The role of Tlr7 in cutaneous and neuropathic pain states

by

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Abstract

The immune system interacts reciprocally with the nervous system in order to accomplish facets of innate immunity via various mechanisms including toll-like receptors (TLRs). TLR activity leads to the release of cytokines which can effect acute and chronic pain states by promoting afferent neuron hypersensitivity. TLR7 is one such receptor that has been implicated in pathways related to inflammation and pain in mice, making it a relevant subject for understanding how these processes are modulated. This project assesses the function of TLR7 with regard to murine nociception in an acute cutaneous inflammatory, chronic cutaneous inflammatory, and neuropathic autoimmune model.

To assess the acute cutaneous inflammatory response, mice were placed in a clear plexiglass container after being injected with 1% formalin into the left hindpaw and observed for nocifensive responses over a period of 60 minutes. To model a chronic cutaneous inflammatory pain state, 0.1 ml of Complete Freund's Adjuvant (CFA) was injected into the left hindpaw and the von Frey assay was performed over 28 days, until recovery of normal mechanical sensitivity was observed. A mouse model of Multiple Sclerosis (MS), Experimental Autoimmune Encephalomyelitis (EAE), was generated in mice using a subcutaneous injection of myelin oligodendrocyte glycoprotein (MOG) to induce a neuropathic pain state in an autoimmune context. Mechanical hypersensitivity was assessed using the von Frey assay over a period of 14 days, at which point accurate responses were no longer reliable due to advanced disease progression, and mice were observed for clinical motor function for 21 days to score disease severity.

The formalin assay showed an increase in overall pain behaviour in Tlr7-/- mice compared to wildtypes, with a significant difference in the female group. In both sexes, wildtype mice recovered from mechanical hypersensitivity induced by CFA while Tlr7-/- mice remained

hypersensitive. EAE disease onset occurred at a similar time point in both Tlr7-/- and wildtype mice, although the Tlr7-/- group demonstrated consistently higher clinical scores over the course of 21 days post-induction. Von Frey testing revealed that while both Tlr7-/- and wildtype mice developed tactile hypersensitivity by 14 days post-induction, this hypersensitivity was accelerated in Tlr7-/- mice. Tlr7-/- mice of both sexes exhibited significant reduction of their paw withdrawal thresholds several days before the wildtype controls.

Following these experiments, the spinal cord (SC) and dorsal root ganglion (DRG) were assessed for Tlr7 RNA and ionized calcium binding receptor molecule 1 (Iba1) protein expression in EAE, while SC cFos and hind paw cluster of differentiation (CD) 45, Iba1, and CD4 protein expression levels were measured in the formalin and CFA responses. For the CFA model specifically, the SC was explored further in terms of peptidergic calcitonin gene-related peptide (CGRP) and non-peptidergic isolectin-B4 (IB4) protein expression levels for nociceptor density and projection length within the dorsal horn. In all models, certain significant changes were found in the SC and hind paw which indicate greater inflammation in Tlr7-/- tissue. With regard to localizing Tlr7, RNAScope combined with immunohistochemistry (IHC) showed that it is colocalized with neurons in the DRG, and thus far we are not able to detect meaningful differences in expression level of Tlr7 in EAE.

In order to test the effect of exogenous agonism on TLR7 and the resolution of mechanical hypersensitivity after cutaneous inflammatory insult, we injected imiquimod into the mouse hind paw following an initial CFA injection. While both vehicle and treatment groups ultimately showed a trend toward recovery, the treatment group demonstrated significantly less mechanical hypersensitivity altogether throughout the course of the experiment in addition to displaying a quicker recovery to baseline levels, suggesting that TLR7 agonism facilitates pain resolution.

Overall, we show that TLR7 is a critical regulator of the resolution of mechanical hypersensitivity and the progression of disease course in the neuropathic pain model of EAE and in cutaneous inflammatory models. We propose a resolutionary functional role of TLR7 and the potential to consider it a novel therapeutic target for clinical studies.

Preface

This thesis is an original work by Olivia Rose La Caprara. This research project was conducted at the University of Alberta and was co-supervised by Dr. Kerr and Dr. Taylor. Much of the data I collected and included in this project is also shown in Figures 3, 4, 7, 8, and 9 of a paper currently under review to be published in Brain, Behaviour, and Immunology, entitled "Sex differences in peripheral immune cell activation: neuro-immune interactions with consequences for pain," authored by PhD graduate Timothy N. Friedman.

The data collection and analysis for these figures was performed mainly by myself along with the incredible contributions of undergraduate student Celine Zhang for the formalin and CFA experiments (Figure 1; Figure 2), PhD student Christian Faig for the CFA hind paw injections (Figure 2; Figure 6), and laboratory technician Gustavo Tenorio for certain data points during von Frey testing in the EAE (Figure 4) and imiquimod (Figure 6) experiments. In addition, Figure 4-5 in this thesis document includes data from Timo Friedman, and also appears in the aforementioned paper as part of Figure 6.

Acknowledgements

This thesis was only possible through community. Even before I stepped foot in the University of Alberta for the first time, I was welcomed online during the covid pandemic by the students and supervisors of the Kerr, Taylor, and Plemel labs. Thank you to Dr. Kerr, for teaching me to maintain a flexible perspective on my research, encouraging me to understand complex ideas, and for always celebrating my successes. Thank you to Dr. Taylor, for listening carefully, providing invaluable insight into my proposed experiments and research findings and for offering critical, innovative guidance throughout my degree. To Dr. Plemel, thank you for inspiring me by demonstrating methodical research work ethic and scientific spirit. And to Dr. Fouad, thank you for never missing an opportunity to challenge Dr. Kerr or myself on our ideas; it has been both engaging and educational.

Thank you especially to my fellow students, researchers, and collaborators. To Gustavo and Kelly, who validated every one of my questions and ensured the lab stayed functional. To Aislinn, who taught me how to present effectively and to Timo, who succeeded in imparting to me most everything I needed to know about being a good lab scientist. To Madelene, for forging friendship at every opportunity, and to Brady, Sameera, Charbel, and Sharmistha for making the lab a place full of laughter. To Zoë, for taking the time to share her graduate story with me, and to Caylin, Christian, Julia, and Adam for supporting me emotionally at every turn. To Anastasia, for late nights having long conversations while working side by side. Finally, thank you especially to Celine, the undergraduate who not only was extremely dedicated to our projects, but who also became my confidante for all things lab and life. And to Andrea, whom I consider my first official lab student, her enthusiasm and joy for learning reminded me why I cared so much after all. I know she is going on to a future full of exciting scientific endeavours.

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List of Abbreviations

ANOVA: Analysis of variance

AUC: Area under the curve

BBB: Blood-brain barrier

BMDM: Bone marrow-derived macrophages

CD4: Cluster of differentiation 4

CD45: Cluster of differentiation 45

CD8: Cluster of differentiation 8

CFA: Complete Freund's adjuvant

CGRP: Calcitonin gene-related peptide

CM: Conditioned media

CNS: Central nervous system

CSF: Cerebrospinal fluid

DAMP: Damage-associated molecular pathogens

DAPI: 4′,6-diamidino-2-phenylindole

DRG: Dorsal root ganglion

EAE: Experimental autoimmune encephalomyelitis

FISH: Fluorescent in situ hybridization

GABA: Gamma aminobutyric acid

IFN-I: Type 1 interferon

IFN-II: Type II interferon

IL: Interleukin

i.p: intraperitoneal

IB4: Isolectin-B4

Iba1: Ionized calcium-binding adaptor molecule 1

MAP: Mitogen-activated protein kinase

miRNA: Micro ribonucleic acid

MOG: Myelin oligodendrocyte glycoprotein

mRNA: Messenger ribonucleic acid

MS: Multiple Sclerosis

NDS: Normal donkey serum

NF-κB: nuclear factor kappa B

NGF: Nerve growth factor

PAMP: Pathogen-associated molecular pattern

PBS: Phosphate-buffered saline

PFA: Paraformaldehyde

PGE2: Prostaglandin E2

PNS: Peripheral nervous system

PRR: Pattern recognition receptor

50% PWT: 50% Paw withdrawal threshold

SC: Spinal cord

SEM: Standard error of the mean

RNA: Ribonucleic acid

TLR7: Toll-like receptor 7

TNFα: Tumour necrosis factor alpha

TRPA1: Transient receptor potential ankyrin 1

TRPV1: Transient receptor potential vanilloid 1

VEH: Vehicle

CHAPTER 1: Introduction

1.1 General Overview

Pain is a common symptom of diseases of the nervous system, and a Canadian national survey found that over a third of people with neurological conditions reported experiencing pain with a prevalence of almost twice that of the general population (Cragg et al., 2018). Despite being subjective, pain often results from the culmination of immune messenger cascades which promote an inflammatory state locally or systemically (Woolf, 2010). This inflammation effectively communicates to nerve endings in the periphery and primary sensory neurons in the dorsal root ganglion (DRG) to fire and transduce pain signals to the brain via the spinal cord (SC) (Argoff, 2011).

In the experimental autoimmune encephalomyelitis (EAE) mouse model of multiple sclerosis (MS), DRG sex-dependent inflammatory profiles and mechanical hypersensitivity are clearly observed even before classical motor symptoms manifest, at disease onset (Thorburn et al., 2016; Maguire et al., 2022). Similarly, in the autoimmune disease of MS, affected persons report pain symptoms in its prodrome phase up to 5 years before the first demyelinating event (Yusuf et al., 2021), which are associated with fatigue, depression, anxiety, sleep problems, and impaired cognitive status (Shahrbanian et al., 2018).

The incidence of MS has increased in females over the past 40 years, with a shift from a 2:1 to a 3:1 female-to-male ratio; women with MS experience more relapses and worse lesions, and their MS onset occurs earlier compared to men (Ysrraelit & Correale, 2019). This increase in female diagnoses may be due to more effective research methods and disease awareness, in addition to changes over the years in nutrition, obesity levels, and environmental factors (Harbo et al., 2013). It is also thought that regardless of these trends, female diagnoses in general may be hormone-dependent, with estrogen and progesterone known to have immunomodulatory impacts

in terms of both anti-inflammatory and pro-inflammatory effects (Khan & Ansar Ahmed, 2016). It has also been postulated that females are more susceptible to chronic pain development due to immune response mechanisms that are more inclined to facilitate pro-nociceptive inflammation rather than resolution due to their complex interplay with hormones and genetics (Nicotra et al., 2012).

In the case of chronic pain, innate immune inflammation related to acute pain can lead to nociceptor hypersensitivity and central sensitization (Verma et al., 2015). Acute pain becomes chronic when, instead of noxious stimuli lessening as healing progresses, cytokines in the peripheral nervous system (PNS) stimulate first-order neurons, which then influence second-order neurons in the central nervous system (CNS) via neuroglia and neuron receptors to ultimately hypersensitize the CNS (Voscopoulos & Lema, 2010).

Inflammation is associated with deleterious effects such as pain, but active inflammation may in fact be necessary for pain resolution to occur (Buckley et al., 2013). For example, certain immune cells called macrophages were found to release a chemical messenger, interleukin-10, which inhibit nociceptor sensitization (Ji et al., 2020), but this is only one piece of the puzzle. By studying different players in the immune system, even those not characteristically thought to contribute to the development of pain, we can better understand the story of pain development and resolution. Members of the TLR family have been recognized to play a role in pain, such as TLR4, which is expressed on various non-immune and immune cell types, including resident macrophages and microglia in the central nervous system (Vaure & Liu, 2014). TLR4 has been studied for its role in neuropathic pain and maintenance of chronic pain states (Bruno et al., 2018), where it was found that antagonizing this receptor attenuated mechanical hypersensitivity (Christiansen et al., 2011; Wu et al., 2010). Additionally, Sorge et al. (2011) discovered that TLR4

mediates inflammatory and neuropathic pain in a sex-specific manner in the spinal cord, specifically with regard to pain behaviours in male mice. Another TLR that may act similarly TLR4 to trigger inflammatory cascades (Schapovalova et al., 2022), toll-like receptor 7 (TLR7), is known to identify exogenous, single-stranded ribonucleic acid (RNA) and mount an antiviral response.

TLR7 binds single-stranded enveloped virions which have released their capsids by acidification of the endosomal vesicle; this process was found to be required for the antiviral response mounted by TLR7, confirming its endosomal location (Crozat and Beutler, 2004; Lund et al., 2004). Furthermore, viral and bacterial nucleic acids that are known to be endocytosed are detected by TLR7 once this protein has undergone a chaperoned shuttling process from the endosplasmic reticulum to the endosome in response to pro-inflammatory cytokine signalling (Petes et al., 2017). In terms of its cellular distribution, TLR7 is expressed predominantly in antigen-presenting immune cells such as plasmacytoid and myeloid dendritic cells, macrophages, and B cells (Petes et al., 2017), but TLR7 is involved in more than binding pattern-associated molecular patterns and mounting an immune response. It has also been postulated to be involved in various inflammatory pain states, such as MS (Gambuzza et al., 2011).

Since pain is poorly managed in MS with current treatment options (Urits et al., 2019), we sought to examine the role of TLR7 to study whether or not it is a viable therapeutic target, and to further contextualize the immune mechanisms of chronic inflammatory pain as a whole.

1.2 Literature Research

1.2.1   Chronic pain and the immune system

To understand the mechanisms of chronic inflammatory pain, we need to first understand the role of the immune system in facilitating chronic pain. Chronic pain is the result of peripheral

and central nervous system pathways becoming dysfunctional, causing a heightened response to noxious stimuli, hyperalgesia, and a painful response to innocuous stimuli, allodynia (Malcangio, 2020). Immune cells play an important role in generating positive feedback loops that support chronic pain by sensitizing critical neurons, such as those in the SC dorsal horn, through the release of inflammatory mediators like cytokines (Malcangio, 2020).

The continuous crosstalk between immune mediators and nociceptor neurons is foundational to both acute and chronic inflammation (Pinho-Ribeiro et al., 2017), but not necessarily to acute pain. Notably, modulated activity of the immune system aside from basic innate inflammatory responses have not been found in acute nociception, but appreciable changes have been observed in persistent pain states (Marchand et al., 2005), suggesting that the immune system is a critical component to the development and maintenance of chronic pain.

Immune cells release mediators at peripheral nerve terminals and centrally in the SC that affect mechanical and thermal sensitivity, and nociceptors release neuropeptides and neurotransmitters that, in turn, regulate the immune response (**Figure 1-1**) (Pinho-Ribeiro et al., 2017). Intracellular signalling pathways including specific cytokine, lipid, and growth factor receptors can phosphorylate or gate ion channels TRPV1, TRPA1, $Na_v1.7$, $Na_v1.8$, and $Na_v1.9$, increasing the probability of neuronal firing and causing hypersensitivity. Immune cells can also sensitize nociceptor neurons by releasing interleukins (ILs), interferons, serotonin, histamine, nerve growth factor (NGF), tumour necrosis factor alpha (TNFα), and prostaglandins (Pinho-Ribeiro et al., 2017).
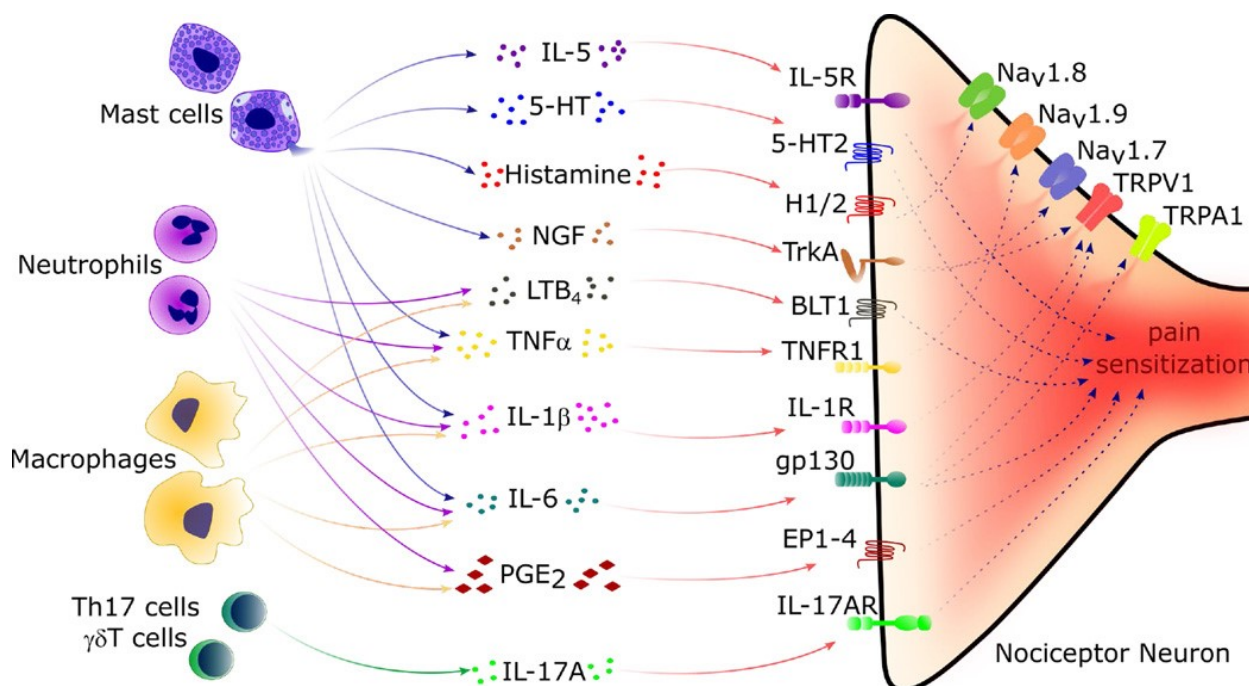
Figure 1-1. *Immune cells sensitize nociceptors through various pathways.* During inflammation, immune cells secrete molecular mediators that sensitize nociceptor neurons, leading to increased pain sensitivity. These mediators include cytokines, growth factors, and neurotransmitters such as interleukins, prostaglandins, serotonin, histamine, and nerve growth factor. Specific receptors on nociceptors are activated by these mediators, leading to increased action potential generation and pain signal transduction. (Pinho-Ribeiro et al., 2017).

Among the immune cells most commonly associated with inflammation and chronic pain are mast cells, macrophages, and T cells. Mast cells originate from precursors in hematopoietic tissue, migrate to peripheral tissue at tissue-environment interfaces (Chatterjea & Martinov, 2015) such as the dermal layer (Wilgus & Wulff, 2014), and differentiate under various stimuli to contribute to tissue homeostasis and pathological regulation in the microenvironment (Frossi et al., 2018). In chronic inflammatory pain conditions, mast cells accumulate (Li et al., 2012) and contribute to nociceptor sensitization by releasing cytokines, serotonin, histamine, and nerve growth factor (Chatterjea & Martinov, 2015). Like mast cells, macrophages play an important role

in the immune system defense against pathogens (Lampiasi, 2022) as well as in the inflammatory processes associated with chronic pain (Chen et al., 2020).

Macrophages are phagocytic cells that engulf and digest foreign bodies or debris and can present antigens to adaptive immune T-lymphocytes, or T cells (Underhill et al., 1999). In general, macrophages generate and release pro-inflammatory cytokines such as TNF when tissue damage occurs, and prolonged release of these cytokines can lead to persistent pain (Silva et al., 2021). They also play an immunoregulatory role due to their ability to transition functions depending on signals from the wider immune system (Lavin et al., 2014).

Macrophages are normally classified as blood monocyte-derived macrophages, which enter into tissues in response to inflammatory cues, or tissue resident macrophages, which are specialized for the specific tissue they live within (Watanabe et al., 2019). Sensory neuron-associated macrophages are a type of resident macrophage that play a role in pathophysiological processes stimulated by the activation of pattern recognition receptors (PRRs) (Silva et al., 2021). Following traumatic nerve injury, resident macrophages facilitate a pronociceptive state via interluekin-1β (IL1β) at the site of injury (Schuh et al., 2014). In other neuropathic models like trigeminal pain and EAE, monocyte-derived macrophages target transient receptor potential ankyrin 1 (TRPA1) via oxidative stress byproducts (Trevisan et al., 2016) and participate in ongoing crosstalk between microglia and T cells to ultimately increase the incidence of lesions in the CNS via downstream effects of cytokine release (Nally et al., 2019), respectively. Conversely, macrophages have also been found to promote pain resolution through interleukin-10 expression, which is believed to dampen neuroinflammation by affecting cytokine signalling: after mild nerve damage in spinal macrophages (Niehaus et al., 2021), and during transient inflammatory pain in dorsal root ganglion macrophages (Willemen et al., 2014).

The immediate phagocytic action of macrophages is part of the innate immune response, but macrophages also participate in the adaptive immune response due to their ability to regulate T cell activity, through antigen presentation and cytokine signalling (Guerriero, 2019). The specific cytokines released by macrophages depends on the context of the immune response, and overall play an important role in shaping the T cell response in inflammatory pain models. Recruitment of T cells can maintain the inflammatory response (Marchand et al., 2005), but a wide body of evidence suggests that T cells play a dual role in chronic pain, with the ability to both promote and suppress instances of chronic pain (Kavelaars & Heijnen, 2021).

Specifically, the role of T cells is being studied in terms of its subsets: cluster of differentiation 8 (CD8)+ T-cytotoxic cells and cluster of differentiation 4 (CD4) + T-helper cells. These T cell types are involved in the suppression of pain or the promotion of its resolution after inflammation by producing interleukin 10 (IL-10) and opioid peptides which message nociceptors to diminish pain signalling (Kavelaars & Heijnen, 2021). Interluekin 4 (IL-4) has also been identified as a key cytokine in prolonged pain relief, typically released by CD4+ T cells (Celik et al., 2020). While there have been contradictory findings on the role of T cells in pain (Marchand et al., 2005), recent research implies that pharmacologically intervening to support rather than dampen T cell function would likely improve outcomes of pain resolution (Ji et al., 2016).

A discussion about chronic pain and the immune system would not be complete without introducing microglia, the resident immune cell of the CNS, likened in function to that of the macrophage (van Rossum & Hanisch, 2004). Like macrophages, microglia phagocytose cellular debris and pathogens, and can incite an immune response by presenting antigens and producing cytokines (Amor et al., 2010). Microglia also have additional roles unique to the CNS such as supporting the blood-brain barrier (BBB), participating in neurogenesis, and helping to maintain

synapses (Graeber, 2010). All these roles combined make microglia another likely actor in the development and maintenance of chronic pain.

Microglia are activated in response to events such as CNS injury, leading to the production of inflammatory cytokines, chemokines, and other substances, triggering a cascade of immune signalling (Marchand et al., 2005). Microglial activation is thought to be beneficial in certain contexts (De Nicola et al., 2003), but other studies have shown that it can contribute to chronic pain states (Marchand et al., 2005); importantly, microglia are believed to be involved in the initiation rather than the maintenance of chronic pain (Raghavendra et al., 2003).

Specifically, microglia release substances including IL1β, tumour necrosis factor α (TNFα), prostaglandin E2, and nitric oxide, which are known to elicit pain symptoms in otherwise healthy animals (Sung et al., 2004). In connection with the innate immune system, TLR4 was found to contribute to neuropathy by increasing microglial activation along with the production of inflammatory mediators (Tanga et al., 2005). TLRs as a whole, in fact, have been implicated in the biological processes related to pain (Nicotra et al., 2012), potentially by their impact on the aforementioned immune cells. As innate immune receptors, their scope extends past emergent immune responses to realize downstream effects on the nervous system (Kong & Le, 2011).

1.2.2 Toll-like receptors in the innate immune system

One of the earliest disease-modifying therapies for MS was interferon beta therapy (Madsen, 2017). Interferons are a group of cytokines that are involved in the innate immune system and help to regulate antiviral and immune modulatory functions (Sen, 2000). There are three types of interferons comprising classes of interferon α (IFNα), interferon γ (IFNγ), and interferon β (IFNβ) (Imanishi, 1994). We know now that the release of signalling interferons, predominantly

IFNβ, is tied to TLR activity (Hertzog et al., 2003), which is involved in autoimmune neuropathy, neurodegenerative disorders, and autoimmunity-induced neuroinflammation (Kong & Le, 2011).

Host defense primarily operates on the premise of PRRs to identify pathogens which express pattern-associated molecular patterns in addition to molecules released from damaged tissues (Kato et al., 2016). PRRs are expressed in peripheral immune cells as well as neurons and glial cells in the nervous system, which can contribute to pain development (Kidd & Urban, 2001). Endogenous ligands called damage-associated molecular pathogens and pathogen-associated molecular pathogens (DAMPS and PAMPs), which are released from damaged cells or foreign cells, activate PRRs and result in an inflammatory cascade that ultimately clears pathogens and necrotic cells (Takeuchi & Akira, 2010). PRRs were previously thought to exist solely on immune cells, but importantly, DAMPS can induce pain signalling by directly activating PRRs found on neurons in the PNS (Kato et al., 2016).

An important family of these PRRs are TLRs, a type I transmembrane receptor (Hemmi & Akira, 2005; J. Li et al., 2013). TLRs were one of the first families of PRRs discovered (Vijay, 2018), and of these evolutionarily ancient receptors (Fitzgerald & Kagan, 2020), mammals have 13 different TLRs responsible for recognizing damage-associated molecular pathogens and other molecular patterns from certain microbes, pathogen-associated molecular patterns, which leads to immune activation and adaptive immune processes (Takeda & Akira, 2015). Overactivation or disruption of proper TLR functioning is related to poor immune homeostasis and is associated with autoimmune disorders (El-Zayat et al., 2019).

TLRs bridge innate and adaptive functions by recruiting a variety of immune cells to drive attacks on pathogens and to induce adaptive immune responses (Kawai & Akira, 2006). While previously thought to respond mainly to microbes and viruses (Takeda & Akira, 2004), TLRs are

also activated by cytokines (Mogensen, 2009). Thus, certain cytokines are ligands of TLRs; at the same time, cytokine release from leukocytes such as macrophages can be induced by TLR-activated pathways (Kawai & Akira, 2006).
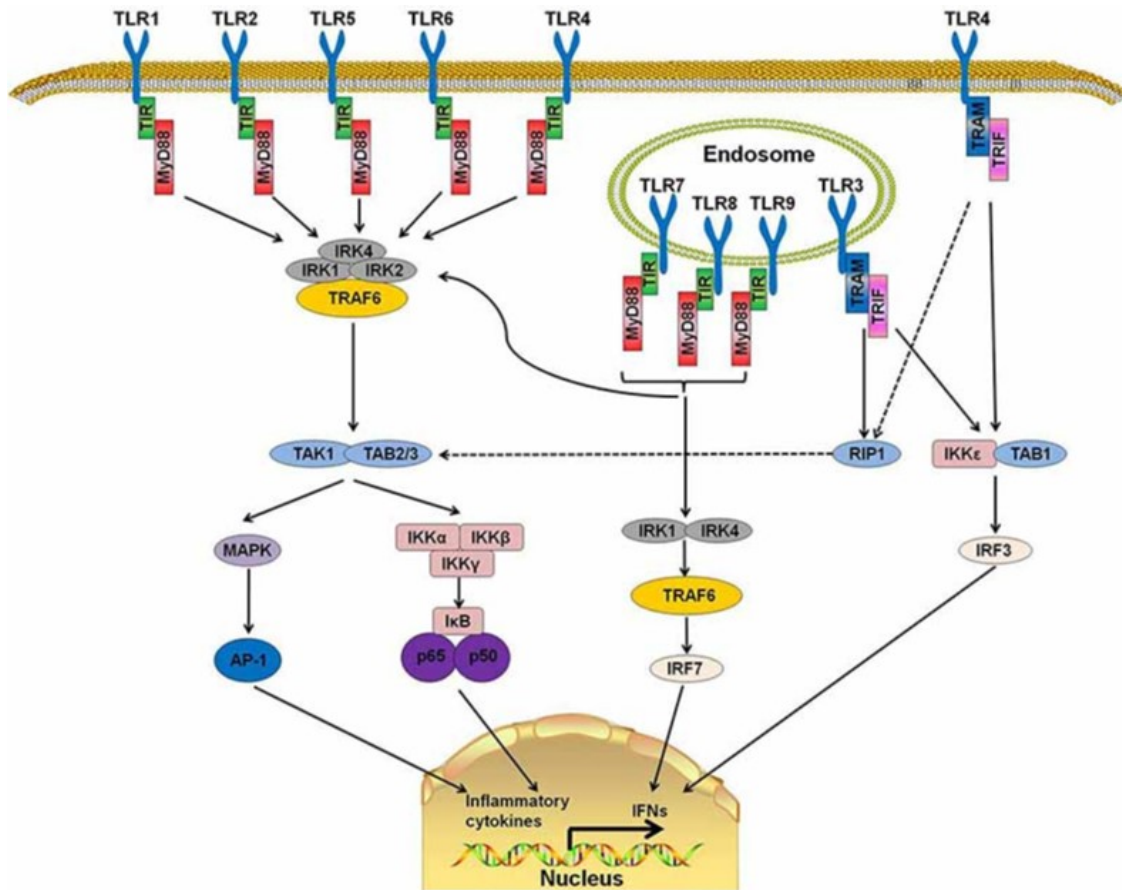
Figure 1-2. *TLR family signalling pathways showing the adaptor protein MyD88 at transmembrane TLRs.* TLR intracellular signalling results in the transcription of interferons (IFNs) (Zheng et al., 2020).

TLRs are therefore crucial effectors of cytokine-induced inflammation and consequent neuropathic pain. To expand, TLRs are expressed on neurons in the DRG, and their activation can lead to the release of pro-inflammatory cytokines (Qi et al., 2011). Certain pro-inflammatory cytokines like IL-1β are not effective at inducing pain in the absence of the TLR adaptor protein MyD88 (Deguine & Barton, 2014; Liu et al., 2016) (**Figure 1-2**); this data points to the necessity

of cytokine signalling through TLRs for the development of hypersensitivity in afferent neurons. This is achieved when the conductivity of glutamate and gamma aminobutyric acid (GABA) receptors in the spinal cord is altered by pro-inflammatory cytokines including IL-1β and TNFα, which are released from glial cells after peripheral nerve injury (Malta et al., 2019).

In autoimmune disease, the theory that pro-inflammatory cytokines mediate hypersensitivity was reviewed by examining the significance of TLRs in the EAE model (Zheng et al., 2020). TLRs are involved in gatekeeping leukocyte entry into the BBB because they can affect the expression of cell adhesion molecules that are necessary for transendothelial migration. In MS, peripheral leukocytes that have been stimulated by cytokines can migrate into the CNS by damaging the integrity of the BBB, which can exacerbate the disease and lead to pain (Ellwardt & Zipp, 2014).

There are a variety of TLRs that have been shown to be pivotal in the development of chronic pain in autoimmune disease models like EAE and nerve trauma models like peripheral nerve injury via cytokine signalling, such as TLR2 and TLR4. In EAE mice, levels of TLR2 along with TNFα were increased in the brain (Zekki et al., 2006), and mice lacking TLR2 showed a protective phenotype against the development of EAE (Rocca et al., 2017). TLR2, therefore, is likely communicating via cytokines to activate leukocytes involved in the advancement of EAE.

TLRs are expressed on adaptive immune cells such as T cells, and TLR4 expression on T cells is another factor that may be required for the development of EAE (Reynolds et al., 2012); although, the role of TLR4 demands further research as its exact function in autoimmune conditions is still debated (Zheng et al., 2020). Additionally, increased levels of TLR2 in the peripheral nerve injury model are related to increased levels of TNFα, and in terms of neuropathic pain behaviour, TLR2 knockout mice lacked a nociceptive response to thermal stimuli (Shi et al.,

2011). There is a body of research devoted to understanding the function of TLR2 and TLR4, but recently, TLR7 has become another receptor of interest in the story of pain.

1.2.3 TLR7 signalling pathways

TLR7 is a lesser-known receptor that may also serve a purpose in the development of chronic pain. TLR7 is coupled to the TRPA1 ion channel in a subset of afferent neurons, and this is important because TRPA1 is imperative for pain signalling (Meseguer et al., 2014). Studies have shown that certain microRNAs act as TLR7 ligands and elicit pain by activating nociceptor neurons (Park et al., 2014). Of the 13 mammalian TLR7 paralogues, 10 are functional in humans (Takeda et al., 2003) and 12 are functional in mice (Sameer & Nissar, 2021).

Regarding TLR7, researchers have found that rat cells respond differently to inflammatory stimulation than human cells (Clarke et al., 2009). In addition, the TLR7 expressed on human CD4+ T cells plays a role in infection-induced anergy (Dominguez-Villar et al., 2015), which may be similar to the function of murine TLR7 (Wiedemann et al., 2016). Both human TLR7 and mouse TLR7 bind imidazoquinoline derivatives like the pharmacological agent imiquimod (Kawasaki & Kawai, 2019). Imiquimod, however, is also a potent agonist of toll-like receptor 8 (TLR8) (Angelopoulou et al., 2020), another major TLR that is phylogenetically and structurally similar to TLR7 (Roach et al., 2005).

Together with toll-like receptor 9 (TLR9) to make up the endosomal TLR subfamily (Maeda & Akira, 2016), TLR7 and TLR8 are similar in their common ability to detect single-stranded RNA, but they actually bind different endogenous ligands due to minute structural differences (Barrat, 2018). In autoimmune diseases such as lupus, it has been proposed that TLR8 and TLR9 each act separately to control the TLR7 response on dendritic cells and B cells, respectively (Desnues et al., 2014). In addition, the function of TLR7 is further complicated by the

fact that diseases related to TLR7 functioning are very different from those related to TLR8 (Guiducci et al., 2013).

Overall, due to the varied cellular distribution of these receptors among species, it is difficult to study their exact role; however, one study in mice concluded that TLR8 activation leads to a general proinflammatory response, whereas TLR7 activation is more closely tied to the specific release of type 1 IFN (Guiducci et al., 2013). This confirms an earlier study which found that TLR7-selective agonists effectively induced IFN-related chemokines compared with the proinflammatory cytokines and chemokines stimulated by TLR8 agonists (Gorden et al., 2005). Moreover, using distinct synthetic human TLR7 and TLR8 agonists, it was reported that plasmacytoid dendritic cell activation was related to TLR7 while monocyte-derived dendritic cell activation was related to TLR8 (Gorden et al., 2005).

Although it was once believed that there was a lack of functional TLR8 in mice (Barrat, 2018), it was recently discovered that murine DRG TLR8 is involved in the development of neuropathic pain after nerve injury independently of the MyD88 pathway, making it distinct from the TLR7 mechanism (Zhang et al., 2018). Understanding the TLR7 signalling mechanism is foundational to this thesis work, although its specificity varies with disease.

In MS, there is a connection to adaptive B cells and TLR7 activity. Particularly, compared with other viruses, infection with Epstein-Barr virus was found to substantially increase the risk of developing MS (Bjornevik et al., 2022); this virus is known to manipulate TLR7 signalling with the result of augmenting B cell proliferation (Martin et al., 2007). B cells are involved in the production of inflammatory cytokines, antigen presentation to T cells, and other actions that may contribute to MS disease progression (Comi et al., 2021). In the EAE model of MS, there is a significant increase of Tlr7 RNA in the female DRG at disease onset (**Figure 1-3**).
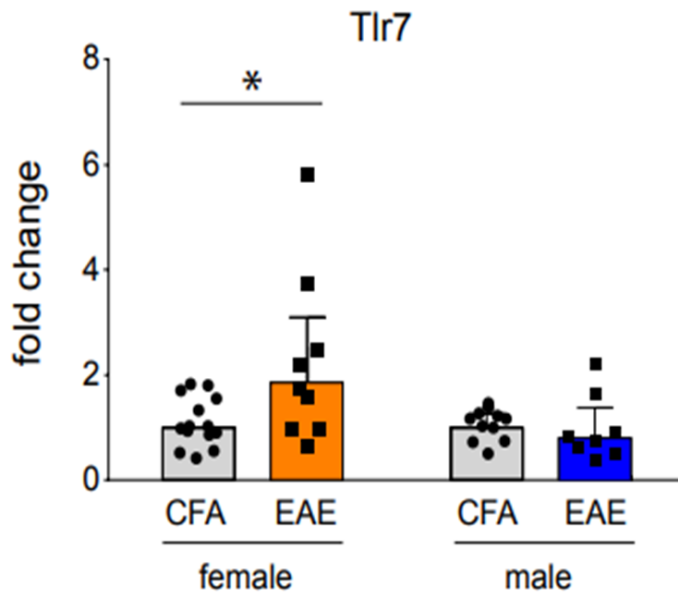
Figure 1-3. *Tlr7 RNA is upregulated in the DRG from female EAE mice.* Differential expression of genes as measured by qPCR (Friedman et al., 2019)**.**

This evidence alludes to the sex difference reported in human MS epidemiology, and suggests that TLR7 plays an important role in MS pathogenesis. Taken together with previous reports in the literature, it also gives an indication that TLR7 may be involved in inflammatory processes in general. Some of those inflammatory processes include itch sensations, deemed pruriception, and nociception; therefore, studying the role of TLR7 in these contexts is critical to the foundation of this work.

1.2.4 TLR7 in pruriception and nociception

Pruriception and nociception may seem vastly different, but they share many similar mechanisms, including peripheral and central sensitization of sensory neurons and spinal projection and interneurons, respectively, and certain neuroimmune interactions (Liu & Ji, 2013). Due to these similarities, studying certain signalling pathways of pruritus may influence our

understanding of pain. Like pain, itch is translated to the CNS mostly via small diameter, unmyelinated C-fibers (Schmelz et al., 2000). These fibers are activated by compounds deemed pruritogens which stimulate cytokine receptors, G-protein coupled receptors, TLRs, and histamine receptors (Lerner, 2016). Although antihistamines are often prescribed to attempt to control chronic itch, these are not always successful due to histamine-independent pathways that are active in pruriception such as TLR-mediated signalling (Liu et al., 2012).

TLR7 specifically has been implicated in pruriception and has been found to be expressed on TRPV1-positive neurons in the DRG (Liu & Ji, 2012). Tlr7-/- mice exhibit normal thermal and mechanical pain sensitivity, but show a reduced response to both non-histamine pruritogens and to the synthetic TLR7 agonist imiquimod (Liu et al., 2010). It has been suggested that TLR7 is functionally coupled with ion channel activation in primary sensory neurons like TRPA1 (Park et al., 2014), which aligns with previous literature describing increased neuronal excitability in the DRG due to application of TLR7 agonists (Liu et al., 2010). Furthermore, TLR7 expression in the DRG may affect neuronal excitability by inducing prostaglandin E2 (PGE2), calcitonin gene-related peptide (CGRP), and IL1β (Qi et al., 2011), common inflammatory mediators which are also implicated in pain states.

Additionally, TLR7 signalling through the MyD88 pathway stimulates cytokines and chemokines through the nuclear factor kappa B (NF-κB) and mitogen-activated protein (MAP) kinase pathways for the initiation of innate immune processes, and MyD88 is expressed in the majority of DRG neurons where infiltrating immune cells such as T cells and macrophages play a surveillance role (Liu et al., 2014). Both T cells and macrophages have been shown to contribute to the maintenance of pain in the presence of MyD88 in a chemotherapy-induced neuropathic pain model in mice (Liu et al., 2014). TLR7 was again found to be crucial to this pathway in a recent

study involving a spinal nerve ligation or constriction model of pain; TLR7 expression was increased in the DRG which activated the NF-κB pathway in primary sensory neurons; the authors postulate that this may result in hypersensitivity to mechanical and thermal stimuli (He et al., 2020).

Overall, researchers have learned much about the role of TLR7 in mediating pruritus which have added to the body of knowledge surrounding TLR7 signalling mechanisms involved in pain. Still, the lack of direct research on TLR7 in this field demanded that we explore a variety of murine models to better identify if and how this innate immune receptor plays a role in inflammatory pain contexts.

1.3   Models of inflammatory pain

Inflammatory pain manifests itself in many forms. We aimed to research three different models of inflammation in order to better define the role of TLR7 in various pain states using a Tlr7-/- mouse. First, we tested the formalin model, with the active ingredient formaldehyde, to incite acute cutaneous inflammation in the hind paw. This model is well-characterized, with intraplantar formalin administration eliciting a distinct biphasic response for around 60 minutes post-injection of pain behaviour accompanied by activation of afferent neurons and induced cFos expression in the spinal cord (Sawynok & Liu, 2003). For this research, the goal of the formalin model was to study short-term pain induced in the mouse hind paw. We also wanted to investigate long-term pain in the hind paw, so we then tested the CFA model of inflammation.

Intraplantar CFA induces tissue injury and hyperalgesia, but its effects on behaviour and cellular correlates have historically only been studied for a few days post-injection. Due to the robust local inflammation caused by CFA, we aimed to carry out this experiment until the hallmark

hypersensitivity was resolved. Of the few reports that have studied mechanical hypersensitivity throughout chronic time frames with CFA, one reports pain recovery in wildtype mice around 30 days after the injection (Laumet et al., 2020), but none have examined this time course in a Tlr7-/- mouse model. Finally, we sought to expand our research of chronic inflammation by extending it to a model of chronic neuropathic pain at the systemic level.

Chronic pain in general is not necessarily neuropathic; neuropathic pain is a subset of chronic pain that is based on a lesion or disease of the nervous system itself and leads to sensory, or afferent, neuron hypersensitivity (Colloca et al., 2017). Neuropathic pain is an abnormal state of the nervous system (Thacker et al., 2007), so that the very system meant to communicate instances of pain to the brain is flawed (Colloca et al., 2017). Pain in MS is deemed neuropathic due to the direct damage to the myelin sheath surrounding neurons (Iannitti et al., 2014). There are many mouse models aimed at effectively replicating facets of MS, but the most commonly utilized one is EAE (Procaccini et al., 2015). This allowed us to study the Tlr7-/- mouse in the context of an autoimmune disease that causes inflammation in the CNS and PNS (Yousuf et al., 2019), resulting in neuropathic pain.

1.3 Research Aims and Hypotheses

The research for this thesis began with years of graduate work from many different students focused on understanding neuroimmune mechanisms influencing pain in MS. At the start of my research journey, there was much literature pointing toward the innate immune system, specifically TLR7, as a main player in pain signalling in addition to its classical role in viral defense. As such, this project was designed to localize neuronal TLR7 expression and to explore the role of TLR7

in chronic pain development and resolution across various models; namely, the formalin, CFA, and EAE models of pain.

In order to test this, I visualized Tlr7 RNA levels in the SC and DRG in EAE mice. I then evaluated mechanical sensitivity of male and female Tlr7-/- mice throughout the duration of each model, from peak hypersensitivity until resolution to baseline levels using the von Frey method. I also extracted and examined tissues of interest including the hind paw glabrous skin, SC, and DRG for inflammation and other relevant markers of inflammation: cluster of differentiation 45 (CD45) for leukocytes, ionized calcium-binding adaptor molecule 1 (Iba1) for macrophages, and CD4 for T cells. Finally, I assessed TLR7 agonism on the CFA inflammatory pain time course to test the effect of exogenous pharmacological therapeutic intervention. Ultimately, the findings described here lay the groundwork for further research in this field regarding TLR7 expression and function in MS in addition to other manifestations of chronic inflammatory pain.

CHAPTER 2: Materials and Methods

2.1 Animals and Ethics

All animal experiments and procedures were conducted in compliance with the Canadian Council on Animal Care Guidelines and Policies with approval from the University of Alberta Health Sciences Animal Care and Use Committee. For all experiments described here, male (N=51) and female (N=63) were used in total. C57BL/6 mice aged 8-12 weeks old (N=26 male; N=36 female) were used in these experiments, in addition to wildtype mice from a KDM5C knockout line used for EAE controls in the Tlr7 RNAScope DRG experiment (N=6 male; N=5 female). Tlr7-/- mice aged 8-12 weeks old (B6.129S1-*Tlr7tm1Flv*/J; Jackson Laboratories, Bar Harbor, ME, USA, Stock #008380) were used for all experiments except for the imiquimod recovery and Tlr7 RNAScope experiments (N=19 male; N=22 female), in which 8-12 week-old mice from Jackson Laboratories were used. The Tlr7-/- mice did not display abnormal behavioural phenotypes, and in terms of physiology, Jackson Labs reports no obvious developmental issues with a reduced response to infection. For the formalin, CFA, and EAE experiments, the control groups were wildtype mice while the experimental groups were Tlr7-/- mice. Mice were housed in standard wire-top cages in a temperature and humidity-controlled environment with food and water available ad libitum. Mice were kept on a 12 hour light cycle and all experimental procedures were performed during light cycles only. Prior to handling or behavioural testing, mice were allowed an acclimation period of 1 week after arrival to the animal housing facility.

2.2 Inflammatory Pain Models

2.2.1 Formalin Model

Mice (N=6 wildtype + 5 Tlr7-/- males; N=6 wildtype + 6 Tlr7-/- females) were placed in a clear plexiglass container after a 30uL injection of 1% formalin (formaldehyde solution in sterile saline) into the left hind paw and observed for nocifensive responses organized in 5 minute increments over a total period of 60 min. Nocifensive responses were subjectively defined as any scratching, biting, licking, or guarding behaviours directed at the injected paw and all behavioural testing was performed by a single trained experimenter.

2.2.2 CFA Model

Mice (N=8 wildtype + 9 Tlr7-/- males; N=8 wildtype + 9 Tlr7-/- females) were injected with 100uL of CFA (1mg/ml, Sigma) into the left hind paw. Baseline von Frey measurements were taken 24 hours prior to injection and reassessed at 3, 7, 10, 14, 16, 21, and 28 days post-injection.

2.2.3 EAE Model

Mice (N=6 wildtype + 5 Tlr7-/- males; N=5 wildtype + 7 Tlr7-/- females) were habituated for 2 weeks in the housing facility with baseline handling and behavioral testing. After this period, EAE was induced by dual subcutaneous injections of myelin oligodendrocyte glycoprotein MOG 35–55 into the base of the tail and neck, 50µg total (Hooke Labs, Cat. No. EK-2110, https://hookelabs.com/products/EK-2110/), emulsified in CFA. An intraperitoneal injection of 100ng of pertussis toxin was given on the day of induction as well as 24 hours later. All mice were briefly anesthetized for induction using isoflurane. Mice were assessed daily for clinical progression of EAE following a four-point scale: grade 0, normal mouse; grade 1, flaccid tail

(disease onset); grade 2, mild hindlimb weakness with quick righting reflex; grade 3, severe hindlimb weakness with slow righting reflex; grade 4, hindlimb paralysis in one hindlimb or both.

2.2.4 Imiquimod Injection

Mice (N=6 vehicle injection + 6 treatment condition females) were injected with 100uL of imiquimod solution (Imiq, 500uM in sterile saline) or vehicle (VEH, 0.21% DMSO) into the left hind paw at 3, 6, 8, 10, and 12 days post-injection of CFA. Imiquimod solutions were prepared by preparing stock solutions in DMSO and further dilution in sterile saline.

2.3 Behavioural Assays

2.3.1 Mechanical Paw Withdrawal Thresholds

Evoked mechanical paw withdrawal thresholds were measured with von Frey filaments using the up-down method (Chaplan et al., 1994). Filaments of increasing force, beginning with the 0.4g filament, were applied to the paw for a maximum of 5 seconds unless a pain response was observed. Once a pain response was observed, an additional four filaments were tested using increasing or decreasing force depending on whether each elicited a pain response. A lesser force followed a painful stimulus, while a greater force followed a non-painful stimulus. These responses were used to calculate the 50% PWT, which is the force required to elicit a pain response to 50% of stimulus exposures as described in Dixon (Dixon, 1980). For testing, mice were habituated to individual, clear plexiglass chambers on an elevated wire mesh screen for 30 min per day for 3 consecutive days, followed by a baseline assessment and subsequent experimental behaviour testing.

2.4 Tissue Analysis

2.4.1   Extraction and Preparation

Hind paw glabrous skin and SC tissue was collected after completion of the in vivo behavioural experiments of formalin and CFA recovery. Animals were euthanized and intracardially perfused with ice-cold 0.9% saline followed by ice-cold 4% PFA. EAE animals which were intended for DRG tissue collection underwent the same procedure except for the 4% PFA perfusion step, since they were required to be fresh-frozen rather than fixed-frozen for the intended RNAScope analysis. DRG tissue therefore was cryopreserved immediately following extraction in Optimal Cutting Temperature (OCT Tissue-Tek, Sakura Ref. No. 4583) in molds atop dry ice, then placed in a -80°C freezer. For the hind paw glabrous skin, 2mm biopsy samples of the injected area were collected utilizing a biopsy punch, with contralateral hind paws collected as controls. SC tissue was collected at the L4 and L5 level using a variety of surgical instruments. Samples were post-fixed in 4% PFA for an additional day, followed by cryopreservation for 48 hours in a 30% sucrose solution in 0.1M phosphate buffer. Finally, cryopreserved samples were frozen in Optimal Cutting Temperature (OCT Tissue-Tek, Sakura Ref. No. 4583) in molds atop dry ice and sectioned into 12μm (hind paw), 20μm (SC), and 10μm (DRG) sections on a Cryostat, collected on Superfrost Plus (Fisherbrand, Cat. No. 2-0370246) slides, and stored at -80° C.

2.4.2   Immunohistochemistry

For IHC, slides underwent a classical IHC protocol. Slides were thawed for 2 min, then washed in 1x phosphate buffered saline (PBS) for 5 minutes, repeated three times. Tissue sections

were incubated with a blocking media of 10% Normal Donkey Serum (NDS) in PBS containing 0.2% Triton X for one hour at rvoom temperature to prevent nonspecific antibody binding. Blocking media was then removed and replaced with an antibody solution with optimized dilutions of the antibody of interest overnight at 4°C. For the hind paw experiments, the primary antibodies used were rat anti-CD45 at 1:500 from BD Pharmingen, monoclonal rabbit anti-CD4 at 1:200 from BioRad, and rabbit anti-Iba1 at 1:500 from Wako. For spinal cord staining, I used rabbit anti-cFos at 1:500 from Cell Signaling, rabbit anti-Iba1 at 1:500 from Wako, rabbit anti-CGRP at 1:500 from Millipore Sigma; and IB4-biotin at 1:500 from Millipore Sigma. Specifically for the IB4 protocol, the primary antibody solution also included 1:1000 magnesium chloride, 1:1000 calcium chloride, and 1:100 manganese chloride. For all protocols, the following day, slides were triple-washed in 1x PBS and incubated with antibody solution containing corresponding fluorescent conjugated secondary antibodies and DAPI from Jackson ImmunoResearch for one hour at room temperature. Slides were washed a final three times and mounted with coverslips using FluoromountG (Invitrogen, Cat. No. 00-4958-02).

2.4.3 Fluorescent *in situ* hybridization

DRG tissue was extracted from non-diseased and EAE mice (N=6 non-diseased + 6 EAE males; N=5 non-diseased + 5 EAE females) immediately after euthanization and perfusion with an ice-cold 0.9% saline solution and then frozen into OCT (Tissue-Tek) molds. Tissue was stored at -80°C. The RNAScope kit for Version 1 for fresh-frozen tissue from ACDBio was used in this protocol. Slides were removed from the freezer and immediately immersed in 4% PFA in 0.1M phosphate buffer for 15 minutes at 4°C, followed by a dehydration process of five minutes each in 50%, 70%, and twice in 100% ethanol. Slides were allowed to dry before a hydrophobic barrier

was drawn around the tissue samples (ImmEdge® Hydrophobic Barrier PAP Pen). Tissue was then incubated for 15 minutes with RNAscope Protease IV (ACDBio) at room temperature. Slides were then washed 3 times in fresh 1x PBS for 2 minutes each time, and incubated at 40 C for 2 hours with RNAScope probe Mm-Tlr7-C3. Slides were then sequentially incubated with amplification reagents: AMP 1-FL for 30 minutes, AMP 2-FL for 15 minutes, AMP 3-FL for 30 minutes, and AMP 4-FL for 15 minutes, all at 40 C. Following each incubation period, slides were washed in RNAScope Wash Buffer (ACDBio). The slides were then fixed again in 4% PFA in 0.1M phosphate buffer for 15 minutes at 4°C to fix the fluorescent in situ hybridization (FISH) stain before undergoing IHC as described previously using rat anti-NeuN 1:500 from Abcam.

2.4.4   Image Acquisition and Analysis

Following staining, slides were imaged on a Zeiss AxioObserver.Z1 at 20x. Images were saved as .tif files for analysis using ImageJ. Image acquisition and analyses were performed blind to experimental conditions and sex, and total measurements are the average of 2 images from different slides for each animal. For the IHC image analysis in the formalin and CFA models, total positive cells for each marker were counted within the total section area and expressed as a ratio of cells/area. CFA SC CGRP and IB4 were measured as percent density in ImageJ after certain areas of interest were selected, similar to the Iba1 IHC for EAE SC.

2.5 Statistical Analysis

Statistical analyses were performed using GraphPad Prism software version 9.5.1. In all experiments, data were tested for normality and parametric or non-parametric statistics were used accordingly. For comparison of two groups, unpaired two-tailed Student's t-test or MannWhitney

test was performed. Two and three-way ANOVAs with appropriate post-hoc tests were used for comparisons of data sets with two or three nominal variables, respectively. Significance threshold was set at $P < 0.05$ in all experiments. All data were expressed as mean ± standard error of the mean (SEM).

CHAPTER 3: Results

3.1 Tlr7 -/- Formalin Response

3.1.1 Nocifensive behaviour time is increased in Tlr7-/- mice during the formalin response

Male and female mice were recorded for the total time of nocifensive responses such as licking and biting the affected paw following a 1% formalin injection, measured as the amount of seconds within each 5-minute interval over the course of 60 minutes. We observed that wildtype mice followed the characteristic response as described in (Dubuisson & Dennis, 1977), with a biphasic pattern separated by an interphase, consisting of phase I and phase II: activation of nociceptors and central sensitization, respectively. Tlr7-/- mice followed this pattern in general and a significantly different response was found with regard to genotype (3way ANOVA, $F_{genotype(1, 19)}$=10.52, *p=0.0043*, $F_{time(3.101, 58.91)}$=17.45, *p<0.0001*, $F_{sex(1, 19)}$=3.511, *p=0.0764*; **Figure 3-1 B**). As evidenced by the AUC as well (**Figure 1 C**), genotype has a significant effect on nocifensive response time (2way ANOVA, $_{(1, 19)}$=10.48, *p=0.0043*, $F_{sex(1, 19)}$=3.867, *p=0.0640*, $F_{interaction(1, 19)}$=0.0002138, *p=0.9885*; **Figure 3-1 C**). Overall, female Tlr7-/- mice displayed a significantly greater nocifensive response time than the wildtype counterparts (t-test, t=2.464, *p=0.0334*).
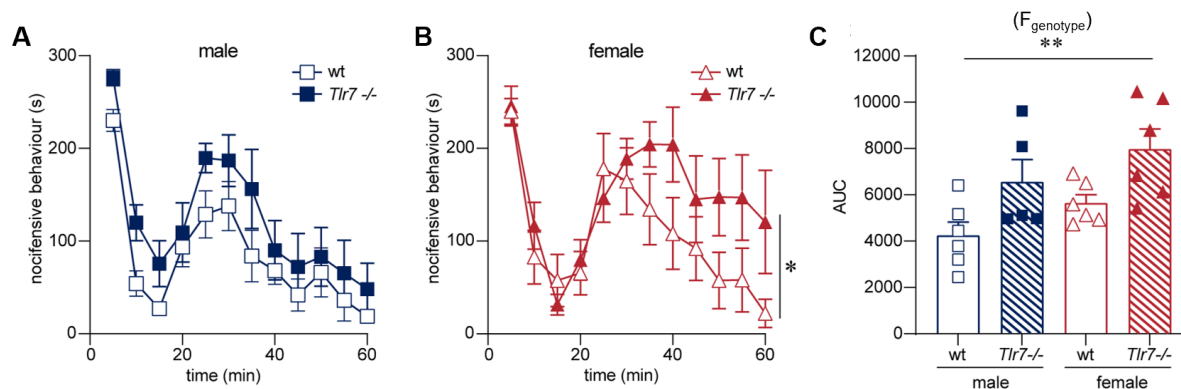
Figure 3-1. *Nocifensive behaviour time is increased in Tlr7-/- mice during the formalin response.* **(A)** Male Tlr7-/- mice do not exhibit significant differences with regard to nocifensive behaviour in the formalin response compared with wildtype mice (p=0.0692). **(B)** Female Tlr7-/- mice demonstrate significantly greater nocifensive behaviour time than wildtype mice (p=0.0043). **(C)** Area under the curve of male and female formalin response (p=0.0043). Post-hoc analyses by Tukey's multiple comparisons test. Data presented as mean $\pm$ SEM. AUC, Area Under the Curve.

3.1.2 Spinal cord dorsal horn cFos protein level expression does not change between Tlr7-/- and wildtype mice 1-hour post-injection of formalin

Since a significant result was found in the female formalin response (**Figure 3-1 B**), we evaluated female spinal cord dorsal horns ipsilateral to the formalin injection for the number of cFos-positive cells following the formalin behaviour test. While there is an indication that cFos expression may be increased in the Tlr7-/- tissue, there is no evidence of a significant difference (t-test, t=0.7378, *p=0.4794*; **Figure 3-2 C**).
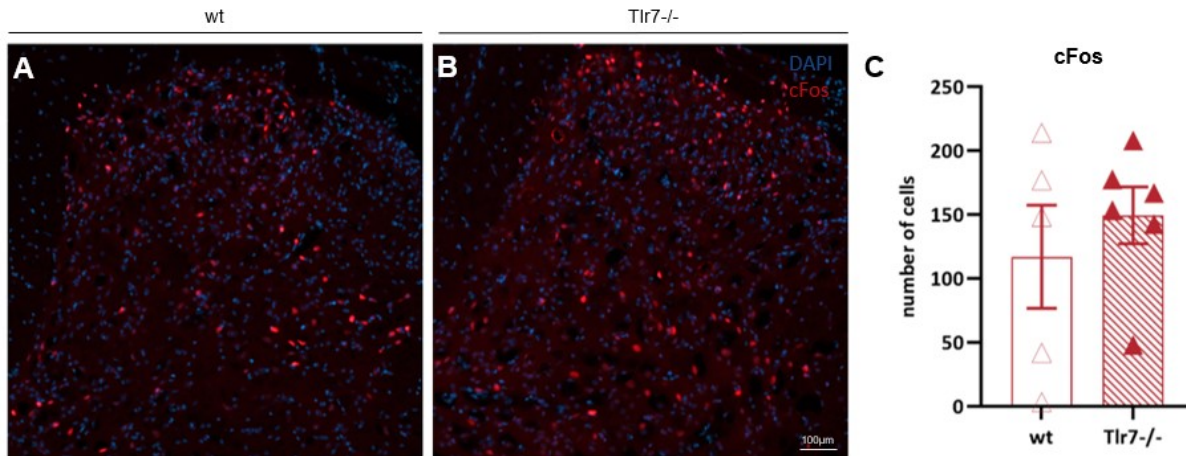
Figure 3-2. *Spinal cord dorsal horn cFos expression does not change between Tlr7-/- and wildtype mice 1-hour post-injection of formalin.* **(A)** Representative 20X images of cFos immunostaining in female wildtype and **(B)** Tlr7-/- spinal cord dorsal horn ipsilateral to the formalin injection. Scale bar = 100 µm, applies to all images. **(C)** Total number of cFos+ cells per image (p=0.3000). Data presented as mean $\pm$ SEM.

3.1.3   CD45 and Iba1 expression is reduced in the male Tlr7-/- hind paw, while CD4 expression is are reduced in the female Tlr7-/- hindpaw 1-hour post-injection of formalin

To assess inflammation in the hind paw resulting from the formalin injection, glabrous hind paw skin was treated to visualize CD45, Iba1, and CD4 markers on the ipsilateral side to the injection. In males, CD45 (t-test, t=3.665, *p=0.0052*; **Figure 3-3 C**) and Iba1 (t-test, t=7.674, *p<0.0001*; **Figure 3-3 I**) levels were significantly reduced in the Tlr7-/- tissue, while no significant difference was observed in CD4 levels (t-test, t=1.931, *p=0.0855*; **Figure 3-3 O**). In females, CD45 (t-test, t=0.2879, *p=0.7807*; **Figure 3-3 F**) and Iba1(t-test, t=0.02720, *p=0.9789*; **Figure 3-3 L**) levels were not significantly different between Tlr7-/- and wildtypes, but CD4 levels were significantly reduced in Tlr7-/- tissue (t-test, t=3.985, *p=0.0040*; **Figure 3-3 R**).
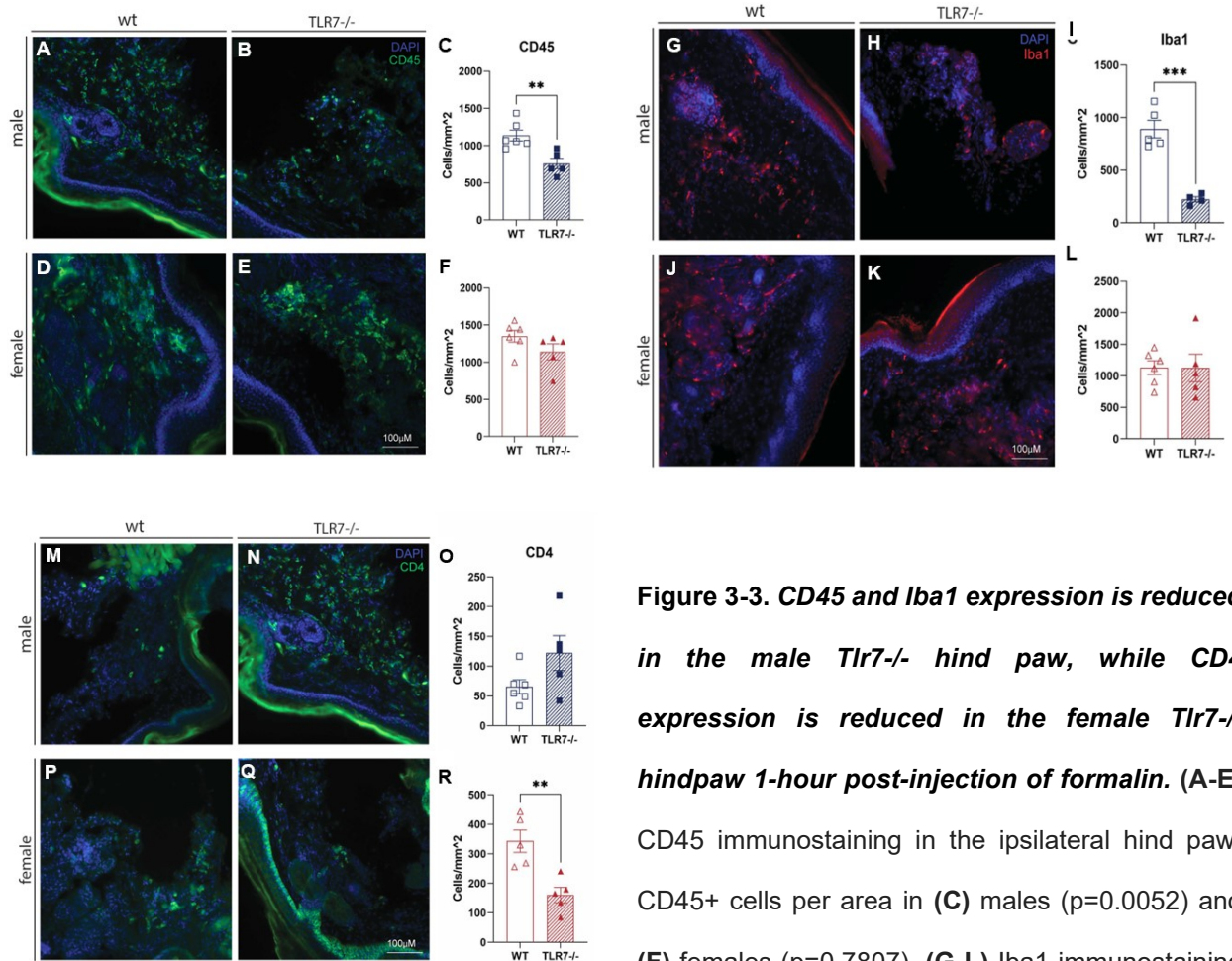
Figure 3-3. *CD45 and Iba1 expression is reduced in the male Tlr7-/- hind paw, while CD4 expression is reduced in the female Tlr7-/- hindpaw 1-hour post-injection of formalin.* **(A-E)** CD45 immunostaining in the ipsilateral hind paw. CD45+ cells per area in **(C)** males (p=0.0052) and **(F)** females (p=0.7807). **(G-L)** Iba1 immunostaining in the ipsilateral hind paw. Iba1+ cells per area in **(I)** males (p<0.0001) and **(L)** females (p=0.9789). **(M-R)** CD4 immunostaining in the ipsilateral hind paw. CD4+ cells per area in **(O)** males (p=0.0855) and **(R)** females (p=0.0040). Representative 20X images. Scale bar = 100 µm, applies to all images.

3.2 Tlr7-/- CFA Recovery

3.2.1   Tlr7-/- mice do not return to mechanical sensitivity baseline threshold after a CFA injection into the hindpaw at 28 days post-injection

The CFA hind paw injection results in severe hypersensitivity as measured by the von Frey test at 3 days post-injection, reliably replicated by multiple sources including (Ling et al., 2020). Since the CFA test was historically used only to induce acute inflammation and nociception in the

hindpaw, we aimed to expand on this research by evaluating mechanical sensitivity throughout the entire potential time course of this effect. Therefore, we injected CFA into the hind paw and continued taking von Frey measurements until either the wildtype or Tlr7-/- groups recovered to baseline threshold. Our results show that in males and females, Tlr7-/- mice remained hypersensitive at 28 days post-injection, while the wildtype mice recovered from the CFA-induced mechanical hypersensitivity at this same time point (**Figure 3-4 A; Figure 3-4 B**). The data shows that there is a significant effect of genotype (3way ANOVA, $F_{genotype(1, 30)}$=19.30, **p=0.0001**, $F_{time(2.484, 74.51)}$=22.51, **p<0.0001**, $F_{sex(1, 30)}$=4.087, **p=0.0522, Figure 3-4 B**) throughout the CFA time course. The AUC also shows a significant effect of genotype (2way ANOVA, $F_{genotype(1, 30)}$=14.95, **p=0.0006**, $F_{sex(1, 30)}$=5.179, **p=0.0302**, $F_{interaction(1, 30)}$=14.95, **p=0.7993, Figure 3-4 C**).
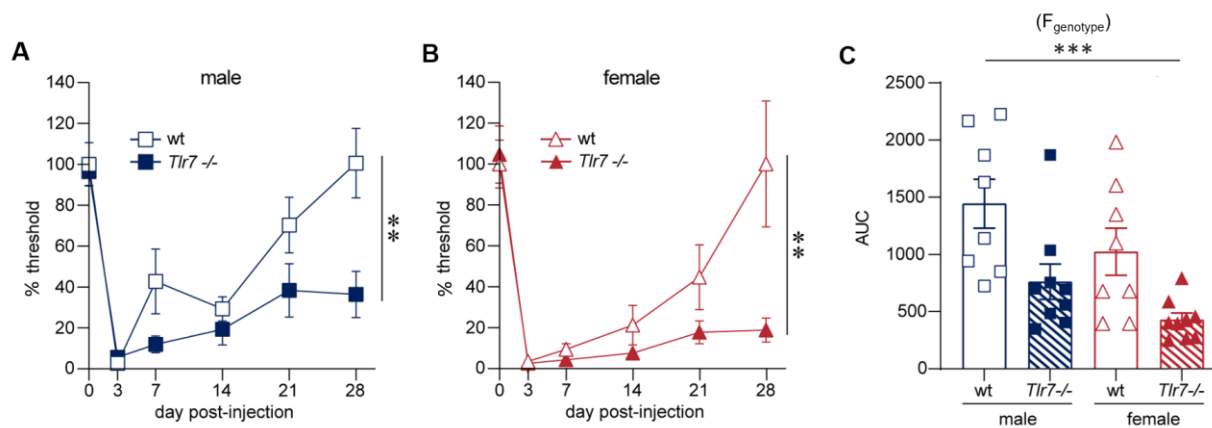
Figure 3-4. *Tlr7-/- mice do not return to mechanical sensitivity baseline threshold after a CFA injection into the hindpaw at 28 days post-injection.* Mechanical sensitivity as measured by von Frey in **(A)** males (p=0.0062) and **(B)** females (p=0.0025). **(C)** Area under the curve of male and female 50% paw withdrawal thresholds throughout the CFA time course (p=0.006). Post-hoc analyses by Sidak's multiple comparisons test. Data presented as mean $\pm$ SEM.

The hind paw swells dramatically in the days following the CFA injection, an inflammatory response that can be appreciated qualitatively by observation as well as with quantitative evidence.

We measured hind paw thickness at the time point of assumed maximal swelling based on the literature (3 days post-injection) and repeated this measurement at the end of the experiment (28 days post-injection). Interestingly, within both wildtype and Tlr7-/- groups, the same significant reduction in hind paw thickness was observed over time. Although the wildtype group demonstrated recovery from mechanical hypersensitivity while the Tlr7-/- group did not, there is no significant effect of genotype in the paw thickness (2way ANOVA, $F_{genotype(1, 63)}$=0.1532, *p=0.6968*, $F_{time(1, 63)}$=135.1, *p<0.0001*, $F_{interaction(1, 63)}$=1.788, *p=0.1859*; **Figure 3-5).**
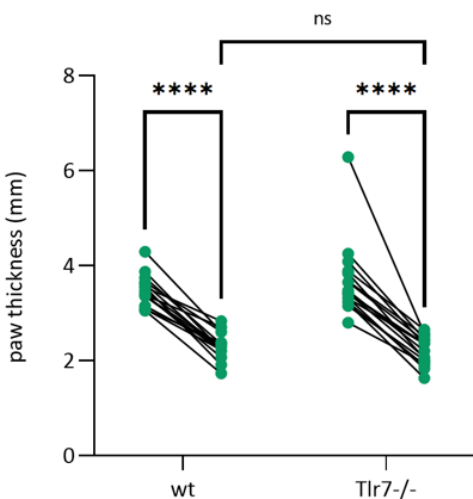
Figure 3-5. *The change in hind paw thickness does not differ between day 3 post-injection of CFA and day 28 post-injection of CFA in wildtype and Tlr7-/- groups.* Caliper measurements of hind paw thickness at its widest point at day 3 post-injection of CFA and at day 28 post-injection of CFA in wildtype and Tlr7-/- groups (p=0.6968). Post-hoc analyses by Tukey's multiple comparisons test. Data presented as mean ± SEM.

3.2.2   Spinal cord dorsal horn cFos protein level expression is increased in male Tlr7-/- mice at 28 days post-injection of CFA

As a follow-up to the significant differences observed between wildtype and Tlr7-/- behaviour in males and females (**Figure 3-4 A; Figure 3-4 B**), we assessed the spinal cord dorsal horn ipsilateral to the CFA hind paw injection for the number of cFos-positive cells at the end of the experiment (28 days post-injection), when mechanical hypersensitivity was still present in the Tlr7-/- groups. In males, there was a significant difference between the wildtype and Tlr7-/- groups (t-test, t=3.126, *p=0.0167*; **Figure 3-6 C**), with the Tlr7-/- tissue showing greater numbers of cFos-

positive cells in the spinal cord dorsal horn. On the contrary, females showed no significant difference (t-test, t=0.3410, *p=0.7431*; **Figure 3-6 F**).
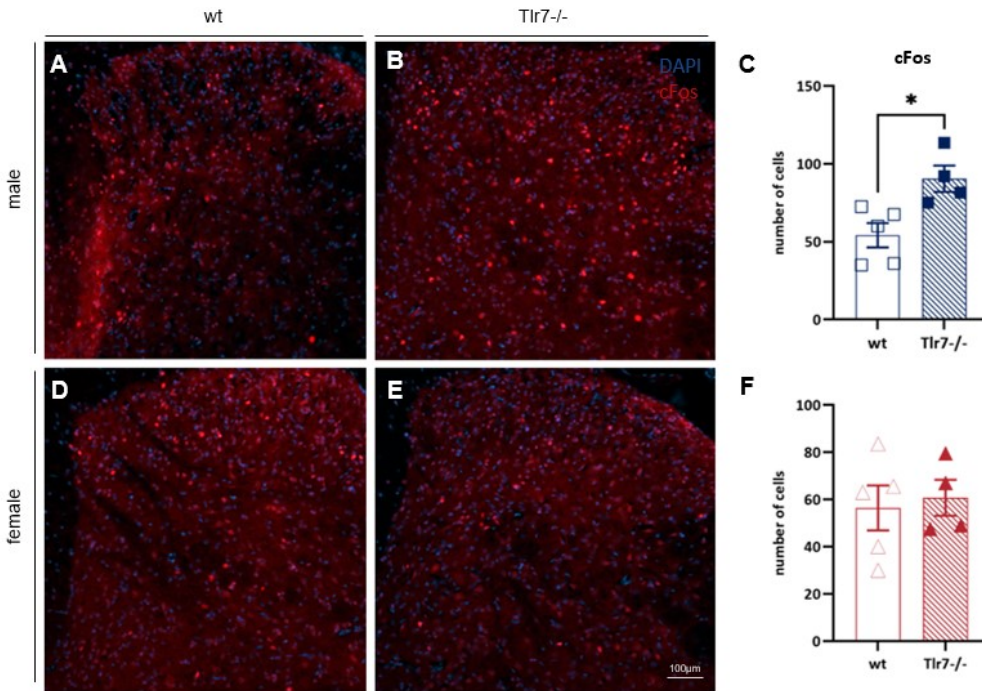
Figure 3-6. *Spinal cord dorsal horn cFos expression is increased in male Tlr7-/- mice 28 days post-injection of CFA*. Representative 20X images of cFos immunostaining in male **(A, B)** and female **(D, E)** ipsilateral dorsal horn of the spinal cord. **(C)** Total number of male cFos+ cells per image (p=0.0167) and **(F)** female cFos+ cells per image (p=0.7431). Data presented as mean $\pm$ SEM.

3.2.3   CD45, Iba1, and CD4 expression in the hindpaw do not differ between Tlr7-/- and wildtype mice at 28 days post-injection of CFA

To assess inflammation in the hind paw resulting from the CFA injection, glabrous hind paw skin was treated to visualize CD45, Iba1, and CD4 markers on the ipsilateral side to the injection. This tissue was obtained at the end of the experiment (28 days post-injection), when the

hind paw thickness had diminished since peak inflammation (3 days post-injection) (**Figure 3-5**) but mechanical hypersensitivity was still present in the Tlr7-/- groups (**Figure 3-4**). In males, we did not calculate any significant differences between wildtype and Tlr7-/- hind paw tissue in terms of CD45 (t-test, t=0.2965, *p=0.7715*; **Figure 3-7 C**), Iba1(t-test, t=0.8803, *p=0.3960*; **Figure 3-7 I**), or CD4 (t-test, t=0.1240, *p=0.9032*; **Figure 3-7 O**). In females, we did not observe significant differences for CD45 (t-test, t=0.6950, *p=0.5003*; **Figure 3-7 F**), Iba1(t-test, t=1.583, *p=0.1445*; **Figure 3-7 L**), or CD4 (t-test, t=1.470, *p=0.1673*; **Figure 3-7 R**).
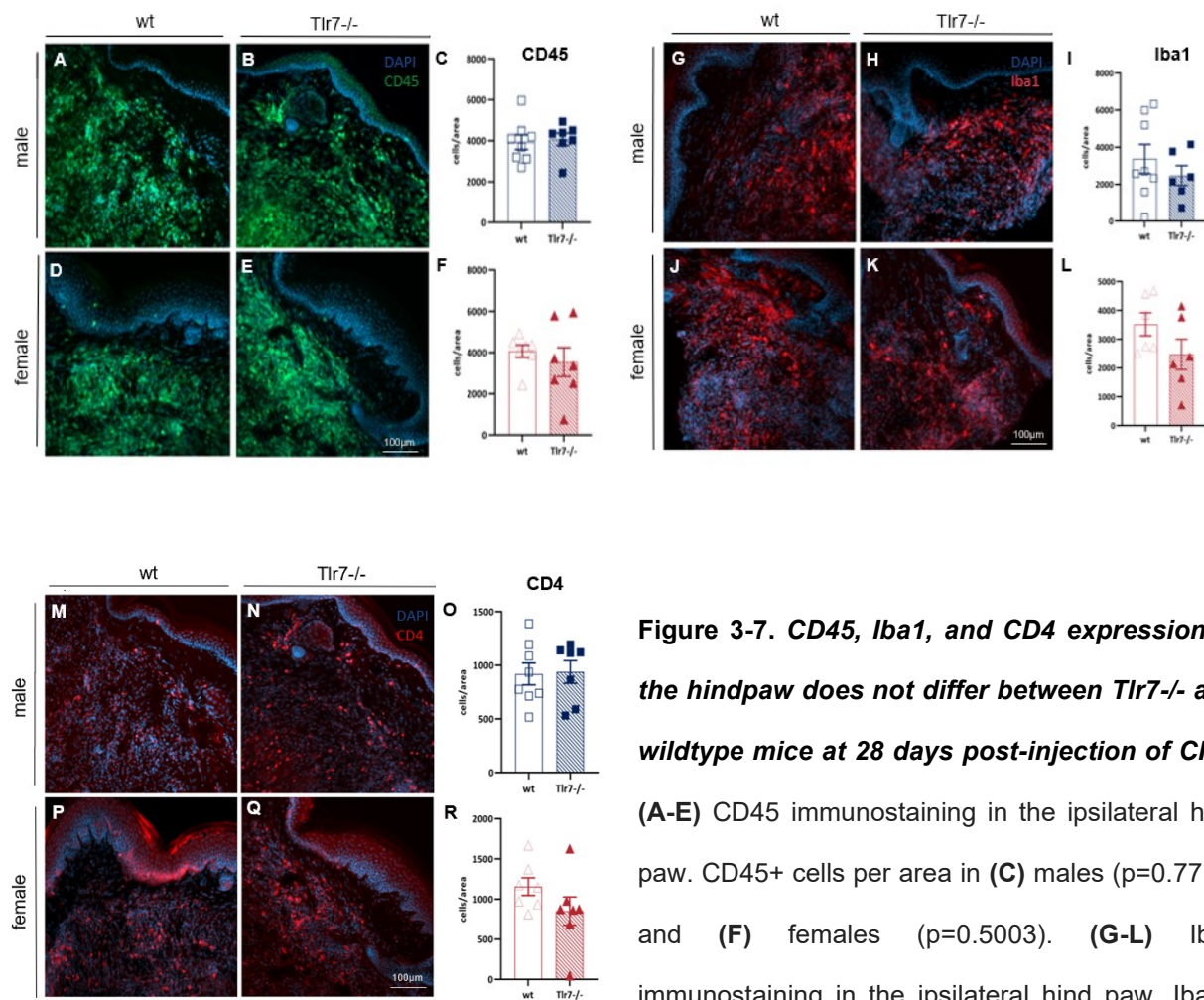
Figure 3-7. *CD45, Iba1, and CD4 expression in the hindpaw does not differ between Tlr7-/- and wildtype mice at 28 days post-injection of CFA.* **(A-E)** CD45 immunostaining in the ipsilateral hind paw. CD45+ cells per area in **(C)** males (p=0.7715) and **(F)** females (p=0.5003). **(G-L)** Iba1 immunostaining in the ipsilateral hind paw. Iba1+ cells per area in **(I)** males (p=0.3960) and **(L)** females (p=0.1445). **(M-R)** CD4 immunostaining in the

ipsilateral hind paw. CD4+ cells per area in **(O)** males (p=0.9032) and **(R)** females (p=0.1673). Representative 20X images. Scale bar = 100 µm, applies to all images.

3.2.4   CGRP expression in the superficial and deep laminae of the spinal cord dorsal horn are reduced in female Tlr7-/- mice, while the intensity and length of IB4 expression and projections are unchanged at 28 days post-injection of CFA

CGRP and IB4 in the spinal cord dorsal horn are indicative of non-peptidergic and peptidergic nociceptive fibers, respectively. In general, CGRP is involved in pain signals from the muscles and skin to the SC, and CGRP interneurons in the dorsal horn are known to contribute to mechanical hypersensitivity (Löken et al., 2021). While CGRP neurons terminate in lamina I and outer lamina II, IB4-positive neurons are mainly characteristic of unmyelinated nociceptive fibers which terminate in inner lamina II (Stucky, 2007). It has been theorized that CGRP and IB4 neurons play different roles in inflammatory nociceptive signalling (Cowie et al., 2018; Breese et al., 2005), so we aimed to investigate whether there were changes in the expression of these markers in response to chronic inflammation from CFA.

To assess CGRP, we measured pixel density in the superficial and deep laminae from images of the SC dorsal horn. In female tissue, we found a significant difference between wildtype and Tlr7-/- groups in superficial (t-test, t=2.892, *p=0.0178*; **Figure 3-8 C**) and deep (t-test, t=4.487, *p=0.0015*; **Figure 3-8 F**) laminae. In contrast, we did not find any significant change in either genotype in the female IB4 measurements of pixel density in lamina II (t-test, t=1.264, *p=0.2286*; **Figure 3-9 C**) or in the length of IB4-positive neurons (t-test, t=0.2107, *p=0.8364*; **Figure 3-9 D**).
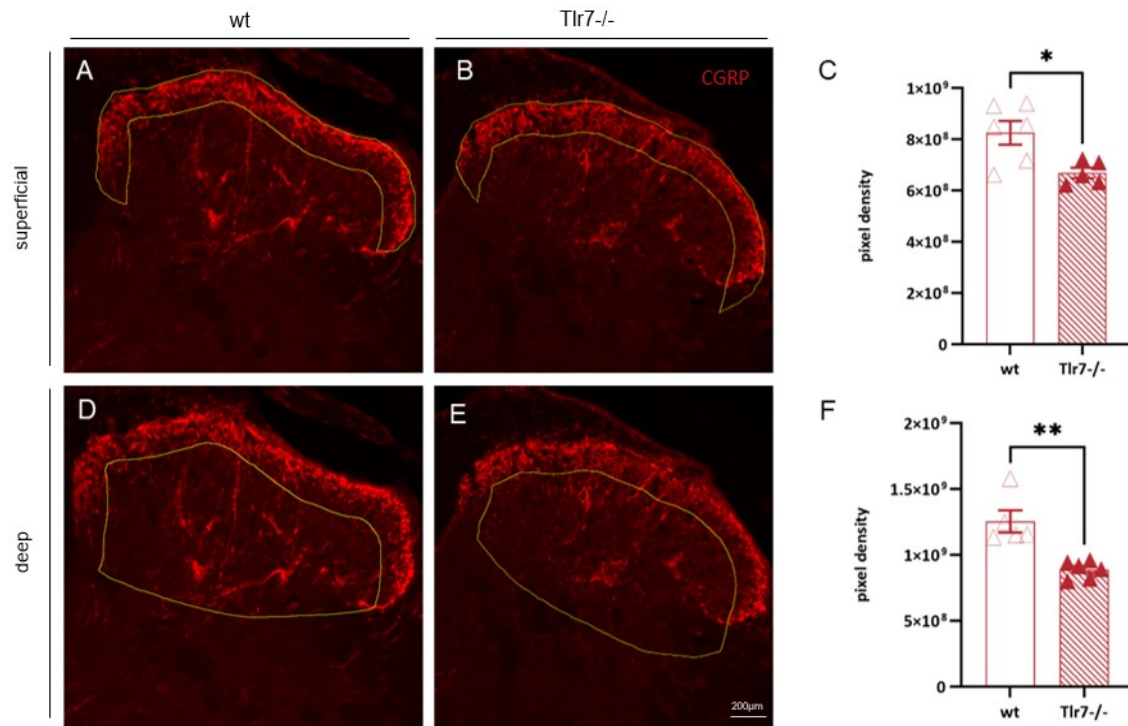
Figure 3-8. *CGRP expression in the superficial and deep laminae of the spinal cord dorsal horn are reduced in female Tlr7-/- mice at 28 days post-injection of CFA.* Representative 10X images of CGRP immunostaining in female superficial **(A, B)** and deep **(D, E)** laminae of the ipsilateral spinal cord dorsal horn. **(C)** CGRP pixel density of CGRP+ staining in the superficial laminae region of interest (p=0.0178) and **(F)** deep laminae region of interest (p=0.0015). Data presented as mean $\pm$ SEM.
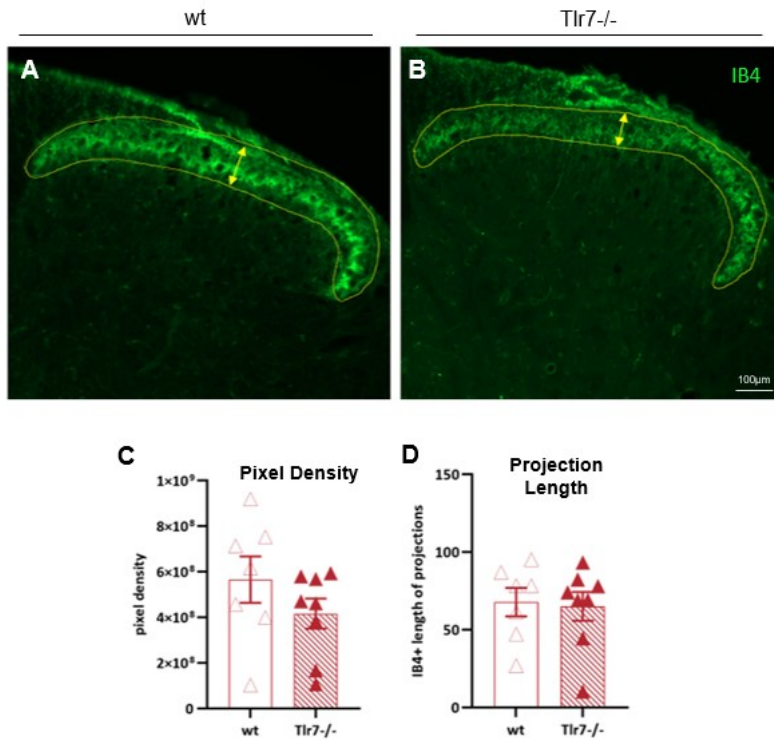
Figure 3-9. *The intensity and length of IB4 expression and projections are unchanged at 28 days post-injection of CFA.* Representative 20X images of IB4+ immunostaining showing projection length in female ipsilateral spinal cord dorsal horn in female **(A)** wildtype **(B)** Tlr-/- groups. **(C)** IB4+ pixel density (p=0.2286) in the region of interest and **(D)** projection length (p=0.8364). Data presented as mean $\pm$ SEM.

3.3 Tlr7-/- EAE Model

3.3.1   EAE disease progression is exacerbated in Tlr7-/- mice

We monitored the progression of EAE by assigning clinical scores to all mice while blinded from wildtype and Tlr7-/- genotypes. We found overall that both sexes showed significant differences in regard to the effect of time and genotype; ultimately, EAE disease progression was exacerbated in Tlr7-/- mice in males and females (3way ANOVA, $F_{genotype(1, 38)}$=9.062, ***p=0.0075***, $F_{time(12, 216)}$=46.63, ***p<0.0001***, $F_{sex(1, 18)}$=0.2041, ***p=0.6568,*** **Figure 3-10 A; Figure 3-10 B**). In the AUC, we observed a significant effect of genotype (2way ANOVA, F(1, 18)=8.191, p=0.0104)

2way ANOVA, $F_{genotype(1, 18)}$=8.191, *p=0.0104*, $F_{time(1, 18)}$=26.34, *p=0.6140*, $F_{interaction(1, 18)}$=0.1231, *p=0.7298*; **Figure 3-10 C**).

3.3.2 Mechanical hypersensitivity occurs earlier in Tlr7-/- mice with EAE

Mechanical hypersensitivity in response to the von Frey test is known to occur in EAE at disease onset (Olechowski et al., 2009). We observed that all EAE mice exhibited mechanical hypersensitivity at 14 days post-induction, but a significant difference was found between wildtype and Tlr7-/- mice within that time period (3way ANOVA, $F_{genotype(1, 16)}$=8.039, *p=0.0119*, $F_{time(2.479, 39.67)}$=28.87, *p<0.0001*, $F_{sex(1, 16)}$=0.0027, *p=0.9593,* **Figure 3-10 D; Figure 3-10 E**). Specifically, 2way ANOVAs for each time course relative to sex showed that the Tlr7-/- mice displayed mechanical hypersensitivity earlier than wildtypes at day 11 post-induction for males (*p=0.0004*; **Figure 3-10 D**) and at day 7 post-induction for females (*p=0.0172;* **Figure 3-10 E**).
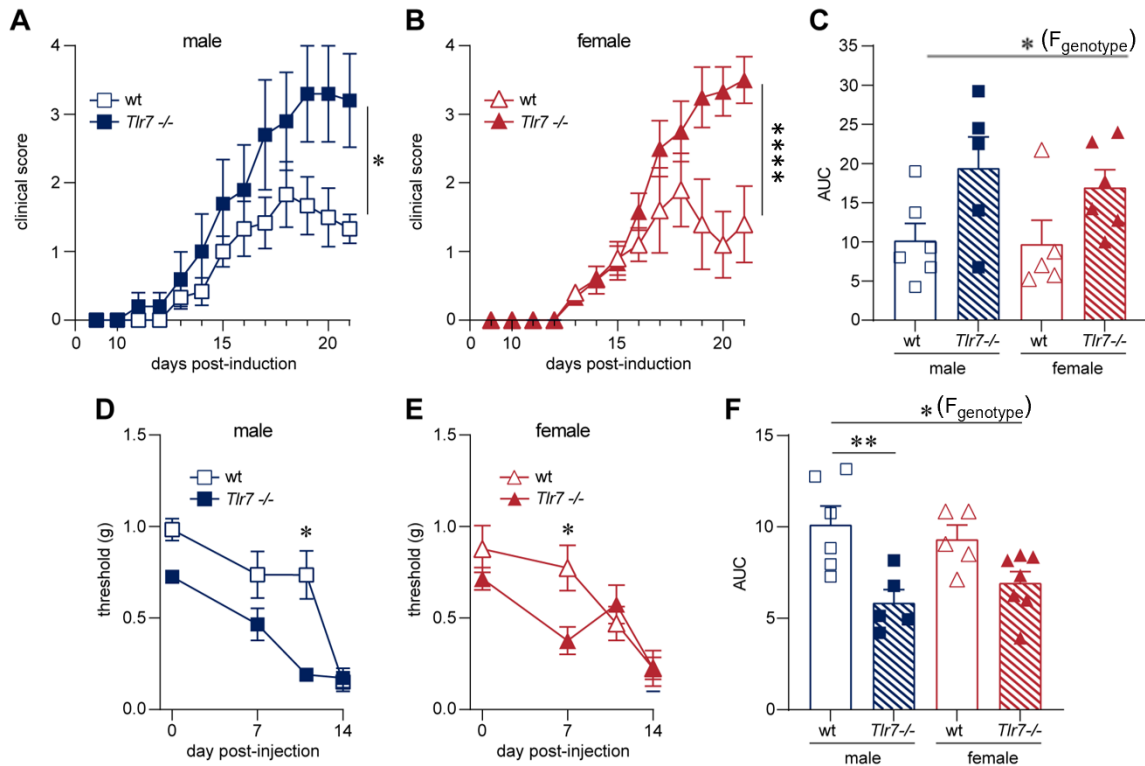
Figure 3-10. *EAE disease progression is exacerbated and mechanical hypersensitivity occurs earlier in Tlr7-/- mice*. Clinical score throughout the disease time course in **(A)** males (p=0.0420) and **(B)** females (p<0.0001). **(C)** Area under the curve of male and female clinical scores (p=0.0104). Mechanical sensitivity as measured by von Frey in **(D)** males (day 11, p=0.0004) and **(E)** females (day 7, p=0.0172). **(F)** Area under the curve of mechanical sensitivity 50% paw withdrawal thresholds (p=0.0006). Post-hoc analyses by Sidak's multiple comparisons test. Data presented as mean $\pm$ SEM.

3.3.3 Spinal cord dorsal horn Iba1 expression does not change between Tlr7-/- and wildtype mice with EAE at 21 days post-induction

SC Iba1 microglia up-regulation has been implicated in neuropathic pain mechanical hypersensitivity (Leinders et al., 2013), so we analyzed images of the SC dorsal horn of EAE wildtype and Tlr7-/- mice. In measurements of average pixel density, neither males (t-test,

t=0.5820, *p=0.5788*; **Figure 3-11 C**) nor females (t-test, t=1.617, *p=0.1444*; **Figure 3-11 F**) showed any significant difference (**Figure 3-11**).
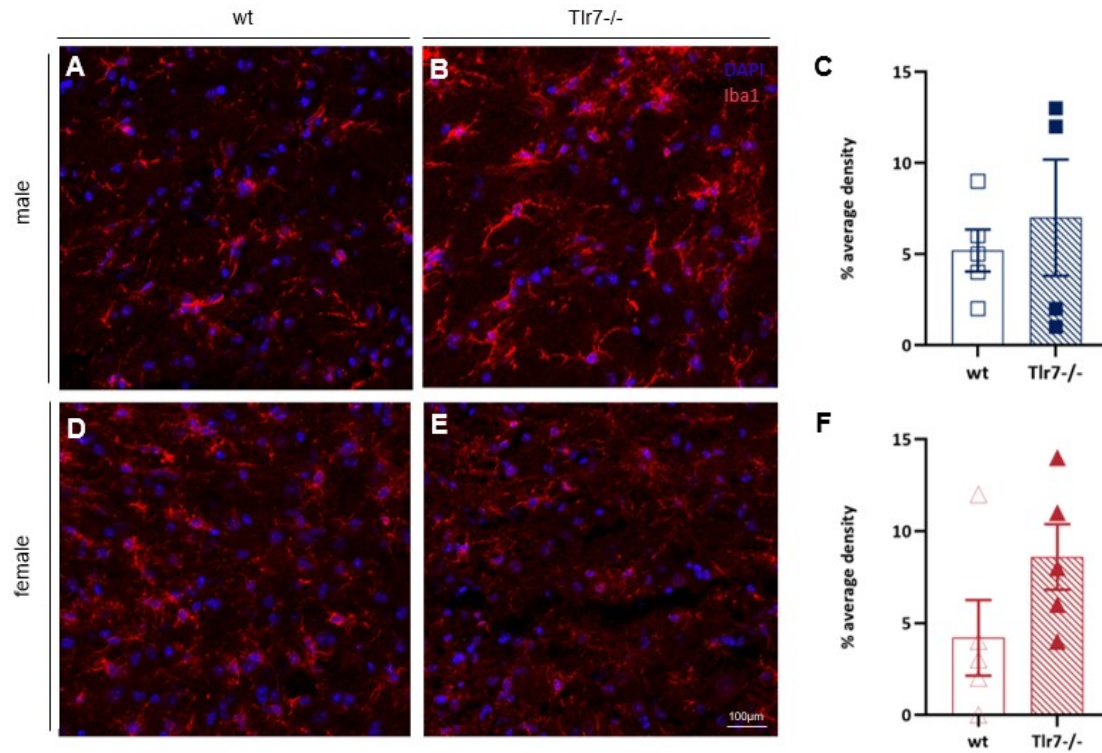
Figure 3-11. *Spinal cord dorsal horn Iba1 expression does not change between Tlr7-/- and wildtype mice with EAE at 21 days post-induction.* Representative images of 20X Iba1+ immunostaining in the spinal cord dorsal horn in **(A, B)** males and **(D, E)** females. Average pixel density between left and right dorsal horns in **(C)** male (p=0.5788) and **(F)** female (p=0.1444). Data presented as mean $\pm$ SEM.

3.3.4   The proportion of Tlr7+ NeuN+ cells to NeuN+ cells in the DRG does not differ between EAE and non-diseased wildtype mice at 21 days post-induction as measured by Tlr7 mRNA and NeuN expression

Once we verified that Tlr7 was present in the DRG using RNAScope, we aimed to test whether levels of Tlr7+ neurons in the DRG changed in the autoimmune context of EAE. Using

RNAScope to detect Tlr7 and IHC to detect NeuN for neurons, we measured the ratio of Tlr7+ neurons to total neurons. We found that this ratio did not significantly differ between EAE and non-diseased mice (t-test, t=0.3894, *p=0.7011*; **Figure 3-12 C**). While it seems that all Tlr7 observed here was present in neurons, we did not stain markers of other cell types to validate this. Additionally, the majority of the Tlr7 staining appears in large-diameter neurons.
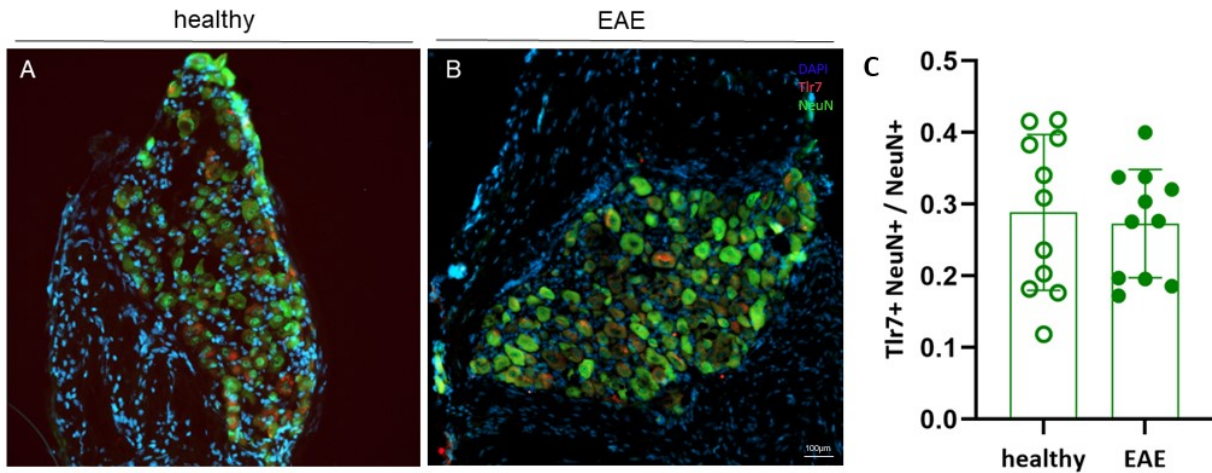
Figure 3-12. *The proportion of Tlr7+ NeuN+ cells to NeuN+ cells in the DRG does not differ between Tlr7-/- and wildtype mice at 21 days post-induction of EAE.* Representative images of 20X Tlr7+ RNAScope and NeuN+ immunostaining of the dorsal root ganglion in **(A)** healthy control and **(B)** EAE wildtype mice. **(C)** The ratio of Tlr7+ NeuN+ cells to total NeuN+ cells does not differ between groups (p=0.7011). Data presented as mean ± SEM.

3.4 CFA Recovery after TLR7 Agonism via Imiquimod Treatment

3.4.1   Following mechanical hypersensitivity induced by CFA injection into the hindpaw, imiquimod-treated wildtype mice recovered to 50% of baseline threshold before vehicle-treated wildtype mice

We repeated the CFA experiment with the goal of assessing mechanical sensitivity throughout the time course of the known hypersensitivity effect. Here, we used wildtype mice and treated them with either VEH or imiquimod via injection into the hind paw following the initial CFA injection into the hind paw (**Figure 3-13 A)**. By day 54 post-injection of CFA, it was observed that both groups displayed mechanical sensitivity close to their baseline levels, demonstrating recovery. However, the von Frey measurements throughout the entire time course of imiquimod-treated mice significantly differed from VEH-treated mice (2way ANOVA, $F_{treatment(1, 100)}$=4.741, **$p=0.0318$**, $F_{time(9, 100)}$=31.05, **$p<0.0001$**, $F_{interaction(9, 100)}$=1.726, **$p=0.0028$**; **Figure 3-13 B**).

Importantly, imiquimod-treated mice displayed a much greater 50%PWT at 47 (**$p=0.0247$**) and 49 days post-injection (**$p=0.365$**) (**Figure 3-13**) than VEH-treated mice which remained hypersensitive, indicating that imiquimod treatment quickened the time to recovery after the CFA inflammatory insult. In addition, the AUC of VEH and imiquimod treatment groups significantly differed (t-test, t=2.289, **$p=0.0451$**; **Figure 3-13 C**), with the imiquimod-treated group displaying overall less mechanical hypersensitivity.
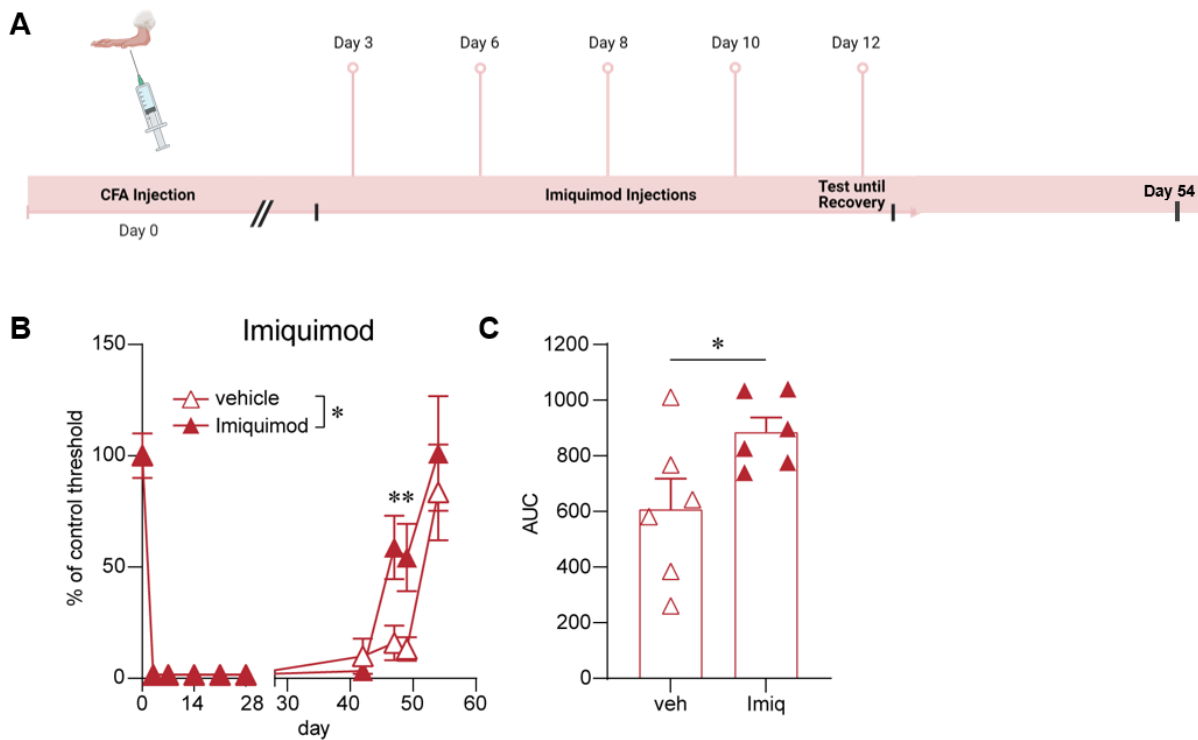
Figure 3-13. *Following mechanical hypersensitivity induced by CFA injection into the hindpaw, imiquimod-treated wildtype mice recovered to 50% of baseline threshold before vehicle-treated wildtype mice.* **(A)** Schematic of injection schedule. **(B)** Mechanical sensitivity in females as measured by von Frey throughout the time course of the experiment (p=0.0168). **(C)** Area under the curve of mechanical sensitivity for both groups (p=0.0451) with significant differences at day 47 (p=0.0247) and day 49 (p=0.0365). Post-hoc analyses by Sidak's multiple comparisons test. Data presented as mean ± SEM.

CHAPTER 4: Discussion

4.1 Summary of Results

This study was conducted in response to the work currently being performed on the relationship between the immune system, nervous system, and pain development, resolution, and management. TLR7 is an immune system receptor that has been implicated in inflammation and nociception in mice; therefore, we aimed to analyze its role in various models of inflammatory pain in order to inform our understanding of TLR7 function. This project takes into account murine nociception in an acute cutaneous inflammatory, chronic cutaneous inflammatory, and neuropathic autoimmune model and presents strong behavioural evidence which shows that TLR7 is a critical regulator of inflammatory pain resolution.

Our behavioural data shows supports the hypothesis that TLR7 plays an important role in the resolution of inflammatory pain. To summarize, the formalin assay resulted in an increase in the overall duration of pain behaviour in Tlr7-/- mice compared to wildtypes, with a significant difference in the female group. In the CFA experiment, wildtype mice of both sexes recovered from mechanical hypersensitivity induced by CFA while Tlr7-/- mice remained hypersensitive. In EAE, the Tlr7-/- group demonstrated consistently higher clinical scores over the course of the experiment and von Frey testing showed that mechanical hypersensitivity was accelerated in Tlr7-/- mice. These behavioural experiments involving Tlr7-/- mice highlighted the necessity of TLR7 to achieve inflammatory pain resolution. When we injected imiquimod into the mouse hind paw following a CFA injection, the imiquimod treatment group demonstrated a quicker recovery to baseline levels. This suggests that TLR7 agonism facilitates pain resolution.

In terms of the cellular correlates to this behavioural data, effects in the SC dorsal horn were largely inconclusive. The main significant findings were in the formalin assay with increased cFos levels in Tlr7-/- males, and in the CFA assay with decreased CGRP levels in females.

Furthermore, the hind paw glabrous skin revealed interesting data; we observed decreased levels of CD45 and Iba1 in Tlr7-/- males and decreased levels of CD4 in Tlr7-/- females in the formalin assay, but saw no changes in inflammatory markers due to genotype or sex in the CFA assay along with no change in hind paw thickness. It should be noted that no tissue was dissected out of mice in the CFA-imiquimod experiment.

The behavioural data combined with our tissue analysis provides grounds for further research in this field, specifically with regard to markers of inflammation and changes in nociceptor outgrowth and signalling, in addition to assessing a wider scope of behavioural tests to expand this story beyond mechanical sensitivity. In terms of localizing Tlr7 with RNAScope, we found that it is colocalized with neurons in the DRG, and thus far we are not able to detect significant changes in expression levels of Tlr7 due to EAE. This does not mean, however, that Tlr7 is inconsequential in EAE; it simply means that there may be measurements other than RNA expression which are more significant and yet to be studied, such as miRNA messengers.

4.2 TLR7 and miRNAs in pain signalling

Studies have shown that micro RNAs (miRNAs), short, non-coding RNAs, not only regulate gene expression after transcription, but can also move between cells and act as ligands of TLRs (Chen et al., 2013). The miRNAs let7b and miR21 are endogenous ligands of TLR7 (Winkler et al., 2014; Liu et al., 2015). Let7b is found in the CNS and targets TLR7 with varying functional outcomes that are dependent on the location of the interaction (Winkler et al., 2014). In the brain, let7b induced TLR7-mediated apoptosis of healthy neurons, resulting in neurodegeneration; notably, mice with genetic deletion of Tlr7 were protected against this effect (Lehmann et al., 2012). In the DRG, however, let7b interaction with TLR7 on nociceptors resulted

in depolarization via TRPA1, suggesting that TLR7 and TRPA1 interact to mediate pain signalling via let7b (Park et al., 2014).

It has been proposed that let7b binds TLR7 at the extracellular surface rather than the endosome, a theory based on data from the inside-out patch clamp technique and an analysis of cell-surface localization on human embryonic kidney 293 cells (Park et al., 2014). This signalling pathway is likely independent of MyD88, since MyD88 is associated with endosomes. The question of how TLR7 becomes available for ligand binding at the cell surface remains, but in the case of nociceptive signalling in the DRG, TRPA1 may play a role in trafficking TLR7 from the endosome to the cell surface (Winkler et al., 2014).

In terms of neuropathic pain, TRPA1 in the SC is hypothesized to be involved in the development of the hypersensitivity behaviours that are measurable in EAE (Dalenogare et al., 2020; Ritter et al., 2020). TRPA1 is also activated by another known TLR7 ligand, miR21, to mediate neuronal excitation in the DRG (Park et al., 2014). At EAE onset, miR21 was found to be upregulated in the DRG (**Figure 4-1**), and a preliminary study from our lab shows evidence of miR21 upregulation at the trigeminal root entry zone (TREZ) at EAE onset as well (**Figure 4-2**). MiR21 is released from DRG neurons in exosomes following TRPV1 activation and peripheral nerve injury, which may lead to neuronal hypersensitivity (Simeoli et al., 2017). Exosomes allow cell-cell communication, so this research supports the idea that crosstalk between neurons and support cells in the PNS and CNS is an essential research avenue in the study of TLR7 function in pain.
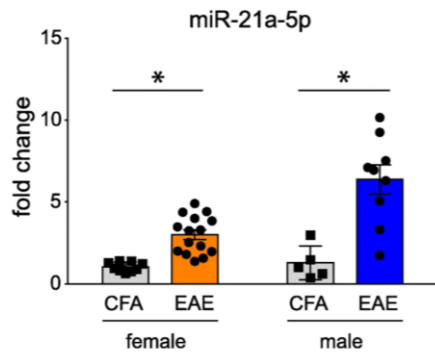
Figure 4-1. *miR21 is upregulated in the female DRG in EAE.* qPCR validation of miR-21a-5p in EAE DRG tissue. Data presented as mean $\pm$ SEM. (Friedman et al., 2019).
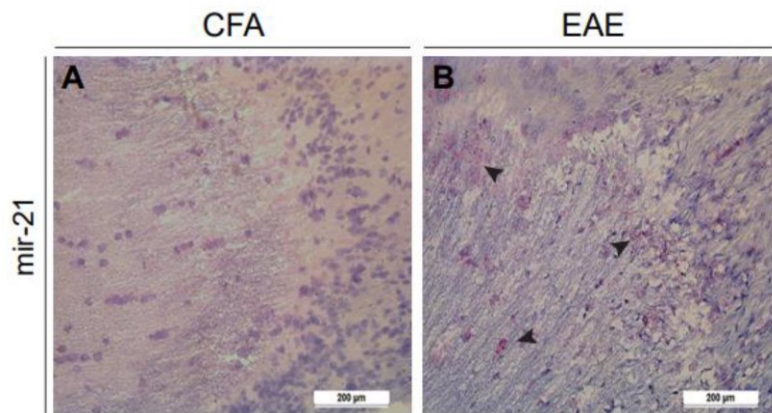
Figure 4-2. *RNAScope for miR21 in the female TREZ at EAE onset.* **(A)** EAE control tissue compared with **(B)** EAE tissue at disease onset in the trigeminal root entry zone (TREZ) for the presence and level of miR21. The higher level of staining at EAE onset, shown by the arrowheads in B, can be appreciated in this preliminary study.

It has been shown that TLR7 signalling can affect nervous system processes (Acioglu et al., 2022; Dieu et al., 2021; Hoshikawa et al., 2020), but still, the literature regarding TLR7 in neurons points to its role in the development of pain rather than the resolution. Since our recent data postulates that TLR7 is an important mediator of pain resolution, perhaps we must look outside of neurons in order to understand this phenomenon. TLR7 may function depending on its location; for example, researchers found that stimulation of brain TLR7 led to an influx of

inflammatory molecules in cerebrospinal fluid (CSF), but in microglia, TLR7 activation suppressed the inflammatory response that was initiated by agonizing TLR2, 4, and 9 (Rosenberger et al., 2014).

In the DRG, we observed that Tlr7 appeared mainly in large-diameter neurons (**Figure 3-12**), which are considered to be nonnociceptive while small-diameter neurons are considered to be nociceptive. The Gate Control Theory originally published by Melzack and Wall (1965) frames nociception as the culmination of inputs from large and small-diameter neurons from the DRG to spinal cord dorsal horn interneurons to spinal cord projection neurons, although the specificity of the signalling pathways and various neuronal subtypes involved remains to be studied in detail (Basbaum et al., 2014). The activation of large-diameter neurons is theorized to stimulate inhibitory interneurons which inhibit projection neuron firing, and our finding that Tlr7 is expressed in large-diameter neurons may contribute to the mechanism underlying this inhibition in inflammatory pain states.

The lack of change in Tlr7 RNA expression we discovered in EAE DRGs could mean that neuronal TLR7 is not the main source of the behavioural changes we observed in EAE. There is a strong possibility that non-neuronal TLR7 plays a role as well, and neuron-macrophage communication has already been proposed as a method of nociceptor sensitization (Simeoli et al., 2017). Furthermore, it was recently reported that TLR7 is not expressed by sensory neurons, but rather by support cells such as satellite glia and macrophages (Proskocil et al., 2021). Currently unpublished research by Friedman et al. (2023) posits that macrophage TLR7 regulates cytokine release which leads to the resolution of inflammatory nociception in a sex-dependent manner.

4.3   The case for non-neuronal TLR7 in pain signalling

Bone marrow-derived macrophages (BMDMs) are innate immune cells involved in acute inflammatory processes which aid in tissue recovery from exogenous insults (Kratochvill et al., 2015). Stimulation of BMDMs *in vitro* by TNFα can influence cultured BMDMs toward this phenotype (Parameswaran & Patial, 2010) and lead to the production of cytokines in media. Friedman et al. (2023) compared the behavioural effects of TNFα-stimulated conditioned media (T-MCM), with control macrophage condironed media (MCM).

Both female T-MCM and MCM injected into the female mouse hind paw resulted in mechanical hypersensitivity in wildtype mice immediately following the injection**.** At 48 hours post-injection, however, mice that received the T-MCM treatment had recovered to their baseline levels while mice that received the MCM treatment remained hypersensitive **(Figure 4-3 B)**. It follows that upon analysis of the hind paw tissue, the MCM group showed elevated levels of CD45+ cells **(Figure 4-4 E)** while the T-MCM group did not (**Figure 4-4 F**). This demonstrates that gross inflammation evidenced by increased leukocyte counts in the hind paw is correlated with mechanical hypersensitivity following this MCM-induced pain phenotype. Leukocytes are stimulated by cytokines to promote bidirectional communication with vascular cells (Mantovani & Dejana, 1989). Cytokines also play a role in the persistence of pathological pain through diverse effects on the immune and nervous systems, perhaps even by directly activating nociceptor sensory neurons (J.-M. Zhang & An, 2007).

Among male and female CM, Friedman et al. (2023) found that cytokine profiles did not differ greatly overall, meaning there may be more to our understanding of inflammatory pain than cytokine levels alone. The prolonged nociception and corresponding increased CD45+ leukocyte counts that were measured in the MCM-injected females may be indicative of generalized

increased cytokine receptor sensitivity in females rather than the differential production of cytokine levels in CM compared with males. However, Friedman et al. (2023) did find one major sex-specific factor in female T-MCM: a significant reduction in IL-16 levels.
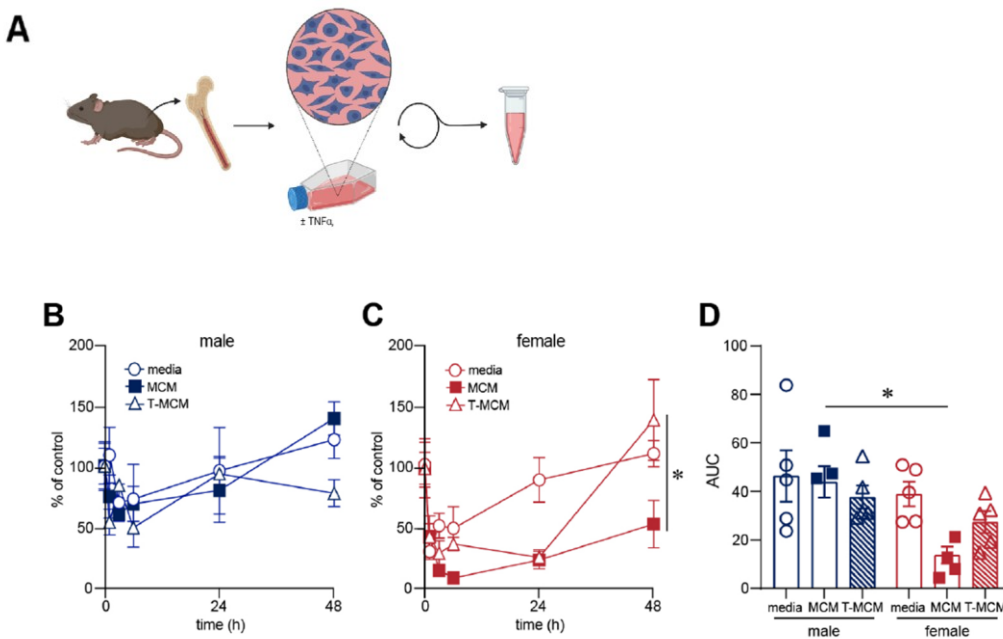
Figure 4-3. *T-MCM injected into the hind paw induces a recovery phenotype in female mice.* **(A)** Schematic of the development of CM. BMDMs were cultured with either TNFα or control solution. **(B)** Male and **(C)** female von Frey measurements throughout the time course of the experiment, from baseline to 48 hours post-injection of media, MCM, or T-MCM. Male mice did not demonstrate prolonged hypersensitivity, but females in the treatment conditions demonstrated hypersensitivity that did not recover in the MCM condition and that recovered by 48 hours post-injection in the T-MCM condition. **(D)** AUC of von Frey time courses showing a sex difference. (Friedman et al., 2023).
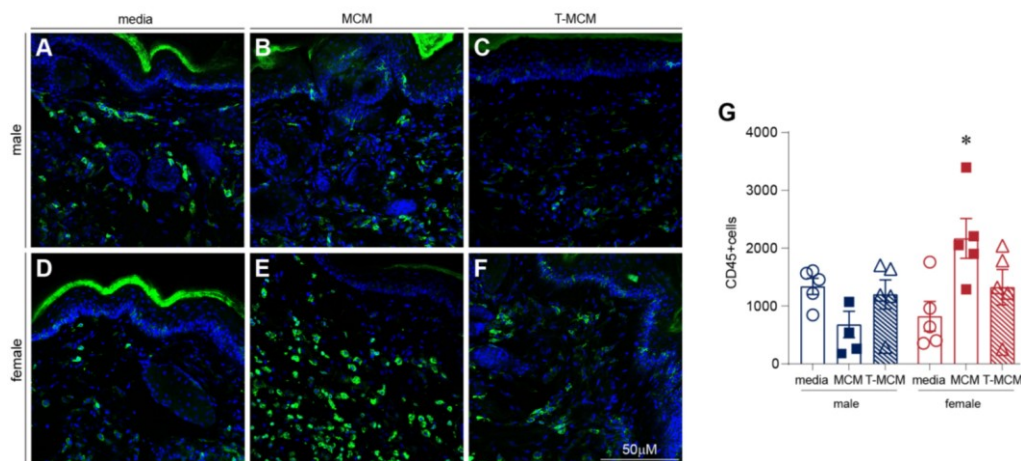
Figure 4-4. *MCM induces greater inflammation by CD45+ cells in the hind paw.* **(A-C)** Male and **(D-F)** female CD45+ hind paw sections in each injection condition. Female hind paw skin in the MCM condition shows significantly greater CD45 levels than comparative tissue in the alternate conditions **(G)**, correlating with the prolonged hypersensitivity measured in female MCM-treated mice. (Friedman et al., 2023).

Based on the literature reporting that TLR7 can act as negative regulator of cytokines like IL-16, Friedman et al. (2023) assessed the role of TLR7 in the TNFα-driven BMDM response. Applying the TLR7 agonist, imiquimod, to female BMDMs in vitro decreased their IL-16 output in CM samples, supporting the theory that TLR7 is a negative cytokine regulator. Applying both TNFα and imiquimod to female wildtype macrophages decreased IL-16 to almost negligible levels, demonstrating that TLR7 activation may support the effects of TNFα. Strikingly, IL-16 levels are higher in all Tlr7-/- conditions compared with wildtypes, suggesting that TLR7 is necessary for proper cytokine regulation in this case (**Figure 4-5**).

When we injected imiquimod into the hind paws of female mice, they showed a speedier recovery from CFA-induced inflammatory pain than vehicle-treated female mice (**Figure 3-13**). Similarly, in our experiments that measured nociceptive behaviour in various inflammatory in vivo conditions including EAE, we observed exacerbated pain responses in Tlr7-/- mice, providing

further evidence for the role of TLR7 in pain resolution. The phenotype seen in the female T-MCM group (**Figure 4-3 C**) illustrates that early inflammatory processes, here induced by TNFα-stimulated macrophage media, may lay the foundation for subsequent pain resolution in females (Parisien et al., 2022) and reveals a potential function of TLR7 via cytokine signalling. The mechanism by which this process occurs is still unclear, but another class of cytokines, IFNs, may be a critical regulator.
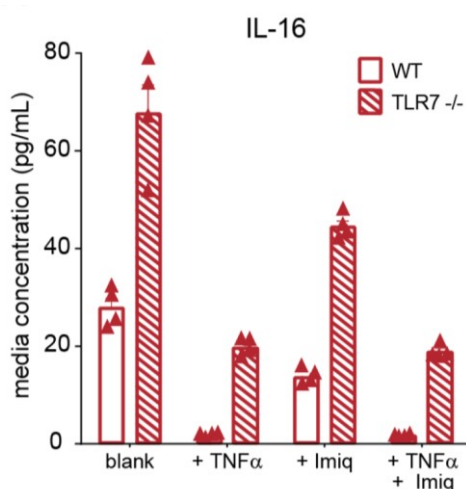
Figure 4-5. *IL-16 levels in female macrophage conditioned media are affected by TNFα and imiquimod.* Levels of IL-16 in media samples from wildtype and Tlr7-/- macrophages stimulated by TNFα, imiquimod, and costimulation with both TNFα and imiquimod. (Friedman et al., 2023).

TLR7 signalling through MyD88 can lead to the production of type 1 interferon (IFN-I) (Hemmi et al., 2003), which is considered one of the most important antiviral agents. IFN-I can be produced by almost any cell type (Trinchieri, 2010), but plasmacytoid dendritic cells specifically have been identified as a main bulk producer of IFN-I to support natural killer cell-mediated control of viruses via endosomal TLR7 signalling (Colonna et al., 2004). Just as widespread

neuropathic pain is a hallmark of severe viral infection, so too is it for autoimmune diseases like MS. One link between these two disease contexts is inflammation involving the overproduction or dysregulation of IFN-I, and it has been shown that these molecules stimulate receptors expressed in the DRG which leads to nociceptor hypersensitivity (Barragán-Iglesias et al., 2020). Importantly, this mechanism may change depending on the environment of a viral infection or an autoimmune response like EAE. In EAE, increasing IFN-I signalling alleviated symptoms of brain damage and reduced clinical disease scores by negatively regulating the activity of T helper cells (Kalinke & Prinz, 2012), but this does not directly address the mechanism of documented hypersensitivity in this context.

Considering the behavioural data from our EAE model (**Figure 3-10**), the heightened clinical scores and accelerated time course to allodynia in Tlr7-/- mice may be due to an immune system inability to regulate IFN-I. Furthermore, another innate immune regulator, STING, is known to modulate the production of IFN-I and produce antinociception via peripheral nociceptors, with intrathecal activation of STING resulting in antinociception in mice and non-human primates (Donnelly et al., 2021). A recent study in humans demonstrated that monocytes are the main source of STING-mediated IFNα production as induced by whole-blood stimulation by relevant PRR ligands to simulate the conditions of viral infection (Congy-Jolivet et al., 2022). In response to TLR7 stimulation, the female IFNα response was amplified across all age groups. To summarize, researchers posit that IFN-I suppresses nociceptor hyperexcitability and that monocytes are a key origin of IFN-I production through TLR7. Taken with our research, perhaps TLR7 also drives IFN-I production in inflammatory and autoimmune responses in order to dampen neurological injury and promote pain resolution.

While we did attempt to visualize IFN1β in the SC and DRG using RNAScope, we were unable to optimize the protocol under the current logistical constraints on this project; additional experiments are recommended for future research in order to identify IFN-I correlates with chronic inflammatory pain. An additional avenue is to analyze IFNγ, part of the type 2 interferon (IFN-II) class, which has been associated with pain as well. Despite the fact that the relationship between TLRs and IFN-I is more well-characterized than with IFN-II, IFNγ does play a role in TLR signal integration in macrophages by priming their response to TLR agonists, regulating TLR signalling components, and coordinating TLR transcription factors (Schroder et al., 2006).

IFNγ is also involved in the endocannabinoid system to mitigate manifestations of neuropathic pain after injury, but amplified levels increased iNOS measurements (Racz et al., 2008), potentially stimulating a proinflammatory environment via macrophages and microglia. Correspondingly, IFNγ-stimulated microglia in the SC produced robust hypersensitivity (Tsuda et al., 2009). These studies identifying markers of inflammation are valuable to our understanding of the immune mechanisms at work across different models of pain. We looked for cellular correlates of pain as well in our work, and the results that we identified in our experiments are interesting because they propose so many research possibilities.

4.4   Research limitations and future directions

In the formalin model, we observed that female Tlr7-/- mice exhibited a prolonged pain response, but upon analysis of the SC dorsal horn, we did not find increased counts of cFos (**Figure 3-2**). Cfos is an accepted marker of neuronal activation following noxious stimulation (Bullitt, 1990). It is also generally used as evidence of central sensitization in the formalin model and its magnitude is attenuated with analgesic drugs (Abbadie et al., 1997; Gogas et al., 1991), so we expected that this pain phenotype in Tlr7-/- female mice would be accompanied by significantly increased cFos. We may not have used the ideal dose or had adequate numbers of subjects in our experimental groups to measure the anticipated cellular effect. Additionally, although we examined a basic neural activation marker, it is possible that we did not measure the most meaningful marker in this context.

There are many studies demonstrating that nociceptive behaviour does not always correlate with changes in SC cFos levels, and that not all neurons express cFos when activated, especially in areas known to play a role in pain processing such as the DRG and the thalamus (Harris, 1998). Due to these discrepancies, our statistically insignificant results here do not necessarily equate to a lack of difference in neuronal activity. Taking electrophysiological recordings from DRG or SC dorsal horn neurons and analyzing later time points are avenues for experimentation should this study be repeated. Furthermore, we were unable to compare cFos levels from the end of the experiment, at 60 minutes post-injection, to baseline levels or at other points throughout the experiment since we only extracted tissue samples from one set of animals: the same animals which were tested in the full formalin time course. Our results in hind paw tissue for CD45, Iba1, and CD4 were also drawn from this set of animals, meaning that they were taken at the end of the experiment.

Our results for hind paw tissue inflammatory markers demonstrated a sex-specific effect (**Figure 3-3**). Formalin induces cell apoptosis, leading to tissue damage, vascular leak, and inflammation (Winn & Harlan, 2005). Therefore, we predicted that both Tlr7-/- males and females would show either no change or an increase in all inflammatory markers compared to wildtypes, but we did not expect that male Tlr7-/- tissue would show a significantly decreased amount of CD45 and Iba1 accompanied by no change in CD4. In this case, we suspect that the change in overall CD45+ lymphocytes and specific Iba+ macrophages is related to a TLR7 regulatory mechanism of action.

Females had an opposite response, with no change in CD45 and Iba1, but a significant decrease in CD4. Relating this back to the increased nocifensive behaviour time in Tlr7-/- females, perhaps we can interpret a protective effect by CD4+ T cells that is absent in these mice. CD4 binds IL-16 in order to generate T cell activation and migration at sites of inflammation (Lynch et al., 2003); interestingly and somewhat conversely, IL-16 is the same cytokine identified by Friedman et al. (2023) which was decreased in the pain-resolving female T-MCM group (**Figure 4-3 C**). There, TNFα-stimulated macrophages produced media containing decreased IL-16 which was injected into the hind paw, resulting in a pain resolving phenotype. In our formalin experiment, female Tlr7-/- mice did not recover from the initial formalin insult, which was accompanied by decreased CD4 and unchanged Iba1 in the hind paw tissue. Macrophages in females might not be influencing the pain phenotype in the same way here, conceivably since formalin is not a strict inflammatory model per se. In the future, it would make sense to examine macrophage activity in this model.

In addition, we did not have a vehicle injection as a control, which means we cannot be sure about the effect of the physical hind paw injection itself on nocifensive behaviours and local

inflammation. This limitation extends into our other models involving hind paw injections, namely our CFA experiments. CFA is a robust inflammatory stimulant, so it makes sense that there was no change in hind paw inflammatory markers between wildtype and Tlr7-/- mice (**Figure 3-7**), accompanied by no change in paw swelling (**Figure 3-5**) even though wildtype mice demonstrated pain recovery. In the CFA recovery experiment, we only saw an increase in cFos in the Tlr7-/- male SC despite females demonstrating prolonged pain as well. One factor here that plays an important role in measurable biological markers is time. We only analyzed tissue at the endpoint of the experiment at 28 days post-injection, but there could be changes occurring in various tissues throughout the entire time course. This is also true for the CGRP and IB4 that was measured in the SC at 28 days post-injection to assess the influence of peptidergic and non-peptidergic C fibers in the pain response, respectively.

CGRP is a neuropeptide involved in pain transmission and is found in the superficial laminae of the SC dorsal horn and is known to be found in deeper laminae as well following spinal cord injury (Christensen & Hulsebosch, 1997). Laminae II of the SC, the substantia gelatinosa, is a main hub of nociceptive nerve fibers expressing CGRP which signal to the brain, and stimulation of this region by CGRP incites nociceptive behaviour (Sun et al., 2003). It was also found that CGRP in the SC facilities neuronal excitability and consequent nociception (Bird et al., 2006), so it was initially surprising that we measured a significant decrease in the CGRP marker in female Tlr7-/- tissue (**Figure 3-8**), one of the groups which demonstrated prolonged pain. However, a recent review proposed that CGRP may actually act as a context-dependent neuromodulator rather than a reliable marker of pain signal transmission (Schou, 2017). In the context of cutaneous inflammatory pain mechanisms, one study also found a loss of CGRP, although in the skin, following an acute model of nociception by capsaicin injection (Simone et al., 1998).

There are few studies concerning the effect of CGRP on the CFA model of pain, however, it was demonstrated that antagonizing the CGRP receptor peripherally but not centrally resulted in decreased neuronal activity in deep SC laminae along with reversal of the pain phenotype during an acute time frame (Hirsch et al., 2013). It is important to note that CGRP levels in the SC dorsal horn likely fluctuate over time, as they were shown to decrease immediately after CFA injection only to greatly increase in the days following at 8 days post-injection (Seybold et al., 1995). Notably, there has not been much research on the pattern of CGRP expression after CFA hind paw injection at chronic time points.

Our results suggest a few opposing points: CGRP may be more influential during the development of chronic pain rather than the resolution, since our non-resolving Tlr7-/- group showed decreased CGRP compared with wildtypes, or perhaps decreased CGRP may actually be indicative of chronic pain in the CFA model without the potential immunoregulatory function of TLR7. Again, we cannot compare to earlier time points to confidently draw these conclusions. Furthermore, pixel density may be a sub-optimal measurement in this case, and a more in-depth study could involve testing refined methods of quantifying neuronal CGRP such as using confocal image stacks. In addition, while CGRP is a peptidergic neurotransmitter, IB4 is a marker of non-peptidergic DRG neurons with terminals primarily in lamina II of the SC dorsal horn and is also implicated in inflammatory pain (Gerke & Plenderleith, 2004).

Two days after a CFA hind paw injection, researchers found that IB4+ neurons in the SC were more responsive to TRPV1 stimulation (Breese et al., 2005). A nerve injury model revealed mechanical allodynia and a decrease in IB4+ terminal in superficial laminae ipsilateral to the injury (Bailey & Ribeiro-da-Silva, 2006). In terms of pain, mice recovering from a SC contusion injury began an exercise program which ameliorated mechanical and thermal hypersensitivity, but was

not accompanied by a considerable change in IB4 labelling in superficial and deep laminae (Sliwinski et al., 2018).

Moreover, there is a relationship between pain and central plasticity, meaning that IB4+ afferent terminals in the SC change in their outgrowth and density in response to inflammatory stimuli (Casals-Díaz et al., 2009). We assessed IB4 pixel density and length of projection from the substantia gelatinosa into the deeper laminae and did not find any changes between wildtype and Tlr7-/- groups within the female mice (**Figure 3-9**). It may simply be the case that non-peptidergic neurons are not influential in the maintenance of inflammatory pain at this chronic time point. Similar to CGRP, data from earlier time points would be necessary in order to compare changes in IB4+ neurons. It is recommended to assess this data in the future using neuron tracing for a more accurate understanding of outgrowth.

Although we obtained various tissue samples from our CFA experiment, we did not extract any tissues from animals in our CFA-imiquimod experiment, where we observed an increased time to recovery from CFA-induced mechanical hypersensitivity by imiquimod treatment (**Figure 3-13**). This is a limitation in our research because it prevents us from consolidating our previous findings about hind paw skin inflammatory profiles and neuronal changes with this therapeutic data. Another limitation here is that we only tested the effects of one dose of imiquimod which was based on significant findings from Friedman et al. (2023), so we cannot be sure if there is a dose-dependent effect on recovery time.

Next, our EAE model demonstrated that Tlr7-/- mice developed mechanical hypersensitivity more quickly than their wildtype counterparts, but they also experienced a worsened disease course as measured by motor symptoms (**Figure 3-10**). While we analyzed inflammation in the SC dorsal horn by Iba1 staining and did not measure significant changes, we

did observe increased Iba1 in the male Tlr7-/- ventral horn. Unfortunately, we did not have the resources at that stage to delve further into the possible underlying mechanisms of these motor symptoms. There is evidence in the literature of a relationship between TLRs and motor dysfunction by neurodegeneration (Abg Abd Wahab et al., 2019), so it would be interesting to attempt to clarify this connection in terms of TLR7.

Finally, another system that we did not include in our study of TLR7 and pain is the opioid system. TLRs have been shown to be associated with opioid-induced neuroinflammation, particularly with TLR4 (Wang et al., 2018). TLRs may mediate increased sensitivity to pain following opioid exposure by affecting neuroimmune signalling cascades via glia and neurons (Grace et al., 2016). The role of TLR7 with regard to the opioid system in terms of chronic pain is a valuable research avenue for the future of this field.

4.5 Conclusions

This thesis demonstrates evidence for a novel role of TLR7 in the relationship between the immune system and the nervous system, and how this relationship might underlie chronic pain resolution. It is because TLR7 is an immune system receptor, not despite it, that makes it so important in the cellular mechanisms leading to nociception. We have shown in our models of murine nociception in acute cutaneous inflammatory, chronic cutaneous inflammatory, and neuropathic autoimmune states, that TLR7 is a critical regulator of inflammatory pain resolution, specifically in terms of mechanical hypersensitivity. Consideration of TLR7 as a therapeutic target for clinical studies would be a next step in understanding treatment avenues for chronic inflammatory conditions such as MS, among many others.

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