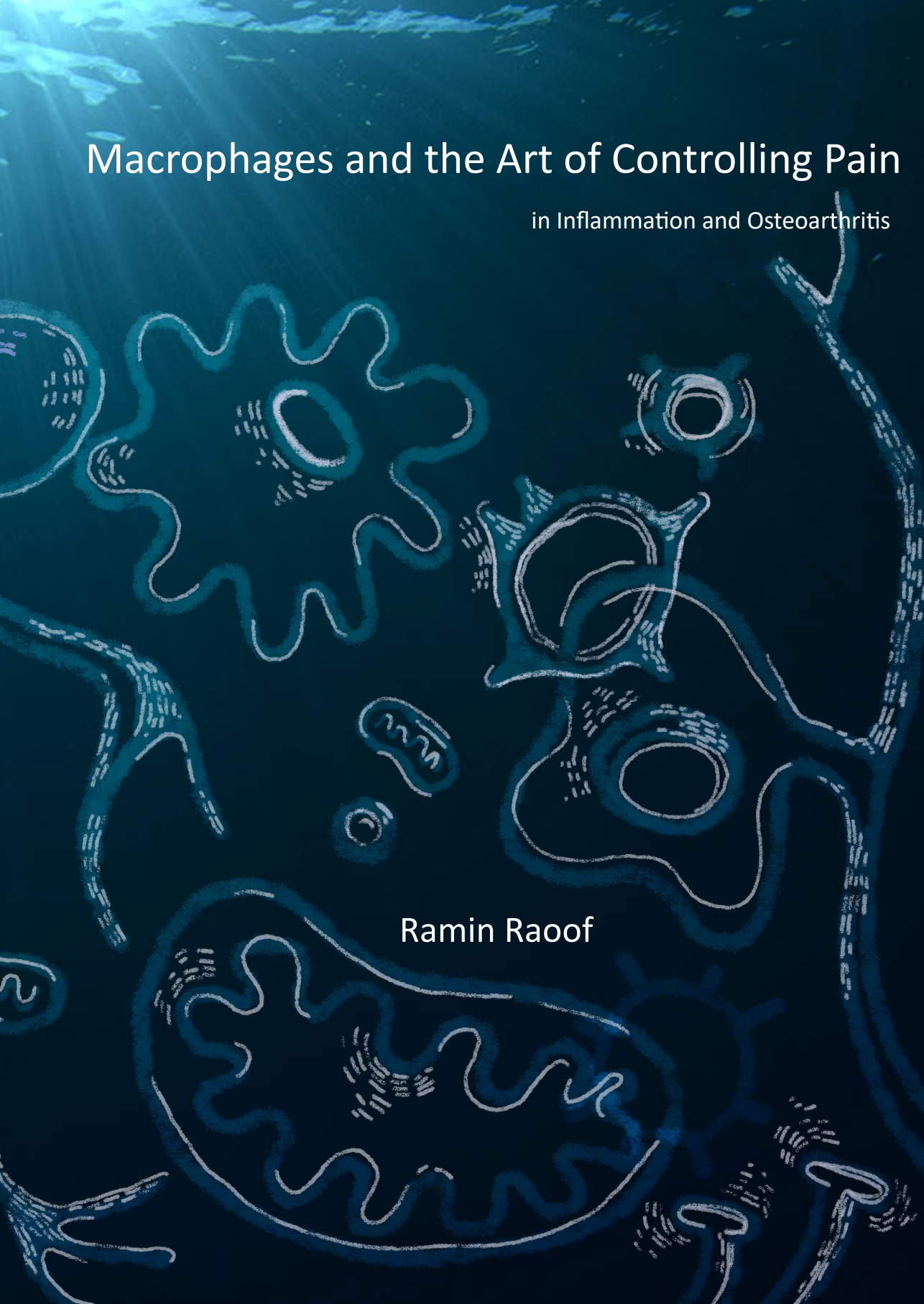
Macrophages and the Art of Controlling Pain

in Inflammation and Osteoarthritis



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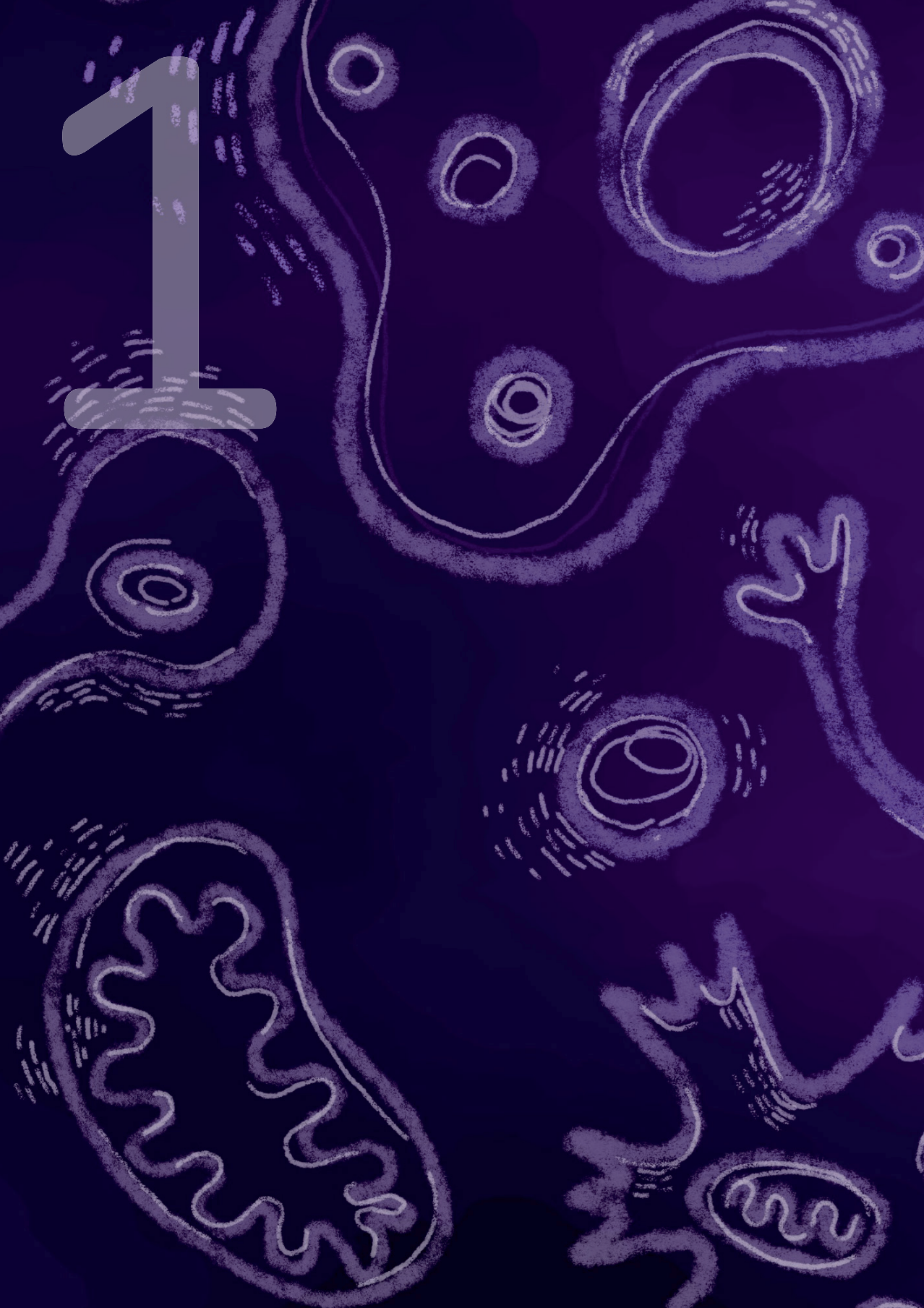
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CHAPTER 1

General Introduction

Pain

Pain is defined by the The International Association for the Study of Pain (IASP) as 'An unpleasant sensory and emotional experience associated with, or resembling that associated with, actual or potential tissue damage'[1]. The World Health Organisation (WHO), recognizes pain as an important global, public health concern, that there are urgent needs for improved, standardized management of pain.

The sensation of pain involves communication between your nerves, spinal cord, and brain. There are different types of pain, depending on the underlying cause. Three most prominent types are described below. The most common type of pain is that concerned with sensing noxious stimuli and is called 'nociceptive pain'. It is the pain that we sense when something is sharp or hot. It is an early-warning and physiological protective system, essential to detect and minimize contact with damaging or noxious stimuli to prevent further damage[2-4]. Nociceptive pain is transient, and fades away if the noxious stimulus is gone. Another type of pain is inflammatory pain, which is also adaptive and protective. 'Inflammatory pain' is associated with tissue damage and tissue inflammatory responses and aids in protecting the damaged/inflamed tissue, until healing occurs. A third type of pain is 'pathological pain', that persists even when the original insult/noxious stimulus or inflammation/tissue damage have disappeared. Such pain loses its protective function and is considered as pathologic[5-8]. The most well-known pathological pain also is caused by damage to the nervous system and is also known as 'neuropathic pain'. Neuropathic pain, is associated with the sensations of pain regardless of the presence of any noxious stimuli[9,10]. Pathological pain may also occur in chronic inflammatory disease such as arthritis. In arthritis, pain often does not correlate well with the magnitude of joint inflammation or joint damage. In a large proportion of rheumatoid arthritis patients (up to 70%) and osteoarthritis patients (~40%), pain persists even with minimal disease activity or even sustained remission[11-15]. Pathological pain, whether caused by nerve damage or inflammation, is often chronic and as such referred to as 'chronic pain'. Chronic pain affects ~20% of the population word-wide, with even a higher prevalence in Europe (25-30%). Chronic pain is more prevalent among women and in developed countries[16-20].

The prevalence of chronic pain is expected to increase due to the effects of aging population, increased levels of obesity, and lack of physical activity[20,21].

Nociceptive pain can be controlled relatively well pharmacologically with for example opioid analgesics such as oxycodone, nonsteroidal anti-inflammatory drugs (NSAIDs) e.g. ibuprofen and paracetamol (acetaminophen)[22-24]. However, these drugs demonstrate modest efficacy in chronic pain[24-26], and they have substantial side effects associated with prolonged use during the chronic pain. For example, chronic use of paracetamol causes liver damage[27], and NSAIDS are associated with potentially serious renal, gastrointestinal, and cardiovascular side effects[28]. Opioids have well-known side effects such as constipation and sedation, long term treatment with opioids can also lead to tolerance, hyperalgesia, addiction, and misuse[26,29,30]. Opioid prescriptions to treat pain have increased opioid use and misuse in many countries, particularly in the US, and has led to the current opioid epidemic[31-33]. Thus, chronic pain is considered as a major debilitating condition that is difficult to treat and needs urgent improved and new therapeutic strategies for a better pain management. Hence, understanding the processes governing pain pathophysiology, specifically chronic pain, is crucial to the development of novel and effective therapeutics that treat chronic pain but leave the nociceptive pain responses intact.

Pain pathways

Nociceptors, like other primary somatosensory neurons, are pseudounipolar with the cell bodies located in the dorsal root ganglia (DRG) for the body organs or located in the trigeminal ganglion for the head and face. Nociceptors have a peripheral axonal branch that innervates peripheral tissues in the body and a central axon that synapse on second-order neurons in the dorsal horn of the spinal cord (Fig. 1). Nociceptive information is processed in the spinal cord and then sent to the brain where complex circuits eventually lead to the perception of pain[34]. A variety of receptors and ion channels on the peripheral terminal of the nociceptor are activated, often by modality-specific stimuli, to transduce the noxious input into a depolarising electrical signal. For example, ligand-gated channels that confer sensitivity to heat (e.g. TRPV1), cold (e.g. TRPM8), pressure (e.g. TRPV4), chemical irritants (e.g. TRPA1), or acidity (ASICs). When the depolarisation is strong enough to reach the threshold to activate voltage-gated

sodium channels (e.g. Nav1.7, Nav1.8, Nav1.9) an action potential is triggered[3,35,36].

Historically, sensory neurons have been categorized by their degree of myelination and associated conduction velocity. Based on this classification, there are three major classes of sensory neurons, Aβ, Aδ and C fibres. 'Aβ' fibers, are large-diameter low-threshold fibers that primarily transmit innocuous stimuli such as touch, vibration, and pressure, but a small fraction of Aβ fibers also transmit pain, particularly in chronic pain conditions such as nerve injury[37-39]. 'Aδ' fibers are the medium diameter myelinated neurons that mediate acute, well-localized "first" or fast pain[38,39]. The third class of nociceptor includes small diameter unmyelinated 'C' fibers that convey poorly localized, "second" or slow pain[38,39].

Sensory neurons can also be subdivided based on their neurochemical features. A major division is between peptidergic and nonpeptidergic neurons[40]. Peptidergic sensory neurons release neuropeptides such as substance P, and calcitonin-gene related peptide (CGRP) and express the TrkA neurotrophin receptor, which respond to nerve growth factor (NGF). The non-peptidergic class of neurons express the c-Ret neurotrophin receptor that is targeted by glial-derived neurotrophic factor (GDNF), neurturin, and artemin.
Sensory neurons can also be distinguished in a function-based manner by their differential expression of channels that convey sensitivity to heat (TrpV1, TrpM2, TrpM3), cold (TRPM8), acidic milieu (ASICs), or chemical stimuli (TrpA1, Mrgprd)[41,42]. However, with the more recent use of single-cell RNA sequencing, sensory neurons can be more comprehensive and unbiased classified. Intriguingly, associating the single-cell RNA sequencing data with the established functions of sensory neurons allows for the prediction of response to various types of stimuli, that identified 3 main categories: 1) Noxious cold-sensing neurons that are further subdivided in Trpm8.1, Trpm8.2, Trpm8.3; 2) Mechano-noxious heat neurons further subdivided in peptidergic C fibers PEP1.1, PEP1.2, PEP1.3, PEP1.4 and Aδ peptidergic fiber PEP2; and 3) Noxious itch-mechano-heat neurons including nonpeptidergic C fibers NP1, NP2, NP3[43-45].

Stimulus intensity of the noxious stimuli is encoded by frequency of the fired action potentials that travel along the axons of the sensory neurons to the dorsal horn of spinal cord. First order sensory neurons conveying noxious stimuli,

predominantly synapse in the superficial dorsal horn of the spinal cord in laminae I-II with ascending second order neurons including nociceptive specific neurons, wide dynamic range (WDR) neurons and interneurons. The second order neurons project to the higher brain centers such as thalamus and brainstem nuclei[46]. In the brain, projections follow two major tracts: those projecting into the lateral thalamus and to the somatosensory cortex and those projecting to more medial regions that then project to areas such as the inferior insula and the anterior cingulate, correlated with processing and mediating of pain components[3,47]. Beside ascending noxious tracts, a descending pain modulatory neural tract, that originates in midbrain and brainstem regions and projects to the dorsal horn of the spinal cord, allows for inhibition of transmission of the pain signal in the spinal cord. Several neurotransmitters, such as serotonin and norepinephrine, release from synapses of descending pain modulatory neurons in the dorsal horn of the spinal cord. Serotonin binds with 5-hydroxytryptamine (5-HT) receptors and activate GABA-ergic interneurons, whereas norepinephrine binds with alpha-2-adrenoreceptors, causing modulation of potassium ion channels. Dysregulation of these descending inhibitory systems is also thought to play a role in chronic pain states[48-50].

Inflammatory Pain

Inflammation is characterized by five typical signs: *rubor* (redness), *calor* (increased heat), *tumor* (swelling), *dolor* (pain), and *functio laesa* (loss of function). Tissue injury whether by trauma or surgical intervention, metabolic dysfunction, ischemia, or pathogens, evoke inflammation and cause an inflammatory response[51]. An inflammatory response induces immune cell recruitment and release of inflammatory mediators[52]. Pain is the primary feature of an inflammatory response that results from activation and/or sensitization of sensory neurons by inflammatory mediators. In the acute stages, inflammation may aid protective reflexes, such as pain and wound healing. However the protective aspects of pain caused by inflammation are thought to be lost when inflammation and the associated pain persist well beyond the need for tissue repair or survival. In various (auto)inflammatory diseases inflammation is persistent or recurrent which cause ongoing pain. Pain may also precede the other signs of inflammation. For example, in rheumatoid arthritis, pain often precedes stiffness or swelling[53,54]. In some cases, pain persist even after the

inflammation has been treated or subdued[11-14]. This persistent inflammatory pain is observed in rheumatoid arthritis, inflammatory bowel disease but also after infections like herpes zoster or bacterial infections[51,55]. It is well established that during the course of the inflammatory response, pro-inflammatory mediators released locally after tissue injury including classic mediators (e.g. bradykinin, prostaglandins, H^+, ATP, nerve growth factor), cytokines, and chemokines but also microRNAs act synergistically to stimulate nociceptors[56,57]. These mediators contribute in pain pathways by directly activating sensory neurons to generate action potentials or by sensitizing nociceptors and reduce their activation threshold[42,58,59]. Bacterial pathogens also induce pain by directly activating sensory neurons that modulate inflammation via releasing bacterial N-formylated peptides[60].

The first indications that cytokines could induce pain came from studies using intraplantar injections of TNF and IL-1β causing hyperalgesia[58,61,62]. The central role of the cytokines in inflammatory pain is corroborated by reports showing that neutralization of TNF inhibits pain in patients with rheumatoid arthritis much faster than it improves the signs of inflammation such as a reduction in joint swelling[63]. These temporal differences emphasize that in some anti-inflammatory treatments, such as anti-TNF treatment, the nociceptive effect precede the anti-inflammatory responses, potentially because the underlying mechanism is distinct in controlling inflammation versus controlling inflammatory pain[56,63]. Indeed, as mentioned above, pain sometimes remains even after the inflammation is waned[11-13]. It is still not clear why pain can persist beyond the resolution of the inflammation that induced it. More precisely, the mechanisms on how inflammatory pain is resolved are not well understood. Therefore, in this thesis we describe our study on mechanisms involved in endogenous resolution of inflammatory pain by using transient inflammatory pain models.

Sensitisation

Sensory neurons are not only static detectors, they can modulate their receptor threshold and the efficacy of their synaptic contacts to regulate the perception of pain. For example, following injury nociceptors become sensitized, leading to enhanced activity and pain responsiveness after exposure to normally innocuous (allodynia) and noxious (hyperalgesia) stimuli[64,65]. Sensitization is caused by a

reduction in the threshold of activation and/or an increase in the magnitude of response of sensory neurons to pungent stimuli[64]. Sensitization of the sensory system may occur at different levels within the nervous system, including sensory neurons (peripheral sensitization) or higher-order neurons in spinal cord and brain (central sensitization) (Fig. 1).

Generally it is thought that acute sensitization serves a protective function and that senbsitisation which persists past the healing phase of an injury is maladaptive[66-68]. However, studies in squid showed that fin crush injuries induce long-lasting sensitization of afferent neurons that provide long-term information about the injury location. Intriguingly, Crook & Walters showed that minor injury increases the risk of predatory attack, but that the injury-induced sensitized state promotes enhanced responsiveness to threats, increasing the survival (Darwinian fitness) of injured animals during subsequent predatory encounters[69]. Moreover, recent evidence showed that nerve injury produces hypervigilance to predator odour in mice, suggesting that even chronic pain may serve some adaptive responses[70].

Peripheral sensitization

Peripheral sensitization occurs in response to chemical mediators released in tissue during inflammation or after tissue injury. A wide range of molecules induce peripheral sensitization, including ATP, PGE2, growth factors (such as NGF, G-CSF, GM-CSF), cytokines (like IL6, IL1β, TNF), chemokines, neuropeptides (like CGRP, substance P, bradykinin, histamine), lipids, and diverse proteases[41,71-73]. Peripheral nerve terminals of sensory neurons express receptors that detect these mediators[42,74]. Upon activation of these receptors specific signalling cascades are activated that modify the gating properties of several transduction ion channels including, transient receptor potential (TRP) channels such as TRPV1, TRPA1 or sodium channels like $Na_v1.7$, $Na_v1.8$, and $Na_v1.9$ that contribute to action potential generation[74-76].

Central sensitization

Central sensitization contributes to the persistence of pain[77,78]. Central sensitisation is induced by several mechanisms that alter synaptic connectivity in e.g. the spinal cord. As example, synaptic release of various neurotransmitters,

such as glutamate, may induce long term potentiation (LTP) at spinal synapseia[56,79,80]. Astrocytes also contribute by producing cytokines like CCL2 and CXCL1 that may activate spinal neurons and induce central sensitization[74,84]. Moreover, in the dorsal horn of the spinal cord, resident microglia and astrocytes, and infiltrating immune cells produce pro-inflammatory cytokines and growth factors such as TNF, IL1β, IL17A, PGE2. These factors act presynaptically at synapses from DRG neurons, or post-synaptically at second order neurons to modulate membrane excitability and synaptic transmission. Glia cells also release factors including ATP, glutamate, prostaglandins, and NO that facilitate heterosynaptic LTP inducing central sensitization and hyperalgesia[81-83].

Pathological pain

As mentioned earlier, various causes, such as chronic inflammation or nerve damage, may lead to pathological pain[55,85,86]. As such, specific mechanisms may contribute to different types of pathological pain, although sometimes there are considerable overlaps too[5,87]. Therefore, understanding the differences and similarities in these pain mechanisms in various diseases may help clinicians to better manage pathological pain. A growing amount of evidence suggests that neuropathic pain may develop in inflammatory disease such as osteoarthritis or rheumatoid arthritis[88-90]. Indeed, inflammation may cause damage to the neurons and as result lead to neuropathic pain. Likewise, neuronal injury is often associated with inflammatory responses which may induce responses similar as observed in inflammatory pain[3,91]. Osteoarthritis is a great example of a chronic pain condition that involves a mix of both inflammatory and neuropathic components[88,92,93]. Although various types of pathological pain may exist, in this thesis we focussed on osteoarthritis pain.

Osteoarthritis pain

Osteoarthritis (OA) is the most common degenerative joint disorder. Pain is the major symptom and as such OA represents one of the most frequently occurring painful condition worldwide[94]. In Europe, OA is the cause of ~20% of all chronic pain conditions. OA results from a combination of factors such as joint malalignment, increased biomechanical loading of joints, genetics and low-grade

systemic inflammation, with increasing age and obesity (metabolically and mechanically) being the most prominent[95].

For long OA was thought as a cartilage-limited disease. This concept has evolved and OA is now considered a disease of the whole joint, including alterations in the articular cartilage, subchondral bone, ligaments, capsule, and synovial membrane, ultimately leading to joint failure[96-98]. Disease progression in OA is usually slow and can take (several) years to develop[94]. The structural damages to the joint are loss of cartilage, osteophyte formation, subchondral bone changes, synovial inflammation, and meniscal alterations (structural OA). These alterations are normally associated by joint pain (symptomatic OA)[92,94]. To date, OA and its pain remain challenging to treat. Pharmacological treatments are mostly related to relief of symptoms such as pain which are insufficiently effective or have severe side effects, specifically because of the long-term need for treatment. Until now there is no clinically approved disease-modifying OA drug providing tissue structure repair and pain relief[92,94].

The mechanism that govern OA pain are still poorly understood. Although, evidence from experimental models suggests that (1) OA-like joint damage is associated with sensitization and pain-related behaviours; (2) joint damage appears to be driving pain; (3) multiple mediators in the OA joint can sensitize nociceptors through binding specific receptors; (4) joint damage causes neuroinflammation in the peripheral and central nervous system; and (5) mechanisms of pain might be different in early vs. late stages of the disease[99,100]. However, in OA patients the severity of joint pain poorly correlates with the actual joint changes[101-104], suggesting that other mechanisms, in addition to local joint damage and inflammation, contribute to the experienced OA pain. Inflammation in synovial tissue and bone (e.g. bone marrow lesions) plays a role in the development of OA in a subset of patients[105], and may as such contribute to pain development. In the OA joint, local inflammation induces the release of inflammatory mediators such as prostaglandins, bradykinin, H^+, nerve growth factor (NGF), pro-inflammatory cytokines (TNF, IL-1β), and pro-inflammatory chemokines such as CCL2. Receptors for all these mediators are expressed by nociceptors. Activation of these receptors generate second messengers such as Ca^{2+} and cAMP, which in turn activates several kinases, such as the PKA, PKC, CaMK, PI3K, and MAPKs (ERK, p38, and JNK). This modulates key ion channels, such as transient receptor potential ion channel V1 (TRPV1) and voltage-gated

sodium-channels like $Na_V1.7$, $Na_V1.8$ and $Na_V1.9$, causing hypersensitivity and hyperexcitability of nociceptors. Rodent studies have demonstrated that cytokines like TNF, IL1β, IL6, IL18, IL7, IL15, and IL21, and chemokines such as CCL2 generate joint pain by acting directly on joint nociceptors[106-108]. Joint tissues, except cartilage, are richly innervated[106,109,110]. However, new innervation of normally non-innervated cartilage has been demonstrated in both human OA joints and animal models. Nerve sprouting occurs by osteochondral channels carrying blood vessels and CGRP positive neuronal fibres breaching into articular cartilage[106,109]. The neovascularization of articular cartilage, including the new sensory nerves formed in the cartilage, have been shown to further promote OA pain[111-113].

There are reports indicating neuronal damage in OA, suggesting development of a neuropathic pain phenotype[88,90,114]. Clinical features that distinguish neuropathic from inflammatory pain such as paraesthesia, mechanical and thermal hyperalgesia, allodynia, burning and paroxysmal pain, and numbness [114], support signs of neuropathic pain in OA patients [88,90]. Although the mechanism causing neuropathy in OA remain to be elucidated, a recent study found that the lipid mediator lysophosphatidic acid (LPA) is upregulated in OA synovial fluid. LPA induces demyelination of peripheral nerves, resulting in joint neuropathic-like pain [115]. A recent systematic review showed that the prevalence of neuropathic pain in persons with knee or hip OA is 23%[116]. Moreover, neuropathic pain, like OA pain, is unresponsive to common analgesics such as NSAIDs[114,117,118]. Overall, OA is a heterogeneous disease that has different phenotypes based on inflammation, structural damage, and pain. In OA, most studies focussed on joint architecture and local degradation rather than the mechanism that drive pain. However, there is often discordance between the degree of articular pathology and pain experienced by patients with OA, suggesting the presence of individual variability in the central processing of nociceptive stimuli, such as central sensitization. Therefore, in this thesis we investigated other pain involving factors than joint damage which may explain the dissociation of pain with joint damage[92].

Immune system in control of pain

Recently it has becomes clear that immune cells regulate pain not only at the inflamed or injured site[3,119], but at other pain-relevant areas such as DRG which contain somata of sensory neurons or the dorsal horn of the spinal cord which receives peripheral input (fig. 1)[11,120,121]. Normally, a small number of immune cells reside in DRG, with their numbers increasing in chronic pain conditions[11]. In models of inflammatory and neuropathic pain the number of macrophages, monocytes, neutrophils, and T cells increase in the DRG[11,74,122,123]. The accumulation of immune cells in areas remote from the site of inflammation or injury, which induce pain sensitivity, explains why in some diseases like rheumatoid arthritis and osteoarthritis pain can be dissociated from the local inflammation or injury. Therefore, in this thesis first we evaluated the role of immune cells and their mediators in regulation of pain in different stages of several diseases by reviewing current literature. Next, we focused on the role of macrophages in regulation and resolution of persistent pain.

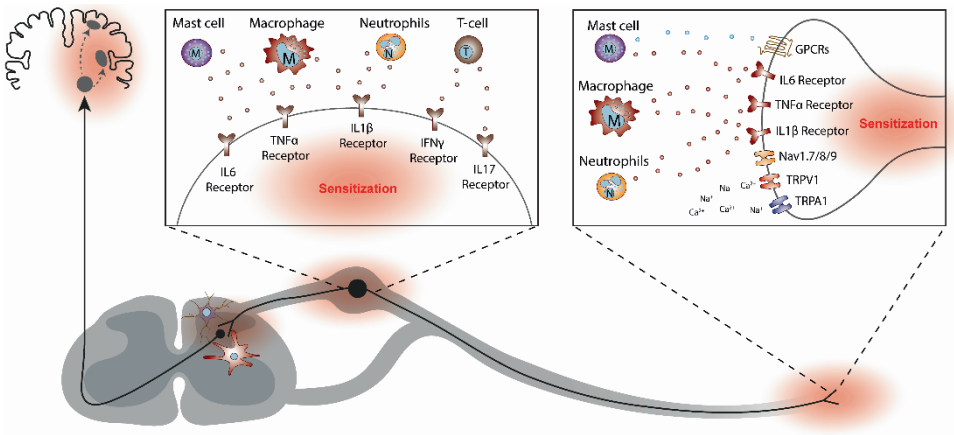

Figure 1. Immune cells release mediators that induce neuronal sensitization

During inflammation or injury, tissue resident and recruited immune cells accumulate to the inflamed or damaged site or innervating DRG and secrete inflammatory mediators that act on the sensory neurons which cause pain sensitization. In the sensory neurons, specific cytokine receptors lead to phosphorylation of ion channels such as Nav1.7, Nav1.8, Nav1.9, TRPV1 and TRPA1, which increase action potential generation and pain sensitivity.

Macrophages

Macrophages were originally identified by Ilya Metchnikoff more than 100 years ago, which resulted in the Nobel Prize for Medicine in 1908. Macrophages are a highly heterogeneous population of innate myeloid cells. A characteristic feature of macrophages is their plasticity. They are the most functionally diverse cells of the hematopoietic system, and present in all tissues.

Macrophages produce many cytokines, growth factors, and lipids that can act directly on sensory neurons to induce pain[74]. The pain promoting role of macrophages during chronic pain conditions, in particular at the site of inflammation or injury, has been extensively demonstrated[121,124-126]. However, there is evidence indicating expansion of macrophages in the DRG in various chronic pain conditions, such as in chemotherapy- and sciatic nerve ligation-induced neuropathic pain[122,123]. However, the function and the mechanisms on how macrophages contribute to pain pathways in the DRG is not well understood.

Macrophages differentiate into distinct subsets, depending on the local tissue milieu. In the 1990s it was discovered that IL4 induced different changes in macrophage gene expression when compared to IFNγ and LPS[127,128]. A few years later, Mills et al. proposed a new classification of subsets of macrophages as either M1 or M2, that differ in their cell markers, secreted cytokines and biological functions[129]. STAT signalling is a central pathway in controlling macrophage polarization. The balance between activation of STAT1/NF-κB and STAT3/STAT6, tightly regulates macrophage polarization and activity. A predominance of NF-κB and STAT1 activation increase the expression of M1 markers such as iNOS and promotes the pro-inflammatory function of M1 macrophages. In contrast, activation of STAT3 and STAT6 by IL-4, IL13 or IL-10 increase the expression of M2 markers like CD206 and induce M2 macrophage polarization and promotes the expression of anti-inflammatory molecules such as IL10 and TGFβ (Fig. 2)[130-133].

After a painful stimulus, macrophages accumulate in different areas through the pain pathway, in peripheral and central nervous system[11,134,135]. In peripheral nervous system, IL-34, an alternative ligand for CSF1R, governs the numbers and maintenance of macrophages[136]. In the neuronal environment, inflammatory

mediators such as cytokines or sensory neuron-derived neuropeptides and neurotransmitters regulate the phenotype and function of macrophage, whereas factors produced by macrophages mediate the neuronal activation[11,137]. For example, sensory neurons release exosomes containing microRNAs that program DRG macrophages into a M1 phenotype[138]. Moreover, activated satellite or Schwann cells release cytokines like TNF and IL-1β, and prostaglandins that program macrophages into a M1-like phenotype[139-141]. On the other hand, nociceptors release neuropeptides such as CGRP and substance P that program macrophages into an anti-inflammatory M2-like phenotype[76,142]. Thus, this reciprocal interaction between neurons and macrophages defines a regulatory system that modifies both neuron and macrophage function.

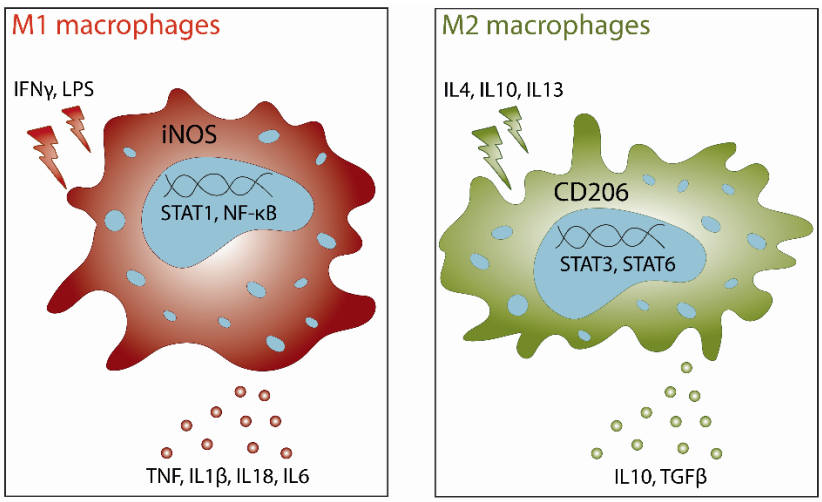

Figure 2. Programming of macrophage

STAT signalling controls macrophages programming. Activation of STAT1/NF-κB promotes M1 macrophage polarization, resulting in expression inflammatory genes including TNF, IL1β, IL6, and IL18. Activation of STAT3 and STAT6 by IL-4, IL13 or IL-10 increases M2 macrophage polarization and promotes expression of anti-inflammatory molecules such as IL10 and TGFβ. M1 and M2 macrophages differ in their cell markers (iNOS vs. CD206), secreted cytokines and biological functions (proinflammatory vs. anti-inflammatory).

Experimental pain models

Pain perception in humans is considered more complex than in animals since human pain perception encompasses psychosocial, cultural, developmental, and environmental variables. Although animal models do not simulate every aspect of chronic pain symptoms, they do model some key features of human pain. Thus, studies in animals give insight into certain aspects of human pain conditions and lead to improved insight in pain management for patients. For example, in preclinical rodent studies ziconotide, that blocks N-type voltage-gated calcium channels, produced anti-hyperalgesic effects in models of chronic inflammatory[143] and neuropathic[144] pain. After successful clinical trials, ziconotide has been approved as a non-opioid analgesic for the management of chronic inflammatory and neuropathic pain[145]. Successful drug development with animal studies, like ziconotide, makes a strong case for the importance of preclinical pain research despite the species differences.

Patients can self-report the severity of their pain, in contrast to animals, which makes assessing the severity of pain a challenge to all animal pain models. There are two main ways to assess pain in animal models: 1) Evoked measurements of pain; and 2) Spontaneous measurements of pain. In evoked measurements, the assumption in each model is that the animal's behaviour in response to noxious stimuli can be reliably and objectively evaluated. In this thesis sensitivity to different sensory modalities (e.g. mechanical, heat) are used as readouts of evoked pain behaviours. For example, the latency to withdraw or escape from a thermal or mechanical stimulus, where a longer latency equates to a higher nociceptive threshold. In this thesis we used Von Frey assay to measure mechanical hypersensitivity[146] and Hargreaves assay to measure thermal hypersensitivity[147] as readouts of evoked pain. In this thesis, dynamic weight bearing (DWB) is used to assess postural deficits caused by ongoing pain or sensitisation, by measuring the weight bearing on the affected paw[148]. Spontaneous pain measurements are important because clinically spontaneous pain is a debilitating feature of chronic pain. However, study of spontaneous pain has been limited due to lack of assessment tools[149]. Recently a few techniques have been developed that may assess spontaneous pain to a better extend, for example by testing whether animals can be conditioned to a pain-relieving stimulus. In this thesis we use conditioned place preference (CPP) which is used

to assess an animal's motivation to seek pain relief and indirectly assess the effectiveness of the treatment[150]. This approach takes advantage of the fact that pain relief is rewarding and, therefore, analgesic treatments should only be rewarding in the presence of pain.

Outline of this thesis

The aim of this thesis is to determine the role of DRG infiltrating macrophages in modulation of pain. **Chapter 2** focuses on how the immune system regulates pain and discusses the emerging roles of immune cells in the initiation, maintenance and resolution of chronic pain. We highlighted which immune cells infiltrate the peripheral damaged nerves, DRG, spinal cord, and tissues around free nerve endings and discuss through which mechanisms they control pain. Finally, we discuss emerging roles of the immune system in resolving pain and how the immune system contributes to the transition from acute to chronic pain. These findings pointed to a key role of macrophages in various pain condition in particular neuropathic pain. However, knowledge on macrophages in pain resolution and in osteoarthritis pain was limited. Therefore, in **Chapter 3**, mechanistic insight are provided as how inflammatory pain is resolved and identify a contribution of macrophages in this process. We showed how macrophages actively control resolution of inflammatory pain remotely from the site of inflammation. Moreover, a mechanistic frame work as to how macrophages resolve inflammatory pain is presented. In **chapter 4**, in contrast with the previous chapter, we studied the contribution of macrophages in maintenance of osteoarthritis pain and whether targeting macrophages represent a way to treat OA pain. In **chapter 5**, we elucidated what drives the DRG macrophage accumulation in OA pain and evaluate whether these cells are required for the initiation of OA pain. Finally, in **chapter 6,** the findings of this thesis are summarized and discussed by placing them in the context of current knowledge of neuro-immune crosstalk in pain pathways. Furthermore, concluding remarks and implications for future research and possible therapeutic investigations are presented.

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2

CHAPTER 2

Divergent Roles of Immune Cells and Their Mediators in Pain

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Abstract

Chronic pain is a major debilitating condition that is difficult to treat. Although chronic pain may appear to be a disorder of the nervous system, crucial roles for immune cells and their mediators have been identified as important contributors in various types of pain. This review focuses on how the immune system regulates pain and discusses the emerging roles of immune cells in the initiation or maintenance of chronic pain. We will highlight which immune cells infiltrate damaged nerves, the dorsal root ganglia, spinal cord, and tissues around free nerve endings and discuss through which mechanisms they control pain. Finally we discuss emerging roles of the immune system in resolving pain and how the immune system contributes to the transition from acute to chronic pain. We propose that targeting some of these immune processes may provide novel therapeutic opportunities for the treatment of chronic pain.

Keywords: Chronic Pain, Immune cells, Cytokines, Arthritis, Neuropathic, Inflammatory, Dorsal root ganglia, Spinal cord

Key messages:

- Immune cells contribute to chronic pain but have different roles in the initiation, maintenance and resolution of pain.
- Modulating immune cells or immune mediators can attenuate chronic pain.

Introduction

Pain-related problems are the main reason for physician consults[1,2]. Chronic pain affects more than 20% of the population[3,4]. Current therapies to relief pain (e.g. NSAIDs, opioids) often fail or produce treatment-limiting side-effects[5,6]. Different causalities may be at root of chronic pain development. Inflammation causes inflammatory pain (e.g. rheumatoid arthritis, inflammatory bowel diseases), whilst nerve injury as consequence of an operation/trauma, metabolic disorders (e.g. diabetic mellitus), or auto-immune diseases (e.g. multiple sclerosis) may cause neuropathic pain. Moreover, cancer itself or its treatment (chemotherapy) may result in painful neuropathies[7].

Rheumatoid arthritis (RA) and osteoarthritis (OA) are common causes of chronic pain and combined substitute ~42% of the chronic pain patients in Europe[8]. Although inflammation and damage are closely linked to pain, chronic pain may not be the direct consequence of ongoing inflammation or damage because arthritis pain does not correlate well with the magnitude of inflammation or joint damage [9,10]. Moreover, in a substantial proportion (ranging from 12-70%) of RA patients pain persists even with minimal disease activity or with sustained remission[9,11,12]. Finally, ~20% of OA patients with total knee replacement surgery report severe/extreme pain 3 to 4 years after the operation[13,14].

Chronic pain may result from aberrant neuronal activity including ectopic discharges, peripheral sensitisation of primary sensory neurons, and sensitization of neurons in the central nervous system (CNS)[15]. However, the immune system is also involved in pain regulation[16]. Microglia, the resident macrophages of the CNS, play important roles in multiple rodent models of chronic pain, including neuropathic pain, cancer-induced bone pain, and chronic inflammatory pain. In these models, resident microglia switch from a quiescent inactive state to an activated phenotype that is associated with production of inflammatory mediators that increase the sensitivity of the pain system[17]. However, evidence indicates that peripheral immune cells and their mediators are also involved in regulating pain[18-20].

During inflammation or tissue damage, infiltrating or resident peripheral immune cells at the site of inflammation/damage produce mediators that trigger sensory neurons to produce action potentials or sensitize neurons by enhancing

sensory transduction and neuronal excitability[21,22]. However, peripheral inflammation or tissue damage also induces infiltration of immune cells into other pain-relevant sites such as peripheral nerves, dorsal root ganglia (DRG) containing the somas of the sensory neurons, or the dorsal horn of the spinal cord that receives peripheral input to modulate pain sensitivity[19,23,24]. These peripheral immune cells and their mediators play different roles in the initiation and maintenance of different types of pain, and evidence exists for a role in the resolution of pain[21,25-27]. These intricate contributions of immune cells at different stages of pain induced by inflammation (e.g. arthritis), damage (osteoarthritis), or nerve damage (neuropathic) will be discussed in the following paragraphs and is summarized in figure 1.

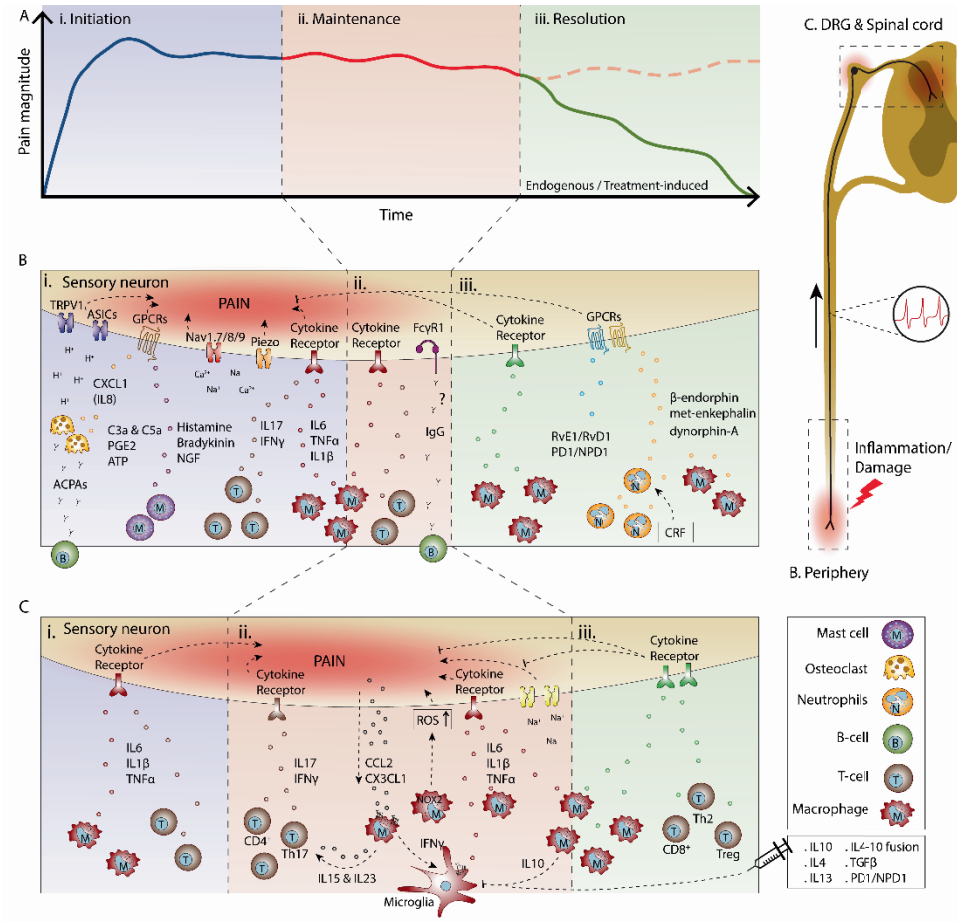

Figure 1: Overview of the role of immune cells and their mediators at different stages of pain

*(**A**) Time course of chronic pain induced by inflammation or damage visualising the different stages of pain: (i) initiation, (ii) maintenance, and (iii) resolution that are used in B/C. (**B/C**) Schematic overview of the different types of immune cells and mediators modulating pain at different sites (B/C) and during the different stages (i-iii) of pain. During inflammation or tissue damage, resident and immune cells recruited to the inflamed/damaged site secrete inflammatory mediators that act on (**B**) peripheral nerves innervating the affected tissue. Similarly, different immune cells migrate to (**C**) the spinal cord and/or to the dorsal root ganglia to modulate pain sensitivity during the different phase of pain.*

Immune-derived mediators in pain initiation and maintenance

Several inflammatory mediators such as bradykinin, histamine, adenosine triphosphate, neurotrophins, and cytokines but also protons or damage-associated molecular patterns (DAMPs) activate sensory neurons to generate action potentials and/or enhance neuronal excitability and sensory transduction through neuronal receptors leading to pain and hyperalgesia[21,22]. The contribution of cytokines in initiating pain is supported by evidence that the development of inflammatory pain is attenuated by neutralizing cytokines or blocking cytokine receptors at the site of inflammation. Neutralization of TNFα with TNFα antibodies or soluble TNF-receptors attenuates the development of pain in various experimental arthritis models[28-31]. Inhibition of IL6 or IL1β signaling by an intra-articular injection of soluble gp130 or with IL1R antagonist Anakinra, respectively, attenuates the development of pain in experimental arthritis[32,33]. Pro-inflammatory cytokines may also maintain pain through modulating the central terminals of primary afferent neurons and/or spinal cord neurons because spinal administration of neutralizing TNFα antibodies also reduces experimental arthritis pain[31]. Some RA patients continue to experience pain after systemic anti-TNFα treatment[11], but this may be explained because systemically administered antibodies do not efficiently block spinal TNFα. Indeed, spinal TNFα neutralization is more effective in treating arthritis pain than when administered systemically[31]. Other cytokines such as IL15, IFNγ, IL18, IL22, and IL17 or damage associated molecules such as high mobility group box 1 (HMGB1) or S100 initiate or maintain pain[34,35]. IL15 contributes to the development of neuropathic pain by promoting infiltration of macrophages and T-cells into the sciatic nerve and spinal cord whilst IFNγ induces spontaneous neuronal firing and activates spinal microglia[36]. Nerve injury-induced allodynia is reduced after genetic or pharmacological inhibition of IL17 or IL18 and intrathecal injections of these cytokines induce pain, probably through activating spinal glial cells[37,38]. Finally, IL22 expression is increased during the onset of experimental arthritis pain and inhibiting IL22 reduces pain[39]. Intriguingly, pro-inflammatory cytokines levels such as IL1β, IL6, and IL18 are increased in the spinal cerebral fluid of fibromyalgia, non-diabetic polyneuropathy and post-traumatic neuralgia patients[40,41].

The functional capacity of inflammatory mediators such as cytokines to produce pain is highly dependent on the expression and composition of their receptors in

sensory neurons. Indeed, a wide range of cytokine receptors are expressed on sensory neurons, allowing cytokines to act directly on sensory neurons[42,43]. During development of pain expression of these receptors may be modulated, affecting the functional consequences of inflammatory mediators released. After peripheral nerve injury TNF receptors (TNFR1 and TNFR2) and their ligand TNFα are upregulated in sensory neurons[44]. However, in several models of chronic pain TNFR1 is the main pain promoting receptor[43], yet some reports indicate involvement of TNFR2 in pain induction[45].

Sensory neurons also express IL1R and IL1β induces activation of sensory neurons[46]. In models of neuropathic and inflammatory pain, sensory neuron IL1R1 expression is increased[43,47,48] and in an experimental adjuvant-induced arthritis model the proportion of IL1R1 expressing neurons almost doubles[33]. Finally, after nerve injury IL6R and IL6 are upregulated, however, expression of the signal-transducing component gp130 remained unchanged[49,50]. Nevertheless, sensory neuron-specific depletion of gp130 attenuates inflammatory, tumor, and arthritis pain[51,52], indicating an important role of the receptor component in pain development.

Complement components appear to be also important contributors to pain and are increased in the affected knee of rheumatoid and osteoarthritis patients[53,54]. Moreover, C1q, factor B, and C3 are increased in sciatic nerves of humans with traumatic nerve lesion-induced pain and after spinal cord injury in rodents[55,56]. Components from the classical and non-classical pathway (e.g. C5, C3, C1q) cause pain through triggering sensory neurons or recruiting and activating immune cells (e.g. C5a, C3a) to facilitate pain[57-59]. Intrathecal as well as intraplantar injections of C3a and C5a induce pain hypersensitivity in rodents, whilst blocking C5aR reduces pain in inflammatory, neuropathic and postoperative pain models[57,60,61].

Evidence exists that short-lived episodes of acute inflammation can induce long-lasting sensory neuron plasticity that may contribute to chronic pain development[62,63]. This neuronal plasticity includes changes in the expression of neurotransmitters, receptors, signalling cascades, and ion channels causing long-lasting altered sensory response to subsequent inflammatory mediators[64,65]. For example, rodents challenged with a transient inflammatory stimulus such as IL6, IL1β, bradykinin, or carrageenan develop short-lasting hyperalgesia. However,

when signs of inflammation have resolved, even weeks later, and animals are challenged with a second inflammatory stimulus that normally induces a transient hyperalgesia (e.g. PGE_2), this inflammatory mediator now induces a hyperalgesia that lasts much longer[66,67]. Possibly, such neuronal plasticity may explain why some RA patients after arthritis flares experience pain that outlast the inflammatory response.

Immune-derived mediators resolving pain

First evidence of immune-derived mediators that can resolve pain came from work of the Watkin's group that showed that intrathecal administration of the anti-inflammatory cytokine IL10 reduces neuropathic pain[68]. Intriguingly, IL10 is important in endogenous pain resolution pathways that occur in naturally resolving transient pain conditions. For example, intrathecal injection of neutralizing IL10 antibodies prolongs transient inflammatory pain and inhibition of IL10 signalling either genetically ($IL10^{-/-}$ mice) or pharmacologically (neutralizing IL10 antibodies) delays recovery from chemotherapy-induced neuropathy[25,69]. Finally, in neuropathic pain patients cerebrospinal fluid IL10 levels are reduced compared to healthy controls[40]. The pain resolving action of IL10 may be explained by its inhibitory effects on spinal glia that maintain chronic pain[17]. IL10 inhibits glia cell activation in both inflammatory and neuropathic models of pain[69,70]. Moreover, IL10-mediated attenuation of paclitaxel-induced mechanical allodynia is associated with decreased CD11b, TNFα, and IL1β expression in the DRG, suggesting that IL10 inhibits activation and/or DRG recruitment of $CD11b^+$ immune cells and subsequent pro-inflammatory cytokine production[71]. Nevertheless, IL10 also inhibits TTX-sensitive sodium channels in sensory neurons and reduces chemotherapy-induced spontaneous firing of sensory neurons *in vitro*, indicating IL10 modulates sensory neurons directly[69,72]. Importantly, sensory neurons do express other anti-inflammatory cytokine receptors such as IL4R, IL13R, and TGFβR, and potentially these regulatory cytokines modulate sensory function and pain as well[42]. Indeed IL10 is not the only pain resolving cytokine since mice deficient for IL4 display mechanical allodynia and increased neuronal excitability, and patients with painful neuropathy have reduced IL4 serum levels, indicating that IL4 plays some role in controlling pain[73,74]. Moreover, intrathecal injections of TGFβ, IL13, or sensory neuron specific overexpression of IL4 have analgesic effects in neuropathic and inflammatory pain models[75-78]. The efficacy of these anti-inflammatory cytokines

to inhibit pain is dependent on receptor expression/signalling. To our knowledge there are no data available on whether expression and/or receptor signalling are regulated in sensory neurons during chronic pain. Nevertheless, expression of IL10Rα is reduced in synovial tissue of rheumatoid arthritis patients, enabling the possibility that in such chronic inflammatory conditions sensory neurons IL10R signalling may be affected rendering these neurons less susceptible to IL10-mediated pain inhibition[79].

Despite analgesic actions of anti-inflammatory cytokines, the therapeutic potential of unmodified anti-inflammatory cytokines is limited because these cytokines work most optimal in concert of each other and their relatively small size causes rapid clearance, reducing their bioavailability[80-82]. More recently these limitations have been overcome by fusion of IL4 and IL10 into one molecule which was more effective in inhibiting persistent inflammatory and neuropathic pain than the combination of the individual cytokines[83]. Moreover several viral gene therapy or non-viral transduction vectors have been employed to induce prolonged production of native cytokines to resolve chronic pain conditions[68,78,84,85]. Overall these strategies show a promising perspective for the use of anti-inflammatory cytokines to treat chronic pain.

Other immune-derived mediators known to be involved in the termination program of inflammation, such as resolvins (e.g. RvE1, RvD1) and protectins (e.g. NPD1/PD1) have strong analgesic actions. RvE1 and RvD1 suppress pain through inhibiting TRP channels activity in sensory neurons and NMDA receptors post-synaptically in the dorsal horn[86,87]. Similarly, intrathecal NPD1/PD1 injections reduce established neuropathic pain by blocking nerve injury-induced spinal glia activation and spinal synaptic plasticity[88].

Immune cells regulating pain

Myeloid cells

Pain initiation

Monocytes/macrophages are linked to the development of pain by the production of inflammatory mediators. In neuropathic (e.g. nerve injury-induced) and inflammatory pain models (e.g. arthritis, intraplantar Complete Freund's Adjuvant (CFA) injections) elevated numbers of monocytes/macrophages are observed in pain-relevant tissues such as the

injured nerve, the inflamed skin, or the DRG[23,89-92] at the time pain is developing. Depletion of macrophages locally after CFA-induced paw inflammation attenuates the development of inflammatory pain, whilst depletion of macrophages at the site of inflammation during established persistent inflammatory pain does not affect pain[89,93]. Some evidence exists for a role of myeloid cells in the initiation of pain in neuropathic pain models. Macrophages infiltrate the injured nerve after chemotherapy or sciatic nerve injury and depletion of these cells suppresses the development of neuropathic pain[23,92].

Osteoclasts are derived from myeloid progenitors and play some roles in the initiation of pain. In chronic inflammatory and degenerative disease such as RA and OA, osteoclasts are increased in number and display increased bone resorption activities[94]. These enhanced bone resorption activities cause local acidification, activating acid-sensing ion channels (ASICs) and transient receptor potential channel vanilloid subfamily member 1 (TRPV1) in sensory neurons, leading to pain[95-97]. Inhibitors of osteoclast activity reduce pain in models of osteoarthritis, inflammatory pain, and cancer-induced bone pain[95,97,98]. Similarly, in humans inhibitors of osteoclast activity reduce pain in patients with bone disorders or rheumatoid arthritis[99-101].

Although some studies have shown a role for myeloid cells or myeloid-derived osteoclasts in the initiation of pain, the majority of studies indicate roles for myeloid cells in pain maintenance.

Pain maintenance

In rodent models of neuropathic pain, either induced surgically or by chemotherapy, monocytes/macrophages appear in the DRG and spinal cord at time points when pain is already established and these cells remain present for several weeks[24,102-104]. In several chronic inflammatory pain models, including CFA–induced arthritis and experimental arthritis, macrophages are found in the DRG and spinal cord when pain is established[90,91,105,106]. In a surgical model of OA, macrophages infiltrate the DRG 8 weeks after the destabilization of the medial meniscus and persist within the DRG for at least 16 weeks[107]. Similarly, 4 weeks after intra-articular administration of monosodium iodoacetate (MIA) that induces long-lasting pain by damaging the knee joints[108], the number of $CD68^+$ macrophages in the lumbar DRG triples, suggesting a role of these cells in the maintenance of OA pain (Figure 2). During antigen-induced arthritis, DRG-

infiltrating macrophages exert a phenotype that resembles TNFα-stimulated macrophages[106]. *In vitro*, TNFα skewed macrophages promote CRGP release by sensory neurons, which could explain their pro-nociceptive properties[105]. Macrophage-derived IL6, TNFα and IL1β are described as important drivers of chronic pain[21]. In addition, macrophages also release reactive oxygen species (ROS) that may contribute to the maintenance of pain, since Nox2^{+} macrophages migrate to the DRG and contribute to neuropathic pain in a reactive oxygen species-dependent mechanism[109,110].

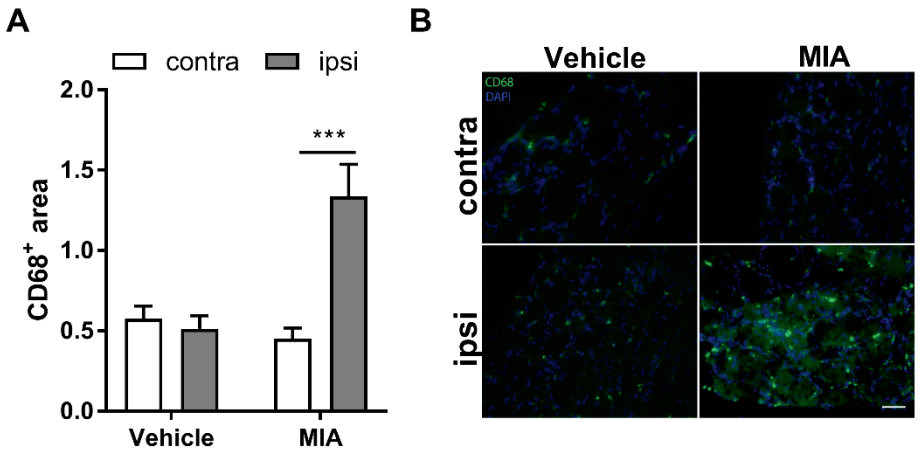

Figure 2: Macrophages infiltrate the DRG in a model of osteoarthritis

*(**A**) Unilateral intra-articular monosodium iodoacetate (MIA) injections in rats significantly increased the number of CD68^{+} macrophages in the lumbar DRG compared to the contralateral knee or vehicle injected rats at 4 weeks after injections (n=6). (**B**) Exemplar images of the DRGs innervating the affected knee (ipsi) or the contral lateral knee (contra). Scale bar is 50 µm. Data are represented as mean ± SEM. ***P < 0.001, statistical analyses were performed by 2-way ANOVA with Bonferroni's post-hoc test.*

The strongest evidence of the involvement of monocytes/macrophages in the maintenance of chronic pain comes from monocyte/macrophage depletion studies. Depletion of peripheral macrophages by intravenous injections of clonodrate liposomes partially reverses established paclitaxel-induced or nerve ligation-induced mechanical hyperalgesia and reduced TNFα expression in DRG[111,112]. Moreover, monocyte/macrophage depletion with clodronate

liposomes delays the progression of diabetes-induced mechanical allodynia[113]. Systemic depletion of monocyte/macrophage *after* sciatic nerve ligation attenuates axonal damage and hyperalgesia, whereas depletion *prior to* L5 spinal nerve transection has no effect on the development of neuropathic pain, indicating that macrophages play a role in the maintenance of chronic pain[114,115].

The presence of macrophages at pain-relevant sites question why these cells migrate to these tissues that are distant from the site of actual damage or inflammation. After peripheral inflammation sensory neurons produce chemokines CCL2 and CX3CL1 that may drive the attraction of macrophages[116,117]. Similarly, after chemotherapy-induced nerve injury or after knee damage in an experimental osteoarthritis model, expression of CCL2 is increased in the DRG and spinal cord, and the increase in CCL2 production is associated with elevated numbers of macrophages in the DRG and spinal cord[107,112]. CX3CL1 is anchored to the plasma membrane, but is liberated after cleavage by proteases (e.g. cathepsin S) produced by activated microglia[118]. After nerve injury soluble CX3CL1 levels are increased in the DRG, whilst membrane-bound CX3CL1 is decreased[119].

In mice deficient for CCR2 and CX3CR1, pain and the number of monocyte/macrophages in the injured nerve or DRG are markedly reduced after a peripheral inflammation, experimental osteoarthritis, or chemotherapy-induced neuropathy[92,107,120,121]. Moreover, inhibition of spinal and DRG CX3CL1 or CCL2 during established paclitaxel-induced neuropathy inhibits macrophage recruitment to the DRG and attenuates allodynia[122,123]. In patients with lumbar disk herniation with sciatic pain the severity of pain is correlated with increased local expression of CX3CL1 and CCL2 in the soft tissues around nerve root. Moreover, intrathecal administration of a CCR2 antagonist inhibits neuropathic pain in a rat model of lumbar disc herniation[124,125].

Sensory neurons also produce other chemokines after nerve injury, such as CCL21, CXCL13, CCL7[120,126,127]. Whether similar chemokines are produced during chronic inflammatory pain remains to be determined. Nevertheless, all these factors may contribute to macrophage infiltration in the DRG to regulate pain. However, it should be noted that many chemokines including CCL2 also act on chemokine receptors expressed by sensory neurons to produce pain[128].

Pain resolution

Depletion of monocytes prior to the induction of transient inflammatory pain with IL1β or carrageenan prevents the resolution of inflammatory pain, that normally last 1-2 days but now persists for more than 1 week. This prevention of the resolution of a transient inflammatory hyperalgesia is dependent on IL10 production by monocytes/macrophages[25]. Moreover, reduction of G protein–coupled receptor kinase 2 (GRK2), an ubiquitously expressed negative regulator of G protein–coupled receptors and other signalling molecules (e.g. p38) in monocytes/macrophages increases production of TNFα whilst reducing IL10 and prevents the resolution of transient inflammatory pain[25]. The existence of pain-resolving macrophages is further supported by evidence that perineural injection of IL4-skewed macrophages reduces neuropathic pain through the production of opioid peptides including Met-enkephalin, dynorphin A and β-endorphin[129].

In conclusion, myeloid cells have distinct roles in the initiation, maintenance and resolution of pain. The functional plasticity of macrophages enables these cells to mediate both pro- and anti-nociceptive effects following injury or inflammation. As such, regulating macrophage phenotype by promoting polarization into anti-nociceptive or blocking polarization into pro-nociceptive phenotype might represent interesting avenues for potential new therapeutic strategies for chronic pain.

Neutrophils and mast cells

Pain initiation and maintenance

After an inflammation/damage, neutrophils are one of the first cells recruited to the affected tissue and may act as potential initiators of pain. However, the majority of studies indicate that there is no substantial role for neutrophils in pain induction, since the development of inflammatory pain or incisional wound pain is not affected by neutrophil depletion[61,89,130]. Moreover, local recruitment of polymorphonuclear cells with CXCL1 and CXCL2/3 does not induce pain[131].

Given that mast cells are frequently found in close proximity to nerve endings, they are in a unique position to activate sensory neurons and induce pain. IgE-dependent activation of human mast cells induces itch. However, upon activation mast cells also rapidly release cytokines, NGF, proteases and histamine and bradykinin that induce pain[132,133]. In patients with chronic pain, such as

inflammatory bowel syndrome, RA and fibromyalgia, increased mast cell numbers are found in the inflamed tissues that correlated with the severity of pain symptoms[134,135]. In rodents, degranulation of mast cells causes immediate hyperalgesia in wild-type but not in mast-cell deficient mice[136]. Although these results point to some role of mast cells and granulocytes in the initiation of pain, potential roles in maintaining pain are thus far unknown.

Pain resolution

Neutrophils can release opioid peptides (β-endorphin, met-enkephalin, and dynorphin-A) that have anti-nociceptive effects through μ, δ or κ opioid receptors expressed by sensory neurons[137]. An anti-nociceptive role of neutrophils is evoked by corticotrophin releasing factor (CRF) injections that induce opioid secretion by neutrophils[138]. CRF attenuates CFA-induced inflammatory-hyperalgesia in rats in an opioid and granulocyte-dependent manner and intra-articular injections of CRF relieve post-operative pain in patients after arthroscopic knee surgery[138,139].

T-cells

Pain initiation and maintenance

Some evidence suggests that T-cells control the *initiation* of neuropathic pain, because T-cell infiltration into damaged nerves coincidences with the time when allodynia is developing[23,27]. In some neuropathic pain models mechanical allodynia is reduced in T-cell deficient mice at time points during the development of allodynia (day 3)[140,141]. There is some evidence that T-cells infiltrate the inflamed tissue after intraplantar CFA injections. However in T-cell deficient mice the pain sensitivity after CFA is not altered, suggesting that T-cells do not contribute to the initiation of inflammatory pain[89].

The majority of studies indicate that T-cells infiltrate spinal cord and DRGs during the *maintenance* of neuropathic pain and are thus more likely to contribute to the maintenance of pain[18,140-142]. T-cells are present in spinal cord starting from 1-2 weeks after nerve injury in different models of neuropathic pain[20,142]. The majority of infiltrating T-cells are $CD4^+$ and produce IL17 and IFNγ[140,142,143]. IL15 and IL23 produced by macrophages and dendritic cells drive T helper 17 cells to the spinal cord during the maintenance of neuropathic pain[141]. In T-cell deficient mice nerve injury-induced neuropathic pain is attenuated when pain has already

developed, whilst the initial development phase of neuropathic pain is intact[27,142]. Depletion of $CD4^+$ T-cells with intravenous CD4 antibodies also reduces hyperalgesia and allodynia once pain has already developed[23]. Similarly, in $CD4^{-/-}$ mice neuropathic pain is reduced only during the maintenance of neuropathic pain and is rescued by adoptive transfer of $CD4^+$ T-cells[140].

Some recent evidence suggests that the involvement of T-cells in pain maintenance is sex dependent. In female mice the expression of several T-cell markers in the spinal cord is almost 2-fold higher than in males after nerve injury and nerve injury-induced neuropathic pain is reduced in female but not in male T-cell deficient mice[144]. This immune system-related sex difference may be explained by sex-dependent IFNγ and IL-17A expression by CD4 T-cells[143,144]. The contribution of T-cells might not only be limited by sex but also by age, because the large T-cell infiltration and upregulation of IFNγ in the dorsal horn after spared nerve injury is only observed in adult and not in infant rats and mice[142]. This age-dependent involvement of T-cells in neuropathic pain could explain the clinical observation that in children neuropathic pain is less often observed[145]. However, increased production of IL4 and IL10 in the spinal cord also contributes to the diminished neuropathic pain development in neonatal rats and mice[146].

Resolution of pain

Adoptive transfer of T helper 2 cells reduces established neuropathic pain in an IL10-dependent manner, indicating that this T-cell subset has some anti-nociceptive roles[27]. Similarly, regulatory T-cells (Tregs) resolve pain. Systemic application of a CD28 superagonist, a Treg population expander, reduces the development of nerve injury-induced neuropathic pain and number of infiltrating T-cells in the damaged nerve[26]. Conversely, depletion of Tregs with cytotoxic CD25 antibodies or by using transgenic mice to selectively deplete $FOXP3^+$ T-cells prolong nerve injury-induced mechanical hypersensitivity[26,147]. In mice deficient for T-cells, transient chemotherapy-induced allodynia does not resolve and the resolution is rescued by reconstitution of $CD8^+$ T-cells but not by $CD4^+$ T-cells[69]. Importantly, this $CD8^+$ T-cell mediated resolution of chemotherapy-induced pain required IL10 signalling not by direct secretion of IL10 but rather through upregulating IL10 receptor expression in the DRG[69]. Thus, T-cells also control resolution of chemotherapy-induced neuropathic pain.

Overall, T-cells clearly have roles in development of neuropathic pain. However, specific T-cell subtypes and their secreted inflammatory products determine whether T-cells have a pro- or anti-nociceptive role. Whether T-cells also regulate inflammatory or other type of pain remains to be determined.

B-cells

Pain initiation and maintenance

Evidence for the involvement of B-cells in the initiation of pain mainly comes from studies that show that autoantibodies can induce pain[148]. Autoantibodies against citrullinated antigens (ACPAs; e.g. against citrullinated fibrinogen, vimentin, α-enolase, collagen type II, immunoglobulin-binding protein and histone 4) are increased in patients with RA[149]. Intravenous injection of purified ACPAs from RA patients or that from arthritic mice to healthy mice induces pain and increased heat and cold sensitivity without inducing inflammation[150]. ACPAs can be present years before RA diagnosis and may explain the pain-related problems of RA patients before the onset of clinical symptoms[151]. Mechanistically, ACPAs bind to osteoclasts to induce the release of CXCL1 (equivalent to human IL8) that activates sensory neurons and induces pain[152]. The majority of ACPAs are IgGs and the Fcγ receptor type 1 (FcγR1) is expressed by some sensory neuron subsets. Intradermal injection with IgG immune complexes produces hyperalgesia dependent of FcγR1 expression, indicating that IgG immune complexes also produce pain through activating neurons directly[153]. Moreover, during experimental arthritis, the number of sensory expressing FcγR1 is increased suggesting that during inflammation the sensory system becomes more sensitive for painful IgGs[153,154]. Autoantibodies are also detected in other autoimmune diseases associated with pain such as multiple sclerosis, Guillian-Barre syndrome and complex regional pain syndrome (CRPS)[155,156]. Moreover, anti-neuronal antibodies are detected in patients with CRPS and B-cell depletion in a mouse model of CRPS reduced pain[157,158]. Finally, treatment of neuroblastoma with antibodies against disialoganglioside produces severe pain as side-effect, indicating that some IgGs can induce pain[159].

Concluding Remarks

Immune cells and their mediators have important but distinct roles in regulating different types of pain (figure 1) indicating that the immune system and nervous system are intimately intertwined. The diverse roles of myeloid cells and T-cells in the initiation, maintenance and resolution of inflammatory and neuropathic pain are intriguing and question whether chronic pain conditions may be the results of defects in the immune system rather than merely nervous system defects. The intricate involvement of the immune system in pain regulation also highlights possibilities of using immune approaches for the treatment of pain. Regulating the subsets of these cells by inducing anti-nociceptive phenotypes may represent a strategy to prevent debilitating chronic pain conditions. In some clinical studies strategies have been tested to interfere with myeloid cell for treating neuropathic pain (e.g. CCR2, CSF1R antagonists), however these compounds failed to reduce pain scores[160]. Other approaches including targeting B-cells to prevent the production of autoantibodies (e.g. B-cell depletion strategies with anti-CD20) reduce arthritis disease onset, however this study only showed a limited improvement of VAS pain scores[161]. Although systemic anti-inflammatory strategies may have the risk of introducing infections, local (spinal) and/or transient administration of immunomodulatory compounds may reduce these risks. Finally, the use of anti-inflammatory cytokines for pain treatment remains a very promising strategy, but the best of our knowledge, clinical trials are yet to be conducted.

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3

CHAPTER 3

Macrophages Transfer Mitochondria to Sensory Neurons to Resolve Inflammatory Pain

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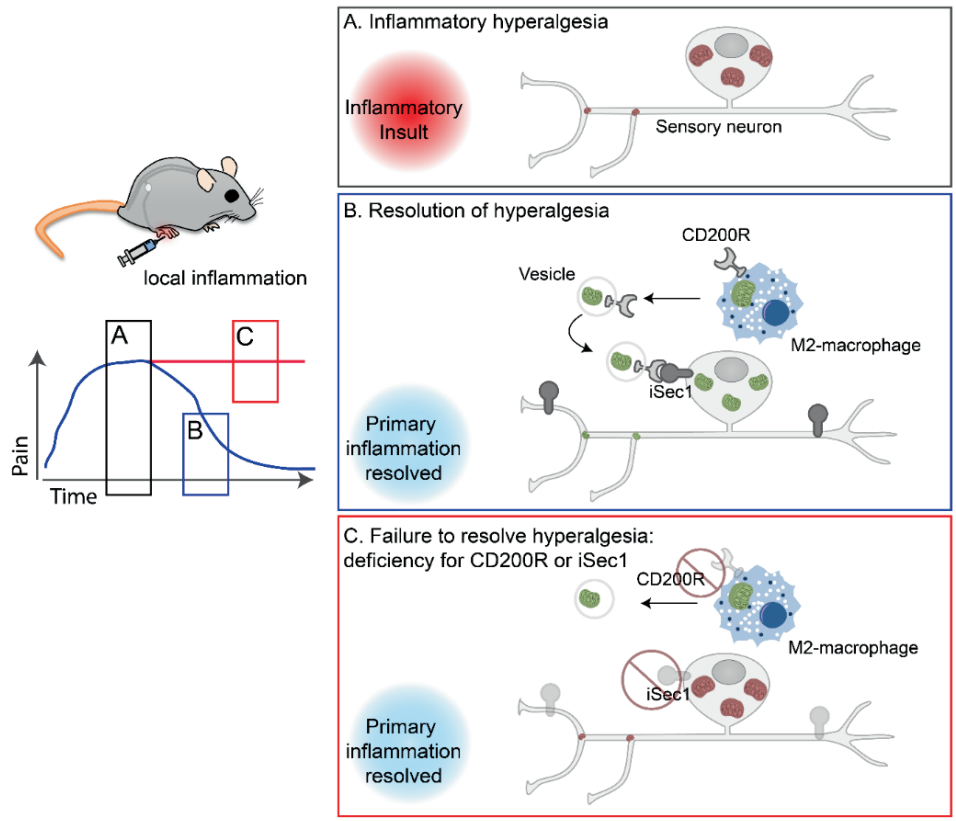
Graphical Abstract



Summary

The current paradigm is that inflammatory pain passively resolves following the cessation of inflammation. Yet, in a substantial proportion of patients with inflammatory diseases, resolution of inflammation is not sufficient to resolve pain, resulting in chronic pain. Mechanistic insight as to how inflammatory pain is resolved is lacking. Here we show that macrophages actively control resolution of inflammatory pain remotely from the site of inflammation by transferring mitochondria to sensory neurons. During resolution of inflammatory pain in mice, M2-like macrophages infiltrate the dorsal root ganglia that contain the somata of sensory neurons, concurrent with the recovery of oxidative phosphorylation in sensory neurons. The resolution of pain and the transfer of mitochondria requires expression of CD200 Receptor (CD200R) on macrophages and the non-canonical CD200R-ligand iSec1 on sensory neurons. Our data reveal a novel mechanism for active resolution of inflammatory pain.

Keywords

Inflammatory pain, Macrophage, CD200R, CD200, iSec1, Mitochondria, Sensory neurons, Pain resolution, Carrageenan, CFA

Introduction

Pain and pain hypersensitivity (hyperalgesia) are functional features of inflammation that serve to protect the tissue from further damage. At the site of inflammation, immune cells and inflammatory mediators, such as IL-1β, TNF, and bradykinin, sensitize and activate sensory neurons, which cause pain and hyperalgesia[1,2]. While the initiation of inflammatory pain is relatively well understood[3,4], the mechanisms of inflammatory pain resolution are less well characterized. Resolution of inflammatory pain is often considered to be the direct result of waning of inflammation. However, in a substantial proportion of patients with inflammatory diseases, such as rheumatoid arthritis and inflammatory bowel disease, spontaneous or treatment-induced resolution of inflammation does not reduce pain[5-9]. Basic discovery research to understand mechanisms of endogenous pain-resolution may help us understand how chronic pain develops when resolution pathways fail[10].

Macrophages are one of the most plastic cells of the immune system and are well known for their ability to induce tissue healing and resolution of inflammation[11]. Macrophages are strongly imprinted by their tissue of residence[12,13]. Peripheral nervous tissue shapes resident macrophages to have unique features compared to microglia and/or macrophages outside the nervous system[14-16]. After nerve damage, monocyte-derived macrophages engraft nervous tissue[14], are skewed by sensory neurons into an M1-like phenotype[17], and accumulate in the DRG to initiate and maintain neuropathic pain[18]. Thus, nervous tissue macrophages contribute to neuropathic pain. However, because macrophages can contribute to tissue healing and resolution of inflammation, we here set out to better understand the endogenous mechanisms for resolution of inflammatory pain and the role of macrophages in this process, using transient inflammatory pain models.

Results

We injected carrageenan into the hind paw of mice (intraplantar; i.pl.) as a model for transient inflammatory pain (Supplemental fig. 1A)[19]. Treated mice displayed signs of pain hypersensitivity, such as allodynia/hyperalgesia as assessed by the von Frey and Hargreaves tests, and postural changes measured with dynamic weight bearing. Carrageenan-induced hyperalgesia resolved within ~3-4 days (Fig. 1A). We analysed the cellular composition of lumbar (L3-L5) dorsal root ganglia (DRG) which contain the somata of sensory neurons innervating the hind paw and observed an accumulation of macrophages. Macrophage numbers peaked at day 3 and returned to baseline levels after resolution of inflammatory hyperalgesia (Fig. 1B/C, supplemental figs. 1B, and supplemental movies 1 and 2). Infiltration of macrophages was specific to the DRG that innervate the inflamed paw, and was not observed at the contralateral side (supplemental fig. 1C). In contrast, during the entire course of inflammatory hyperalgesia, T cells, B cells or other $CD45^+$ immune cell numbers in the DRG did not change significantly (Figs. 1B and supplemental fig. 1B). To address the function of these macrophages in pain resolution, we selectively depleted monocytes and macrophages by intraperitoneal (i.p.) injection of diphtheria toxin (DT) in *Lysm*^{cre} x *Csf1r* $^{LsL\text{-}DTR}$ mice [20] (from here on referred to as 'MM^{dtr}', supplemental fig. 2). DT administration depleted monocytes and macrophages in the DRG and blood (Supplemental figs. 2A-E) but did not affect the number and morphology of microglia in spinal cord (Supplemental figs. 2G-J). The induction and magnitude of carrageenan-induced hyperalgesia in these mice was normal. However, MM^{dtr} mice failed to resolve inflammatory mechanical hyperalgesia (Fig. 1D), thermal hyperalgesia (Supplemental fig. 3A) and postural changes related to inflammatory pain for at least six days (Fig. 1E) in both male and female mice (Fig. 1F). Similarly, MM^{dtr} mice failed to resolve Complete Freund's Adjuvant (CFA)-induced transient inflammatory hyperalgesia for at least 12 days (Fig. 1G, and supplemental fig. 3B).

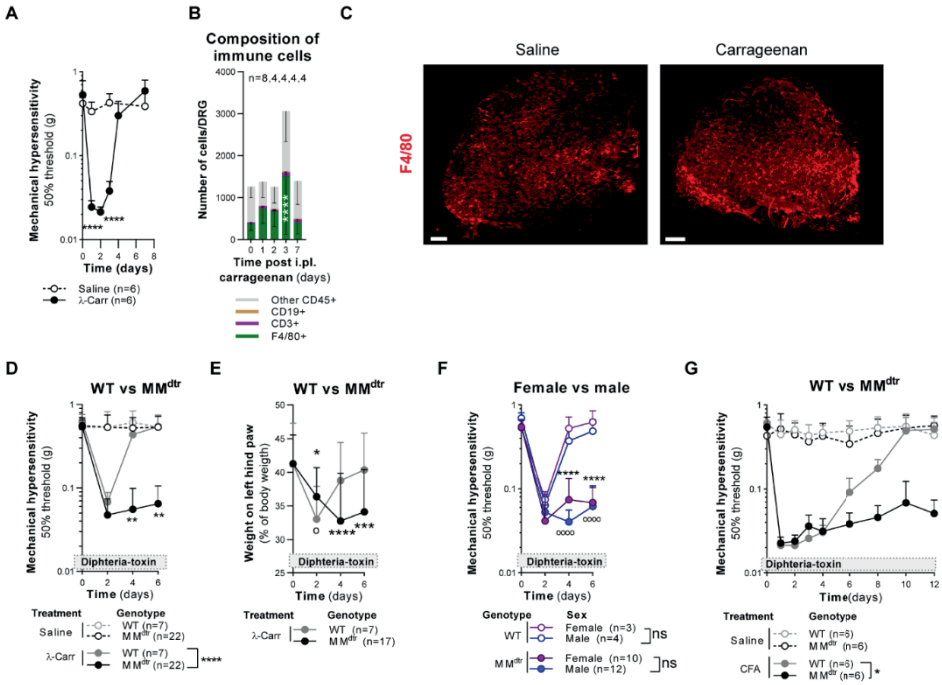


Figure 1. Monocytes/macrophages are required to resolve inflammatory pain

(A) *Course of mechanical hyperalgesia after i.pl. injection of 1% carrageenan in the left hind paw and saline in the right hind paw. Statistics tested by multiple t test.* ***(B)*** *Absolute number of CD45^{+} cells classified to subset per lumbar dorsal root ganglia (DRG, L3-L5) of mice that received 1% carrageenan. See Supplemental fig. 1C for gating strategy. 2-way ANOVA with Dunnett post-hoc.* ***(C)*** *Light-sheet render showing macrophage (F4/80, red) dispersed throughout an ipsilateral lumbar DRG isolated 1 day after saline or 1% carrageenan injection. See supplemental movies S1 and S2. Scale bar: 150µm.* ***(D)*** *Course of mechanical hyperalgesia in WT and MM^{dtr} littermates injected with 1% carrageenan in one and saline in the other hind paw. Mixed-effects model (REML), with Sidak post-hoc.* ***(E)*** *Course of weight bearing of the left hind paw (as % of total body weight) in WT and MM^{dtr} littermates injected with 1% carrageenan in one and saline in the other hind paw. 2-way repeated-measures ANOVA, comparing WT (o) and MM^{dtr} (*) versus day 0.* ***(F)*** *Course of carrageenan-induced mechanical hyperalgesia in male versus female in WT and MM^{dtr} littermates. 2-way repeated-measures ANOVA, astrics indicate significance between male(o)/female(*) WT vs male/female MM^{dtr} mice.* ***(G)*** *Course of CFA-induced mechanical hyperalgesia in WT and MM^{dtr} littermates. 2-way repeated-measures ANOVA, Sidak post-hoc comparing carrageenan conditions. See supplementary files for all related statistical values. Related data is available in supplemental figures 1-3.*

To directly target monocytes to the DRG[21], we intrathecally (i.t.) injected wildtype (WT) bone-marrow-derived CD115^{+} monocytes into MM^{dtr} mice

(Supplemental fig. 4A), which reconstituted macrophages in the ipsilateral DRG (Supplemental fig. 4B). Within hours, i.t. injection of WT monocytes sustainably rescued the defective resolution of hyperalgesia in MM^{dtr} mice (Fig. 2A and supplemental fig. 4C). The pain-resolving capacity of monocytes was independent of their origin (bone marrow or spleen; supplemental fig. 4D-F) or Ly6C expression ('classical' $Ly6C^+$ or 'non-classical' $Ly6C^-$; supplemental figs. 4D-F). These data show that monocytes are essential to resolve inflammatory pain.

Macrophages that reside in peripheral nerve tissue are different from microglia and non-nervous residing macrophages[16,22]. It was shown that after nerve injury, monocyte-derived macrophages engraft in the pool of resident peripheral nervous system macrophages[14], and are programmed by vesicles secreted by sensory neuron[17]. We determined whether monocytes/macrophages that infiltrate the DRG during inflammatory pain, had an inflammatory ('M1') - or resolution ('M2')-like phenotype. At day 3, the number of $CD206^+$ M2-like or tissue-repair macrophages[23,24] was increased in the DRG, whereas the number of $iNOS^+$ M1-like or inflammatory macrophages did not significantly change (Fig. 2B). Consistent with the dominant presence of $CD206^+$ macrophages, i.t. injection of *in vitro* differentiated bone-marrow derived macrophages ('M0', from here on referred to as 'macrophages') and macrophages subsequently differentiated with IL-4 ('M2') rescued resolution of hyperalgesia in MM^{dtr} mice (Figs. 2C-D, supplemental fig. 4G-H). In contrast, inflammatory macrophages differentiated with LPS and IFNγ ('M1') induced a transient hyperalgesia in the saline treated paws and were incapable of resolving inflammatory hyperalgesia in the carrageenan treated paws (Fig. 2D and supplemental fig. 4H). Macrophages resolved pain through a pathway independent of neuronal IL10 receptor (*Il10r*) signalling because $Nav1.8^{Cre}Il10r^{flox}$ mice, which are deficient for the IL10 receptor in pain-sensing sensory neurons that mediate inflammatory hyperalgesia[25], recovered normally (Supplemental fig. 4I).

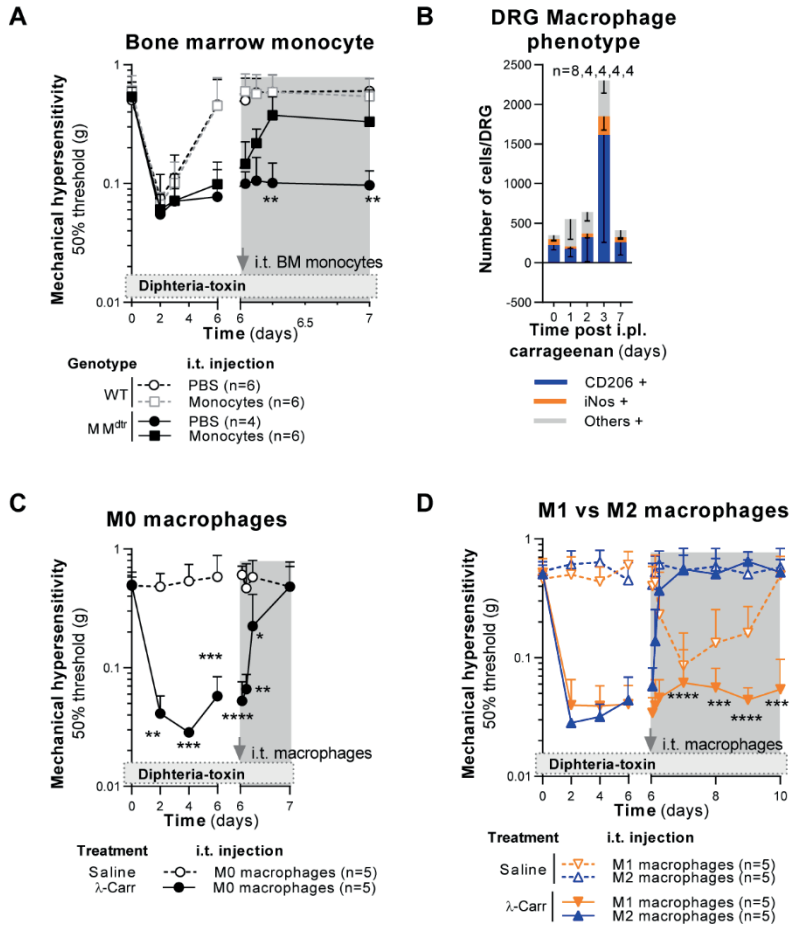


Figure 2. Monocytes/macrophages are required to resolve inflammatory pain

(A) *Course of mechanical hyperalgesia in WT and MM^{dtr} mice after i.pl. injection of 1% carrageenan in the hind paws and i.t. injection of PBS or WT CD115^{+} monocytes. 2-way repeated measure ANOVA, Sidak post-hoc comparing MM^{dtr} conditions.* ***(B)*** *Phenotype of F4/80 positive macrophages in DRG of mice i.pl injected with carrageenan in the hind paw at indicated time points. Gating strategy is indicated in Fig. S1C. 2-way ANOVA with Dunnett post-hoc.* ***(C)*** *Course of mechanical hyperalgesia in MM^{dtr} mice after i.pl. injection of 1% carrageenan in one and saline in the other hind paw, followed by i.t. injection of M0 macrophages. 2-way repeated measures ANOVA, with Sidak post-hoc.* ***(D)*** *Course of mechanical hyperalgesia in MM^{dtr} mice after i.pl. injection of 1% carrageenan in one and saline in the other hind paw, followed by i.t. injection of M1 or M2 macrophages. 2-way repeated measures ANOVA, with Sidak post-hoc comparing carrageenan conditions. See supplementary files for all related statistical values. Related data is available in supplemental figures 1 and 4.*

Metabolically, M2 macrophages depend on oxidative phosphorylation (OxPhos), while M1 macrophages are glycolytic (Supplemental fig. 5)[26,27]. Neurons have a high metabolic demand[28]. We previously demonstrated that a deficiency in mitochondrial function in sensory neurons prevents the resolution of inflammatory pain[21]. Moreover, in chronic pain neuronal mitochondrial functions, such as OxPhos and Ca^{2+} buffering, are impaired[29,30]. Indeed, oxygen consumption in DRG neurons was reduced with ~30% during the peak of inflammatory pain and resolved preceding pain resolution at day 3 (Fig. 3A). Therefore, we posited that to enable resolution of inflammatory pain, sensory neurons have to re-establish OxPhos by restoring a functional mitochondrial pool. Given that after ischemic stroke neurons can take up mitochondria released by adjacent astrocytes[31], we hypothesized that during inflammatory pain resolution monocytes/macrophages aid neurons by supplying mitochondria.

We stained mitochondria from macrophages with MitoTracker Deep Red (MTDR) that binds covalently to mitochondrial proteins [32] and co-cultured live macrophages, or an equivalent volume of sonicated macrophages, with the neuronal cell line Neuro2a (N2A). After 2 hours, macrophage-derived $MTDR^{+}$-mitochondria were detectable in N2A cells by flow cytometry and image stream (Supplemental figs. 6A/B). Macrophages transduced with mitochondria-targeted DsRed (mitoDsRed) also transferred mitochondria to primary sensory neurons upon co-culture *in vitro*, excluding that the signal was due to MTDR leak from macrophages to neurons (Supplemental fig. 6C). Transfer of mitochondria from macrophages to neurons also occurred *in vivo*. During the resolution of pain, we detected a significantly higher percentage of MitoDentra2 positive sensory neurons in the lumbar ipsilateral DRG of *LysM*^{Cre}-MitoDendra2^{flox} mice compared to the contralateral DRG, suggesting that that monocytes/macrophages transfer mitochondrial content to neurons during resolution of inflammatory pain (Figs. 3B/C). In contrast, MitoDendra2^{flox} mice or *LysM*^{Cre}-GFP^{flox} mice did not have any MitoDendra2 or GFP positive neurons, suggesting that MitoDentra2 positivity in neurons was not because of a leaky LysM promotor (Supplemental fig. 6D). In addition, intrathecal injection of MTDR-labelled macrophages in MM^{dtr} mice at day 6 after carrageenan injection increased the MTDR labelling (MFI and percentage) of sensory neurons of mice with persisting inflammatory hyperalgesia (Figs. 3D/E, supplemental fig. 6E), but not in control treated mice or

in WT mice that had resolved inflammatory hyperalgesia, which confirms mitochondrial transfer to sensory neurons. Injection of sonicated MTDR-labelled macrophages did not result in accumulation of MTDR in sensory neurons (Fig. 3D), excluding that the signal in sensory neurons was due to MTDR leak from macrophages. Using flow cytometry, we found that macrophages released $CD45^+$ extracellular vesicles that stained positive for macrophage plasma membrane proteins, such as MHC class II, CD11b and CD200 Receptor 1 (CD200R), and the mitochondrial dye MTDR (Supplemental fig. 7A/B). In line with the MTDR staining in vesicles, in the supernatant of $MitoDendra2^+$ macrophages ~17% of $CD45^+$ vesicles were also $MitoDentra2^+$ positive (Supplemental fig. 7C). The vesicles had a broad range in size (Supplemental fig. 7D).

We hypothesized that the mitochondria-containing vesicles released by macrophages were sufficient to resolve pain. Indeed, i.t. administration of mitochondria-containing extracellular vesicles isolated from macrophage supernatant rapidly but transiently resolved inflammatory hyperalgesia in MM^{dtr} mice (Fig. 3F, supplemental fig. 7E). However, injection of extracellular vesicles that were destroyed by sonication did not affect hyperalgesia (Fig. 3F). Taken together, this suggests that intact vesicles with mitochondria, but not their individual components such as lipids and proteins, are sufficient to resolve pain. In support for the need of functional mitochondria, monocytes that have distressed mitochondria and significantly reduced mitochondrial DNA (mtDNA) content due to a heterozygous deletion of the Transcription Factor A/*Tfam*[33] failed to resolve inflammatory hyperalgesia in MM^{dtr} mice (Supplemental figs. 8A/B). Finally, we isolated artificial vesicles containing mitochondria from M0 macrophage cell bodies (MitoAV). MitoAV stained positive for macrophage plasma membrane markers and MTDR and had active OxPhos (Supplemental figs. 8C and 11B). Intrathecal injection of MitoAV rapidly but transiently resolved inflammatory hyperalgesia in MM^{dtr} mice (Fig. 3G and supplemental fig. 8D). In contrast, MitoAV in which oxidative phosphorylation was blocked by complex III inhibitor myxothiazol[34] failed to resolve hyperalgesia (Fig. 3G and supplemental fig. 8D). Thus vesicles secreted by or isolated from macrophages contain mitochondria and resolve inflammatory pain.

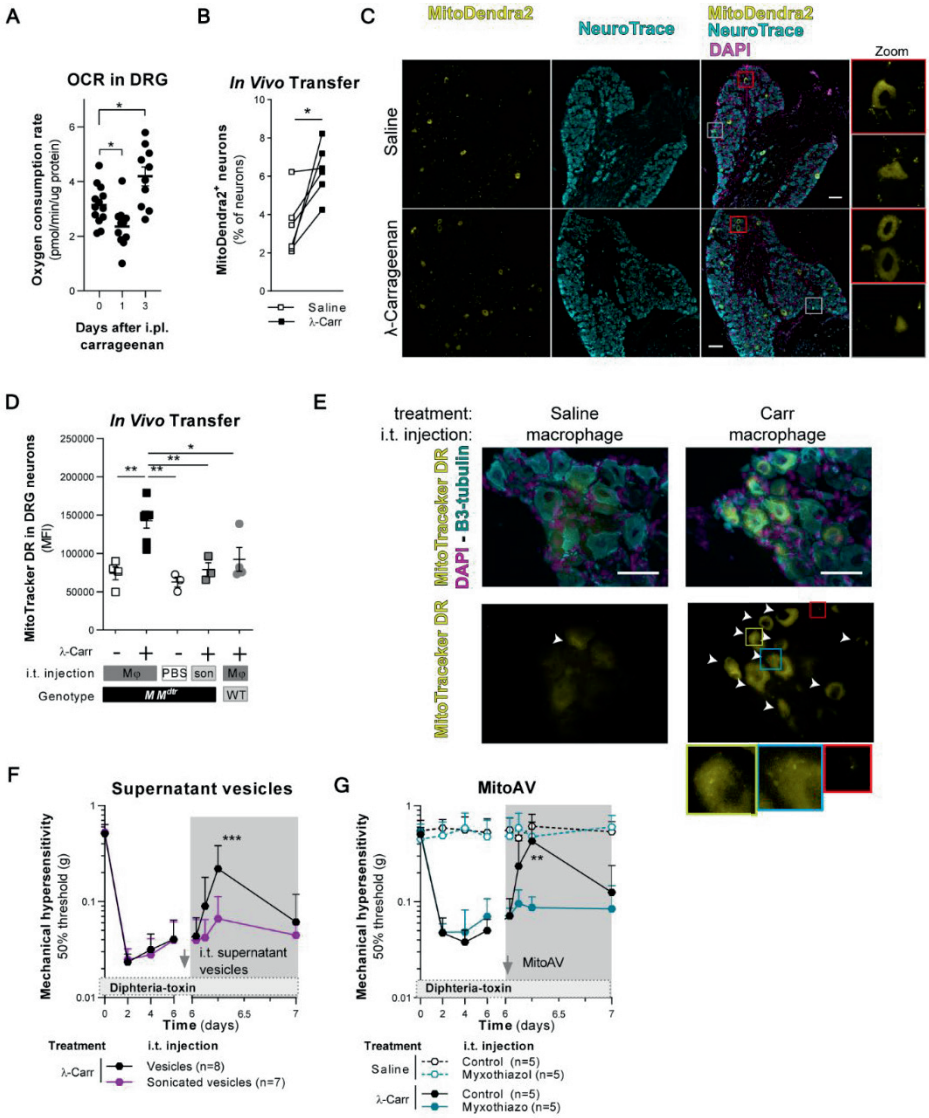


Figure 3. Macrophages migrate into the DRG and transfer mitochondria to neurons

(A) *Basal oxygen consumption rates in sensory neuron cultures obtained from lumbar DRG isolated from mice at indicated days post carrageenan injection in the hind paw. DRG from 1 or 2 mice were pooled per experiment and divided over 3-5 wells. Each dot is a well from a total of 5 mice assessed in 3 experiments. ANOVA with Hold-Sidak post-hoc.* ***(B, C) (B)*** *Quantification and* ***(C)*** *example images of percentage of MitoDendra2+ neurons in the contra- or ipsilateral DRG of LysMcre-MitoDendra2flox mice three days after carrageenan injection. scale bar: 150 µm. n=6. Paired-t Test.* ***(D, E) (D)*** *Quantification and* ***(E)*** *example images of MTDR signal in sensory neurons in the DRG of MM^{dtr} and WT litter*

mates. At day 6 after 1% carrageenan (ongoing pain in MM^{dtr}, resolved pain in WT mice) or saline injection, we injected i.t. PBS, MTDR-labelled macrophages (Mφ), or sonicated MTDR-labelled macrophages (son). After 18h, lumbar DRG were isolated for immunofluorescence analysis and counter-stained with β3-tubulin (cyan, neurons) and DAPI (magenta, nuclei). White arrowheads indicate MTDR^{+} (yellow) neurons. Scale bar: 50µm. ANOVA with Sidak's post-hoc. ***(F)*** *Course of mechanical hyperalgesia in MMdtr mice injected with carrageenan. At day 6 mice were injected i.t. with intact or sonicated macrophage-derived vesicles. 2-way ANOVA, Sidak post-hoc comparing carrageenan conditions.* ***(G)*** *Course of mechanical hyperalgesia in MMdtr mice injected with carrageenan in the left hind paw, and saline in the right hind paw. At day 6 mice were injected i.t. with artificially generated vesicle containing mitochondria (MitoAV) with functional or myxothiazol (complex III)-inhibited mitochondria. 2-Way ANOVA with Dunnett post-hoc. See supplementary files for all related statistical values. Data are represented as mean±SD, and mean±SEM in graphs showing individual data points. Related data is available in supplemental figures 5-8.*

For efficient transfer of mitochondria, we hypothesized that docking of extracellular vesicles to sensory neurons is facilitated by receptor-ligand interactions. Macrophages, predominantly those with an M2 phenotype[35], and macrophages-derived extracellular vesicles expressed CD200R (Fig Supplemental fig. 7A), while neurons are known to express its ligand CD200 (ref.[36]). In line with this reasoning, *Cd200r*$^{-/-}$ mice failed to resolve inflammatory hyperalgesia, which persisted for at least 16 days (Fig. 4A and Supplemental fig. 9A). Place-preference conditioning with the fast-working analgesic gabapentin[37], a drug that relieves neuropathic and inflammatory pain[38,39], confirmed ongoing spontaneous pain in *Cd200r*$^{-/-}$ mice for at least 2 weeks after carrageenan injection (Fig. 4B, supplemental fig. 9B). Of note, acute inflammation and the resolution of inflammation at the site of carrageenan injection in *Cd200r*$^{-/-}$ mice did not differ from that of WT mice (Figs. 4C and 4D). This further supports the role of CD200R in the resolution of acute inflammatory pain and prevention of chronic pain.

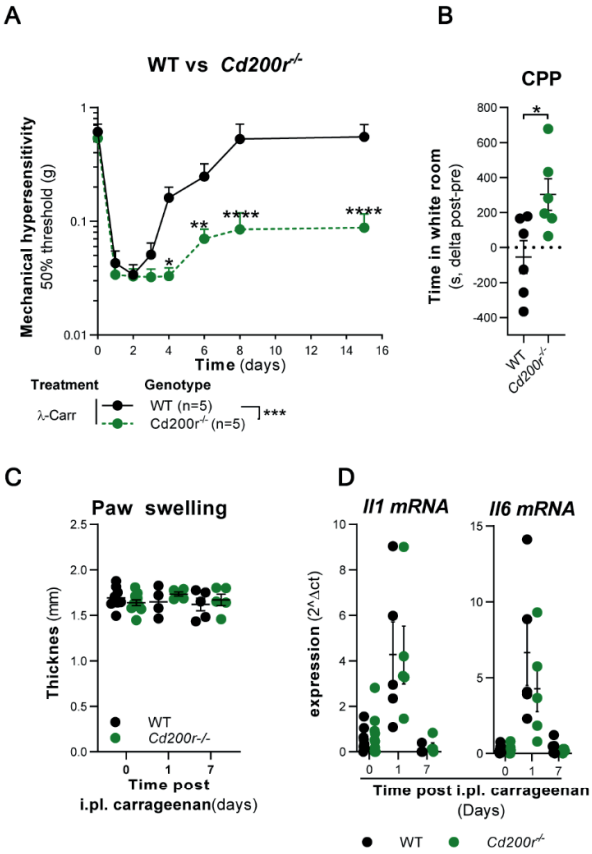


Figure 4. Monocytes require CD200R to resolve inflammatory pain

(A) *Course of mechanical hyperalgesia in Cd200r$^{-/-}$ or WT mice that were unilateral injected with 1% carrageenan in the hind paws. 2-way ANOVA, Sidak post-hoc.* ***(B)*** *Gabapentin-induced place preference conditioning at day 16 after unilateral 1% carrageenan injection in the hind paws. Conditioning efficiency is depicted as the difference in time (seconds, s) spent in a white room pre- and post-conditioning. Unpaired t-test.* ***(C)*** *Paw swelling of the carrageenan-injected paw in WT and Cd200r$^{-/-}$ mice. 2-way repeated measures ANOVA, Sidak.* ***(D)*** *mRNA expression of cytokines Il1 and Il6 in the carrageenan-injected paw of WT and Cd200r$^{-/-}$ mice. 2-way repeated measures ANOVA, Sidak. See supplementary files for all related statistical values. Summarized data are represented as mean±SD, and mean±SEM in graphs showing individual data points. Related data is available in supplemental figure 9.*

Intrathecal injection of *Cd200r*$^{-/-}$ monocytes did not resolve inflammatory hyperalgesia in MM^{dtr} mice (Fig 5A, supplemental fig. 10A). Consistent with these data, WT monocytes or macrophages resolved persisting inflammatory hyperalgesia in *Cd200r*$^{-/-}$ mice, whereas injection of additional *Cd200r*$^{-/-}$ monocytes or macrophages did not (Fig. 5B; supplemental fig. 10B). These data indicate an intrinsic defect in the pain-resolution capacity of *Cd200r*$^{-/-}$ monocytes and macrophages independent from effects of macrophages at the site of primary inflammation.

We found no evidence for a defect in mitochondrial respiration or vesicle release in *Cd200r*$^{-/-}$ macrophages (Supplemental figs. 11A-C) and *Cd200r*$^{-/-}$ macrophages were normal in their capacity to migrate into the DRG and had a similar phenotype to WT macrophages (Supplemental figs. 11D-H). This suggested instead that there was a defect in mitochondrial transfer between *Cd200r*$^{-/-}$ macrophages and neurons. MTDR-labelled mitochondria did transfer from intrathecally injected MTDR-labelled wild type macrophages to neurons from *Cd200r*$^{-/-}$ mice (Fig. 5C). In contrast, *Cd200r*$^{-/-}$ macrophages failed to transfer MTDR-labelled mitochondria to sensory neurons of *Cd200r*$^{-/-}$ mice (Fig. 5C; supplemental fig. 12A). Thus, CD200R expression on macrophages but not on neurons is required for transfer of mitochondria from macrophages to neurons. In addition, since no MTDR staining was observed in neurons upon injection of MDTR-labelled *Cd200r*$^{-/-}$ macrophages, we exclude MTDR leaking from macrophages. Extracellular vesicles isolated from *Cd200r*$^{-/-}$ macrophage culture supernatant or supernatant from WT vesicle-pellets did not resolve inflammatory hyperalgesia in MM^{dtr} mice (Fig. 5D; supplemental fig. 12B). Thus, CD200R expression on extracellular vesicles, and not soluble factors produced by macrophages, is required for the resolution of inflammatory pain.

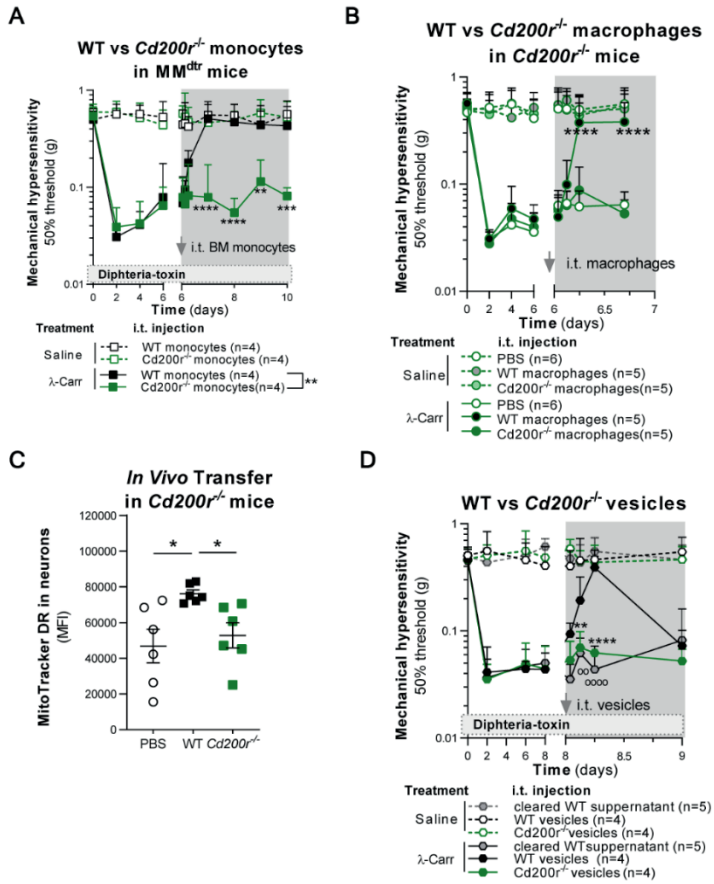


Figure 5. Cd200r deficient monocytes/macrophages fail to resolve inflammatory pain

(A) *Course of mechanical hyperalgesia in MMdtr mice that were injected unilateral with 1% carrageenan and saline. At day 6, WT or Cd200r$^{-/-}$ CD115^{+} monocytes were i.t. injected. 2-way repeated measures ANOVA, Sidak post-hoc comparing carrageenan conditions.* ***(B)*** *Course of mechanical hyperalgesia in Cd200r$^{-/-}$ mice that were injected unilateral with 1% carrageenan and saline. At day 6, WT or Cd200r$^{-/-}$ macrophages were i.t. injected. 2-way repeated measures ANOVA, Sidak post-hoc comparing carrageenan conditions.* ***(C)*** *In vivo MTDR transfer from WT or Cd200r$^{-/-}$ MTDR-labelled macrophages to DRG neurons in Cd200r$^{-/-}$ mice. At day 6 after carrageenan injection, macrophages or PBS were injected i.t. and after 18h DRG were isolated and stained as described for Fig. 3D/E. ANOVA with Holm-Sidak post-hoc.* ***(D)*** *Course of mechanical hyperalgesia in MM^{dtr} mice injected unilateral with 1% carrageenan and saline. At day 6, vesicles isolated from WT or Cd200r$^{-/-}$ macrophage culture supernatant, or the supernatant of the vesicle pellet (cleared supernatant) was i.t. injected. 2-way repeated measures ANOVA, Dunnett post-hoc comparing carrageenan conditions. See supplementary files for all related statistical values. Data are represented as mean±SD, and mean±SEM in graphs showing individual data points. Related data is available in supplemental figures 9-12.*

CD200 is the best-known ligand for CD200R and in inflammatory models, such as arthritis, *Cd200*$^{-/-}$ and *Cd200r*$^{-/-}$ mice have a similar phenotype[40,41]. However, in sharp contrast to *Cd200r*$^{-/-}$ mice, *Cd200*$^{-/-}$ mice completely resolved inflammatory pain with similar kinetics to WT mice (Fig. 6A; supplemental fig. 13A). This suggests the involvement of an alternative CD200R ligand. In 2016, iSec1/*Gm609* was described as a CD200R ligand expressed specifically in the gut[42]. We found that iSec1*/Gm609* mRNA is also expressed in DRG, along with *CD200* mRNA (Supplemental figs. 13B/C). Repetitive intrathecal injections of iSec1*/Gm609* targeting antisense oligodeoxynucleotides (ASO)[43] silenced iSec1 mRNA expression in the DRG of WT mice (Fig. 6B and supplemental fig. 13D) and partially prevented resolution of inflammatory hyperalgesia (Fig. 6C; Supplemental fig. 13E). In *Cd200*$^{-/-}$ mice, i.t. injections of *iSec1/Gm609*-ASO completely prevented the resolution of hyperalgesia (Fig. 6D; supplemental fig. 13F). Next, we injected Herpes Simplex Virus (HSV) encoding iSec1 intraplantar to specifically target sensory neurons innervating the inflamed area[21]. Expression of iSec1*/gm609* that was mutated to resist ASO treatment in sensory neurons (HSV-iSec1^{res}, Fig. 6E) completely rescued the ability of iSec1*/Gm609*-ASO treated *Cd200*$^{-/-}$ mice to resolve pain, while an empty vector (HSV-e) did not (Fig. 6F; Supplemental fig. 13G). We conclude that monocyte/macrophage expression of CD200R and sensory neuron expression of the ligand iSec1 is required *in vivo* to resolve inflammatory pain.

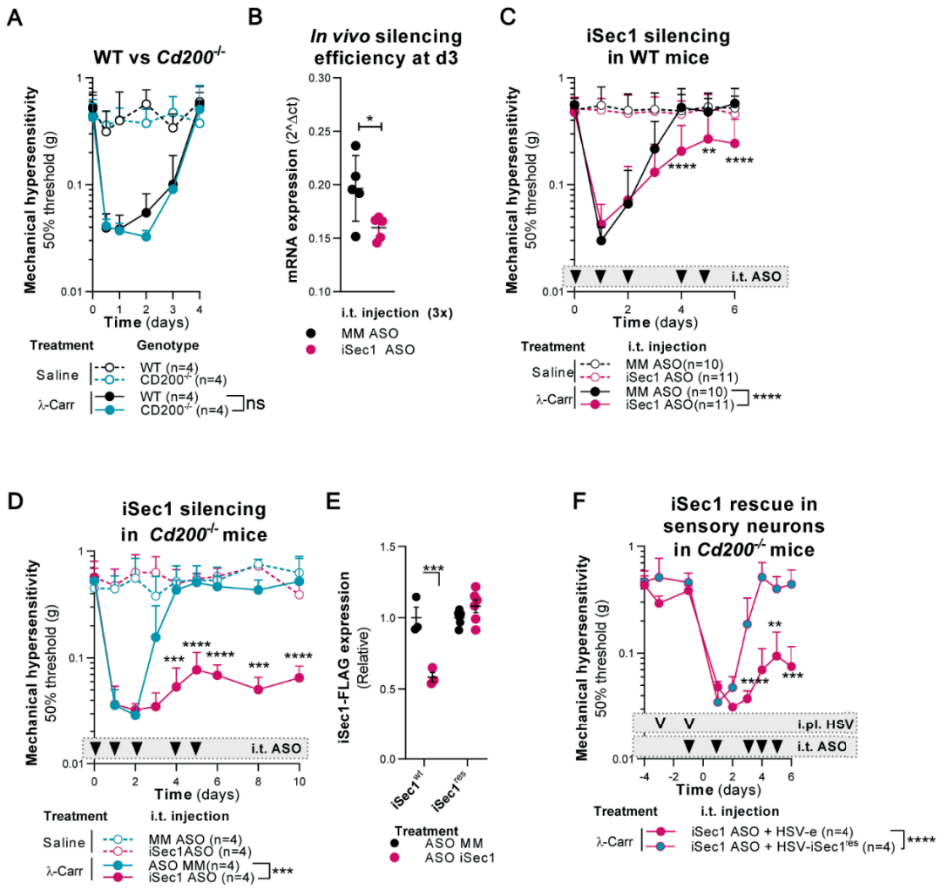


Figure 6 – iSec1 is required for resolution of pain

(A) *Course of carrageen-induced mechanical hyperalgesia in WT or Cd200$^{-/-}$ littermates. 2-way repeated measures ANOVA, Sidak post-hoc comparing carrageenan conditions.* ***(B)*** *iSec1/gm609 mRNA expression after silencing of iSec1/gm609 in DRG of WT mice treated with mismatch (MM-ASO) or iSec1-targeting Antisense Oligo nucleotides (iSec1-ASO). Unpaired t-test.* ***(C-D)*** *Course of carrageen-induced mechanical hyperalgesia in WT* ***(C)*** *or Cd200$^{-/-}$* ***(D)*** *mice injected with mismatch (MM-ASO) or iSec1-specific antisense oligonucleotides (iSec1-ASO). 2-way repeated measures ANOVA, Sidak post-hoc comparing carrageenan conditions.* ***(E)*** *Expression of iSec1^{wt}-flag (n=3 from 1 experiment) and iSec1^{res}-flag (n=6 from 2 experiments) as assessed by flow cytometry. 2-way ANOVA, Sidak post-hoc.* ***(F)*** *Course of carrageen-induced mechanical hyperalgesia in Cd200$^{-/-}$ mice that received intraplantar (i.pl) with HSV-e or HSV-iSec1^{res} before i.pl. carrageenan injection, treated with iSec1-specific ASO injected i.t. 2-way repeated measures ANOVA, Sidak post-hoc. See supplementary files for all related statistical values. Data are represented as mean ±SD. Related data is available in supplemental figure 13.*

Discussion

We identified a previously unappreciated role for macrophages which transfer mitochondria to somata of sensory neurons to resolve inflammatory pain. Previous studies showed that respiratory competent mitochondria are present in human whole blood[44], and that tissue-resident cells can transfer mitochondria[31,45]. We now show that non-tissue resident monocytes are recruited to the DRG, acquire a M2/tissue-repair like phenotype and transfer mitochondria to sensory neurons via a CD200R:iSec1 interaction to facilitate resolution of inflammatory pain. In contrast to M2 macrophages, inflammatory M1 macrophages induced pain. Thus, a DRG-milieu that skews local macrophages towards a M1 phenotype could contribute to the development of chronic pain[46-49].

Previous studies have implicated macrophages in resolution of inflammatory pain at the site of the primary inflammatory insult by secretion of IL-10[50,51], or by clearance of the inflammatory agent zymosan[52]. We now found that macrophages are necessary to resolve pain distant from the primary inflammatory insult independent of the anti-inflammatory capacities of macrophages at the site of the primary insult. Importantly, resolution of inflammatory pain was independent of IL-10 Receptor signalling in sensory neurons, excluding a direct effect of IL-10 on neurons in resolution of inflammatory pain.

Our data show that transfer of mitochondria by macrophages in the DRG is required for resolution of inflammatory pain. However, it is possible that macrophages have additional roles in other areas of the nervous system, including nerves or nerve endings. Also, we cannot exclude other cells to contribute to resolution of inflammatory pain. For example, Csf1r/LysM negative macrophages at the site of the primary inflammatory insult or satellite glial cells in the DRG that surround the soma of sensory neurons may play additional roles[50-52].

Previously intercellular transfer of cellular organelles was shown[53]. We show with the use of chemical and genetic approaches that macrophages transfer of mitochondria to neurons *in vitro* and *in vivo*. These experiments indicated that ~3-20% of neurons acquired mitochondria from macrophages during

inflammation, which is concordant with the percentage of somata of neurons in the DRG that innervate the hind paw [54-56]. Intriguingly, also under non-inflammatory conditions, we observed that neurons acquire some mitochondria, yet to a lesser extent than after induction of inflammatory pain. It remains to be determined what determines which neurons acquire macrophage-derived mitochondria, and whether this transfer is induced due to control injection or that transfer occurs in naïve conditions. Unfortunately, we cannot answer this question in the current experimental set-up.

Various chronic pain states, such as chemotherapy-induced pain and neuropathic pain caused by trauma or diabetes, are associated with mitochondrial defects[57-60]. We show here that oxidative phosphorylation is reduced during the peak of transient inflammatory pain but is restored when inflammatory hyperalgesia resolves. Mitochondrial respiration had restored at day 3 whilst full pain resolution in the carrageenan model occurs at day 4. Hence, we postulate that to enable resolution programs in sensory neurons mitochondrial homeostasis in sensory neurons first needs to be restored, a process facilitated by DRG macrophages.

Given that the injection of isolated extracellular vesicles transiently resolves pain, a more durable resolution of pain requires a prolonged flux of mitochondria and/or additional signals from intact macrophages. These mitochondria could replace mitochondria in neurons that have incurred mitochondrial damage. Future work should assess how exactly neuronal mitochondrial homeostasis is restored by macrophage-derived mitochondria.

Although diverse structures, such as tunnelling nanotubes, can mediate intercellular mitochondrial transfer[61,62], our data show that mitochondria containing vesicles are sufficient to resolve pain. We cannot exclude that other mechanisms such as tunnelling nanotubes or cytoplasmic fusions may contribute to the observed transfer of mitochondria to sensory neurons.

CD200 has long been thought of as the only ligand for CD200R. Although previous studies implicate CD200 as a checkpoint for microglia cell activation in neuropathic pain by ligating microglial CD200R[63,64], we show that *Cd200*$^{-/-}$ mice fully resolve inflammatory pain. Furthermore, we found that iSec1/*gm609* is expressed in DRG neurons and we demonstrated that sensory neuron-iSec1 is required to resolve inflammatory pain. Of note, iSec1/*gm609* knockdown did

have a greater effect on pain resolution in *Cd200*$^{-/-}$ mice than in WT mice, suggesting that the function of these ligands is partially redundant.

Why would sensory neurons require external help to restore the integrity of their mitochondrial network? Sensory neurons face unique challenges in maintaining a functional mitochondrial network because of their exceptional architecture and their intense demand for energy to support energetically expensive processes such as resting potentials, firing action potentials and calcium signalling[28,65]. Stressed neurons, e.g. during inflammatory pain, turn to anabolic metabolism[66]. In the face of this high energy demand during stress, an energy consuming process such as rebuilding the mitochondrial network would not be favourable. Moreover, maintaining an excess mitochondrial pool that is capable of handing the stress of inflammatory pain would come at a fitness cost. Thus, we propose that it would be more energy favourable for the organism to fulfil peak energy demands in indispensable sensory neurons by mitochondrial transfer from dispensable monocytes/macrophages.

Together, our data show that pain is actively resolved by an interaction between the immune and neuronal systems that is separate from the cessation of inflammation within the peripheral tissue. Novel therapeutic strategies to resolve chronic pain may focus on the restoration of mitochondrial homeostasis in neurons or on enhancing the transfer of mitochondria from macrophages.

Acknowledgments

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Author contributions: Conceptualization (LM, MV, NE), Methodology (NE, LM, MV, HW, RR, JP, RL), Formal Analysis (MV, NE, HW, RR, JP), Investigation (LM, NE, MN, RR, HW, MVos, WKH, MV, SV, RL), Resources (RJP), Writing original draft (MV, NE, RR), Writing reviewing and editing (MV, NE, LM, RR, JP, HW, WKH), Visualization (MV, NE, RR), Supervision (MV, HW, NE, LM, RR), Project Administration (NE, LM), Funding Acquisition (HW, MV, NE, LM).

Competing interests: Authors declare no competing interests.

Materials and Methods

Animals

All experiments were performed in accordance with international guidelines and approved by the experimental animal committee of University Medical Center Utrecht (2014.I.08.059) or by the local experimental animal welfare body and the national Central Authority for Scientific Procedures on Animals (CCD, AVD115002015323).

Adult (age 8–15 weeks) male and female C57Bl/6J, *Lysm*^{Cre} x *Csf1r*$^{LsL\text{-}DTR}$(Jackson laboratories #024046)(MM^{dtr}), *Nav1.8*^{Cre}*Il10r*^{flox}, *Cd200*$^{-/-}$(ref.[40]), *Cd200r*$^{-/-}$ (ref.[67]), *Lysm*^{Cre} x PhAM^{flox} (LysM^{MitoDendra2}, Jackson laboratories #18397) mice in a CD57Bl/6 background were used and maintained in the animal facility of the University of Utrecht. *Nav1.8*^{cre} mice were kindly donated by Dr. Wood (University College London, UK). *Il10r*^{flox} mice were backcrossed from *Lysm*^{cre}*Il10r*^{flox} mice that were kindly donated to us by Dr. Muller (University of Manchester, UK). *Cd200r*$^{-/-}$ were kindly donated by Dr. R.J. Soberman (Harvard Medical School). Mice were housed in groups under a 12h:12h light:dark cycle, with food and water available *ad libitum*. The cages contained environmental enrichments including tissue papers and shelter. To minimize bias, animals were randomly assigned to the different groups prior to the start of experiment, and experimenters were blinded for the treatments and genotypes. For all genetic backgrounds the littermates of heterozygous breeding's with the specified genotype were used. In all experiments, we used both sexes based on availability to correctly control for genetic background and age.

Transient inflammatory pain models

For the carrageenan model, mice received an intraplantar injection of 5 µl λ-carrageenan (1% w/v, Sigma-Aldrich) in one or both hind paws[68]. For the transient complete Freund's adjuvant (CFA) model, mice received intraplantar injection of 2.5 µl mix of 1:1 saline and CFA (Sigma-Aldrich). In experiments where mice received a unilateral intraplantar injection, latency times and 50% thresholds of each paw was considered as an independent measure, while in experiments with bilateral intraplantar injection the average of the left and right paw were considered as an independent measure.

Pain behavioral tests

Prior to the start of experiments, mice were first acclimatized to the testing environment by placing the mice in the in test environment for at least 3 times for 1 hour, 1-2 weeks before starting of the experiments. Behavioral assays are performed at the same time point at the day per individual experiment, preferably between 9-15h. At least 3 baselines measures are performed at different days one week before starting the experiment, with the last one at the day of the start of the experiment. Experiments were performed in the same room and same test setup over the duration of the experiment. Experimenters were well trained to perform the assays and were blinded for the experimental groups and genotypes. Experimenters did not wear any type of perfume or musk-based deodorants at the time of assessing behavior. Mice were put into the testing environment (von Frey and Hargreaves) at least 30 minutes before performing measurements with the experimenter present in the same room. The outside of the plexiglass chambers (Size: 13.0 × 25.0 × 15.0 cm) used for the von Frey and Hargreaves setup were covered to avoid that mice could see the experimenters.

Mechanical thresholds were assessed in both hind paws using the von Frey test (Stoelting, Wood Dale, IL, US) with the up-and-down method to determine the 50% threshold[69,70]. In short, von Frey filaments were applied for 5 seconds to the plantar surface of the paw. After applying the first filament (0.4 g), in case of a non-response the next filament with a higher force was used. In case of a response, the next lower force filament is used. A minimum of 30 seconds between the application of filaments was taken. Four readings were obtained after the first change of direction. Experimenters applied the von Frey filaments perpendicularly, smoothly, without moving the filaments horizontally during application. If the respond was ambiguous, the experimenter waited for a minute and reproved the mouse. Heat withdrawal latency times were determined in both hind paws using the Hargreaves test (IITC Life Science)[70,71]. Briefly, the Hargreaves test was carried out using a perspex enclosure on a heated (32°C) glass bottom enclosure. A radiant heat source is positioned underneath the animals and aimed at the plantar surface of the hind paw. The time taken to withdraw from the heat stimulus is recorded as the withdrawal latency. Each paw is measured at least 3 times with at least 30 seconds between measurements. The intensity of the light source was adjusted to produce withdrawal latencies of

8-10 seconds in naive C57Bl/6 mice, with a pre-determined cut off at 20 seconds to prevent tissue damage.

Changes in weight bearing were evaluated using the dynamic weight bearing (DWB) apparatus (Bioseb, Vitrolles, France)[72] and the following parameters were used for the analysis. (i) Low weight threshold of 0.5g, (ii) High weight threshold of 1g, (iii) Surface threshold of 2 cells, (iv) Minimum 5 images (0.5 seconds) for stable segment detections. The device consists of a small Plexiglas chamber (11.0 × 19.7 × 11.0 cm) with a sensor mat containing pressure transducers. The system records the average weight that each limb exerts on the floor. Mice were placed in the chamber at least 1 minute prior to starting the measurements and were allowed to move freely within the chamber. Each mouse was recorder for period of 5 minutes. A camera at the top of the enclosure was used to record all movements to ensure accurate validation of the position of the mouse by the experimenter. The weight bearing of the affected paw (ipsilateral paw) was expressed as percentage of body weight.

Spontaneous pain was assessed by the conditioned place preference test (CPP, Stoelting, Wood Dale) as described previously[37,73]. The CPP apparatus consisted of 2 visually distinct chambers (18.0 × 20.0 cm) connect by a smaller neutral chamber (10.0 × 20.0 cm). The two chambers had differing visual cues, one being darker ('dark chamber') and one brighter ('bright chamber'). Prior to the start of CPP, mice were acclimatized to the setup. At the baseline measurement day, the animals were placed in the center hallway with free access to both chambers. The time spent in each chamber was recorded for 15 min to determine preconditioning baseline. In case a mouse preferred the bright chamber they were excluded from further testing. Mice underwent three trial conditionings the following 3 day. In the morning of the conditioning day, the animals received intraperitoneal saline for 20 min in the preferred 'dark' chamber, with no access to the other chambers. In the afternoon approximately 4 hours later, mice received intraperitoneal gabapentin (100 mg/kg, Sigma-Aldrich) and were placed in the non-preferred 'bright' chamber for 20 min, with no access to the other chambers. After the three trial conditioning animals were placed back into the hallway between the CPP chambers with free access to all chambers for 15 min. The time spent in each chamber was again recorded. CPP was calculated by subtracting the mean time spent in the bright chamber during preconditioning

(day 1) from the time spent in the bright chamber after 3 days of conditioning (day 5).

Depletion of monocytes and macrophages

To deplete monocytes and macrophages in vivo, MM^{dtr} mice received a first intraperitoneal injection of 20 ng/g body weight diphtheria toxin (DT) (Sigma-Aldrich) followed by daily intraperitoneal injections of 4 ng/g body weight on all subsequent days as described previously[20].

Monocyte isolation and in vitro differentiation

To obtain bone marrow-derived monocytes, the tibia and femur bones were flushed with phosphate buffered saline (PBS). For spleen-derived monocytes, spleens were mechanically excised and minced in PBS and passed through a 70 µm cell strainer (Corning). After erythrocyte lysis with RBC lysis buffer (eBioscience), cells were centrifuged on a Ficoll density gradient (GE Healthcare) for 22 min at 1100RCF at 22°C to obtain mononuclear cells. Finally, $CD115^{+}$ monocytes were isolated with biotin labeled anti-CD115 antibody and streptavidin-coupled magnetic beads according to the manufacturer's instructions (Miltenyi Biotec).

To obtain classical ($Ly6c^{hi}$) or non-classical ($Ly6c^{low}$) monocytes for adoptive transfer, monocytes were FACS sorted (FACS Aria III, BD) using CD115 and Ly6c antibodies (see subheading 'antibodies'). *Tfam*$^{+/-}$ splenocytes and bone-marrow cells were isolated at Yale School of Medicine and shipped frozen in 10% DMSO to the Netherlands.

For monocyte-derived macrophage generation, 10 million bone-marrow cells were seeded in a 75 cm^2 non-treated tissue culture flasks (VWR, Radnor, PA) for 7 days in macrophage medium (High-glucose Dulbecco's Modified Eagle medium (DMEM; Cat# 31966-021, Gibco) and DMEM/F12 (Cat#31331-028, Gibco) (1:1), supplemented with 30% L929 cell-conditioned medium (see cell lines and primary cell cultures), 10% fetal bovine serum (FBS; Cat# 10270-106, Gibco), 1% Penicillin/Streptomycin (Gibco) and 1% L-Glutamine (200 mM, ThermoFisher).

To polarize macrophages toward M1- or M2-like macrophages, cells were stimulated with 20 ng/ml IFNγ and 100 ng/ml LPS, or 20 ng/ml of IL-4 for 24 hours, respectively.

Adoptive Transfer of monocytes and macrophages

Cells were injected intrathecal (30.000 cells/5 µl per mouse) under light isoflurane anesthesia as described previously[70,74]. For some experiments, 2 million macrophages were labeled with 20nM of MitoTracker DeepRed FM (MTDR; Thermo Fisher Scientific) in 400µl macrophage-medium for 30 minutes at 37°C, followed by 3 washes before intrathecal injection of 30.000 cells. For some experiments, cells were sonicated with a sonicator (soniprep150, MSE, UK) at a frequency of 23 kHz, 3 times for 15 seconds on ice before injection.

Isolation and injection of extracellular vesicles

To isolate macrophages-derived extracellular vesicles, 10 million macrophages were in a 75 cm^2 flask the day prior to the isolation of vesicles. The supernatant from 10 flasks were collected and centrifuged at 2000RCF for 10 minutes to pellet large debris[31]. The supernatant was divided in 1.5 ml Eppendorf tubes followed by a centrifugation at 17,000RCF for 30 minutes at 4°C. Supernatant was discarded, except the last 50 µl to resuspend the pellets and pool them together. The pooled supernatant was centrifuged at 17.000RCF for 30 minutes. The final supernatant was collected as 'cleared supernatant', and the pellet was resuspended in 100 µl PBS and injected intrathecally (5 µl per mouse).

To destroy vesicles, supernatant vesicles were sonicated 3 times at a frequency of 23 kHz for 15 seconds on ice before injection.

NTA analysis of extra cellular vesicles

For analysis of extracellular vesicles, 4 million macrophages were seeded in a T75 flask and cultured for 4 days, then they were washed and 7 ml of plain Opti-Mem (Gibco, 31985062) was added for an additional 24h. As control, mock-treated medium that was treated identically except for no macrophages had been present. Supernatant was harvested, spun down at 2000G to remove cell debris, followed by centrifugation at 17.000G to pellet the vesicles. The pellets were resuspended in PBS and measured in a Nanosight NS500 analyser (Melvern Intruments) equipped with a 405 nm laser. NTA analysis was acquired in NTA 3.3 Dev Build 3.3.104, the camera level was set at 10, and detection threshold was set at 5, all other settings were automated.

MitoAV: isolation and injection of mitochondria

As described before[75], in brief: 10 million macrophages were seeded in a 75 cm^2 flask the day prior to the isolation of mitochondria. To inhibit mitochondrial oxidative phosphorylation, macrophages were cultured for 10 minutes with 1µM myxothiazol (Sigma-Aldrich). Subsequently, macrophages were detached, and pelleted macrophages were solved in MIB buffer (210 mM D-mannitol, 70 mM Sucrose, 5 mM HEPES, 1 mM EGTA, and 0.5% (w/v) fatty acid-free BSA, pH 7.2), transferred into a glass tube, and disrupted by 30 strokes with a homogenizer. After centrifugation (600g, 10 min, 4°C), the supernatant is collected into a new tube and centrifuged at 8000g (10 min 4°C). Pellet is washed once with MIB and once with PBS. Finally the pellet, containing the mitochondria, is resuspended in 200 µl of PBS and injected intrathecally (5 µl per mouse).

Cell lines and primary cell cultures

Mouse neuroblastoma N2A cells (ATCC) were kept in cell culture-medium: DMEM (Cat# 31966-021, Gibco) plus 10% FBS (Cat# 10270-106, Gibco) and 1% Penicillin/Streptomycin (Gibco) and 1% L-Glutamine (200 mM, ThermoFisher).

DRG were collected, and subsequently digested in an enzyme mixture containing $Ca2^+$- and $Mg2^+$-free HBSS, 5 mM HEPES, 10 mM glucose, collagenase type XI (5 mg/ml) and dispase (10 mg/ml) for 40 min before mechanical trituration in DMEM+10% heat-inactivated fetal bovine serum. Cells were centrifuged for 5 min at 140 RCF, resuspended in DMEM containing 4.5 g/l glucose, 4 mM L-glutamine, 110 mg /l sodium pyruvate, 10% fetal bovine serum, 1% penicillin–streptomycin (10,000 i.u./ml), 1% glutamax, 125 ng/ml nerve growth factor, and plated on poly-l-lysine- (0.01 mg/ml) and laminin- (0.02 mg/ml) coated 35-mm dishes. Neurons were used 24 h after plating.

To obtain L929 cell-conditioned medium, 10 million L929 cells were seeded in a 75 cm^2 flask with cell culture-medium supplemented with 1% non-essential amino acids (Sigma-Aldrich) for a week. L929 cells were passaged to a 162 cm^2 flask with 50 ml medium and after a week the supernatants were collected and filtered through a 0.2-µm filter and stored at −20°C (L929-drived M-CSF).

Over-expression and cloning

We amplified CD200 cDNA derived from DRG (mCD200-BamHI-fwd: TAAGCAGGATCCGCCGCCACCATGGGCAGTCTGGTATTCAG; mCD200-SalI-rev: TGC TTAGTCGACTCATTATTTCATTCTTTGCATCC; mCD200-NotI-rev:TGCTTAGCGGCCG CTCATTATTTCATTCTTTGCATCC) and ligated the PCR product into pMXc after digestion with BamHI and ApoI, or into pLenti-MP2 (ref.[76]) after digestion with BamHI and SalI. All cDNA inserts were verified using sanger sequencing.

Standard transduction protocols were performed to generate stable CD200 and iSec1 expression in N2A using pMX-iSec1-IRES-GFP[42] and pLenti-MP2-CD200. MitoDsRed expression in macrophages were made by standard transduction protocols using pLV-MitoDsRed[77].

We generated a bicistronic herpes simplex virus (HSV) construct by cloning iSec-Flag, under control of the α4 promotor and GFP under control of the α22 promoter (HSV-iSec). We used sewing-PCR to introduce silent mutations in iSec1-Flag to resist antisense oligonucleotide-mediated knockdown (see table below for sequence of primers). The first PCR products were made by combining primers '*iSec1-FLAG forward*' with 'iSec1^{resASO1}_mid_reverse', and '*iSec1^{resASO1}_mid_forward*' with '*iSec1^{resASO2}-reverse*'. The right length products were excised from agarose gel, purified and combined in a next PCR using primers '*iSec1-FLAG forward*' with '*iSec1^{resASO2}-reverse*'. The resulting iSec1^{res} was digested with HINDIII and purified from agarose gel and ligated into HSV as described before[78], and validated using sanger sequencing. Control empty HSV (HSV-e) only expresses GFP. HSV was produced as previously described[78]. Mice received 35000 pfu/paw (8 µl) intraplantar HSV-e or –iSec1^{res} at days -3 and -1 prior to carrageenan.

Isec1^{mut} was tested for ASO resistance *in vitro* by transfecting Isec1^{wt} or iSec1^{res} into Neuro 2A cells with Lipofectamin 2000, followed by transfection with mis-match (MM) ASO or iSec-targetting ASO (See below: *Antisense oligonucleotide-mediated knockdown*).

Mutagenesis primer	**sequence**
iSec1-FLAG forward	cgcgcgcg*AAGCTT*ccaccgcatggctcctgccatggaatc
iSec1^{resASO2}-reverse	gcgcgcgc*AAGCTT*ttaAGTCTCGCTCTGACAGTGATGTCGCAttaagtt ctcagtctctgttgatgg
iSec1^{resASO1}_mid_reverse	TGTCAAACATGTCTGCCCTCCatggctgccatgtggaaaagtg
iSec1^{resASO1}_mid_forward	GGAGGGCAGACATGTTTGACAattataactgtatctgaactagtaactga

Italic capital sequence: HINDIII site, underlined capital: mutated sequence to resist iSec-ASO.

Antisense oligonucleotide-mediated knockdown

For in vitro knockdown, we used lipofectamine 2000 (Thermofisher) to transfect N2A-Cd200-iSec1 cells with Mismatch (MM) or iSec1-targetting phosphorothioated antisense oligonucleotides (ASO) according to manufacturer protocol. After 24h, mRNA was isolated with a RNeasy mini kit (Qiagen) and cDNA was generated with iScript (Biorad) according to manufacturer's protocol. To knockdown iSec1 in sensory neurons in vivo, mice received intrathecal injections of 5 µl iSec-ASO mix (total concentration of 3 µg/µl constituting 1:1 mix of iSec-ASO 1 and 2; Sigma-Aldrich) at day 0, 1, 2, 4, and 5. A MM-ASO mix was used as control[21,74]. The following phosphorothioated ASO sequences, that specifically target iSec1/gm609, were used:

Name	**Sequence (5'-3')**
iSec-ASO 1	[mG]*[mG]*[mU]*[mG]*[mA]*G*G*C*A*G*G*T*T*T*G*[mU]*[mC]*[mC]*[mU]*[mC]
iSec-ASO 2	[mA]*[mC]*[mU]*[mG]*[mG]*C*A*G*T*G*A*T*G*T*C*[mU]*[mC]*[mA]*[mU]*[mU]
MM-ASO 1	[mG]*[mU]*[mG]*[mC]*[mG]*A*T*G*T*A*T*G*C*C*G*[mG]*[mU]*[mG]*[mC]*[mU]
MM-ASO 2	[mG]*[mU]*[mA]*[mU]*[mU]*G*T*T*A*A*C*G*C*G*T*[mA]*[mU]*[mG]*[mC]*[mC]

[mA] [mG] [mC] [mU] = 2'OMethyl nucleotides to prevent breakdown/toxicity

Flow cytometry analysis

DRGs (L3–L5) were collected to analyze infiltrating immune cells. In brief, tissues were gently minced and digested at 37 °C for 30 minutes with an enzyme cocktail (5 mg collagenase type I with 2.5 mg trypsin, Sigma Aldrich) in 5 ml DMEM. Cells were stained with various combinations of fluorochrome-labeled antibodies (see subheading 'antibodies').

Blood was collected in EDTA tubes (Greiner Bio-One) following heart puncture and erythrocytes were lysed (RBC lysis buffer, eBioscience) before FACS staining.

For vesicles (see isolation of extracellular vesicles), pellets were pooled and stained for CD45, CD11b and CD200 Receptor 1 (CD200R), and MTDR (see subheading 'antibodies'). Before samples were acquired by LSRFortessa flow cytometer (BD Biosciences) and analyzed with FACSDIVA software, counting beads were added. On average the recovery rate of counting beads (eBioscience) was 44%±3. For all cellular analysis, we used FSC as trigger to identify events. For vesicle analysis, we used FSC or CD45-PB as trigger to identify events.

Transfer of mitochondria

Bone marrow-derived macrophages were harvested and 2 million cells were labelled with 20 nM MTDR in 500 µl culture medium. Cells were washed 3 times, counted (NucleoCounter NC-200; Chemometec) and resuspended at a concentration of 120.000 cells/ml medium.

N2As (30.000 cells) were seeded in a 24-well plate and 24h later co-cultured with 12.000 MTDR pre-stained macrophages for 2h and harvested using 1X Trypsin-EDTA (Gibco). Cells were stained for F4/80 and CD11b (see subheading 'antibodies'). MTDR signal in N2A's was assessed using the ImageStream MkII (Millipore, Burlington, MA) or flow cytometer (4 laser BD Fortessa, 3 laser BD Canto II).

Primary DRG neurons were cultured as described before (see Cell lines and primary cell cultures)[79] and co-cultured with MitoDsRed-expressing macrophages. After 16h, co-cultures were fixed and imaged with a Zeiss Axio Observer microscope (Zeiss, Oberkochen).

Immunofluorescent staining and detection of mitochondrial transfer in vivo

To monitor mitochondrial transfer in vivo, 24 hours after intrathecal injection of MTDR-labelled macrophages, mice were killed by cervical dislocation and lumbar spinal cords and DRGs were collected. Tissues were post-fixed in 4% paraformaldehyde (PFA), cryoprotected in sucrose overnight and embedded in optimal cutting temperature (OCT) compound (Sakura, Zoeterwoude, the Netherlands), and frozen at −80°C.

For immunofluorescence, cryosections (10 µm) of lumbar DRGs or spinal cords, were stained with primary antibodies overnight at 4°C followed by 2 hours incubation with fluorescent-tagged secondary antibodies (see subheading 'antibodies'). Nuclei were counterstained with or without 4,6-diamidino-2-phenylindole (DAPI). Immunostaining images were captured with a Zeiss Axio Observer microscope (Zeiss, Oberkochen, Germany) using identical exposure times for all slides within one experiment. Fluorescence intensity was analyzed with ImageJ software.

iDisco, clearing procedure and light sheet imaging

DRGs from adult mice were cleared using iDISCO protocol as described before[80]. Briefly, animals were perfused with 4% PFA, lumbar dorsal root ganglia were dissected and samples were dehydrated in increasing concentrations (20%, 40%, 60%, 80%, 100%) of methanol solutions. Samples were bleached and rehydrated in decreasing concentrations of methanol solutions. After blocking for 48 hours, samples were incubated with the primary antibodies for 48 hours followed by incubation with secondary antibody for another 48 hours (see subheading 'antibodies'). After samples were embedded in agarose, they were dehydrated in increasing concentrations of methanol solutions. Samples were incubated overnight in 1 volume of 100% methanol/2 volumes 100% dichloromethane (DCM) anhydrous, washed with 100% DCM and incubated in 100% dibenzyl ether (DBE) for at least one day before imaging. Samples were imaged with an Ultramicroscope II (LaVision BioTec) lightsheet microscope equipped with Inspector (version 5.0285.0) software (LaVision BioTec). The microscope consists of an Olympus MVX-10 Zoom Body (0.63-6.3x) equipped with an Olympus MVPLAPO 2x Objective lens, which includes, dipping cap correction optics (LV OM DCC20) with a working distance of 5.7mm. Images where taken with a Neo sCMOS camera (Andor) (2560x2160 pixels. Pixel size: 6.5 x 6.5 μm^2).

Samples were scanned with a sheet NA of 0.148348 (results in a 5 µm thick sheet) and a step-size of 2.5 µm using the horizontal focusing light sheet scanning method with the optimal number of steps and using the contrast blending algorithm. The following laser filter combinations were used: Coherent OBIS 561-100 LS Laser with 615/40 filter and Coherent OBIS 647-120 LX with 676/29 filter.

Real-time RT-PCR

Total RNA was isolated from freshly isolated DRGs (L3-L5) or hind paws using TRizol and RNeasy mini kit (Qiagen, Hilden, Germany). cDNA was synthesized using iScript reverse transcription supermix, according to manufacture protocol (Bio-Rad, Hercules, CA). Quantitative real-time PCR reactions were performed using an I-cycler iQ5 (Bio-Rad, Hercules, CA) as described[81] or on a QuantStudio 12K Flex or a StepOnePlus Realtime PCR system (AB Instruments) with SYBR Select Master Mix (Life Technologies). We used 1-5 ng cDNA input per qPCR reaction.

mRNA expression is represented as relative expression = 2^(Ct(average of reference genes)-Ct(target)). For N2As we used the average Ct values of Gapdh and B2M as reference, for mRNA expression in DRG we used the average of 18S, TBP and Rictor as reference. #1 primers were used for silencing validation in vitro, #2 primers were used for ex vivo mRNA.

Target	**Fwd primer**	**Rev primer**
Cd200 #1	AAGGATGGGCAGTCTGGTATTC	CATGCCCCAAATCAGGCTGT
Cd200 #2	GGGGTGAATCATCACAGGGG	CAAATCCCTCACAGGCTCGT
iSec1/Gm609 #1	TCAAGGAGGTACCACGAATCC	TGATGGCTCGGGCATGTTAT
iSec1/Gm609 #2	CTCTTTGAAAACTGCGAGGTC	CAGTTTAAACAAGGATTCGTGGTA
18S	GTAACCCGTTGAACCCCATT	CCATCCAATCGGTAGTAGCG
Gapdh	TGAAGCAGGCATCTGAGGG	CGAAGGTGGAAGAGTGGGAG
Tnf	GCGGTGCCTATGTCTCAG	GCCATTTGGGAACTTCTCATC
Tgfb1	CAGAGCTGCGCTTGCAGAG	GTCAGCAGCCGGTTACCAAG
Il10	GCACCCACTTCCCAGTCG	GCATTAAGGAGTCGGTTAGCAG

Il1	CAACCAACAAGTGATATTCTCCATG	GATCCACACTCTCCAGCTGCA
Il6	TCTAATTCATATCTTCAACCAAGAGG	TGGTCCTTAGCCACTCCTTC
TBP	CCTTGTACCCTTCACCAATGAC	ACAGCCAAGATTCACGGTAGA
B2M	TGGTCTTTCTGGTGCTTGTCT	ATTTTTTTCCCGTTCTTCAGC
Rictor	TGCGATATTGGCCATAGTGA	ACCCGGCTGCTCTTACTTCT

Measurement of mitochondrial respiration by Seahorse

Macrophages of WT and *Cd200r*$^{-/-}$ mice were seeded on non-coated XF24 (125k cells) or XF96 (50k cells) plates (Seahorse Bioscience), and grown overnight at 37°C. Next day, cells were washed and placed in Seahorse XF-assay base media (pH 7.4) supplemented with 10 mM glucose and 1 mM sodium pyruvate at 37°C to degas. The Seahorse Bioscience XFe24 Analyzer (Seahorse Bioscience) was used to measure oxygen consumption rates (OCR) under basal conditions, and after sequential addition of oligomycin (1 µM), FCCP (0.2 µM), and rotenone (0.5 µM), which were injected after cycle 4, 8, and 12, respectively. Each assay cycle consisted of 1.5 minute of mixing, 2 minutes waiting, and 3 minutes of OCR measurements. For each condition, three cycles were used to determine the average OCR under given condition. The measured OCR was normalized for protein content. Five independent experiments were run, each consisting of 3 or more replicates.

DRG OCR analysis as described before[82]. In brief, DRGs were isolated day 0, 1 and 3 after intraplantar carrageenan. Primary DRG neurons were cultured as described before[79] and seeded on poly-d-ornithine/laminin coated XF24 wells plate (15K) and grown overnight at 37°C. Next day, cells were washed and placed in Seahorse XF-assay base media (pH 7.4) supplemented with 4 mM Glutamine, 25 mM glucose and 1 mM sodium pyruvate. OCR was measured under basal conditions.

Mitochondria from WT and *Cd200r*$^{-/-}$ macrophages were isolated according to Iuso et al.[75]. To measure complex I and complex II driven respiration, 15 µg and 5 µg mitochondria were added in a non-coated XF24 plates, respectively. To measure complex II driven respiration, MAS buffer (220 mM d-Mannitol, 70 mM

sucrose, 10 mM KH2PO4, 5 mM MgCl2, 2 mM HEPES, 1 mM EGTA, and 0.2% (w/v) of fatty acid-free BSA, pH 7.2) was supplemented with 10 mM succinate and 2 µM rotenone. For complex I specific respiration, MAS buffer was supplemented with 5 mM malate and 10 mM glutamate. OCR levels were measured under basal conditions, and after sequential addition of ADP (2 mM), oligomycin (3,2 µM), FCCP (4 µM), and antimycin A (4 µM). Each assay cycle consisted of 1 minute of mixing and 3 minutes of OCR measurements. For each condition, three cycles were used to determine the average OCR under given condition.

Statistical analysis

All data are presented as mean ±SD and were analyzed with GraphPad Prism version 8.3 using unpaired two-tailed t tests, one-way or two-way ANOVA, or as appropriate two-way repeated measures ANOVA, followed by post-hoc analysis. The used post-hoc analyses are indicated in each figure. A p value less than 0.05 was considered statistically significant, and each significance is indicated with * or °: $p < .05$; ** or °° :$p < .01$; *** or °°°: $p < .001$; **** or °°°°: $p <.0001$. All results from statistical analysis are available in the supplemental.

Supplementary Materials:

Figures S1-S13

Movies S1-S2: *https://www.biorxiv.org/content/10.1101/2020.02.12.940445v2*

Antibodies

Target	Clone	Fluorophore	Vendor	Catalogue #
Flow cytometry and ImageStream				
CD115	AFS98	APC	eBioscience	1277550
CD115	AFS98	PE-eF610	eBioscience	61-1152-80
CD11b	M1/70	PerCP-Cy5.5	BioLegend	101227(8)
CD11b	M1/70	PE	BD Bioscience	553311
CD11c	N418	BV785	BioLegend	117336
CD19	6D5	PE	BioLegend	115508
CD200	OX90	PE	eBioscience	12-5200-82
CD200R	OX110	AF488	Bio-Rad	MCA2281A488
CD206	C068C2	BV650	eBioscience	1308615
CD3	17A2	APC	BioLegend	100236
CD45	30-F11	BV711	BioLegend	103147
CD45	30-F11	PB	BioLegend	103126
CD45	30-F11	APC-eF780	eBioscience	47-0451-82
F4/80	BM8	FITC	BioLegend	123108
F4/80	BM8	BV510	BioLegend	123135
iNOS	CXNFT	APC	eBioscience	17-5920-80
Ly6C	AL-21	BV421	BD Bioscience	562727
Ly6G	1A8	BV785	BioLegend	127645
MHCII	M5/114.15.2	PerCP	BioLegend	107624
FLAG	L5	APC	BioLegend	637308
Immune fluorescent microscopy and iDISCO				
F4/80	C1:A3-1 (rat)	None	Cedarlane	CL8940AP
CD206	Poly. goat	None	R&D	AF2535
IbaI	Poly. rabbit	None	Wako	019-19741
β3-Tubulin	Poly. rabbit	None	Abcam	ab18207
Neurofilament-M	Poly. rabbit	None	Biolegend	841001
Anti-Goat	Donkey	AF488	LifeTech	A11055
Anti-Rabbit	Donkey	AF594	LifeTech	A21207
Anti-Rabbit	Donkey	AF568	LifeTech	A10042
Anti-Rat	Goat	AF647	LifeTech	A21247

Supplemental figures for:

Chapter 3: Macrophages transfer mitochondria to sensory neurons to resolve inflammatory pain

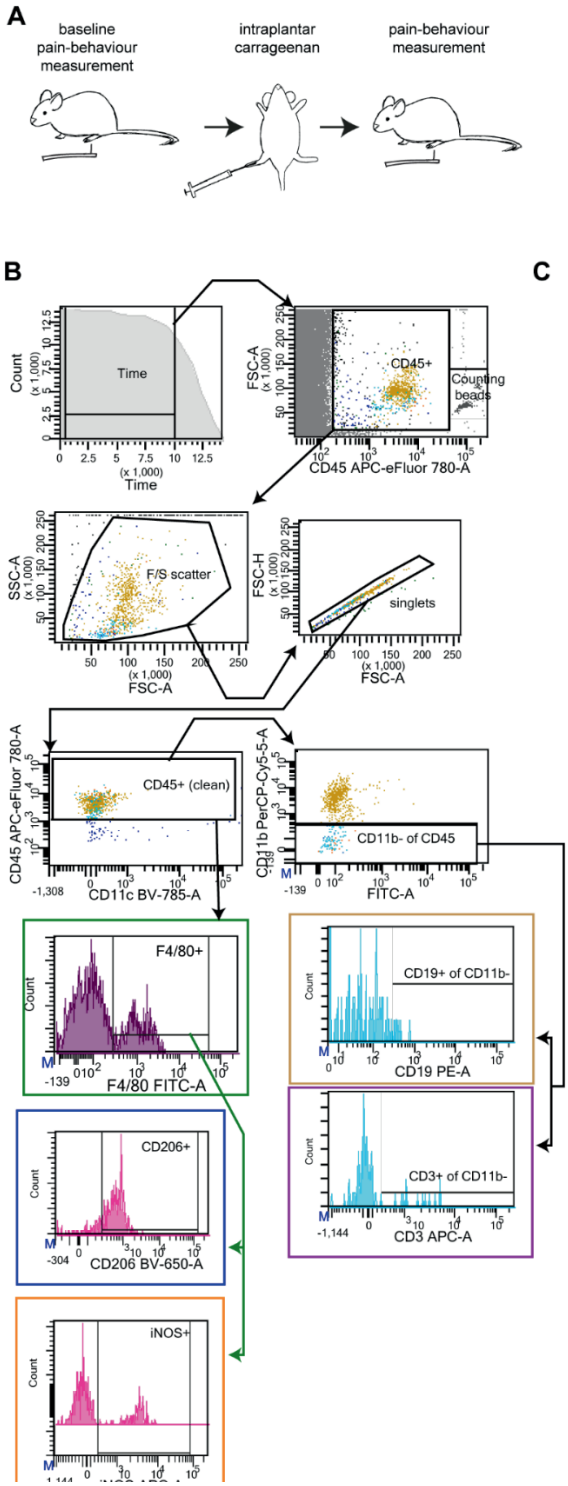

A
baseline pain-behaviour measurement
intraplantar carrageenan
pain-behaviour measurement
B
Time
Count
CD45+
Counting beads
CD45 APC-eFluor 780-A
F/S scatter
SSC-A
FSC-A
singlets
FSC-H
CD45+ (clean)
CD11c BV-785-A
CD11b- of CD45
CD11b PerCP-Cy5-5-A
FITC-A
F4/80+
F4/80 FITC-A
CD19+ of CD11b-
CD19 PE-A
CD206+
CD206 BV-650-A
CD3+ of CD11b-
CD3 APC-A
iNOS+
C
Number of F4/80+ cells/DRG
no pain
Cntr

Fig. S1. Carrageenan-induced transient inflammatory pain model

(A) *Schematic representation of the carrageenan-induced transient inflammatory pain model. Baseline thresholds were determined three times the first week prior to carrageenan injection and averaged. After intraplantar (i.pl.) carrageenan injection, we measured pain-associated behaviors and signs.* ***(B)*** *Gating strategy for monocyte /macrophage phenotyping and T- and B-cells in the DRG. A time gate unified the acquisition analysis, followed by a rough separation of events based on CD45 expression but excluding the counting beads. Cells were further gated by FCS/SSC, single cells and CD45 expression before analysis of CD11b, and F4/80. F4/80+ cells were further assessed for expression of iNOS and CD206; or excluding CD11b+ cells and assessing CD3 and CD19 expression on the CD11b negative cells.* ***(C)*** *F4/80 positive macrophage in DRG of control animals or the contralateral lumbar DRG at day 3 after i.pl. injection of carrageenan. Unpaired t-test (ns). All statistical information can be found in the supplement data files.*

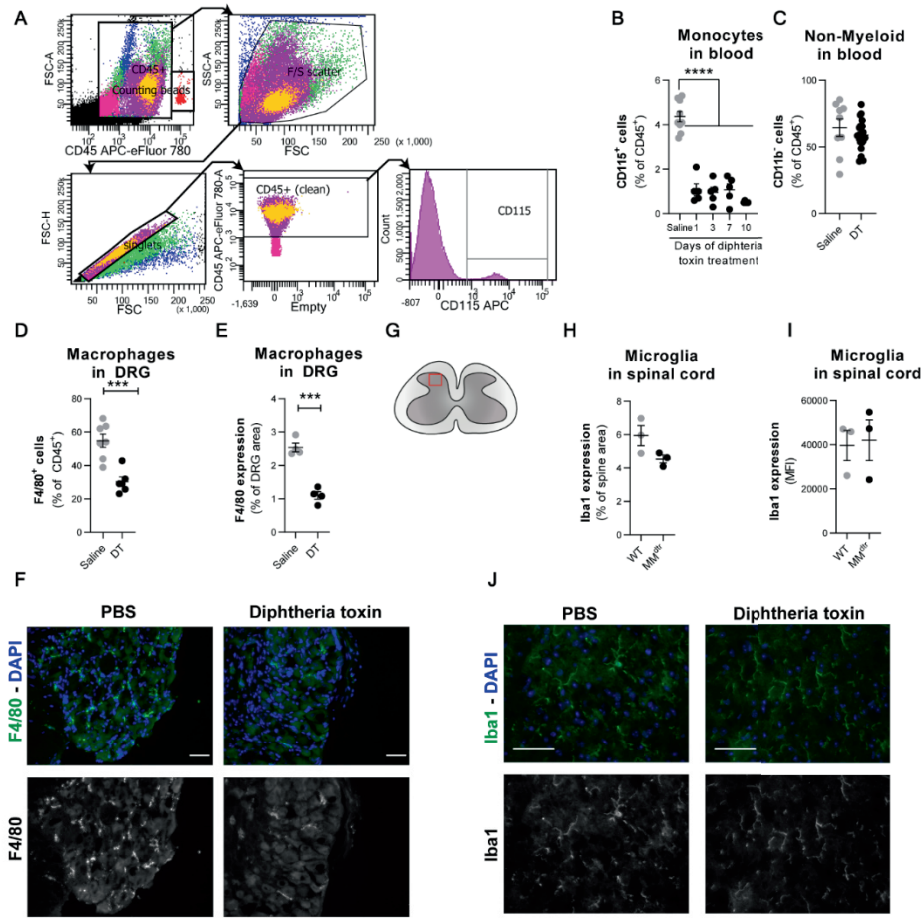


Fig. S2. Depletion controls in MM^{dtr} mice

(A) *Gating strategy for depletion analysis in blood.* ***(B, C)*** *Flow cytometry data to validate monocyte depletion in MM^{dtr} mice treated with Diptheria toxin (DT).* ***(B)*** *Percentage of CD115^{+} monocytes in blood to confirm depletion of monocytes, and* ***(C)*** *percentage of non-myeloid cells in blood (CD45^{+}CD11b^{-} cells) to confirm depletion specificity. (B) one-way ANOVA, Dunnett; (C) Unpaired t-test with Welch's correction.* ***(D)*** *Percentage of F4/80^{+} macrophages in the DRG to confirm partial depletion of tissue resident macrophages, for gating see Fig. S1B. Unpaired t-test.* ***(E, F)*** *Immunofluorescent microscopy analysis of depletion specificity of macrophages versus microglia cells in MM^{dtr} mice after DT treatment.* ***(E)*** *Quantification and* ***(F)*** *example images of F4/80 expression in DRG of MM^{dtr} mice treated with saline or DT. Blue: nuclei, Green: F4/80. Scale bar: 50µM. Unpaired t-test.* ***(G)*** *Red square in schematic drawing of lumbar spinal cord indicates the area of dorsal horn analyzed for subfigures H, I and J.* ***(H-J)*** *Spinal microglia are not depleted after DT treatment.* ***(H, I)*** *Quantification and* ***(J)*** *example images of Iba1^{+} microglia in the spinal cord of MM^{dtr} mice treated with saline or DT. Blue: nuclei, Green: Iba1. Scale bar: 50µM. Significance tested: Unpaired t-test. All statistical information can be found in the supplemental data files.*

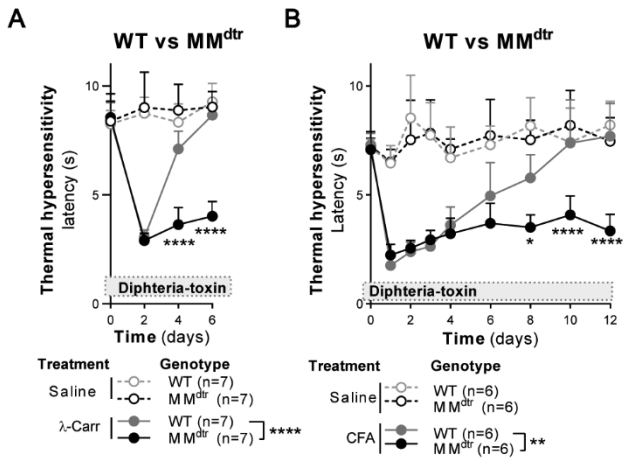
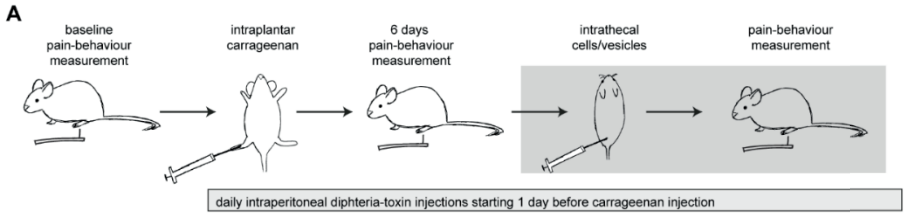


Fig. S3. Course of pain hypersensitivity in MM^{dtr} mice

(A) *Course of thermal hyperalgesia in WT and MM^{dtr} mice injected with 1% carrageenan in one saline in the other hind paw. 2-way repeated measures ANOVA, Sidak post-hoc comparing carrageenan conditions.* ***(B)*** *Course of unilateral CFA-induced thermal hyperalgesia in WT and MM^{dtr} mice. 2-way repeated measures ANOVA, Sidak post-hoc comparing CFA conditions. All statistical information can be found in the supplemental data files.*


A
baseline pain-behaviour measurement
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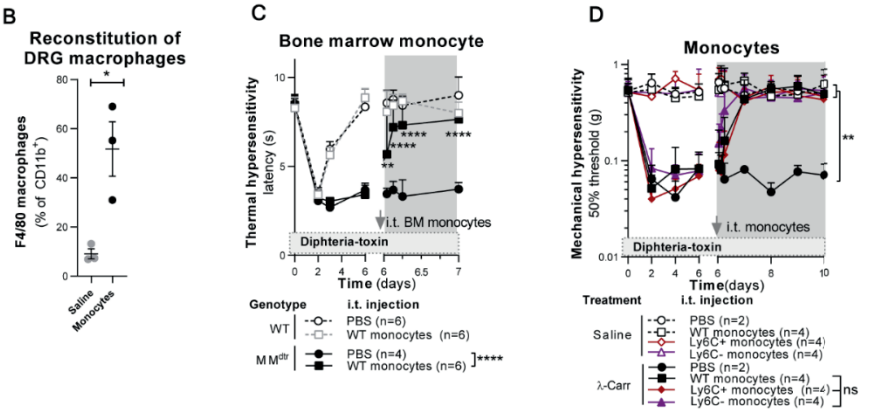
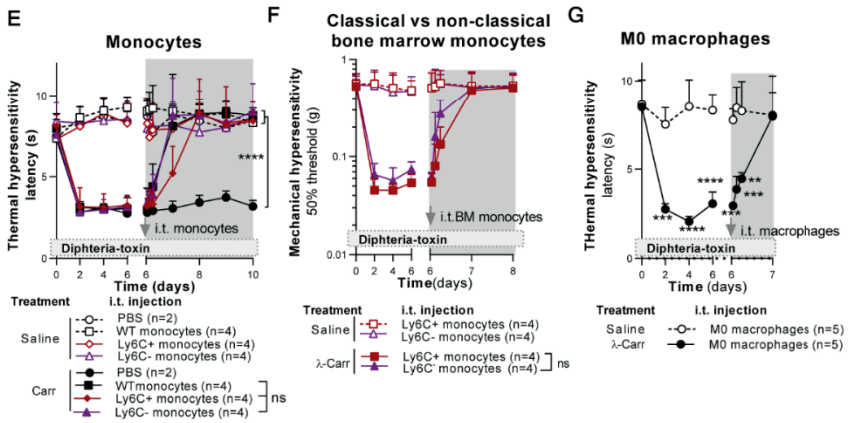
6 days pain-behaviour measurement
intrathecal cells/vesicles
pain-behaviour measurement
daily intraperitoneal diphteria-toxin injections starting 1 day before carrageenan injection


B
Reconstitution of DRG macrophages
F4/80 macrophages (% of CD11b+)
Saline
Monocytes
C
Bone marrow monocyte
Thermal hypersensitivity latency (s)
i.t. BM monocytes
Diphteria-toxin
Time (days)
Genotype
i.t. injection
WT
PBS (n=6)
WT monocytes (n=6)
MMdtr
PBS (n=4)
WT monocytes (n=6)
****
D
Monocytes
Mechanical hypersensitivity 50% threshold (g)
i.t. monocytes
Diphteria-toxin
Time(days)
Treatment
i.t. injection
Saline
PBS (n=2)
WT monocytes (n=4)
Ly6C+ monocytes (n=4)
Ly6C- monocytes (n=4)
λ-Carr
PBS (n=2)
WT monocytes (n=4)
Ly6C+ monocytes (n=4)
Ly6C- monocytes (n=4)
**
ns


E
Monocytes
Thermal hypersensitivity latency (s)
i.t. monocytes
Diphteria-toxin
Time (days)
Treatment
i.t. injection
Saline
PBS (n=2)
WT monocytes (n=4)
Ly6C+ monocytes (n=4)
Ly6C- monocytes (n=4)
Carr
PBS (n=2)
WTmonocytes (n=4)
Ly6C+ monocytes (n=4)
Ly6C- monocytes (n=4)
ns
F
Classical vs non-classical bone marrow monocytes
Mechanical hypersensitivity 50% threshold (g)
i.t.BM monocytes
Diphteria-toxin
Time(days)
Treatment
i.t. injection
Saline
Ly6C+ monocytes (n=4)
Ly6C- monocytes (n=4)
λ-Carr
Ly6C+ monocytes (n=4)
Ly6C- monocytes (n=4)
ns
G
M0 macrophages
THermal hypersensitivity latency (s)
i.t. macrophages
Diphteria-toxin
Time (days)
Treatment
i.t. injection
Saline
M0 macrophages (n=5)
λ-Carr
M0 macrophages (n=5)

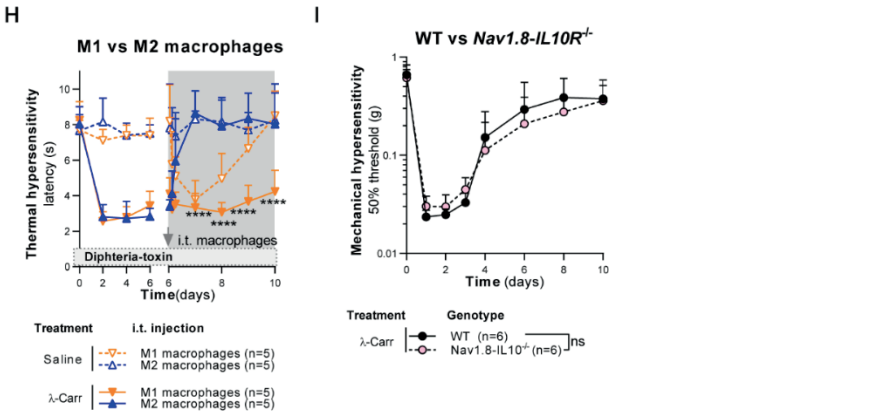

H
M1 vs M2 macrophages
Thermal hypersensitivity latency (s)
i.t. macrophages
Diphteria-toxin
Time(days)
Treatment
i.t. injection
Saline
M1 macrophages (n=5)
M2 macrophages (n=5)
λ-Carr
M1 macrophages (n=5)
M2 macrophages (n=5)
I
WT vs Nav1.8-IL10R-/-
Mechanical hypersensitivity 50% threshold (g)
Time (days)
Treatment
Genotype
λ-Carr
WT (n=6)
Nav1.8-IL10-/- (n=6)
ns

Fig. S4. Depletion of monocytes/macrophages prevents resolution of inflammatory pain

(A) *Schematic representation of the depletion, intraplantar (i.pl.) and intrathecal (i.t.) injections in the pain models.* ***(B)*** *Flow cytometry analysis of lumbar DRG (L3-L5) after i.t injection of WT monocytes in MM^{dtr} mice. Gating strategy is indicated in Fig. S2. Unpaired T test.* ***(C)*** *Course of carrageenan-induced thermal hyperalgesia in MM^{dtr} mice injected i.t. with $CD115^{+}$ bone marrow monocytes at day 6. 2-way repeated measures ANOVA, Sidak post-hoc comparing MM^{dtr} conditions.* ***(D, E)*** *Course of carrageenan-induced mechanical (D) and thermal(E) hyperalgesia in MM^{dtr} mice injected i.t. with splenic $CD115^{+}$ monocytes, 'classical' $Ly6C^{+}$ or 'non-classical' $Ly6C^{-}$ monocytes or PBS at day 6. 2-way repeated measures ANOVA, Sidak post-hoc comparing carrageenan conditions.* ***(F)*** *Course of carrageenan-induced mechanical hyperalgesia in MM^{dtr} mice injected i.t. with 'classical' $Ly6C^{+}$ or 'non-classical' $Ly6C^{-}$ bone marrow monocytes. 2-way repeated measures ANOVA, Sidak post-hoc comparing carrageenan conditions.* ***(G)*** *Course of thermal hyperalgesia in MM^{dtr} mice after i.pl. injection of 1% carrageenan in the left hind paw and i.t. injection of M0 macrophages. 2-way repeated measures ANOVA, with Sidak post-hoc. (H) Course of carrageenan-induced thermal hyperalgesia in MM^{dtr} mice injected i.t. with LPS/IFNγ-treated 'M1', IL4-treated 'M2' macrophages or PBS. 2-way repeated measures ANOVA, Sidak post-hoc comparing carrageenan conditons.* ***(I)*** *Course of carrageenan-induced mechanical hypersensitivity in $Nav1.8^{cre}$-$Il10r^{-/-}$ litter mates. 2-way repeated measurs ANOVA, Sidak post-hoc. All statistical information can be found in the supplemental data files.*

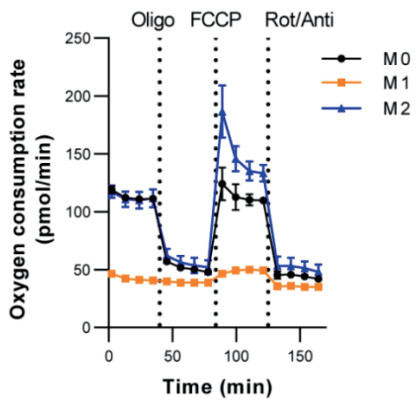


Fig. S5. Macrophage oxidative phosphorylation

Oxygen consumption rate of macrophages (M0), macrophages differentiated with LPS and IFNγ (M1), or with IL4 (M2), as was described before[27].

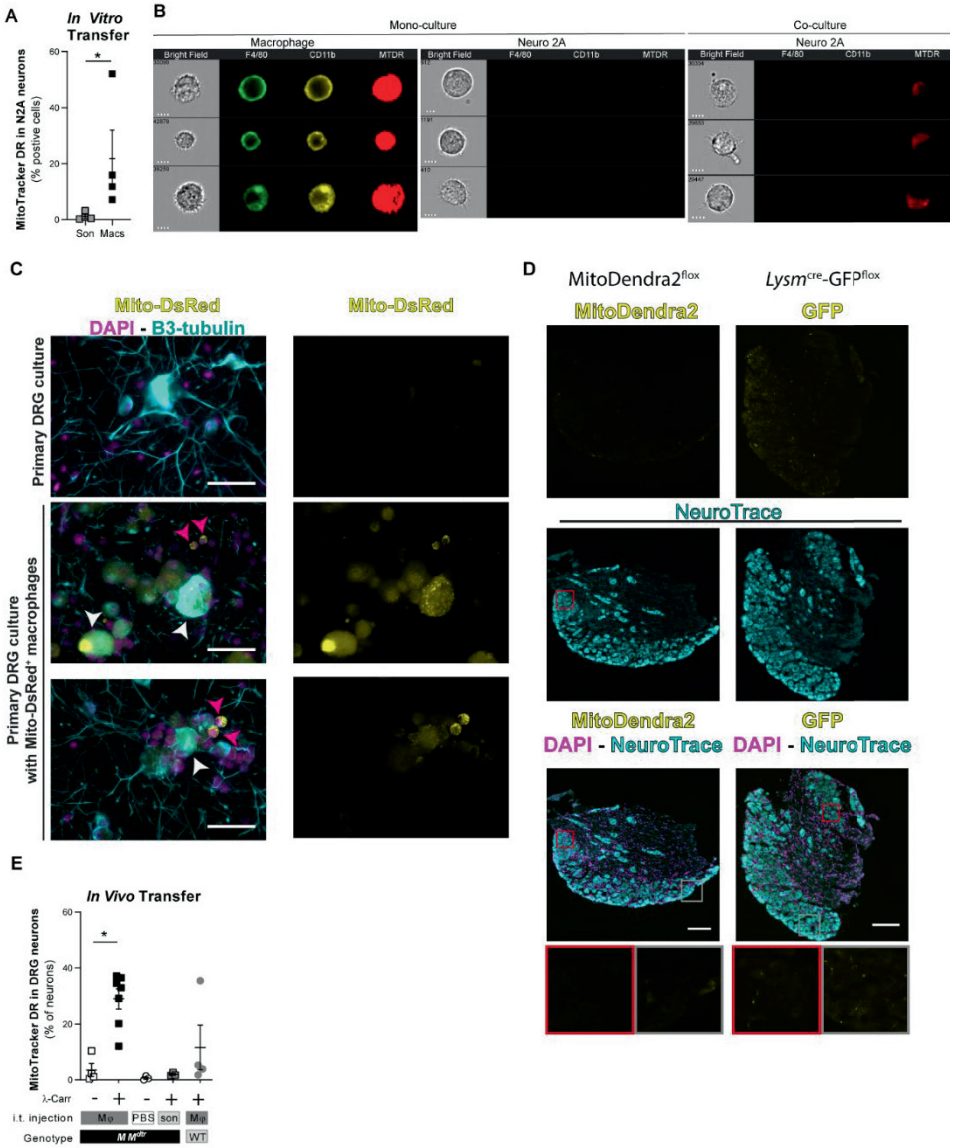


Fig. S6. Macrophages transfer mitochondria to neurons

(A) *Analysis of MitoTracker Deep Red (MTDR) signal in N2A-neurons after co-culture of MTDR-labelled macrophages (macs), or sonicated MTDR-labelled macrophages (Son.) with MTDR-negative N2A-neurons. Co-cultures were stained with CD11b and CD45 to identify macrophages and N2A cells and analyzed by flow cytometry. Mann Whitney test.* ***(B)*** *Macrophages labeled with MTDR were co-cultured with N2A for 2 hours and analyzed by image stream (n=2). Co-cultures were stained for CD11b and F4/80 to identify*

macrophages, and neurons were identified by negative selection. Scale bar: 7µm. ***(C)*** *Macrophages transduced with Mito-dsRed were co-cultured with primary mouse sensory neuron cultures for 16h. Co-cultures were analyzed with immune fluorescence for macrophage-derived mitochondria (red), neurons (β3 tubulin, green), and nuclei (DAPI, blue). Macrophages are indicated with pink arrow heads. Neurons positive for Mito-dsRed are indicated by white arrow heads. n=1. Scale bar: 50µM.* ***(D)*** *Example image of MitoDenra2^{+} presence in naïve MitoDendra2^{flox} mouse, or GFP^{+} neurons in Lysm^{cre}-GFP^{flox} mouse.* ***(E)*** *Analysis of MTDR signal in sensory neurons in the DRG of MMdtr and WT mice. At day 6 after 1% carrageenan (ongoing pain in MMdtr, resolved pain in WT mice) we i.t. injected PBS, MTDR-labelled macrophages (Mφ), or sonicated MTDR-labelled macrophages (son). After 18h, lumbar DRG were isolated for immunofluorescence analysis and counter-stained with β3-tubulin (cyan, neurons) and DAPI (magenta, nuclei). White arrowheads indicate MTDR^{+} (yellow) neurons. Scale bar: 50µm. Kruskal-Wallis with Dunn post-hoc. All statistical information can be found in the supplemental data files.*

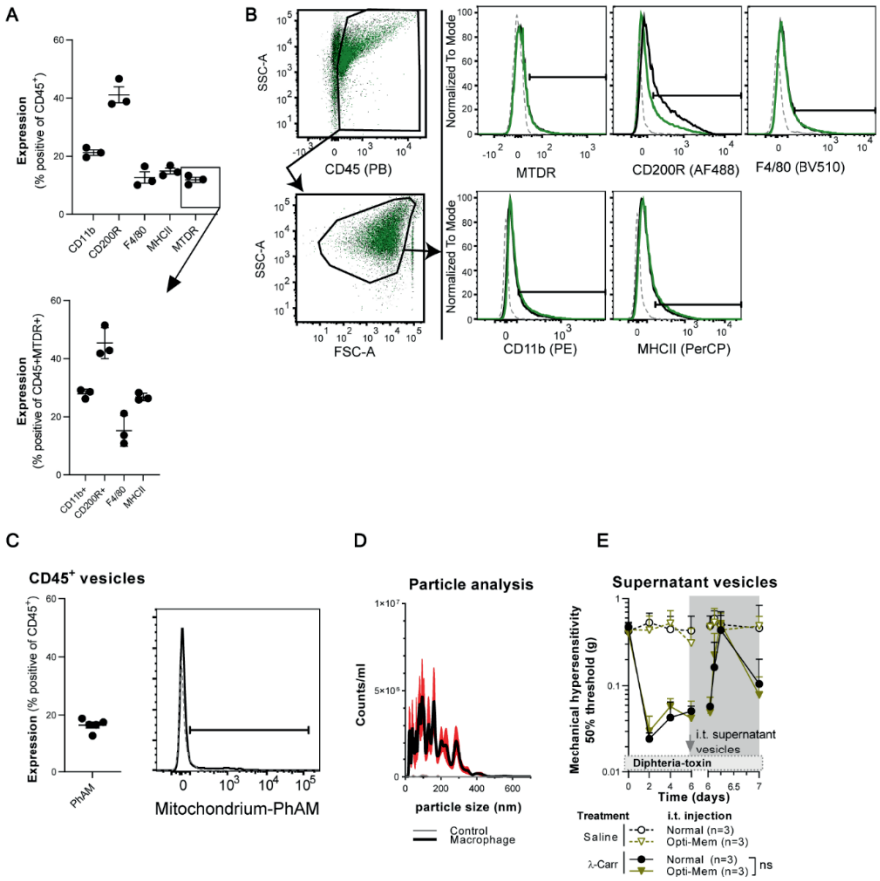


Fig. S7. Characterization of macrophage-derived extra cellular vesicles.

(A, B) (A) *Expression of CD11b, F4/80, CD200R and MHCII on CD45⁺ and below CD45⁺MTDR⁺ vesicles.* ***(B)*** *Gating strategy: Events were captured based on FSC or CD45-PB-signal and gated for CD45 expression, followed by an FFC/SSC gate. Dotted line: unstained, solid black: WT, solid green: Cd200r$^{-/-}$.* ***(C)*** *Expression of Mitochondrion-targeted MitoDendra2 in CD45⁺ vesicles of macrophages, quantified and example histogram.* ***(D)*** *Nanoparticle Tracking Analysis (NTA) of supernatant vesicles from WT macrophages. Mean+SEM from 6 technical replicates (red lines are SEM) . Macrophages were cultured in plain Opti-Mem to prevent contamination of FCS particles. As control, medium was 'cultured' at 37C in flasks without macrophages.* ***(E)*** *Course of mechanical hyperalgesia in MM^{dtr} mice injected with carrageenan in the left hind paw, and saline in the right hind paw. At day 6 mice were injected i.t. with intact macrophage-derived vesicles harvested from macrophage cultured in normal medium (see M&M) or plain Opti-mem. 2-way repeated measures ANOVA, Sidak post-hoc. All statistical information can be found in the supplemental data files.*

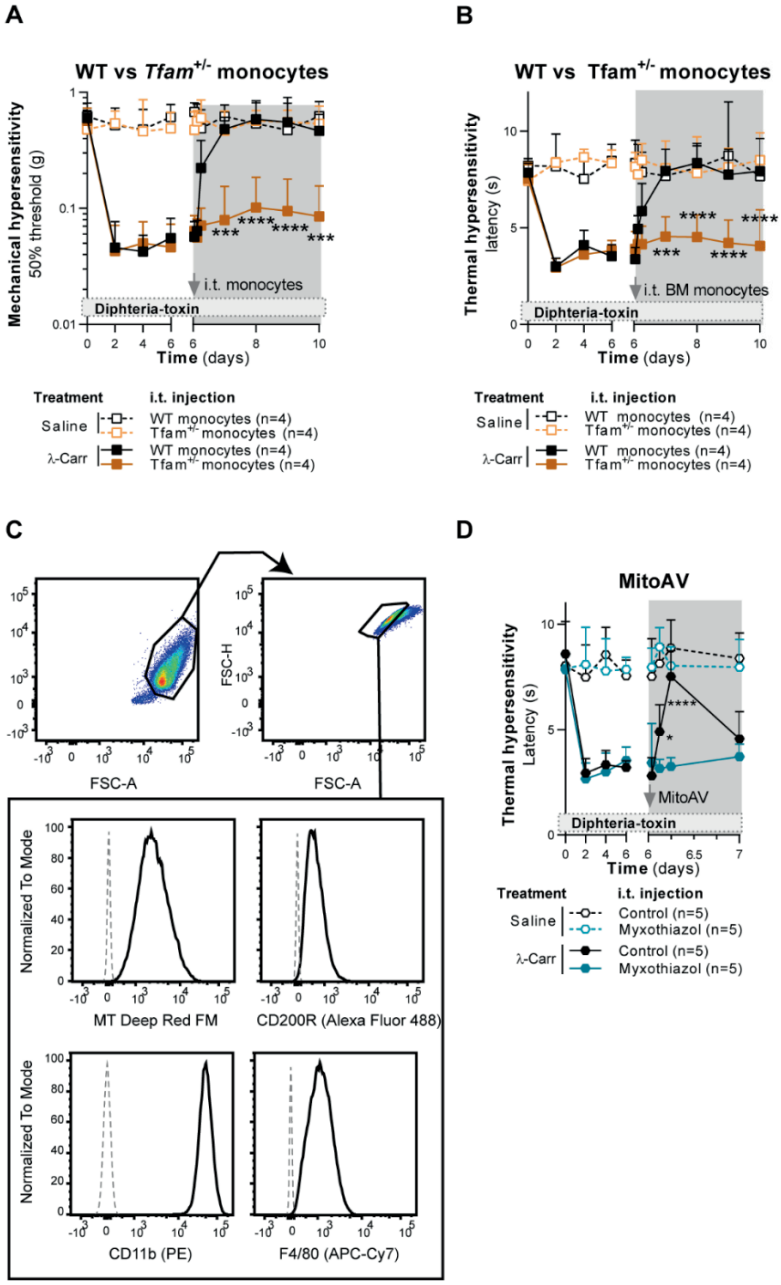


Fig. S8. Functional mitochondria are required to resolve inflammatory pain

(A, B) *Course of carrageenan-induced* ***(A)*** *mechanical and* ***(B)*** *thermal hyperalgesia in MM^{dtr} mice injected intra thecal (i.t.) with WT or Tfam$^{+/-}$ CD115^{+} monocytes. 2-way repeated measures ANOVA, Sidak post-hoc comparing carrageenan conditions.* ***(C)*** *Flow*

cytometry analysis of isolated mitochondria. Events were gated on FSC/SSC, followed by FCS/FCS-H for singles. Dotted line: unstained, solid black: specific stain. ***(D)*** *Course of carrageenan-induced thermal hyperalgesia in MM^{dtr} mice i.t. injected with functional isolated mitochondria, or mitochondria inhibited with complex III inhibitor myxothizaol. 2-way repeated measures ANOVA, Sidak post-hoc comparing carrageenan conditions. All statistical information can be found in the supplemental data files.*

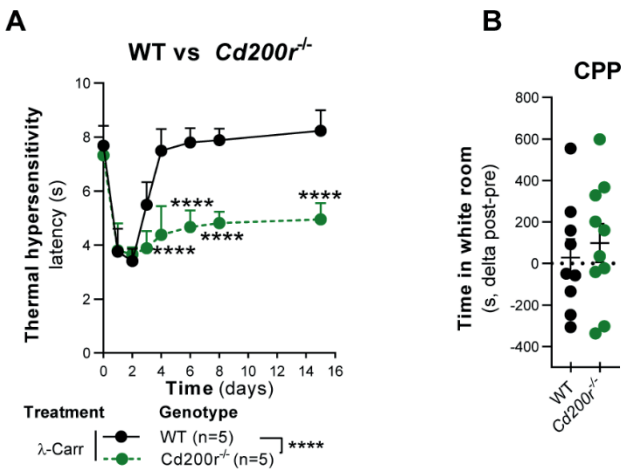


Fig. S9. CD200R is required for the resolution of inflammatory pain

(A) *Course of carrageenan-induced thermal hyperalgesia in WT and Cd200r$^{-/-}$ littermates. 2-way repeated measures ANOVA, Sidak post-hoc.* ***(B)*** *Gabapentin-induced place preference conditioning at day 16 after unilateral saline injection in the hind paws. Conditioning efficiency is depicted as the difference in time (seconds, s) spent in a white room pre- and post-conditioning. Unpaired t-test. All statistical information can be found in the supplemental data files.*

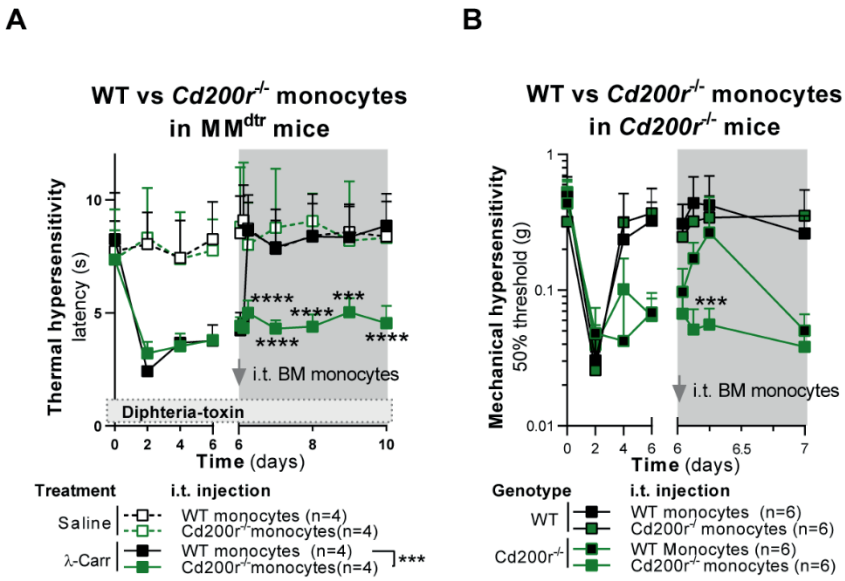


Fig. S10. CD200R is required for the resolution of inflammatory pain

(A) *Course of carrageenan-induced thermal hyperalgesia in MM^{dtr} mice intra thecal (i.t.) injected with WT of Cd200r$^{-/-}$ CD115^{+} monocytes. 2-way repeated measures ANOVA, Sidak post-hoc comparing carrageenan conditions.* ***(B)*** *Course of carrageenan-induced mechanical hyperalgesia in Cd200r$^{-/-}$ mice i.t injected with WT of Cd200r$^{-/-}$ CD115^{+} monocytes. 2-way repeated measures ANOVA, Sidak post-hoc comparing carrageenan conditions. All statistical information can be found in the supplemental data files.*

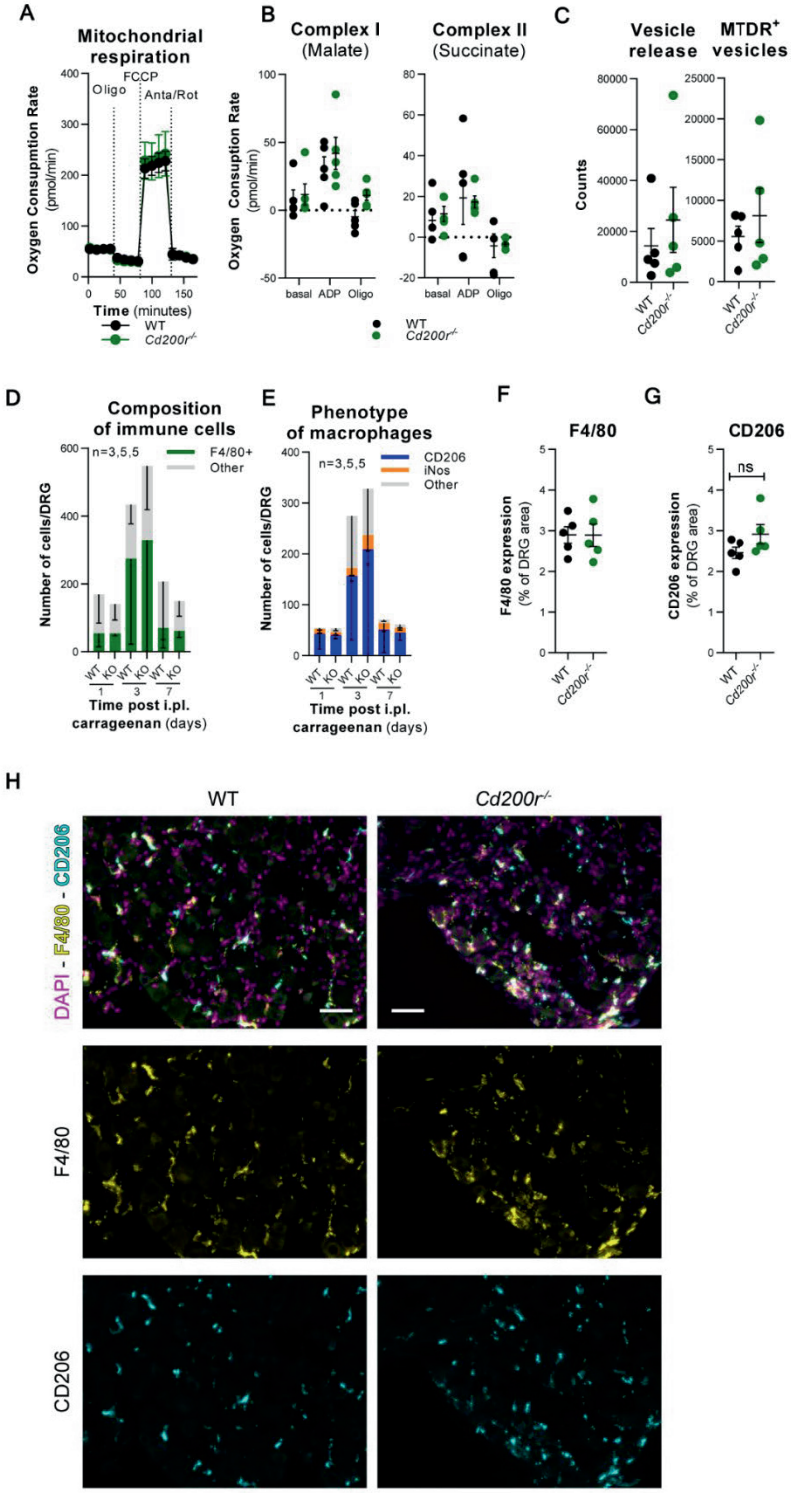

A
Mitochondrial respiration
Oligo
FCCP
Anta/Rot
Oxygen Consupmtion Rate (pmol/min)
Time (minutes)
WT
Cd200r-/-
B
Complex I (Malate)
Complex II (Succinate)
Oxygen Consuption Rate (pmol/min)
basal ADP Oligo
WT
Cd200r-/-
C
Vesicle release
MTDR+ vesicles
Counts
WT
Cd200r-/-
D
Composition of immune cells
n=3,5,5
F4/80+
Other
Number of cells/DRG
WT KO WT KO WT KO
1 3 7
Time post i.pl. carrageenan (days)
E
Phenotype of macrophages
n=3,5,5
CD206
iNos
Other
Number of cells/DRG
Time post i.pl. carrageenan (days)
F
F4/80
F4/80 expression (% of DRG area)
G
CD206
ns
CD206 expression (% of DRG area)
H
WT
Cd200r-/-
DAPI - F4/80 - CD206
F4/80
CD206

Fig. S11. CD200R$^{-/-}$ have normal vesicles, mitochondrial respiration and macrophage infiltration into DRG

(A) *Mitochondrial respiration in WT or Cd200r$^{-/-}$ macrophages. 2-way repeated measures ANOVA, Bonferroni post-hoc.* ***(B)*** *Mitochondrial respiration in MitoAV from WT or Cd200r$^{-/-}$ macrophages assessed by extracellular flux assay. 2-way repeated measures ANOVA, Bonferroni post-hoc.* ***(C)*** *Analysis of vesicle content of WT or Cd200r$^{-/-}$ macrophage-conditioned medium. Unpaired t-test.* ***(D, E)*** *Flow cytometry analysis of monocytes/macrophages infiltrating the DRG. 2-way repeated measures ANOVA, Bonferroni post-hoc.* ***(F, G, H)*** *Quantification and example image of F4/80 and CD206 expression in DRG by immune fluorescence in naïve WT and Cd200r$^{-/-}$ mice. Scale bars: 50µm. Unpaired t-test. All statistical information can be found in the supplemental data files.*

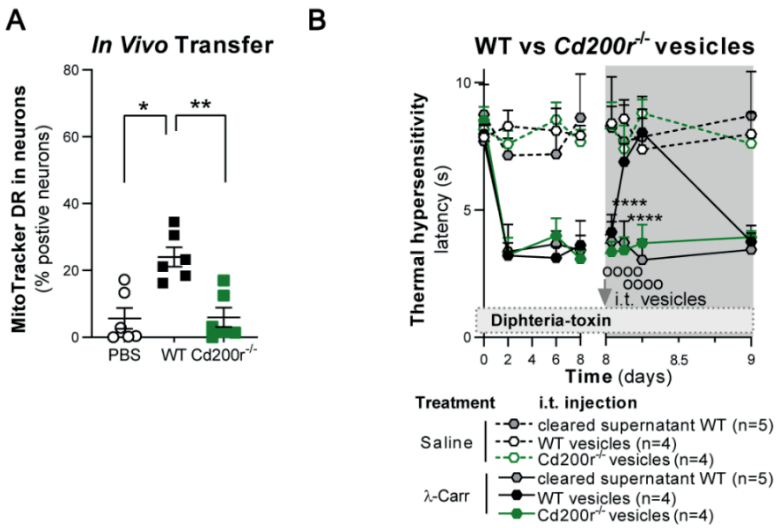


Fig. S12. *Cd200r$^{-/-}$* macrophages cannot resolve inflammatory pain.

(A) *In vivo MTDR transfer from WT or Cd200r$^{-/-}$ MTDR-labelled macrophages to DRG neurons of Cd200r$^{-/-}$ mice. At day 6 after carrageenan injection, macrophages or PBS were injected i.t. and after 18h DRG were isolated and stained as described for Fig. 2C. Kruskal-Wallis, Dunn post-hoc.* ***(B)*** *Course of carrageenan-induced thermal hyperalgesia in MMdtr mice i.t injected with vesicles derived from WT of Cd200r$^{-/-}$-macrophage-conditioned medium. 2-way repeated measures ANOVA, Dunnet post-hoc comparing carrageenan conditions. All statistical information can be found in the supplemental data files.*

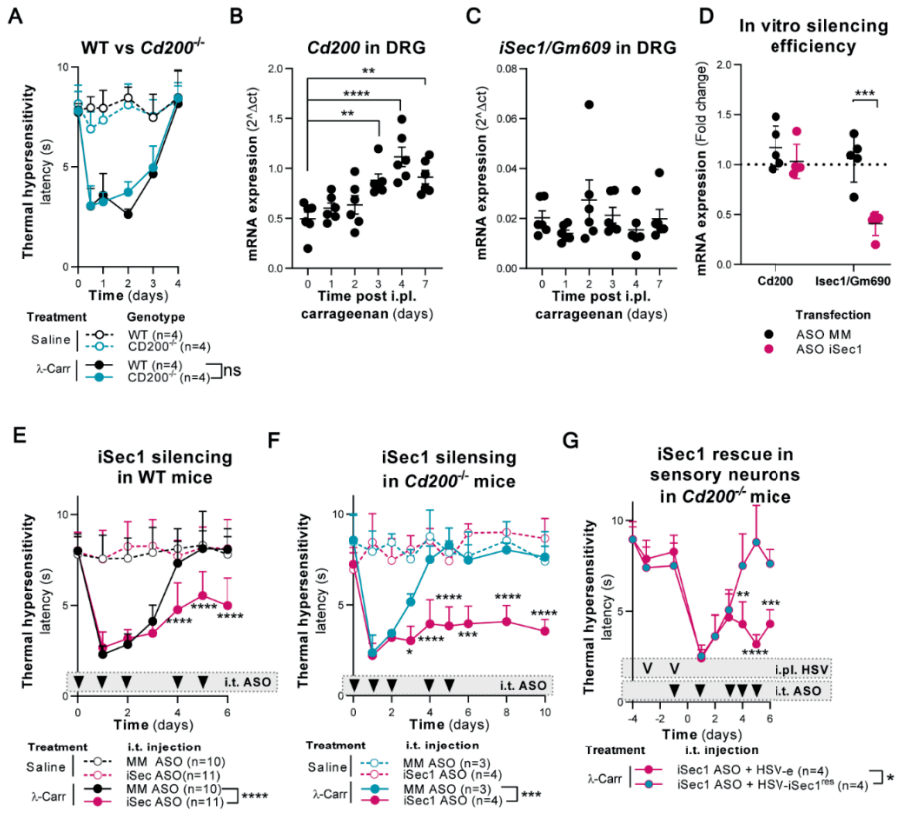


Fig. S13. iSec1 is required for resolution of inflammatory pain

(A) Course of carrageenan-induced thermal hyperalgesia in WT and *Cd200*$^{-/-}$ littermates. 2-way repeated measures ANOVA, Sidak post-hoc comparing carrageenan conditions. **(B)** Cd200 mRNA expression in DRG of WT mice at indicated days after intra plantar (i.pl) injection of carrageenan. Ordinary ANOVA, Dunnett post-hoc comparing vs time point 0. **(C)** *iSec1/gm690* mRNA expression in DRG of WT mice at indicated days after intra plantar (i.pl) injection of carrageenan. Ordinary ANOVA, Dunnett post-hoc comparing vs time point 0. **(D)** *Cd200* and *iSec1/gm609* mRNA expression after silencing of iSec1/gm609 in neuronal N2A cells ectopically expressing Cd200 and iSec1/gm609. n=5 from 2 experiments. 2-way repeated measures ANOVA, Sidak post-hoc. **(E)** Course of carrageenan-induced thermal hyperalgesia in WT mice that are control- or iSec1/*gm609* silenced by ASO. 2-way repeated measures ANOVA, Sidak post-hoc comparing carrageenan conditions. **(F)** Course of carrageenan-induced thermal hyperalgesia in *Cd200*$^{-/-}$mice that are control- or iSec1/*gm609* silenced by ASO. 2-way repeated measures ANOVA, Sidak post-hoc comparing carrageenan conditions. **(H)** Course of carrageenan-induced thermal hyperalgesia in *Cd200*$^{-/-}$ mice that are iSec1/gm609 silenced by ASO, while i.pl. infected with HSV-e or HSV-iSec1. 2-way repeated measures ANOVA, Sidak post-hoc. All statistical information can be found in the supplemental data.

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4

CHAPTER 4

Dorsal Root Ganglia Macrophages Maintain Osteoarthritis Pain

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Abstract

Pain is the major debilitating symptom of osteoarthritis (OA), which is difficult to treat. In OA patients joint tissue damage only poorly associates with pain, indicating other mechanisms contribute to OA pain. Immune cells regulate the sensory system, but little is known about their involvement in OA pain. Here we report that macrophages accumulate in the dorsal root ganglia (DRG) distant from the site of injury in two rodent models of OA. DRG macrophages acquired a M1-like phenotype and depletion of DRG macrophages resolved OA pain. Sensory neurons innervating the damaged knee joint shape DRG macrophages into a M1-like phenotype. Persisting OA pain, accumulation of DRG macrophages, and their programming into M1-like phenotype was independent of $Na_V1.8$ nociceptors. Inhibition of M1-like macrophages in the DRG, by intrathecal injection of a IL4-IL10 fusion protein or M2-like macrophages resolved persistent OA pain. In conclusion, these findings reveal a crucial role for macrophages in maintaining OA pain independent of the joint damage and suggest a new direction to treat OA pain.

Keywords: Osteoarthritis, Chronic pain, Macrophage, Sensory neuron

SIGNIFICANCE STATEMENT

In osteoarthritis (OA) patients pain poorly correlates with joint tissue changes indicating mechanisms other than only tissue damage cause pain in OA. We identified that DRG containing the somata of sensory neurons innervating the damaged knee are infiltrated with macrophages that are shaped into a M1-like phenotype by sensory neurons. Targeting the phenotype of DRG macrophages with either M2-like macrophages or a cytokine fusion protein that skews macrophages into M2-like phenotype OA pain resolves pain. Our works reveals a mechanism that contributes to the maintenance of OA pain distant from the affected knee joint and suggest that dorsal root ganglia macrophages are a target to treat osteoarthritis chronic pain.

Introduction

Osteoarthritis (OA) is a highly prevalent disease affecting one in eight adults with a large and increasing societal burden[1]. Joint pain is the main symptom, leading to functional limitations and reduced quality of life[2]. With disease progressing, pain often becomes worse and manifests even at rest[3]. Current treatment options to relieve pain, such as NSAIDS and opioids, are insufficiently effective or fail and have substantial risks of side effects, specifically during the needed long-term treatment[4,5].

OA pain may be driven by peripheral input from the affected joint, because intra-articular anaesthetics and total joint replacement in the majority of cases limits the pain[6,7]. OA is frequently associated with low-grade synovitis[8]. Locally produced inflammatory mediators such as IL-1β, TNF, IL-6, and PGE_2 directly excite and sensitize sensory neurons innervating the affected joint and cause pain[9]. However, in OA patients the severity of joint pain poorly correlates with the actual joint damage and/or synovitis[10-13]. Moreover, in more than 20% of patients pain remains after total joint replacement[14,15]. Overall these data suggest that other mechanisms, in addition to joint damage and inflammation, contribute to the experienced OA pain.

Both preclinical and clinical evidence support the hypothesis that central mechanisms contribute to OA pain[16,17]. OA patients show signs of central sensitisation. Patients with a painful knee have lower pressure pain thresholds in areas remote from the site of damage, increased temporal summation, and reduced descending inhibition of pain pathways[18]. In rodent models of OA, glial cell activation, including astrocytes and microglia in the spinal cord and satellite cells in the DRG, are associated with pain[19]. Activated glial cells produce pro-inflammatory cytokines, chemokines, and extracellular factors such as proteases, nitric oxide, and kinins that contribute to persisting pain[20]. Intriguingly, recent evidence in rodent models of neuropathic and inflammatory pain shows that immune cells modulate pain in peripheral and central pain pathways[5,21]. Activated sensory neurons produce factors including CCL2, CX3CL1 and CSF1 that attract immune cells to nervous tissue[17,22,23]. In various chronic pain models, such as nerve injury-induced neuropathic pain or inflammatory pain models, macrophages infiltrate the DRG[5,22,24]. In models of OA evidence is more sparse, but examples exist that macrophages accumulate DRG after knee joint

damage[5,17]. However, the pain regulatory function of the macrophages in OA is still poorly understood[5]. Macrophages are highly heterogeneous and their phenotype and functions are regulated by the surrounding micro-environment[25]. Indeed, macrophages in nervous tissue have a unique phenotype and neurons can drive programming of these cells. Classically activated M1 macrophages express high levels of iNOS and have a pro-inflammatory profile. In contrast, alternatively activated M2 macrophages express high levels of CD206 and have an inflammation controlling tissue regenerative profile[26]. Although the precise function of these macrophage is not clear, some evidence suggests that because of their different transcriptional profiles, M1 and M2 macrophages have opposing roles in pain regulation[5]. In the current study we investigated the role of macrophages in the regulation of OA pain and whether nociceptors drive the accumulation of these macrophages.

Results

Macrophages accumulate in the DRG during osteoarthritis pain

Joint damage was induced by a unilateral intra-articular injection of mono-iodoacetate (MIA) in mice[27]. MIA injection induced joint pathology that was present at week one and persisted for at least four weeks (Figs. 1A and 1B). Intra-articular MIA injection induced pain-like behaviour such as mechanical hypersensitivity (Fig. 1C) and a reduction of weight bearing (Fig. 1D) of the affected leg that persisted for at least for four weeks.

To identify whether the persisting pain was associated with immune cell infiltration, the lumbar (L3-L5) DRG containing the somata of sensory neurons that innervate the knee joint were analysed using flow cytometry (See figure 1E for the gating strategy). The number of $CD11b^{+}F4/80^{+}$ macrophages was increased at one week after intra-articular MIA injection and peaked at three to four weeks (Fig. 1F). $CD11b^{-}CD3^{+}$ T cell and $CD11b^{-}CD19^{+}$ B cell numbers in the DRG did not significantly change over time (Fig. 1F). In contrast, the number of $CD45^{+}Ly6G^{+}$ neutrophils were increased at week two and three after MIA injection, but returned to baseline numbers four weeks after MIA injection (Fig. 1F). To further identify where the macrophages were localized within the tissue, we performed iDISCO tissue clearing followed by staining of the lumbar spinal cord with the spinal nerves and DRG attached. At three weeks after MIA injection, an increased number of macrophages was present throughout the ipsilateral DRG and the dorsal horn of the spinal cord. Infiltration of macrophages into the DRG was specific for the ipsilateral side, because there was no evidence of macrophage accumulation at the contralateral side (Fig. 1G and Movie 1).

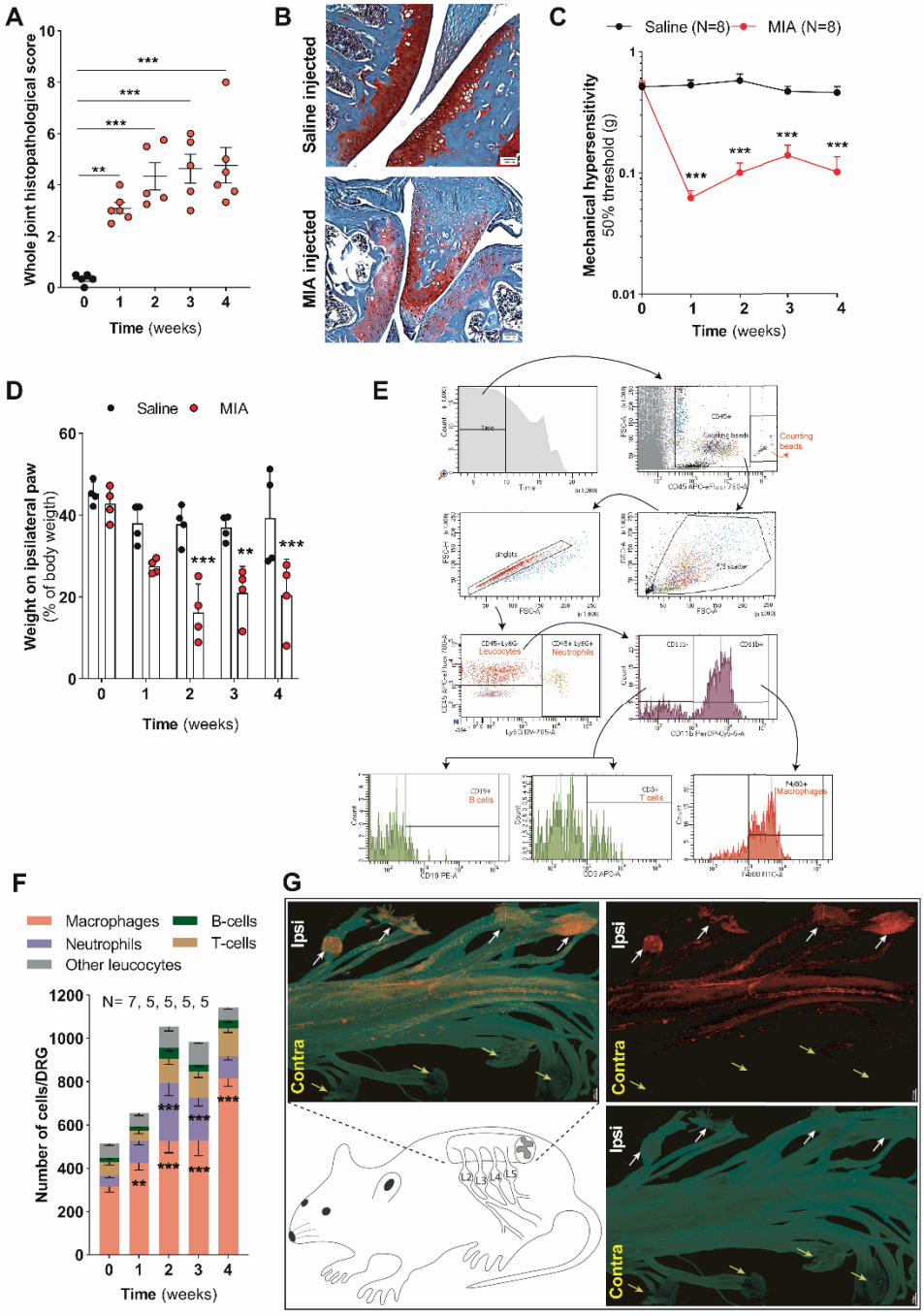
A
Whole joint histopathological score
Time (weeks)
B
Saline injected
MIA injected
C
Saline (N=8)
MIA (N=8)
Mechanical hypersensitivity
50% threshold (g)
Time (weeks)
D
Saline
MIA
Weight on ipsilateral paw
(% of body weight)
Time (weeks)
E
Counting beads
Leucocytes
Neutrophils
B cells
T cells
Macrophages
F
Macrophages
Neutrophils
Other leucocytes
B-cells
T-cells
N= 7, 5, 5, 5, 5
Number of cells/DRG
Time (weeks)
G
Ipsi
Contra
L2
L3
L4
L5

Figure 1. Macrophages infiltrate DRG in MIA-induce OA pain

(A) *Quantification of histopathological changes in the knee joints over 4 weeks after intra-articular MIA injection in mice. Knee joints were dissected for histology at indicated time points and scored according to the Histological-Histochemical Grading System. 1-way ANOVA with Dunnett post-hoc.* ***(B)*** *Exemplar image of mice knee joints at week 3 after intra-articular injection of MIA or Saline. The knee joints were stained with Safranin-O. Femur is on the left side and tibia is at the right. The left picture shows the normal cartilage structure (red staining) of the joint after saline injection. The right picture shows MIA-induced knee joint lesion that was observed in the joint of the MIA-injected mice. Scale bar: 200µM.* ***(C)*** *Course of mechanical hyperalgesia after injection of MIA in the ipsilateral or saline in the contralateral knee. Mechanical hyperalgesia was followed over time using Von Frey. 2-way ANOVA with Sidak post-hoc.* ***(D)*** *Course of weight distribution determined after intraarticular injection of MIA or saline in the contralateral knee using dynamic weight bearing apparatus. 2-way repeated-measures ANOVA with Sidak post-hoc.* ***(E)*** *Gating strategy to identify the immune cells (as displayed in Fig. 1FE) in the lumbar DRG (L3-L5) after MIA injection. A time gate unified the acquisition analysis, followed by a rough separation of events based on CD45 expression but excluding the counting beads. Cells were further gated by FCS/SSC (to identify the size of each cell), single cells, CD45 and Ly6G expression before analysis of CD11b, and F4/80. CD3 and CD19 cells were identified from the CD11b negative cells.* ***(F)*** *Lumbar DRG (L3-L5), from male and female mice, containing the sensory neurons innervating the knees that received MIA were isolated at each time point to determine the absolute number of CD45+ cells classified to subset per DRG. The control group (0 weeks) is pulled from DRG of mice that only received saline from all the time points (1, 2, 3, and 4 weeks). 2-way ANOVA with Dunnett post-hoc.* ***(G)*** *Light-sheet render of lumbar spinal cord with the spinal nerves and DRG attached of mice injected at one knee intra-articular with MIA (ipsilateral), and the other knee with saline (contralateral). Neurons are stained with Neurofilament M (green) and Macrophages with F4/80 (red). See movie 1 for 3D render. Scale bar: 500µm.*

Obesity is associated with developing OA pain. To test whether macrophages accumulation in the DRG is a generic mechanism that occurs in when OA is induced differently and in different species, we used the tibia-femoral ‘groove’ model in obese rats. To test whether macrophages accumulation in the DRG extends beyond MIA-induced OA model, we used the tibia-femoral ‘groove’ model in obese rats. The groove model in obese rats, combines systemic metabolic alterations with local cartilage damage and results in mild knee joint degeneration, without permanent joint instability and only limited joint inflammation[28].

At twelve weeks post groove surgery, histological OA scores were significantly increased (Figs. 2A and 2B). Groove surgery significantly decreased mechanical withdrawal thresholds (Fig. 2C) and weight bearing of the affected leg (Fig. 2D) at twelve weeks compared to the sham-operated controls. Macrophages (in rats $CD68^{+}$) in the lumbar DRG innervating the grooved OA knees were significantly enhanced compared to DRG related to the non-grooved contralateral knees at twelve weeks after surgery (Figs. 2E and 2F).

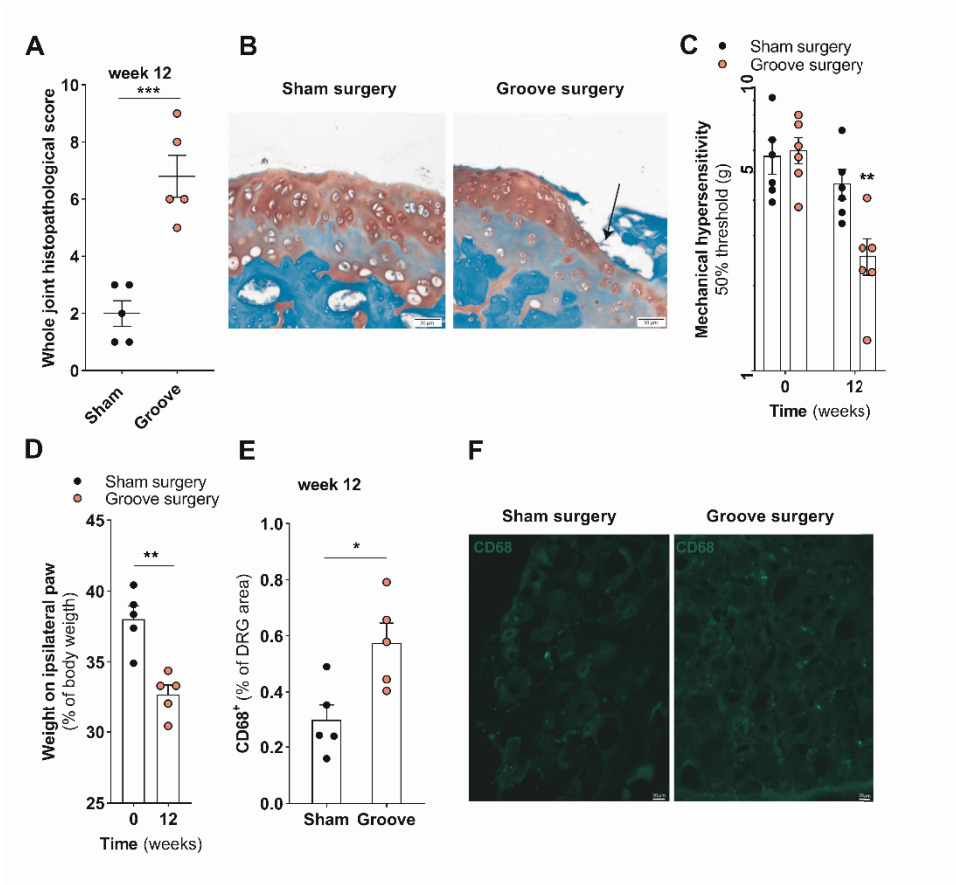

Figure 2. Groove surgery induced OA-like pain and macrophage accumulation into DRG

(A) *Quantification and of histopathological changes in the obese rat knee joints 12 weeks after groove or sham surgery. The cartilage degeneration is scored according to Histological-Histochemical Grading System. Unpaired two-tailed t tests.* ***(B)*** *Representative picture of histopathological changes in the rat knee joints 12 weeks after groove or sham surgery. The knee joints were stained with Safranin-O. The arrow indicates the cartilage degeneration induced by groove surgery. Scale bar: 50µM.* ***(C)*** *Mechanical hyperalgesia at the hind paw after groove (ipsilateral) or sham*

(contralateral) operation in rat knee joints before and 12 weeks after surgery. 2-way ANOVA with Sidak post-hoc. ***(D)*** *Paw weight distribution of the affected ipsilateral leg was evaluated before and 12 weeks after groove surgery in rat knee joints using dynamic weight bearing. Unpaired two-tailed t tests.* ***(E)*** *Quantification of $CD68^+$ macrophages in infiltrated lumbar (L3-L5) DRG containing the sensory neurons innervating the knees at 12 weeks after groove or sham operation. Unpaired two-tailed t tests.* ***(F)*** *Example immunofluorescent microscopy images of $CD68^+$ macrophages (green fluorescent) infiltrated lumbar (L3-L5) DRG in rats at 12 weeks after groove or sham operations. Scale bar: 20µM.*

Macrophages maintain osteoarthritis pain

To address the function of DRG-infiltrating macrophages in OA pain, we selectively depleted monocytes and macrophages three weeks after intra-articular MIA injection. Cells were depleted by injection of diphtheria toxin (DT) in mice that specifically express the diphtheria toxin receptor in monocytes and macrophages ($Lysm^{cre}$ x Csfr1-Stop-DTR mice; MM^{dtr}). Daily intraperitoneal injections of DT reduced the number of $F4/80^+$ macrophages in the DRG (Fig. 3A) as well as $CD115^+$ blood circulating monocytes (Fig. 3B). The number of other immune cells including $CD11b^+F4/80^-$ myeloid cells and $CD11b^-$ non-myeloid cells in the DRG did not change (Fig. 3A). Intraperitoneal DT administration to MM^{dtr} mice completely resolved the ongoing MIA-induced mechanical hyperalgesia (Fig. 3C) and resolved the deficits in weight bearing of the affected knee (Fig. 3D). In control mice, sensitivity to mechanical stimuli was not affected by intraperitoneal DT administration (Figs. 3C and 3D).

To address whether local depletion of DRG macrophages is sufficient to resolve OA-induced persisting pain behaviours, DT was intrathecally injected to MM^{dtr} mice. Intrathecal DT injection selectively depleted macrophages from the DRG without affecting blood circulating monocytes and other $CD45^+F4/80^-$ leukocytes in the DRG (Figs. 3A and 3B). Importantly, local depletion of macrophages in the DRG completely resolved established persistent MIA-induced mechanical hyperalgesia (Fig. 3E) and reduced weight bearing of the affected knee (Fig. 3F). Depletion of monocytes/macrophages abolished pain in both male and female mice (Figs. 3C and 3E). Overall, these data show that macrophages infiltrating DRG are responsible for maintaining OA pain.

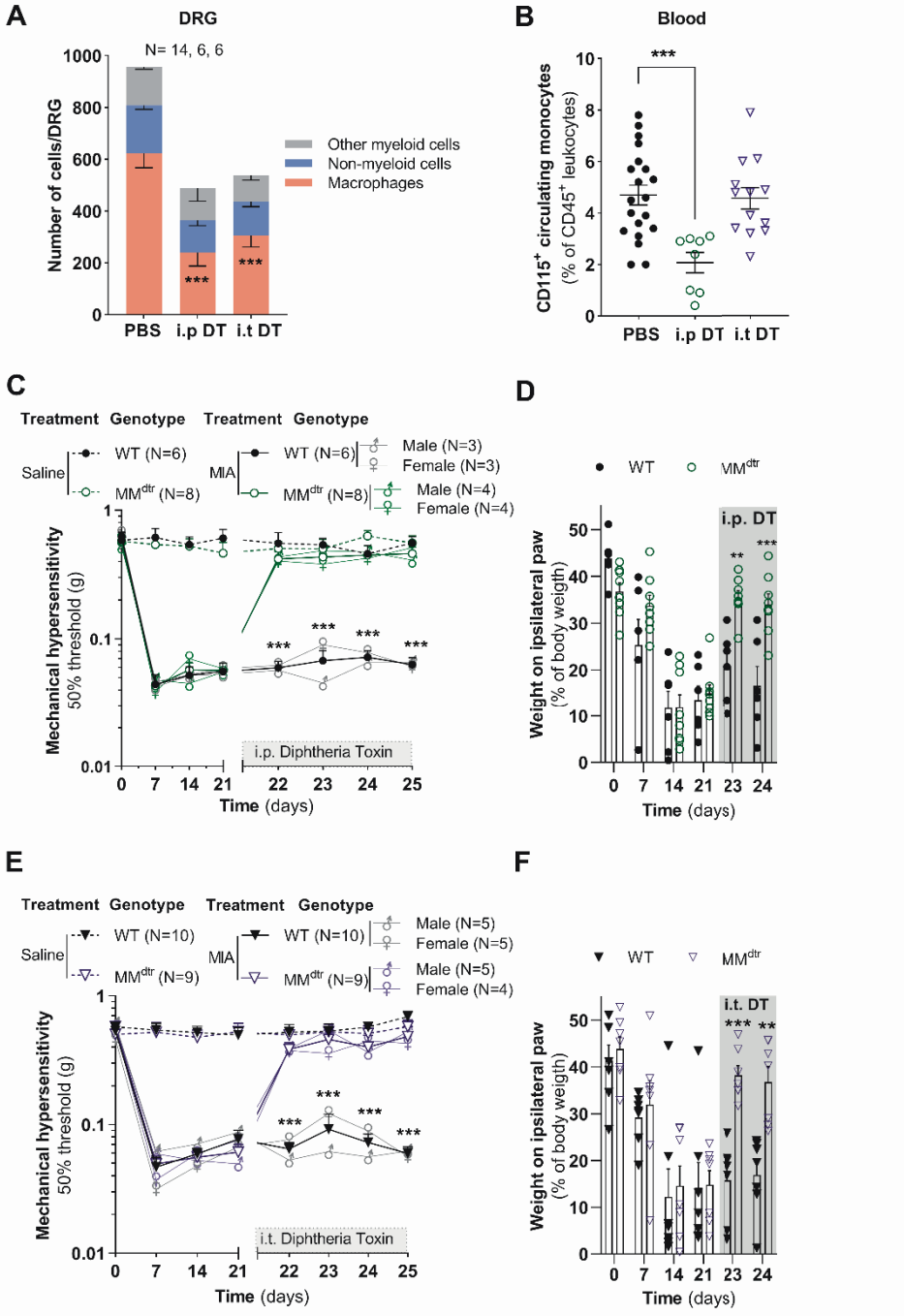
A
DRG
N= 14, 6, 6
Number of cells/DRG
1000
800
600
400
200
0
***
***
PBS
i.p DT
i.t DT
Other myeloid cells
Non-myeloid cells
Macrophages
B
Blood
***
CD115+ circulating monocytes
(% of CD45+ leukocytes)
10
8
6
4
2
0
PBS
i.p DT
i.t DT
C
Treatment Genotype Treatment Genotype
Saline
WT (N=6)
MMdtr (N=8)
MIA
WT (N=6)
MMdtr (N=8)
Male (N=3)
Female (N=3)
Male (N=4)
Female (N=4)
Mechanical hypersensitivity
50% threshold (g)
1
0.1
0.01
***
***
***
***
i.p. Diphtheria Toxin
0
7
14
21
22
23
24
25
Time (days)
D
WT
VIMdtr
i.p. DT
Weight on ipsilateral paw
(% of body weigth)
50
40
30
20
10
0
**
***
0
7
14
21
23
24
Time (days)
E
Treatment Genotype Treatment Genotype
Saline
WT (N=10)
MMdtr (N=9)
MIA
WT (N=10)
MMdtr (N=9)
Male (N=5)
Female (N=5)
Male (N=5)
Female (N=4)
Mechanical hypersensitivity
50% threshold (g)
1
0.1
0.01
***
***
***
***
i.t. Diphtheria Toxin
0
7
14
21
22
23
24
25
Time (days)
F
WT
MMdtr
i.t. DT
Weight on ipsilateral paw
(% of body weigth)
50
40
30
20
10
0
***
**
0
7
14
21
23
24
Time (days)

Figure 3. DRG infiltrating macrophages maintain osteoarthritis pain

(A, B) *Flow cytometry analysis of single cell suspension of DRG and blood isolated from MM^{dtr} mice treated with diphtheria toxin (DT) intraperitoneal (i.p.) or intrathecal (i.t.) to validate monocyte and macrophage depletion at week four after MIA injection.* ***(A)*** *Number of $F4/80^{+}$ macrophages, non-myeloid cells ($CD45^{+}CD11b^{-}$), and other myeloid cells ($CD11b^{+}F4/80^{-}$) in the DRG of MM^{dtr} mice after i.p. and i.t. injection of DT. 2-way ANOVA with Dunnett post-hoc.* ***(B)*** *Flow cytometry analysis of $CD45^{+}CD11b^{+}CD115^{+}$ monocytes in blood after i.t. and i.p. injection of DT in MM^{dtr} mice. 1-way ANOVA with Dunnett post-hoc.* ***(C)*** *Course of mechanical hyperalgesia in wild type (WT) versus MM^{dtr} mice, in female and male mice, after intra-articular injection of MIA in the ipsilateral knee and saline in the contralateral knee. Monocytes/macrophages were systemically depleted by daily i.p. injections of DT starting at day 21. 2-way ANOVA with Tukey post-hoc comparing MMdtr to WT in each timepoint. Statistical analyses using a repeated measures ANOVA indicated that the course of mechanical hyperalgesia was not significant different between males and females.* ***(D)*** *Weight distribution of the ipsilateral hind paws at indicated time points in WT versus MM^{dtr} mice after intra-articular injection of MIA in the left hind paw and saline in the right hind paw. Mice were treated with daily i.p. injection of DT starting at day 21 to deplete monocytes/macrophages. 2-way ANOVA with Sidak post-hoc.* ***(E)*** *Course of mechanical hyperalgesia in WT versus MM^{dtr} mice, in female and male mice, after intra-articular injection of MIA in the ipsilateral knee and saline in the contralateral knee. Mice received daily i.t. injections of DT starting at day 21 to depletion macrophages in the lumbar DRG. 2-way ANOVA with Tukey post-hoc comparing MMdtr to WT in each timepoint. Statistical analyses using a repeated measures ANOVA indicated that the course of mechanical hyperalgesia was not significant different between males and females.* ***(F)*** *Weight distribution of the ipsilateral hind paws at indicated time points in WT versus MM^{dtr} mice after intra-articular injection of MIA in the ipsilateral knee and saline in the contralateral knee. Mice received daily i.t. injection of DT starting at day 21 to deplete macrophages in the lumbar DRG. 2-way ANOVA with Sidak post-hoc.*

DRG macrophages recruitment and polarization is independent of Nav1.8 nociceptors

We next phenotypically analysed DRG macrophages using M1 and M2 markers. Expression of iNOS (a M1 macrophage marker) significantly increased in DRG infiltrating macrophages from week 1 after MIA injection and remained elevated until at least four weeks (Fig. 4A). In contrast, expression of CD206 (a M2 macrophage marker) remained similar (or even tended to be decreased) in MIA-injected mice compared to control mice during the four weeks (Fig. 4B). Thus, during OA pain, macrophages acquire a M1-like phenotype. To assess whether sensory neurons in the DRG drive this M1 skewing of macrophages, we co-

cultured bone marrow-derived macrophages with isolated sensory neurons that innervated the affected joint (ipsilateral) and those innervating the healthy joint (contralateral) one week after MIA injection. Macrophages cultured for 24 hours with sensory neurons innervating ipsilateral knee expressed more iNOS than macrophages cultured with sensory neurons innervating the contralateral knee joint (Fig. 4C). Expression of the M2 macrophage marker CD206 did not change significantly (Fig. 4D). Thus, sensory neurons from osteoarthritis mice program macrophages into a M1-like phenotype.

$Na_v1.8$ nociceptors are required for development of inflammatory pain but not neuropathic pain[29]. To test whether $Na_v1.8$ nociceptors are required for MIA-induced OA pain and associated with infiltration and skewing of macrophages in the DRG, we used mice in which $Na_V1.8$ nociceptors are ablated from birth by expression of diphtheria toxin A in $Na_V1.8$ neurons ($Na_v1.8$-DTA mice). MIA-induced persistent pain in $Na_V1.8$-DTA mice was indistinguishable compared to wild type mice for either mechanical sensitivity of the ipsilateral hind paw (Fig. 4E) or weight bearing of the affected leg (Fig. 4F). Moreover, the number and phenotype of DRG infiltrating macrophages were similar between $Na_V1.8$-DTA and wild type mice (Figs. 4G-4I). No significant differences were observed in DRG infiltrating $CD11b^-$ non-myeloid cells or other $CD11b^+F4/80^-$ cells (fig. 4G). Overall, these data indicate that $Na_v1.8$ nociceptors do not mediate OA pain. Moreover, sensory neurons, but not $Na_v1.8$ nociceptors, control macrophages recruitment and M1 differentiation of DRG macrophages during MIA-induced OA pain.

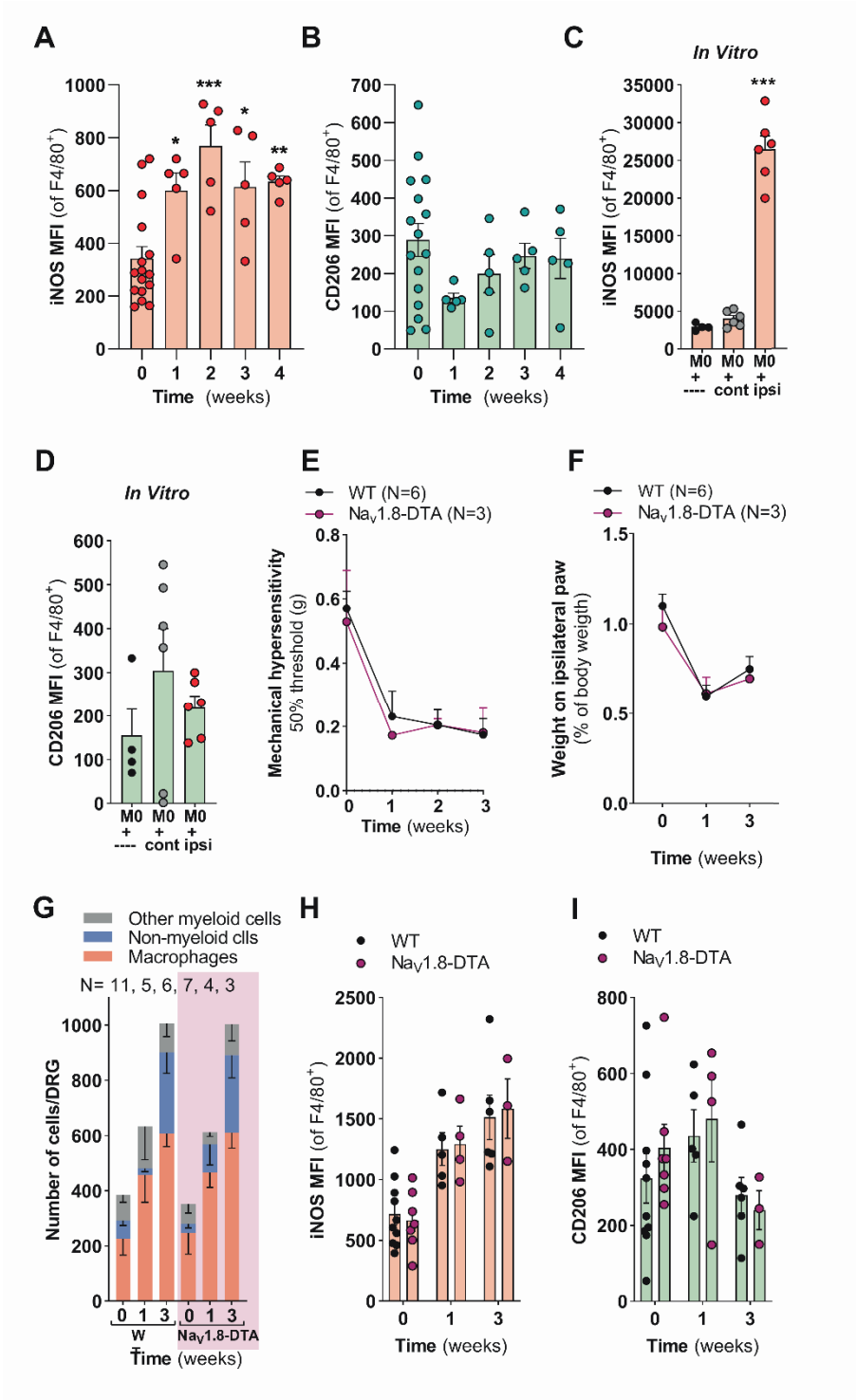
A
iNOS MFI (of F4/80+)
Time (weeks)
B
CD206 MFI (of F4/80+)
Time (weeks)
C
In Vitro
iNOS MFI (of F4/80+)
M0 + ---- cont
M0 + ipsi
D
In Vitro
CD206 MFI (of F4/80+)
M0 + ---- cont
M0 + ipsi
E
WT (N=6)
Nav1.8-DTA (N=3)
Mechanical hypersensitivity
50% threshold (g)
Time (weeks)
F
WT (N=6)
Nav1.8-DTA (N=3)
Weight on ipsilateral paw
(% of body weigth)
Time (weeks)
G
Other myeloid cells
Non-myeloid clls
Macrophages
N= 11, 5, 6, 7, 4, 3
Number of cells/DRG
W
Nav1.8-DTA
Time (weeks)
H
WT
Nav1.8-DTA
iNOS MFI (of F4/80+)
Time (weeks)
I
WT
Nav1.8-DTA
CD206 MFI (of F4/80+)
Time (weeks)

Figure 4. DRG macrophages acquire a M1-like phenotype independent of nociceptors

(A, B) *Median fluorescence intensity (MFI) of* ***(A)*** *iNOS and* ***(B)*** *CD206 of the F4/80$^+$ macrophage population in the DRG of mice at the indicated time points after intra-articular injection of MIA. 1-way ANOVA with Dunnett post-hoc.* ***(C, D)*** *Median fluorescence intensity (MFI) of* ***(C)*** *iNOS and* ***(D)*** *CD206 of bone marrow-derived macrophages in vitro co-cultured for 24 hours with isolated sensory neurons that innervated the MIA injected joint (ipsilateral) or healthy joint (contralateral) one week after MIA injection. 1-way ANOVA with Dunnett post-hoc.* ***(E)*** *Course of mechanical hyperalgesia after intra-articular injection of MIA in wild type (WT) versus Na$_V$1.8-DTA mice. 2-way ANOVA with Sidak post-hoc.* ***(F)*** *Weight distribution of wild type (WT) versus Na$_V$1.8-DTA mice before and at 1 and 3 weeks after intra-articular injection of MIA using static weight bearing apparatus. 2-way ANOVA with Sidak post-hoc.* ***(G)*** *Absolute number of CD45$^+$ leukocytes classified to subset per lumbar DRG of wild type (WT) compared to Na$_V$1.8-DTA mice (purple background) at indicated time points after intra-articular MIA injection. 2-way ANOVA with Dunnett post-hoc, comparing DRG infiltrated cells in WT versus NaV1.8 mice, in each timepoint.* ***(H, I)*** *Median fluorescence intensity (MFI) of* ***(H)*** *iNOS and* ***(I)*** *CD206 of F4/80$^+$ macrophage population accumulated in the DRG of wild type (WT) versus Na$_V$1.8-DTA before and at 1 and 3 weeks after intra-articular injection of MIA. 2-way ANOVA with Sidak post-hoc.*

Macrophages induce or resolve pain depending on their phenotype

To address whether the pain regulatory role of macrophages in the DRG is determined by their phenotype, we programmed macrophages in vitro into a M1-like phenotype before injecting them close to the DRG and spinal cord by intrathecal injection of naïve mice. Macrophages stimulated with 20 ng/ml IFNγ and 100 ng/ml LPS for 24 hours strongly upregulated the M1 marker iNOS (Fig. 5B). In contrast to M0 macrophages, intrathecal injection of M1-like macrophages into naive mice induced transient mechanical hypersensitivity at the hind paws that resolved within four days, indicating the M1-like phenotype of macrophages governs their pain promoting role (Fig. 5A).

M2 macrophages may inhibit the proinflammatory M1 macrophages and as such could dampen OA pain. To test whether M2 macrophages modulate macrophage M1 polarization, we co-cultured M1 macrophages with mCherry2 expressing M2 macrophages to enable analyses of each population separately. Macrophages stimulated with 20 ng/ml IL-4 for 24 hours had increased expression of M2 marker CD206 compared to unstimulated M0 macrophages (Fig. 5C). Co-culture of M1 macrophages with mCherry2-expressing M2 macrophages reduced the iNOS expression in the M1 macrophages compared to non-treated M1

macrophages (Fig. 5B). M2 macrophages did not affect CD206 expression in M1 macrophages (Fig. 5C).

To verify whether M2 macrophages also inhibit M1 macrophages *in vivo*, we injected 30.000 M2 macrophages intrathecally at day 21 during established MIA-induced pain. Injection of M2 macrophages reduced iNOS expression of the total F4/80^{+} macrophage population in the DRG compared to mice that received M0 macrophages (Fig. 5D). Intrathecal injection of M2 macrophages resolved the persistent MIA-induced pain, whilst M0 macrophages did not have any effect (Figs. 5E and 5F).

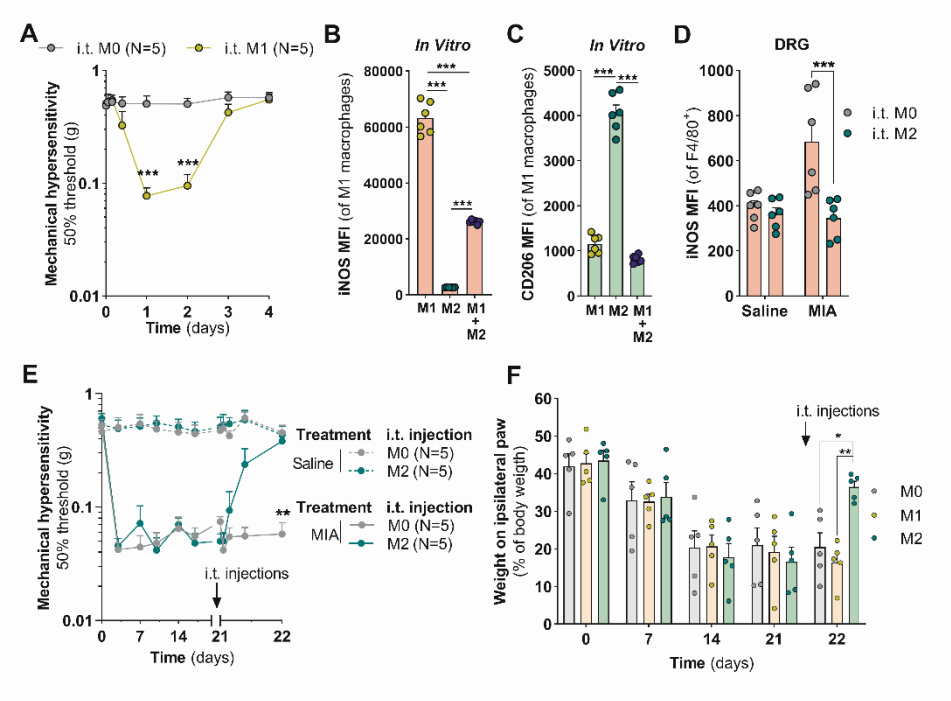

Figure 5. The M1 phenotype of DRG macrophages regulate their pain promoting function

(A) *Course of mechanical hyperalgesia in naïve mice injected intrathecally (i.t.) with 30.000 M0 or M1-like macrophages. 2-way ANOVA with Sidak post-hoc.* ***(B, C)*** *Median fluorescence intensity (MFI) of* ***(B)*** *iNOS and* ***(C)*** *CD206 of macrophages that were differentiated in vitro into a M1-like phenotype or M2-like phenotype, and M1 macrophages co-cultured for 24 hours with mCherry2 expressing M2-like macrophages. Expression of iNOS and CD206 was determined after gating out the mCherry2 positive macrophages. 1-way ANOVA with Tukey post-hoc.* ***(D)*** *iNOS Median fluorescence intensity (MFI) of F4/80^{+} macrophage population in the DRG of mice 24 hours after i.t.*

injection of M2-like macrophages. All animals received intra-articular injection of MIA or saline at day 0, and a single i.t injection of macrophages at day 21. DRG were isolated at day 22. 2-way ANOVA with Sidak post-hoc. ***(E)*** *Course of mechanical hyperalgesia after intra-articular injection of MIA in the left hind paw and saline in the right hind paw. Mice were i.t. injected with M0 macrophages or M2-like macrophages at day 21. Arrow indicates the moment of intrathecal injection of macrophages. 2-way ANOVA with Sidak post-hoc.* ***(F)*** *Weight distribution of ipsilateral hind paws during the course of MIA-induced OA in mice treated with i.t. injection of M0 macrophages, M1-like macrophages or M2-like macrophages at day 21. Arrow indicates the moment of intrathecal injection of macrophages. 2-way ANOVA with Sidak post-hoc.*

Inhibition of M1 macrophages resolves OA pain

To further assess whether pharmacological inhibition of M1 macrophages in the DRG resolves persistent OA pain, we tested if a fusion protein of the human anti-inflammatory cytokines IL4 and IL10 (IL4-10 FP), that has anti-nociceptive effect in inflammatory and neuropathic mouse pain models, could reskew DRG pain promoting macrophages and resolve OA pain[30,31]. Intrathecal injection of 1 µg IL4-10 FP protein in mice with established MIA-induced OA pain, significantly reduced expression of iNOS in DRG infiltrating macrophages 48 hours after the injection (Fig. 6A). IL4-10 FP also resolved the persistent mechanical hyperalgesia and deficit in weight bearing at 48 hours after IL4-10 FP administration (Figs. 6B and 6C). Overall, these data indicate that inhibiting DRG infiltrated M1 macrophages using the IL4-10 FP resolves OA-induced persistent pain.

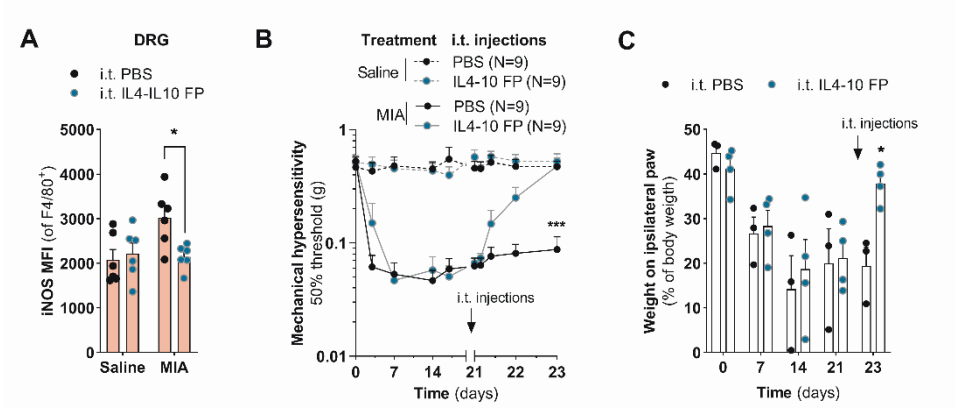

Figure 6. Inhibiting inflammatory DRG macrophages with a fusion protein of IL4-10 resolves OA pain

(A) *iNOS Median fluorescence intensity (MFI) of F4/80^{+} macrophages in the DRG of mice 48 hours after intrathecal injection of the fusion protein of IL4-10 (IL4-10 FP). All animals received intra-articular injection of MIA or saline at day 0, intrathecal (i.t.) injections are done at day 21 and DRG were isolated at day 23. 2-way ANOVA with Sidak post-hoc.* ***(B)*** *Course of mechanical hyperalgesia after intra-articular injection of MIA in the left hind paw and saline in the right hind paw. Mice were i.t. injected with IL4-10 FP or PBS. 2-way ANOVA with Sidak post-hoc.* ***(C)*** *Weight distribution of hind paws during the course of MIA-induced OA in mice received i.t. injection of IL4-10 FP or PBS at day 21. 2-way ANOVA with Sidak post-hoc.*

Discussion

In this study we identified a key role for DRG macrophages in the maintenance of OA pain. We unravelled that during the course of OA macrophages accumulate in the DRG innervating the damaged knee, and acquire an M1 phenotype. Importantly, these DRG macrophages maintain pain independent from the damage in the knee joint. We found that DRG sensory neurons that innervate the damaged knee program macrophages toward an M1 phenotype, a phenomenon which is independent of $Na_v1.8$ nociceptors. Intriguingly, established OA pain is treated by local depletion of DRG macrophages, or by inhibition of M1 macrophages either using M2 macrophages or a fusion protein of IL4-10 (IL4-10 FP). Overall these data indicate that macrophages are key players in the regulation of osteoarthritis pain and that these cells can be modulated to treat pain.

Over the recent years it has become clear that macrophages regulate neuropathic and inflammatory pain at various levels. Macrophages contribute to pain at the site of inflammation in a complete Freund's adjuvant (CFA) model of inflammatory pain, or at the site of injury after an incision[22,24]. Here we show for the first time that macrophages also accumulate in the DRG after joint damage in both mice and rats and contribute to the maintenance of OA pain. Importantly, we identified that DRG macrophages control OA pain as local depletion of macrophages was sufficient to resolve pain. These data are in line with recent findings that in neuropathic pain DRG macrophages are critical contributors to maintenance of pain[22]. Although some earlier reports indicate that infiltration of macrophages is sex-dependent[32,33], we found that DRG macrophages control OA pain in a sex-independent manner, similar to that in neuropathic pain[22]. Thus, osteoarthritis pain, similar to neuropathic pain, is controlled by DRG macrophages in both sexes.

The accumulation of DRG macrophages is either the result of proliferation of resident macrophages or infiltration of circulating monocytes. During nerve injury-induced pain, macrophage proliferation contributes to DRG macrophages expansion[22]. After induction of OA, the major accumulation of DRG macrophages occurred within the first week after knee damage and remained stable over the following four weeks. The major part of these macrophages was $CD115^+$ (data not shown) suggesting that the increase in macrophages, at least in part, is

caused by infiltration of circulating monocytes into the DRG in OA pain. In support of this hypothesis, in models of OA, sensory neurons express chemokines like CCL2 that attract monocyte-derived macrophages[17].

Macrophages are highly plastic and have marked functional heterogeneity[25]. Although we show here that DRG macrophages maintain OA pain, findings also point toward a pain resolving role of macrophages[21,34-36]. Intrathecal injection of M2 macrophages inhibited OA pain. In contrast, M1 macrophages injected intrathecally induce pain hypersensitivity. How M1 macrophages promote pain is not completely understood. Various studies support the idea that M1 macrophages may promote pain through the release of pro-inflammatory cytokines that activate sensory neurons[37,38]. For example, in neuropathic pain, macrophages in the DRG release reactive oxygen species (ROS) but also IL-1β that contribute to the maintenance of pain[22,39,40]. Importantly, we show that local inhibition of M1 macrophages with IL4-10 FP in the DRG prevents the maintenance of OA pain. Indeed, IL4-10 FP inhibits release of pro-inflammatory mediators by macrophages[31]. Thus, limiting pro-inflammatory M1 DRG macrophages prevents the maintenance of pain.

The question remains what drives the M1-like phenotype of DRG macrophages in OA pain? Activated sensory neurons in the DRG or spinal cord release factors such as neuropeptides, neurotransmitters or cytokines like IL-1β and TNF that may promote macrophage programming to a M1 phenotype[41-44]. Moreover, following peripheral axon injury, sensory neurons release exosomes containing microRNAs, including miR-21-5p, that program DRG macrophages toward a M1 phenotype[45]. We show that *in vitro* sensory neurons isolated from OA mice drive the M1 programming of macrophages. Unexpectedly, $Na_v1.8$ nociceptors, that are important for inflammatory pain, do not contribute to the attraction nor to the M1 programming of DRG macrophages, suggesting other sensory neurons, similar to neuropathic pain, drive macrophage attraction and polarization. Future research will need to identify the factors that program DRG macrophages in OA pain.

Beside macrophages, we observed infiltration of neutrophils in the DRG 2-3 weeks after MIA injection. A majority of studies indicate that there is no substantial role for neutrophils in the regulation of pain, since neutrophil depletion or recruitment of neutrophils into pain relevant areas did not change

the pain thresholds[24,46]. In our study the number of neutrophils were returned to the baseline numbers in the DRG four weeks after MIA injection while pain was still present, suggesting that DRG neutrophils do not contribute to maintaining OA pain. Neutrophils infiltrate the DRG probably because they are the first cells to respond to acute tissue damage. Some evidence exists that sensory neurons express damage markers such as ATF3 after joint damage[47]. ATF3 expression in sensory neurons is mainly elevated early after induction of OA, whilst four weeks after MIA injection when we did not observed DRG neutrophils ATF3 expression is also absent. Thus, possibly neutrophils migrate to the DRG in response to nerve damage caused by injury in the knee joint.

The importance of macrophages in maintenance of OA-pain, indicates that targeting DRG macrophages may be a therapeutic potential to treat OA pain. We showed that OA pain resolved following the depletion of macrophages. OA pain was also attenuated after intrathecal administration of either M2 macrophages or a fusion protein of IL4-10 that both reduced iNOS expression in inflammatory macrophages in the DRG. Similarly, reprogramming macrophages with IL-13 shift M1 macrophages into a M2 phenotype and reverses neuropathic pain[48]. However, both M2 and IL4-10 FP may also directly target sensory neurons. For example, IL-10 inhibits spontaneous activity of sensory neurons[49] and the IL4-10 fusion protein acts on microglia and sensory neurons[30]. Moreover, M2 macrophages resolve pain by transferring mitochondria to sensory neurons[34]. Thus possibly IL4-10 FP or M2 macrophages directly control sensory neurons and as consequence affect macrophage phenotype. The IL4-10 FP has also been shown to have direct pain relieving and chondroprotective effects in large animal models[50,51]. Clearly, these data point to important novel strategies to control OA pain.

The current treatments to resolve OA pain often fail or have significant side effects. OA pain can become independent from the pathological changes in the joint suggesting that other mechanisms than the joint damage itself contribute to pain. Here we provide a conceptual framework that DRG macrophages control OA pain independent of the damage in the joint. As such these macrophages represent an interesting target to treat OA chronic pain. Such novel therapeutic strategies should focus on the reprogramming the DRG macrophages and may include, the fusion protein of IL4-10 or potentially even cell-based therapies that reprogram inflammatory macrophages in the DRG.

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Data and materials availability: All data is available in the manuscript or supplementary materials. Raw data and materials will be made available upon reasonable request. Some materials used in this manuscript are subject to a Material Transfer Agreement (MTA).

Materials and Methods

Animals

All experiments were performed in accordance with international guidelines and approved by the experimental animal committee of University Medical Center Utrecht (DEC 2014.I.03.019) or by the local experimental animal welfare body and the national Central Authority for Scientific Procedures on Animals (AVD115002015323 and AVD115002016490).

Adult (age 8–15 weeks) male and female, *Lysm*^{Cre} x *Csf1r* $^{LsL\text{-}DTR}$ (Jackson laboratories #024046) mice in a C57Bl/6 background (MM^{dtr}) were used and maintained at the animal facility of the Utrecht University and University Medical Center Utrecht. Experiments with Nav1.8^{Cre} x eGFP-DTA ($Na_V1.8$-DTA) mice were performed at University College London, UK[52]. Wistar rats (Charles-River, Sulzfeld, Germany), 16 weeks of age, were housed two per cage. Mice were housed in groups. All animals were kept under a 12h:12h light:dark cycle, with food and water available *ad libitum*. The cages contained environmental enrichments including tissue papers and shelter. Animals were acclimatized to the experimental setup for at least 1 week prior to the start of each experiment and baseline measurements were at least performed three times. To minimize bias, animals were randomly assigned to the different groups prior to the start of experiment, and experimenters were blinded for the treatments and genotypes.

Osteoarthritis (OA) pain models

Mice received an intra-articular injection of 10 µl mono-iodoacetate (MIA) (10% w/v, Sigma-Aldrich) in one knee joint (ipsilateral) under isoflurane anesthesia. The other knee joint (contralateral) received 10 µl sterile saline (0.9%). Knee joints was flexed at a 90° angle and MIA or sterile saline was injected with a 30-gauge needle[27].

In rats 'groove surgery' was performed in one knee joint (ipsilateral), under isoflurane anesthesia, to induce local cartilage damage. Prior to groove surgery, rats received a high fat diet (D12492i, Research Diets Inc., NJ, USA) for a period of 12 weeks. Groove surgery was done as described previously[53]. In short, surgery was performed in one knee joint of rats at baseline, under general Isolflurane anesthesia, to induce local cartilage damage. The knee joint cavity was opened

with a small longitudinal incision through the ligamentum patellae of the knee joint. The tip of an enhanced surgical tool was bent 90° at 150–180 µm from the tip, to ensure the underlying subchondral bone was not damaged, because the articular cartilage of the rat is approximately 200–250 µm thick. Five longitudinal grooves were applied on the weight bearing area of the articular cartilage of both the medial and lateral femoral condyle, and three longitudinal grooves on the non-weight bearing surface of the articular cartilage of the femoral trochlea. After the surgical procedure, all animals quickly recovered after surgery and no wound healing problems were observed.

Pain behavioral tests

Mechanical thresholds were assessed using the von Frey test (Stoelting, Wood Dale, IL, US) with the up-and-down method to determine the 50% threshold[54]. In short, von Frey filaments were applied for a maximum of 5 second to the plantar surface of the paw. After applying the first filament (0.4 g). In case of a non-response the next filament with a higher force is used. In case of a response the next lower force filament is used. Four readings are obtained after the first change of direction.

Changes in weight bearing were evaluated using the dynamic weight bearing (DWB) apparatus (Bioseb, Vitrolles, France). For mice analysis we used (i) Low weight threshold of 0.5g and (ii) High weight threshold of 1g. For rat analysis we used (i) Low weight threshold of 1g and (ii) High weight threshold of 2g. The following parameters were used in all the analysis (iii) Surface threshold of 2 cells, (iv) Minimum 5 images (0.5 seconds) for stable segment detections[55]. The weight bearing of the affected knee is expressed as percentage of body weight.

Depletion of monocytes and macrophages

To deplete monocytes and macrophages in vivo, MM^{dtr} mice received a first intraperitoneal injection of 20 ng/g body weight diphtheria toxin (DT) (Sigma-Aldrich) followed by daily intraperitoneal injections of 4 ng/g body weight on all subsequent days. For local DRG macrophage depletion, each mouse received 5 µl of (5 ng/µl) DT by daily intrathecal injections[56,57].

Monocyte isolation and in vitro differentiation into macrophages

For monocyte-derived macrophage generation, 10 million bone-marrow cells were seeded in a 75 cm^2 non-treated tissue culture flasks (VWR, Radnor, PA) for

7 days in macrophage medium (High-glucose Dulbecco's Modified Eagle medium (DMEM; Cat# 31966-021, Gibco) and DMEM/F12 (Cat#31331-028, Gibco) (1:1), supplemented with 30% L929 cell-conditioned medium, 10% fetal bovine serum (FBS; Cat# 10270-106, Gibco), 1% Penicillin/Streptomycin (Gibco) and 1% L-Glutamine (200 mM, ThermoFisher).

To differentiate macrophages toward M1- or M2-like macrophages, cells were stimulated with 20 ng/ml IFNγ and 100 ng/ml LPS, or 20 ng/ml of IL-4 for 24 hours, respectively.

To obtain L929 cell-conditioned medium (L929-drived M-CSF), 10 million L929 cells were seeded in a 75 cm^2 flask with cell culture-medium supplemented with 1% non-essential amino acids (Sigma-Aldrich) for a week. L929 cells were passaged to a 162 cm^2 flask with 50 ml medium and after a week the supernatants were collected and filtered through a 0.2-µm filter and stored at −20°C (L929-drived M-CSF).

Adoptive transfer of macrophages

Macrophages were dissociated from the 75 cm^2 non-treated tissue culture flasks (VWR, Radnor, PA) using 1% Trypsin-EDTA solution and washed in 10 ml macrophage medium and dissolved at a concentration of 6 million cells in 1 ml of sterile PBS. Cells were injected intrathecally (30.000 cells/5 µl per mouse) under light isoflurane anesthesia using a 30G needle as described previously[34,54].

Flow cytometry analysis

DRGs (L3–L5) were collected to analyze infiltrating immune cells. In brief, tissues were gently minced and digested at 37 °C for 30 minutes with an enzyme cocktail (1 mg collagenase type I with 0.5 mg trypsin in 1 ml DMEM; Sigma Aldrich). Cells were stained with various combinations of fluorochrome-labeled antibodies (see table 1 for used antibodies).

Blood was collected in EDTA tubes (Greiner Bio-One) following heart puncture and erythrocytes were lysed (RBC lysis buffer, eBioscience) before FACS staining. Samples were acquired by LSRFortessa flow cytometer (BD Biosciences) and analyzed with FACSDIVA software, counting beads were added. For all cellular analysis, we used FSC as trigger to identify events.

Immunofluorescent staining and histological preparations

Lumbar DRG or knees were collected. DRGs were post-fixed in 4% paraformaldehyde (PFA), cryoprotected in 30% sucrose in PBS overnight and embedded in optimal cutting temperature (OCT) compound (Sakura, Zoeterwoude, the Netherlands), and frozen at −80°C. Knees were fixed in 4% paraformaldehyde, decalcified in 20% EDTA. Dehydrated and embedded in paraffin wax.

For immunofluorescence, cryosections (10 µm) of lumbar DRG, were stained with primary antibodies overnight at 4°C followed by 2 hours incubation with fluorescent-tagged secondary antibodies (see table 1 for used antibodies). Immunostaining images were captured with a Zeiss Axio Observer microscope (Zeiss, Oberkochen, Germany) using identical exposure times for all slides within one experiment.

For histological analysis of the knee, specimens were sagittal sectioned at 6 µm and stained with safranin-O and imaged with a Zeiss Axio Lab A1 microscope. Damage to the joint were evaluated using Histological-Histochemical Grading System (HHGS) score on a scale of 0–14 points[58]. The scores were evaluated by three trained independent observers which were blinded to the groups and treatments.

iDisco, clearing procedure and light sheet imaging

Lumbar spinal cord with DRG attached, from adult mice, were cleared using iDISCO protocol as described before[59]. Briefly, animals were perfused with 4% PFA, lumbar spinal cord with the spinal nerves and DRG attached were dissected and samples were dehydrated in increasing concentrations (20%, 40%, 60%, 80%, 100%) of methanol solutions. Samples were bleached with 5% H2O2 in methanol overnight at 4°C with shaking and rehydrated in decreasing concentrations of methanol solutions. After blocking for 48 hours at 37°C, samples were incubated with the primary antibodies for 48 hours followed by incubation with secondary antibody for another 48 hours all at 37°C with shaking. While rotating- shaking (see subheading 'antibodies'). After samples were embedded in 1.5% agarose, they were dehydrated in increasing concentrations of methanol solutions. Samples were incubated overnight in 1 volume of 100% methanol/2 volumes 100% dichloromethane (DCM) anhydrous, washed with 100% DCM for 1 hour and incubated in 100% dibenzyl ether (DBE) for at least one day before imaging. Samples were imaged with an Ultramicroscope II

(LaVision BioTec) light sheet microscope equipped with Imspector (version 5.0285.0) software (LaVision BioTec). The microscope consists of an Olympus MVX-10 Zoom Body (0.63-6.3x) equipped with an Olympus MVPLAPO 2x Objective lens, which includes, dipping cap correction optics (LV OM DCC20) with a working distance of 5.7mm. The effective magnification for the images was 1.36x (zoombody * objective+dipping lens = 0.63x*2.152x). Images where taken with a Neo sCMOS camera (Andor) (2560x2160 pixels. Pixel size: 6.5 x 6.5 um2). Samples were scanned with a sheet NA of 0.148348 (results in a 5 µm thick sheet) and a step-size of 2.5 µm using the horizontal focusing light sheet scanning method with the optimal number of steps and using the contrast blending algorithm. The following laser filter combinations were used: Coherent OBIS 561-100 LS Laser with 615/40 filter and Coherent OBIS 647-120 LX with 676/29 filter.

Statistical analysis

All data are presented as mean ± SEM and were analyzed with GraphPad Prism version 8.3 using unpaired two-tailed t tests, one-way or two-way ANOVA, or as appropriate two-way repeated measures ANOVA, followed by post-hoc analysis. The used statistical and post-hoc analyses are indicated in each figure. A p value less than 0.05 was considered statistically significant, and each significance is indicated with *: $p < .05$; **:$p < .01$; ***: $p < .001$.

Table 1. Antibodies used for flow cytometry, immune fluorescent microscopy and iDISCO

Target	Clone	Fluorophore	Vendor	Catalogue #
Flow cytometry				
CD115	AFS98	APC	eBioscience	1277550
CD115	AFS98	PEeF610	eBioscience	61-1152-80
CD11b	M1/70	PerCPCy5.5	BioLegend	101227(8)
CD11c	N418	BV785	BioLegend	117336
CD19	6D5	PE	BioLegend	115508
CD206	C068C2	BV650	eBioscience	1308615
CD3	17A2	APC	BioLegend	100236
CD45	30-F11	APCeF780	eBioscience	47-0451-82
F4/80	BM8	FITC	BioLegend	123108
iNOS	CXNFT	APC	eBioscience	17-5920-80
Ly6C	AL-21	BV421	BD Bioscience	562727
Ly6G	1A8	BV785	BioLegend	127645
MHCII	M5/114.15.2	PerCP	BioLegend	107624
Immune fluorescent microscopy and iDISCO				
F4/80	C1:A3-1 rat	None	Cedarlane	CL8940AP
β3-Tubulin	Poly. rabbit	None	Abcam	ab18207
CD68 (anti-Rat)	ED1 mouse	None	BIO-RAD	MCA341GA
Anti-Mouse	Donkey	AF488	LifeTech	A21202
Anti-Rabbit	Donkey	AF568	LifeTech	A10042
Anti-Rat	Goat	AF647	LifeTech	A21247

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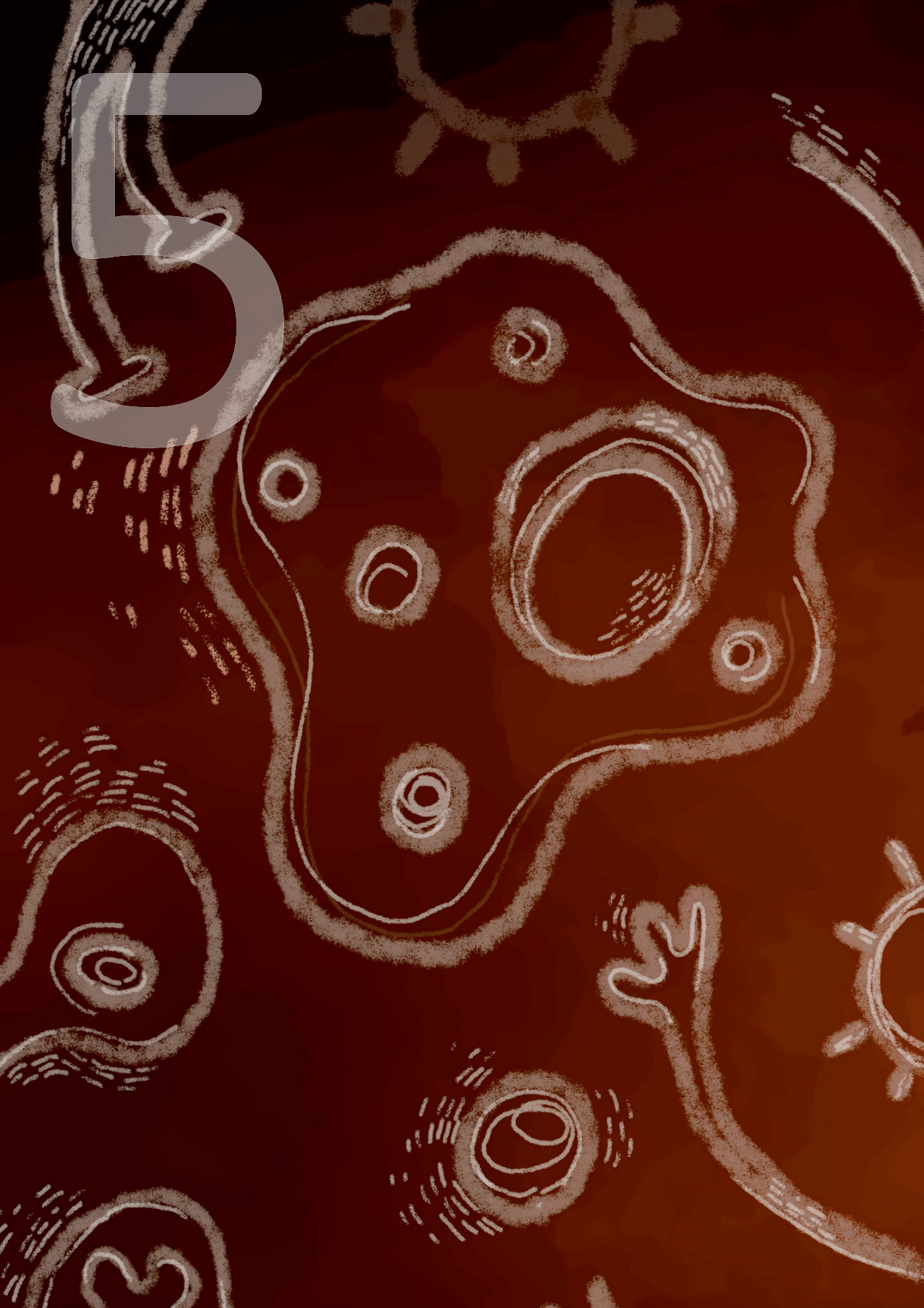
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CHAPTER 5

CXCL11 Controls Accumulation of DRG Macrophages in Osteoarthritis Pain

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To be submitted

Abstract

Pain is the major debilitating symptom of osteoarthritis (OA), which is difficult to treat. In OA patients joint tissue damage only poorly associates with pain, indicating other mechanisms contribute to the experienced OA pain. Macrophages accumulate in dorsal root ganglia (DRG) that innervate the affected joint and maintain OA pain independent from the damage in the joint. Here we aimed to elucidate what factors drive DRG macrophage accumulation and whether DRG macrophages, in addition to maintenance, also contribute to initiation of OA pain.

To induce OA, mice received unilateral mono-iodoacetate (MIA) in the knee joint. MIA injection into the knee joint induced pain behaviors such as mechanical hyperalgesia and deficits in weight bearing of affected leg within one week. The development of pain behavior was associated with accumulation of DRG macrophages with a M1-like phenotype starting from day 3. Analysis of mRNA from the DRG identified 19 upregulated genes and 36 downregulated genes at day 3 after MIA injection. Of the upregulated genes, CXCL11 was most prominently increased. Blockade of CXCL11 by intrathecal injection of neutralizing CXCL11 IgG antibodies, prevented the accumulation of DRG macrophages at day 7. CXCL11 blockade did not affect development of pain-like behaviors at days 1-3, however, at day 7 pain had completely resolved. Similarly, selective depletion of monocytes and macrophages by daily intraperitoneal injection of diphtheria toxin (DT) in $Lysm^{cre}$ x $Csf1r^{LsL\text{-}DTR}$ mice (MM^{dtr}) from day -1 to 7 did not affect development of pain but recovered mice from OA pain starting from day 7. Intrathecal injection of CXCL11 in naive mice was sufficient to increase the number of DRG macrophages. However, exogenous CXCL11 was not associated with M1 programming of macrophages and did not induce any sign of pain behaviors. Together, our data show that CXCL11 is a driver of DRG macrophage accumulation, but not sufficient to induce pain. DRG macrophages contribute to persistence, but not initiation of OA pain.

Keywords: Osteoarthritis pain, CXCL11, Macrophage, DRG, Chemokine, Cytokine

Introduction

Osteoarthritis (OA) is the most common joint disorder and is the leading cause of chronic pain[1]. Worldwide, 250 million people are affected by knee OA[2]. Approximately 12% of the adult population has symptomatic OA, and this number is expected to increase to 50% over the next 20 years[3]. The most prominently clinical symptom of OA is joint pain. Treatments that alter the progression of the structural damage in the joint are still in development and none is yet approved. Options for treating the OA pain such as opioids, nonsteroidal anti-inflammatory drugs, or steroids are often inadequate to effectively control pain. Moreover, prolonged treatment as needed in OA is undesirable because of side effects. Uncontrolled pain is the number one reason that people with OA undergo joint-replacement surgery[3].

Despite the enormous health and economic burden of OA, the molecular mechanisms of OA pain are still poorly understood[1]. OA pain can become independent from the pathological changes in the joint suggesting that other mechanism contribute to pain[4-7]. For example, peripheral and central sensitization plays an important role in persistent pain in some OA patients[8-10]. and is present in patients that suffer from severe pain but in absence of significant radiographic changes[11]. Immune cells regulate pain in peripheral and central pain pathways[12,13]. In rodent models of OA, including rat and mouse monoiodoacetate (MIA)-induced OA[12,14] and surgical-induced models of OA[1,14], macrophages accumulate in the lumbar dorsal root ganglia (DRG) innervating the damaged knee. Importantly, these DRG macrophages maintain OA pain independent from the level of damage in the knee joint. Evidence supports a key role for chemokines that control the trafficking of macrophages into the DRG. During neuropathic pain a range of chemokines, including CCL2, CX3CL1, CXCL9 , CXCL10, and CXCL12 are upregulated in DRG, that have shown to attract macrophages to nervous tissue[15-18]. Moreover, after nerve injury sensory neurons express CSF1 that promotes macrophage expansion in the DRG[19] What drives macrophage accumulation in the DRG during osteoarthritis is still poorly understood. Evidence supporting a role for chemokines in OA is that in a surgical mouse model of OA, CCR2 deficiency prevents macrophage infiltration in DRG two months after surgery. Of note, CCR2 deficiency may also affect pain through direct loss of CCL2-CRR2 mediated excitation of DRG neurons.[1] We have shown

that macrophages accumulate DRG already 7 days after injury and that these macrophages are required for the maintenance of OA pain three weeks after OA induction[14]. Therefore, in the current study we aimed to identify putative factors that drive the early accumulation of DRG macrophages in MIA-induced osteoarthritis and whether these macrophages contribute to initiation of OA pain.

Results

Monocyte-derived M1 macrophages accumulate DRG in an early stage of osteoarthritis

OA was induced by a single unilateral mono-iodoacetate (MIA) injection into the knee joint of mice. MIA injection induced pain-like behaviors such as reduced mechanical sensitivity (Fig. 1A) and deficits in weight bearing (Fig 1B) of the affected knee within the first week after MIA injection. We analyzed the joint innervating DRG at days three and seven, to identify whether the initiation of OA pain was associated with expansion of $CD11b^+$ myeloid cells in the DRG using flow cytometry. The number of $F4/80^+$ macrophages was increased at days 3 and 7 after MIA injection (Fig. 1C). In contrast, $Ly6G^+$ neutrophils and other $CD11b^+F4/80^-$ myeloid cells did not change significantly compared to control group at days 3 and 7 (Fig. 1C). Next, we determined whether DRG macrophages expressed the CSF1 receptor CD115 as a marker to differentiate between tissue resident macrophages and macrophages derived from infiltrating blood monocytes[20,21]. At day 3, a significantly higher percentage of DRG macrophages expressed CD115 compared to naive mice, indicating that the increase in DRG macrophages is likely caused by infiltration of circulating monocytes (Fig. 1D). Previously we showed that the M1-like phenotype of macrophages in the DRG is essential for their pain promoting role. At days 3 and 7, expression of the M1 marker iNOS was significantly increased, while the M2 marker CD206 remained indistinguishable in DRG macrophages of MIA-injected mice compared to control mice (Figs. 1E and 1F). These data show that in the early stage of OA, monocyte-derived macrophages accumulate in the DRG and adapt a pain promoting M1-like phenotype.

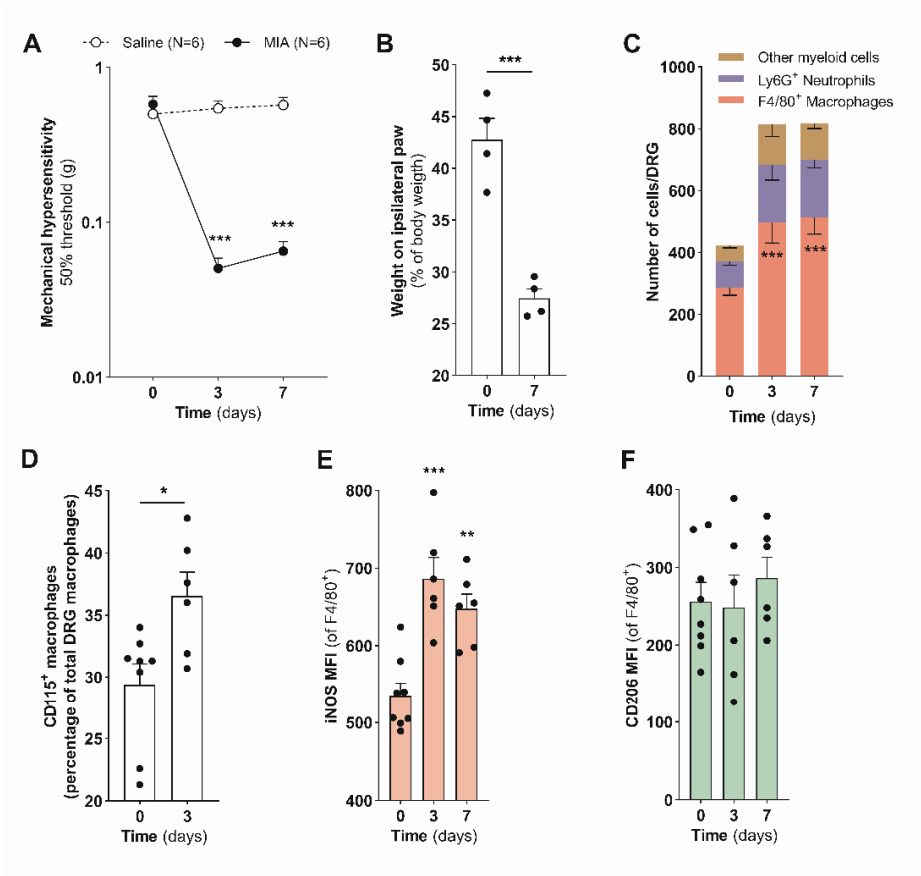

Figure 1. Monocyte derived DRG macrophages acquire a M1 phenotype in early stage of OA

(A) *Course of mechanical hyperalgesia during one week after intra-articular injection of MIA in the ipsilateral knee. The contralateral knee received an intra-articular saline injection as control. The course of mechanical hyperalgesia was measured using Von Frey. 2-way repeated measure ANOVA, Sidak post-hoc* ***(B)*** *Weight distribution determined before and 7 days after a unilateral intraarticular injection of MIA measured using dynamic weight bearing apparatus. Unpaired two-tailed t tests.* ***(C)*** *Lumbar DRG containing the sensory neurons innervating the knees that received MIA were isolated at days 3 and 7 after MIA injection to determine the absolute number of CD11b+ myeloid cells classified to subsets per DRG. 2-way repeated measure ANOVA, Sidak post-hoc* ***(D)*** *The percentage of CD115+ macrophages (from total F4/80+ DRG macrophages) in the DRG innervating the MIA injected knees at day 3 after MIA injection. Unpaired two-tailed t tests.* ***(E, F)*** *Median fluorescence intensity (MFI) of* ***(E)*** *iNOS and* ***(F)*** *CD206 of the F4/80+ DRG macrophages innervating the affected knees at days 3 and 7 after MIA injection. 1-way ANOVA, Dunnett post-hoc. Data represent mean ±SEM; *p<0.05; **p<0.01; ***p<0.001.*

Endogenous CXCL11 contributes to DRG accumulation of macrophages and the maintenance of osteoarthritis pain

To identify which factors cause DRG macrophages accumulation in OA, we analyzed changes in mRNA expression of 90 cytokine and chemokines in the DRG of MIA-treated mice at day 3 (Table 1). Innervating DRG samples from 5 mice that received intra-articular MIA injection or from naive mice were pooled per group and analyzed using a RT^2 profiler PCR array. We identified 19 genes that were upregulated, whilst 36 genes were downregulated (Fig. 2A and Table 1). From the upregulated genes, CXCL11 was the most elevated with a 7.6 fold increase (Table 1). Validation of CXCL11 expression by qPCR confirmed that CXCL11 was increased at day three post MIA injection. However, 1 week after MIA injection CXCL11 expression had returned to the baseline levels (Fig. 2B). Monocytes and macrophages highly express the CXCL11 receptor (CXCR3). Therefore, we hypothesized that CXCL11 may facilitate macrophages accumulation in the DRG during onset of OA. To test this hypothesis, male and female mice received intrathecal injections of CXCL11 neutralizing antibodies from day 0 until day 4 after MIA injection. Neutralizing anti-CXCL11 IgG injections reduced the number of DRG macrophages in MIA-treated mice compared to mice that received control IgG. This effect was observed in both female and male animals. At day 7 the number of macrophages were indistinguishable from baseline numbers (Fig. 2C). Expression of the M1 marker iNOS was significantly decreased after treatment with CXCL11 neutralizing antibodies compared to control IgG (Fig. 2D). The M2 marker CD206 in DRG macrophages was not affected (Fig 2E). Intrathecal injections of anti-CXCL11 IgG resolved MIA-induced pain in both male and female mice at day 7, with the first inhibition of pain-like behaviors starting at day 4 (Fig. 2F). In contrast, anti-CXCL11 IgG did not affect the development of MIA-induced pain from day 0 until day 3 compared to mice treated with control IgG (Fig. 2F) These data show that CXCL11 is required for DRG accumulation of macrophages and the maintenance but not initiation of OA pain.

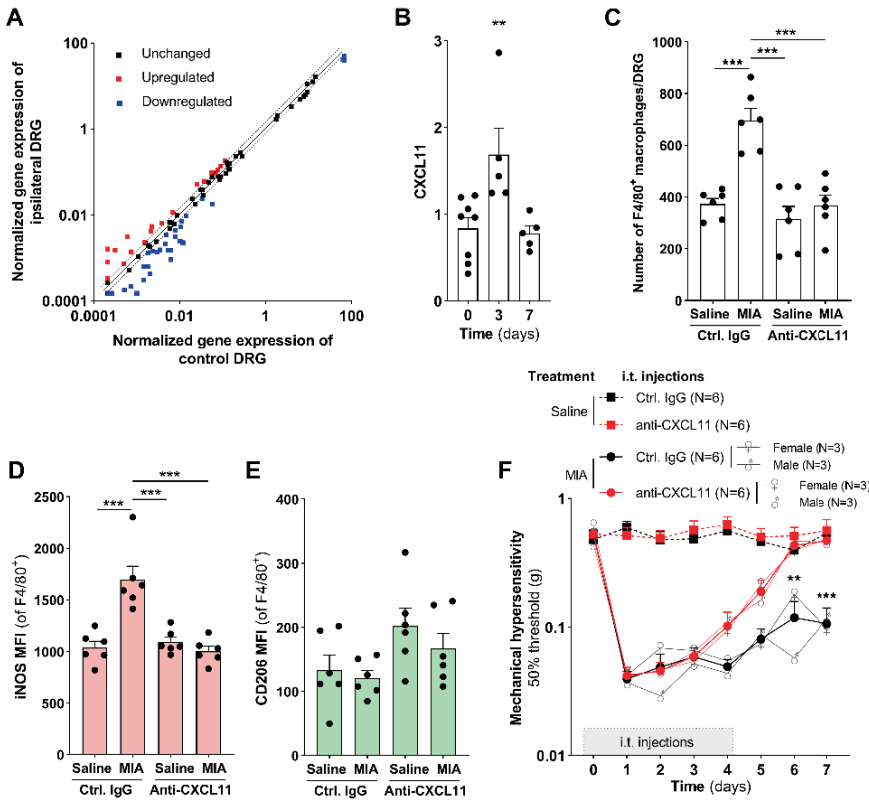

Figure 2. CXCL11 is required for development of osteoarthritis pain

(A) *Scatter plot comparing the normalized expression of 90 chemokine and cytokine genes in the DRG innervating the MIA injected knees versus knees from non-injected control animals at day 3 after MIA injection. DRG samples for each group were pooled from 5 mice and analysed using RT² profiler PCR array. The central line indicates unchanged gene expression. The dotted lines indicate the 1.4 fold up- and down-regulation threshold. Data points above the dotted lines are up-regulated genes whilst below the dotted line are down-regulated genes in MIA-treated mice compared to control treated mice.* ***(B)*** *Expression of CXCL11 mRNA in the lumbar DRG before and at days 3 and 7 after intra-articular MIA injection. 1-way ANOVA, Dunnett post-hoc.* ***(C)*** *Number of F4/80⁺ macrophages per lumbar DRG at day 7 after intraarticular MIA injection. Mice received daily intrathecal injections of anti-CXCL11 or control IgG starting from day 0 until day 4 after MIA injection. 1-way ANOVA, Dunnett post-hoc.* ***(D, E)*** *Median fluorescence intensity (MFI) of* ***(D)*** *iNOS and* ***(E)*** *CD206 of the F4/80⁺ macrophage population in lumbar DRG (L3-L5) at day 7 after intraarticular MIA or saline injection. Mice received daily i.t. injection of anti-CXCL11 or control IgG from day 0 to 4. 1-way ANOVA, Dunnett post-hoc.* ***(F)*** *Course of mechanical hyperalgesia after injection of MIA in the ipsilateral and saline in the contralateral knee of male and female Mice received daily intrathecal (i.t.) injection*

*of anti-CXCL11 IgG or control IgG starting from day 0 for 4 days. 2-way repeated measure ANOVA, Sidak post-hoc. Data represent mean ±SEM; *p<0.05; **p<0.01; ***p<0.001.*

Table 1. Fold changes in expression of 90 chemokine/cytokine genes

Unchanged genes		Up-regulated* genes		Down-regulated* genes	
Gene	Fold Regulation	Gene	Fold Regulation	Gene	Fold Regulation
Bmp2	-1,3122	Adipoq	1,4123	Bmp7	-3,0147
Bmp4	-1,2501	Ccl1	1,8251	Ccl11	-2,2222
Bmp6	1,0338	Ccl2	1,4221	Ccl12	-1,5605
Ccl17	-1,3031	Ccl3	1,5999	Ccl22	-6,6438
Ccl19	1,0267	Ccl4	1,7148	Ccl5	-1,426
Ccl20	1,3641	Cd40lg	1,6335	Cd70	-1,6609
Ccl24	1,238	Cntf	1,4123	Csf1	-1,426
Ccl7	1,108	Cxcl11	7,558	Ctf1	-3,6604
Csf2	1,0556	Cxcl5	1,5241	Cxcl1	-3,4153
Csf3	1,1313	Il11	2,0251	Cxcl10	-2,2689
Cx3cl1	-1,1745	Il13	1,611	Cxcl12	-2,2222
Cxcl13	1,0852	Il18	4,7833	Cxcl3	-5,6256
Cxcl16	1,1712	Il1b	1,5562	Cxcl9	-1,426
Fasl	-1,3031	Il3	2,3916	Hc	-6,9741
Gpi1	-1,0084	Il3	2,3916	Ifna2	-2,3817
Ifng	-1,1034	Il6	4,952	Il10	-1,4064
Il12b	-1,2075	Ltb	1,6795	Il12a	-2,9322
Il15	1,1157	Mstn	3,779	Il16	-4,1756
Il17a	1,0056	Spp1	1,9697	Il17f	-1,7679
Il1a	1,238	Tgfb2	1,4825	Il1rn	-6,9741
Il4	1,2041			Il2	-1,426
Lif	-1,2329			Il21	-1,4162
Lta	-1,2852			Il22	-1,5497
Nodal	-1,2588			Il23a	-1,805
Pf4	-1,0295			Il24	-1,9889
Ppbp	1,1392			Il27	-4,2047
Thpo	1,3268			Il5	-4,8299
Tnf	-1,1909			Il7	-6,8781
Tnfrsf11b	-1,2329			Il9	-2,5704
Tnfsf11	1,1876			Mif	-1,456
Tnfsf13b	1,1004			Osm	-3,2989
Vegfa	-1,2763			Tnfsf10	-5,2854
Xcl1	1,108			MGDC	-4,7305
Actb	-1,2941				
B2m	1,1876				
Gapdh	1,3177				
Gusb	1,0267				
Hsp90ab1	-1,2414				

* The table provides the fold regulation of genes using a minimal threshold of 1.4 for regulation cut off. Positive values indicate up-regulated genes and negative values indicate the down-regulated genes compared to control mice.

DRG macrophages do not initiate pain in osteoarthritis

Next we addressed whether DRG macrophages are required for the initiation of OA pain. $Lysm^{cre}$ x $Csf1r^{LsL\text{-}DTR}$ (MM^{dtr}) mice that selectively express DT receptor in monocyte/macrophages were depleted from monocytes and macrophages by intraperitoneal (i.p.) injection of diphtheria toxin (DT) from day -1 until day 7. Intra-articular injection of MIA, increased the number of F4/80+ macrophages in the DRG at day 7, that was prevented by daily intraperitoneal injections of DT (Fig. 3A). In monocyte/macrophage depleted MM^{dtr} mice, mechanical hyperalgesia developed with similar kinetics to WT mice until day 3. However, in contrast to non-depleted littermate mice, MM^{dtr} mice had resolved from MIA-induced mechanical hypersensitivity at day 7 (Fig. 3B). To assess whether reconstitution of blood monocytes would return pain-like behaviours, DT administration was stopped at day 7. After stopping DT administration, blood monocytes reach to normal levels within 3 days[22]. However, after stopping DT injections the number of DRG macrophages remained similar to the contralateral DRG, equalling to baseline numbers, (Fig 3C). Moreover, after stopping depletion the sensitivity to mechanical stimuli remained similar to that of the contralateral side (Fig. 3B). Thus, DRG accumulation of macrophages do not contribute to pain initiation, but only to the maintenance.

Exogenous CXCL11 recruits DRG macrophages accumulation, but it does not induce pain

Next we assessed whether CXCL11 is sufficient to attract macrophages to the DRG and induce mechanical hypersensitivity. Intrathecally injection of CXCL11 in naive mice at a dose of 100 ng did not induce a significant DRG macrophage expansion (Fig. 3D). However, injecting 500 ng of CXCL11 significantly increased the number of DRG macrophages compared to vehicle-injected mice (Fig. 3D). Intrathecal injection of either 100 ng or 500 ng CXCL11 did not induce mechanical hyperalgesia (Fig. 3E). Phenotypic analysis of CXCL11-induced DRG macrophages showed that iNOS and CD206 expression in DRG macrophages were indistinguishable between CXCL11 and saline injected mice (Fig. 3F and 3G). Overall, intrathecal CXCL11 recruits' macrophages to the DRG, but it does not program macrophages and is neither sufficient to induces pain-like behaviors.

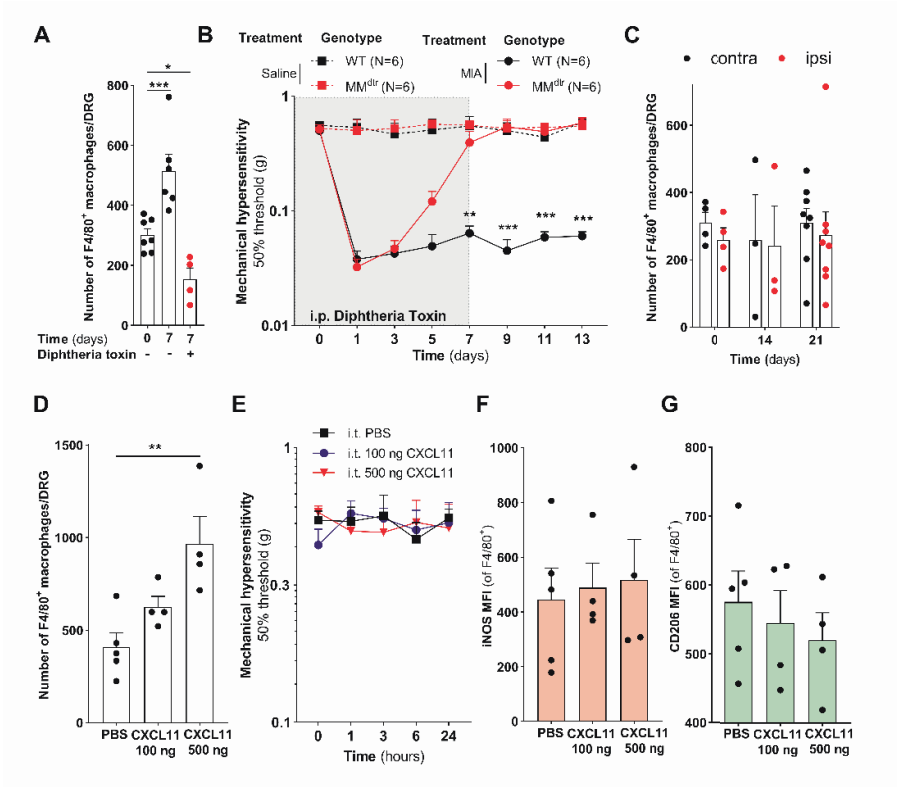

Figure 3. DRG macrophages maintain OA pain, although CXCL11 recruits DRG macrophages do not contribute to pain pathways

(A) *Flow cytometry analysis of DRG F4/80⁺ macrophages isolated from MM^{dtr} mice intraperitoneally injected with diphtheria toxin (DT) or saline to determine DRG macrophage depletion at day 7 after MIA injection. 1-way ANOVA, Dunnett post-hoc.* ***(B)*** *Course of mechanical hyperalgesia in WT versus MM^{dtr} mice after intra-articular injection of MIA in ipsilateral or saline in the contralateral knees. Monocytes/macrophages were systemically depleted by daily i.p. injections of DT starting at day -1 until day 7. 2-way repeated measure ANOVA, Sidak post-hoc.* ***(C)*** *Number of F4/80⁺ macrophages per lumbar DRG (L3-5) at days 14 and 21 after intra-articular injection of MIA in ipsilateral or saline in contralateral knees. DT was injected intraperitoneally from day -1 until day 7. 2-way repeated measure ANOVA, Sidak post-hoc.* ***(D)*** *Number of F4/80⁺ macrophages per lumbar DRG (L3-5) at 24 hours after intrathecal injection of PBS, 100 ng CXCL11, or 500 ng of CXCL11 in naïve mice. 1-way ANOVA, Dunnett post-hoc.* ***(E)*** *Course of mechanical hyperalgesia after intrathecal (i.t.) injections of PBS, 100 ng CXCL11, or 500 ng of CXCL11 in naïve mice. . 2-way repeated measure ANOVA, Sidak post-hoc.* ***(F, G)*** *Median fluorescence intensity (MFI) of* ***(F)*** *iNOS and* ***(G)*** *CD206 of the F4/80⁺ macrophage population in the lumbar DRG at 24 hours after intrathecal injections of PBS, 100 ng CXCL11, or 500 ng of CXCL11 in naïve mice. 1-way ANOVA, Dunnett post-hoc. Data represent mean ±SEM; *p<0.05; **p<0.01; ***p<0.001*

Discussion

In this study we identified that macrophages accumulate in the DRG early during OA induction. Although DRG macrophages are present at early-stage of OA, they did not contribute to development of pain, but rather for the maintenance of OA pain. We identified CXCL11 as a key driver for the accumulation of these DRG macrophages in MIA-induced OA. Strikingly, although exogenous administered CXCL11 was sufficient to increase macrophages in the DRG it did not induce pain. Altogether, our data indicate that CXCL11 contributes to macrophages accumulation and pain in OA, but that CXCL11-mediated macrophage accumulation in the DRG alone is not sufficient to induce pain.

In this study we observed infiltration of macrophages into lumbar DRG innervating damaged knees, which confirm our previous findings[14]. We show here that increased number of macrophages in the DRG were already present at an early stage of MIA-induced OA. Despite their early presence in the DRG, depletion of DRG macrophages prior to and during the development of OA did not prevent the initiation of pain, but only the maintenance of pain. Intriguingly, after stopping depletion monocytes return to the blood[22], but they do not infiltrate the DRG anymore, neither did they return pain behaviors. In line with these findings, we only found upregulation of CXCL11 at day three after MIA injection. Thus, we propose that macrophages are only recruited early in the onset of OA, but that they remain present in the DRG for longer periods to maintain pain.

A growing amount of evidence suggests that pain in OA has a neuropathic component in some patients[23]. Indeed, MIA-induced OA pain, similar to neuropathic pain, is independent from $Na_V1.8$ nociceptors[14,24]. Moreover, both OA and neuropathic pain are controlled by DRG macrophages[14,19]. Despite these similarities, there are also mechanistic differences between OA and neuropathic pain. In neuropathic pain, proliferation of tissue resident macrophages mainly contributes to DRG macrophages expansion[19]. Here we show that DRG macrophages expressed CD115, indicating that DRG accumulation in OA is likely the result from infiltration of blood monocyte into the DRG. In neuropathic pain macrophages in the DRG contribute to both initiation and maintenance of pain[19]. However, we show here that macrophages only control maintenance but not the development of OA pain. In neuropathic pain, DRG macrophages expansion in

male but not female mice depends on CSF1-expressing sensory neurons[19]. In contrast, our data did not suggest a putative sexual dimorphism because neutralizing CXCL11 in both females and males prevented pain maintenance. Overall these data shows that different pathways drive the accumulation of DRG macrophage in OA pain versus neuropathic pain.

Neutralizing endogenous CXCL11 was sufficient to block macrophage accumulation and pain maintenance. A question arises why exogenous administration of CXCL11 was not sufficient to induce pain, whilst it increased the number of macrophages in the DRG. These findings indicate that CXCL11 does not provide nociceptive signaling to sensory neurons, despite that sensory neurons express the CXCL11 receptor CXCR3[25,26]. Although CXCL11 increased the number of macrophages in the DRG, it is possible that the localization of these macrophages is different to that during OA. We found that the phenotype of macrophages after intrathecal CXCL11 is indeed different to that in OA mice. The CXCL11 induced DRG macrophages expansion, but it was not associated with M1-like programming of macrophages. We therefore postulate that something else than CXCL11 is required to program macrophages into a pain-promoting phenotype. Sensory neurons can indeed release factors such as neuropeptides or cytokines that modulate the phenotype and function of immune cells, including macrophages[27,28]. Upon activation by noxious stimuli, sensory neurons release cytokines like IL-1β, IL-6, CX3CL1 and TNF, and microRNAs, such as miR-21-5p, from cell bodies that may program DRG macrophages toward a M1 phenotype that promotes pain[27,29-31]. Moreover, we recently showed that sensory neurons that innervate the damaged knee joint program macrophages to M1. However, future research has to identify the factors released by sensory neurons, or other cells that program DRG macrophages in OA models.

CXCL11 is a well-known chemoattractant of monocytes, which activates various chemokine receptors including CXCR3, CXCR4 and CXCR7[32]. All these receptors are expressed by monocytes[32,33], however CXCL11-CXCR3 signaling is likely the key axis for monocyte attraction because CXCL11 has the highest affinity to CXCR3[34,35]. However, it is not clear which cells express CXCL11 in DRG. Current antibody based staining failed to show where CXCL11 is expressed due to an excessive non-specific signal. Spinal cord astrocytes, but not neurons, express CXCL11 following spinal nerve injury[36-38]. Thus possibly, non-neuronal cells like

satellite glial cells in the DRG may be candidate for CXCL11 expression. It remains to be determined which cell type is the origin of DRG CXCL11 expression.

Our result point towards CXCL11 as a putative therapeutic target to limit OA pain. Currently no human studies have been performed to test whether CXCL11 is produced in OA patients, for example in cerebrospinal fluid. Moreover, the question arises whether targeting DRG chemokines to prevent macrophage attraction is a good approach to reduce OA pain. In humans, OA progresses slowly and it is unknown whether this is associated with continues production of chemokines in the DRG. Here we show that the attraction of macrophages is time restricted and occurs only at an early stage of OA. If this is translatable to humans, than with respect to the time window for treatment, we think a better therapeutic approach would be to target the phenotype of DRG macrophages instead of just blocking the infiltration which inherently may be difficult. Indeed, reprogramming M1 macrophages in the DRG with an IL4-10 fusion protein or even cell-based therapies like M2 macrophages also resolves OA pain[14]. Overall, DRG macrophages are key for maintaining pain in OA, whilst they do not play a role in the initiation of pain. Macrophages are attracted to the DRG early during the onset of OA (in part) through the expression of CXCL11 in the DRG. In case translatable to humans, a new avenue for highly needed therapeutic approaches to treat OA pain may therefore be targeting these DRG infiltrating macrophages already in the early stage of osteoarthritis.

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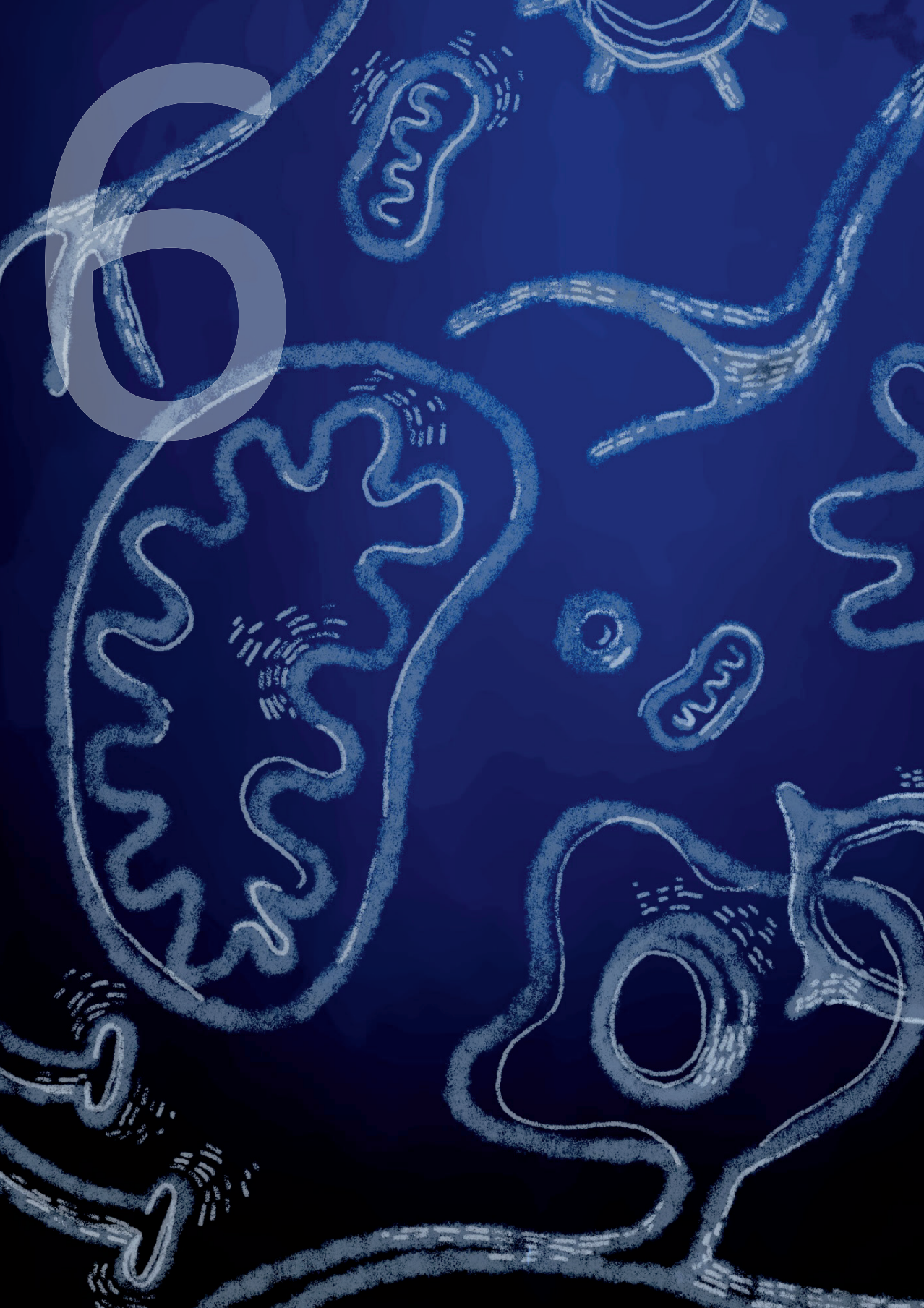
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CHAPTER 6

Summary & Discussion

Summary

At first sight chronic pain might seem a pure neurological problem. However, evidence indicates that the immune system contributes in pain pathways. In this thesis, the focus was on elucidating the role of macrophages in 1) transient inflammatory pain where endogenous resolution pathways are active, and 2) chronic osteoarthritis (OA) pain, a pathophysiological condition where pain resolution fails. By understanding how resolution of transient pain is regulated, and learning what goes wrong during chronic pain, we aim to identify highly needed novel options to treat chronic pain.

In this thesis, macrophages were identified as key regulators of pain and that these cells control pain remotely from the site of inflammation or damage. We identified that macrophages, particularly those with the M2 phenotype, control the resolution of pain in a transient inflammatory pain model by a previously unrecognised mechanism, independent of their cytokine release. On the other hand, we showed in a persistent osteoarthritis pain model that macrophages, in this case with the M1 phenotype, have a completely different function and maintain pain. Finally, by inhibiting M1 macrophages in the DRG persisting OA pain was suppressed, indicating that targeting these macrophages is a potential therapeutic approach to treat debilitating pain conditions.

In **chapter 2,** the role of immune cells and their mediators in regulation of pain is reviewed and evaluated. Here we identified, based on what was already known, that the immune and nervous system closely interact, and that immune cells have important but distinct roles in regulating different types of pain. In this chapter, the role of immune cells is outlined at the different stages during the course of pain including, (i) initiation, (ii) maintenance and (iii) resolution of pain. An overview of their divergent roles in different types of pain and where immune cells modulate pain at different anatomic site in the nervous system is discussed. The contribution of the immune system in pain pathways highlights potential use of immunological approaches to treat chronic pain. However, to optimally develop such strategies we need to have better understanding as to how immune cells control pain. In the following chapters we focused on macrophages and studied whether and how they contribute to endogenous pain resolution pathways, and conversely, whether these plastic cells regulate the initiation and

maintenance of chronic osteoarthritis pain. In **chapter 3,** we showed macrophages accumulate in the dorsal root ganglia during the resolution of inflammatory pain and acquire a M2-like phenotype. By cell-specific depletion and adoptive transfer studies we determined that macrophages actively resolve inflammatory pain. The pain resolving phenotype of macrophages is restricted to M2-like macrophages. To resolve pain, macrophages transfer functional mitochondria in vesicles to DRG sensory neurons. This transfer requires expression of CD200R on vesicles released by macrophages and the CD200R-ligand iSec1 on sensory neurons. Our data in this chapter revealed a mechanism governing the active resolution of inflammatory pain.

The finding that macrophage resolve pain made us wonder whether macrophages, the other way around, also contribute to chronic pain, in particular persisting pain caused by osteoarthritis. In **chapter 4**, we investigated the role of macrophages in the regulation of persistent OA pain. We unravelled that during the course of OA, macrophages accumulate in the DRG innervating the damaged OA affected knee, and acquire a M1-like phenotype and maintain OA pain. *In vitro*, DRG sensory neurons that innervate the damaged knee program macrophages toward the pain promoting M1 phenotype. Importantly, these DRG macrophages maintain pain independent from the damage of the knee joint. $Na_V1.8$ sensory neurons, so-called nociceptors, that are important for mediating inflammatory pain, were not required for OA pain, nor for the accumulation of M1-like DRG macrophages, indicating that sensory neurons other than nociceptors drive the programming of these macrophages. Finally, inhibition of DRG macrophages by intrathecal injection of either M2 macrophages or a novel fusion protein of IL4 and IL10 (IL4-10 FP), inhibited OA pain. Thus, during OA polarised macrophages maintain pain, and by therapeutically targeting these macrophages OA pain can be treated.

To further understand what causes the accumulation of DRG macrophages we searched for potential factors that drive the attraction of DRG macrophages. In **chapter 5**, we identified CXCL11 as a main driver for the accumulation of DRG macrophages in OA. We found that macrophages are attracted by CXCL11 already early during development of OA. However, despite their presence very early during the initiation of OA pain, these DRG macrophages are not required for the initiation of pain. CXCL11 is required to attract macrophages to the DRG but not sufficient to program these DRG macrophages into a pain maintaining

phenotype. Thus, we postulated that other factors in addition to CXCL11 are required to program macrophages into a pain-promoting phenotype before they will maintain OA pain. Future research has to identify these putative factors required for the programming of the DRG macrophages in OA.

Altogether, in this thesis we elucidated unprecedented roles of macrophages in the regulation of pain. DRG macrophages can have pain promoting or pain resolving functions depending on their phenotype that are likely governed by the DRG milieu. Finally, we showed that in case of chronic pain targeting the polarized DRG macrophages gives an opportunity for novel therapeutic strategies to potentially treat variety types of chronic pain.

Discussion

Macrophages control pain

Over the recent years, evidence is accumulating that the number of macrophages increase in nervous tissue after various types of insults[1-3]. In this thesis we identified that macrophages expand in the DRG either after a transient inflammation in the hind paw or after damage of the knee joint. Similarly, emerging evidence indicates that in various pain models macrophage numbers increase not only at the site of damage but also at pain relevant sites such as the sciatic nerve, DRG, dorsal horn of spinal cord, and brain[3-6]. This indicates that macrophages accumulation in neuronal tissue appears to be a common phenomenon[3,7,8]. But why would macrophages distant from the site of injury, accumulate in the DRG? Potentially this might be a part of macrophages natural response to an insult. Evidence indicate that after a painful insult, e.g. inflammation or OA, neuronal damage may occur in a distinct site of innervating nerves, such as axons or even the cell bodies in the DRG[9-13]. Neuronal damage attracts and activates macrophages in order to remove the injurious stimuli and initiate the healing process, e.g. by initiating a beneficial inflammatory response[14-18]. Thus, a small danger signal is sufficient to attract macrophages. Indeed, after a painful insult (e.g. inflammation or tissue damage) cell bodies of the neurons that innervate the affected tissue release factors, such as CSF-1 and CCL2, that can attract macrophages[1,19,20]. Importantly, neuron-derived chemokines convey injury signals to perineuronal macrophages to take on a pro-regenerative phenotype that may induce long-lasting neuronal regenerative processes[21,22]. Thus, after a painful insult, the cell bodies of damaged innervating neurons attract macrophages to enhance the regenerative capacity of the sensory neurons by their beneficial inflammatory response. Indeed, in chapter 3, we observed that macrophages accumulate in DRG in transient inflammatory pain condition to actively resolve pain. Thus, after a relative mild stimulus of peripheral inflammation, macrophages are actively attracted by neurons in order to aid neurons to resolve pain.

If initially macrophages are to help neurons and resolve pain, then why would macrophages maintain pain as well? Pain is a side effect or feature of the inflammatory response of macrophages[23,24]. The inflammatory mediators

released by macrophages in an inflammatory response induce pain by directly activating sensory neurons or sensitize nociceptors and reduce their activation threshold[25-27]. As such, in a more persisting inflammatory condition or tissue damage such as OA, potentially macrophages that initially were attracted to support the regenerative capacity of neurons are skewed toward a different phenotype that now induces pain. Indeed, a difference in phenotype between pain resolving macrophages in transient inflammatory pain and pain promoting macrophages in persistent OA pain was observed. This alternate skewing of macrophage may be due to a different nature of mediators released by sensory neurons in inflammatory and OA pain (see 'programming of macrophages'). Thus, in chronic pain conditions macrophages may ultimately fail to resolve pain for reasons that we do not yet fully understand, but may be driven by factors in the DRG that influence the phenotype and function of DRG macrophages. Although, currently it is unclear what determines whether DRG macrophages promote or resolve pain, including cause or consequence, in the next paragraphs we will discuss how macrophages expand and how their pain regulatory functions are determined.

Macrophages accumulation in the DRG: proliferation or infiltration

Despite the general agreement on DRG expansion of macrophages in various types of pain, some controversies exist whether the accumulation of these macrophages is caused by infiltrating monocytes or proliferation of the existing tissue resident macrophages. In this thesis we observed that DRG macrophages expressed CD115, a monocyte marker, suggesting the accumulation of macrophages in OA is likely due to infiltration of circulating monocytes rather than proliferation of existing tissue-resident macrophages. More importantly, CXCL11 caused accumulation of macrophages, suggesting chemotaxis is driving the infiltration of monocytes. There are also other studies indicating infiltration of macrophages into the DRG[2,19,20]. However, in neuropathic pain proliferation of tissue resident macrophages is thought to be the main reason of DRG macrophage expansion[1,28]. In neuropathic pain CSF-1 is a major driver for proliferation of tissue resident macrophages in the DRG[1,28]. However, it is known that CSF-1 may also attract macrophages[29-31]. Although it remains unclear why the origin of macrophages varies (infiltration versus proliferation) in different pain conditions, it will be important to know whether the origin of these cells has

any consequences on their pain regulatory roles, or whether they both can be targeted with drugs to promote a pain resolving role.

Recent studies using fate-mapping techniques, or markers to distinguish recruited macrophages from tissue-resident macrophages, have revealed a much higher plasticity of recruited monocytes compared with resident macrophages or microglia[32-35]. For example, when microglia were compared with recruited monocytes in the brain[35] or during experimental autoimmune encephalomyelitis (EAE)[33,34], inflammation had a smaller effect on the gene expression profiles of resident macrophages and microglia compared with the newly recruited monocyte. This indicates the higher plasticity of infiltrated monocytes compared to resident macrophage and microglia. Thus, possibly DRG infiltrated monocytes are more plastic than resident macrophages, and therefore therapeutically there is a greater possibility to reprogram them in order to resolve pain.

Programming of DRG macrophages determines their pain regulatory role

Although DRG accumulation of macrophages may be commonly observed after various insults that induce pain[1-3], we identified that macrophages have opposing functions in the regulation of pain. We showed that DRG macrophages resolve pain after a transient inflammation (chapter 3), but maintain pain after damage (chapters 4 and 5). It appeared that macrophages acquired a M1-like phenotype during the course of persistent OA pain, while in transient inflammatory pain they adopted a M2-like phenotype. The phenotype, and associated function, of macrophages is regulated by their surrounding micro-environment[36]. Sequencing data showed that macrophages of nervous tissue acquire unique phenotypes. These data indicate that possibly neurons, or other cells such as glia, shape macrophage phenotype and function[37,38]. Similarly, by co-culturing naïve macrophages with DRG of sensory neurons innervating either the arthritic or healthy joint, we showed that neurons innervating the arthritic knee induce a M1-like phenotype, while the phenotype of macrophages co-cultured with neurons innervating the healthy joint did not change. Thus, the local DRG milieu determines the phenotype of macrophages likely by factors released from sensory neurons. Future studies have to identify whether during transient inflammatory pain sensory neurons drive the M2 programming of macrophages,

or that other non-neuronal cells such as satellite or Schwann cells contribute to the programming of these macrophages.

If the local nervous tissue programs macrophages, the question is what mediators are involved in programming of DRG macrophages? Evidence shows that after different inflammatory or damaging stimuli in the periphery, the somata of these neurons produce different mediators. Upon activation by noxious stimuli, sensory neurons release factors such as neuropeptides, neurotransmitters, chemokines, and cytokines that all have been shown to modulate the function of macrophages[39,40]. For example, in OA or neuropathic pain, sensory neurons release pro-inflammatory mediators such as IL1β and TNF[41,42], that may skew macrophages towards the M1 phenotype. Similarly, following peripheral injury, sensory neurons release exosomes containing microRNAs, including miR-21-5p, that program DRG macrophages toward a M1 phenotype[43]. However, sensory neurons may also release neurotransmitters like CGRP and substance P after a noxious stimulus including after transient inflammation that affects macrophages function[44,45]. For example, substance P programs macrophages into M2 phenotype through activation of the PI3K/Akt/mTOR/S6kinase pathway and induction of Arginase-1 and CD206, even in the presence of IFNγ[46,47]. CGRP skews macrophages to a M2 phenotype via activating RAMP1 and downstream PKA activation which promotes IL-10 production and downregulates TNF expression[39,48]. Overall, the surrounding nervous tissue may determine the phenotype of macrophages by releasing different mediators.

But, why do in some pain conditions sensory neurons release pro-inflammatory mediators, leading to M1 phenotype of macrophages, while in other conditions they produce mediators that program macrophages into a M2 phenotype. First, the difference in programming of macrophages in inflammatory compared to OA pain may origin from different types of neurons innervating the affected tissue. Distinct sets of neurons innervate the deep tissue, such as knee and hip, compared to skin. DRG neurons that innervate skin are isolectin B4 (IB4) positive and GDNF-sensitive neurons, while in deep tissue the key neurons are peptidergic IB4 negative and NGF-sensitive neurons[49,50]. Theses deep tissue innervating peptidergic neurons, in contrast to skin innervating IB4 neurons, can release inflammatory mediators such as IL1β that potentially program macrophages into a M1 phenotype[51,52]. Another explanation for divergence in

the programming of macrophages, is that the nature of the stimulus which activate sensory neurons determines the local DRG milieu and thus programming of macrophages. After different painful stimuli different sets of neurons become activated[53-55]. Possibly certain sensory neuron subsets are more prone to produce mediators that either program macrophages into the M1 or M2 phenotype. Thus, the potential of programming macrophages into M1 or M2 phenotype, may be restricted to specific subsets of sensory neurons activated by different stimuli. Indeed, specific sensory neuron subtypes are involved in different types of pain[56,57]. Using single-cell RNA sequencing, until now 13 subtype sensory neurons have been identified that each produce unique sets of mediators[58]. For example, $Na_v1.8$ neurons that contribute to inflammatory pain, of which we showed in chapter 4 that they do not contribute to OA chronic pain, produce substance P and CGRP whilst other subtype of sensory neurons do not[44,45]. Thus, after an inflammatory stimulus, it is possible that these mediators, specifically produced by $Na_v1.8$ nociceptors, drive the M2 programming of macrophages. On the other hand, after tissue damage in OA, pro-inflammatory cytokines such as IL18, IL-1β, and IL-6 were significantly increased in the DRG of OA mice (chapter 5) skewing them to a M1 phenotype. These findings are in line with other evidence indicating that after peripheral joint damage, inflammatory mediators are upregulated in the DRG. Neuronal increase of pro-inflammatory cytokines and chemokines (e.g., IL-1β, RANTES, CINC2α/β, IL-17, Thymus chemokines (TC), TNF, L-Selectin and VEGF) was shown in OA mice models[3,41,59], that possibly induce the M1 programming of macrophages[39,42]. Although, it is not clear which subset of neurons contribute to OA pain[60], potentially during development of OA the local tissue changes, activate a certain set of sensory neurons to release factors that program macrophages into the M1-like phenotype.

How do macrophages maintain pain?

Intrathecal injection of M1 macrophages was sufficient to induce pain in naïve mice (chapter 3). Moreover, local depletion of DRG M1 macrophages, during OA induction, resolved pain (chapter 3), indicate that M1 macrophages promote pain. But how do M1 macrophages maintain OA pain independent from the damage at the joint? Various studies support that M1 macrophages may promote pain through the release of pro-inflammatory cytokines[43,61-64]. In

neuropathic pain, macrophages in the DRG release reactive oxygen species (ROS) but also IL-1β that contribute to the maintenance of pain[1,65,66]. M1 macrophages express high levels of angiotensin II (AngII), that can trigger neuronal sensitization through PKA-mediated TRPV1[67], resulting in sensory neuron sensitization[68,69]. The importance of these cytokines in maintaining pain is highlighted by the fact that inhibiting the release of pain promoting cytokines by e.g. anti-inflammatory cytokines inhibits pain. Anti-inflammatory cytokines like IL4, IL10 and IL13 shown to have pain resolving functions through inhibiting macrophages[70,71]. Moreover, TGF-β and IL4-10 FP suppresses cytokine production of macrophages and prevent pain[72,73]. Finally, we showed that inhibition of M1 macrophages with IL4-10 FP prevents the maintenance of OA pain (chapter 3). Thus, macrophages can promote pain via release of pro-inflammatory cytokines and limiting their cytokine production inhibits their pain regulatory role.

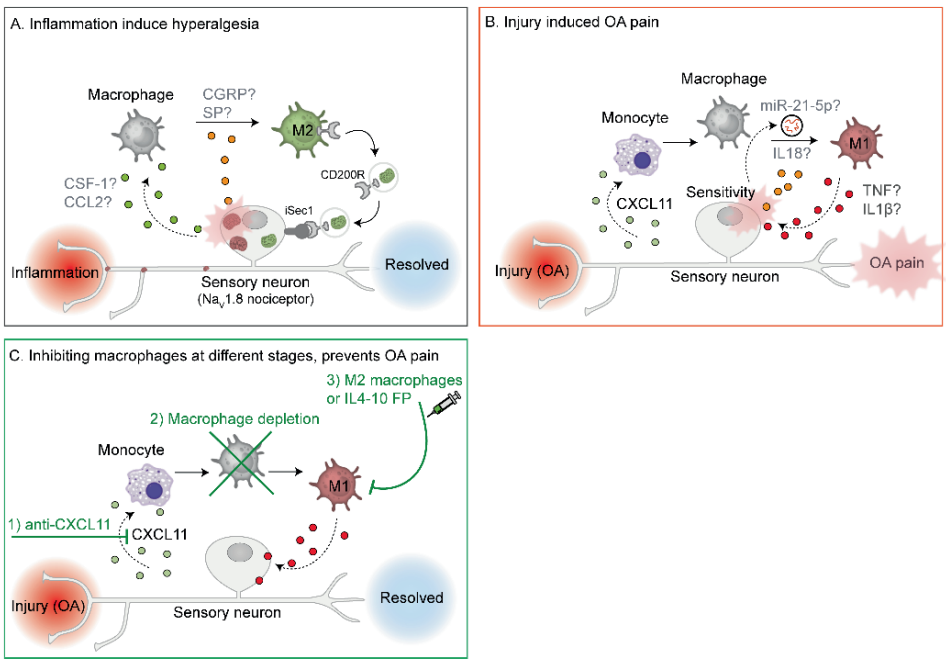

Figure 1. Stages of inflammatory and osteoarthritis pain

(A) *Inflammatory insult induce transient pain hypersensitivity (hyperalgesia). During the inflammatory pain neuronal mitochondrial functions, such as oxidative phosphorylation (OXPHOS) and Ca2+ buffering, are impaired. Possibly after inflammation macrophages*

infiltrate DRG through release of factors by neurons that may attract macrophages such as CCL2 and CSF-1. DRG sensory neurons ($Na_V1.8$ nociceptors) may also express and release neuropeptides that may determine the phenotype of macrophages, including substance-P (SP) and CGRP. As described in chapter 3, M2 macrophages transfer mitochondria in vesicles to DRG sensory neurons in a receptor (CD200R) and ligand (isec1) depend manner to actively resolve inflammatory pain. ***(B)*** *In early stage of osteoarthritis (OA), CXCL11 induce infiltration of circulating monocytes into the DRG (chapter 5). Sensory neurons, have been shown to release inflammatory cytokines such as IL18 or exosomes containing microRNAs like miR-21-5p that may program macrophages toward a M1 phenotype. M1 DRG macrophages releasing pro-inflammatory cytokines such as TNF and IL-1β, that are known to promote pain.* ***(C)*** *Established OA pain is inhibited by 1) blockade of CXCL11 in early stage of OA, which prevents the accumulation of DRG macrophages 2) local depletion of DRG macrophages, or 3) inhibition of M1 macrophages by either using M2 macrophages or a fusion protein of IL4-10 (IL4-10 FP).*

Sex differences in pain

Chronic pain is more prevalent in females than males[74,75]. Moreover, laboratory experimentation has demonstrated that women are more sensitive to pain than men[76-79]. Over the recent years, attention has shifted to investigating mechanisms underlying sex differences in pain. The Mogil's Lab was the first to show female mice require T cells to maintain neuropathic pain, whilst males are depended on microglia[80]. Subsequent studies further support presence of sex differences in neuro-immune modulation of pain[81,82]. However, until now there is no evidence that macrophages regulate pain differently in males and females[7,81]. In support, we did not observe relevant sex differences in the number of macrophages that accumulated in the DRG in transient inflammatory and in OA pain. However, small changes in number of macrophages could possibly not have been detected due to limited sample size. Nevertheless, depletion of macrophages, during transient inflammatory pain (chapter 3) and OA pain (chapter 4), prevented pain resolution or resolved pain respectively in both males and females.

Despite that macrophage accumulation in the nervous system and their pain regulatory role appears to be independent of sex, but the mechanisms that govern the increase of macrophages in the nervous tissue may be different between sexes. For example, DRG macrophages expansion after spinal nerve

injury in male mice depends on sensory neurons expressing CSF-1 but not in females[1]. Similarly, in a CFA-induced inflammatory pain model, macrophage migration to the site of injury was dependent on COX-2 pathway in females, but not males[20]. Evidence indicates that chemokines that can be produced by sensory neurons are differentially expressed in males and females following nerve injury[71,83-85]. RNAseq analysis of lumbar DRG showed that following nerve injury cytokines (e.g. IL6 and IL1R1) and chemokines (e.g. CXCL13 and CXCL9) were differentially regulated between males and females[83]. In chapter 5, CXCL11 identified as driver of macrophages accumulation in the DRG, after OA pain. However, in contrast to the previous mentioned studies our data did not suggest a sexual dimorphism in macrophages accumulation pathways in OA pain, because neutralizing CXCL11 in both females and males prevented pain maintenance (chapter 5). Thus, mechanistically sex differences may depend on the type of chemokine that govern the migration of macrophages in a specific pain condition. Therefore, sex-dependent mechanisms that govern the accumulation of macrophages, like CSF-1, Cxcl13 and Cxcl9, are likely restricted to neuropathic pain, in contrast to CXCL11 in OA pain.

Mitochondria and pain

In chapter 3, we identified a previously unknown bioenergetic crosstalk between macrophages and sensory neurons. We identified that macrophages transfer mitochondria to sensory neurons in order to resolve pain. This intriguing finding highlighted that neurons require help to resolve from pain and that this is likely through restoring their metabolism. These findings also opened up many more questions such as: why would sensory neurons need help to resolve pain? What is the role of mitochondria in pain? What happens with the mitochondria that are transferred to sensory neuron? Why do macrophages provide mitochondria while other cells that are present in DRG in contact with neurons, do not? Although still mostly unresolved, but in the following paragraphs we will discuss some of these questions.

Neurons display high energy consumption relative to other cells, and they are highly sensitive to energy limitations[86,87]. For example, a decrease in ATP leads to a fast and significant reduction in electrical activity[86]. Various chronic pain states, such as chemotherapy-induced pain and neuropathic pain caused by trauma or diabetes, are associated with mitochondrial defects[88-91]. An important

question is whether extracellular mitochondria from macrophages entering into neurons may generate additional benefits beyond ATP energetics per se? A deficiency in mitochondrial function in sensory neurons, e.g. deficiency in FAM173B, a mitochondrial lysine methyltransferase, prevents the resolution of inflammatory pain[92], suggesting that at least proper functioning of neuronal mitochondria is required for pain to resolve. Moreover, in neuropathic pain, neuronal oxidative phosphorylation (OXPHOS) is diminished and Ca^{2+} buffering by mitochondria is impaired[93,94]. Here we also identified that after a transient inflammatory pain, mitochondrial OXPHOS is reduced during the peak of inflammatory pain. However, neuronal OXPHOS was restored after resolution of pain (Chapter 3). Therefore, we postulated that macrophages at least support neurons to restore their mitochondrial OXPHOS by donating mitochondria that represent active signals to the neurons.

Why do macrophages donate mitochondria, and other cells already present in the DRG and in close contact with sensory neurons such as Schwann or satellite cells, and that are known to communicate with sensory neurons[95-97], do not? These cells are likely capable of donating mitochondria, because brain astrocytes transfer mitochondria to neurons to promote neuronal survival after stroke[98]. Glia cells are less obvious to donate mitochondria since they are important to maintain the integrity of the nervous system such as the architecture and survival of neurons, development and modulation of synaptic transmission, propagation of nerve impulses, and many other physiological functions[99,100]. If glia cells would give away their mitochondria, this might impact their own health and as such the integrity of the nervous system. Conversely, macrophages, that can increase quickly in numbers, can provide mitochondria when needed and they are expendable. Another benefit of macrophages as compared to glial cells may be that they can accumulate at different neuroanatomic sites of the sensory system, depending where they are required, e.g. in sciatic nerve, or peripheral damaged or inflamed tissues[3,101-103]. Thus, theoretically replaceable macrophages, virtually found in almost all tissues, are suitable cell types to provide help to sensory neurons wherever needed.

We found macrophages transfer mitochondria through vesicles. Intercellular cargo exchanges may occur through various mechanisms including: 1) tunnelling nanotubules, 2) extracellular vesicles, and 3) cellular fusion[104-107]. Thus, although we showed that macrophages transfer mitochondria through vesicles, we could

not exclude transfer through these other mechanisms. Mitochondria typically range in size from 500 to 1000 nm[108,109]. Extracellular vesicles size is from 50 nm up to 5000 nm in diameter, and nanotubules are from 50 nm to 1500 nm[110,111]. Thus, theoretically mitochondria could be transferred through vesicles and nanotubes. Mesenchymal stem cells, use nanotubes for mitochondria transfer[112-115]. Moreover, macrophages also use nanotubes for intercellular organelle trafficking[116,117]. However, a benefit of vesicles over other organelle transport mechanisms, is the range in which these vesicles may acts. Nanotubules are limited in their length and cellular fusion is restricted to cell-cell contact[110,118]. Thus, although macrophages can form nanotubes, to date there are no evidence exists indicating macrophages use nanotubes to transfer mitochondria.

The question remains why macrophages don't transfer mitochondria in OA to resolve pain? We showed here, that macrophages require CD200R in order to transfer mitochondria to sensory neurons that express iSec1. We also identified that the phenotype of macrophages is different in inflammatory pain, with an M2 phenotype, compared to OA pain where macrophages acquire an M1 phenotype. Intriguingly, CD200R is highly expressed on M2 macrophages and not M1 macrophages[119-122]. Therefore, possibly in OA the M1 macrophages are not capable to transfer mitochondria because they lack CD200 receptor. Additionally it is possible that M1 macrophages cannot donate mitochondria that function properly, because M1 macrophages shut down their OXPHOS/mitochondria and depend on glycolysis[123,124]. Otherwise, it is also possible that only $Na_V1.8$ neurons express iSec1, a ligand for CD200R that we identified to be essential for the resolution of inflammatory pain (chapter 3). $Na_V1.8$ neurons mediate inflammatory pain, but as shown in chapter 4, they do not contribute to OA pain. Thus, possibly in OA pain sensory neurons do not express the required molecules to receive vesicles. It will be important to identify what types of sensory neurons express iSec1 in future studies.

Clinical perspectives

Chronic pain affects >20% of the population word-wide and 25-30% in Europe[125-128]. Current treatment options to relieve chronic pain, such as NSAIDS, steroids, and opioids are often inadequate and have substantial risks of side effects[3,129]. In this thesis we elucidate that DRG macrophages have two pain regulatory sides, one that promote pain and one that actively resolve pain. From a therapeutic perspective reducing/limiting pain promoting (M1) macrophages and increase pain resolving (M2) macrophages may be desirable. Thus, exogeneous administration of pain-resolving macrophages or re-education of existing DRG macrophage to treat pain is of interest. Autologous macrophages were already used in clinical trials to treat a variety of human tumors[130,131]. However, regardless of the dose and methods of administration, most of the clinical trials were unsuccessful, mainly because of macrophages plasticity[130,131]. These inflammatory macrophages can kill tumor cells. However, the tumor environment is able to reprogram these macrophages into a tumorigenic phenotype that instead of killing tumor cells will promote tumor growth, limiting the effectiveness of the therapy[131-133]. Therefore, exogeneous administration of pain resolving macrophages in chronic pain conditions would be challenging, because it is possible that the phenotype of the injected macrophages switch in vivo, due to high plasticity of macrophages[131,134]. Indeed, injecting M2 macrophages only transiently treated established OA pain (chapter 4).

Although exogenous administration of macrophages may have limitation as discussed above, another approach is to switch the phenotype of existing nervous tissue macrophages *in vivo*. For example, IL13 switches M1 macrophages into a M2 phenotype *in vivo* and reverses neuropathic pain[135]. Moreover, we showed in chapter 4 that the IL4-10 FP inhibit M1 macrophages in the DRG. Moreover, there are clinical trials indicating successful re-educating of macrophages in humans[134,136]. Thus, clinically reprogramming the accumulated DRG macrophages appears to be more efficient compared to injecting macrophages, to control pain.

In this thesis we unveiled the importance of macrophages derived mitochondria in pain resolution pathways, indicating that novel therapeutic strategies may involve administration of mitochondria to neurons. In fact, only injection of

vesicles containing mitochondria was sufficient to resolve the pain, albeit transiently, probably due to unsustained delivery. Supplementation of healthy mitochondria to damaged neurons has been reported to promote neuronal viability, activity, and neurite re-growth[137-139]. To date, a few registered clinical trials of mitochondrial transplantation for treating neurodegenerative diseases, stroke, or traumatic brain injury have been launched (not completed yet)[137,139]. A possibility is to generate artificial vesicles with mitochondria to treat chronic pain[137,140,141]. Indeed, pilot studies in which vesicles with mitochondria were administered during established persistent inflammatory pain resolved pain transiently (data not shown), providing proof of concept that mitochondrial supplementation may be a feasible approach to treat persistent pain. However, ideally such vesicles would target the stressed neurons in need. In Chapter 3, CD200R on vesicles was identified to be required to deliver mitochondria to sensory neurons to resolve pain. Thus, potentially by synthetic cell surface receptors, such as CD200R, artificial vesicles can be used as a platform for membrane-associated therapeutic mitochondria delivery to sensory neurons. Thus, mitochondria transplantation may be a potential therapy to clinically manage chronic pain, although we first need to find a way to provide mitochondria sustainably.

Altogether, we demonstrate the pain regulatory role of DRG macrophages is highly dependent on the state these cells. We provided a conceptual framework that DRG macrophages control pain independent of the peripheral changes. As such, these macrophages represent an interesting target to treat chronic pain. Such novel therapeutic strategies should focus on reprogramming the DRG macrophages, which may include the fusion protein of IL4-10 or even cell-based therapies to reprogram inflammatory macrophages, or restoration of mitochondrial homeostasis in neurons or on enhancing the transfer of mitochondria from macrophages.

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Netherlandse Samenvatting

Pijn is een waarschuwingssignaal, essentieel om te beschermen tegen schade. Pijn wordt veroorzaakt door ontsteking en schade. Normaliter verdwijnt de pijn als de ontsteking of schade is opgelost. Echter, in sommige gevallen blijft de pijn aanhouden, zelfs als de oorspronkelijke oorzaak (bijvoorbeeld bij een ontsteking of weefselschade) is verdwenen. Wereldwijd heeft ~20% van de bevolking last van deze zogenaamde chronische pijn. Chronische pijn kan dan ook als ziekte worden beschouwd, en is zeer lastig te behandelen.

Op het eerste gezicht lijkt chronische pijn een probleem van de zenuwcellen die pijnprikkels detecteren. Echter, recente aanwijzingen laten zien dat het immuunsysteem mogelijk bijdraagt aan het ontstaan van chronische pijn. Om het ontstaan van chronische pijn beter te begrijpen, hebben we in dit proefschrift onderzocht wat de rol is van een bepaald type ontstekingscel - de macrofaag - bij het reguleren van pijn. Ten eerste hebben we onderzocht wat de rol van de macrofaag is ten tijde van pijn veroorzaakt door een tijdelijke ontsteking en waarbij de pijn weer vanzelf oplost (tijdelijk ontstekingspijn). Ten tweede bij pijn die ontstaat als gevolg van schade aan het kniegewricht vergelijkbaar met artrose en waar de pijn chronisch aanwezig blijft (chronische pijn). Door te begrijpen óf en hoe macrofagen betrokken zijn bij een tijdelijke ontstekingspijn die vanzelf overgaat, hopen we te leren wat de mechanismen zijn die normaliter pijn uitzetten. Door dit te combineren met begrijpen van wat er misgaat tijdens chronische pijn, willen we hoognodige nieuwe opties identificeren om chronische pijn te behandelen.

In hoofdstuk 2 wordt door middel van een literatuur review de rol onderzocht van ontstekingscellen en de ontstekingsstoffen die ze maken bij het reguleren van pijn. Uit dit review blijkt dat het immuunsysteem en het zenuwstelsel met elkaar communiceren. Ontstekingscellen spelen een belangrijke rol in het reguleren van verschillende soorten pijn. Echter, de rol van deze cellen varieert afhankelijk van het stadium van de pijn. In dit hoofdstuk wordt de precieze rol van ontstekingscellen beschreven in deze verschillende stadia van pijn, waaronder (i) initiatie, (ii) handhaving en (iii) oplossing van pijn. Ontstekingscellen kunnen afhankelijk van de verschillende stadia van pijn verschillende functies uitoefenen op verschillende plekken van het zenuwstelsel. De bijdrage van het immuunsysteem aan reguleren van pijn benadrukt het

mogelijke gebruik van immunologische benaderingen om chronische pijn te behandelen. Om dergelijke strategieën optimaal te ontwikkelen, moeten we echter beter begrijpen hoe ontstekingscellen cellen pijn reguleren. In hoofdstuk 3 hebben we onderzocht óf en hoe macrofagen bijdragen aan endogene mechanismen bij pijn resolutie. In hoofdstuk 4-5 onderzoeken we of deze cellen ook betrokken zijn bij het ontstaan van chronische artrose pijn.

In hoofdstuk 3 laten we zien dat ten tijde van een lokale tijdelijke pijnlijke ontsteking macrofagen zich ophopen in de spinale ganglia waar de cellichamen van sensorische neuronen zitten en waarvan de axonen het ontstoken weefsel innerveren. De toename in macrofagen in deze spinale ganglia vindt plaats, precies op het moment dat de ontstekingspijn aan het verdwijnen is. Door middel van verschillende studies waarin we deze cellen specifiek verwijderen of weer terug brengen, hebben we aangetoond dat deze macrofagen nodig zijn om de pijn veroorzaakt door de ontsteking te laten verdwijnen. Met deze studie laten we zien dat in tegenstelling tot het huidige dogma dat pijn passief verdwijnt door het verdwijnen van de ontsteking, het uitzetten van de pijn actief gebeurt en onafhankelijk is van het verdwijnen van de ontsteking zelf. Macrofagen kunnen verschillende fenotypes hebben die zijn gelinkt aan de verschillende functies die ze kunnen uitoefenen. We zagen dat de macrofagen in de spinale ganglia een zogenaamd (weefsel helende) M2-fenotype hadden. Alleen macrofagen met een (naïeve) M0 of M2 fenotype, maar niet (ontsteking-stimulerende) M1 fenotype kunnen de pijn uitzetten. Om deze pijn op te lossen, dragen macrofagen hun energiefabrieken, zogenaamde mitochondriën, in blaasjes over naar het cellichaam van pijnzenuwen. Deze overdracht vereist een soort ‘docking systeem’ wat bestaat uit de CD200-receptor (CD200R) welke op de blaasjes zit die worden afgegeven door macrofagen en de CD200R-ligand iSec1 op sensorische neuronen. Onze bevindingen in dit hoofdstuk onthulden een verrassend mechanisme betrokken bij het actief uitzetten van ontstekingspijn.

Deze bevinding deed ons vervolgens de vraag stellen wat de rol van macrofagen is als pijn niet meer verdwijnt en dus chronisch is geworden. Daarvoor hebben we specifiek gekeken in modellen voor knieartrose. Artrose is een degeneratieve gewrichtsaandoening waarbij met name het kraakbeen in het gewricht in kwaliteit achteruit gaat. Dit gaat gepaard met pijn die lang aanwezig blijft. Echter, de pijn is vaak niet geassocieerd met de mate van schade en er is nog

onvoldoende kennis wat deze aanhoudende pijn veroorzaakt. In hoofdstuk 4 tonen we aan dat tijdens artrose pijn, macrofagen zich eveneens ophopen in de spinale ganglia die de zenuwcellen bevat die de beschadigde knie innerveren. Een interessante bevinding is dat deze macrofagen juist een ander fenotype hebben dan de macrofagen die we zagen als ze pijn uitzetten bij tijdelijke ontstekingspijn, namelijk die van het M1 soort. Deze macrofagen onderhouden de pijn onafhankelijk van de schade aan het kniegewricht. Door middel van *in vitro* studies laten we zien dat zenuwcellen ervoor zorgen dat deze macrofagen een M1 fenotype krijgen. Omdat we in hoofdstuk 3 hadden geleerd dat M2 macrofagen in tegenstelling tot M1 macrofagen pijn uitzetten, hebben we op twee manieren geprobeerd de artrose pijn uit te zetten door deze M1 macrofagen te modificeren. De eerste strategie was macrofagen van het pijn dempende M2 subtype in het ruggenmerg spuiten. De tweede strategie was een nieuw door ons ontwikkelde stof in het ruggenmerg spuiten om de activiteit van M1 macrofagen te dempen. Deze stof is een fusie-eiwit dat bestaat uit twee ontstekingsremmende stoffen IL4 en IL10 en momenteel klinisch verder ontwikkeld wordt. In beide gevallen zagen we dat de artrose pijn tijdelijk minder werd en wat gepaard ging met macrofagen in de spinale ganglia die minder M1 werden. Deze bevindingen geven aan dat artrose pijn kan worden behandeld door macrofagen in het de spinale ganglia te manipuleren.

Om verder te begrijpen wat de oorzaak is van de ophoping van macrofagen in spinale ganglia tijdens artrose, hebben we gezocht naar mogelijke factoren die macrofagen aantrekken in de spinale ganglia. In hoofdstuk 5 hebben we CXCL11 geïdentificeerd als belangrijke factor die macrofagen naar de spinale ganglia aantrekt tijdens artrose. We ontdekten dat macrofagen al vroeg tijdens de ontwikkeling van artrose door CXCL11 worden aangetrokken. Ondanks de aanwezigheid van macrofagen in spinale ganglia in het beginstadium van artrose, zijn deze macrofagen niet betrokken bij het ontstaan van de artrose pijn, echter alleen bij het in standhouden van de pijn. CXCL11 brengt dus macrofagen naar de spinale ganglia, maar dat alleen is niet voldoende om deze macrofagen aan te zetten tot het in standhouden van pijn. Daarom hebben we verondersteld dat naast CXCL11 andere factoren nodig zijn om macrofagen in een pijn bevorderend fenotype te veranderen. In toekomstig onderzoek proberen we deze factoren verder te identificeren, omdat dit mogelijk interessante therapeutische targets kunnen zijn voor de behandeling van artrose pijn.

Conclusie

In dit proefschrift hebben we een nog onbekende rol van macrofagen in de regulatie van pijn opgehelderd. Macrofagen kunnen pijn bevorderende of pijn dempende functies hebben, afhankelijk van hun fenotype. Dit fenotype wordt waarschijnlijk bepaald door de zenuwcellen in spinale ganglia. Ten slotte laten we zien dat het dempen van pijn bevorderende macrofagen of het geven van pijn dempende macrofagen artrose pijn kan verminderen.

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List of Publications

Ramin Raoof, Michiel van der Vlist, Hanneke L.D.M. Willemen, Judith Prado, Sabine Versteeg, Martijn Vos, Roeland Lockhorst, R. Jeroen Pasterkamp, William Khoury-Hanold, Linde Meyaard, Niels Eijkelkamp "Macrophages Transfer Mitochondria to Sensory Neurons to Resolve Inflammatory Pain" *(Submitted; Biorxiv).*

Ramin Raoof, Simon C. Mastbergen, Huub M. de Visser, Judith Prado, Sabine Versteeg, Mirte Pascha, Anne L.P. Heinemans, Youri Adolfs3Jeroen Pasterkamp, John N. Wood, Floris P.J.G. Lafeber, Niels Eijkelkamp "Dorsal Root Ganglia Macrophages Maintain Osteoarthritis Pain" *(Submitted).*

Raoof Raoof, Christian M. Gil, Simon C. Mastbergen, Floris P.J.G. Lafeber, Niels Eijkelkamp "CXCL11 Controls Accumulation of DRG Macrophages in Osteoarthritis Pain" *(To be submitted).*

Ramin Raoof, Hanneke L.D.M. Willemen and Niels Eijkelkamp, review, "Divergent Roles of Immune Cells and Their Mediators in Pain", *Rheumatology (Published).*

Judith Prado, Jelena Popov-Celeketic, Cristine Steen-Louws, **Ramin Raoof**, Erik Hack, Niels Eijkelkamp "Development of Recombinant Proteins to Treat Chronic Pain" *JOVE (Published).*

Ramin Raoof, Saeed Esmaeili Mahani, Mehdi Abbasnejad, Maryam Raoof, Vahid Sheibani, Kooshki R, Amirkhosravi L, Rafie F. "Changes in Hippocampal Orexin 1 Receptor Expression involved in Tooth Pain-Induced Learning and Memory Impairment in Rats" *Neuropeptides (Published).*

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Curriculum vitae

Ramin Raoof was born on March 23rd 1987 in Kerman, Iran. In 2005 he obtained his high school diploma in Experimental Sciences. From 2006-2010 he studied Biology at Shahid-Bahonar University of Kerman. Subsequently, in 2011 he enrolled in Master of Biology with specialization in Physiology and pathophysiology at Shahid-Bahonar University of Kerman, Iran. During his internship from 2011 till 2013 he studied the contribution of hippocampal orexin receptor in cognition and pain under supervision of Profs. Saeed Esmaeili Mahani and Mehdi Abbasnejad at the University of Medical Sciences of Kerman. He graduated in 2013 as the 1st ranked student with a MSc thesis grade (19.90 out of 20). After his Master he moved to the Netherlands. Subsequently, he started as PhD student at Center for Translational Immunology and Rheumatology & Clinical Immunology, UMC Utrecht in 2015. Ramin was also embedded in European Union's Horizon 2020 Marie Curie Training Network, BonePain, as an Early Stage Researcher (www.bonepain.eu). Under the supervision of Dr. Niels Eijkelkamp, Prof. Floris P.J.G. Lafeber and Dr. Simon C. Mastbergen he focused on fundamental understanding of the role of macrophages in the regulation of pain pathways. He conducted a part of his PhD research in University College London (UCL), in Molecular Nociception Group of Prof. John N. Wood. During his PhD, Ramin supervised several Master's students. He received several conference grants including The Challenge of Chronic Pain Conference (Cambridge, 2017), 5th European Congress of Immunology (Amsterdam, 2018), and 17th IASP World Congress on Pain (Boston, 2018). Moreover, Ramin received the 'Bright Sparks in Immunology' award at the 5th European Congress of Immunology for his oral presentation.