not required for IL-10^{F129S} efficacy, and 2) exogenous mIL-10^{F129S} produces profound stable and long-lasting pain suppressive effects in the absence of endogenous wildtype IL-10.

4.2 The immune system has built-in anti-inflammatory redundancy.

An interesting behavioral finding in the current report is that loss of endogenous IL-10 may not lead to dramatic susceptibility to injury-induced pain pathology. This is surprising

given that IL-10 deficiency and IL-10R mutations observed in humans and animal models (i.e. IL-10 KO mice and IL-10R KO mice) are associated with increased risk of enterocolitis (Kuhn et al., 1993; Ward et al., 1996; Spencer et al., 1998). In these cases, absence of IL-10 signaling leads to loss of intestinal mucosal immune homeostasis and consequent enhanced intestinal inflammation in response to the presence of gut microbiota (Shouval et al., 2014). In the current report, no sensory differences were observed between WT and IL-10 KO mice at BL, which is consistent with previous findings that IL-10 KO mice do not differ in their basal light touch sensitivity thresholds (da Silva et al., 2015; Siqueira Mietto et al., 2015; Krukowski et al., 2016), though there is some evidence that uninjured IL-10 KO mice may differ in their thermal nociceptive response (Tu et al., 2003). There are mixed reports regarding intensity and duration of allodynia in IL-10 KO mice following various models of injury (da Silva et al., 2015; Siqueira Mietto et al., 2015; Krukowski et al., 2016). For example, in a paclitaxel model of peripheral neuropathic pain, WT and IL-10 KO mice exhibited both a similar onset of hindpaw mechanical allodynia and a similar peak intensity of allodynia. However, the duration of the timecourse of allodynia was extended in paclitaxel-treated IL-10 KO compared to their WT counterparts (Krukowski et al., 2016). In a model of intramuscular carrageenan, no differences in IL-10 KO vs. WT were observed for allodynic intensity or timecourse of duration in response to non-noxious cutaneous or muscular stimulation (da Silva et al., 2015). In a sciatic nerve crush model, the onset and duration of allodynia in WT vs. IL-10 KO mice was similar, though IL-10 KO mice were significantly more allodynic on the last day (Day 28 post-surgery) of the reported timecourse (Siqueira Mietto et al., 2015). Data from the current report indicate a similar onset, intensity, and duration of allodynia following peripheral

nerve damage in IL-10 KO mice compared to WT mice, and both WT and IL-10 KO CCI mice given control gene therapy remain stably responsive throughout the entire timecourse. However, it is possible that the intensity of allodynia could be greater in IL-10 KO mice relative to WT mice, as the behavioral assessment assay may be limited in detecting maximal hindpaw sensitivity.

The similar levels of allodynia observed between IL-10 KO and WT mice following CCI may be a result of the functional redundancy that occurs within the immune system. Compensatory anti-inflammatory mechanisms could be engaged that reduce the enhanced allodynia one would have predicted in an IL-10 KO model of CCI. While the data reported here reveal a potent pro-inflammatory mRNA and protein environment at the level of the sciatic nerve congruent with previous characterizations of WT mice following sciatic CCI (Okamoto et al., 2001; Kleinschnitz et al., 2006; Uceyler et al., 2007), a clear concurrent upregulation of anti-inflammatory TGF-β1 mRNA was observed in the ipsilateral DRG of pain relieved DM/pDNA-IL-10 co-therapy treated mice.

One possible mechanism by which i.t. non-viral DM/pDNA-IL-10 co-therapy generates enduring pain suppression is via consequent elevated TGF-β1 expression in the DRG. TGF-β1 and IL-10 have bidirectional regulation, as prior work demonstrates astrocytes previously stimulated *in vitro* with lipopolysaccharide (LPS; a potent TLR-4 agonist) can be redirected by incubation with IL-10 to produce TGF-β (Norden et al., 2014). Additionally, IL-10 prevents enterocolitis through increased TGF-β production (Fuss et al., 2002). In turn, TGF-β promotes IL-10 upregulation in macrophages (Maeda et al., 1995) and is known to induce differentiation of IL-10-producing regulatory T cells (iTregs) and T regulatory type 1 cells (Tr1) (Kleinewietfeld and Hafler, 2014).

Furthermore, TGF-β1 non-viral gene therapy administered intranasally for treatment of enterocolitis promotes beneficial effects via enhanced production of IL-10 (Kitani et al., 2000). In line with the anti-inflammatory function of TGF-β, prior work reveals that i.t. recombinant TGF-β1 attenuates neuropathic pain following partial ligation of the sciatic nerve in rats (Echeverry et al., 2009). However, in contrast to IL-10, which exerts predominantly anti-inflammatory actions (Saxena et al., 2015), TGF-β actions are determined by the nearby cellular and receptor milieu (Bottner et al., 2000). For example, TGF-β can act as either a dynamic tumor promoter or a tumor suppressor, thereby making the modulation of TGF-β alone a challenging and unpredictable therapeutic approach (Bottner et al., 2000; Colak and Ten Dijke, 2017). The possibility that the IL-10 transgene works synergistically with TGF-β1 for control of allodynia while potentially attenuating the untoward effects of TGF-β upregulation warrants further investigation.

4.3 Gene therapeutic modulation of the ipsilateral DRG is sufficient for enduring relief of bilateral allodynia

It is increasingly apparent in the literature that cytokine changes at the level of the DRG are deeply involved in the modulation of sensory information and the development of neuropathic pain (Krames, 2015). Therefore, it is noteworthy in this report that IL-10 transgene mRNA expression was detected in the ipsilateral DRG but not in the lumbar spinal cord where centrally projecting nociceptive terminals are communicating to pain projection neurons. What is most striking about the current data is that anti-inflammatory cytokine expression in the DRG is sufficient to drive anti-inflammatory changes at the level

of the lumbar spinal cord (i.e. decreased mRNA for TNF and CCL2). The spinal anti-inflammatory bias following DM/pDNA-IL-10 co-therapy is further supported by decreased lumbar mRNA levels of the microglial marker TMEM119 (upregulates upon activation/proliferation), concurrent with bilateral decreases in GFAP IR in the lumbar spinal cord dorsal horn. In combination, the data reveal a profound reduction of lumbar spinal cord GFAP IR following DM/IL-10 combination therapy, but with less striking mRNA decreases in spinal TNF and CCL2 and DRG TNF, suggesting that the role of other factors critical for ongoing astrocyte activation may generate greater effects on spinal pain processing. Most importantly, the pro- vs. anti-inflammatory rebalancing in the lumbar spinal cord is reflected in the behavioral responses of stably pain-relieved mice treated with non-viral IL-10 gene therapy. These findings further suggest that the protein product of the IL-10 transgene does not require direct contact with pain projection neurons in the dorsal spinal cord, but rather, interaction with cell bodies in the DRG alone is sufficient to induce pain relief.

There are several potential mechanisms by which IL-10 expression at the DRG leads to changes in neuronal signaling. For instance, several key mediators of neuropathic pain, such as TNF, increase neuronal excitability (Kagan et al., 1992; Watkins and Maier, 2005; Leung and Cahill, 2010; Grace et al., 2014; Ji et al., 2016). The current report demonstrates decreased TNF mRNA expression in the ipsilateral DRG of pain relieved DM/pDNA-IL-10 treated mice. This, in conjunction with synergistic IL-10/TGF-β1 actions, may lead to a decrease in TNF protein actions resulting in decreased neuronal excitability. While prior reports document neuronal IL-10R1 is present in the DRG and direct activation of neuronal IL-10R1 may help mediate decreases in pathologic neuronal

signaling (Shen et al., 2013; Alvarez et al., 2017), it is speculated in the current report that immune- and glial cell-derived IL-10R1 is predominantly responsible for the anti-allodynic effect because neurons do not produce the cytokines measured here (i.e. TNF, IL-10 and TGF-β1). Currently, the source of pain-controlling TGF-β1 and IL-10 is thought to be from glial and immune cells following IL-10R1 activation. However, whether IL-10R1 activation on DRG neurons is required for the beneficial effects of DM/pDNA-IL-10 co-therapy will provide an intriguing avenue of future study.

In the current report, we demonstrate that unilateral DRG IL-10 transgene expression is sufficient for relief of bilateral allodynia. Our behavioral observations in the context of absent contralateral DRG pro-inflammatory cytokine changes support that bilateral relief is mediated through events occurring at the level of the lumbar spinal cord. Unfortunately, the mechanisms that underlie allodynia contralateral to the side of injury, clinically referred to as "mirror pain," are not completely understood (Huang and Yu, 2010), though spinal glial activation is strongly implicated. For instance, work by Spataro et al. revealed that astrocyte communication via gap junctions may be critical for contralateral allodynia (Spataro et al., 2004). They elegantly demonstrated that i.t. administration of the gap junction decoupler carbenoxolone relieves contralateral, but not ipsilateral, mechanical allodynia following sciatic CCI in rats. They further argue that: 1) astrocytic gap junctions far outnumber those found on neurons, 2) though astrocytes are slow to activate, gap junctions are quickly upregulated (within 45 minutes post-nerve injury) and dynamically modifiable, and 3) astrocytes possess extensive gap junction-mediated glial connectivity throughout the spinal cord (Spataro et al., 2004). A competing hypothesis is that contralateral allodynia results from diffusion of ipsilaterally released pro-

inflammatory cytokines through cerebrospinal fluid to act on contralateral pain-relevant anatomic structures (Milligan et al., 2001; Cheng et al., 2014). Alternatively, neurons of the ipsilateral spinal cord dorsal horn may directly communicate with neurons, and likely glia, in the contralateral spinal cord dorsal horn (Fitzgerald, 1982; Fitzgerald and Woolf, 1982). In the current report, the astrocyte activation marker GFAP revealed bilateral upregulation in the spinal cord dorsal horn in chronically bilaterally allodynic CCI animals. Interestingly, the presence of DM/pDNA-IL-10 co-therapy in bilaterally pain-relieved animals was sufficient to reduce bilateral CCI-induced GFAP IR to basal levels. These findings support a role for the actions of spinal astrocytes in the production and resolution of contralateral allodynia. Though mRNA levels for the microglial activation marker TMEM119 were decreased in pain-relieved mice compared to allodynic controls, we cannot draw conclusions about laterality as both ipsilateral and contralateral spinal cord segments were analyzed together as single mRNA samples. However, the literature so far demonstrates that microglia are not dramatically altered in the contralateral spinal cord, as assessed by common markers of microglial activation (Ji et al., 2013).

4.4 Changes in TMEM119 expression as a possible marker of microglial activation

It is well understood that nervous tissue insults, such as damage to peripheral nerves, lead to CNS "microgliosis," a condition that describes microglial proliferation as well as changes in microglial morphology, gene expression profiles, and behavior (Calvo and Bennett, 2012). Additionally, microglial proliferation has been clearly linked to neuropathic pain pathology (Liu et al., 2000; Inoue and Tsuda, 2009; Zhuo et al., 2011).

While increased microglial activation can occur without proliferation (Nimmerjahn et al., 2005; Chen et al., 2012), increased microglial proliferation does not occur without stimulation, reflecting the diverse responses microglia generate under healthy or pathological conditions (Ulland et al., 2015).

TMEM119 was recently identified as a microglial specific marker in the mouse and human CNS (Bennett et al., 2016; Satoh et al., 2016). Work by Satoh *et al.* reveal TMEM119 mRNA levels are elevated in microglia from humans with Alzheimer's disease. Further, they report that TMEM119 exhibits a positive correlation with Iba-1 mRNA expression (Satoh et al., 2016), a microglial/monocyte marker known to increase in the CNS under pathological conditions (Echeverry et al., 2008). Another recent report used TMEM119 to distinguish microglia and macrophages in lesions found in post-mortem brain samples from humans diagnosed with multiple sclerosis (Zrzavy et al., 2017). They identified TMEM119 expression in activated microglia present within early active lesions. Such lesions are known to have increased microglial populations and are associated with demyelination, oxidative injury, and antigen presentation (Zrzavy et al., 2017). However, Bennett *et al.* report increased immunoreactivity for Iba-1 (a widely-used marker for microglial activation) but not TMEM119 in the CNS following either sciatic nerve crush injury or LPS-induced systemic inflammation (Bennett et al., 2016). Additionally, Satoh *et al.* did not find an association between TMEM119 immunoreactivity and markers of "M1" classical activation (CD80) or "M2" alternative activation (CD163), speculated to reflect neuroprotective conditions (Satoh et al., 2016). However, it is worth considering that the applicability of the M1/M2 polarization paradigm to microglia is still under dispute (Ransohoff, 2016). Taken together, TMEM119 may not readily reflect the activation state

of diversely functional microglia, changes in TMEM119 mRNA levels can be an indicator of a shift from homeostatic microglial behavior.

The current report provides evidence that TMEM119 mRNA is elevated in the lumbar spinal cord following sciatic CCI, which is consistent with prior reports of increased expression of microglial markers following peripheral nerve damage (Eriksson et al., 1993; Echeverry et al., 2008). Further work must be performed to determine whether increases in TMEM119 mRNA levels reliably predict increases in TMEM119 protein expression and whether these increases positively correlate with other markers of microglial activation.

4.5 Mannose receptor structure, expression, and activation

MR is a c-type lectin receptor commonly associated with endocytosis and pathogen recognition, and possesses an extracellular cysteine rich domain (CR; binds sulfated oligosaccharides), a fibronectin II domain (FNII; binds collagen fragments), and a carbohydrate recognition domain (CRD; binds mannose, fucose, and N-acetyl-D-glucosamine) (Martinez-Pomares, 2012; Sedaghat et al., 2014). MR is expressed by macrophages, dendritic cells, and microvascular endothelial cells (Taylor et al., 2005), as well as in the CNS by astrocytes, microglia, and some neurons (Burudi et al., 1999; Burudi and Regnier-Vigouroux, 2001), and in the PNS by Schwann cells (Baetas-da-Cruz et al., 2009). Increased MR expression is often associated with anti-inflammatory macrophages (Gordon, 2003). It is important to note that macrophages are known to infiltrate the DRG following sciatic CCI (Hu et al., 2007).

While partial ligation of the sciatic nerve in WT mice increases MR in the ipsilateral DRG, (Komori et al., 2011), the data in the current report offer critical new information because elevated MR mRNA in the ipsilateral DRG of all CCI conditions occurs independently of IL-10 actions. Additionally, it is interesting to note that CCI mice given the MR agonist (DM/pDNA-IL-10 and DM/pDNA-Ctrl) exhibit significantly less elevation in MR mRNA expression compared to CCI mice that did not receive the MR agonist (Saline/pDNA-IL-10). For example, a recent report by Xu *et al.* demonstrated *in vivo* that intravenous mannose treatment in a mouse model of acute lung injury induced by intratracheal instillation of LPS dose-dependently attenuates LPS-induced decreases in MR mRNA expression in lung homogenates 3 hours following mannose administration (Xu et al., 2015). Additionally, in vitro mannose pretreatment of RAW 264.7 macrophages prevents LPS-induced decreases in MR mRNA levels while also dose-dependently increasing MR protein levels as compared to unstimulated cells (Xu et al., 2015). The same group demonstrated increased MR protein expression by primary alveolar macrophages 16 hours after *ex vivo* stimulation with DM plus LPS compared to LPS alone (Xu et al., 2010). Lastly, monocyte-derived dendritic cells differentiated in the presence of glycosylated mucins (tumor-derived MR agonist) for a continuous 7 day exposure exhibit increased MR expression at the cell surface compared to controls (Rughetti et al., 2005). Taken together, these data suggest that acute and chronic exposure to MR agonists increase both MR protein and mRNA expression, suggesting MR agonists do not overtly generate tolerance.

Curiously, DRG from chronically allodynic Saline/pDNA-IL-10-treated mice revealed the highest MR expression levels, suggesting that both the pro-inflammatory or anti-inflammatory peripheral immune cell response to nerve injury can be identified by

local alterations in MR levels. Though MR upregulation is commonly considered a phenotypic marker of alternatively activated "M2" macrophages (Gordon, 2003; Martinez and Gordon, 2014a), the applicability of classically activated "M1" vs. "M2" macrophage phenotypes to the ipsilateral DRG following sciatic nerve injury has not been fully established. Furthermore, while macrophages are known to infiltrate the DRG following sciatic CCI (Hu et al., 2007), the possible contribution of other cell types within the DRG following sciatic CCI, such as satellite glial cells and additional trafficking leukocytes (e.g. T cells), in modulating both MR expression and the "balance" between M1 vs. M2 factors is not well understood. Therefore, changes in MR expression reported here are interpreted within the context of the DRG cytokine profile balance. Specifically, chronically allodynic Saline/pDNA-IL-10 mice express the greatest MR and TNF mRNA levels while simultaneously expressing the lowest TGF-β1 mRNA levels. In contrast, pain-relieved CCI+DM/pDNA-IL-10 mice express significantly elevated IL-10, MR, and TGF-β1 mRNA levels with low TNF mRNA expression. In future studies aimed at distinguishing the biochemical effects of IL-10 gene therapy from DM, analysis of CCL2 in DRG may be useful to elucidate the mechanisms by which DM alone generates transient pain reversal.

Given that the lumbar spinal cord, for which no IL-10 transgene was identifiable, exhibited substantial decreases in key inflammatory markers, the M1-to-M2 changes within the DRG suggest that DM/pDNA-IL-10 alters the ability of injured primary sensory neurons to relay pathological pain information to spinal pain projection neurons. That is, DM/pDNA-IL-10 actions at the level of the DRG diminish the salience of incoming peripheral nociceptive signals, with the level of MR expression potentially serving as a useful biomarker of the degree of influence.

4.6 Non-viral naked plasmid gene therapy for treatment of pathological pain

Non-viral gene therapies provide promising avenues for the treatment of CNS diseases (Jayant et al., 2016), and methods using naked plasmid DNA have been previously explored for treatment of pathological pain. Yao et al. demonstrated pain relief following a single i.t. injection of naked pDNA (25μg) encoding human interleukin-2 in CCI-treated Sprague-Dawley rats. However, anti-nociceptive effects disappeared 5-6 days post-injection (Yao et al., 2002). More recently, Hu et al. demonstrated about ~2 weeks of inflammatory pain reversal in mice following i.t. or intramuscular (i.m.) delivery of naked pDNA (20μg) encoding the human proenkaphalin gene. While promising, their pain model exhibits >3 weeks of allodynia, making the value of ~2 weeks of pain relief unclear. Milligan et al. were the first to explore i.t. delivery of naked pDNA-IL-10 for relief of peripheral neuropathic pain following CCI in rats. While a single i.t. injection of pDNA-IL-10 (100μg) provides only ~2 days of pain relief, an appropriately timed second i.t. injection (100μg) produces long-lasting pain reversal for greater than 40 days (Milligan et al., 2006b). Later work by Sloane et al. further optimized this repeated injection paradigm, revealing that an initial i.t. pDNA injection (100μg) induces a sensitization which when followed by a smaller plasmid load (25μg pDNA-IL-10) provides greater than 3 months of pain relief (Sloane et al., 2009b). Non-viral naked pDNA-IL-10 gene therapy was further improved by Dengler et al., who demonstrated that i.t. co-injection of D-mannose (DM) with naked pDNA-IL-10 (25μg) provides greater than 90 days of pain relief in CCI-treated Sprague-Dawley rats (Dengler et al., 2014). The current report demonstrates that co-injection of DM with just 3μg of naked pDNA-IL-10 in neuropathic mice leads to enduring

pain relief for the remainder of the full behavioral timecourse. Notably, data in the current report reveal spontaneous recovery from CCI allodynia occurs sooner in mice than in rats (45 days). Especially given its long-lasting pain suppression profile, these intriguing findings support that non-viral IL-10 gene therapy formulated with DM is the preferred non-viral naked pDNA approach for treatment of neuropathic pain conditions. Future investigations may support use of this unique formulation in treatment of other pain conditions, and perhaps other diseases of the CNS.

4.7 Naked plasmid DNA co-injected with D-mannose is safe, inexpensive, and easy to use

Naked plasmid DNA must face numerous extra- and intracellular obstacles to achieve transgene expression (Glover et al., 2005). To address this, both physical and chemical (e.g. carrier-based) methods have been utilized in an effort to improve the efficacy of non-viral gene therapy (Jayant et al., 2016). Physical methods of gene delivery, such as electroporation, gene gun, ultrasound, or magnetofection, sidestep the numerous obstacles faced by naked pDNA, but are often both costly and labor intensive (Oliveira et al., 2017). Carrier-based methods of non-viral gene therapy, such as polyethynlamine (PEI) pDNA complexes, liposomes, and PLGA microparticles, work to shield pDNA from cellular degradation and aid in cell entry (Slivac et al., 2017) but are not without their disadvantages. For instance, in addition to traditionally low efficacy and transient expression observed in non-viral approaches, PEI exhibits moderate toxicity, liposomes can have a low-to-moderate inflammatory response, and many polymer-based methods require surface modification for improved targeting (Jayant et al., 2016). The current report

supports that DM co-therapy provides a safe, inexpensive, and easy alternative for improving pDNA uptake and expression.

4.8 Role of MR-activation in non-viral gene therapy

The current report supports that MR activation alone transiently attenuates pathologic sensitivity to light touch stimulation by an IL-10-independent mechanism, but it is unclear how MR activation leads to changes in intracellular signaling. Though we show that this mechanism is IL-10 independent, it is likely that MR-mediated pain relief arises from the potential MR role in anti-inflammatory immunomodulation (Nigou et al., 2001; Pathak et al., 2005; Zhang et al., 2005; Gazi and Martinez-Pomares, 2009; Dengler et al., 2014). Many studies of MR signaling are performed under inflammatory states, with MR-activation associated with enhanced pro-inflammatory signaling (Fernandez et al., 2005; Lopez-Herrera et al., 2005; Tachado et al., 2007). However, the "mode" of MR signaling may be dependent on the cellular activation state, with certain phenotypes leading to MR-activated anti-inflammation (Martinez-Pomares, 2012; Hussell and Bell, 2014). For instance, *in vitro* dendritic cell cultures treated with "activating" anti-MR monoclonal antibodies potently increase IL-10 and IL-1 receptor antagonist (IL-1Ra) secretion with concurrent decreases in IL-1β and TNF production (Chieppa et al., 2003). In line with these findings, MR-activation has also been repeatedly demonstrated to prevent LPS-induced acute lung injury (Zhang et al., 2004; Zhang et al., 2005; Xu et al., 2008; Xu et al., 2015) through downstream activation of the anti-inflammatory transcription factor peroxisome proliferator activated receptor gamma (PPARγ) and decreased production of TNF (Xu et

al., 2015). Furthermore, *in vitro* pretreatment with DM prior to LPS stimulation of RAW 264.7 mouse macrophage cells reduces release of pro-inflammatory TNF, IL-1β, and nitric oxide, while increasing the secretion of IL-10 (Dengler et al., 2014). Zhou et al. recently reported that bone marrow derived monocytes from MR-deficient *Mrc1*$^{-/-}$ mice reveal greater mRNA levels for pro-inflammatory "M1" phenotypic markers (IL-1β, IL-6, and nitric oxide synthase-2) following LPS-challenge, and lower mRNA levels for markers of an "M2" phenotype (Fizz1, Ym1, and Arginase-1) (Zhou et al., 2017). They further demonstrated that overexpression of miR-511-3p, an intronic miRNA encoded by both mouse and human *Mrc1/MRC1* and transcriptionally co-regulated with *Mrc1* in macrophages, reverses the M1 bias and instead promotes an anti-inflammatory M2 phenotype. Moreover, vesicular accumulation of cockroach allergens in macrophages, an MR-dependent process, is not associated with the lysosomal compartment (Zhou et al., 2017). This observation is reminiscent of prior studies illustrating ways MR activation in the presence of intracellular pathogens is appropriated to activate anti-inflammatory signaling with delayed phagosome-lysosome fusion (Shibata et al., 1997; Astarie-Dequeker et al., 1999; Shimada et al., 2006; Garcia-Aguilar et al., 2016). These MR-dependent delays in phagosome-lysosome fusion offer a possible mechanism by which MR activation improves transgene expression, and is worthy of future investigation. The behavioral evidence of transient anti-allodynia reported in the current study supports MR-mediated anti-inflammatory effects, and it is reasonable to imagine that MR itself may serve as a novel target for the development of pain therapeutics.

As MR-targeting for enhanced non-viral gene therapy is explored for other CNS pathologies, consideration must be taken to ensure availability of MR prior to application

of this DM/pDNA approach. While MR is widely expressed in a variety of cell types, there is some evidence that priming by pro-inflammatory processes may be beneficial to this non-viral approach. For instance, significant levels of IL-10 transgene were observed in ipsilateral, but not contralateral, DRG of CCI+DM/pDNA-IL-10 mice. Furthermore, it is possible that the non-significant levels of IL-10 transgene expression in the ipsilateral DRG of Sham+DM/pDNA-IL-10 treated mice are linked to expression of only basal levels of MR. While the current report further validates targeting MR activation to facilitate efficacious non-viral transgene uptake and expression for treatment of neuropathic pain, further investigation is needed to assess the translatability of this gene therapeutic approach for other CNS pathologies.

4.9 Exploration of MR-targeting therapeutics

D-mannose, the MR agonist applied here, is an inexpensive and commonly available dietary supplement (Hu et al., 2016) used in a phase 3 clinical trial for prevention of recurrent urinary tract infections (#NCT01808755, clinicaltrials.gov) (Porru et al., 2014). N-acetyl-D-glucosamine is another MR ligand examined in an early phase 1 clinical trial to improve wound healing of venous stasis ulcers (#NCT00720239, clinicaltrials.gov) (Kelechi et al., 2012). The FDA recently approved the use of the MR-targeting agent γ-Tilmanocept, also known as [^{99m}Tc]-DTPA-mannosyl-dextran, for imaging of sentinel lymph nodes in solid tumor staging (Vera et al., 2001; Azad et al., 2015). Mannitol is another FDA approved mannose derivative considered an osmotic diuretic, though whether it may interact with MR on leukocytes or vascular endothelial cells is not yet known.

Depending on the pain etiology, such as for peripheral neuropathic pain or inflammatory pain, activation of MR by small molecules like D-mannose could be explored for therapeutic treatment, especially for patients that would not be good candidates for intrathecal gene therapy. Furthermore, targeting MR could be harnessed to improve the anti-inflammatory response for treatment of diseases where inflammation has become pathologic.

5. Conclusion

In summary, our findings provide evidence that spinal non-viral DM/pDNA-IL-10 gene delivery for chronic neuropathic pain does not require endogenous IL-10 for enduring pain relief, with uptake and expression of the IL-10 transgene predominantly in the DRG. Reliable and significant anti-inflammatory changes in the DRG and the lumbar spinal cord are observed as a consequence of this safe non-viral gene therapy approach. MR-mediated improvements in transgene efficacy and transient pain relief are IL-10-independent, findings that support further investigation into potential MR-activated intracellular cascades and their consequent modification in the presence or absence of anti-inflammatory cytokines. This therapy may provide a framework upon which other non-viral gene therapy approaches can be adopted to treat central nervous system disease that extend beyond chronic pain.

CHAPTER 2 FIGURES

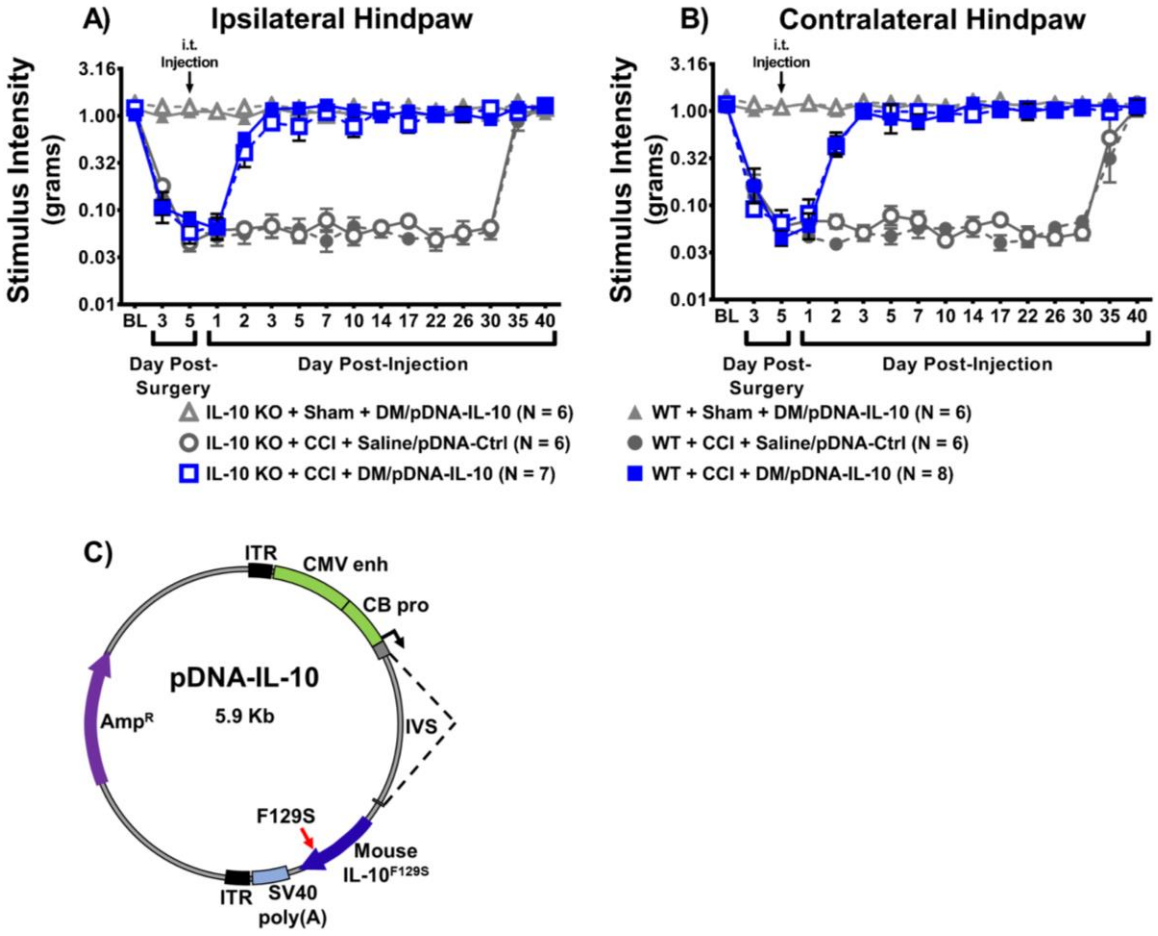


Figure 1: Intrathecal non-viral IL-10 gene therapy provides stable long-lasting relief of allodynia in mice and is independent of endogenous IL-10.
Absolute threshold behavioral responses for (**A**) ipsilateral and (**B**) contralateral hindpaws for WT and IL-10 KO mice are shown (N = 6-8 mice/group as indicated in figure legend). At baseline (BL), responses to low threshold mechanical stimuli were similar between all groups (ipsilateral, $F_{(5, 33)} = 1.01$, $P > 0.05$; contralateral, $F_{(5, 33)} = 1.31$, $P > 0.05$). For data collected between BL through Day 5 post-surgery, a main effect of time (ipsilateral, $F_{(2, 66)} = 170.08$, $P < 0.001$; contralateral, $F_{(2, 66)} = 134.35$, $P < 0.001$) and an interaction between time and surgical manipulation (ipsilateral, $F_{(2, 66)} = 79.76$, $P < 0.001$; contralateral, $F_{(2, 66)} = 64.04$, $P < 0.001$) were observed. After behavioral assessment on Day 5 post-surgery, all mice received an intrathecal (i.t.) co-injection of plasmid DNA encoding interleukin-10 (**pDNA-IL-10**; 3µg in 7.5µL) with D-mannose (**DM**; 25µg in 3µL) vs. pDNA-Control (**Ctrl**; 3µg in 7.5µL) with isotonic sterile saline (saline; 3µL). Following injection, both WT and IL-10 KO CCI-mice given i.t. DM/pDNA-IL-10 reveal bilateral reversal to normal levels of hindpaw sensitivity (ipsilateral, $F_{(2, 66)} = 54.49$, $P < 0.001$; contralateral, $F_{(2, 66)} = 34.34$, $P < 0.001$). This reversal remains stable through Day 30 post-injection (ipsilateral,

$F_{(4.9, 162.5)} = 0.432$, $P > 0.05$; contralateral, $F_{(4.9, 163.1)} = 0.871$, $P > 0.05$). Not surprisingly, control injected CCI-mice demonstrate bilateral spontaneous reversal from allodynia between Days 30-40 post-injection (ipsilateral, $F_{(2, 66)} = 141.30$, $P < 0.001$; contralateral, $F_{(1.5, 48.5)} = 48.54$, $P < 0.001$), with all groups experiencing similar normal levels of light touch sensitivity by Day 40 post-injection (ipsilateral, $F_{(5, 33)} = 0.81$, $P > 0.05$; contralateral, $F_{(5, 33)} = 0.193$, $P > 0.05$). (**C**) Plasmid map of the pDNA-IL-10 construct. The modified plasmid was derived from the expression cassette previously used for adeno-associated virus-2. pDNA-IL-10 is a 5.9 Kb plasmid with gene transcription driven by a chicken β-actin promoter (CB pro) hybridized with a cytomegalovirus enhancer (CMV enh). The expression cassette contains the transgene for mouse IL-10 with a point mutation (serine substitution for phenylalanine at amino acid 129) ($IL\text{-}10^{F129S}$), and a viral SV40 polyadenylation signal (SV40 poly(A)), and is flanked by two inverted terminal repeat (ITR) sequences. The plasmid vector also contains an intervening sequence (IVS; intron) and an ampicillin-resistance (Amp^r) gene.

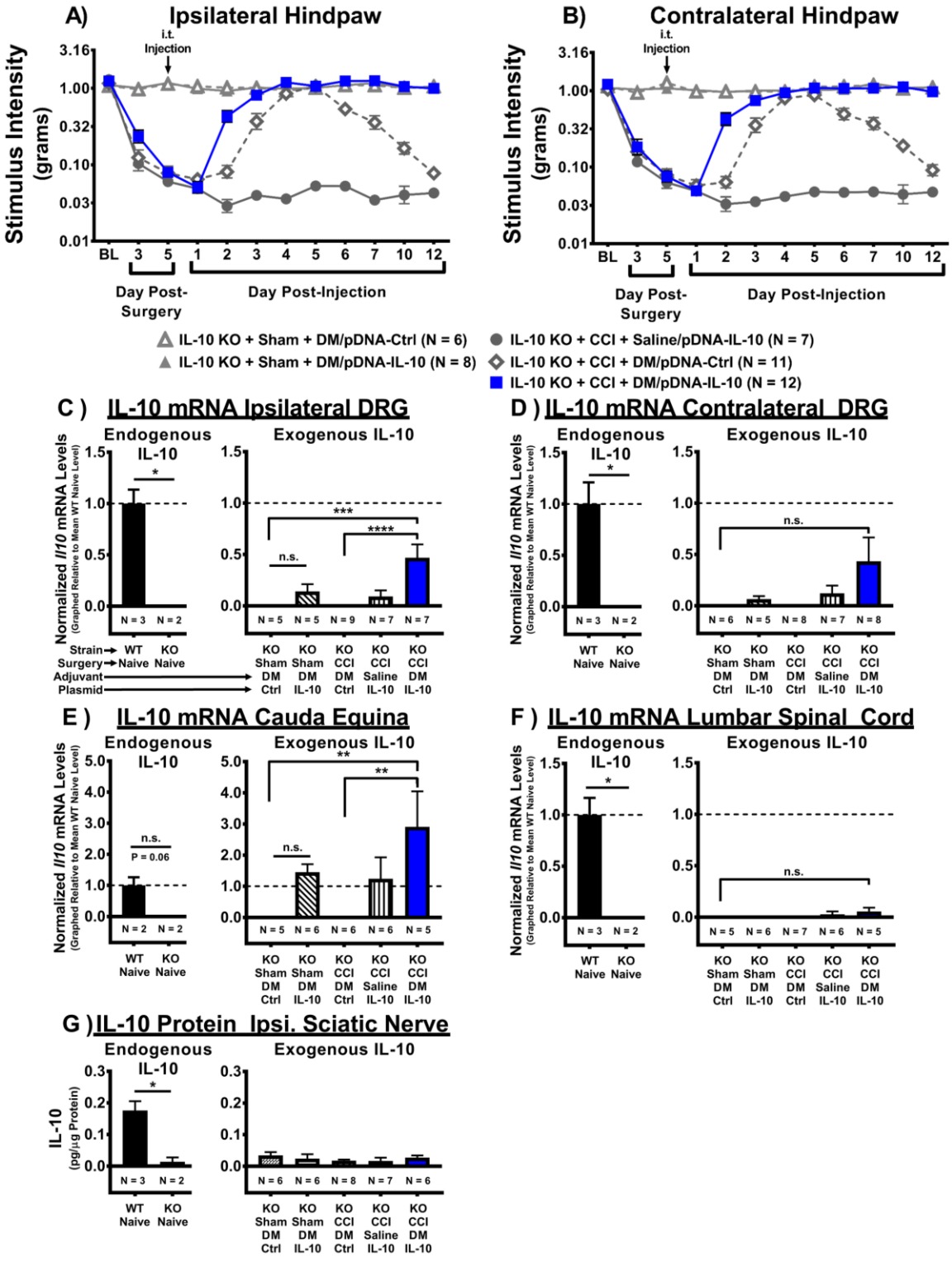


Figure 2: I.t. non-viral IL-10 gene therapy in IL-10 KO mice leads to expression of IL-10 transgene in pain-relevant lumbar dorsal root ganglia.

Absolute threshold behavioral responses for (**A**) ipsilateral and (**B**) contralateral hindpaws are shown. At baseline (BL), responses to low threshold mechanical stimuli were similar

between all groups (ipsilateral, $F_{(4, 39)} = 1.39$, $P > 0.05$; contralateral, $F_{(4, 39)} = 1.04$, $P > 0.05$). For data collected between BL through Day 5 post-surgery a main effect of time (ipsilateral, $F_{(1.7, 66.1)} = 184.69$, $P < 0.001$; contralateral, $F_{(2, 78)} = 186.41$, $P < 0.001$) is observed. After behavioral assessment on Day 5 post-surgery, Sham- or CCI-treated mice received an i.t. co-injection of **DM** (25μg in 3μL) with either **pDNA-IL-10** (3μg in 7.5μL) or pDNA-Ctrl (N = 6-12 mice/group). An additional control group consisted of CCI-treated mice that received i.t. saline with pDNA-IL-10 (N = 7). Following injection, a main effect of time (ipsilateral, $F_{(6.0, 233.1)} = 49.09$, $P < 0.001$; contralateral, $F_{(8, 312)} = 46.2$, $P < 0.001$) and an interaction of time and treatment (ipsilateral, $F_{(23.9, 233.1)} = 29.11$, $P < 0.001$; contralateral, $F_{(32, 312)} = 25.13$, $P < 0.001$) were observed. Comparisons between groups reveal that on Day 2 post-injection, CCI+DM/pDNA-IL-10-treated mice exhibit bilateral reversal from allodynia compared to mice lacking DM treatment (CCI+Saline/pDNA-IL-10) (ipsilateral and contralateral, $P < 0.001$). Surprisingly, on Day 3 post-injection, mice given DM and the control plasmid (CCI+DM/pDNA-Ctrl) reach levels of hindpaw sensitivity similar to Sham+DM/pDNA-IL-10-treated controls (ipsilateral and contralateral, $P > 0.05$). Beginning on Day 6 post-injection, CCI+DM/pDNA-Ctrl mice begin to return to bilateral allodynia (ipsilateral, $F_{(3, 117)} = 19.67$, $P < 0.001$; contralateral, $F_{(3, 117)} = 9.73$, $P < 0.001$), while mice treated with DM/pDNA-IL-10 never return to allodynia. (**C**-**E**) mRNA isolated from DRG and cauda equina tissues collected on Day 12 post-injection (Day 17 post-surgery) reveal IL-10 expression. IL-10 (*Il10*) mRNA levels were observed in WT Naïve positive controls (N = 3) but not in IL-10 KO Naïves (N = 2) for (**C**) ipsilateral, (**D**) contralateral lumbar DRG, and (**F**) lumbar spinal cord tissues (*$P < 0.05$), but not (**E**) cauda equina, despite a strong trend for positive expression ($P = 0.062$). IL-10 mRNA expression was not significantly different between Sham-treated conditions for DRG, cauda equina, and lumbar spinal cord ($P > 0.05$). (**C**) IL-10 transgene mRNA expression was significantly elevated in ipsilateral DRG of pain-relieved CCI+DM/pDNA-IL-10-treated IL-10 KO mice compared to IL-10 KO mice given either CCI+DM/pDNA-Ctrl or Sham+DM/pDNA-Ctrl ($F_{(4, 28)} = 7.72$, $P < 0.001$). (**D**) No significant increase in IL-10 transgene mRNA levels were detected in contralateral DRG following i.t. injection ($F_{(4, 29)} = 2.20$, $P = 0.093$). However, *a priori* comparison revealed that contralateral DRG from CCI-treated mice given DM co-treated with the IL-10 transgene contained significantly greater IL-10 mRNA levels than DRG from CCI-treated mice given DM with the control plasmid ($P < 0.05$) or Sham-treated mice with DM and the control plasmid ($P < 0.05$). (**E**) In the cauda equina, IL-10 transgene mRNA expression was greatly elevated in pain-relieved CCI+DM/pDNA-IL-10 KO mice compared to negative control conditions CCI+DM/pDNA-Ctrl and Sham/DM+pDNA-Ctrl ($F_{(4, 23)} = 4.19$, $P = 0.011$). (**G**) IL-10 protein was detected in ipsilateral sciatic nerve (collected Day 12 post-injection) of WT Naïve but not IL-10 KO Naïve mice ($P < 0.05$), but no significant increase in IL-10 expression was detected in any i.t. injection condition ($F_{(4, 27)} = 0.65$, $P > 0.05$). For mRNA and protein analyses, N = 5-7 mice/group. mRNA levels (Mean ± SEM) are normalized to 18S rRNA and graphically presented relative to mean WT Naïve levels. For protein analyses, protein levels are presented as Mean ± SEM. Post-hoc multiple comparisons via Fisher's LSD ($\alpha = 0.05$; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

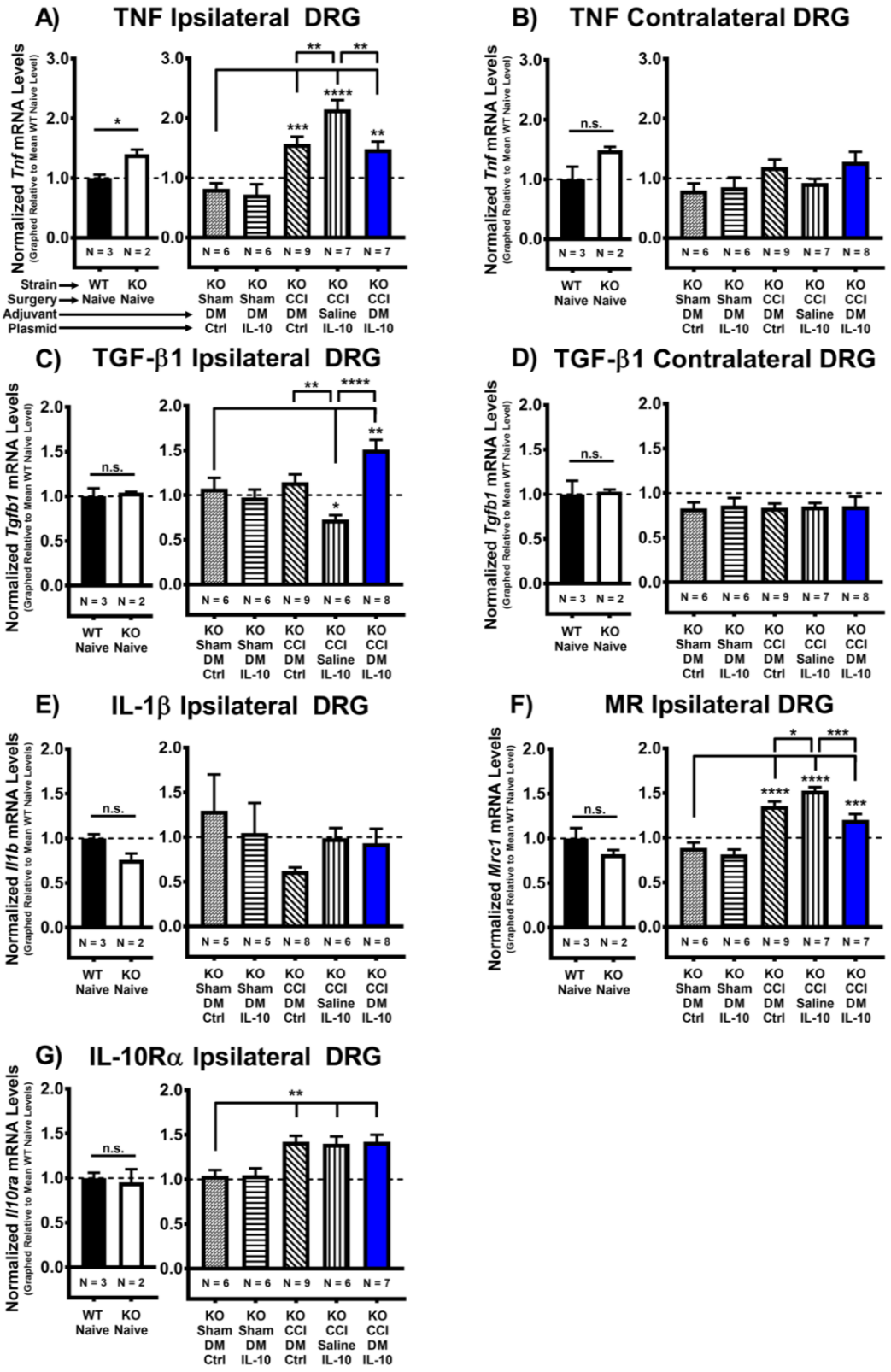


Figure 3: Therapeutic expression of IL-10 transgene mediates pain relief through anti-inflammatory changes in ipsilateral lumbar DRG.

mRNA was isolated from ipsilateral and contralateral Lumbar DRG (L3-5) on Day 12 post-injection (Day 17 post-surgery). (**A-F**) Baseline transcript levels were compared between WT Naïve (N = 3 mice) and IL-10 KO Naïve (N = 2 mice) groups. Transcript levels for TNF mRNA are significantly higher in ipsilateral DRG from IL-10 KO Naïves compared to WT Naïves (*$P < 0.05$), but not in contralateral DRG ($P > 0.05$). *Tgfb1*, *Il1b*, *Mrc1*, and *Il10ra* mRNA transcript levels were not significantly different between WT and IL-10 KO Naïve groups in either ipsilateral or contralateral DRG ($P > 0.05$). (**A**) Post-surgical mRNA levels of pro-inflammatory cytokine TNF are greatly increased in ipsilateral lumbar DRG from all CCI conditions compared to Sham+DM/pDNA-Ctrl treated mice, ($F_{(4, 30)} = 17.17$, $P < 0.0001$). However, TNF mRNA expression was significantly decreased in pain relieved CCI+DM/pDNA-IL-10 mice compared to allodynic CCI+Saline/pDNA-IL-10 controls. Interestingly, TNF mRNA expression was also significantly decreased in CCI+DM/pDNA-Ctrl mice that recently returned to allodynia as compared to CCI+Saline/pDNA-IL-10 controls. (**B**) No statistically significant difference was detected for TNF mRNA levels in contralateral DRG by 1-way ANOVA ($F_{(4, 31)} = 2.4$, $P > 0.05$). (**C**) Transcript levels of the anti-inflammatory cytokine TGF-β1 (*Tgfb1*) are decreased in lumbar DRG collected from chronically allodynic mice (CCI+Saline/pDNA-IL-10) compared to Sham+DM/pDNA-Ctrl treated mice ($F_{(4, 31)} = 11.13$, $P < 0.0001$). In contrast, pain relieved CCI+DM/pDNA-IL-10 mice exhibited elevated TGF-β1 mRNA expression compared to Sham+DM/pDNA-Ctrl treated mice ($F_{(4, 30)} = 8.67$, $P < 0.0001$). TGF-β1 mRNA levels were significantly decreased in chronically allodynic CCI+Saline/pDNA-IL-10 mice as compared to both CCI+DM/pDNA-Ctrl and pain-relieved CCI+DM/pDNA-IL-10 mice. (**D**) No statistically significant difference was detected for TGF-β1 mRNA levels in contralateral DRG ($F_{(4, 31)} = 0.03$, $P > 0.05$). (**E**) No statistically significant difference was detected for pro-inflammatory cytokine IL-1β (*Il1b*) mRNA transcript levels in ipsilateral DRG ($F_{(4, 27)} = 1.36$, $P > 0.05$). (**F**) Post-surgical mRNA levels for the mannose receptor (*Mrc1*; MR; CD206) are increased in ipsilateral lumbar DRG from all CCI conditions compared to Sham+DM/pDNA-Control treated mice, with MR transcript levels significantly decreased in pain-reversed CCI+DM/pDNA-IL-10 mice and recently allodynic CCI+DM/pDNA-Ctrl mice as compared to chronically allodynic CCI+Saline/pDNA-IL-10-treated mice ($F_{(4, 30)} = 29.48$, $P < 0.0001$). (**G**) For IL-10 receptor alpha (*Il10ra*) mRNA levels, all CCI conditions exhibited significant increases compared to Sham+DM/pDNA-Ctrl mice (**$P < 0.01$) ($F_{(4, 30)} = 6.82$, $P < 0.001$). N = 5-9 mice/group for treatment groups, as indicated on graphs. mRNA levels (Mean ± SEM) are normalized to 18S rRNA and graphically presented relative to mean WT Naïve levels. Post-hoc multiple comparisons via Fisher's LSD ($\alpha = 0.05$; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$).

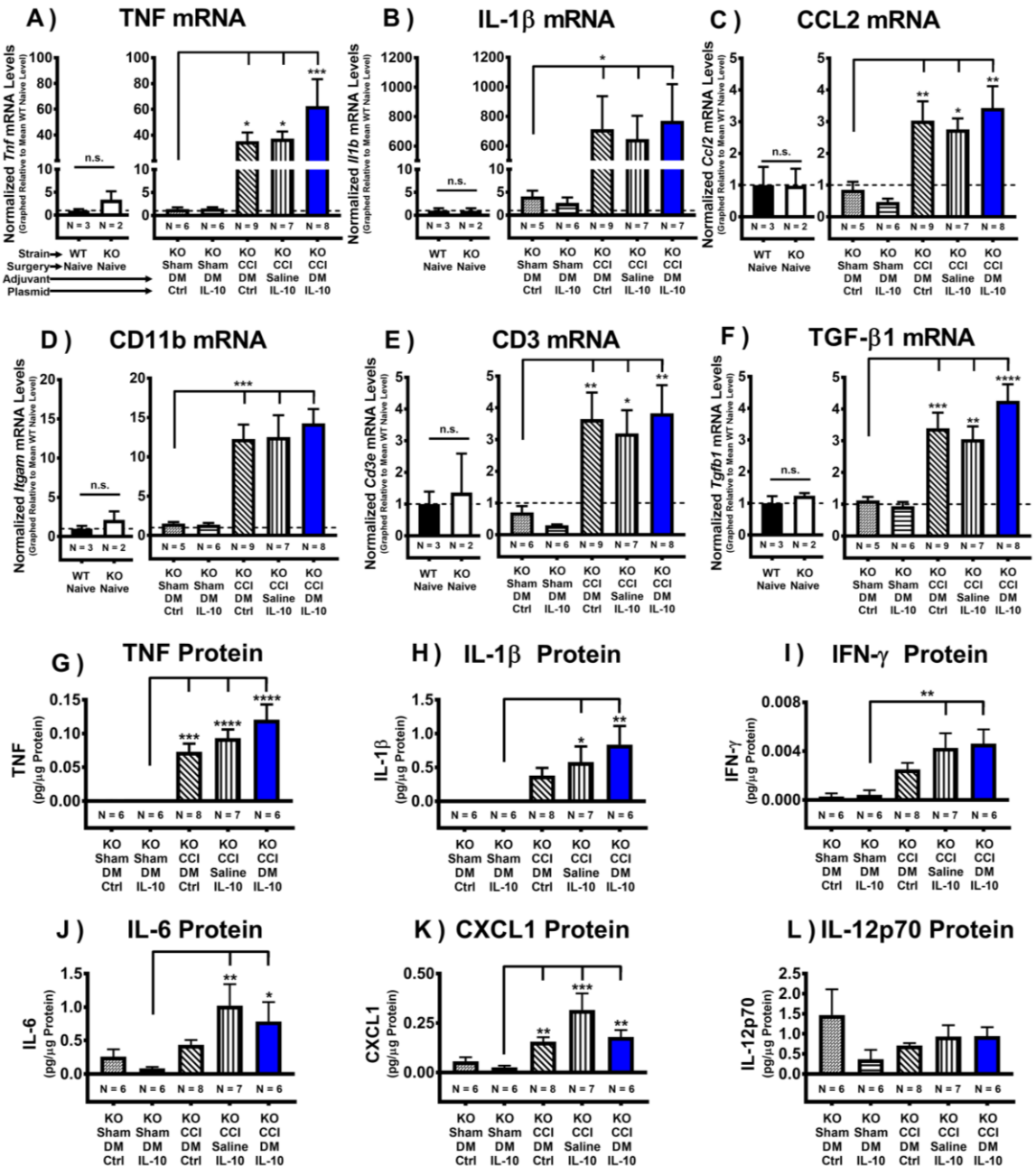


Figure 4: Pro-inflammatory characterization of the CCI-damaged sciatic nerve following i.t. non-viral IL-10 gene therapy in IL-10 KO mice.

mRNA was isolated from ipsilateral sciatic nerve (ipsi SCN) collected on Day 12 post-injection (Day 17 post-surgery) from previously behaviorally verified animals (**Fig 3**). (**A-F**) Baseline transcription levels are not significantly different between WT Naïve and IL-10 KO Naïve groups (N = 2-3 mice/group) for all targets ($P > 0.05$): *Tnf*, *Il1b*, *Tgfb1*, *Ccl2*, *Itgam*, and *Cd3e*. (**A-B**) Post-surgical mRNA levels of pro-inflammatory cytokines TNF (*Tnf*) and IL-1β (*Il1b*) are greatly increased in ipsi SCN from all CCI conditions compared to Sham+DM/pDNA-Control treated mice ($F_{(4, 31)} = 5.07$, $P < 0.01$; $F_{(4, 29)} = 3.769$, $P = 0.0138$, respectively). (**C**) mRNA levels of pro-inflammatory chemokine CCL2 (*Ccl2*) are

greatly increased across all CCI conditions compared to Sham+DM/pDNA-Ctrl treated mice ($F_{(4, 30)} = 5.92$, $P < 0.01$). (**D**) Following CCI surgery, the macrophage activation marker CD11b (*itgam*) is elevated in ipsi SCN, with CD11b mRNA levels significantly increased across all CCI conditions compared to Sham+DM/pDNA-Ctrl treated mice ($F_{(4, 29)} = 3.77$, $P < 0.05$). (**E**) The general T cell marker CD3 (*Cd3e*) is elevated in sciatic tissue following CCI surgery, with CD3 mRNA expression significantly increased across all CCI conditions compared to Sham+DM/pDNA-Ctrl treated mice ($F_{(4, 29)} = 3.77$, $P < 0.05$). (**F**) Post-surgical mRNA transcript levels of anti-inflammatory cytokine TGF-β1 are greatly increased across all CCI conditions compared to Sham+DM/pDNA-Ctrl treated mice ($F_{(4, 31)} = 11.13$, $P < 0.0001$). N = 5-9 mice/group for treatment groups, as indicated on graphs. (**G-L**) Complementary protein analysis for ipsi SCN from the same animals previously assessed for mRNA analysis (**A-F**). Baseline protein levels are not significantly different between WT Naïve and IL-10 KO Naïve groups (N = 2-3 mice/group) for all targets (data not shown; $P > 0.05$): TNF, IL-1β, IFN-γ, IL-6, CXCL1, and IL-12p70. No significant differences were observed between Sham conditions (Sham+DM/pDNA-Ctrl and Sham+DM/pDNA-IL-10) for all protein targets ($P > 0.05$). (**G**) Pro-inflammatory cytokine TNF protein expression levels were elevated across all CCI conditions compared to Sham+DM/pDNA-IL-10 ($F_{(4, 28)} = 15.54$, $P < 0.0001$). (**H**) In most CCI conditions, protein levels for the pro-inflammatory cytokine IL-1β are significantly increased in ipsi SCN compared to Sham+DM/pDNA-IL-10 ($F_{(4, 28)} = 4.33$, $P < 0.01$). (**I**) Protein levels for the pro-inflammatory cytokine IFN-γ are greater in most CCI conditions compared to Sham+DM/pDNA-IL-10 ($F_{(4, 28)} = 5.97$, $P < 0.01$). (**J**) IL-6, a pro-inflammatory cytokine, is significantly increased in ipsi SCN for most CCI conditions compared to Sham+DM/pDNA-IL-10 ($F_{(4, 27)} = 3.47$, $P < 0.05$). (**K**) CXCL1 (a.k.a. KC/GRO), a neutrophil chemoattractant, is significantly elevated following CCI surgery compared to Sham+DM/pDNA-IL-10 ($F_{(4, 27)} = 6.45$, $P < 0.001$). (**L**) No significant increases in IL-12p70, a pro-inflammatory cytokine downstream of CpG-activated TLR-9, were observed for any condition ($F_{(4, 28)} = 1.46$, $P > 0.05$). For protein analyses, N = 6-8 mice/group as indicated, and protein levels are presented as Mean $\pm$ SEM. For mRNA analyses, N = 5-9 mice/group as indicated, and mRNA levels (Mean ± SEM) are normalized to 18S rRNA and graphically presented relative to mean WT Naïve levels. Post-hoc multiple comparisons via Fisher's LSD ($\alpha = 0.05$; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$).

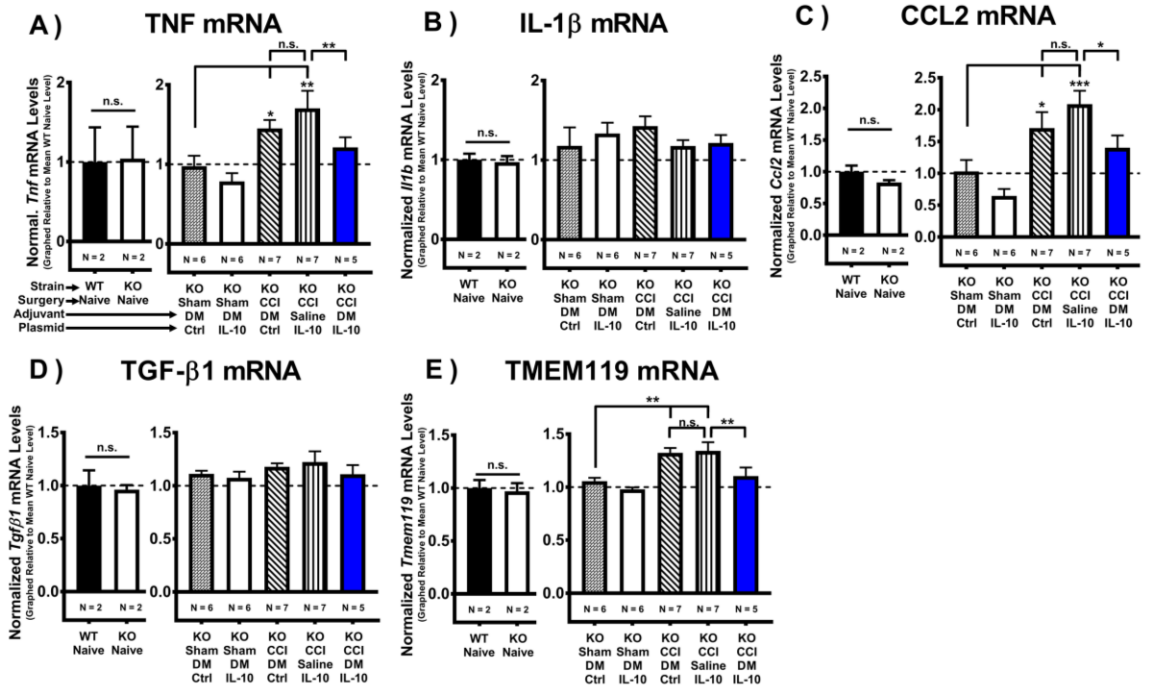


Figure 5: Intrathecal non-viral IL-10 gene therapy alters lumbar cytokines and decreases microglial activation.

Characterization of isolated cytokines and microglial-specific mRNA from the lumbar spinal cord collected on Day 12 after i.t. IL-10 gene therapy injection (Day 17 post-surgery). (**A**-**F**) Baseline transcription levels are not significantly different between WT Naïve and IL-10 KO Naïve groups (N = 2 mice/group) for all targets ($P > 0.05$): *Tmem119*, *Tnf*, *Il1b*, *Ccl2*, and *Tgfb1*. (**A**) mRNA transcript levels for the pro-inflammatory cytokine TNF (*Tnf*) and (**C**) the pro-inflammatory chemokine CCL2 (*Ccl2*) were elevated only in allodynic controls, with significantly less TNF and CCL2 mRNA expression observed in pain-relieved CCI+DM/pDNA-IL-10 vs. allodynic CCI+Saline/pDNA-IL-10 mice (TNF: $F_{(4, 26)} = 6.18$, $P < 0.01$) (CCL2: $F_{(4, 26)} = 8.10$, $P < 0.0001$). (**B**) No significant differences in mRNA transcript levels for the pro-inflammatory cytokine IL-1β (*Il1b*) were detected ($F_{(4, 24)} = 0.6927$, $P > 0.05$). (**D**) No significant differences in mRNA levels for anti-inflammatory cytokine TGF-β1 (*Tgfb1*) were detected for any condition ($F_{(4, 25)} = 0.77$, $P > 0.05$). (**E**) mRNA transcript levels for the microglial marker TMEM119 (*Tmem119*) were elevated in allodynic CCI controls, but not in pain-relieved CCI+DM/pDNA-IL-10 mice. Additionally, TMEM119 mRNA levels in pain-relieved CCI+DM/IL-10 treated mice were significantly less than for allodynic CCI+Saline/IL-10 mice ($F_{(4, 26)} = 8.24$, $P < 0.001$). mRNA levels (Mean ± SEM) are normalized to 18S rRNA and graphically presented relative to mean WT Naïve levels. Post-hoc multiple comparisons via Fisher's LSD ($\alpha = 0.05$; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$).

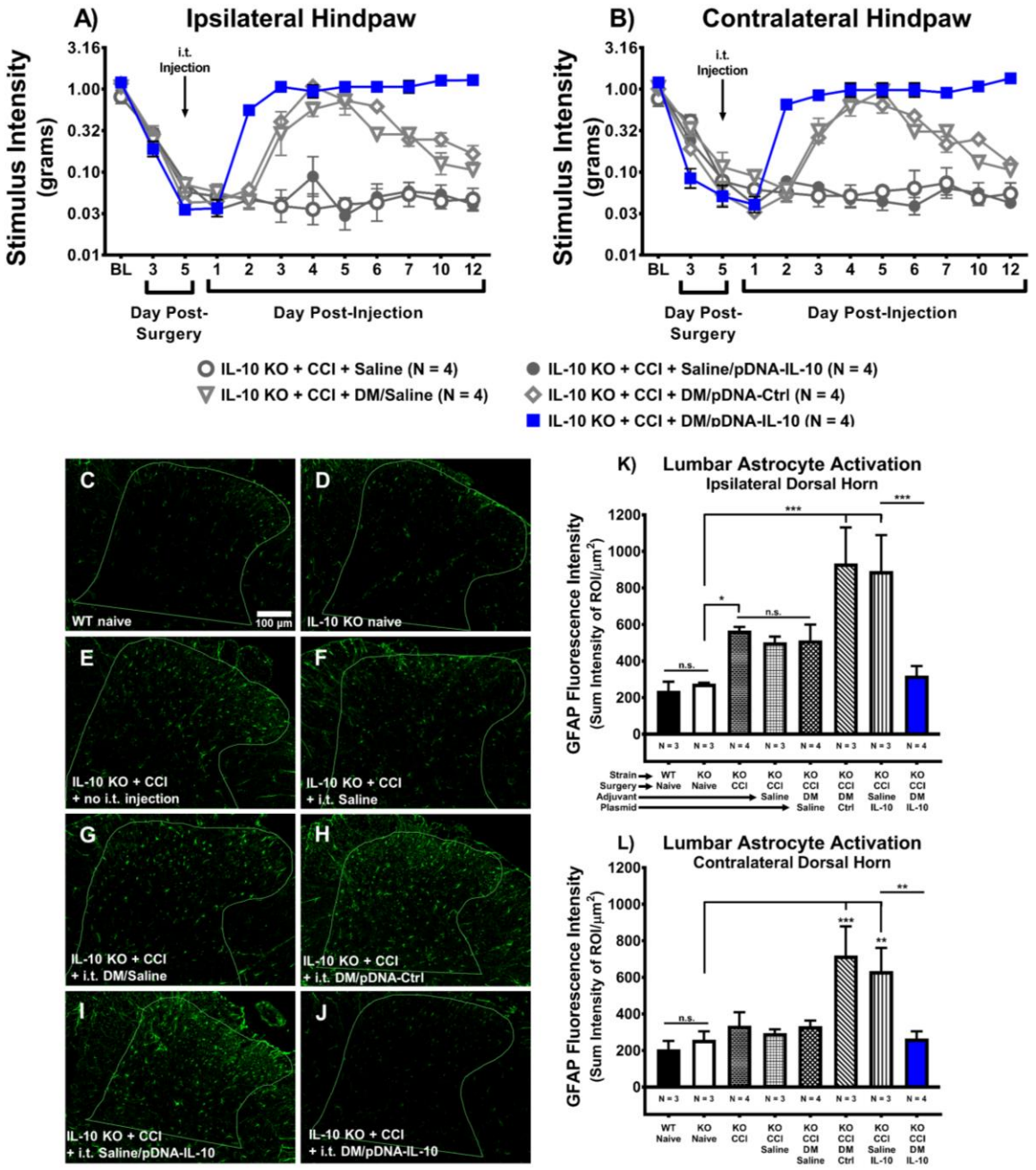


Figure 6: Intrathecal non-viral IL-10 gene therapy decreases astrocyte activation in the lumbar spinal cord dorsal horn.

Absolute threshold behavioral responses for (**A**) ipsilateral and (**B**) contralateral hindpaws are shown. Again replicating our prior results illustrated in **Figs. 1** and **2**, BL responses to low threshold mechanical stimuli were similar between all groups (ipsilateral, $F_{(4, 15)} = 1.25$, $P > 0.05$; contralateral, $F_{(4, 15)} = 48.84$, $P < 0.001$). For data collected between BL through Day 5, a main effect of time (ipsilateral, $F_{(2, 30)} = 306.14$, $P < 0.001$; contralateral, $F_{(2, 30)} = 221.66$, $P < 0.001$) was observed, again replicating our prior data (**Figs. 1** and **2**). After behavioral assessment on Day 5 post-surgery, all mice received an i.t. co-injection of pDNA-IL-10 (3µg in 7.5µL) with DM (25µg in 3µL) vs. DM/pDNA-Control, pDNA-IL-

10 with saline, DM with saline, or saline alone. Following injection, a main effect of time (ipsilateral, $F_{(8, 120)} = 35.817$, $P < 0.001$; contralateral, $F_{(8, 120)} = 42.82$, $P < 0.001$) and an interaction of time and treatment (ipsilateral, $F_{(32, 120)} = 9.81$, $P < 0.001$; contralateral, $F_{(32, 120)} = 13.53$, $P < 0.001$) were revealed. Comparisons between groups show that on Day 2 post-injection, CCI+DM/pDNA-IL-10-treated mice exhibit bilateral reversal from allodynia (ipsilateral, $P < 0.001$; contralateral, $P < 0.001$) compared to CCI+Saline/pDNA-IL-10-treated control mice, and these mice never return to allodynia. Surprisingly, on Day 3 post-injection, DM/Saline and DM/pDNA-Ctrl treated mice reverse from allodynia (ipsilateral, $P < 0.001$; contralateral, $P < 0.001$) but on Day 6 post-injection both control groups begin to return to bilateral allodynia (ipsilateral, $F_{(3, 45)} = 6.27$, $P < 0.01$; contralateral, $F_{(3, 45)} = 5.62$, $P < 0.01$). These transient effects of DM on pain thresholds was previously observed in data from separate experiments represented in **Fig. 2**. N = 4 mice/group for all behavioral conditions. (**C-J**) Representative images (20x objective) of GFAP-stained lumbar spinal cord dorsal horn sections from tissues collected on Day 12 post-injection that were part of the image analysis. (**K-L**) Image analysis of acquired GFAP-stained images of ipsilateral and contralateral lumbar spinal cord dorsal horn revealed a main effect of gene therapy treatment ($F_{(7, 19)} = 6.71$, $P < 0.001$; $F_{(7, 19)} = 5.64$, $P < 0.01$, respectively). Acquired GFAP-stained images of contralateral lumbar spinal cord dorsal horn are not shown, as the images appear highly similar to those from the ipsilateral lumbar spinal cord. Statistical analyses reveal no significant ipsilateral or contralateral differences in GFAP fluorescence intensity between WT Naïve and IL-10 KO Naïve tissues. IL-10 KO+CCI un-injected mice exhibit heightened ipsilateral GFAP fluorescence intensity compared to IL-10 KO Naïves and are not significantly different than CCI+Saline or CCI+ DM/Saline conditions. Bilaterally, GFAP immunoreactive fluorescence intensity levels are greater in IL-10 KO+CCI+DM/pDNA-Ctrl mice and CCI+Saline/pDNA-IL-10 mice compared to IL-10 KO Naïve mice, but do not differ significantly from each other. Pain relieved IL-10 gene therapy treated mice (IL-10 KO+CCI+DM/pDNA-IL-10) reveal significantly less GFAP fluorescence intensity compared to allodynic gene therapy controls (IL-10 KO+CCI mice treated with either DM/pDNA-Ctrl or Saline/pDNA-IL-10), and are not significantly greater than IL-10 KO Naïves. Bar graphs represent Mean $\pm$ SEM. Superimposed outlines in images (**C-J**) demarcate the unique dorsal horn regions of interest for each slice. For immunohistochemical analyses, N = 3-4 mice per group, as indicated. Post-hoc multiple comparisons via Fisher's LSD ($\alpha = 0.05$; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$).

CHAPTER III

Mannose receptor-mediated anti-inflammatory effects are independent of interleukin-10

Abstract

Mannose receptor (MR; CD206) activation by D-mannose (DM) induces anti-inflammatory signaling and has been utilized as an effective adjuvant for improved non-viral interleukin-10 (IL-10) gene therapy for treatment of peripheral neuropathic pain. MR-mediated anti-inflammation is IL-10-independent, but little is known about the cellular mechanisms and intracellular pathways involved. Furthermore, the cellular inflammatory environment required for MR-induced anti-inflammation and transgene adjuvant effects are not well understood. To investigate these questions, combinations of DM and plasmid DNA encoding a therapeutic IL-10 transgene (pDNA-IL-10) were incubated with peritoneal macrophages (PMs) from C57BL/6 (wildtype; WT) and IL-10 deficient (IL-10 KO) mice under the following conditions: a) co-stimulation with lipopolysaccharide (LPS) for 24 or 36 hours, b) 12 hours LPS priming followed by 12 or 24 hours of co-stimulation with or without LPS, or c) 36 hours incubation in the absence of LPS (unstimulated). Unstimulated WT PMs do not reveal any significant differences in nitric oxide (NO), a critical pro-inflammatory mediator and indicator of cell stress, following treatment with DM or DM/pDNA-IL-10. NO levels are effectively decreased by both DM and DM/pDNA-IL-10 following LPS co-stimulation in both WT and IL-10 KO PMs. DM/pDNA-IL-10 treatment following LPS priming resulted in significant decreases in NO

produced by WT PMs. This effect was abolished in both WT and IL-10 KO LPS-primed PMs when subsequently co-stimulated with either LPS+DM or LPS+DM/pDNA-IL-10. IL-10 protein levels following co-stimulation with LPS+DM or LPS+DM/pDNA-IL-10 in WT cells were not significantly higher than LPS alone, and no IL-10 protein was detectable in any IL-10 KO treatment condition. Together, these preliminary data indicate that MR-mediated anti-inflammation potently decreases NO production by an IL-10-independent mechanism. Additionally, the current report indicates that the inflammatory status of MR-expressing cells plays a key role in the efficacy of DM as an anti-inflammatory adjuvant. Future work is required to understand the (1) downstream signaling pathways responsible for both MR-mediated decreases in NO, and (2) optimal DM/pDNA-IL-10 cell culture dose-formulations to explore MR-mediated improvements in non-viral transgene therapy.

1. Introduction

The mannose receptor (MR; CD206) is a C-type lectin receptor known to recognize glycoconjugates that terminate in mannose, fucose, or N-acetyl glucosamine (Taylor et al., 2005). MR is expressed by macrophages, dendritic cells, and microvascular endothelial cells, as well as in nervous tissues by astrocytes, microglia, Schwann cells, and select populations of neurons (Takahashi et al., 1998; Burudi et al., 1999; Linehan et al., 1999; Burudi and Regnier-Vigouroux, 2001; Galea et al., 2005; Baetas-da-Cruz et al., 2009; Martinez-Pomares, 2012). In addition to its role in clearance of endogenous molecules like collagen, lysosomal hydrolases, and tissue-type plasminogen activator (t-PA), it has been implicated in recognition of mannosylated residues present on certain pathogens (Otter et al., 1991; Taylor et al., 2005; Martinez-Pomares, 2012; Sedaghat et al., 2014).

Though many simply consider MR to be an endocytic pattern recognition receptor (PRR), a growing literature supports that MR activation is associated with anti-inflammatory signaling. MR expression is increased on populations of anti-inflammatory "alternatively activated" macrophages (Gordon, 2003; Martinez-Pomares, 2012; Martinez and Gordon, 2014a). Additionally, activation of MR increases anti-inflammatory signaling. For instance, crosslinking of MR on dendritic cells leads to increased secretion of anti-inflammatory interleukin-10 (IL-10) and IL-1 receptor antagonist (IL-1RA) with concurrent decreases in levels of pro-inflammatory cytokines like IL-1β and tumor necrosis factor (TNF) (Chieppa et al., 2003). Administration of D-mannose (DM), a hexose sugar and MR ligand, induces protective ant-inflammatory signaling that attenuates pathology in a rodent model of acute lung injury (Zhang et al., 2004; Zhang et al., 2005; Xu et al., 2015).

Heightened pro-inflammatory signaling is implicated in chronic peripheral neuropathic pain. Following peripheral nerve damage glial cells (i.e. astrocytes, microglia, and satellite glia) in the spinal cord and dorsal root ganglia (DRG), perhaps with help from trafficking immune cells (i.e. macrophages), secrete an abundance of pro-inflammatory mediators which are critical for the induction and maintenance of chronic neuropathic pain symptoms (Milligan and Watkins, 2009; Grace et al., 2014; Ji et al., 2016). Interleukin-10 (IL-10) is a potent anti-inflammatory cytokine shown to reverse chronic pain by decreasing glial activation and the expression of pain-relevant pro-inflammatory mediators like IL-1β, TNF, nitric oxide (NO) (Milligan et al., 2012). One of the most effective routes has been to administer IL-10 via intrathecal (i.t.) non-viral transgene using a naked plasmid encoding the IL-10 gene (pDNA-IL-10) to treat neuropathic pain (Milligan et al., 2012). Recent work has demonstrated that non-viral IL-10 gene therapy is dramatically improved (e.g. enduring pain relief resulting from a single small dose of pDNA-IL-10) when co-injected with DM (Dengler et al., 2014; Vanderwall et al., 2018). Intriguingly, control i.t. injections of DM alone or with a control plasmid are able to induce transient pain relief. It has been hypothesized that DM-mediated transient pain relief occurred through MR activation with consequent increased expression of endogenous IL-10 (Dengler et al., 2014). However, both enduring and transient pain relief were observed following i.t. DM/pDNA-IL-10 or DM alone, in neuropathic mice deficient in endogenous IL-10 (IL-10 KO) (Vanderwall et al., 2018). Together these data indicate that MR activation is associated with both IL-10 dependent and IL-10 independent anti-inflammatory signaling mechanisms.

Macrophages express MR, are highly phagocytic, and are known migrate to the DRG following peripheral nerve damage, making them prime targets for application of both DM and DM/pDNA-IL-10 gene therapy. To better understand the cellular mechanisms of MR-mediated anti-inflammation leading to transient pain relief *and* improved transgene efficacy, MR stimulation was examined *in vitro* using macrophages collected from C57BL/6 (WT) and IL-10 KO mice. While still preliminary, the current report demonstrates that MR-mediated decreases in NO production are IL-10-independent.

2. Materials and Methods

2.1 Animals

All experiments were performed using adult male mice (8-9 weeks of age). C57BL/6J (WT; RRID: IMSR_JAX:000664) or B6.129P2-Il10^{tm1Cgn}/J (IL-10 KO; RRID: IMSR_JAX:002251) mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA) and maintained in specific-pathogen free conditions. Prior to handling, all animals were acclimated to the mouse colony at the University of New Mexico (UNM) Health Sciences Center Animal Facility for a minimum of 7 days. Animals were housed in groups of 3-5 at 23˚ $\pm$ 2˚C in light controlled rooms (12:12 light:dark; lights on at 6:00am) and fed standard rodent chow and water *ad libitum*. All procedures were approved by the Institutional Care and Use Committee (IACUC) of the UNM Health Sciences Center and conducted in accordance to the NIH Guidelines for the Care and Use of Laboratory Animals.

2.2 Cell Culture Media

Ex vivo cell culture experiments were performed with DMEM/F-12 media (Sigma-Aldrich; Cat#: D6421) supplemented with 2mM GlutaMAX™ (Gibco; Cat#: 35050061), 20U/mL penicillin with 20µg/mL streptomycin (Gibco; Cat#: 15140122), and with either 10% fetal bovine serum (FBS) or 10% Cell-Ess® (Bio-Ess Laboratories). Cell-Ess® is a chemically defined serum replacement that is free of any animal components. This approach was initially chosen as an alternative to traditional serum based-supplements in an effort to increase reproducibility and to avoid ill-defined effects of exposure to non-murine proteins. Unfortunately, before full completion of these experiments, Bio-Ess business operations were terminated, so subsequent experiments were performed with traditional 10% FBS instead of 10% Cell-Ess.

2.3 Isolation and ex vivo culture of resident peritoneal macrophages (PMs)

Lavage of the peritoneal cavity provides easy access to a population of mature tissue macrophages, and while anatomically distinct from peri-spinal meningeal tissue macrophage (location of the i.t. route), peritoneal macrophages (PMs) provide an approximation of spinal tissue macrophage responses to DM and/or DM/pDNA-IL-10 gene therapy. Resident PMs were isolated as previously described (Zhang et al., 2008). Briefly, mice were euthanized separately by CO_2 inhalation. The abdomen was sprayed with 70% ethanol. A small incision in the abdominal skin was made using forceps and blunt dissection scissors, then manual retraction of the skin was applied to carefully expose the

peritoneal wall. Using a 25-G needle (Becton Dickinson & Co.; Cat#: 305122) attached to a 10mL syringe, 8-10mL of ice cold sterile PBS (pH = 7.4; Gibco; Cat#: 10010023) was gently injected into the peritoneal cavity, avoiding puncture of internal organs. The distended abdomen was vigorously shaken side-to-side for ~1.5min. A 20-G needle (Becton Dickinson & Co.; Cat#: 305175) attached to a 10mL syringe was used to extract ~6-7mL of peritoneal lavage fluid. The needle was removed from the syringe prior to deposition of the collected lavage fluid into a 50mL conical tube on ice. Lavage samples from 6 animals were pooled per experimental round, such that each experiment represents pooled PMs from 6 mice. Samples were spun at 400 x *g* for 10min at 4˚C. The resulting pellet was resuspended in 1mL Red Blood Cell Lysis Buffer (Sigma; Cat#: R7757) and incubated on ice for 5 minutes, diluted with 15-20mL of complete DMEM/F-12, pelleted at 400 x *g* for 10min at 4˚C, and then resuspended in 1mL of complete DMEM/F-12 media for counting. Live cells were quantified on a hemocytometer using trypan blue staining exclusion criteria. Cell suspensions were then adjusted for plating.

PMs were distributed 2.0-2.5 x 10^5 cells/well on a 24-well tissue culture plate (Corning; Cat#: 353847) in 500µL complete DMEM/F-12 and incubated at 37˚C. After resting 13-14hrs, old media was replaced with fresh complete DMEM/F-12 media and treatment conditions were applied, reaching a total volume of 500µL/well (2-3 well replicates per treatment condition).

2.4 Preparation of plasmid DNA

Plasmid DNA was prepared as previously described (Vanderwall et al., 2018). Briefly, the plasmid vector pDNA-IL-10 (also called pTR2-CB-mIL10^{F129S}) used in these studies is the mouse IL-10 equivalent of a rat IL-10 plasmid that was fully described previously and is derived from an adeno-associated virus-2 (AAV-2) expression cassette (Milligan et al., 2005b; Milligan et al., 2006b). It consists of a 5.9 kilobase pair circular plasmid DNA (pDNA) containing a transcriptional cassette consisting of a hybridized cytomegalovirus enhancer/chicken beta-actin promoter (CMV enh/CB pro) driving expression of the mouse Il10 gene containing a point mutation (mIL-10^{F129S}), and a simian virus 40 (SV40) polyadenylation signal (SV40 poly(A)). The transcription cassette is flanked by 149 bp inverted terminal repeat (ITR) sequences. The plasmid backbone contains an ampicillin resistance (Amp^{r}) gene, as well as components unique to the original AAV-2 expression cassette such as an intervening sequence (IVS; intron). The control plasmid (pDNA-Ctrl) is an analogous plasmid cassette lacking the IL-10 transgene. Plasmids were amplified in SURE2 Supercompetent Cells (Agilent Technologies; Cat#:200152) because the ITR elements are often deleted in conventional E. coli strains (Milligan et al., 2006b) resulting in reduced transgene expression. Plasmid DNA was isolated using an EndoFree Plasmid Giga Kit (Qiagen; Cat#:12391) according to manufacturer's instructions. Purified endotoxin-free plasmids were resuspended in sterile Dulbecco's PBS (1x) (Gibco; Cat#:14190-144) with 3% sucrose, aliquoted, and stored at -20˚C.

2.5 Stimulation and Transfection Experimental Approaches

Experimental Approach #1. Following collection of BL supernatant for NO assessment (see *section 2.5*), media was replaced and WT PMs were incubated for 36 hours at 37˚C in 500mL complete DMEM/F-12 (w/ 10% Cell-Ess) with various stimulation conditions. Treatment conditions are as follows: a) 50mM DM, b) 100mM DM, c) pDNA-Ctrl (5ug/well), d-g) 50mM DM + pDNA-IL-10 (0.005, 0.05, 0.5, or 5.0μg per well), or h) 100mM DM + pDNA-IL-10 (0.5μg/well). Cells were incubated with treatment media for 36 hours. Supernatant samples were collected after 36 hours of treatment for NO detection (see *section 2.5*). D-mannose (Sigma-Aldrich; Cat#:M6020) was dissolved in sterile isotonic sterile saline (0.9% w/v sodium chloride; Ricca Chemical Company; Cat#: 7210-32). N = 2 wells per condition.

Experimental Approach #2. Performed similarly to Experimental Approach #1, but with the following changes. After the initial resting period, PMs from IL-10 KO or WT mice were primed by incubation for 12 hours at 37˚C in 500mL complete DMEM/F-12 (w/ 10% FBS or 10% Cell-Ess as indicated in figure legends) with or without lipopolysaccharide (LPS; 500ng/mL). After this priming step, supernatants were replaced with fresh media containing the following treatment conditions: a) media alone, b) LPS (500ng/mL), c) LPS + 100mM DM, or d) LPS + DM + pDNA-IL-10 (5μg/well). Cells were allowed to incubate with treatment media for 12 or 24 hours. Supernatant was collected for NO detection at BL, following LPS priming, and at 12 and 24 hours treatment. Supernatant was collected for IL-10 protein detection at 24 hours of treatment. N = 2-3 wells per condition.

Experimental Approach #3. Performed similarly to Experimental Approach #2, but with the following changes. After the initial resting period, PMs from IL-10 KO or WT mice were co-stimulated for 24 or 36 hours with the following treatment conditions: a) media alone, b) LPS (1μg/mL), c) LPS (500ng/mL), d) LPS + 100mM DM, or e) LPS + DM + pDNA-IL-10 (5μg/well). Supernatant was collected for NO detection at BL, and at 12, 24, and 36 hours of treatment. Supernatant was collected for IL-10 protein detection at 36 hours of treatment. N = 2-3 wells per condition.

2.6 IL-10 protein and nitric oxide production

Supernatant samples (50μL per biological replicate) for assessment of NO production were collected (see *section 2.4* for timepoints). Samples were immediately measured using a commercially available Griess Reagent System following the manufacturer's instructions (Promega Corp.; Cat#: G2930).

Supernatant samples (120uL per biological replicate, per target) were collected at the terminal timepoint and stored at -20˚C for future protein analysis. Frozen supernatant samples were later thawed at room temperature and assessed in duplicate for IL-10 protein content by enzyme-linked immunosorbent assay (ELISA) via a mouse IL-10 Quantikine ELISA kit (R&D Systems; Cat#: SM1000B) per the manufacturer's instructions.

2.7 Statistical Analyses

Nitric oxide and IL-10 protein data were analyzed by one-way ANOVA in GraphPad PRISM version 7.02 (GraphPad Software Inc.; RRID:SCR_002798). To control the type I error rate during all multiple comparisons, Fisher's LSD test (reported with adjusted P values) was applied for *post hoc* examination of possible group differences selected *a priori*. Unpaired t-tests were applied to NO and IL-10 data for additional group comparisons selected *a priori.* The threshold for statistical significance for all comparisons was set *a priori* to $\alpha = 0.05$. All data are presented as the Mean $\pm$ Standard Error of the Mean (SEM).

3. Results

3.1 MR-mediated modulation of nitric oxide in unstimulated wildtype PMs

Preliminary *in vitro* studies were designed with the goal of understanding *in vivo* parameters under which MR activation induces anti-inflammatory signaling that may aid in non-viral IL-10 transgene uptake. Prior *in vivo* studies indicate that MR-mediated improvements in IL-10 gene therapy may be most efficacious in a pre-existing inflammatory environment (e.g. the DRG of a neuropathic mouse), perhaps due to increased MR availability (Vanderwall et al., 2018). To test this, DM/pDNA-IL-10 treatment was first examined in unstimulated PMs collected from WT mice (**Fig. 1A**). Plated cells were exposed for 36 hours to various combinations and concentrations of DM,

pDNA-IL-10, and pDNA-Ctrl. Nitric oxide levels were within detectable range, but no significant differences were detected by unpaired t-tests for any *a priori* selected comparisons. These results support that exposure to plasmid DNA, whose inherent CpGs could induce a pro-inflammatory response via toll-like receptor 9 (TLR9), is not stimulatory at the chosen concentrations. These data further suggest DM-mediated anti-inflammation and improvement in non-viral IL-10 gene therapy require pre-existing pathology.

3.2 MR-mediated modulation of nitric oxide in LPS stimulated wildtype PMs

To recapitulate *in vitro* the *in vivo* DRG conditions in which DM and DM/pDNA-IL-10 had therapeutic effects in neuropathic rodents (Dengler et al., 2014; Vanderwall et al., 2018), DM/pDNA-IL-10 therapy was examined in the context of LPS co-stimulation and LPS priming (**Fig. 1B-C**). For LPS co-stimulation, WT PMs were treated with either media, LPS (500ng/mL), or co-stimulated with LPS plus DM/pDNA-IL-10 (500mM; 5μ/well) for 24 hours **(Fig. 1B**). Cells stimulated with LPS alone had significantly higher ($P < 0.05$) NO levels than untreated controls at the 24 hour timepoint ($F_{(2, 3)} = 23.11$, $P < 0.05$). Interestingly, co-stimulated cells treated with LPS+DM/pDNA-IL-10 had significantly less NO than cells stimulated with LPS alone ($P < 0.05$).

For LPS priming (**Fig. 1C**), WT PMs were incubated for 12 hours with LPS, at which time the LPS-containing media was replaced with fresh media (no LPS) and incubated for 12 hours with or without DM/pDNA-IL-10. LPS pre-treated cells that did not receive DM/pDNA-IL-10 had significantly higher levels of NO at 12 hours of treatment

($t_{(2)}$ = 5.72, $P < 0.05$). Taken together, these findings further support that DM-mediated effects are not readily discernable outside the context of an inflammatory environment.

3.3 MR activation decreases nitric oxide in an IL-10-independent manner

In preliminary studies (**Fig 1**), the observed decreases in NO production following treatment with DM/pDNA-IL-10 could have resulted from successful IL-10 transgene expression, DM alone, or a synergistic combinatorial effect. To address whether IL-10 transgene expression is critical for the observed suppression of NO, DM vs. DM/pDNA-IL-10 treatment was examined in the presence or absence of endogenous IL-10 by utilizing WT vs. IL-10 KO PMs (**Fig. 2**).

For co-stimulation (**Fig 2A**), cells were incubated for 36 hours with combinations of LPS (0.5μg/mL), DM (100mM), and pDNA-IL-10 (5μg/500μL). Additional cells exposed to either a high dose of LPS (1.0μg/mL) or media alone served as positive and negative controls, respectively, for both NO and IL-10 production. A statistically significant difference in NO production was detected by one-way ANOVA for both WT and IL-10 KO treatment conditions ($F_{(9, 18)}$ = 13.26, $P < 0.0001$). In WT PMs, multiple comparisons reveal a significant decrease in NO production in LPS+DM co-stimulated cells compared to cells that received LPS alone ($P < 0.05$). A significant decrease in NO was also observed in WT PMs treated with LPS+DM/pDNA-IL-10 compared to LPS alone ($t_{(4)}$ = 7.36, $P < 0.01$). Significant decreases in NO were also observed in IL-10 KO PMs given either LPS+DM ($P < 0.0001$) or LPS+DM/pDNA-IL-10 ($P < 0.0001$). Interestingly, while WT and IL-10 KO cells given the high dose LPS were not significantly different (*P*

> 0.05), when given low dose LPS, IL-10 KO cells produced significantly higher levels of NO compared to WT cells (P = 0.001). Despite a dramatic LPS response of NO in the absence of endogenous IL-10, both DM and DM/pDNA-IL-10 returned NO production to basal levels in co-stimulated IL-10 KO cells ($P > 0.05$).

For LPS priming (**Fig. 2B**), WT and IL-10 KO PMs were incubated for 12 hours with or without LPS, at which time the priming media was removed and replaced with fresh media containing no stimulants (media alone), LPS, LPS+DM, or LPS+DM/pDNA-IL-10. These cells were then incubated in these treatment conditions for 24 hours. A statistically significant group difference in NO production was detected by one-way ANOVA for both WT and IL-10 KO treatment conditions ($F_{(7, 16)} = 9.64$, $P < 0.001$). Multiple comparisons reveal increased NO production following LPS stimulation in both WT ($P < 0.01$) and IL-10 KO PMs ($P < 0.0001$). As before, IL-10 KO PMs produce significantly higher NO levels compared to WT ($P < 0.01$). Surprisingly, regardless of WT or IL-10 KO background, neither DM nor DM/pDNA-IL-10 led to significant decreases in NO levels in LPS-primed PMs compared to those given LPS alone ($P > 0.05$).

IL-10 protein production was assessed (**Fig 2C-D**) at the termination of both LPS co-stimulation and LPS priming culture paradigms. For LPS co-stimulated WT PMs (**Fig 2C**), statistically significant group difference in IL-10 protein production was detected by one-way ANOVA ($F_{(7, 16)} = 9.64$, $P < 0.001$). Multiple comparisons reveal significantly elevated IL-10 protein production in PMs treated with high dose LPS ($P < 0.0001$), low dose LPS ($P < 0.05$), LPS+DM ($P < 0.01$), and LPS+DM/pDNA-IL-10 ($P < 0.01$) compared to unstimulated controls. Surprisingly, no significant differences were observed in IL-10 protein levels between LPS, LPS+DM, and LPS+DM/pDNA-IL-10 treated WT

PMs ($P > 0.05$). For LPS pre-treated WT PMs (**Fig 2D**), no statistically significant group difference in IL-10 protein production was detected by one-way ANOVA ($F_{(3, 8)} = 3.18$, $P > 0.05$). IL-10 levels were below the level of detection across all IL-10 KO conditions in both stimulation paradigms (data not shown).

Though still preliminary, these data further support an IL-10-independent anti-inflammatory pathway downstream of MR that decreases expression of nitric oxide. Additionally, current findings indicate that the timing of pro-inflammatory stimulation has a major impact on the ability of MR activation to promote anti-inflammation.

4. Discussion

The current pilot data provide new clues into the cellular mechanisms underlying MR-mediated anti-inflammation. The function of MR activation as an adjuvant for non-viral gene therapy required further refinement of the experimental conditions. Preliminary studies in PMs indicate that MR activation by DM mediates decreased NO in LPS stimulated cells by an IL-10-independent mechanism. Additionally, MR effects were most significant when DM was applied simultaneously with LPS/TLR4 stimulation, as NO was unaffected by DM in LPS pre-treated PMs. DM alone vs. DM/pDNA-IL-10 were indistinguishable regarding modulation of NO. Interestingly, NO production by IL-10 KO and WT PMs were indistinguishable when exposed to high dose LPS, but low dose LPS unmasked significant increased NO production in IL-10 KO PMs. Though not statistically significant, a clear trend was observed for increased IL-10 production in WT PMs co-stimulated with LPS+DM/pDNA-IL-10 or LPS+DM compared to LPS alone, with the

highest IL-10 protein levels observed in gene treated cells (excluding high dose LPS). Thus, these data support that MR activation induces anti-inflammatory signaling that are independent of IL-10, and that further work is required to identify ideal culture conditions by which to unambiguously observe DM/pDNA-IL-10 effects *in vitro*.

4.1 IL-10 regulation of nitric oxide production in stimulated PMs

IL-10 KO mice have previously been shown to have exaggerated pro-inflammatory immune responses following stimulation that predispose this mouse strain to significant disease pathology (Kuhn et al., 1993; Berg et al., 1995). For instance, IL-10 KO mice experience higher mortality levels and increased production of serum pro-inflammatory mediators, including NO, following acute endotoxic shock compared WT mice (Berg et al., 1995). Splenocytes from IL-10 KO mice infected with *Helicobacter hepaticus* produce higher levels of NO than WT counterparts when both cell types were re-challenged *ex vivo* with bacterial antigen (Kullberg et al., 1998). The current data are consistent with prior findings and demonstrate heightened NO production in IL-10 KO vs. WT PMs following LPS stimulation. Interestingly, this effect was not observed following stimulation with a higher dose of LPS. It is possible that a ceiling effect caused by heightened TLR4 stimulation prevents further discrimination between the IL-10 KO and WT backgrounds.

4.2 IL-10 transgene expression in reduced nitric oxide levels

Though no exogenous IL-10 protein was detected by ELISA in IL-10 KO PMs, IL-10 transgene may still have been successfully expressed at low levels. It is possible that the clinically relevant low levels of pDNA-IL-10 applied in these studies might only allow for very low levels of IL-10 protein production. This could hinder the ability to detect IL-10 in IL-10 KO PMs or to distinguish endogenous from exogenous IL-10 in WT PMs. Prior studies in IL-10 KO mice indicate that a very low level of IL-10 transgene expression is necessary for potent therapeutic effects (Vanderwall et al., 2018). For proof of concept, future studies may aim to increase the doses of pDNA-IL-10 or the adjuvant DM applied. Additionally, mRNA analysis may provide a more sensitive assay for detection of IL-10 transcriptional activation.

4.3 Mannose receptor regulation and inflammation

A goal of this report was to examine whether an ongoing pro-inflammatory environment was necessary or beneficial for DM-mediated improvement of non-viral IL-10 gene therapy. Relevant to these culture studies, *in vivo* pro-inflammatory mediators increase in expression at the level of the DRG following peripheral nerve damage. For instance, damaged peripheral neurons express the pro-inflammatory chemokine CCL2 and consequently recruit macrophages to the DRG (Schreiber et al., 2001; Kwon et al., 2015; Zhang et al., 2016). TLR4 signaling has also been implicated in both neuropathic pain pathogenesis and activation of macrophages (Tanga et al., 2005). Prior work by Vanderwall

et al. demonstrate that i.t. injection of DM/pDNA-IL-10 into IL-10 KO mice leads to IL-10 transgene expression in the ipsilateral, but not contralateral, DRG of neuropathic mice while not expressed in sham controls (Vanderwall et al., 2018). Furthermore, ipsilateral DRG of neuropathic IL-10 KO mice have increased mRNA levels for MR and the IL-10 receptor as compared to sham controls (Vanderwall et al., 2018). Therefore, to recapitulate the *in vivo* DRG environment, the TLR4 ligand was applied as either a co-stimulant or a priming agent.

While co-stimulation of WT and IL-10 KO PMs with LPS plus DM or DM/pDNA-IL-10 resulted in decreased NO production compared to those given LPS alone, this effect was not observed in LPS pre-treated cells. This is surprising, as it was hypothesized that the priming paradigm more readily replicates the *in vivo* model in which DM or DM/pDNA-IL-10 produce pain relief in chronically allodynic animals. This incongruity could be explained by evidence that macrophage stimulation, especially via TLR4, can be linked to decreased expression and functionality of MR. For instance, bone marrow-derived macrophages (BMDMs) treated with LPS or LPS/PMA have 60% and 70% less surface binding, respectively, of radiolabeled mannose-BSA (Shepherd et al., 1990; Chroneos and Shepherd, 1995). A similar effect is observed in microglia from neonatal rats, which when cultures with LPS for 48 hours exhibit 45% less MR protein in whole cell lysates (Marzolo et al., 1999). In a more recent study, in a mouse model of acute lung injury, mice given intratracheal LPS have significantly less MR mRNA levels 6 hours later, but this effect is dose-dependently reversed by co-administration with i.v. DM (Xu et al., 2015). These data suggest LPS priming results in decreased availability of MR, thereby preventing efficacy of either DM or DM/pDNA-IL-10. The discrepancy in MR-mediated

anti-inflammation between the two priming experiments (**Figs. 1C** and **2B**) may be due to the change in experimental design by which LPS exposure, which had been previously been completely removed after priming (**Fig 1C**), was extended into the treatment window in **Fig. 2B**. The reprieve from LPS exposure in the initial priming experiment (**Fig. 1C**) may have allowed for less MR downregulation, thereby allowing for DM efficacy. These findings further support that the DM and DM/pDNA-IL-10-mediated decreases in NO production occur through an MR-dependent process. Future investigations aim to unambiguously confirm the role of MR in these effects via blockade with MR antagonists.

4.4 MR and IL-10-independent mechanisms of nitric oxide regulation

The current report provides intriguing evidence that MR-mediated decreases in nitric oxide production is IL-10-independent. TGF-β signaling may provide an alternative to IL-10 downstream of MR-activation. TGF-β is an anti-inflammatory cytokine previously shown to decrease expression of NO. For instance, application of TGF-β prevents increased NO expression in BMDMs co-stimulated with LPS/IFN-γ (Corradin et al., 1993). In an *in vitro* model of myocardial hypoxic-ischemia, TGF-β pretreatment of cardiomyocytes prevents hypoxia-induced increases in both inducible nitric oxide synthase (NOS) mRNA levels and NO production (Chen et al., 2001). TGF-β may also be linked to increased activity of arginase, an enzyme that antagonizes the effect of NOS (Boutard et al., 1995). Interestingly, MR-mediated arginase induction appears to be utilized as an escape mechanism by intracellular pathogens, an effect that may be linked to MR-induced changes in MAPK signaling cascades (Garrido et al., 2011; Nzoumbou-Boko et al., 2017). There is

significant cross-talk between the TGF-β and MAPK signaling pathways (Gui et al., 2012). While no single pathway has been definitively described to be responsible for MR-mediated intracellular signaling, TGF-β may serve as an intriguing target of future investigation.

5. Conclusion

In summary, these data provide evidence that MR activation induces anti-inflammatory effects that are IL-10-independent, and which potently suppress NO production in LPS co-stimulated cells. Additionally, the current report indicates that the timing of cellular activation and MR expression are key determinants of DM-induced anti-inflammatory signaling. Further work is required to better understand the downstream signaling pathways that are critical for MR-mediated anti-inflammatory and transgene adjuvant effects.

CHAPTER III FIGURES

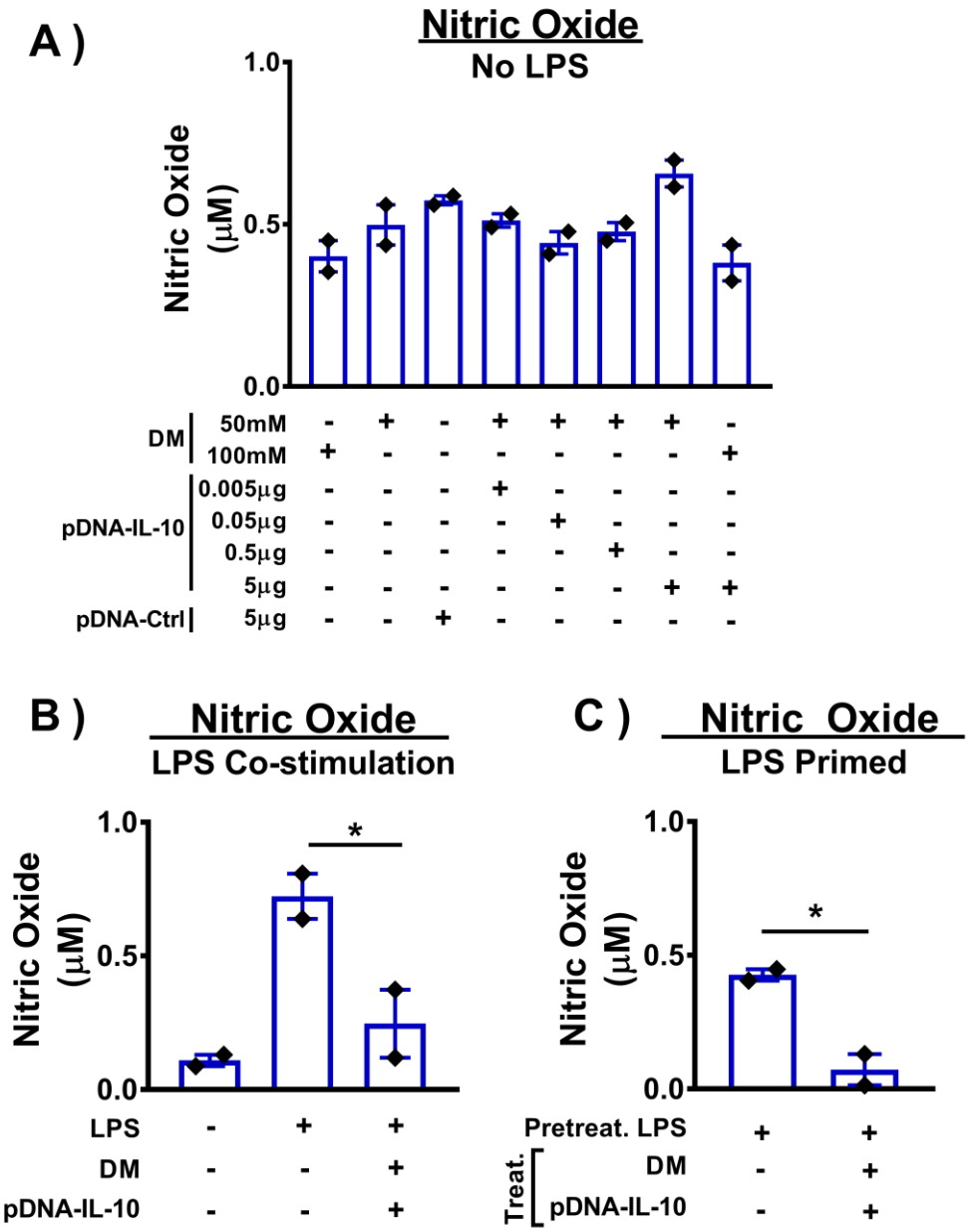


Figure 1: MR activation decreases nitric oxide production in LPS-stimulated WT peritoneal macrophages

Peritoneal macrophages (PMs) were pooled from six WT mice and cultured *ex vivo* for 13-14 hours prior to stimulation or treatment (N = 2 wells per condition; 2-2.5 x 10^5 cells in 500μL/well; 24 well plate). (**A**) Unstimulated PMs were incubated for 36 hours with

combinations of DM (50mM or 100mM), pDNA-IL-10 (0.005μg, 0.05μg, 0.5μg, or 5μg/well), or a control plasmid (pDNA-Ctrl; 5μg/well) as indicated. No significant differences in NO levels were detected by unpaired t-tests for any *a priori* selected comparisons were detected after 36 hours treatment ($P > 0.05$). (**B**) PMs were co-stimulated for 24 hours with combinations of LPS (500ng/mL), D-mannose (DM; 100mM), and non-viral plasmid DNA encoding interleukin-10 (pDNA-IL-10; 5μg/well). A statistically significant difference between groups for NO production was detected ($F_{(2, 3)} = 23.11$, $P < 0.05$). Cells stimulated with either LPS had significantly higher NO levels than untreated controls at 24 hours ($P < 0.05$). Cells co-stimulated with LPS+DM/pDNA-IL-10 had significantly less NO than LPS stimulated PMs ($P < 0.05$). (**C**) PMs were primed for 12 hours with LPS (500ng/mL) prior to stimulation in the absence of LPS for 12 hours with either DM/pDNA-IL-10 or media alone. A statistically significant difference between LPS-primed groups for NO production was detected ($t_{(2)} = 5.72$, $P < 0.05$), with less NO observed following 12 hours of DM/pDNA-IL-10 treatment vs. media. For NO analyses, levels are presented as Mean $\pm$ SEM. Post-hoc multiple comparisons via Fisher's LSD or unpaired t-test ($\alpha = 0.05$; $^*P < 0.05$).

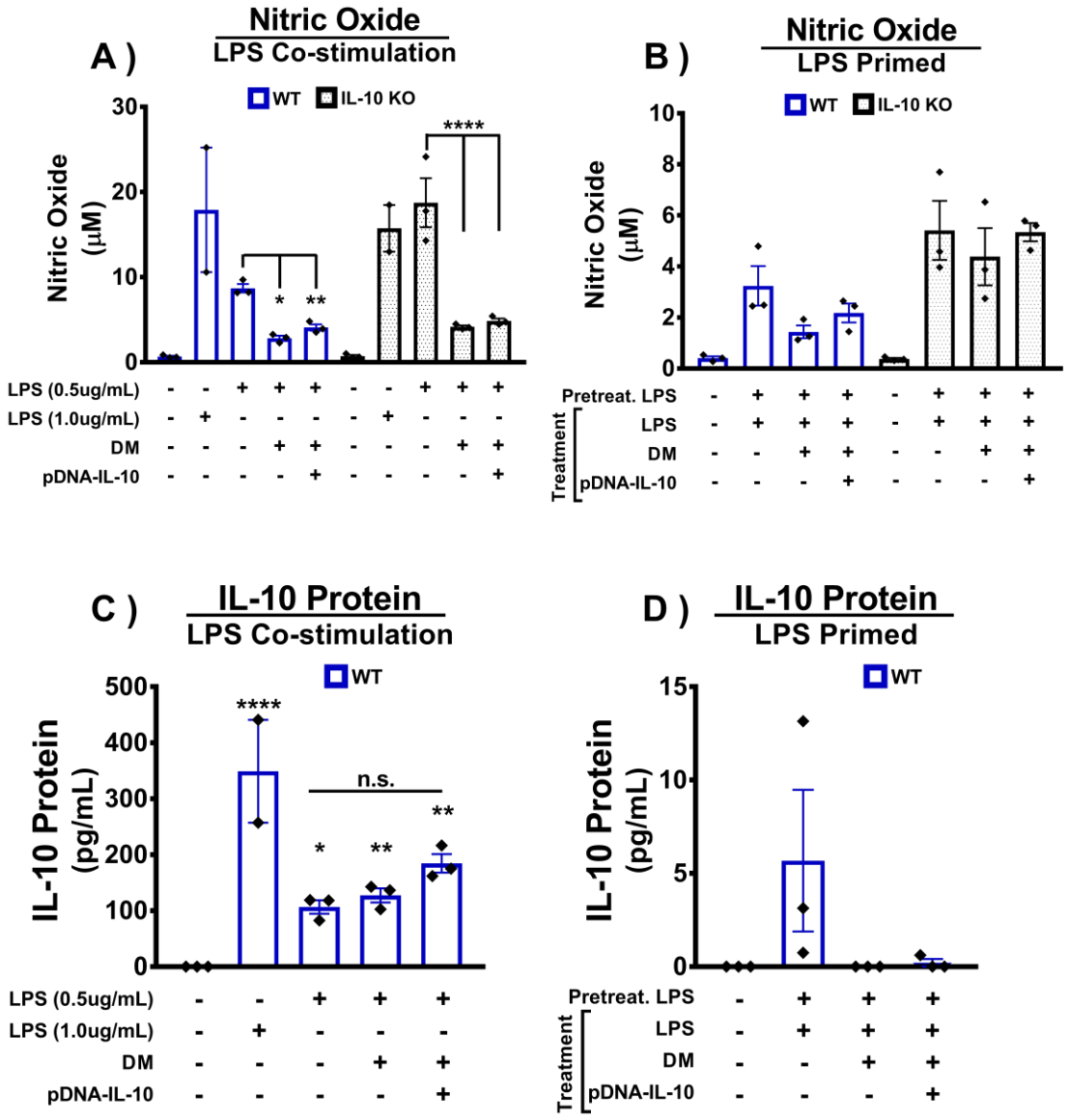


Figure 2: MR-mediated anti-inflammation is independent of interleukin-10
Peritoneal macrophages (PMs) were pooled separately from six WT and six IL-10 KO mice and cultured *ex vivo* for 13-14 hours prior to stimulation or treatment (N = 2-3 wells per condition; 2.5 x 10^5 cells in 500μL/well; 24 well plate). (**A**) PMs were co-stimulated for 36 hours with combinations of LPS (0.5μg/mL), D-mannose (DM; 100mM), and non-viral plasmid DNA encoding interleukin-10 (pDNA-IL-10; 5μg/well). Additional PMs exposed to either a high dose of LPS (1.0μg/mL) or media alone served as positive and negative controls, respectively, for both NO and IL-10 production. At 36 hours of co-stimulation, a statistically significant difference between groups for NO production was detected ($F_{(9, 18)}$ = 13.26, $P < 0.0001$). In WT PMs, multiple comparisons reveal a significant decrease in NO production in LPS+DM co-stimulated cells compared to cells that received LPS alone ($P < 0.05$). WT PMs treated with LPS+DM/pDNA-IL-10 had significantly more NO compared to LPS treatment alone ($t_{(4)}$ = 7.36, $P < 0.01$). Significant decreases in NO were

also observed in IL-10 KO PMs given either LPS+DM or LPS+DM/pDNA-IL-10 also had significantly less NO than LPS treatment alone ($P < 0.0001$). WT and IL-10 KO cells given the high dose LPS were not significantly different ($P > 0.05$), but low-dose LPS-stimulated IL-10 KO PMs produced significantly more NO compared to WT cells ($P = 0.001$). Both DM and DM/pDNA-IL-10 returned NO production to basal levels in co-stimulated IL-10 KO cells ($P > 0.05$). (**B**) PMs were primed with either LPS (0.5μg/mL) or media for 12 hours prior to a 24 hour treatment with either LPS, LPS+DM (100mM), or LPS+DM/pDNA-IL-10 (5μg/well). At 24 hours of treatment, a statistically significant difference was detected between groups for NO production ($F_{(7, 16)} = 9.64$, $P < 0.001$). Multiple comparisons reveal increased NO production following LPS stimulation in both WT ($P < 0.01$) and IL-10 KO PMs ($P < 0.0001$), with IL-10 KO NO levels significantly higher than for WT ($P < 0.01$). Neither DM nor DM/pDNA-IL-10 had significantly different NO levels than LPS alone ($P > 0.05$). (**C**-**D**) Supernatant IL-10 protein levels were assessed at the termination of both LPS co-stimulation and LPS priming paradigms. IL-10 levels were below the level of detection across all IL-10 KO conditions in both stimulation paradigms (data not shown). (**C**) IL-10 protein levels were significantly elevated following LPS co-stimulation in WT PMs ($F_{(7, 16)} = 9.64$, $P < 0.001$), with elevated IL-10 detected for WT PMs treated with high dose LPS ($P < 0.0001$), low dose LPS ($P < 0.05$), LPS+DM ($P < 0.01$), and LPS+DM/pDNA-IL-10 ($P < 0.01$) compared to unstimulated controls. There were no significant differences in IL-10 protein levels between LPS, LPS+DM, and LPS+DM/pDNA-IL-10 treated WT PMs ($P > 0.05$). (**D**) Pre-treated WT PMs reveal no statistically significant group difference in IL-10 protein production ($F_{(3, 8)} = 3.18$, $P > 0.05$). For NO and IL-10 protein analyses, levels are presented as Mean $\pm$ SEM. Post-hoc multiple comparisons via Fisher's LSD or unpaired t-test ($\alpha = 0.05$; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$).

CHAPTER IV

DISCUSSION

1. Chronic pain as a chronic inflammatory disease

1.1 Chronic inflammatory diseases

Acute inflammation is protective, occurs in response to infection or injury, has both rapid onset (minutes to hours) and a short duration (hours to days), and works to help an organism return to homeostasis (Medzhitov, 2008; Kumar et al., 2015). In contrast, chronic inflammation may last weeks to months, often well beyond the regulated phase of protective usefulness (Kumar et al., 2015). Though the mechanisms of chronic inflammation are less well understood than for acute inflammation, chronic inflammatory diseases are typically associated with abnormal or inappropriate activation or accumulation of immune competent cells, such as T cells and macrophages, and expression of pro-inflammatory mediators such as cytokines, chemokines, reactive oxygen species or nitrogen species (Medzhitov, 2008). Pathological chronic inflammatory processes are key components of many immune disorders, including rheumatoid arthritis (RA), inflammatory bowel disease (IBD), psoriasis, multiple sclerosis (MS), cancer, and systemic lupus erythematosus (SLE) (Lassmann et al., 2012; Rubin et al., 2012; Holmdahl et al., 2014; Boehncke, 2015; Joosten et al., 2016).

In the central nervous system (CNS), resident glial cells, including astrocytes and microglia, are implicated in the pathogenesis of chronic neuroinflammatory diseases such

as Alzheimer's disease (AD), amyotrophic lateral sclerosis (ALS), and Parkinson's disease (PD) (Becher et al., 2016). A growing body of evidence supports that chronic pain should also be categorized as a chronic inflammatory disease (De Leo et al., 2006; Scholz and Woolf, 2007; Milligan and Watkins, 2009; Grace et al., 2014). The aim of this section, "Chronic pain as a chronic inflammatory disease", is to explore the etiology of key pain pathologies and the pre-clinical rodent findings that demonstrate underlying chronic neuroimmune dysfunction.

1.2 HIV-associated sensory neuropathies

Human immunodeficiency virus (HIV) has reached pandemic levels, with an estimated 36.7 million people infected as of 2015 and roughly 5,700 possible new infections per day (Aziz-Donnelly and Harrison, 2017). The introduction of highly active antiretroviral therapy (HAART) in 1996 has allowed for prolonged lifespans and improved disease prognosis in many HIV-infected individuals (Chesney, 2003; Wang et al., 2004; Verma et al., 2005). In regions where HAART is accessible, HIV is now considered a chronic illness rather than an acute fatal illness (Verma et al., 2005). Due to increased patient survival, the prevalence of neuropathic disorders resulting from HIV and antiretroviral therapies have increased in incidence. Of the various types of neuropathy observed in HIV-infected patients, HIV sensory neuropathy (HIV-SN) is the most common with an estimated prevalence of 31-50% (Aziz-Donnelly and Harrison, 2017).

Because they are difficult to clinically distinguish, the term "HIV-SN" is generally accepted to encompass both HIV-associated distal sensory polyneuropathy (HIV-DSP) and

antiretroviral toxic neuropathy (ATN) (Hao, 2013; Aziz-Donnelly and Harrison, 2017). The most common complaints of HIV-SN are hyperalgesia and allodynia which typically initiate at the soles of the feet, gradually ascending proximally up the lower extremity and which may eventually involve the fingertips in a bilateral "stocking and glove" distribution (Hao, 2013).

Histologically, HIV-SN is characterized by significant changes at the level of the dorsal root ganglion (DRG). Loss of sensory neurons with Wallerian degeneration of long axons (e.g. those that innervate the extremities) and unmyelinated C fibers is observed alongside infiltration of the DRG by HIV-infected macrophages (Hao, 2013; Aziz-Donnelly and Harrison, 2017). These trafficking leukocytes release pro-inflammatory cytokines and chemokines that serve to activate the surrounding neuroimmune milieu, sensitizing nearby sensory neurons, enabling the appearance of allodynia and hyperalgesia.

There are no naturally occurring rodent lentiviruses, so pre-clinical rodent models of HIV-SN involve application of the HIV viral envelope protein gp120 (Milligan et al., 2000; Herzberg and Sagen, 2001; Milligan et al., 2001; Oh et al., 2001) or use of transgenic mice engineered to express gp120 (Burdo and Miller, 2014). Gp120 interacts with both neurons and glia through binding to both CXCR4 and CXCR5 chemokine receptors leading to neurotoxicity and peripheral axonal damage (Pardo et al., 2001; Jaggi et al., 2011a). Recombinant gp120 administered intrathecally induces rapid onset thermal hyperalgesia and mechanical allodynia (Milligan et al., 2000; Milligan et al., 2001). Similar symptoms were observed following peripheral application directly to the sciatic nerve (Herzberg and Sagen, 2001). In both exposure paradigms, either direct or indirect glial activation resulted in observed pain behaviors. Like macrophages, both astrocytes and microglia can become

HIV infected (Hao, 2013). Further, astrocytic and microglial activation by viral proteins like gp120 lead to additional increases in pro-inflammatory cytokines, like IL-1β, TNF, and IL-6, resulting in enhanced pain states (Milligan et al., 2001; Schoeniger-Skinner et al., 2007).

Though less obvious than for HIV-DSP, ART is also linked with chronic inflammatory processes. Nucleoside reverse transcriptase inhibitors (NRTIs), a key component of the HAART pharmacologic cocktail, are known to be neurotoxic and to cause dose-dependent peripheral neuropathy (Manji, 2000). Systemic administration of NRTIs have been used to model HIV-SN mediated by ATN (Hao, 2013). Pre-clinical studies in rodents and examination of human tissue samples indicate that ATN is closely linked to mitochondrial dysfunction, with NRTIs able to inhibit replication of mitochondrial DNA (mtDNA) (Gardner et al., 2014). The resulting depletion in mtDNA in turn causes neurotoxicity via mitochondrial respiratory chain and oxidative phosphorylation deficits (Yamaguchi et al., 2002). Spinal microgliosis also occurs in response to NRTI administration (Blackbeard et al., 2012). Additionally, increased expression of the astrocyte activation marker glial fibrillary acidic protein (GFAP) is observed in the spinal cord dorsal horn with concurrent increases in colocalization of pro-inflammatory TNF (Zheng et al., 2011).

1.3 Cancer-related and chemotherapy-induced peripheral neuropathy

Cancer-related pain has multiple causes and mechanisms. The tumor, its metastases, or anticancer treatments themselves can induce both nociceptive and neuropathic pain

(Bouhassira et al., 2017). In a recent systematic review with meta-analysis, pain prevalence in cancer patients was found to be 39.3% after curative treatment, 55.0% during anti-cancer treatment, and 66.5% in advanced, metastatic, or terminal disease (van den Beuken-van Everdingen et al., 2016). Additionally, moderate to severe pain was reported in 38% of all cancer patients (van den Beuken-van Everdingen et al., 2016). Another systematic review calculated that the prevalence of cancer patients with neuropathic pain ranges from 19% to 39.1% (Bennett et al., 2012). The World Health Organization (WHO) promotes a three-step analgesic ladder for management of cancer-related pain, starting with non-opioids for mild pain, combinations of weak opioids and non-opioids for mild-moderate pain, and the addition of strong opioids like morphine for severe pain (Dalal and Bruera, 2013). Unfortunately, many patients are unable to achieve adequate pain control, and opioids themselves put patients at risk of a host of unwanted side effects, including tolerance and addiction.

Chronic inflammation is indicated in the pathogenesis of cancer-related neuropathic pain. It has been shown that malignant cells, tumor infiltrating lymphocytes (TILs), and tumor stromal cells release numerous pain-modulating molecules, including pro-inflammatory cytokines like TNF, IL-1β, and IL-6, pro-inflammatory mediators like prostaglandins, and even hydrogen ions from the often hypoxic tumor core (Brown and Ramirez, 2015).

Use of anti-cancer agents themselves are known to cause chemotherapy-induced peripheral neuropathy (CIPN) which presents as a length-dependent peripheral neuropathy characterized by ‘die-back’ of unmyelinated sensory fibers (Brown and Ramirez, 2015). Neuronal cell bodies housed in DRG reside outside the blood-brain-barrier and are

particularly vulnerable to the neurotoxic effects of cancer chemotherapeutics (Carozzi et al., 2015).

Rodent models of CIPN have been critical in understanding the role of chronic inflammation in this pathological pain process. For instance, paclitaxel-mediated CIPN has been shown to induce heightened expression of pain-related pro-inflammatory cytokines IL-1 and TNF mRNA alongside increased expression of the macrophage marker CD11b in the DRG. These markers of inflammation were significantly decreased in pain-relieved rodents treated with anti-inflammatory non-viral interleukin-10 gene therapy (Ledeboer et al., 2007c). Additionally, intravenous paclitaxel-based mouse models have demonstrated activation of satellite glial cells and infiltration of macrophages to the DRG and increased immunoreactivity of astrocytic GFAP in the spinal cord (Peters et al., 2007). Interestingly, paclitaxel-treated rodents also reveal activated satellite glial cells with increased gap-junction mediated coupling, a feature that likely contributes to the spread of neuropathic pain resulting in a diffusive pain pattern (Warwick and Hanani, 2013). Astrocytic gap junctions in the spinal cord have also been implicated in the pathogenesis of oxaliplatin-mediated CIPN (Yoon et al., 2013). Both DRG neurons and spinal astrocytes increase expression of the chemokine CCL2 in paclitaxel-induced CIPN (Zhang et al., 2013).

1.4 Fibromyalgia

Fibromyalgia describes chronic widespread musculoskeletal pain for which no alternative cause can be identified (Sluka and Clauw, 2016). Though fibromyalgia is characterized as having no apparent tissue damage, patients often present with diffuse hyperalgesia and

allodynia (Sluka and Clauw, 2016). This widespread sensitivity suggests that the main disorder may be caused, at least in part, by changes in spinal pain processing machinery as opposed to a localized disease (Clauw, 2009). Fibromyalgia is not considered an inflammatory process, but inflammatory mediators may play a role in this pathologic pain process (Gur and Oktayoglu, 2008; Slade et al., 2011; Sturgill et al., 2014; Mendieta et al., 2016; Sluka and Clauw, 2016). For instance, a systematic review demonstrated consistent increases in serum levels of IL-6, IL-8, and IL-1 receptor antagonist in patients with fibromyalgia (Üçeyler et al., 2011). Other literature supports that serum levels of anti-inflammatory IL-10 are decreased in the patients with chronic widespread pain, a disease category of which fibromyalgia is a major subgroup (Üçeyler et al., 2006). Additionally, a growing body of evidence suggests that individuals with fibromyalgia may have abnormalities in the function of the hypothalamic-pituitary-adrenal (HPA) axis, a system known for potent modulation of the immune system. For instance, females with fibromyalgia have significantly increased circulating levels of 'neuroendocrine-stress' markers, such as cortisol and noradrenaline, compared to healthy controls (Bote et al., 2012).

Unfortunately fibromyalgia does not yet have a well-established "go-to" animal model (DeSantana et al., 2013). The most common and well-characterized models involve repeated insults to musculature. For instance, in a "non-inflammatory model", acidic saline (pH = 4.0-5.0) is repeatedly injected intramuscularly leading to widespread hyperalgesia in the absence of tissue damage or inflammation (DeSantana et al., 2013; Sluka and Clauw, 2016). A related model combines repeated acid saline injections with muscle fatigue, also providing hyperalgesia without overt inflammation or tissue damage (DeSantana et al.,

2013; Sluka and Clauw, 2016). In a different approach, stress, such as exposure to loud sounds, also seems capable of producing muscular and cutaneous hyperalgesia lasting weeks after the initial stressor (DeSantana et al., 2013; Sluka and Clauw, 2016).

Though these models claim to be "non-inflammatory" in nature, a growing body of evidence supports that immune activation is involved in pathogenesis of fibromyalgia-like symptoms. For instance, following repeated intramuscular acid saline injection, depletion of macrophages from the injection site via local application of clodronate liposomes prevents exercise-induced hyperalgesia (Gregory et al., 2016). In a separate model of fibromyalgia, an inflammatory insult is applied intramuscularly (carrageenan or IL-6), inducing acute hyperalgesia. After sensitivity resolves, prostaglandin E2 (PGE2), a pro-inflammatory mediator associated with inflammatory pain, is injected into the same muscle, leading to hyperalgesia that lasts for at least 2 weeks. In contrast, animals that do not receive the initial inflammatory insult experience hyperalgesia that resolves within 4 hours (Dina et al., 2008b; Dina et al., 2008a). Additionally, in the sound stress model of chronic widespread pain, IL-6 and TNF appear to play a critical role in enhancement of muscular hyperalgesia to in response to subsequent immune challenge (Dina et al., 2011).

There is still much unknown about the pathophysiology of fibromyalgia. Despite the lack of overt tissue damage or inflammation, the data from human patients and pre-clinical animal models suggest that pathological pain in this disease may result, at least in part, from inappropriate activation of immune cells with production of pro-inflammatory mediators that may in turn modulate neuronal function in the CNS and/or PNS. Whether resident tissue cells, such as myocytes may also be a pathologic source of these pro-inflammatory mediators is yet to be determined.

1.5 Chronic low back pain

Low back pain is estimated to affect upwards of 80% of all individual at some point in their lifetime and is reported to be the fifth most common reasons for all physician visits in the United States of America (Patrick et al., 2014). Chronic low back pain can be caused by problems with lumbar intervertrebral disc damage (e.g. herniation, displacement, or degeneration), compression of spinal nerve roots, or inflammatory conditions such as autoimmune disorders (Strong et al., 2013). Thus far, no therapy for back pain investigated in randomly assigned clinical trials has shown substantial benefit for symptoms associated with this broad condition (Borenstein, 2013).

Though lower back pain is typically initiated by a mechanical disorder, pro-inflammatory signaling pathways can be engaged that lead to enhanced pain states. This paradigm is commonly observed in discogenic lower back pain. The intervertebral disk (IVD) becomes more vulnerable to damage with age with progressive weakness of the nucleus pulposus and degeneration of the annulus fibrosus. If sufficient pressure is then placed on the IVD, the nucleus pulposus may experience protrusion, herniation, or sequestration (Borenstein, 2013). Radicular pain may develop if a nearby spinal nerve root becomes compressed by extruded disc material (Borenstein, 2013). Interestingly, IVD tissue samples from patients with chronic low back pain show increased expression of pain-related pro-inflammatory molecules, including TNF, IL-1, IL-6, IL-8, PGE2, neutrotrophic growth factor (NGF), and inducible nitric oxide synthase (Ohtori et al., 2015). Beyond exacerbating radiculopathy, these pro-inflammatory mediators could increase pain pathology by directly activating nerve endings present within the IVD itself, the neuronal

cell bodies of which reside in the DRG. Immunohistochemical studies indicate that many different sensory fibers present within the IVD are sensitive to classical pain neurotransmitters like substance P (SP) or calcitonin gene-related peptide (CGRP) (Ohtori et al., 2015).

Animal models support a role for inflammatory processes in the pathogenesis of discogenic low back pain. For example, Li *et al*. demonstrated that posterior puncture of the annulus fibrosus in rats induces 3 weeks of mechanical allodynia (Li et al., 2014). Expression levels of mRNAs for TNF, IL-1β, and IL-6 were significantly elevated for 2-3 weeks following puncture, a timeframe that coincides with observed pain behavior. Immunohistochemical evidence also suggested that TNF and IL-1β protein expression is elevated in the DRG of allodynic animals that received posterior annulus fibrosus puncture (Li et al., 2014). Cytokine signaling is also implicated in radicular back pain models. Sasaki *et al*. developed a model in which nucleus pulposus harvested from a coccygeal disk is applied to the nearby L5 spinal nerve root (Sasaki et al., 2007). This results in at least 4 weeks of mechanical allodynia that could be temporarily relieved by single intravenous administration of TNF-neutralizing antibodies at early timepoints (0 or 6 days post-surgery), but not at a later timepoint (20 days post-surgery) (Sasaki et al., 2007). These data support that the cytokine TNF is important for the initial stages of pain pathology but may not be as important for maintenance of allodynia in this model of radicular back pain.

2. Interleukin-10: Small but mighty

2.1 Cellular sources of IL-10

Discovered nearly 30 years ago by Mosseman and colleagues (Fiorentino et al., 1989; Moore et al., 1990), interleukin-10 (IL-10) was originally called cytokine synthesis inhibitory factor (CSIF). First identified to be produced by type 2 helper T cells (Th2), IL-10 was shown to limit the actions of Th1 cells through potent inhibition of pro-inflammatory cytokines like tumor necrosis factor (TNF) and interferon-γ (IFNγ), while simultaneously limiting proliferative capacity by decreasing levels of IL-2 (Fiorentino et al., 1989). It is now appreciated that this powerful anti-inflammatory cytokine is expressed by almost all leukocytes, including T and B cells, dendritic cells, γδ T cells, NK cells, neutrophils, and eosinophils (Saxena et al., 2015). Microglia and astrocytes are known to express IL-10 in the central nervous system (Kwilasz et al., 2015). Other non-immune sources of IL-10 include keratinocytes, epithelial cells, and some tumor cells (Mosser and Zhang, 2008). It has been hypothesized that the broad cellular capability of expression allows for rapid availability, but that it may also be important for the "compartmentalization" of IL-10's actions (Saxena et al., 2015). This is supported by IL-10's very short half-life (~1-2hrs) (Li et al., 1994; Le et al., 1997; Milligan et al., 2005a; Mosser and Zhang, 2008).

Cell type-specific roles for IL-10 expression seem to be key in the regulation of autoimmune and physiological inflammatory responses. For instance, regulatory T cells (Tregs) are key expressers of IL-10 and suppress inflammation and autoimmunity, a

function that was first appreciated following examination of Treg dysfunction in mouse models of inflammatory bowel disease (Groux et al., 1997; Asseman et al., 1999). IL-10 is also known to promote proliferation of B cells, and regulatory B cell-derived IL-10 is protective against experimental autoimmune encephalomyelitis (EAE) (Fillatreau et al., 2002).

2.2 IL-10 Expression and signaling

The genes for both mouse IL-10 (mIL-10; *Il10*) and human IL-10 (hIL-10; *IL10*) are found on chromosome 1 and encoded by 5 exons (Moore et al., 2001). Several intracellular signaling cascades are important for induction of IL-10 expression, including mitogen activated protein kinase (MAPK), extracellular signal-regulated kinase (ERK), and p38 (Saraiva and O'Garra, 2010). These cascades are commonly initiated by stimulation of key pathogen recognition receptors (PRRs), like toll-like receptor 4 (TLR4), by pathogen associated molecular patterns (PAMPs; e.g. bacterial lipopolysaccharide [LPS]) (Lobo-Silva et al., 2016).

Though not well understood, IL-10 expression may also be stimulated following activation of PRRs by endogenous alarmins, also called danger associated molecular patterns (DAMPs). For example, heat shock protein 70 (HSP70) is a DAMP known to activate TLR2 and TLR4, and appears to have anti-inflammatory activity through induction of IL-10 in a variety of cell types (Luo et al., 2008; Wieten et al., 2009; Wachstein et al., 2012; Borges et al., 2013). Similarly, surfactant protein-A (SP-A) is a possible TLR4 agonist and *in vitro* stimulation of bone marrow-derived macrophages with this DAMP

dose-dependently increased protein expression of both IL-10 and pro-inflammatory tumor necrosis factor (Guillot et al., 2002). High mobility group box protein I (HMGB1), a DAMP known to activate both toll-like receptor 4 (TLR4) and receptor for advanced glycation endproducts (RAGE), increases myeloid-derived suppressor cell (MDSC)-mediated production of IL-10 *in vitro* (Parker et al., 2014). Interestingly, following instances of severe tissue damage (e.g. trauma, major surgery, or pancreatitis) some patients develop an immune suppression similar to sepsis-induced immunoparalysis (Nakos et al., 2002; Allen et al., 2006; Ho et al., 2011; Cornell et al., 2012). It is thought that DAMP activation of PRRs may be linked to increased IL-10 expression, though current data is correlational in nature, e.g. demonstrating increased IL-10 serum levels. While significant room for investigation is evident, these findings suggest that at least some DAMPs may serve to "dampen" the immune system in order to prevent an overzealous immune response to tissue damage.

Following PRR activation, IL-10 expression is regulated both at transcriptional and post-transcriptional stages (Powell et al., 2000; Mosser and Zhang, 2008). IL-10 is the target of many transcription factors, such as Sp1, Sp3, STAT3, C/EBPβ, C/EBPδ, IRF-1, c-Maf, AP-1, CREB, MEF2d, and NF-κB (Mosser and Zhang, 2008; Yang et al., 2015; Lobo-Silva et al., 2016). Post-transcriptional instability of IL-10 mRNA is influenced by the presence of a long segment of 3'-untranslation region (3'-UTR) which contains class II AU-rich elements which can be targeted by the RNA-binding protein tristetraprolin (TTP), leading to rapid degradation (Anderson, 2008). IL-10 is also known to be regulated by microRNAs (miRs) (Lobo-Silva et al., 2016). For example, miR-466l-3p inhibits IL-10 production in microglia following stimulation of the PRR TLR4 (Brown and Yin, 2013).

In contrast, miR-181 overexpression has been shown to increase IL-10 production following lipopolysaccharide (LPS) stimulation (Hutchison et al., 2013).

Following translation and extracellular release, IL-10 monomers homodimerize and bind to the IL-10 receptor, a class II receptor which itself functions as a heterotetramer of IL-10R1 (IL-10Rα) and IL-10R2 (IL-10Rβ) (Moore et al., 2001). IL-10R1 is critical for high affinity binding with the IL-10 homodimer that in turn leads to IL-10R2-mediated intracellular signal transduction via engagement of both Janus kinase 1 (Jak1; associated with IL-10R1) and Jak kinase 2 (Tyk2; associated with IL-10R2) with subsequent phosphorylation and activation of transcription factors like STAT3 (Sabat et al., 2010).

2.3 Biological effects

IL-10 signaling pleiotropically suppresses inflammation. For instance, IL-10 reduces antigen presenting capability in monocytes/macrophages by decreasing trafficking of major histocompatibility complex class II (MHCII) to the cellular membrane and by inhibiting expression of the co-stimulatory molecule CD86 (Kwilasz et al., 2015). IL-10 also suppresses production of numerous pro-inflammatory cytokines, chemokines, reactive oxygen species, and nitrogen species. For example, IL-10-mediated SOCS1 and SOCS3 production prevents nuclear translocation of NF-κB, which thereby prevents transcription of pro-inflammatory cytokines like interleukin-1β (IL-1β) or tumor necrosis factor (TNF; a.k.a. TNFα), and pro-inflammatory chemokines like C-C motif chemokine ligand 2 (CCL2) (Schottelius et al., 1999; Murray, 2005; Yoshimura et al., 2007; Milligan et al., 2012). IL-10 is known to enhance expression of natural antagonists to pro-inflammatory

signaling, for instance, by increasing production of IL-1 receptor antagonist (IL-1RA) and a soluble form of TNF receptor (Moore et al., 2001).

IL-10 is a powerful modulator of immune cell activation and proliferation. IL-10 promotes differentiation of anti-inflammatory macrophages, often called "M2" alternatively activated cells (Mantovani et al., 2004; Martinez and Gordon, 2014b). It also limits CD4+ T cell proliferation and pro-inflammatory cytokine production, thereby diminishing functionality of T_H1 and T_H17 cells (Schuetze et al., 2005; McKinstry et al., 2009). IL-10 is not only a key product of Tregs, but it also promotes differentiation of this anti-inflammatory cell type (Asseman et al., 1999; Palomares et al., 2014; Brockmann et al., 2017).

IL-10 plays a critical role in suppression of innate immune cell function. IL-10 production by stimulated innate immune cells, like macrophages, is considered "late" compared to the initial pro-inflammatory response, with robust expression appearing hours after stimulus exposure (Sabat et al., 2010; Iyer and Cheng, 2012). Delayed expression appears to temper the acute inflammatory response, thereby preventing uncontrolled inflammation. In addition to decreasing pro-inflammatory signaling, IL-10 acts on local innate immune cells to promote "scavenger" functions, such as increased phagocytic activity, which consequently aid in peripheral tolerance against persistent antigen exposure (Buchwald et al., 1999; Saxena et al., 2015).

2.4 Role of IL-10 in disease

IL-10 expression is tightly regulated, and inappropriate under- or overproduction can result in a host of immune diseases. For instance, IL-10 overproduction is pathologic in systemic lupus erythematosus (SLE), EBV-associated lymphomas, and skin malignancies such as melanoma (Stewart et al., 1994; Yue et al., 1997; Grondal et al., 1999). Additionally, IL-10 overexpression can be detrimental to host defense and clearance of infectious agents (Sabat et al., 2010).

Decreased IL-10 expression is often present in diseases for which persistent or aberrant immune activation exists. In humans, polymorphisms in IL-10 have been identified in association with inflammatory bowel disease (IBD) by genome wide association studies (GWAS), and IL-10R mutations have been observed in patients with severe IBD (Schreiber et al., 1995; Franke et al., 2008; Moran et al., 2013). In pre-clinical studies, IL-10 deficient (IL-10 KO) mice develop spontaneous enterocolitis over the course of months, reflecting a loss of mucosal homeostasis in the normal response to commensal bacteria (Kuhn et al., 1993; Berg et al., 1996; Ward et al., 1996; Sartor, 1997; Sellon et al., 1998; Asseman et al., 1999). The role of IL-10 in intestinal homeostasis by Tregs is critically important for maintenance of healthy responses to gut microbiota that interface with intestinal mucosa, and when lost can result in IBD (Asseman et al., 1999). Other diseases for which decreased IL-10 expression has been identified, often linked to enhanced clinical severity, include neuropathic pain, multiple sclerosis, rheumatoid arthritis, psoriasis, type 1 diabetes (T1D), and multiple sclerosis (Sabat et al., 2010; Kwilasz et al., 2015; Saxena et al., 2015). Interestingly, decreased IL-10 expression is

observed in pain-relevant neural tissues in animal models and is correlated with neuropathic pain behaviors, as further discussed below.

2.5 Utilization of IL-10 therapeutics for treatment of neuropathic pain and other chronic inflammatory diseases

Given that decreased IL-10 expression is linked to a variety of immune pathologies, numerous therapeutic approaches to increase IL-10 protein levels have been examined. Unfortunately, due to IL-10's short half-life and blood brain barrier impermeability, systemic administration of IL-10 is not likely to be clinically feasible for most diseases (Li et al., 1994; Kastin et al., 2003). Psoriasis is a disease for which direct IL-10 application by subcutaneous injection of IL-10 protein at the site of psoriatic plaques has had promising results in a phase 2 clinical trial (Asadullah et al., 1999; Khusru et al., 2004). While direct application of IL-10 protein to the site of pathology is capable of producing immediate symptom relief, effects are often transient and may require repeated injections (Milligan et al., 2005a).

A hallmark of efficacious IL-10-based therapeutics is the ability to increase local concentrations of IL-10 in a timely manner (Saxena et al., 2015). A novel approach for the treatment of IBD has been administration of transgenically modified bacteria, such as *Lactococcus lactis*, that secrete IL-10 directly into the intestinal lumen. This method successfully prevented the onset of colitis in IL-10 KO mice, reduced inflammation in other mouse models of IBD (Steidler et al., 2000), and was successfully applied in a phase 1 and 2a clinical trials (http://ClinicalTrials.gov identifier NCT00729872) (Braat et al., 2006;

Marlow et al., 2013). While intriguing, use of bacterial vectors for local IL-10 production may not be feasible for all other inflammatory diseases, though recent work suggests that it may be translatable for the treatment of T1D (Robert and Steidler, 2014).

IL-10 gene therapy has also proven to be a promising approach, especially for treatment for chronic pain. For instance, non-replicating adeno virus, adeno-associated virus, and herpes simplex viral vectors carrying an IL-10 transgene have all been examined for intrathecal (i.t.) treatment of neuropathic pain. While potent pain relief was observed in animal models of chronic pain, effects were short-lived (Wood et al., 1996; Milligan et al., 2005a; Milligan et al., 2005b; Beutler and Reinhardt, 2009; Lau et al., 2012; Zheng et al., 2014b, a). It is hypothesized that transient pain relief is a result of complications associated with the use of a viral vector, such as an immune response following exposure to viral antigens (Kwilasz et al., 2015). Non-viral gene therapy is a safer, easier, and less expensive approach that avoids many of the problems associated with the use of viral vectors (Glover et al., 2005). Paradoxically, though non-viral gene therapy is considered the least efficient method of gene transfer, it has been repeatedly demonstrated to provide profound and long-lasting pain relief in a variety of animal models (Milligan et al., 2006b; Milligan et al., 2006a; Ledeboer et al., 2007b; Sloane et al., 2009b; Sloane et al., 2009a; Soderquist et al., 2010b; Milligan et al., 2012; Dengler et al., 2014; Grace et al., 2017; Vanderwall et al., 2018).

An interesting new approach in IL-10-oriented chronic pain treatments is the enhanced or stabilized production of the body's own endogenous IL-10 to basal or homeostatic levels. Cannabinoids offer one potential option, especially those targeting the cannabinoid 2 receptor (CB_2R). CB_2R agonists have been shown to provide profound pain

relief in two rodent pain models: sciatic chronic constriction injury (CCI) (Wilkerson et al., 2012a; Wilkerson et al., 2012b) and human immunodeficiency virus (HIV)-1 glycoprotein-120 (gp120)-induced spinal inflammation (Wilkerson et al., 2012a). For example, AM1710 prevented IL-10 protein deficiency in the spinal cord dorsal horns and dorsal root ganglia (DRG) of pain-relieved CCI-rats, achieving similar to the levels found in non-neuropathic sham controls (Wilkerson et al., 2012a). This was observed in concert with significantly decreased protein expression of the pain-associated pro-inflammatory mediators IL-1β, phospho-p38MAPK, and glial markers of activation. Similarly, AM1710 decreased IL-1β production in the DRG of pain-relieved gp120-rats (Wilkerson et al., 2012a). Interestingly, AM1710 failed to reverse light touch sensitivity in CCI-treated IL-10 KO mice, supporting a requirement for IL-10 in AM1710-mediated pain relief (unpublished observations by Erin D. Milligan).

The therapeutic benefit for inducement of basal IL-10 expression levels has been demonstrated by Dengler and colleagues. They demonstrated that i.t. D-mannose (DM), a known mannose receptor ligand, induces transient pain relief following a single low-dose injection in CCI-treated rats. Additional *in vitro* examination of DM applied to cultured RAW 264.7 mouse macrophages under conditions mimicking a pro-inflammatory neuropathic environment (i.e. co-stimulation with LPS) reveal enhanced IL-10 protein expression when compared to LPS stimulation alone (Dengler et al., 2014). These data provided rationale for the hypothesis that DM-mediated activation of the mannose receptor leads to temporary increases in endogenous IL-10 production that underlies transient pain relief (Dengler et al., 2014).

The role of increased IL-10 downstream of MR activation has been both supported and contradicted by investigations of MR signaling in IL-10 KO mice. The NLRP3 inflammasome, which can be induced experimentally by co-stimulation with ATP and LPS, activates caspase-1 that in turn leads to production of bioactive pro-inflammatory IL-1β protein. In a preliminary study, DM pretreatment prior to LPS/ATP co-stimulation decreased the population of cells that were double positive for NLRP3 protein and caspase-1 activation in peritoneal macrophages from wildtype but not IL-10 KO mice (Vanderwall unpublished data; Appendix A). This early report suggests that IL-10 may be important for MR-mediated inhibition of the NLRP3-inflammasome. However, IL-10 is not required for all MR-mediated anti-inflammation. For instance, a recent report by Vanderwall *et al*. provides unexpected evidence that i.t. DM continues to provide transient pain relief in IL-10 KO mice in the absence of endogenous IL-10 (Vanderwall et al., 2018), refuting the hypothesis that IL-10 is the required for MR-mediated transient pain relief in IL-10-sufficient neuropathic rats. While these data do not exclude a role for enhanced IL-10 production following D-mannose application in wildtype animals, it proves that D-mannose engages other powerful anti-inflammatory mediators sufficient for pain relief.

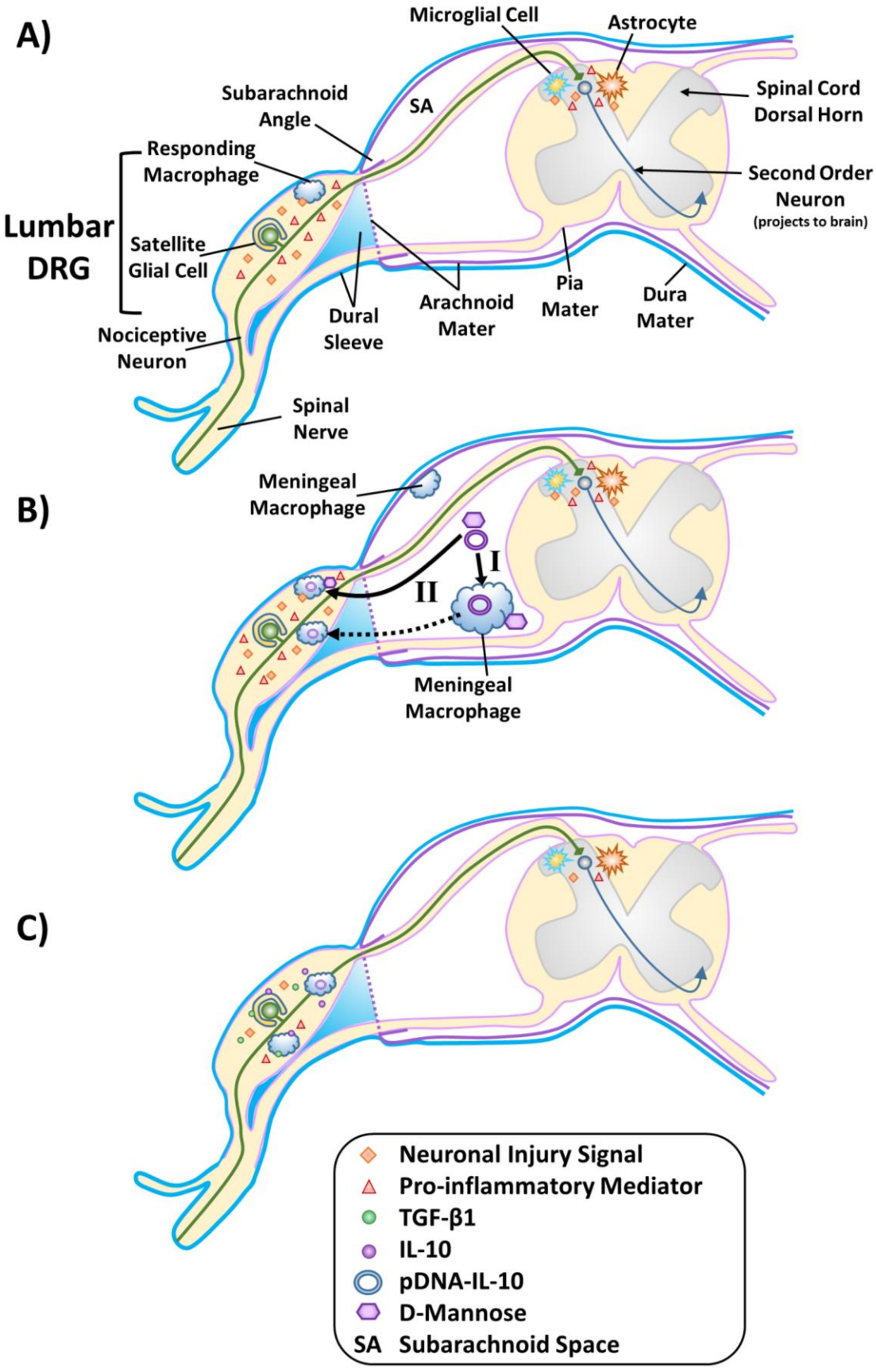

A)
Microglial Cell
Astrocyte
Subarachnoid Angle
SA
Spinal Cord Dorsal Horn
Responding Macrophage
Second Order Neuron
(projects to brain)
Lumbar DRG
Satellite Glial Cell
Dural Sleeve
Arachnoid Mater
Pia Mater
Dura Mater
Nociceptive Neuron
Spinal Nerve
B)
Meningeal Macrophage
I
II
Meningeal Macrophage
C)
Neuronal Injury Signal
Pro-inflammatory Mediator
TGF-β1
IL-10
pDNA-IL-10
D-Mannose
SA Subarachnoid Space

Figure 1: Possible IL-10 transgene therapeutic effects and the DRG. (**A**) The cell bodies of primary nociceptive neurons reside in the dorsal root ganglia (DRG), with peripheral terminals in target tissues, and central projections in the spinal cord dorsal horn. Primary nociceptors communicate with second order neurons that in turn project to the brain. DRG are encased within a dural sleeve, but reside outside of the subarachnoid space (SA). A small volume of cerebrospinal fluid (CSF) exits the SA by draining along the spinal nerves. Lumbar DRG are in close proximity to the cauda equina (not shown), caudal to the spinal cord proper. Following peripheral damage to the sciatic nerve (e.g. chronic constriction injury), sensory neurons release neuronal injury signals, such as the chemokine CCL2, at the level of the DRG and the spinal cord dorsal horn. These injury signals induce activation of glia (i.e. astrocytes, microglia, and satellite glia), leading to increased production of pro-inflammatory mediators in both locations. Pro-inflammatory mediators and neuronal injury signals in the DRG leads to local accumulation of macrophages. Recruited macrophages may facilitate further production of pro-inflammatory mediators in the DRG. (**B**) Intrathecal (subarachnoid) injection of plasmid DNA encoding anti-inflammatory IL-10 transgene (pDNA-IL-10) with D-mannose (DM) induces enduring relief of peripheral neuropathic pain. In potential pathway I, meningeal macrophages may locally internalize pDNA-IL-10. Chemokines produced in the DRG could attract such macrophages, leading to local therapeutic expression of the IL-10 transgene. Alternatively, as in potential pathway II, pDNA-IL-10 and D-mannose may diffuse to the DRG, traveling with draining CSF. Once present in the DRG, pDNA-IL-10 may be internalized and expressed by previously recruited macrophages. (**C**) Local expression of anti-inflammatory IL-10 transgene in the DRG leads to increased production of anti-inflammatory TGF-β1 as well as decreased production of pro-inflammatory mediators. This in turn may decrease production of neuronal injury signals (e.g. CCL2) at the DRG and at the spinal cord dorsal horn, thereby decreasing activation of both satellite glia and spinal glia and allowing a return to physiologic pain signaling.

3. D-mannose: More than just a gene therapeutic adjuvant

3.1 Mannose receptor-mediated anti-inflammatory effects: IL-10 independent pathways

A growing body of evidence supports that mannose receptor (MR; CD206) activation leads to anti-inflammatory effects. These include increased expression of anti-inflammatory mediators, like IL-10 or IL-1R antagonist, and decreased expression of pro-inflammatory mediators, like IL-12, TNF, IL-1β, and NO (Nigou et al., 2001; Chieppa et al., 2003; Pathak et al., 2005; Zhang et al., 2005; Gazi and Martinez-Pomares, 2009; Allavena et al., 2010; Dengler et al., 2014). However, though MR activation has been linked with increased IL-10, recent work by Vanderwall *et al*. indicates that IL-10 is not required for a MR-mediated anti-inflammatory signaling. They demonstrated that intrathecal application of DM in neuropathic IL-10 KO mice induces transient pain relief (Vanderwall et al., 2018). Given that pro-inflammatory signaling is critical for the pathogenesis of peripheral neuropathic pain, this evidence indicates that other anti-inflammatory mediators must be engaged downstream of MR.

Transforming growth factor-β (TGF-β) may offer a potential avenue for MR-mediated anti-inflammation. Virtually all cell types are responsive to TGF-β, which is expressed by numerous tissues types, including mesenchyme, connective tissue, endothelium, platelets, glia, and immune cells (Bottner et al., 2000; Sanjabi et al., 2009). TGF-β signaling, while less predictable than IL-10, can often lead to potent anti-inflammatory effects (Bottner et al., 2000; Colak and Ten Dijke, 2017). One of TGF-β's most important roles is in generation of regulatory T cells (Tregs). TGF-β1 is important for

development of natural thymic-derived (nTregs), as well as peripherally derived Tregs (pTregs) like T regulatory 1 (T_R1) cells or T helper 3 (T_H3) cells, and *in vitro* induced Tregs (iTregs) (Mills, 2004; Hadaschik and Enk, 2015). Tregs in turn help control autoimmune responses and regulate normally inflammatory processes by producing anti-inflammatory cytokines, like TGF-β and IL-10, and other suppressive mechanisms such as conversion of pro-inflammatory ATP to AMP and adenosine by ectoenzymes (Safinia et al., 2015).

An intriguing new study by Zhang *et al.* implicates MR signaling in the generation of Tregs, both *in vivo* and *in vitro (Zhang et al., 2017)*. They initially observed that naive $CD4^+CD25^-$ T cells cultured with 25mM DM for 3 days significantly increase their co-expression of the Treg markers CD25 and Foxp3. They also found that ingestion of D-mannose beginning at 7.5 weeks of age by NOD mice (autoimmune model of type 1 diabetes) prevents the onset of diabetic symptoms until at least 23 weeks of age, whereas 80-90% of control-treated NOD mice developed diabetes by the same age. Interestingly, they further demonstrated that DM increases Treg production via TGF-β. However, this does not appear to be accomplished by increasing TGF-β mRNA or total TGF-β protein, but rather by increasing conversion of latent TGF-β to its active form by an integrin $\alpha_V\beta_8$-dependent mechanism (Zhang et al., 2017).

The report by Vanderwall *et al.* provides complementary evidence that MR signaling may be associated with Treg production (Vanderwall et al., 2018). Intrathecal injection of DM plus a control plasmid (DM/pDNA-Ctrl) or DM plus a plasmid encoding an IL-10 transgene (DM/pDNA-IL-10) in neuropathic IL-10 KO mice led to transient or enduring pain relief, respectively, with significantly increased expression of TGF-β mRNA detected in the ipsilateral lumbar DRG of pain relieved mice that received DM/pDNA-IL-

10 (Vanderwall et al., 2018). Though mice that received DM/pDNA-Ctrl had TGF-β mRNA levels similar to sham controls, it is tantalizing to hypothesize that DM/pDNA-Ctrl mice might also have exhibited elevated TGF-β mRNA levels at an earlier timepoint, perhaps near the peak of transient pain relief. In this study, DM was administered at only one timepoint, whereas the most significant effects of DM observed by Zheng *et al.* occurred following prolonged exposure to DM (days *in vitro*, weeks *in vivo*). This suggests that the transient pain relief observed in DM treated animals in the absence of IL-10 transgene may have been more stable should repeated injections been applied.

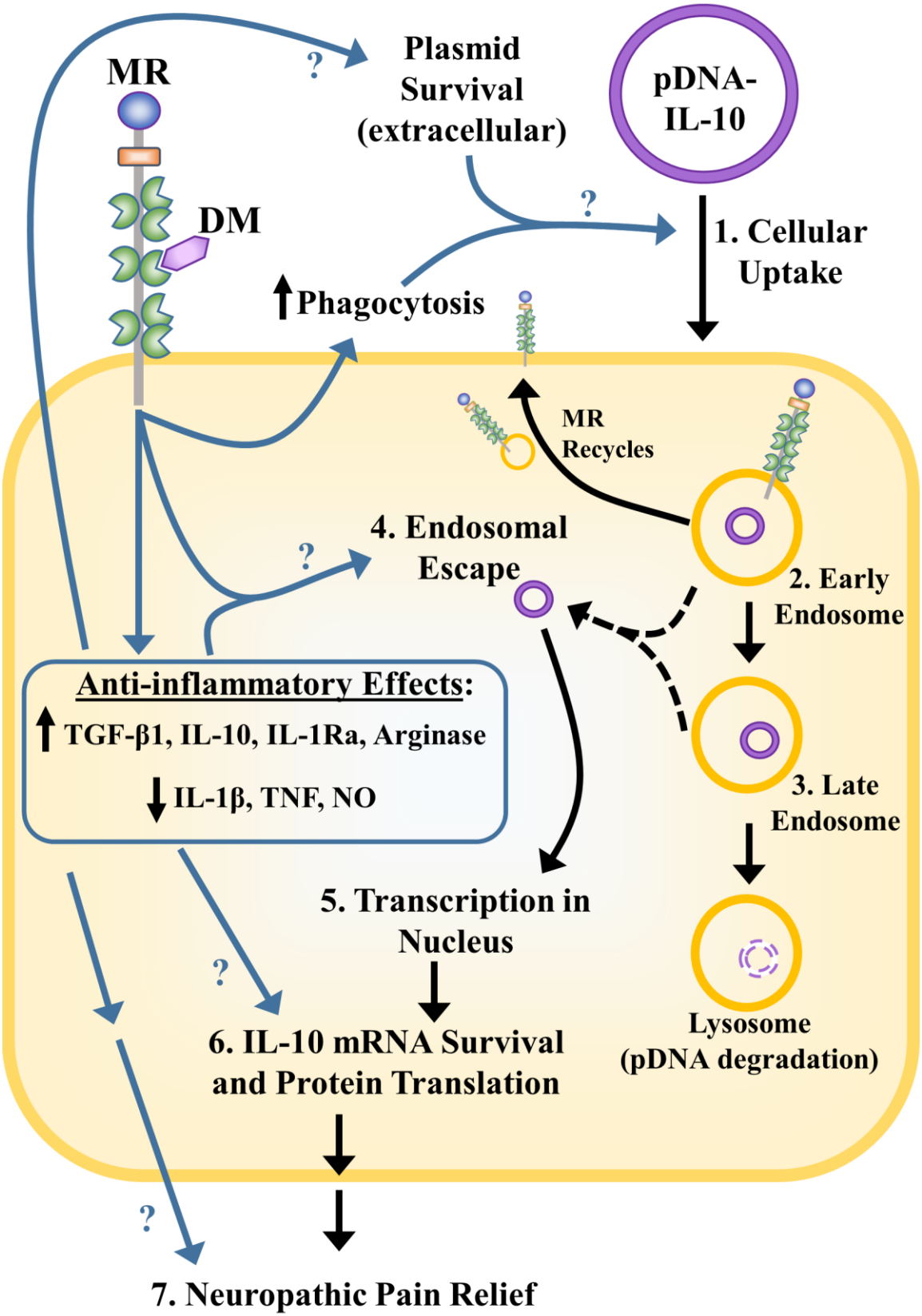


Figure 2: Pleiotropic effects of D-mannose in spinal non-viral IL-10 gene therapy for

neuropathic pain. (**1**) In the absence of gene therapeutic adjuvants like D-mannose (DM), naked plasmid DNA encoding IL-10 (pDNA-IL-10) likely enters cells by endocytosis. (**2-3**) Following internalization, early endosomes containing pDNA-IL-10 may mature to late endosomes. (**4**) To avoid degradation by the lysosome, pDNA-IL-10 must escape the endosome. (**5**) pDNA-IL-10 must access transcription machinery in the nucleus to produce mRNA. (**6**) Transgenic IL-10 mRNA must then survive the intracellular environment long enough to be translated into therapeutic protein. (**7**) IL-10 protein expression decreases pro-inflammatory signaling allowing for relief of neuropathic pain. Free DM is recognized by the mannose receptor (MR). This interaction may increase phagocytic activity, possibly improving likelihood of pDNA internalization. MR itself is internalized but recycles from the early endosome to the plasma membrane, avoiding lysosomal degradation. This mechanism may improve pDNA endosomal escape (**4**). Endosomal escape may also be influenced by signaling effects downstream of MR activation, including increases in anti-inflammatory mediators including TGF-β1, IL-10, IL-1 receptor antagonist (IL-1Ra), and arginase, with decreased expression of pro-inflammatory IL-1β, TNF, and NO (**4**). These anti-inflammatory effects may in turn improve survival of transgenic IL-10 mRNA (**6**). Anti-inflammatory signaling downstream of MR also likely mediates transient pain relief when DM is administered in the absence of pDNA-IL-10 (**7**). Extracellular plasmid survival may be improved by MR-mediated anti-inflammatory signaling. This, in combination with MR-mediated increases in phagocytosis, could allow for increased likelihood of plasmid internalization (**1**).

3.2 D-mannose for treatment of chronic pain

D-mannose and MR-mediated anti-inflammatory signaling may be clinically relevant for the treatment of a variety of chronic pain conditions, not just sciatic peripheral neuropathy, as explored in this dissertation. DM's potentially broad applicability stems from its engagement of both IL-10-independent and IL-10-mediated anti-inflammation (Dengler et al., 2014; Vanderwall et al., 2018). This means DM may be effective for pain relief in patients with known IL-10 gene polymorphisms that would prevent normal IL-10 production in response to other therapeutic approaches. DM may also be beneficial for treatment of pain diseases linked to decreased IL-10 expression, like fibromyalgia (Üçeyler et al., 2006; Milligan et al., 2012).

DM may be especially helpful in the treatment of pain pathologies that involve pro-inflammatory signaling at the level of the DRG, such as HIV-SN, discogenic lower back pain, and CIPN. The DRG is a unique nervous tissue in that it can be therapeutically accessed by direct, intrathecal, and systemic approaches (Sapunar et al., 2012; Berta et al., 2017). For instance, DRG lack protection from a capsule, have a very high blood capillary density, and possess vascular endothelial cells with large fenestrations (Jimenez-Andrade et al., 2008; Berta et al., 2017). Though prior studies in animal models have treated neuropathic pain with intrathecal DM, the unique features of the DRG may make systemic DM treatment a useful approach for patients who are not yet candidates for intrathecal interventions (Dengler et al., 2014; Saulino et al., 2014; Vanderwall et al., 2018). Systemic DM may also offer a successful approach for chronic pain processes where the source of

pathologic pro-inflammatory cytokine production is not well understood or may not be localized, such as observed in fibromyalgia (Clauw, 2009).

While DM holds promise for the treatment of many pain pathologies, not all pains or inflammatory processes are equal. For instance, though acute injury may result in significant physiological pain signaling that may be amplified by local inflammation, it is unclear whether DM would be useful, or even desirable, for pain control in this scenario. Non-steroidal anti-inflammatory agents (NSAIDs) are often utilized for their analgesic actions in acute pain, though whether they are detrimental to normal wound healing that require inflammatory processes remains controversial (Anderson and Hamm, 2012; Chen and Dragoo, 2013; Geusens et al., 2013). Therefore, consideration of DM for modulation of acute inflammatory pain should be approached cautiously. Additionally, it is unclear whether the same inflammatory processes modified by DM in treatment of chronic pathological pain would be relevant during acute inflammation. Pain associated with muscle spasm is another pain pathology that may not be amenable to DM therapy. Muscle spasm is characterized by a painful brief involuntary tonic contraction that can result from overstretching of a previously weakened muscle, protective splinting of an injury, muscle fatigue, or as a centrally mediated phenomenon such as dystonia (Skogseid, 2014; Fricton, 2016). Muscle spasm does not yet appear linked with any inflammatory processes and therefore DM may not be a likely therapeutic option.

Another therapeutic application for DM may exist as an adjuvant analgesic to improve efficacy and tolerability of other pain medications, like opioids. An analgesic adjuvant is a molecule that may not itself possess classical analgesic properties, but is found to have beneficial properties when used with opioids (Khan et al., 2011). This may allow

for decreases in opioid tolerance, dosing frequency, or dose size (Khan et al., 2011). There are a variety of clinically available adjuvant analgesics (e.g. NSAIDs, GABA agonists, antidepressants, antiepileptics, benzodiazepines, etc.), however many of these induce side effects that may limit their safety and utility. For instance, benzodiazepines may potentiate opioid neuropsychological side effects, and NSAIDs are connected with increased risk of gastrointestinal and renal toxicity (Khan et al., 2011; Harirforoosh et al., 2013). Opioids are known to induce spinal pro-inflammatory signaling in glial cells through activation of TLR4, and this signaling has been linked to opioid tolerance (Hutchinson et al., 2008; Hutchinson et al., 2011). Naloxone, especially the (+)-naloxone isomer, is a TLR4 antagonist that has been shown in pre-clinical and clinical studies to act as an analgesic opioid adjuvant, reducing tolerance associated with opioidal pain management (Hutchinson et al., 2008; Hutchinson et al., 2011; Lewis et al., 2012; Leppert, 2014; Firouzian et al., 2016; Wang et al., 2016). Similarly, it is possible that DM co-treatment may be useful in the mitigation of opioid-induced pro-inflammatory signaling via its demonstrated anti-inflammatory effects. Further investigations are required to better understand the full range of clinical applicability of DM in the treatment of chronic pain disorders.

4. Research Limitations

While promising, there are several key limitations that must be considered in the interpretation of this dissertation: (a) use of IL-10 KO mice, (b) mRNA vs. protein

analyses, (c) utilization of *in vitro* experimental approaches, and (d) small sample sizes during *in vitro* cell culture experiments.

IL-10 KO mice served as a powerful tool to unambiguously assess the therapeutic biodistribution of exogenous IL-10 transgene expression following intrathecal injection. They were also critical for investigating the role of endogenous IL-10 in both the efficacy of non-viral IL-10 gene therapy and in the effects of DM as an adjuvant for non-viral transgene therapy. However, the developmental absence of IL-10 could lead to compensatory changes in similar or alternative biological pathways (Keubler et al., 2015). Therefore, it is possible that pain relief observed IL-10 KO mice given non-viral IL-10 gene therapy engages alternative mechanisms that may not be available in wildtype or conditional Il-10 knockout mice. The same must be considered for the "IL-10-independent" adjuvant and anti-inflammatory effects of DM, both *in vitro* and *in vivo*.

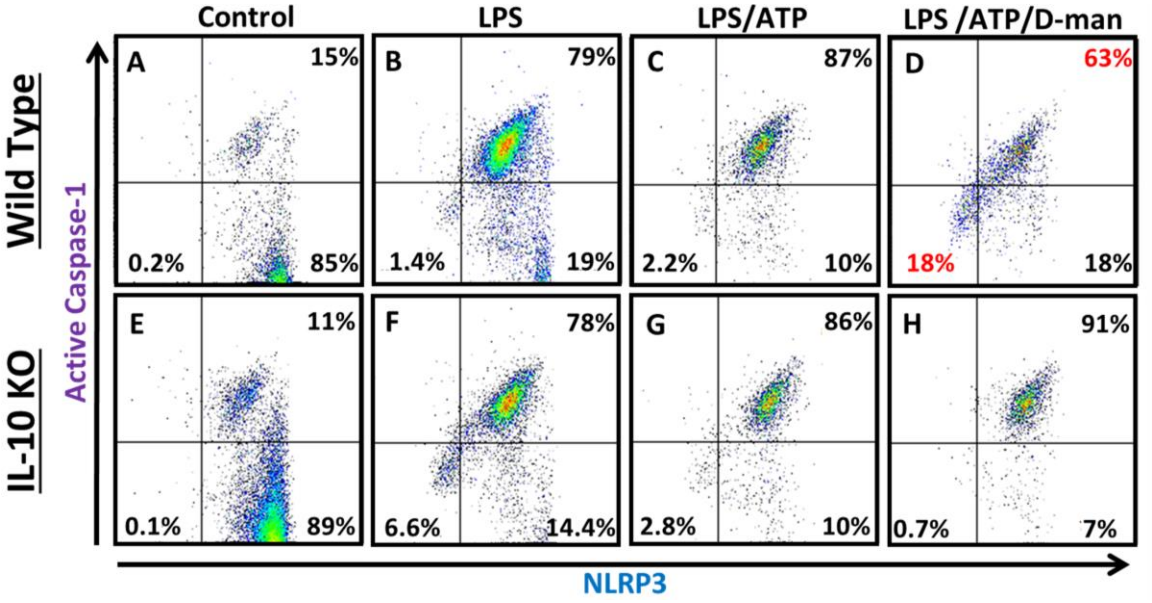
Assessment of mRNA levels offered key insight regarding the molecular and cellular changes that occur following intrathecal non-viral IL-10 gene therapy. While this approach allows for assessment of many targets despite small tissue volumes (e.g. DRG), it must be recognized that changes in mRNA levels do not always reflect changes in levels of corresponding proteins. Production of cytokines is often tightly regulated, as for IL-1β and TGF-β1, with a pro- or latent forms that must be converted to a mature bioactive forms. Therefore, following mRNA analysis, assessment of protein levels can offer a more comprehensive understanding of the systems in question. For instance, while pain relieved IL-10 KO mice given CCI+DM/pDNA-IL-10 express elevated mRNA levels for TGF-β1, whether this results in increased levels of bioactive TGF-β1 protein has not been assessed.

In vitro primary cell culture offers numerous advantages, including control of chemical conditions (e.g. pH, temperature, osmolarity, etc.), control of the physiological exposures (e.g. nutrients, hormones, growth factors, etc.), ease of treatment manipulation (e.g. drug exposure or intervention), enrichment of specific cell types (e.g. macrophage vs. T cell), and similarity in phenotype of primary cells to *in vivo* cells (Stansley et al., 2012). However, this approach is not without its limitations. For instance, cells *in vivo* rarely exist in single cell-type populations and instead co-exist in a diverse population of cells within a given tissue type. Furthermore, cells *in vitro* are often grown in a 2-dimensional (2D) monolayer which is not particularly representative of the natural *in vivo* microenvironment of cells (Fennema et al., 2013). Additionally, while great care is taken to develop culture media that is beneficial for maintenance of cellular viability, experimenters cannot possibly replicate all the variables present in the *in vivo* cellular milieu. Media is commonly supplemented with serum (e.g. fetal bovine serum) to provide supportive growth factors, hormones, lipids and other undefined components. However, use of serum is plagued by lot-to-lot variability as well as presentation of xenobiotic antigens that may unintentionally alter immune cell phenotypes (Vogel et al., 1988; Gstraunthaler, 2003). Therefore, while primary cell culture offers a powerful investigative tool, cell culture conditions may not always be reflective of *in vivo* mechanisms and could explain different dosage for successful non-viral IL-10 gene therapy *in vitro* vs. *in vivo*.

Finally, small sample sizes must be taken into consideration. The sample sizes utilized for investigations in Chapter 3 consisted of 2-3 wells per condition within a single experimental round. A power analysis was performed (G*Power 3.0.10 software) using preliminary data (Ch.3, Fig. 2A and 2C), separately for NO and for IL-10 data. For NO

analyses, assuming a pooled standard deviation (across WT and IL-10 KO cells) of 1.828, $\alpha = 0.05$, power set at 95%, and an effect size of 3.01, n = 2 wells per condition are required for ANOVA statistical analyses to detect significance. This supports that while replicate studies ought to be performed, the presented data were sufficiently powered for assessment of NO. For IL-10 protein power analysis, only WT data was considered. Assuming a pooled WT standard deviation of 23.955, $\alpha = 0.05$, power set at 95%, and an effect size of 1.37, n = 4 wells per condition in IL-10 protein analyses. Future analyses of IL-10 protein expression will therefore require greater sample sizes, including replicates, in order to reject the null hypothesis.

APPENDIX A



D-mannose disrupts the NLRP3-inflammasome in peritoneal macrophages: Peritoneal macrophages (PMs) were separately pooled from 3 WT or 3 IL-10 KO mice and cultured *ex vivo* for 13 hours prior to stimulation or treatment (N = 2 wells per condition; 2 x 10^5 cells in 500µL/well; 24 well plate). At time zero, cells were stimulated 4hrs with LPS (1µg/mL), then 1hr with D-mannose (D-man; 100mM), and finally 1hr with adenosine triphosphate (ATP; 25mM). Cells were collected and stained for flow cytometric analysis. To assess caspase-1 activation in stimulated PMs, a Fluorochrome-Labeled Inhibitor of Caspases Assay (FLICA) was used per manufacturer's instructions with a FAM-FLICA Assay Kit (ImmunoChemistry Tech) prior to other staining steps. Staining was performed, with modification, as previously described (Clark et al., 2011; Noor et al., 2017). PMs were stained with a fixable viability dye, then incubated with Fc block. Surface staining antibodies included: CD11b-PE-Cy7 and F4/80-PerCP-Cy5.5, CD45-APC-Cy7. Cells were fixed in 4% PFA prior to intracellular staining with NLRP3-Alexa Fluor 700. Flow cytometric analysis was performed with a BD LSRFortessa (BD Bioscience), and the data were analyzed with FlowJo software (Tree Star). Macrophages were identified as $CD45^+/CD11b^+/F4/80^+$ and analyzed for Active Caspase-1 vs. NLRP3. Control stimulated cells (**A**, **E**) are $NLRP3^+$, but few are positive for Caspase-1 activation. LPS only (**B**, **F**) cells exhibit increases in the proportion of cells that are double positive for NLRP3 and Active Caspase-1, and this increase is enhanced in LPS/ATP treated cells (**C**, **G**). These trends are consistent in both WT and IL-10 KO cells. D-mannose prior to ATP stimulation (LPS/ATP/D-man) dramatically decreases the proportion of double positive cells and increases the proportion of double negatives in WT cells (**D**), but these effects are not observed in IL-10 KO cells (**H**). Representative plots are shown.

References

Agalave NM, Larsson M, Abdelmoaty S, Su J, Baharpoor A, Lundbäck P, Palmblad K, Andersson U, Harris H, Svensson CI (2014) Spinal HMGB1 induces TLR4-mediated long-lasting hypersensitivity and glial activation and regulates pain-like behavior in experimental arthritis. PAIN® 155:1802-1813.

Allavena P, Chieppa M, Bianchi G, Solinas G, Fabbri M, Laskarin G, Mantovani A (2010) Engagement of the mannose receptor by tumoral mucins activates an immune suppressive phenotype in human tumor-associated macrophages. Clin Dev Immunol 2010:547179.

Allen ML, Hoschtitzky JA, Peters MJ, Elliott M, Goldman A, James I, Klein NJ (2006) Interleukin-10 and its role in clinical immunoparalysis following pediatric cardiac surgery*. Critical Care Medicine 34:2658-2665.

Alvarez P, Bogen O, Green PG, Levine JD (2017) Nociceptor interleukin 10 receptor 1 is critical for muscle analgesia induced by repeated bouts of eccentric exercise in the rat. Pain 158:1481-1488.

Anderson K, Hamm RL (2012) Factors That Impair Wound Healing. The Journal of the American College of Clinical Wound Specialists 4:84-91.

Anderson P (2008) Post-transcriptional control of cytokine production. Nature immunology 9:353-359.

Asadullah K, Döcke W, Ebeling M, et al. (1999) Interleukin 10 treatment of psoriasis: Clinical results of a phase 2 trial. Archives of Dermatology 135:187-192.

Asseman C, Mauze S, Leach MW, Coffman RL, Powrie F (1999) An Essential Role for Interleukin 10 in the Function of Regulatory T Cells That Inhibit Intestinal Inflammation. The Journal of Experimental Medicine 190:995-1004.

Astarie-Dequeker C, N'Diaye EN, Le Cabec V, Rittig MG, Prandi J, Maridonneau-Parini I (1999) The mannose receptor mediates uptake of pathogenic and nonpathogenic mycobacteria and bypasses bactericidal responses in human macrophages. Infect Immun 67:469-477.

Azad AK, Rajaram MV, Metz WL, Cope FO, Blue MS, Vera DR, Schlesinger LS (2015) gamma-Tilmanocept, a New Radiopharmaceutical Tracer for Cancer Sentinel Lymph Nodes, Binds to the Mannose Receptor (CD206). J Immunol 195:2019-2029.

Aziz-Donnelly A, Harrison TB (2017) Update of HIV-Associated Sensory Neuropathies. Current Treatment Options in Neurology 19:36.

Baetas-da-Cruz W, Alves L, Pessolani MC, Barbosa HS, Regnier-Vigouroux A, Corte-Real S, Cavalcante LA (2009) Schwann cells express the macrophage mannose receptor and MHC class II. Do they have a role in antigen presentation? J Peripher Nerv Syst 14:84-92.

Basbaum AI, Bautista DM, Scherrer G, Julius D (2009) Cellular and molecular mechanisms of pain. Cell 139:267-284.

Becher B, Spath S, Goverman J (2016) Cytokine networks in neuroinflammation. Nature Reviews Immunology 17:49.

Bennett GJ, Xie YK (1988) A peripheral mononeuropathy in rat that produces disorders of pain sensation like those seen in man. Pain 33:87-107.
Bennett MI, Rayment C, Hjermstad M, Aass N, Caraceni A, Kaasa S (2012) Prevalence and aetiology of neuropathic pain in cancer patients: A systematic review. PAIN 153:359-365.
Bennett ML, Bennett FC, Liddelow SA, Ajami B, Zamanian JL, Fernhoff NB, Mulinyawe SB, Bohlen CJ, Adil A, Tucker A, Weissman IL, Chang EF, Li G, Grant GA, Hayden Gephart MG, Barres BA (2016) New tools for studying microglia in the mouse and human CNS. Proc Natl Acad Sci U S A 113:E1738-1746.
Berg DJ, Kühn R, Rajewsky K, Müller W, Menon S, Davidson N, Grünig G, Rennick D (1995) Interleukin-10 is a central regulator of the response to LPS in murine models of endotoxic shock and the Shwartzman reaction but not endotoxin tolerance. Journal of Clinical Investigation 96:2339-2347.
Berg DJ, Davidson N, Kuhn R, Muller W, Menon S, Holland G, Thompson-Snipes L, Leach MW, Rennick D (1996) Enterocolitis and colon cancer in interleukin-10-deficient mice are associated with aberrant cytokine production and CD4(+) TH1-like responses. J Clin Invest 98:1010-1020.
Berta T, Qadri Y, Tan PH, Ji RR (2017) Targeting dorsal root ganglia and primary sensory neurons for the treatment of chronic pain. Expert Opin Ther Targets 21:695-703.
Beutler AS, Reinhardt M (2009) AAV for pain: steps towards clinical translation. Gene Therapy 16:461.
Blackbeard J, Wallace VCJ, O'Dea KP, Hasnie F, Segerdahl A, Pheby T, Field MJ, Takata M, Rice ASC (2012) The correlation between pain-related behaviour and spinal microgliosis in four distinct models of peripheral neuropathy. European Journal of Pain 16:1357-1367.
Boehncke W-H (2015) Etiology and Pathogenesis of Psoriasis. Rheumatic Disease Clinics of North America 41:665-675.
Bonin RP, Bories C, De Koninck Y (2014) A simplified up-down method (SUDO) for measuring mechanical nociception in rodents using von Frey filaments. Mol Pain 10:26.
Borenstein D (2013) Mechanical low back pain—a rheumatologist's view. Nature Reviews Rheumatology 9:643.
Borges TJ, Lopes RL, Pinho NG, Machado FD, Souza APD, Bonorino C (2013) Extracellular Hsp70 inhibits pro-inflammatory cytokine production by IL-10 driven down-regulation of C/EBPβ and C/EBPδ. International Journal of Hyperthermia 29:455-463.
Bote ME, Garcia JJ, Hinchado MD, Ortega E (2012) Inflammatory/stress feedback dysregulation in women with fibromyalgia. Neuroimmunomodulation 19:343-351.
Bottner M, Krieglstein K, Unsicker K (2000) The transforming growth factor-betas: structure, signaling, and roles in nervous system development and functions. J Neurochem 75:2227-2240.
Bouhassira D, Luporsi E, Krakowski I (2017) Prevalence and incidence of chronic pain with or without neuropathic characteristics in patients with cancer. PAIN 158:1118-1125.
Boutard V, Havouis R, Fouqueray B, Philippe C, Moulinoux JP, Baud L (1995) Transforming growth factor-beta stimulates arginase activity in macrophages.

Implications for the regulation of macrophage cytotoxicity. The Journal of Immunology 155:2077-2084.
Braat H, Rottiers P, Hommes DW, Huyghebaert N, Remaut E, Remon JP, van Deventer SJH, Neirynck S, Peppelenbosch MP, Steidler L (2006) A Phase I Trial With Transgenic Bacteria Expressing Interleukin-10 in Crohn's Disease. Clinical Gastroenterology and Hepatology 4:754-759.
Breivik H, Collett B, Ventafridda V, Cohen R, Gallacher D (2006) Survey of chronic pain in Europe: prevalence, impact on daily life, and treatment. Eur J Pain 10:287-333.
Brockmann L, Gagliani N, Steglich B, Giannou AD, Kempski J, Pelczar P, Geffken M, Mfarrej B, Huber F, Herkel J, Wan YY, Esplugues E, Battaglia M, Krebs CF, Flavell RA, Huber S (2017) IL-10 Receptor Signaling Is Essential for TR1 Cell Function In Vivo. J Immunol 198:1130-1141.
Brown MR, Ramirez JD (2015) Neuroimmune mechanisms in cancer pain. Current opinion in supportive and palliative care 9:103-111.
Brown PN, Yin H (2013) PNA-based microRNA inhibitors elicit anti-inflammatory effects in microglia cells. Chem Commun (Camb) 49:4415-4417.
Buchwald UK, Geerdes-Fenge HF, Vöckler J, Ziege S, Lode H (1999) Interleukin-10: effects on phagocytosis and adhesion molecule expression of granulocytes and monocytes in a comparison with prednisolone. Eur J Med Res 4:85-94.
Burdo TH, Miller AD (2014) Animal models of HIV peripheral neuropathy. Future virology 9:465-474.
Burudi EM, Regnier-Vigouroux A (2001) Regional and cellular expression of the mannose receptor in the post-natal developing mouse brain. Cell Tissue Res 303:307-317.
Burudi EM, Riese S, Stahl PD, Regnier-Vigouroux A (1999) Identification and functional characterization of the mannose receptor in astrocytes. Glia 25:44-55.
Calvo M, Bennett DLH (2012) The mechanisms of microgliosis and pain following peripheral nerve injury. Experimental Neurology 234:271-282.
Carozzi VA, Canta A, Chiorazzi A (2015) Chemotherapy-induced peripheral neuropathy: What do we know about mechanisms? Neuroscience Letters 596:90-107.
Chaplan SR, Bach FW, Pogrel JW, Chung JM, Yaksh TL (1994) Quantitative assessment of tactile allodynia in the rat paw. J Neurosci Methods 53:55-63.
Chen H, Li D, Saldeen T, Mehta JL (2001) TGF-β1 modulates NOS expression and phosphorylation of Akt/PKB in rat myocytes exposed to hypoxia-reoxygenation. American Journal of Physiology - Heart and Circulatory Physiology 281:H1035-H1039.
Chen MR, Dragoo JL (2013) The effect of nonsteroidal anti-inflammatory drugs on tissue healing. Knee Surgery, Sports Traumatology, Arthroscopy 21:540-549.
Chen Z, Jalabi W, Shpargel KB, Farabaugh KT, Dutta R, Yin X, Kidd GJ, Bergmann CC, Stohlman SA, Trapp BD (2012) Lipopolysaccharide-induced microglial activation and neuroprotection against experimental brain injury is independent of hematogenous TLR4. J Neurosci 32:11706-11715.
Cheng CF, Cheng JK, Chen CY, Lien CC, Chu D, Wang SY, Tsaur ML (2014) Mirror-image pain is mediated by nerve growth factor produced from tumor necrosis factor alpha-activated satellite glia after peripheral nerve injury. Pain 155:906-920.
Chesney M (2003) Adherence to HAART regimens. AIDS patient care and STDs 17:169-177.

Chieppa M, Bianchi G, Doni A, Del Prete A, Sironi M, Laskarin G, Monti P, Piemonti L, Biondi A, Mantovani A, Introna M, Allavena P (2003) Cross-linking of the mannose receptor on monocyte-derived dendritic cells activates an anti-inflammatory immunosuppressive program. J Immunol 171:4552-4560.

Chiu IM, von Hehn CA, Woolf CJ (2012) Neurogenic Inflammation – The Peripheral Nervous System's Role in Host Defense and Immunopathology. Nature neuroscience 15:1063-1067.

Chroneos Z, Shepherd VL (1995) Differential regulation of the mannose and SP-A receptors on macrophages. The American journal of physiology 269:L721-726.

Clark RT, Philip Nance J, Noor S, Wilson EH (2011) T-cell production of matrix metalloproteinases and inhibition of parasite clearance by TIMP-1 during chronic Toxoplasma infection in the brain. ASN NEURO 3:e00049.

Clauw DJ (2009) Fibromyalgia: An Overview. The American Journal of Medicine 122:S3-S13.

Colak S, Ten Dijke P (2017) Targeting TGF-beta Signaling in Cancer. Trends Cancer 3:56-71.

Colburn RW, Rickman AJ, DeLeo JA (1999) The effect of site and type of nerve injury on spinal glial activation and neuropathic pain behavior. Exp Neurol 157:289-304.

Colleoni M, Sacerdote P (2010) Murine models of human neuropathic pain. Biochim Biophys Acta 1802:924-933.

Colloca L, Ludman T, Bouhassira D, Baron R, Dickenson AH, Yarnitsky D, Freeman R, Truini A, Attal N, Finnerup NB, Eccleston C, Kalso E, Bennett DL, Dworkin RH, Raja SN (2017) Neuropathic pain. Nat Rev Dis Primers 3:17002.

Cornell TT, Sun L, Hall MW, Gurney JG, Ashbrook MJ, Ohye RG, Shanley TP (2012) Clinical implications and molecular mechanisms of immunoparalysis after cardiopulmonary bypass. The Journal of Thoracic and Cardiovascular Surgery 143:1160-1166.e1161.

Corradin SB, Buchmuller-Rouiller Y, Smith J, Suardet L, Mauel J (1993) Transforming growth factor beta 1 regulation of macrophage activation depends on the triggering stimulus. J Leukoc Biol 54:423-429.

Costa B, Comelli F, Bettoni I, Colleoni M, Giagnoni G (2008) The endogenous fatty acid amide, palmitoylethanolamide, has anti-allodynic and anti-hyperalgesic effects in a murine model of neuropathic pain: involvement of CB(1), TRPV1 and PPARgamma receptors and neurotrophic factors. Pain 139:541-550.

da Silva MD, Bobinski F, Sato KL, Kolker SJ, Sluka KA, Santos AR (2015) IL-10 cytokine released from M2 macrophages is crucial for analgesic and anti-inflammatory effects of acupuncture in a model of inflammatory muscle pain. Molecular neurobiology 51:19-31.

Dalal S, Bruera E (2013) Access to opioid analgesics and pain relief for patients with cancer. Nature Reviews Clinical Oncology 10:108.

De Leo JA, Tawfik VL, LaCroix-Fralish ML (2006) The tetrapartite synapse: Path to CNS sensitization and chronic pain. PAIN 122:17-21.

Dengler EC, Alberti LA, Bowman BN, Kerwin AA, Wilkerson JL, Moezzi DR, Limanovich E, Wallace JA, Milligan ED (2014) Improvement of spinal non-viral IL-10 gene delivery by D-mannose as a transgene adjuvant to control chronic neuropathic pain. Journal of neuroinflammation 11:92.

Dengler EC, Liu J, Kerwin A, Torres S, Olcott CM, Bowman BN, Armijo L, Gentry K, Wilkerson J, Wallace J, Jiang X, Carnes EC, Brinker CJ, Milligan ED (2013) Mesoporous silica-supported lipid bilayers (protocells) for DNA cargo delivery to the spinal cord. J Control Release 168:209-224.

DeSantana JM, da Cruz KML, Sluka KA (2013) Animal models of fibromyalgia. Arthritis Research & Therapy 15:222-222.

Dina OA, Levine JD, Green PG (2008a) Muscle inflammation induces a protein kinase C ε–dependent chronic-latent muscle pain. The journal of pain : official journal of the American Pain Society 9:457-462.

Dina OA, Green PG, Levine JD (2008b) Role of IL-6 in Chronic Muscle Hyperalgesic Priming. Neuroscience 152:521-525.

Dina OA, Levine JD, Green PG (2011) Enhanced cytokine-induced mechanical hyperalgesia in skeletal muscle produced by a novel mechanism in rats exposed to unpredictable sound stress. European journal of pain (London, England) 15:796-800.

Dworkin RH, O'Connor AB, Backonja M, Farrar JT, Finnerup NB, Jensen TS, Kalso EA, Loeser JD, Miaskowski C, Nurmikko TJ, Portenoy RK, Rice ASC, Stacey BR, Treede R-D, Turk DC, Wallace MS (2007) Pharmacologic management of neuropathic pain: Evidence-based recommendations. PAIN 132:237-251.

Echeverry S, Shi XQ, Zhang J (2008) Characterization of cell proliferation in rat spinal cord following peripheral nerve injury and the relationship with neuropathic pain. Pain 135:37-47.

Echeverry S, Shi XQ, Haw A, Liu H, Zhang ZW, Zhang J (2009) Transforming growth factor-beta1 impairs neuropathic pain through pleiotropic effects. Mol Pain 5:16.

Eriksson NP, Persson JK, Svensson M, Arvidsson J, Molander C, Aldskogius H (1993) A quantitative analysis of the microglial cell reaction in central primary sensory projection territories following peripheral nerve injury in the adult rat. Experimental brain research 96:19-27.

Fennema E, Rivron N, Rouwkema J, van Blitterswijk C, de Boer J (2013) Spheroid culture as a tool for creating 3D complex tissues. Trends in Biotechnology 31:108-115.

Fernandez N, Alonso S, Valera I, Vigo AG, Renedo M, Barbolla L, Crespo MS (2005) Mannose-containing molecular patterns are strong inducers of cyclooxygenase-2 expression and prostaglandin E2 production in human macrophages. J Immunol 174:8154-8162.

Fields HL, Basbaum AI (1978) Brainstem Control of Spinal Pain-Transmission Neurons. Annual review of physiology 40:217-248.

Fillatreau S, Sweenie CH, McGeachy MJ, Gray D, Anderton SM (2002) B cells regulate autoimmunity by provision of IL-10. Nature immunology 3:944.

Fiorentino DF, Bond MW, Mosmann TR (1989) Two types of mouse T helper cell. IV. Th2 clones secrete a factor that inhibits cytokine production by Th1 clones. J Exp Med 170:2081-2095.

Firouzian A, Gholipour Baradari A, Alipour A, Emami Zeydi A, Zamani Kiasari A, Emadi SA, Kheradmand B, Hadadi K (2016) Ultra-low-dose Naloxone as an Adjuvant to Patient Controlled Analgesia (PCA) With Morphine for Postoperative Pain Relief Following Lumber Discectomy: A Double-blind, Randomized, Placebo-controlled Trial. Journal of neurosurgical anesthesiology.

Fitzgerald M (1982) Alterations in the ipsi- and contralateral afferent inputs of dorsal horn cells produced by capsaicin treatment of one sciatic nerve in the rat. Brain Res 248:97-107.

Fitzgerald M, Woolf CJ (1982) The time course and specificity of the changes in the behavioural and dorsal horn cell responses to noxious stimuli following peripheral nerve capsaicin treatment in the rat. Neuroscience 7:2051-2056.

Fleischmann RM, Tesser J, Schiff MH, Schechtman J, Burmester GR, Bennett R, Modafferi D, Zhou L, Bell D, Appleton B (2006) Safety of extended treatment with anakinra in patients with rheumatoid arthritis. Annals of the Rheumatic Diseases 65:1006-1012.

Franke A et al. (2008) Sequence variants in IL10, ARPC2 and multiple other loci contribute to ulcerative colitis susceptibility. Nat Genet 40:1319-1323.

Fricton J (2016) Myofascial Pain: Mechanisms to Management. Oral and maxillofacial surgery clinics of North America 28:289-311.

Fuss IJ, Boirivant M, Lacy B, Strober W (2002) The interrelated roles of TGF-beta and IL-10 in the regulation of experimental colitis. J Immunol 168:900-908.

Galea I, Palin K, Newman TA, Van Rooijen N, Perry VH, Boche D (2005) Mannose receptor expression specifically reveals perivascular macrophages in normal, injured, and diseased mouse brain. Glia 49:375-384.

Garcia-Aguilar T, Espinosa-Cueto P, Magallanes-Puebla A, Mancilla R (2016) The Mannose Receptor Is Involved in the Phagocytosis of Mycobacteria-Induced Apoptotic Cells. J Immunol Res 2016:3845247.

Gardner K, Hall PA, Chinnery PF, Payne BAI (2014) HIV Treatment and Associated Mitochondrial Pathology:Review of 25 Years of in Vitro, Animal, and Human Studies. Toxicologic Pathology 42:811-822.

Garrido VV, Dulgerian LR, Stempin CC, Cerbán FM (2011) The Increase in Mannose Receptor Recycling Favors Arginase Induction and Trypanosoma Cruzi Survival in Macrophages. International Journal of Biological Sciences 7:1257-1272.

Garrison CJ, Dougherty PM, Carlton SM (1994) GFAP Expression in Lumbar Spinal Cord of Naive and Neuropathic Rats Treated with MK-801. Experimental Neurology 129:237-243.

Garrison CJ, Dougherty PM, Kajander KC, Carlton SM (1991) Staining of glial fibrillary acidic protein (GFAP) in lumbar spinal cord increases following a sciatic nerve constriction injury. Brain Res 565:1-7.

Gazi U, Martinez-Pomares L (2009) Influence of the mannose receptor in host immune responses. Immunobiology 214:554-561.

Geusens P, Emans PJ, de Jong JJ, van den Bergh J (2013) NSAIDs and fracture healing. Current opinion in rheumatology 25:524-531.

Glover DJ, Lipps HJ, Jans DA (2005) Towards safe, non-viral therapeutic gene expression in humans. Nat Rev Genet 6:299-310.

Gordon S (2003) Alternative activation of macrophages. Nat Rev Immunol 3:23-35.

Goss JR, Goins WF, Glorioso JC (2007) Gene therapy applications for the treatment of neuropathic pain. Expert Review of Neurotherapeutics 7:487-506.

Grace PM, Hutchinson MR, Maier SF, Watkins LR (2014) Pathological pain and the neuroimmune interface. Nat Rev Immunol 14:217-231.

Grace PM, Loram LC, Christianson JP, Strand KA, Flyer-Adams JG, Penzkover KR, Forsayeth JR, van Dam AM, Mahoney MJ, Maier SF, Chavez RA, Watkins LR (2017) Behavioral assessment of neuropathic pain, fatigue, and anxiety in experimental autoimmune encephalomyelitis (EAE) and attenuation by interleukin-10 gene therapy. Brain Behav Immun 59:49-54.

Gregory NS, Brito RG, Oliveira Fusaro MCG, Sluka KA (2016) ASIC3 is required for development of fatigue-induced hyperalgesia. Molecular neurobiology 53:1020-1030.

Grondal G, Kristjansdottir H, Gunnlaugsdottir B, Arnason A, Lundberg I, Klareskog L, Steinsson K (1999) Increased number of interleukin-10-producing cells in systemic lupus erythematosus patients and their first-degree relatives and spouses in Icelandic multicase families. Arthritis Rheum 42:1649-1654.

Groux H, O'Garra A, Bigler M, Rouleau M, Antonenko S, de Vries JE, Roncarolo MG (1997) A CD4+T-cell subset inhibits antigen-specific T-cell responses and prevents colitis. Nature 389:737.

Gstraunthaler G (2003) Alternatives to the use of fetal bovine serum: serum-free cell culture. Altex 20:275-281.

Gui T, Sun Y, Shimokado A, Muragaki Y (2012) The Roles of Mitogen-Activated Protein Kinase Pathways in TGF-β-Induced Epithelial-Mesenchymal Transition. Journal of Signal Transduction 2012.

Guillot L, Balloy V, McCormack FX, Golenbock DT, Chignard M, Si-Tahar M (2002) Cutting Edge: The Immunostimulatory Activity of the Lung Surfactant Protein-A Involves Toll-Like Receptor 4. The Journal of Immunology 168:5989-5992.

Gur A, Oktayoglu P (2008) Status of immune mediators in fibromyalgia. Current Pain and Headache Reports 12:175-181.

Hadaschik EN, Enk AH (2015) TGF-β1-induced regulatory T cells. Human Immunology 76:561-564.

Hanani M (2015) Role of satellite glial cells in gastrointestinal pain. Front Cell Neurosci 9:412.

Hao S (2013) The Molecular and Pharmacological Mechanisms of HIV-Related Neuropathic Pain. Current Neuropharmacology 11:499-512.

Harirforoosh S, Asghar W, Jamali F (2013) Adverse effects of nonsteroidal antiinflammatory drugs: an update of gastrointestinal, cardiovascular and renal complications. Journal of pharmacy & pharmaceutical sciences : a publication of the Canadian Society for Pharmaceutical Sciences, Societe canadienne des sciences pharmaceutiques 16:821-847.

Hart BL (1988) Biological basis of the behavior of sick animals. Neurosci Biobehav Rev 12:123-137.

Hashizume H, DeLeo JA, Colburn RW, Weinstein JN (2000) Spinal glial activation and cytokine expression after lumbar root injury in the rat. Spine (Phila Pa 1976) 25:1206-1217.

Herzberg U, Sagen J (2001) Peripheral nerve exposure to HIV viral envelope protein gp120 induces neuropathic pain and spinal gliosis. Journal of Neuroimmunology 116:29-39.

Ho Y-P, Chiu C-T, Sheen IS, Tseng S-C, Lai PC, Ho S-Y, Chen W-T, Lin T-N, Lin C-Y (2011) Tumor necrosis factor–α and interleukin-10 contribute to immunoparalysis in patients with acute pancreatitis. Human Immunology 72:18-23.

Holmdahl R, Malmström V, Burkhardt H (2014) Autoimmune priming, tissue attack and chronic inflammation — The three stages of rheumatoid arthritis. European Journal of Immunology 44:1593-1599.

Hu P, Bembrick AL, Keay KA, McLachlan EM (2007) Immune cell involvement in dorsal root ganglia and spinal cord after chronic constriction or transection of the rat sciatic nerve. Brain Behav Immun 21:599-616.

Hu X, Shi YN, Zhang P, Miao M, Zhang T, Jiang B (2016) d-Mannose: Properties, Production, and Applications: An Overview. Compr Rev Food Sci F 15:773-785.

Huang D, Yu B (2010) The mirror-image pain: an unclered phenomenon and its possible mechanism. Neurosci Biobehav Rev 34:528-532.

Hughes TS, Langer SJ, Johnson KW, Chavez RA, Watkins LR, Milligan ED, Leinwand LA (2009) Intrathecal injection of naked plasmid DNA provides long-term expression of secreted proteins. Mol Ther 17:88-94.

Hussell T, Bell TJ (2014) Alveolar macrophages: plasticity in a tissue-specific context. Nat Rev Immunol 14:81-93.

Hutchinson MR, Bland ST, Johnson KW, Rice KC, Maier SF, Watkins LR (2007) Opioid-Induced Glial Activation: Mechanisms of Activation and Implications for Opioid Analgesia, Dependence, and Reward. TheScientificWorldJOURNAL 7.

Hutchinson MR, Shavit Y, Grace PM, Rice KC, Maier SF, Watkins LR (2011) Exploring the Neuroimmunopharmacology of Opioids: An Integrative Review of Mechanisms of Central Immune Signaling and Their Implications for Opioid Analgesia. Pharmacological Reviews 63:772-810.

Hutchinson MR, Zhang Y, Brown K, Coats BD, Shridhar M, Sholar PW, Patel SJ, Crysdale NY, Harrison JA, Maier SF, Rice KC, Watkins LR (2008) Non-stereoselective reversal of neuropathic pain by naloxone and naltrexone: involvement of toll-like receptor 4 (TLR4). Eur J Neurosci 28:20-29.

Hutchinson MR, Loram LC, Zhang Y, Shridhar M, Rezvani N, Berkelhammer D, Phipps S, Foster PS, Landgraf K, Falke JJ, Rice KC, Maier SF, Yin H, Watkins LR (2010) Evidence that tricyclic small molecules may possess Toll-like receptor and MD-2 activity. Neuroscience 168:551-563.

Hutchison ER, Kawamoto EM, Taub DD, Lal A, Abdelmohsen K, Zhang Y, Wood WH, 3rd, Lehrmann E, Camandola S, Becker KG, Gorospe M, Mattson MP (2013) Evidence for miR-181 involvement in neuroinflammatory responses of astrocytes. Glia 61:1018-1028.

Hylden JL, Wilcox GL (1980) Intrathecal morphine in mice: a new technique. Eur J Pharmacol 67:313-316.

Inoue K, Tsuda M (2009) Microglia and neuropathic pain. Glia 57:1469-1479.

Institute of Medicine Committee on Advancing Pain Research C, Education (2011) The National Academies Collection: Reports funded by National Institutes of Health. In: Relieving Pain in America: A Blueprint for Transforming Prevention, Care, Education, and Research. Washington (DC): National Academies Press (US)

National Academy of Sciences.

Iyer SS, Cheng G (2012) Role of Interleukin 10 Transcriptional Regulation in Inflammation and Autoimmune Disease. Critical reviews in immunology 32:23-63.
Jaggi AS, Jain V, Singh N (2011a) Animal models of neuropathic pain. Fundamental & clinical pharmacology 25:1-28.
Jaggi AS, Jain V, Singh N (2011b) Animal models of neuropathic pain. Fundamental & clinical pharmacology 25:1-28.
Jamison RN, Mao J (2015) Opioid Analgesics. Mayo Clinic proceedings 90:957-968.
Jancalek R, Dubovy P, Svizenska I, Klusakova I (2010) Bilateral changes of TNF-alpha and IL-10 protein in the lumbar and cervical dorsal root ganglia following a unilateral chronic constriction injury of the sciatic nerve. Journal of neuroinflammation 7:11.
Jancalek R, Svizenska I, Klusakova I, Dubovy P (2011) Bilateral changes of IL-10 protein in lumbar and cervical dorsal root ganglia following proximal and distal chronic constriction injury of peripheral nerve. Neurosci Lett 501:86-91.
Jayant RD, Sosa D, Kaushik A, Atluri V, Vashist A, Tomitaka A, Nair M (2016) Current status of non-viral gene therapy for CNS disorders. Expert Opin Drug Deliv 13:1433-1445.
Ji RR, Berta T, Nedergaard M (2013) Glia and pain: Is chronic pain a gliopathy? Pain 154:S10-S28.
Ji RR, Xu ZZ, Gao YJ (2014) Emerging targets in neuroinflammation-driven chronic pain. Nat Rev Drug Discov 13:533-548.
Ji RR, Chamessian A, Zhang YQ (2016) Pain regulation by non-neuronal cells and inflammation. Science 354:572-577.
Jimenez-Andrade JM, Herrera MB, Ghilardi JR, Vardanyan M, Melemedjian OK, Mantyh PW (2008) Vascularization of the dorsal root ganglia and peripheral nerve of the mouse: implications for chemical-induced peripheral sensory neuropathies. Mol Pain 4:10.
Johnson JL, Rolan PE, Johnson ME, Bobrovskaya L, Williams DB, Johnson K, Tuke J, Hutchinson MR (2014) Codeine-induced hyperalgesia and allodynia: investigating the role of glial activation. Translational Psychiatry 4:e482.
Joosten LAB, Abdollahi-Roodsaz S, Dinarello CA, O'Neill L, Netea MG (2016) Toll-like receptors and chronic inflammation in rheumatic diseases: new developments. Nature Reviews Rheumatology 12:344.
Kagan BL, Baldwin RL, Munoz D, Wisnieski BJ (1992) Formation of ion-permeable channels by tumor necrosis factor-alpha. Science 255:1427-1430.
Kastin AJ, Akerstrom V, Pan W (2003) Interleukin-10 as a CNS therapeutic: the obstacle of the blood-brain/blood-spinal cord barrier. Brain Res Mol Brain Res 114:168-171.
Kelechi TJ, Mueller M, Hankin CS, Bronstone A, Samies J, Bonham PA (2012) A randomized, investigator-blinded, controlled pilot study to evaluate the safety and efficacy of a poly-N-acetyl glucosamine-derived membrane material in patients with venous leg ulcers. Journal of the American Academy of Dermatology 66:e209-215.
Keubler LM, Buettner M, Häger C, Bleich A (2015) A Multihit Model: Colitis Lessons from the Interleukin-10–deficient Mouse. Inflammatory Bowel Diseases 21:1967-1975.

Khan J, Ramadan K, Korczeniewska O, Anwer MM, Benoliel R, Eliav E (2015) Interleukin-10 levels in rat models of nerve damage and neuropathic pain. Neurosci Lett 592:99-106.

Khan MIA, Walsh D, Brito-Dellan N (2011) Opioid and Adjuvant Analgesics: Compared and Contrasted. American Journal of Hospice and Palliative Medicine® 28:378-383.

Khusru A, Robert S, Markus F, Hans Dieter V, Wolfram S (2004) Interleukin-10: An Important Immunoregulatory Cytokine With Major Impact on Psoriasis. Current Drug Targets - Inflammation & Allergy 3:185-192.

Kitani A, Fuss IJ, Nakamura K, Schwartz OM, Usui T, Strober W (2000) Treatment of experimental (Trinitrobenzene sulfonic acid) colitis by intranasal administration of transforming growth factor (TGF)-beta1 plasmid: TGF-beta1-mediated suppression of T helper cell type 1 response occurs by interleukin (IL)-10 induction and IL-12 receptor beta2 chain downregulation. J Exp Med 192:41-52.

Kleinewietfeld M, Hafler DA (2014) Regulatory T cells in autoimmune neuroinflammation. Immunol Rev 259:231-244.

Kleinschnitz C, Hofstetter HH, Meuth SG, Braeuninger S, Sommer C, Stoll G (2006) T cell infiltration after chronic constriction injury of mouse sciatic nerve is associated with interleukin-17 expression. Exp Neurol 200:480-485.

Komori T, Morikawa Y, Inada T, Hisaoka T, Senba E (2011) Site-specific subtypes of macrophages recruited after peripheral nerve injury. Neuroreport 22:911-917.

Krames ES (2015) The dorsal root ganglion in chronic pain and as a target for neuromodulation: a review. Neuromodulation 18:24-32; discussion 32.

Krukowski K, Eijkelkamp N, Laumet G, Hack CE, Li Y, Dougherty PM, Heijnen CJ, Kavelaars A (2016) CD8+ T Cells and Endogenous IL-10 Are Required for Resolution of Chemotherapy-Induced Neuropathic Pain. J Neurosci 36:11074-11083.

Kuhn R, Lohler J, Rennick D, Rajewsky K, Muller W (1993) Interleukin-10-deficient mice develop chronic enterocolitis. Cell 75:263-274.

Kullberg MC, Ward JM, Gorelick PL, Caspar P, Hieny S, Cheever A, Jankovic D, Sher A (1998) Helicobacter hepaticus Triggers Colitis in Specific-Pathogen-Free Interleukin-10 (IL-10)-Deficient Mice through an IL-12- and Gamma Interferon-Dependent Mechanism. Infection and Immunity 66:5157-5166.

Kumar V, Abbas AK, Aster JC (2015) Inflammation and Repair. In: Robbins and Cotran pathologic basis of disease, Ninth edition. Edition, pp 69-111. Philadelphia, PA: Elsevier/Saunders.

Kwilasz AJ, Grace PM, Serbedzija P, Maier SF, Watkins LR (2015) The therapeutic potential of interleukin-10 in neuroimmune diseases. Neuropharmacology 96:55-69.

Kwon MJ, Shin HY, Cui Y, Kim H, Thi AHL, Choi JY, Kim EY, Hwang DH, Kim BG (2015) CCL2 Mediates Neuron–Macrophage Interactions to Drive Proregenerative Macrophage Activation Following Preconditioning Injury. The Journal of Neuroscience 35:15934-15947.

Lassmann H, van Horssen J, Mahad D (2012) Progressive multiple sclerosis: pathology and pathogenesis. Nature Reviews Neurology 8:647.

Latremoliere A, Woolf CJ (2009) Central Sensitization: A Generator of Pain Hypersensitivity by Central Neural Plasticity. The journal of pain : official journal of the American Pain Society 10:895-926.

Lau D, Harte SE, Morrow TJ, Wang S, Mata M, Fink DJ (2012) Herpes simplex virus vector-mediated expression of interleukin-10 reduces below-level central neuropathic pain after spinal cord injury. Neurorehabil Neural Repair 26:889-897.

Le T, Leung L, Carroll WL, Schibler KR (1997) Regulation of Interleukin-10 Gene Expression: Possible Mechanisms Accounting for Its Upregulation and for Maturational Differences in Its Expression by Blood Mononuclear Cells. Blood 89:4112-4119.

Ledeboer A, Hutchinson MR, Watkins LR, Johnson KW (2007a) Ibudilast (AV-411). A new class therapeutic candidate for neuropathic pain and opioid withdrawal syndromes. Expert Opin Investig Drugs 16:935-950.

Ledeboer A, Sloane EM, Milligan ED, Frank MG, Mahony JH, Maier SF, Watkins LR (2005) Minocycline attenuates mechanical allodynia and proinflammatory cytokine expression in rat models of pain facilitation. Pain 115:71-83.

Ledeboer A, Jekich BM, Sloane EM, Mahoney JH, Langer SJ, Milligan ED, Martin D, Maier SF, Johnson KW, Leinwand LA, Chavez RA, Watkins LR (2007b) Intrathecal interleukin-10 gene therapy attenuates paclitaxel-induced mechanical allodynia and proinflammatory cytokine expression in dorsal root ganglia in rats. Brain Behav Immun 21:686-698.

Ledeboer A, Jekich BM, Sloane EM, Mahoney JH, Langer SJ, Milligan ED, Martin D, Maier SF, Johnson KW, Leinwand LA, Chavez RA, Watkins LR (2007c) Intrathecal Interleukin-10 Gene Therapy Attenuates Paclitaxel-Induced Mechanical Allodynia and Proinflammatory Cytokine Expression in Dorsal Root Ganglia in Rats. Brain, behavior, and immunity 21:686-698.

Lee JC, Mayer-Proschel M, Rao MS (2000) Gliogenesis in the central nervous system. Glia 30:105-121.

Leppert W (2014) Oxycodone/naloxone in the management of patients with pain and opioid-induced bowel dysfunction. Current drug targets 15:124-135.

Leung L, Cahill CM (2010) TNF-alpha and neuropathic pain--a review. Journal of neuroinflammation 7:27.

Lewis SS, Loram LC, Hutchinson MR, Li C-M, Zhang Y, Maier SF, Huang Y, Rice KC, Watkins LR (2012) (+)-Naloxone, an opioid-inactive toll-like receptor 4 signaling inhibitor, reverses multiple models of chronic neuropathic pain in rats. The Journal of Pain 13:498-506.

Li L, Elliott JF, Mosmann TR (1994) IL-10 inhibits cytokine production, vascular leakage, and swelling during T helper 1 cell-induced delayed-type hypersensitivity. J Immunol 153:3967-3978.

Li Z, Liu H, Yang H, Wang J, Wang H, Zhang K, Ding W, Zheng Z (2014) Both expression of cytokines and posterior annulus fibrosus rupture are essential for pain behavior changes induced by degenerative intervertebral disc: An experimental study in rats. Journal of Orthopaedic Research 32:262-272.

Linehan SA, Martinez-Pomares L, Stahl PD, Gordon S (1999) Mannose receptor and its putative ligands in normal murine lymphoid and nonlymphoid organs: In situ expression of mannose receptor by selected macrophages, endothelial cells,

perivascular microglia, and mesangial cells, but not dendritic cells. J Exp Med 189:1961-1972.
Littlejohn G (2015) Neurogenic neuroinflammation in fibromyalgia and complex regional pain syndrome. Nature Reviews Rheumatology 11:639.
Liu L, Rudin M, Kozlova EN (2000) Glial cell proliferation in the spinal cord after dorsal rhizotomy or sciatic nerve transection in the adult rat. Experimental brain research 131:64-73.
Liu L, Yin Y, Li F, Malhotra C, Cheng J (2017) Flow cytometry analysis of inflammatory cells isolated from the sciatic nerve and DRG after chronic constriction injury in mice. J Neurosci Methods.
Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25:402-408.
Lobo-Silva D, Carriche GM, Castro AG, Roque S, Saraiva M (2016) Balancing the immune response in the brain: IL-10 and its regulation. Journal of neuroinflammation 13:297.
Lopez-Herrera A, Liu Y, Rugeles MT, He JJ (2005) HIV-1 interaction with human mannose receptor (hMR) induces production of matrix metalloproteinase 2 (MMP-2) through hMR-mediated intracellular signaling in astrocytes. Biochim Biophys Acta 1741:55-64.
Luo X, Zuo X, Zhang B, Song L, Wei X, Zhou Y, Xiao X (2008) Release of heat shock protein 70 and the effects of extracellular heat shock protein 70 on the production of IL-10 in fibroblast-like synoviocytes. Cell Stress and Chaperones 13:365-373.
Maeda H, Kuwahara H, Ichimura Y, Ohtsuki M, Kurakata S, Shiraishi A (1995) TGF-beta enhances macrophage ability to produce IL-10 in normal and tumor-bearing mice. J Immunol 155:4926-4932.
Maier SF, Wiertelak EP, Watkins LR (1992) Endogenous pain facilitory systems Antianalgesia and hyperalgesia. APS Journal 1:191-198.
Manji H (2000) Neuropathy in HIV infection. Current opinion in neurology 13:589-592.
Mansfield JR (2014) Multispectral imaging: a review of its technical aspects and applications in anatomic pathology. Vet Pathol 51:185-210.
Mantovani A, Sica A, Sozzani S, Allavena P, Vecchi A, Locati M (2004) The chemokine system in diverse forms of macrophage activation and polarization. Trends in Immunology 25:677-686.
Marchand F, Perretti M, McMahon SB (2005) Role of the immune system in chronic pain. Nat Rev Neurosci 6:521-532.
Marlow GJ, van Gent D, Ferguson LR (2013) Why interleukin-10 supplementation does not work in Crohn's disease patients. World Journal of Gastroenterology : WJG 19:3931-3941.
Martinez-Pomares L (2012) The mannose receptor. J Leukoc Biol 92:1177-1186.
Martinez FO, Gordon S (2014a) The M1 and M2 paradigm of macrophage activation: time for reassessment. F1000Prime Rep 6:13.
Martinez FO, Gordon S (2014b) The M1 and M2 paradigm of macrophage activation: time for reassessment. F1000Prime Reports 6:13.
Martucci C, Trovato AE, Costa B, Borsani E, Franchi S, Magnaghi V, Panerai AE, Rodella LF, Valsecchi AE, Sacerdote P, Colleoni M (2008) The purinergic antagonist PPADS reduces pain related behaviours and interleukin-1 beta, interleukin-6, iNOS

and nNOS overproduction in central and peripheral nervous system after peripheral neuropathy in mice. Pain 137:81-95.
Marzolo MP, von Bernhardi R, Inestrosa NC (1999) Mannose receptor is present in a functional state in rat microglial cells. J Neurosci Res 58:387-395.
Maxwell JR, Denson JL, Joste NE, Robinson S, Jantzie LL (2015) Combined in utero hypoxia-ischemia and lipopolysaccharide administration in rats induces chorioamnionitis and a fetal inflammatory response syndrome. Placenta 36:1378-1384.
McKinstry KK, Strutt TM, Buck A, Curtis JD, Dibble JP, Huston G, Tighe M, Hamada H, Sell S, Dutton RW, Swain SL (2009) IL-10 deficiency unleashes an influenza-specific Th17 response and enhances survival against high-dose challenge. J Immunol 182:7353-7363.
Medzhitov R (2008) Origin and physiological roles of inflammation. Nature 454:428.
Meller ST, Dykstra C, Grzybycki D, Murphy S, Gebhart GF (1994) The possible role of glia in nociceptive processing and hyperalgesia in the spinal cord of the rat. Neuropharmacology 33:1471-1478.
Mellios N, Woodson J, Garcia RI, Crawford B, Sharma J, Sheridan SD, Haggarty SJ, Sur M (2014) beta2-Adrenergic receptor agonist ameliorates phenotypes and corrects microRNA-mediated IGF1 deficits in a mouse model of Rett syndrome. Proc Natl Acad Sci U S A 111:9947-9952.
Mendieta D, la Cruz-Aguilera DLD, Barrera-Villalpando MI, Becerril-Villanueva E, Arreola R, Hernández-Ferreira E, Pérez-Tapia SM, Pérez-Sánchez G, Garcés-Alvarez ME, Aguirre-Cruz L, Velasco-Velázquez MA, Pavón L (2016) IL-8 and IL-6 primarily mediate the inflammatory response in fibromyalgia patients. Journal of Neuroimmunology 290:22-25.
Mika J (2008) Modulation of microglia can attenuate neuropathic pain symptoms and enhance morphine effectiveness. Pharmacol Rep 60:297-307.
Mika J, Zychowska M, Popiolek-Barczyk K, Rojewska E, Przewlocka B (2013) Importance of glial activation in neuropathic pain. Eur J Pharmacol 716:106-119.
Milligan ED, Watkins LR (2009) Pathological and protective roles of glia in chronic pain. Nat Rev Neurosci 10:23-36.
Milligan ED, Penzkover KR, Soderquist RG, Mahoney MJ (2012) Spinal interleukin-10 therapy to treat peripheral neuropathic pain. Neuromodulation 15:520-526; discussion 526.
Milligan ED, Mehmert KK, Hinde JL, Harvey LO, Martin D, Tracey KJ, Maier SF, Watkins LR (2000) Thermal hyperalgesia and mechanical allodynia produced by intrathecal administration of the human immunodeficiency virus-1 (HIV-1) envelope glycoprotein, gp120. Brain Res 861:105-116.
Milligan ED, O'Connor KA, Nguyen KT, Armstrong CB, Twining C, Gaykema RP, Holguin A, Martin D, Maier SF, Watkins LR (2001) Intrathecal HIV-1 envelope glycoprotein gp120 induces enhanced pain states mediated by spinal cord proinflammatory cytokines. J Neurosci 21:2808-2819.
Milligan ED, Soderquist RG, Malone SM, Mahoney JH, Hughes TS, Langer SJ, Sloane EM, Maier SF, Leinwand LA, Watkins LR, Mahoney MJ (2006a) Intrathecal polymer-based interleukin-10 gene delivery for neuropathic pain. Neuron Glia Biol 2:293-308.

Milligan ED, Langer SJ, Sloane EM, He L, Wieseler-Frank J, O'Connor K, Martin D, Forsayeth JR, Maier SF, Johnson K, Chavez RA, Leinwand LA, Watkins LR (2005a) Controlling pathological pain by adenovirally driven spinal production of the anti-inflammatory cytokine, interleukin-10. Eur J Neurosci 21:2136-2148.

Milligan ED, Sloane EM, Langer SJ, Cruz PE, Chacur M, Spataro L, Wieseler-Frank J, Hammack SE, Maier SF, Flotte TR, Forsayeth JR, Leinwand LA, Chavez R, Watkins LR (2005b) Controlling neuropathic pain by adeno-associated virus driven production of the anti-inflammatory cytokine, interleukin-10. Mol Pain 1:9.

Milligan ED, Sloane EM, Langer SJ, Hughes TS, Jekich BM, Frank MG, Mahoney JH, Levkoff LH, Maier SF, Cruz PE, Flotte TR, Johnson KW, Mahoney MM, Chavez RA, Leinwand LA, Watkins LR (2006b) Repeated intrathecal injections of plasmid DNA encoding interleukin-10 produce prolonged reversal of neuropathic pain. Pain 126:294-308.

Mills KHG (2004) Regulatory T cells: friend or foe in immunity to infection? Nature Reviews Immunology 4:841.

Moore KW, de Waal Malefyt R, Coffman RL, O'Garra A (2001) Interleukin-10 and the interleukin-10 receptor. Annu Rev Immunol 19:683-765.

Moore KW, Vieira P, Fiorentino DF, Trounstine ML, Khan TA, Mosmann TR (1990) Homology of cytokine synthesis inhibitory factor (IL-10) to the Epstein-Barr virus gene BCRFI. Science 248:1230-1234.

Moran CJ, Walters TD, Guo CH, Kugathasan S, Klein C, Turner D, Wolters VM, Bandsma RH, Mouzaki M, Zachos M, Langer JC, Cutz E, Benseler SM, Roifman CM, Silverberg MS, Griffiths AM, Snapper SB, Muise AM (2013) IL-10R polymorphisms are associated with very-early-onset ulcerative colitis. Inflamm Bowel Dis 19:115-123.

Mosser DM, Zhang X (2008) Interleukin-10: new perspectives on an old cytokine. Immunological reviews 226:205-218.

Murray PJ (2005) The primary mechanism of the IL-10-regulated antiinflammatory response is to selectively inhibit transcription. Proc Natl Acad Sci U S A 102:8686-8691.

Nakos G, Malamou-Mitsi VD, Lachana A, Karassavoglou A, Kitsiouli E, Agnandi N, Lekka ME (2002) Immunoparalysis in patients with severe trauma and the effect of inhaled interferon-gamma. Crit Care Med 30:1488-1494.

Nash TP (2005) What use is pain? Br J Anaesth 94:146-149.

Nigou J, Zelle-Rieser C, Gilleron M, Thurnher M, Puzo G (2001) Mannosylated lipoarabinomannans inhibit IL-12 production by human dendritic cells: evidence for a negative signal delivered through the mannose receptor. J Immunol 166:7477-7485.

Nimmerjahn A, Kirchhoff F, Helmchen F (2005) Resting microglial cells are highly dynamic surveillants of brain parenchyma in vivo. Science 308:1314-1318.

Nitzan-Luques A, Devor M, Tal M (2011) Genotype-selective phenotypic switch in primary afferent neurons contributes to neuropathic pain. PAIN® 152:2413-2426.

Noor S, Sanchez JJ, Vanderwall AG, Sun MS, Maxwell JR, Davies S, Jantzie LL, Petersen TR, Savage DD, Milligan ED (2017) Prenatal alcohol exposure potentiates chronic neuropathic pain, spinal glial and immune cell activation and alters sciatic nerve and DRG cytokine levels. Brain Behav Immun 61:80-95.

Norden DM, Fenn AM, Dugan A, Godbout JP (2014) TGFbeta produced by IL-10 redirected astrocytes attenuates microglial activation. Glia 62:881-895.
Nzoumbou-Boko R, De Muylder G, Semballa S, Lecordier L, Dauchy FA, Gobert AP, Holzmuller P, Lemesre JL, Bras-Goncalves R, Barnabe C, Courtois P, Daulouede S, Beschin A, Pays E, Vincendeau P (2017) Trypanosoma musculi Infection in Mice Critically Relies on Mannose Receptor-Mediated Arginase Induction by a TbKHC1 Kinesin H Chain Homolog. J Immunol 199:1762-1771.
Oh SB, Tran PB, Gillard SE, Hurley RW, Hammond DL, Miller RJ (2001) Chemokines and Glycoprotein120 Produce Pain Hypersensitivity by Directly Exciting Primary Nociceptive Neurons. The Journal of Neuroscience 21:5027-5035.
Ohtori S, Inoue G, Miyagi M, Takahashi K (2015) Pathomechanisms of discogenic low back pain in humans and animal models. The Spine Journal 15:1347-1355.
Okamoto K, Martin DP, Schmelzer JD, Mitsui Y, Low PA (2001) Pro- and anti-inflammatory cytokine gene expression in rat sciatic nerve chronic constriction injury model of neuropathic pain. Exp Neurol 169:386-391.
Oliveira AV, Rosa da Costa AM, Silva GA (2017) Non-viral strategies for ocular gene delivery. Materials science & engineering C, Materials for biological applications 77:1275-1289.
Otter M, Barrett-Bergshoeff MM, Rijken DC (1991) Binding of tissue-type plasminogen activator by the mannose receptor. Journal of Biological Chemistry 266:13931-13935.
Palomares O, Martín-Fontecha M, Lauener R, Traidl-Hoffmann C, Cavkaytar O, Akdis M, Akdis CA (2014) Regulatory T cells and immune regulation of allergic diseases: roles of IL-10 and TGF-β. Genes And Immunity 15:511.
Pardo CA, McArthur JC, Griffin JW (2001) HIV neuropathy: Insights in the pathology of HIV peripheral nerve disease. Journal of the Peripheral Nervous System 6:21-27.
Parker K, Sinha P, Horn LA, Clements VK, Yang H, Li J, Tracey KJ, Ostrand-Rosenberg S (2014) HMGB1 enhances immune suppression by facilitating the differentiation and suppressive activity of myeloid-derived suppressor cells. Cancer research 74:5723-5733.
Pathak SK, Basu S, Bhattacharyya A, Pathak S, Kundu M, Basu J (2005) Mycobacterium tuberculosis lipoarabinomannan-mediated IRAK-M induction negatively regulates Toll-like receptor-dependent interleukin-12 p40 production in macrophages. J Biol Chem 280:42794-42800.
Patrick N, Emanski E, Knaub MA (2014) Acute and chronic low back pain. The Medical clinics of North America 98:777-789, xii.
Peters CM, Jimenez-Andrade JM, Kuskowski MA, Ghilardi JR, Mantyh PW (2007) AN EVOLVING CELLULAR PATHOLOGY OCCURS IN DORSAL ROOT GANGLIA, PERIPHERAL NERVE AND SPINAL CORD FOLLOWING INTRAVENOUS ADMINISTRATION OF PACLITAXEL IN THE RAT. Brain research 1168:46-59.
Porreca F, Ossipov MH, Gebhart GF (2002) Chronic pain and medullary descending facilitation. Trends in Neurosciences 25:319-325.
Porru D, Parmigiani A, Tinelli C, Barletta D, Choussos D, Franco CD, Bobbi V, Bassi S, Miller O, Gardella B, Nappi R, Spinillo A, Rovereto B (2014) Oral D-mannose in

recurrent urinary tract infections in women: a pilot study. Journal of Clinical Urology 7:208-213.
Powell MJ, Thompson SA, Tone Y, Waldmann H, Tone M (2000) Posttranscriptional regulation of IL-10 gene expression through sequences in the 3'-untranslated region. J Immunol 165:292-296.
Raghavendra V, Rutkowski MD, DeLeo JA (2002) The Role of Spinal Neuroimmune Activation in Morphine Tolerance/Hyperalgesia in Neuropathic and Sham-Operated Rats. The Journal of Neuroscience 22:9980-9989.
Raghavendra V, Tanga FY, DeLeo JA (2003) Attenuation of Morphine Tolerance, Withdrawal-Induced Hyperalgesia, and Associated Spinal Inflammatory Immune Responses by Propentofylline in Rats. Neuropsychopharmacology 29:327.
Ransohoff RM (2016) A polarizing question: do M1 and M2 microglia exist? Nat Neurosci 19:987-991.
Rider P, Carmi Y, Cohen I (2016) Biologics for Targeting Inflammatory Cytokines, Clinical Uses, and Limitations. International Journal of Cell Biology 2016:9259646.
Robert S, Steidler L (2014) Recombinant Lactococcus lactis can make the difference in antigen-specific immune tolerance induction, the Type 1 Diabetes case. Microbial Cell Factories 13:S11-S11.
Robinson S, Winer JL, Berkner J, Chan LAS, Denson JL, Maxwell JR, Yang Y, Sillerud LO, Tasker RC, Meehan WP, Mannix R, Jantzie LL (2016) Imaging and serum biomarkers reflecting the functional efficacy of extended erythropoietin treatment in rats following infantile traumatic brain injury. Journal of neurosurgery Pediatrics 17:739-755.
Romero-Sandoval EA, Horvath RJ, DeLeo JA (2008) Neuroimmune interactions and pain: focus on glial-modulating targets. Curr Opin Investig Drugs 9:726-734.
Rothwell NJ, Luheshi G (1994) Pharmacology of interleukin-1 actions in the brain. Adv Pharmacol 25:1-20.
Rowitch DH, Kriegstein AR (2010) Developmental genetics of vertebrate glial-cell specification. Nature 468:214-222.
Rubin DC, Shaker A, Levin MS (2012) Chronic intestinal inflammation: inflammatory bowel disease and colitis-associated colon cancer. Frontiers in immunology 3:107.
Rughetti A, Pellicciotta I, Biffoni M, Backstrom M, Link T, Bennet EP, Clausen H, Noll T, Hansson GC, Burchell JM, Frati L, Taylor-Papadimitriou J, Nuti M (2005) Recombinant tumor-associated MUC1 glycoprotein impairs the differentiation and function of dendritic cells. J Immunol 174:7764-7772.
Sabat R, Grutz G, Warszawska K, Kirsch S, Witte E, Wolk K, Geginat J (2010) Biology of interleukin-10. Cytokine Growth Factor Rev 21:331-344.
Safinia N, Scotta C, Vaikunthanathan T, Lechler RI, Lombardi G (2015) Regulatory T Cells: Serious Contenders in the Promise for Immunological Tolerance in Transplantation. Frontiers in immunology 6.
Sanjabi S, Zenewicz LA, Kamanaka M, Flavell RA (2009) Anti- and Pro-inflammatory Roles of TGF-β, IL-10, and IL-22 In Immunity and Autoimmunity. Current opinion in pharmacology 9:447-453.
Sapunar D, Kostic S, Banozic A, Puljak L (2012) Dorsal root ganglion – a potential new therapeutic target for neuropathic pain. Journal of Pain Research 5:31-38.

Saraiva M, O'Garra A (2010) The regulation of IL-10 production by immune cells. Nat Rev Immunol 10:170-181.
Sartor RB (1997) Pathogenesis and immune mechanisms of chronic inflammatory bowel diseases. Am J Gastroenterol 92:5S-11S.
Sasaki N, Kikuchi S-i, Konno S-i, Sekiguchi M, Watanabe K (2007) Anti-TNF-α Antibody Reduces Pain-Behavioral Changes Induced by Epidural Application of Nucleus Pulposus in a Rat Model Depending on the Timing of Administration. Spine 32:413-416.
Satoh J, Kino Y, Asahina N, Takitani M, Miyoshi J, Ishida T, Saito Y (2016) TMEM119 marks a subset of microglia in the human brain. Neuropathology 36:39-49.
Saulino M, Kim PS, Shaw E (2014) Practical considerations and patient selection for intrathecal drug delivery in the management of chronic pain. Journal of Pain Research 7:627-638.
Saxena A, Khosraviani S, Noel S, Mohan D, Donner T, Hamad AR (2015) Interleukin-10 paradox: A potent immunoregulatory cytokine that has been difficult to harness for immunotherapy. Cytokine 74:27-34.
Schmittgen TD, Livak KJ (2008) Analyzing real-time PCR data by the comparative C(T) method. Nat Protoc 3:1101-1108.
Schoeniger-Skinner DK, Ledeboer A, Frank MG, Milligan ED, Poole S, Martin D, Maier SF, Watkins LR (2007) Interleukin-6 mediates low-threshold mechanical allodynia induced by intrathecal HIV-1 envelope glycoprotein gp120. Brain, behavior, and immunity 21:660-667.
Scholz J, Woolf CJ (2007) The neuropathic pain triad: neurons, immune cells and glia. Nat Neurosci 10:1361-1368.
Schottelius AJG, Mayo MW, Sartor RB, Baldwin AS (1999) Interleukin-10 Signaling Blocks Inhibitor of κB Kinase Activity and Nuclear Factor κB DNA Binding. Journal of Biological Chemistry 274:31868-31874.
Schouenborg J, Sjolund BH (1983) Activity evoked by A- and C-afferent fibers in rat dorsal horn neurons and its relation to a flexion reflex. Journal of Neurophysiology 50:1108-1121.
Schreiber RC, Krivacic K, Kirby B, Vaccariello SA, Wei T, Ransohoff RM, Zigmond RE (2001) Monocyte chemoattractant protein (MCP)-1 is rapidly expressed by sympathetic ganglion neurons following axonal injury. Neuroreport 12:601-606.
Schreiber S, Heinig T, Thiele H-G, Raedler A (1995) Immunoregulatory role of interleukin 10 in patients with inflammatory bowel disease. Gastroenterology 108:1434-1444.
Schuetze N, Schoeneberger S, Mueller U, Freudenberg MA, Alber G, Straubinger RK (2005) IL-12 family members: differential kinetics of their TLR4-mediated induction by Salmonella enteritidis and the impact of IL-10 in bone marrow-derived macrophages. Int Immunol 17:649-659.
Sedaghat B, Stephenson R, Toth I (2014) Targeting the mannose receptor with mannosylated subunit vaccines. Curr Med Chem 21:3405-3418.
Sellon RK, Tonkonogy S, Schultz M, Dieleman LA, Grenther W, Balish E, Rennick DM, Sartor RB (1998) Resident enteric bacteria are necessary for development of spontaneous colitis and immune system activation in interleukin-10-deficient mice. Infect Immun 66:5224-5231.

Shen KF, Zhu HQ, Wei XH, Wang J, Li YY, Pang RP, Liu XG (2013) Interleukin-10 down-regulates voltage gated sodium channels in rat dorsal root ganglion neurons. Exp Neurol 247:466-475.

Shepherd VL, Abdolrasulnia R, Garrett M, Cowan HB (1990) Down-regulation of mannose receptor activity in macrophages after treatment with lipopolysaccharide and phorbol esters. The Journal of Immunology 145:1530-1536.

Shibata Y, Metzger WJ, Myrvik QN (1997) Chitin particle-induced cell-mediated immunity is inhibited by soluble mannan: mannose receptor-mediated phagocytosis initiates IL-12 production. J Immunol 159:2462-2467.

Shimada K, Takimoto H, Yano I, Kumazawa Y (2006) Involvement of mannose receptor in glycopeptidolipid-mediated inhibition of phagosome-lysosome fusion. Microbiol Immunol 50:243-251.

Shimoyama M, Tanaka K, Hasue F, Shimoyama N (2002) A mouse model of neuropathic cancer pain. Pain 99:167-174.

Shouval DS, Ouahed J, Biswas A, Goettel JA, Horwitz BH, Klein C, Muise AM, Snapper SB (2014) Interleukin 10 receptor signaling: master regulator of intestinal mucosal homeostasis in mice and humans. Adv Immunol 122:177-210.

Siqueira Mietto B, Kroner A, Girolami EI, Santos-Nogueira E, Zhang J, David S (2015) Role of IL-10 in Resolution of Inflammation and Functional Recovery after Peripheral Nerve Injury. J Neurosci 35:16431-16442.

Skogseid IM (2014) Dystonia – new advances in classification, genetics, pathophysiology and treatment. Acta Neurologica Scandinavica 129:13-19.

Slade GD, Conrad MS, Diatchenko L, Rashid NU, Zhong S, Smith S, Rhodes J, Medvedev A, Makarov S, Maixner W, Nackley AG (2011) Cytokine biomarkers and chronic pain: Association of genes, transcription, and circulating proteins with temporomandibular disorders and widespread palpation tenderness. PAIN 152:2802-2812.

Slivac I, Guay D, Mangion M, Champeil J, Gaillet B (2017) Non-viral nucleic acid delivery methods. Expert Opin Biol Ther 17:105-118.

Sloane E, Ledeboer A, Seibert W, Coats B, van Strien M, Maier SF, Johnson KW, Chavez R, Watkins LR, Leinwand L, Milligan ED, Van Dam AM (2009a) Anti-inflammatory cytokine gene therapy decreases sensory and motor dysfunction in experimental Multiple Sclerosis: MOG-EAE behavioral and anatomical symptom treatment with cytokine gene therapy. Brain Behav Immun 23:92-100.

Sloane E, Langer S, Jekich B, Mahoney J, Hughes T, Frank M, Seibert W, Huberty G, Coats B, Harrison J, Klinman D, Poole S, Maier S, Johnson K, Chavez R, Watkins LR, Leinwand L, Milligan E (2009b) Immunological priming potentiates non-viral anti-inflammatory gene therapy treatment of neuropathic pain. Gene Ther 16:1210-1222.

Sluka KA, Clauw DJ (2016) Neurobiology of fibromyalgia and chronic widespread pain. Neuroscience 338:114-129.

Soderquist RG, Milligan ED, Harrison JA, Chavez RA, Johnson KW, Watkins LR, Mahoney MJ (2010a) PEGylation of interleukin-10 for the mitigation of enhanced pain states. J Biomed Mater Res A 93:1169-1179.

Soderquist RG, Milligan ED, Sloane EM, Harrison JA, Douvas KK, Potter JM, Hughes TS, Chavez RA, Johnson K, Watkins LR, Mahoney MJ (2009) PEGylation of

brain-derived neurotrophic factor for preserved biological activity and enhanced spinal cord distribution. J Biomed Mater Res A 91:719-729.
Soderquist RG, Sloane EM, Loram LC, Harrison JA, Dengler EC, Johnson SM, Amer LD, Young CS, Lewis MT, Poole S, Frank MG, Watkins LR, Milligan ED, Mahoney MJ (2010b) Release of plasmid DNA-encoding IL-10 from PLGA microparticles facilitates long-term reversal of neuropathic pain following a single intrathecal administration. Pharm Res 27:841-854.
Sommer C, Schafers M (1998) Painful mononeuropathy in C57BL/Wld mice with delayed wallerian degeneration: differential effects of cytokine production and nerve regeneration on thermal and mechanical hypersensitivity. Brain Res 784:154-162.
Song P, Zhao Z-Q (2001) The involvement of glial cells in the development of morphine tolerance. Neuroscience Research 39:281-286.
Spataro LE, Sloane EM, Milligan ED, Wieseler-Frank J, Schoeniger D, Jekich BM, Barrientos RM, Maier SF, Watkins LR (2004) Spinal gap junctions: potential involvement in pain facilitation. J Pain 5:392-405.
Spencer SD, Di Marco F, Hooley J, Pitts-Meek S, Bauer M, Ryan AM, Sordat B, Gibbs VC, Aguet M (1998) The orphan receptor CRF2-4 is an essential subunit of the interleukin 10 receptor. J Exp Med 187:571-578.
Standring S (2016a) Pectoral girdle and upper limb: Overview and surface anatomy. In: Gray's Anatomy: The Anatomical Basis of Clinical Practice, Forty-first edition. Edition (Standring S, ed), pp 774-793.e771. New York: Elsevier Limited.
Standring S (2016b) Brainstem. In: Gray's Anatomy: The Anatomical Basis of Clinical Practice, Forty-first edition. Edition (Standring S, ed), pp 309-330.e301. New York: Elsevier Limited.
Stansley B, Post J, Hensley K (2012) A comparative review of cell culture systems for the study of microglial biology in Alzheimer’s disease. Journal of neuroinflammation 9:115-115.
Steidler L, Hans W, Schotte L, Neirynck S, Obermeier F, Falk W, Fiers W, Remaut E (2000) Treatment of murine colitis by Lactococcus lactis secreting interleukin-10. Science 289:1352-1355.
Stewart JP, Behm FG, Arrand JR, Rooney CM (1994) Differential expression of viral and human interleukin-10 (IL-10) by primary B cell tumors and B cell lines. Virology 200:724-732.
Strong JA, Xie W, Bataille FJ, Zhang J-M (2013) Preclinical studies of low back pain. Molecular Pain 9:17-17.
Sturgill J, McGee E, Menzies V (2014) Unique Cytokine Signature in the Plasma of Patients with Fibromyalgia. Journal of Immunology Research 2014:5.
Sweitzer S, De Leo J (2011) Propentofylline: glial modulation, neuroprotection, and alleviation of chronic pain. Handb Exp Pharmacol:235-250.
Sweitzer SM, Colburn RW, Rutkowski M, DeLeo JA (1999) Acute peripheral inflammation induces moderate glial activation and spinal IL-1beta expression that correlates with pain behavior in the rat. Brain Res 829:209-221.
Tachado SD, Zhang J, Zhu J, Patel N, Cushion M, Koziel H (2007) Pneumocystis-mediated IL-8 release by macrophages requires coexpression of mannose receptors and TLR2. J Leukoc Biol 81:205-211.

Takahashi K, Donovan MJ, Rogers RA, Ezekowitz RA (1998) Distribution of murine mannose receptor expression from early embryogenesis through to adulthood. Cell Tissue Res 292:311-323.

Tanga FY, Nutile-McMenemy N, DeLeo JA (2005) The CNS role of Toll-like receptor 4 in innate neuroimmunity and painful neuropathy. Proceedings of the National Academy of Sciences of the United States of America 102:5856-5861.

Taylor PR, Gordon S, Martinez-Pomares L (2005) The mannose receptor: linking homeostasis and immunity through sugar recognition. Trends Immunol 26:104-110.

Tracey I, Mantyh PW (2007) The cerebral signature for pain perception and its modulation. Neuron 55:377-391.

Tu H, Juelich T, Smith EM, Tyring SK, Rady PL, Hughes TK, Jr. (2003) Evidence for endogenous interleukin-10 during nociception. J Neuroimmunol 139:145-149.

Uceyler N, Tscharke A, Sommer C (2007) Early cytokine expression in mouse sciatic nerve after chronic constriction nerve injury depends on calpain. Brain Behav Immun 21:553-560.

Üçeyler N, Häuser W, Sommer C (2011) Systematic review with meta-analysis: cytokines in fibromyalgia syndrome. BMC Musculoskeletal Disorders 12:245-245.

Üçeyler N, Valenza R, Stock M, Schedel R, Sprotte G, Sommer C (2006) Reduced levels of antiinflammatory cytokines in patients with chronic widespread pain. Arthritis & Rheumatism 54:2656-2664.

Ulland TK, Wang Y, Colonna M (2015) Regulation of microglial survival and proliferation in health and diseases. Semin Immunol 27:410-415.

van den Beuken-van Everdingen MHJ, Hochstenbach LMJ, Joosten EAJ, Tjan-Heijnen VCG, Janssen DJA (2016) Update on Prevalence of Pain in Patients With Cancer: Systematic Review and Meta-Analysis. Journal of Pain and Symptom Management 51:1070-1090.e1079.

Vanderwall AG, Noor S, Sun MS, Sanchez JE, Yang XO, Jantzie LL, Mellios N, Milligan ED (2018) Effects of spinal non-viral interleukin-10 gene therapy formulated with d-mannose in neuropathic interleukin-10 deficient mice: Behavioral characterization, mRNA and protein analysis in pain relevant tissues. Brain Behav Immun 69:91-112.

Vera DR, Wallace AM, Hoh CK, Mattrey RF (2001) A synthetic macromolecule for sentinel node detection: (99m)Tc-DTPA-mannosyl-dextran. J Nucl Med 42:951-959.

Verma S, Estanislao L, Simpson D (2005) HIV-associated neuropathic pain: epidemiology, pathophysiology and management. CNS Drugs 19:325-334.

Vogel SN, Perera PY, Hogan MM, Majde JA (1988) Use of serum-free, compositionally defined medium for analysis of macrophage differentiation in vitro. J Leukoc Biol 44:136-142.

Wachstein J, Tischer S, Figueiredo C, Limbourg A, Falk C, Immenschuh S, Blasczyk R, Eiz-Vesper B (2012) HSP70 Enhances Immunosuppressive Function of CD4+CD25+FoxP3+ T Regulatory Cells and Cytotoxicity in CD4+CD25− T Cells. PLOS ONE 7:e51747.

Wang C, Vlahov D, Galai N, Bareta J, Strathdee SA, Nelson KE, Sterling TR (2004) Mortality in HIV-seropositive versus -seronegative persons in the era of highly

active antiretroviral therapy: implications for when to initiate therapy. The Journal of infectious diseases 190:1046-1054.
Wang X, Zhang Y, Peng Y, Hutchinson MR, Rice KC, Yin H, Watkins LR (2016) Pharmacological characterization of the opioid inactive isomers (+)-naltrexone and (+)-naloxone as antagonists of toll-like receptor 4. British Journal of Pharmacology 173:856-869.
Ward JM, Anver MR, Haines DC, Melhorn JM, Gorelick P, Yan L, Fox JG (1996) Inflammatory large bowel disease in immunodeficient mice naturally infected with Helicobacter hepaticus. Lab Anim Sci 46:15-20.
Warwick RA, Hanani M (2013) The contribution of satellite glial cells to chemotherapy-induced neuropathic pain. European Journal of Pain 17:571-580.
Watkins LR, Maier SF (2000) The pain of being sick: implications of immune-to-brain communication for understanding pain. Annual review of psychology 51:29-57.
Watkins LR, Maier SF (2002) Beyond neurons: evidence that immune and glial cells contribute to pathological pain states. Physiological reviews 82:981-1011.
Watkins LR, Maier SF (2003) GLIA: A novel drug discovery target for clinical pain. Nature Reviews Drug Discovery 2:973.
Watkins LR, Maier SF (2005) Immune regulation of central nervous system functions: from sickness responses to pathological pain. Journal of internal medicine 257:139-155.
Watkins LR, Milligan ED, Maier SF (2001a) Glial activation: a driving force for pathological pain. Trends Neurosci 24:450-455.
Watkins LR, Milligan ED, Maier SF (2001b) Spinal cord glia: new players in pain. Pain 93:201-205.
Watkins LR, Martin D, Ulrich P, Tracey KJ, Maier SF (1997) Evidence for the involvement of spinal cord glia in subcutaneous formalin induced hyperalgesia in the rat. Pain 71:225-235.
Watkins LR, Hutchinson MR, Ledeboer A, Wieseler-Frank J, Milligan ED, Maier SF (2007a) Glia as the “bad guys”: Implications for improving clinical pain control and the clinical utility of opioids. Brain, behavior, and immunity 21:131-146.
Watkins LR, Wieseler-Frank J, Hutchinson MR, Ledeboer A, Spataro L, Milligan ED, Sloane EM, Maier SF (2007b) Neuroimmune Interactions and Pain: The Role of Immune and Glial Cells. In: Psychoneuroimmunology (Fourth Edition) (Ader R, ed), pp 393-414. Burlington: Academic Press.
Wieten L, Berlo SE, ten Brink CB, van Kooten PJ, Singh M, van der Zee R, Glant TT, Broere F, van Eden W (2009) IL-10 Is Critically Involved in Mycobacterial HSP70 Induced Suppression of Proteoglycan-Induced Arthritis. PLOS ONE 4:e4186.
Wilkerson JL, Gentry KR, Dengler EC, Wallace JA, Kerwin AA, Armijo LM, Kuhn MN, Thakur GA, Makriyannis A, Milligan ED (2012a) Intrathecal cannabilactone CB(2)R agonist, AM1710, controls pathological pain and restores basal cytokine levels. Pain 153:1091-1106.
Wilkerson JL, Gentry KR, Dengler EC, Wallace JA, Kerwin AA, Kuhn MN, Zvonok AM, Thakur GA, Makriyannis A, Milligan ED (2012b) Immunofluorescent spectral analysis reveals the intrathecal cannabinoid agonist, AM1241, produces spinal anti-inflammatory cytokine responses in neuropathic rats exhibiting relief from allodynia. Brain Behav 2:155-177.

Wood MJA, Charlton HM, Wood KJ, Kajiwara K, Byrnes AP (1996) Immune responses to adenovirus vectors in the nervous system. Trends in Neurosciences 19:497-501.
Xu X, Xie Q, Shen Y, Lu G, Yao H, Chen Y, Zhou J (2010) Involvement of mannose receptor in the preventive effects of mannose in lipopolysaccharide-induced acute lung injury. Eur J Pharmacol 641:229-237.
Xu XL, Xie QM, Shen YH, Jiang JJ, Chen YY, Yao HY, Zhou JY (2008) Mannose prevents lipopolysaccharide-induced acute lung injury in rats. Inflamm Res 57:104-110.
Xu XL, Zhang P, Shen YH, Li HQ, Wang YH, Lu GH, Zhou JY (2015) Mannose prevents acute lung injury through mannose receptor pathway and contributes to regulate PPARgamma and TGF-beta1 level. Int J Clin Exp Pathol 8:6214-6224.
Yamaguchi T, Katoh I, Kurata S-i (2002) Azidothymidine causes functional and structural destruction of mitochondria, glutathione deficiency and HIV-1 promoter sensitization. European journal of biochemistry 269:2782-2788.
Yang S, Gao L, Lu F, Wang B, Gao F, Zhu G, Cai Z, Lai J, Yang Q (2015) Transcription factor myocyte enhancer factor 2D regulates interleukin-10 production in microglia to protect neuronal cells from inflammation-induced death. Journal of neuroinflammation 12:33.
Yao MZ, Wang JH, Gu JF, Sun LY, Liu H, Zhao ZQ, Liu XY (2002) Interleukin-2 gene has superior antinociceptive effects when delivered intrathecally. Neuroreport 13:791-794.
Yaqub F (2015) Pain in the USA: states of suffering. Lancet 386:839.
Yoon S-Y, Robinson CR, Zhang H, Dougherty PM (2013) Spinal astrocyte gap junctions contribute to oxaliplatin-induced mechanical hypersensitivity. The journal of pain : official journal of the American Pain Society 14:205-214.
Yoshimura A, Naka T, Kubo M (2007) SOCS proteins, cytokine signalling and immune regulation. Nat Rev Immunol 7:454-465.
Yue FY, Dummer R, Geertsen R, Hofbauer G, Laine E, Manolio S, Burg G (1997) Interleukin-10 is a growth factor for human melanoma cells and down-regulates HLA class-I, HLA class-II and ICAM-1 molecules. Int J Cancer 71:630-637.
Zarpelon AC, Rodrigues FC, Lopes AH, Souza GR, Carvalho TT, Pinto LG, Xu D, Ferreira SH, Alves-Filho JC, McInnes IB, Ryffel B, Quesniaux VFJ, Reverchon F, Mortaud S, Menuet A, Liew FY, Cunha FQ, Cunha TM, Verri WA (2016) Spinal cord oligodendrocyte-derived alarmin IL-33 mediates neuropathic pain. The FASEB Journal 30:54-65.
Zhang D, Chia C, Jiao X, Jin W, Kasagi S, Wu R, Konkel JE, Nakatsukasa H, Zanvit P, Goldberg N, Chen Q, Sun L, Chen Z-J, Chen W (2017) D-mannose induces regulatory T cells and suppresses immunopathology. Nat Med 23:1036-1045.
Zhang H, Boyette-Davis JA, Kosturakis AK, Li Y, Yoon S-Y, Walters ET, Dougherty PM (2013) Induction of monocyte chemoattractant protein-1 (MCP-1) and its receptor CCR2 in primary sensory neurons contributes to paclitaxel-induced peripheral neuropathy. The journal of pain : official journal of the American Pain Society 14:1031-1044.
Zhang H, Li Y, de Carvalho-Barbosa M, Kavelaars A, Heijnen CJ, Albrecht PJ, Dougherty PM (2016) Dorsal root ganglion infiltration by macrophages contributes to

paclitaxel chemotherapy induced peripheral neuropathy. The journal of pain : official journal of the American Pain Society 17:775-786.
Zhang J, Zhu J, Imrich A, Cushion M, Kinane TB, Koziel H (2004) Pneumocystis activates human alveolar macrophage NF-kappaB signaling through mannose receptors. Infect Immun 72:3147-3160.
Zhang J, Tachado SD, Patel N, Zhu J, Imrich A, Manfruelli P, Cushion M, Kinane TB, Koziel H (2005) Negative regulatory role of mannose receptors on human alveolar macrophage proinflammatory cytokine release in vitro. J Leukoc Biol 78:665-674.
Zhang X, Goncalves R, Mosser DM (2008) The Isolation and Characterization of Murine Macrophages. Current protocols in immunology / edited by John E Coligan [et al] CHAPTER:Unit-14.11.
Zheng W, Huang W, Liu S, Levitt RC, Candiotti KA, Lubarsky DA, Hao S (2014a) IL-10 mediated by herpes simplex virus vector reduces neuropathic pain induced by HIV gp120 combined with ddC in rats. Mol Pain 10:49.
Zheng W, Huang W, Liu S, Levitt RC, Candiotti KA, Lubarsky DA, Hao S (2014b) Interleukin 10 mediated by herpes simplex virus vectors suppresses neuropathic pain induced by human immunodeficiency virus gp120 in rats. Anesth Analg 119:693-701.
Zheng X, Ouyang H, Liu S, Mata M, Fink DJ, Hao S (2011) TNF α is involved in neuropathic pain induced by nucleoside reverse transcriptase inhibitor in rats. Brain, behavior, and immunity 25:1668-1676.
Zhou Y, Do DC, Ishmael FT, Squadrito ML, Tang HM, Tang HL, Hsu MH, Qiu L, Li C, Zhang Y, Becker KG, Wan M, Huang SK, Gao P (2017) Mannose receptor modulates macrophage polarization and allergic inflammation through miR-511-3p. J Allergy Clin Immunol.
Zhuo M, Wu G, Wu L-J (2011) Neuronal and microglial mechanisms of neuropathic pain. Molecular Brain 4:31-31.
Zrzavy T, Hametner S, Wimmer I, Butovsky O, Weiner HL, Lassmann H (2017) Loss of 'homeostatic' microglia and patterns of their activation in active multiple sclerosis. Brain.