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Potential Role Of DRG Macrophages In Chronic Pain

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POTENTIAL ROLE OF DRG MACROPHAGES IN CHRONIC PAIN

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B.S. Biology and Chemistry, Roger Williams University, 2015

THESIS

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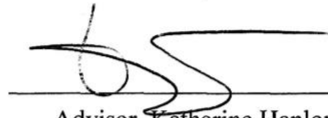
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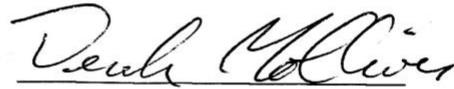
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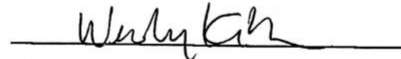CAS Advisor, Geoffrey Ganter, Ph.D.
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DEDICATION

I would like to dedicate this to everyone who has contributed and guided me along this journey. Foremost is Dr. Katherine Hanlon and Dr. Derek Molliver for their guidance and dedication to mentorship. Not only have they provided excellent guidance within my scientific endeavors at UNE, but also encouraged me to pursue my scientific inquiry at the Ph.D. level. Additionally, I would like to thank Kayla Lindros, Moxie Scarneo and Taxia Arabatzis for their friendship during this process.

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FORWARD

       This thesis presents data collected during my master's program. The data collected and presented here describes the role of resident tissue macrophages in the peripheral nervous system with an emphasis on their contribution to chronic pain states. The phenotype of resident tissue macrophages was poorly established especially in context to more widely known blood monocytes and microglia. Further we demonstrate the transient changes in resident tissue macrophages in the dorsal root ganglia were established, highlighting the up regulation of extra cellular proteins such as MHC Class II and CD39. This work will be submitted to a peer review journal upon completion of a few follow up studies.

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ABSTRACT

POTENTIAL ROLE OF DRG MACROPHAGES IN CHRONIC PAIN

by

Scott Scarneo

University of New England, May, 2017

Persistent pain remains a significant burden on the US healthcare system with more than 100 million patients experiencing persistent pain annually. Complex etiology coupled with functional plasticity of cells involved often results in a chronic health concern with limited treatment modalities. To date, most work aimed at describing neuro-immune interactions in pain focuses on the site of injury, leaving the functional significance of resident tissue macrophages in the dorsal root ganglia largely unexplored in both uninjured and peripheral injury states. Here, we systematically evaluate the phenotype of naïve DRG resident tissue macrophages and compare to naïve spinal microglia and bone marrow derived monocytes. We report the upregulation of CD39 and MHC Class II by DRG macrophages 24 and 72 hours following distal insult in multiple models of inflammatory pain. Further, we demonstrate functional changes induced in DRG macrophages in response to peripheral injury by profiling the cytokine proteome: DRG expression of 111 cytokines were evaluated with and without injury. Taken together, these data suggest that resident tissue macrophages actively respond to distant injury stimuli with functional changes and may influence nociception via neuro-immune communication.

CHAPTER 1

INTRODUCTION

Chronic pain remains a prevalent health concern. Despite affecting up to 100 million patients in the U.S. alone, the underlying disease mechanism still remains relatively unresolved [1]. One underlying mechanism contributing to chronic pain may be the synergistic communication between immune cells and neurons. Interactions amid pain and inflammation have established for centuries, however mechanisms of neuro-immune interaction in response to injury remain unclear [2]. Current therapeutics aimed at regulation of inflammation, such as non-steroidal anti-inflammatory drugs (NSAIDs), often fail to alleviate severe pain and are short acting [3]. Understanding how specific genetic, psychological, and physiological properties contribute to the development of chronic pain will aid in the development of novel non-opioid compounds [4].

Neuronal Pathology of Pain

Conscious perception of nociception is essential for life, allowing human beings to detect harmful stimuli [5]. Under normal circumstances, pain is indicative of a potentially harmful stimulus directly affecting tissue. Nociceptors, specialized receptors present on nerve endings, detect thermal, chemical, and mechanical stimuli within their cutaneous and visceral targets [6]. Specific nerve fibers within the peripheral nervous system (PNS) transmit pain: class A (δ) myelinated fibers and unmyelinated class C fibers. Class A (δ) fibers are responsible for carrying sharp and fast initial pain transmissions, whereas class C fibers aid in the transmission of duller and slower pain signals [7]. Transmission of these signals, nociception, is accomplished via synapse of primary afferent neurons with secondary afferent axons in the dorsal horn of the spinal cord. The signal then continues up the contralateral spinothalamic tract to synapse in the ventroposterolateral (VPL) nucleus and

ventroposteroinferior (VPI) nucleus of the thalamus with conscious perception of pain occurring with synapse in the primary somatosensory cortex [9,10].

In some cases, pain persists even after the removal of noxious stimuli and the return to normal cellular physiology [8]. The term neuropathic pain is defined as pain which derives from a direct consequence of damage or disease to the somatosensory system [9]. It remains to be determined whether neuropathic pain and chronic pain are one and the same or distinct subclasses of persistent painful conditions. However, the complexity of these conditions and urgency for development of novel non-opioid therapeutics is consistently agreed upon [8]. Although persistent pain has been documented clinically for many years, the exact cause and pathology continue to be elusive. This in part arises from the plasticity of the primary afferent neurons, in which cellular processes are modified in response to inflammation or direct nerve damage [10]. Hyperalgesia, an increased sensitivity to pain stimulus, is an often observed clinical aspect of persistent pain. Primary hyperalgesia occurs directly at the site of injury within tissue and may be specifically dependent on persistent inflammatory processes, whereas secondary hyperalgesia is mediated a distance from the injury with unknown immune contribution [11]. Changes in neuronal transmission of pain may develop into alloydnia, a condition in which a previous innoculous stimulis is perceived as noxious under persistent pain states [12].

Receptors and ion channels expressed on neuronal cell surface play a large role in the transmission of noxious stimuli within the pain pathway and expression characteristics aid in distinguishing one neuron from another in their biology and transmissory ability [13]. For example, the transient receptor potential (TRP) family of ligand-gated ion channels are a class of receptors frequently expressed on nociceptor neurons that respond to temperature [14] . TRP channel expression isn't the only distinguishing mark of nocicpetors however; there are other varying sodium channels and neuropetptides which help discern neuronal sub type and function [15].

G coupled protein receptors (GPCRs) are another group of receptors with high levels of neuronal expression, though many other tissues express GPCRs as well. The diverse family of GPCRs all share a characteristic 7 transmembrane passing protein domain and play an important role in transducing extracellular signals. Intracellular G protein signaling is induced by binding of a plethora of ligands including hormones, neurotransmitters and cytokines to their respective GPCR [16]. Following extracellular binding, intracellular G protein subunits α, β, and γ are further activated through GTP/ GDP exchanges induced by conformational changes in the corresponding GPCR upon ligand binding. Ligand binding and subsequent G protein initiation lead to a wide variety of downstream intracellular changes dependent on the specific combinations of G protein subunits coupling with the GPCR [17].

Major GPCR classifications

$G\alpha_s$ coupled GPCRs serve to increase neuronal excitability by increasing adenylyl cyclase activity downstream of the α_s subunit [18]. Elevated cAMP levels within the cytoplasm cause activation of protein kinase A (PKA), an immediate effector kinase capable of modulating activation status of other downstream effectors. PKA also regulates transcriptional activity as well as modulates cell membrane compositions [19].

Contrary to $G\alpha_s$ -coupled receptors, $G\alpha_{i/o}$ –coupled receptors inhibit effects of neurotransmitters within neurons. $G\alpha_{i/o}$ subunit activity inhibits adenylyl cyclase, directly opposing $G\alpha_s$ signaling activity [20]. In addition to these effects, $G\alpha_{i/o}$ signal cascades inhibit neurotransmitter release in the synaptic cleft. Reduction in postsynaptic neurotransmitter availability subsequently decreases signaling between neurons; within sensory neurons the result may include increased pain thresholds by reducing flux through afferent signaling pathways.

Finally, $G\alpha_{q/11}$ –coupled receptors biochemical pathway operates through phospholipase C Beta (PLCβ). Down stream effectors include phosphatidylinositol-4,5-bisphosphate PIP2 leading to

(IP3) initiated (Ca^{2+}) release from intracellular calcium storages [21].Alternatively, $G\alpha_{q/11}$ activation activates diacylglycerol (DAG) which may also aid in the increase in intracellular calcium levels. Heightened levels of calcium within the cytoplasm cause activation of protein phosphatase calcineurin further facilitating stimulating the NFAT transcriptional factors [22].

Purinergic receptors

Purinergic receptors are a specialized family of GPCRs of particular importance. P2 receptors are expressed primarily on neurons that respond to extracellular signals such as ATP as well as other purine and pyrimidine nucleotides. Studies have shown both P2X and P2Y receptors participate in the processing of sensory information including pain [23].

Previous work demonstrates via RT-PCR analysis that the mRNAs of three P2Y receptors, P2Y12, P2Y13 and P2Y14, are upregulated 4 days following CFA injection in murine dorsal hind paw [24]. These findings suggest modulation of P2Y expression and potentially functional response to peripheral injury. Further work shows that P2Y12 receptor expression in lumbar ganglia is limited to unmyelinated neurons within the sensory ganglia whereas expression of P2Y13, and P2Y14 expression are seen on both myelinated and unmyelinated neuronal cell bodies [24] . P2Y12, P2Y13, and P2Y14 receptors signal through the $G\alpha_{i/o}$ signaling pathway. It stands to reason then that these receptors may play a role in modulating pain response in murine peripheral nerve injury models. Indeed, unpublished data collected in the Molliver laboratory have shown that treatment with P2Y agonists exert anti-nociceptive responses following forskolin. Thus, P2Y signaling may be of particular importance in restoring neuronal cell homeostasis following nerve injury.

Neuronal support: satellite glial cells

Satellite glial cells (SGC's) tightly adhere and wrap around neuronal cell bodies in ganglia, and can mediate cell signaling. These specialized Schwann cells are primarily found within sensory, sympathetic and parasympathetic ganglia [25]. Originally believed to have complex processes similar

to astrocytes in the brain, it is now known these cells are laminar in morphology with often more than one cell interacting with a sensory neuron [26]. Close proximity of neurons and satellite glial cells, 20 nm apart, provide the potential for robust proximal signaling between the two [23]. Signaling is further enhanced by microvilli which extend from the neuronal cell surface into the extracellular space, and invaginations of the satellite glial cells in some cases increasing the neuronal cell surface by up to 30-40% and the interactions between satellite glial cells and neurons [27].

In addition to their close spatial relation to neuronal cell bodies, it has also been demonstrated that satellite glia share the ability to communicate through intercellular messengers including ATP [28]. The presence of purinergic receptors for ATP, as well as other neurotransmitter receptors, have been explored on satellite glia data suggest neuronal communication with satellite glia [29]. Further work has implicated nitric oxide as a critical mediator of signal transduction between neurons and satellite glial cells. In naïve states 5% of sensory neurons express neuronal nitric oxide synthase (nNOS), however under pathological conditions expression is markedly upregulated, leading to subsequent elevated NO levels in neurons [30]. Further, Aoki et al. demonstrated high levels of cyclic GMP (cGMP), which is upregulated in response to NO, in satellite glial cells[32]. Concurrent elevation of arginine levels found by the same group suggests existence of an inhibitory feedback loop [31]. These discoveries led to the proposal of a mechanism of signal transduction in which neurons, under pathological stress, upregulate nNOS activity and its product NO, which signals in paracrine fashion to upregulate cGMP signaling in satellite glial cells [31].

Relevant immune cells and inflammatory contribution to pain signaling

Macrophages

Macrophages are critical mediators of the innate immune response. While traditionally macrophages have been defined as originating from bone marrow derived monocytes, recent work by

Perdiguero et al. in 2014 demonstrates that populations of yolk sac derived tissue resident macrophages are distinct from macrophages arising from bone marrow derived monocytes [32]. Bone marrow derived monocytes are continuously regenerated in bone marrow throughout adulthood and circulate in the blood stream, activating into macrophages only upon stimulation by extracellular factors such as pathogen associated molecular patterns (PAMPs) or damage associated molecular patterns (DAMPs) [33, 34]. Conversely, tissue resident macrophages differentiate in the prenatal period and persist throughout adulthood in the tissue of origin [35]. It is still unclear whether blood monocytes are able to replenish tissue resident macrophage populations on an as needed basis [36]. Both bone marrow derived and tissue resident macrophages mediate inflammatory response, acting as the cellular bridge between innate and adaptive response by taking on the role of major cytokine and chemokine manufacturing sites and by exhibiting the ability to upregulate and express the antigen presentation complex MHC Class II [37].

An additional macrophage classification system is useful in delineating specific cell functions. M1 macrophages contribute to the pro-inflammatory response whereas M2 macrophages are trophic or anti-inflammatory [38]. The dichotomy is derived from the distinct metabolic processing of arginine. M1 macrophages convert arginine to NO, a reactive oxygen species (ROS), through inducible nitric oxide synthase iNOS whereas metabolic processing of arginine by arginase in M2 macrophages inhibits iNOS activity by out competing substrate availability [39, 40]. This reduces the inflammatory response through reduction of ROS in the cell [38]. These phenotypic classifications are not mutually exclusive but rather they highlight the complexity of macrophages in that macrophages are not terminally differentiating cells, differentiated phenotypes are modifiable by alterations in microenvironment and significant crossover exists between the M1 and M2 phenotypes [41].

Macrophages isolated from mammalian tissues are notoriously difficult to identify due to the overlap of expression of many significant cell surface proteins between various leukocytes. A number

of parameters are described in chapter II and chapter III to define macrophage or monocyte populations or to exclude extraneous populations. For reference, a description of these markers is provided here.

CD11b- A common macrophage marker used to aid in delineating cell type is Cd11b. Cd11b, also known as integrin alpha M (ITGAM), has been implicated in cell adhesion, migration, phagocytosis and cell activation [42].The protein is a subunit of the greater heterodimeric (α_M-β_2) protein known as macrophage-1 antigen (Mac-1) with the common chain β_2 or also known as cluster differentiation molecule CD18 [43]. CD11b is not exclusive to macrophages and monocytes with expression demonstrated on related leukocytes in the innate immune system including dendritic cells, granulocytes and natural killer cells [42].

CD11c- an additional integrin, often used to identify dendritic cells. Similar to Cd11b, Cd11c is a subunit of the heterodimeric protein in conjunction with β_2 [44]. Although expression can often overlap that of Cd11b, high expression has been found on dendritic cells in human and murine species, thus $CD11c^{medium-high}$ expressing Cd11b cells are often described as classical dendritic cells (cDC's). Expression has also been found on naïve alveolar macrophages at levels that mimic cDC's [45].

CD103- Cluster of differentiation protein 103 is used in identification of a small subpopulation of intestinal classical dendritic cells with expression ranging from 20-30% of total leukocyte population in the intestinal track[45]. Hence, these cells are further characterized as $CD103^+$ cDC's. $CD103^+$ cDC's are also found in most connective tissues [46] .

CD115- Macrophage colony stimulating factor receptor or colony stimulating factor 1 receptor (CSF1r; CD115) is a receptor found on the surface of macrophages and other innate immune cells which acts as the receptor to the cytokine colony stimulating factor- 1 ligand [47]. This cytokine plays a role in production, subsequent differentiation, migration, and function of macrophages.

CD169- Siglec-H or CD169 is a surface adhesion molecule found on macrophages which binds the sialic acid residues on other cells [48]. Moreover, it has been seen that CD169 can adhere to red blood

cells and thus is highly expressed in organs which filter blood such as the spleen [49]. Under pro inflammatory states CD169 is upregulated on macrophages [50].

Ly6G- a neutrophil specific cell surface protein; allows distinction from other myeloid derived leukocytes [51]. Ly6G is an extracellular protein expressed in a highly regulated fashion from progenitor bone marrow cells throughout development and differentiation of neutrophils [52]. High Ly6G expression in $Cd11b^+$ cells is used to define neutrophil lineage cells.

Major histocompatibility complex class II (MHC Class II) expression occurs on immune cells capable of presenting antigen to CD4 helper T cells, including macrophages, dendritic cells, and some B lymphocytes [53]. This expression profile contrasts major histocompatibility complex class I (MHC Class I) protein complexes, which are present on all mammalian cells with a nucleus and are viewed as 'self' identifiers by effector immune cells including NK cells and CD8 cytotoxic T lymphocytes [54]. MHC Class I recognition prevents inappropriate NK cell killing response and facilitates proper activated CD8 T lymphocyte response by presenting self antigen on healthy self cells while stress antigens or foreign antigens are complexed with MHC Class I on unhealthy self cells, thus allowing immune response against invaded cells [55, 56]. Under healthy physiological conditions macrophages exhibit MHC class I complexes much like other self cells, however upon stimulation by PAMPs or DAMPs macrophages upregulate expression of MHC Class II membrane proteins [57]. In response to foreign antigens macrophages phagocytize invaders and present protein fragments (antigens) via MHC Class II complex [58]. Hence, MHC Class II expression is often correlated with antigen detection and subsequent antigenic presentation to other immune cells, leading initiation of the adaptive immune response.

CX3CR1- CX3C chemokine receptor 1, or fractalkine receptor, is a membrane bound protein involved in adhesion and migration of mononuclear leukocytes [59]. Expression of this protein has been found on macrophages, monocytes and microglia where its expression is often linked to cell survival [62].

Additionally, in the central nervous system CX3CR1 expression on microglia has been found to play a role in activation and migration of microglia to damaged regions of CNS where they may undergo phagocytosis [60].

CD39- The ectonucleoside triphosphate diphosphohydrolase 1, E-NTPDase1 or cluster of differentiation 39 (CD39), is an extracellular protein with an active domain open to the extracellular milieu [61]. CD39 responds to extracellular purinergic signaling molecules, primarily ATP. CD39 mediates ATP availability by catalyzing the hydrolysis of ATP to AMP, which can be further converted via CD73 into adenosine [62]. In some persistent pain states elevated levels of ATP have been found in the DRG following peripheral injury [102]. Thus, macrophages may have the ability to mediate purinergic signaling by modulation of available ATP/AMP substrate. Interestingly, adenosine has been reported to dampen inflammatory response and allow pro inflammatory cells to return to a more quiescent state [63]. Together, this data leads to the hypothesis that insufficient DRG macrophage CD39 activity may contribute to persistent pain states by limiting flux through the metabolic pathway of ATP to adenosine conversion.

CCR2- Expression of chemokine receptor-2 (CCR2) has been documented on macrophages, monocytes as well as other migrating leukocytes [64]. This extracellular receptor for the ligand (CCL-2) plays an important role in cell migration. Recent data suggest up regulation of the CCR2 ligand, CCL2 in mRNA levels in DRG of peripherally injured rats [65].

	Cd11b	CD11c	CD39	CD103	CD169	MHC II	CX3CR1	CCR2	Ly6G	Ly6C
Macrophages	++	+	+++	-	+	+++	+++	+	-	+
Monocytes	++	NK	+	-	+	+	+	++	-	++

Neutrophils	+	-	-	-	-	-	NK	NK	+++	-
CD103+ cDC's	+/-	+++	NK	+	NK	++	NK	NK	-	NK
cDC's	+	+++	NK	-	NK	++	NK	NK	-	NK
Microglia	+	NK	+	-	NK	-	+	-	-	-

Table 1.1 – Expression of phenotypic lineage markers by leukocyte populations in mice.
*NK= Not currently known, or agreed upon

Macrophage expression of purinergic receptors

Macrophages have also been shown to express P2X and P2Y receptors. P2Y12, P2Y13, and to a greater extent P2Y14 have been demonstrated. P2Y14 receptors act to inhibit adenylyl cyclase activity, further inhibiting the production of cAMP [66] . The endogenous ligand for P2Y14 is UDP-glucose- an important nucleotide sugar involved in glycotransferase reactions in glycogen metabolism. Often found in the cytoplasm, UDP-glucose may also be transferred to the cell membrane and released [67].

Cytokines involved in macrophage function

Cytokines, the most prevalent immune signaling molecules, act as intercellular messengers. These small proteins usually less than 30 kDa in size mediate the carefully choreographed immune response to foreign antigens and are loosely categorized as pro or anti inflammatory, however significant overlap occurs depending on the full cytokine milieu [68, 69]. For context, cytokines involved in macrophage differentiation and function are briefly described.

Pro-inflammatory cytokines polarize bone marrow derived monocytes or tissue resident macrophages into pro-inflammatory effector cells, upregulating migration, phagocytosis and antigen presentation [70]. Pro-inflammatory cytokines include IL-1a, IL-1ra, IFN-γ, CXCL2, CCL2. CD14, Tumor necrosis factor α (TNF-α), and granulocyte macrophage colony stimulating factor (GM-CSF). While

these are primary members of the classical inflammatory milieu, many other inflammatory cytokines have been documented [71, 72].

Trophic cytokines promote function of the trophic macrophage response important for wound healing. A hallmark of trophic macrophage activity is reduced production of the reactive oxygen species nitric oxide, which is a key component of inflammatory macrophage response. The reduction in NO production can be attributed to substrate competition for the arginine precursor achieved by upregulation of the enzyme arginase in M2 macrophages. The net effect of arginase upregulation is significant reduction in arginine availability for activity of the inducible nitric oxide synthase enzyme (iNOS). Cytokines supporting an trophic-inflammatory or trophic macrophage response include IL-4, IL-10, Il-13,serepin E1, MMP3 and TGF-β [73]. These important mediators of the immune system initiate the return to homeostasis once the pro-inflammatory stimulus has been resolved and allow for tissue repair to begin [74] .

Central Nervous System- immune interactions

The discovery of microglial cells by Franz Nissi shed light on the presence of immune entities in the central nervous system [75]. Similar to macrophages in the periphery, microglia act as first responders to injury in the central nervous system by quickly mobilizing and differentiating into functional states reactive to CNS insult [76]. Microglia are capable of polarization rapidly and with high efficiency into pro or trophic-inflammatory phenotypes dependent on extracellular stimuli. Due to their dichotomous nature, tight regulation of microglial activation is needed to prevent unwanted damage in central nervous tissue. Microglial activation is primarily mediated through receptors where either of two categories may be received: "off" and "on" signals [77]. The "off" signal inhibits potentially damaging inflammatory activation. "Off" receptors include CD200R, as well as P_1A_3 which tonically retain the muted state of microglial cells [78]. "On" receptors include purinergic receptors such as P1 adenosine receptors, P2 ATP receptors, as well as receptors for advanced

glycation end products (RAGE) and others [79].In microglia, the existence and expression of P2X4, P2X7, P2Y2, P2Y6 and P2Y12 have been previously investigated [80]. Studies have shown that intrathecal injection of ATP stimulates microglia and initiates mechanical hypersensitivity as well as release of brain derived neurotrophic factor (BDNF) [81]. Overall, regulation of microglial activation plays a critical role in CNS injury response.

Peripheral nervous system- immune interactions.

Previous research has demonstrated some neuro-immune interactions in the periphery. In 1993, Maves and colleagues were able to evoke neuropathic pain in the absence of direct peripheral nerve injury by transplanting chromic gut sutures proximal to the sciatic nerve, leading to immune response and subsequent neuropathy [82]. These data provided the initial suggestion that neuropathic pain may be mediated at least in part by the immune system and that cross cell communication may be taking place. Further studies have shown neuronal communication with immune cells through neurotransmitters [83]. Research conducted by Pacheo, et. al. highlight the role of neurotransmitter receptors, specifically dopamine receptors (DAR's), in immune response [84]. To date five DAR have been identified and are neuronal GPCR's primarily activated through endogenous dopamine and analogs [89]. Interestingly, all five dopamine receptors have been reported to also be expressed on T cells. Findings indicate that of the five, only one, D3R, promotes inflammatory T cell stimulation. Investigation of the other four DAR's have shown they play a critical role in the induction of inhibitory signals. D3R signaling activation promotes production and secretion of IFNγ while also inhibiting IL-10 and IL-4 synthesis in T cells [84]. Evidence of leukocyte response to neurotransmitters may help elucidate the means by which the immune system reacts to nerve tissue damage and signaling. Likewise, research has shown that microglia cells are able to produce and secrete neurotransmitters [84].

The complex interactions between immune cells and neurons remain widely unknown, however recent data suggests that macrophages may be involved in the development of persistent pain [85]. We have confirmed the existence of resident macrophages in the dorsal root ganglia (DRG) of naïve mice of multiple strains; these results are detailed in chapter II. We further explore the function of tissue resident macrophages in sensory ganglia in subsequent chapters.

Overall, the role of macrophages in the peripheral nervous system has yet to be elucidated in response to injury. In order to better understand the significance of resident macrophages in pain response to peripheral nerve stimuli, we characterized the resident immune system in the DRG with use of a transgenic mouse model. Initial findings of resident tissue macrophages were confirmed through fluorescent imaging of eGFP expressing macrophages in the DRG of uninjured mice. Further, we hypothesize that not only do resident tissue macrophages exist in the naïve ganglia but they respond to neurons in persistent pain states though phenotype changes.

CHAPTER 2

IMPACT OF RESIDENT TISSUE MACROPHAGES IN PERSISTENT PAIN

<u>ABSTRACT</u>

Pain is relevant to all clinical settings and a unifying force across specialties, though it remains a burden on the US Health Care System. The development of persistent pain is preceded by sensitization of central nociceptive pathways by mechanisms incompletely understood. Resident tissue macrophages have been previously described in many tissue types, however peripheral nervous system macrophages are less well described. Here we characterized the morphology and naïve phenotype of resident tissue macrophages using flow cytometry and immunohistochemistry. Phenotypically distinct, compared to microglia and blood monocytes, >90% resident tissue macrophages in the dorsal root ganglia express MHC Class II, CX3CR1, and CD39, with few <1% of cells positive for other leukocyte phenotype markers including CD103, Cd11c, and Ly6G. Approximately 50% of resident tissue macrophages express the monocyte chemokine attractant receptor CCR2. Inflammatory pain model complete Freund's adjuvant, show resident tissue macrophage response to peripheral injury by exhibiting increased expression of CD39 and MHC Class II and an altered cytokine proteome profile, suggesting altered macrophage function on detection of distant insult. This taken together indicates a distinct phenotypic immune cell population in naïve dorsal root ganglia with a potentially distinct functional value under persistent pain states.

METHODS

Animals – All mice were housed and cared for in accordance of the University of New England Institution Animal Care and Use Committee (IACUC). Mice were housed in a temperature and humidity controlled facility under 12-hour light/dark cycle (lights on at 7 am) and access to food and water *ad libitum*. All experiments were undertaken under approved protocol in accordance with the institutional guidelines and the Guide for the Care and Use of Laboratory Animals. Csfr1-eGFP mice were purchased from Jackson Laboratory and used for IHC and peripheral inflammatory injury (CFA) as well as primary cell culture. Genotyping was performed by PCR using tail digests. Primers and PCR conditions for Csfr1-eGFP were carried out under the following conditions 95°C for 5:00 minutes, 30 cycles of 95°C for 0:45s, 60°C for 0:45s, 72°C for 0:45s, followed by 72°C for 5:00 minutes. PCR results were imaged on 1.5% agarose gel.

Transgenic Mice- B6N.Cg-Tg (Csf1r-EGFP) 1Hume/J mice were purchased from Jackson Laboratory and used for IHC and peripheral inflammatory injury (CFA) as well as primary cell culture. Genotyping was performed by PCR using tail digests. Primers and PCR conditions for Csfr1-eGFP were carried out under the following conditions 95°C for 5:00 minutes, 30 cycles of 95°C for 0:45s, 60°C for 0:45s, 72°C for 0:45s, followed by 72°C for 5:00 minutes. PCR results were imaged on 1.5% agarose gel. Primers listed in Appendix of Thesis.

Immunohistochemistry- Mice were perfused transcardically with Hanks Balanced Salt Solution (HBSS) followed by 40 mLs of 4% paraformaldehyde (PFA). L3-5 DRG, spinal cord lumbar enlargement, and glabrous and hairy hind paw skin were dissected out and cryo-protected overnight in 30% sucrose, and embedded in Tissue-Tek Optimum Cutting Temperature (OCT) compound (Thermo Fisher Scientific, Waltham, MA). Serial sections of DRG (12 μm), were mounted on Superfrost(+) slides (VWR, Radnor, PA). Cells were stained with antibodies against neuronal specific enolase (NSE) – (Genetex Cat#GTX85462), GFAP- (Biolegend Cat#914501). Slides were incubated

overnight at room temperature, washed 3 times for 15 minutes then stained with the following secondary's, Donkey anti Rabbit IgG – PE (Biolegend Cat#406421), Donkey anti Rabbit IgG –Ax 647 (Biolegend Cat#406414), and Goat anti mouse IgG – Ax488 (Biolegend Cat#405315).

Dorsal Root Ganglia Dissociation- Prepared Collagenase IV (Sigma-Aldrich, C5138) enzyme solution by added 3 mg of collagenase to 3 mL of HBSS (Ca^{2+}/Mg^{2+} free). Enzymes were placed in 37°C water bath. L2-L5 bilateral DRG were removed in less than 20 minutes. Aspirate all solution from DRG solution and added 3mL of Collagenase IV solution via 0.2-micron filter. Incubated in a 37°C incubator for 20 minutes. Centrifuge at 150g for 2 minutes, remove all media, cells were reconstituted in 1 mL of HBSS (Ca^{2+}/Mg^{2+} free) pH 7.2. DRG's were triturated and pipetted through 70 μm filter.

Flow Cytometry- Macrophages were isolated from naïve animals L2-L5 bilateral dorsal root ganglia. Rapidly dissociated as previously described. Cells were suspended in a 1% BSA in PBS solution with Fc Blocked (Biolegend Cat# 101310) for 30 minutes at 4°C. Cells were spun down at 300g for 4 minutes and Fc block solution removed. Cells were re-suspended in 100ul of 1% BSA in PBS solution and stained with pre conjugated antibodies against the following extra cellular proteins, CD11b-V450 (BD Bioscience Cat# 560455), CD39- PE (Biolegend Cat# 143804), MHC Class II- APC-Cy7 (Biolegend Cat#107628), LY6G-PE Tex Red (Biolegend Cat#127648), LY6C-BV570 (Biolegend, Cat#128029), CCR2-APC (Biolegend Cat# 150604), CD11c-Percp (Biolegend, Cat# 117326), CD103-Ax647 (Biolegend Cat# 121410), CD169-Ax488 (Cat# 42419), F4/80 –Percp (Cat# 123125) and CX3CR1-PE Cy 7 (Biolegend, Cat#149016). Cells were gated for lives cells using FSC-A and SSC-A parameters and further gated for singlet's through SSC-A to SSC-H 1:1 ratio. Leukocyte populations were identified through back gating CD11b(+) population.

Sciatic Nerve Crush- Sciatic nerve crush (SNC) was done as described. Briefly, mice were anesthetized by inhalation of isoflurane (4% for induction and 3% for maintenance) in 100% O_2. The

left flank was shaved with surgical clippers and cleaned once with betadine solution. A small incision was made in the mid-thigh region of the left leg. The inner muscle was separated using forceps to expose the sciatic nerve. Using fine hemostatic forceps, the sciatic nerve was lifted out of the muscle with care taken not to stretch the nerve. The nerve was crushed using one click of the hemostatic forceps for 30 seconds. The wound was irrigated with sterile saline, and closed using a 5-0 polyglactin 910 suture (Ethicon). Sham surgery was identical to the sciatic nerve crush surgery except that the sciatic nerve was exposed but not manipulated in any way. After the surgery, all mice were individually housed with food and water readily available.

Injection of Complete Freunds Adjuvant (CFA) - Mice were anesthetized by inhaled isoflurane. Sterile syringe consisting of a 1:1 mixture of saline to CFA was used to inject 20 µl of CFA into the globourous hind paw skin. Mice survived for up to 1 or 3 days' post injection.

Bone Marrow Derived Macrophages – Acutely cultured bone marrow derived macrophages were obtained from tibia and femura of naïve male C57Bl/6J mice. Immature bone marrow cells were culture for 72 hours in L929 culture media to induce macrophage differentiation. Following 72 hour rest period in L929 media cells were treated with Capsaicin and CFA.

Cytokine Assays - 3 Days following injection with either CFA or saline bilaterally into the hind paws, C57Bl/6J male and female mice (n=4 per condition) bilateral L2-L5 DRG were rapidly dissected and dissociated as previously described. Cells were plated for 16 hours in DMEM- High Glucose media supplemented with 10% FBS, 30% L929 conditioned media, and gentamycin. Following incubation for 16 hours' supernatant was removed and spun down to remove any debris in media. Protease inhibitors were added to the supernatant. Protein quantification was performed on all samples to determine protein concentration. Cytokine assay was done as described by manufacturing protocol (R&D, XL mouse Cytokine Array A).

RESULTS.

2.1 *Macrophages exist in DRG of naïve male and female C57Bl/6J and BALB/cJ mice*

The expression of CD11b and Ly6G proteins within naive L2-L5 dorsal root ganglia of female and male C57Bl/6J and BALBc/j mice was analyzed by flow cytometry. CD11b expressing cells were found in both male and female C57Bl/6J naïve L2-L5 DRG (Figure 2.1 B). This finding was confirmed in another mouse strain: male and female BALB/cJ mice also express CD11b+ cell population in L2-L5 naïve DRG (Supplemental Figure). In order to exclude the possibility that the CD11b+ population of cells found in mouse DRG may be a previously unidentified population of neutrophils, dissociated DRG were prepared and stained simultaneously with CD11b and Ly6G, a definitive neutrophil marker. Ly6G was found in an insignificant population of CD11b+ cells (< 1%) in L2-L5 DRG of either C57Bl/6J male or female mice (Figure 2.5). Similarly, dendritic cells were ruled out by evaluating expression of CD11c and CD103 within the sensory ganglia. Very small or no populations of CD11c cells with co-expression of CD11b (<1%) were found in the sensory ganglia (Figure 2.1 C.).

2.2 *CSF1R-eGFP reporter mice enable analysis of DRG macrophage morphology and localization*

Csf1r-eGFP resident tissue macrophages in naïve DRG were obtained from collagenase digested L2-L5 DRG and stained for CD11b. Co-expression of CD11b and CD115-eGFP was found in DRG resident tissue macrophages and spinal cord with the majority (>95%) of CD11b cells co expressing CD115 (Figure 2.2 B, C).

2.3-2.5 *DRG macrophages localize surrounding neuronal cell bodies, independent of vasculature and satellite glia*

Immunohistochemistry was used to determine localization of macrophages within the naïve ganglia. The limited ability to perform IHC staining with currently available anti-CD11b antibodies, which target highly ex-vivo labile cell surface integrin's normally only visible on in tact cells by flow cytometry, distribution of resident tissue macrophages was ascertained by immunostaining L4 naïve

DRG of CD115-eGFP mice. CD115 is the CSF1-R receptor expressed and required for survival of macrophages. Naïve L4 DRG were isolated from male B6N.Cg-Tg (Csf1r-EGFP) 1Hume/J mice. After flow cytometry analysis revealed co-expression of CD115 and CD11b without concurrent expression of Ly6G or CD11c in DRG macrophages, L4 DRG isolated from CD115-eGFP mice were prepared and stained with IHC antibodies against PGP9.5 to identify morphology and position proximal to neuronal cell bodies. A robust population of resident tissue macrophages in the dorsal root ganglia was observed with ramified processes, with close proximity to neuronal cell bodies (Figure 2.3).

Significant satellite glial cell populations also exist within the dorsal root ganglia surrounding neuronal cell bodies, thus, to exclude the possibility of glial expression of CD115 in the B6N.Cg-Tg(Csf1r-EGFP)1Hume/J mouse line, naïve L4 DRG were stained for glial fibrillary acidic protein (GFAP). Satellite glia were found to be exclusive of CD115+ cells (Figure 2.4 D).

Due to the highly vascular nature of the dorsal root ganglia, leukocytes may exist within the vasculature and not as part of the DRG microenvironment; thus it is possible that residual blood leukocytes may have been inadvertently captured at the time of analysis. To exclude this possibility, CD31, which is platelet endothelial cell adhesion molecule and is expressed on endothelial cells, was used to label blood vessels in naïve L4 DRG isolated from CD115 B6N.Cg-Tg (Csf1r-EGFP) 1Hume/J reporter line. CD115 expressing cells were found to be predominately exclusive of vasculature in the sensory ganglia (Figure 2.5 D).

2.6 *Phenotypic analysis of DRG macrophages reveals a distinct population of tissue resident cells*

The phenotype of CD11b+/Ly6G-/CD11c- macrophages isolated from naïve mouse DRG resident tissue macrophage populations was analyzed by flow cytometry by staining with antibodies directed against CX3CR1, MHC Class II, CD39, LY6C, CD169, and CCR2. DRG macrophages were analyzed alongside blood monocytes and spinal microglia isolated from the same animal for

comparison. A single cell suspension of whole dissociated DRG, red blood cell lysed blood leukocytes, and dissociated lumbar spinal cord were prepared and stained prior to running the suspensions through a BD LSRII flow cytometer. Analysis consisted of first sorting by size and granularity to identify a live cell population consistent with macrophages, which are significantly smaller than neuronal cell bodies and satellite glial cells but larger and more granular than lymphocytes. Samples were then gated on CD11b+/Ly6G-/CD11c- cells where 'gating' refers to exclusion of all cells not found to express CD11b or that do express Ly6G or Cd11c (hereafter referred to as DRG macrophages). CX3CR1 (CX3 chemokine receptor 1) was found to be highly expressed in majority (> 96%) of microglia and ~90% DRG macrophages isolated from naïve animals (Figure 2.6 A, C). This high expression of CX3CR1 by DRG macrophages is a stark contrast to blood monocytes isolated from the same animals, in which 45-50% of blood monocytes express medium to low levels of CX3CR1 (Figure 2.6 B). The antigen presentation molecule MHC Class II was found to be highly expressed on DRG resident tissue macrophages (~90%) compared to microglia (~10%) and blood monocytes (~4%) (Figure 2.6 D, F).

Flow cytometric analysis of CD39 on resident tissue macrophages, microglia and blood monocytes revealed that similar to microglia the majority of resident tissue macrophages in the DRG have high expression of CD39, compared to blood monocytes which are CD39 low to null expressing (Figure 2.6 G, H, I).

Low expression of LY6C in DRG resident tissue macrophages and microglia was found compared to blood monocytes which have a higher overall expression of LY6C (Figure 2.6 J, K, L). 50% of resident tissue macrophages in the sensory ganglia expressed CCR2 compared to low to null expression on microglia and approximately 25-30% of blood monocytes (Figure 2.6 M, N, O).

2.7 *Sprague Dawley Rats express significant resident tissue macrophage population in naïve L2-L5 DRG.*

Macrophages isolated from female Sprague Daley rats were analyzed via flow cytometry. Significant population of CD11b$^+$, LY6G$^-$ populations were discovered (Figure 2.7 B, E). Thus resident tissue macrophages are conserved across multiple species. Furthermore, these cells are predominately MHC II positive similar to C57Bl/6J male and female mice (Figure 2.7 E).

2.8 *Transient phenotypic changes induced by hind paw injection of CFA.*

Marked upregulation of CD39 and MHC Class II was recorded in DRG resident tissue macrophages 24 hours following CFA injection (Figure 2.8 A, B, C) (n=4, *p< 0.05). Comparatively, no significant changes in expression of CD39 or MHC Class II were observed in microglia isolated from the lumbar enlargement of matched spinal cord (Figure 2.8 H, I, G). A significant increase in mid-level CD39 expressing blood monocytes was observed (Figure 2.8 D, F), although no concurrent increase in MHC Class II was detected (Figure 2.8 E, F).

2.9 *Evaluation of acutely cultured bone marrow derived macrophages treated with CFA and capsaicin revealed no inducible change of CD39 of MHC II expression in treated cells.* Differentiation of acutely cultured bone marrow cells was established through cytokine induced induction of M1 and M2 macrophages. M1 differentiated cells markedly upregulated MHC Class II and iNOS where as M2 upregulated arginase (Figure 2.9 A-C). Following successful pro and trophic-inflammatory polarization, differentiated cells were treated with high concentrations of CFA and Capsaicin at 100nm or 100ng/mL of capsaicin or CFA, respectively. No notable changes in CD39 or MHC II expression was seen in treated samples over control undifferentiated samples (n=3) (Figure 2.9 D-F).

2.10-2.11 *Cytokine and Chemokine profile in L2-L5 C57Bl/6J DRG 3 days following CFA injection*

Marked changes in the chemokine and cytokine profile of lumbar 2-5 DRG were observed 3 days following intraplantar CFA injection in male and female C57Bl/6J mice. Sex dependence of cytokine expression was variable: serepin-E1 was upregulated in both male and female mice following CFA injection (Figure 2.10, 2.11). Furthermore, female specific upregulation of MMP3, a trophic cytokine,

about 2 fold and pro inflammatory cytokines/chemokines CXCL2, CCL2, and CD14 in CFA treated animals. Down regulation of IFN-γ in female CFA treated animals 72 hours post CFA injection indicate a dynamic cytokine milieu following injury.

Similarly, males also exhibited biphasic cytokine response with both pro and trophic mediators expressed 3 days following peripheral injury with CFA. Stark down regulation of MMP3 in CFA treated animals was contrasted with upregulation of IL-13 and IL-4 in CFA treated mice. Pro inflammatory cytokines, IL-1a, IL-1ra and CD14 were significantly upregulated in CFA treated mice over saline treated. CCL2, an important chemokine in chemotaxis of leukocytes to injury was down regulated in male CFA animals.
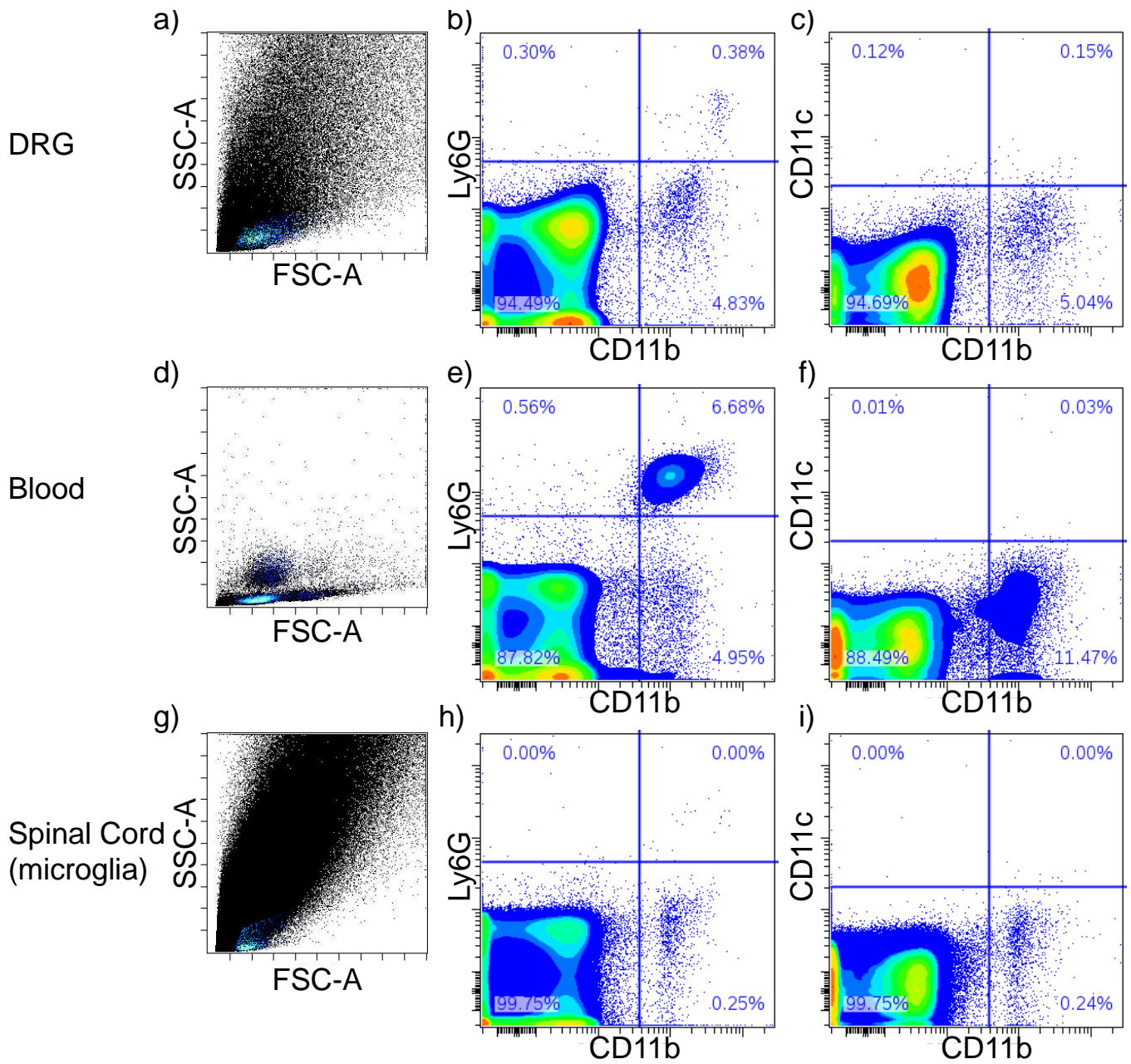
Figure 2.1
Significant CD11b⁺ cell populations are found within naïve L2-L5 DRG. Resident tissue macrophages in naïve DRG were obtained from collagenase digested L2-L5 DRG and stained for CD11b. Both C57Bl/6J male and female mice contain significant CD11b+ cell populations in naïve animals(A,D,G,). Minimal neutrophil populations were identified as seen by minimal to no Ly6G expression of CD11b+ cells (B,H), and dendritic cells CD11c (C,I) in DRG macrophages and microglia.
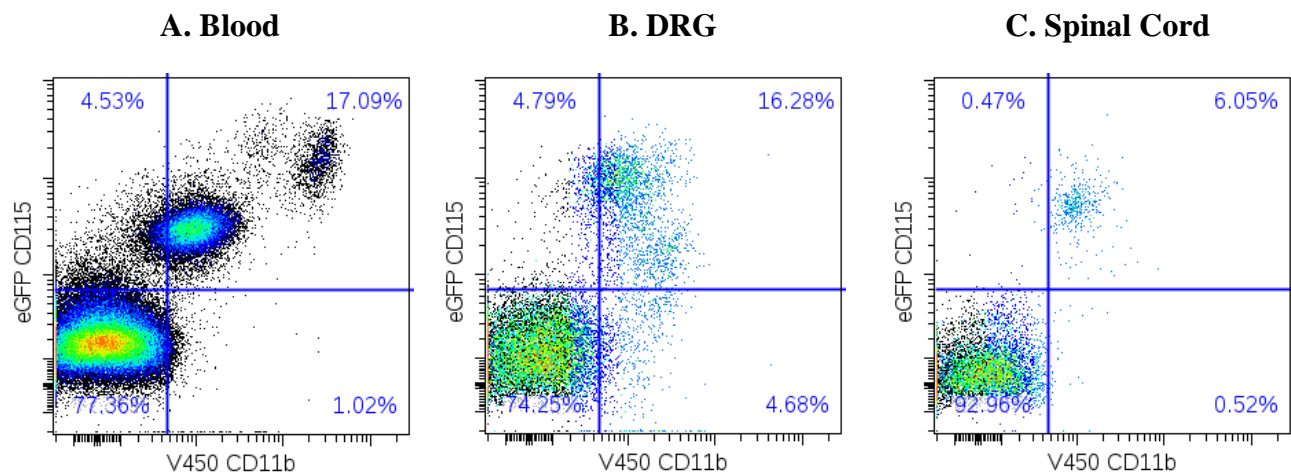
Figure 2.2. CSF1R-eGFP reporter mice enable analysis of DRG macrophage morphology and localization. Resident tissue macrophages in naïve DRG were obtained from collagenase digested L2-L5 DRG in CD115-eGFP transgenic reporter mouse and stained for CD11b (B). Additionally, blood monocytes (A), and spinal cord microglia (C) were isolated and stained against CD11b.   Co-expression of Cd11b and eGFP was seen in a majority of CD11b+ cells.
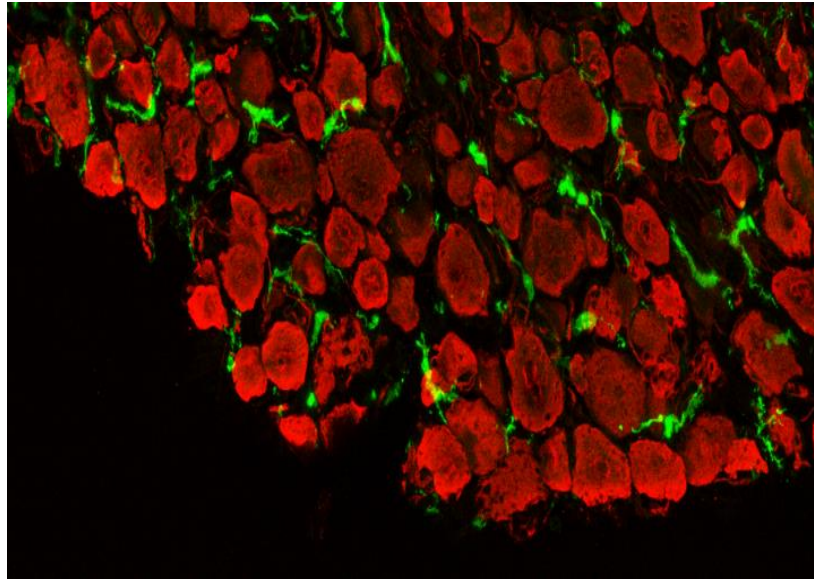
Figure 2.3
L4 DRG section from a naive male mouse expressing eGFP (green) driven by the CSF1R promoter. Double-labeling with antibodies to the neuronal marker PGP9.5 (red) indicates the presence of many eGFP-positive cells closely apposed to neuronal cell bodies.
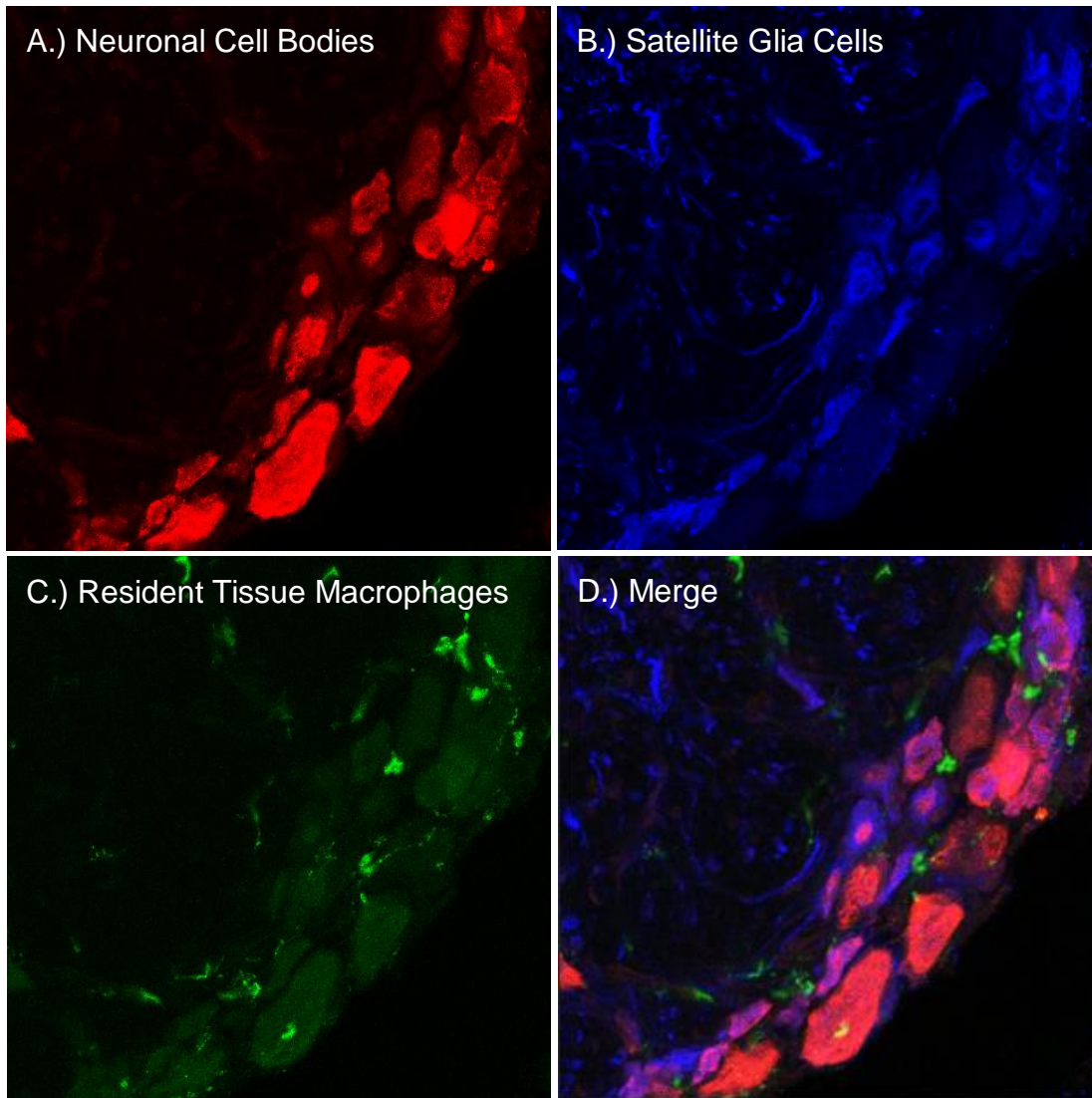
Figure 2.4
Spatial arrangement of CD115-eGFP cells (green) around neuronal cell bodies (red) and GFAP$^+$ cells (blue) indicate absence of co localization between satellite glial cells (GFAP+) and immune cells (CD115 +). **A.** neurons (red, Ax 647), **B.** satellite glial cells (blue, Cy3), **C.** CD115+ cells (green, eGFP) and merged **(D)** within L4 naïve DRG.
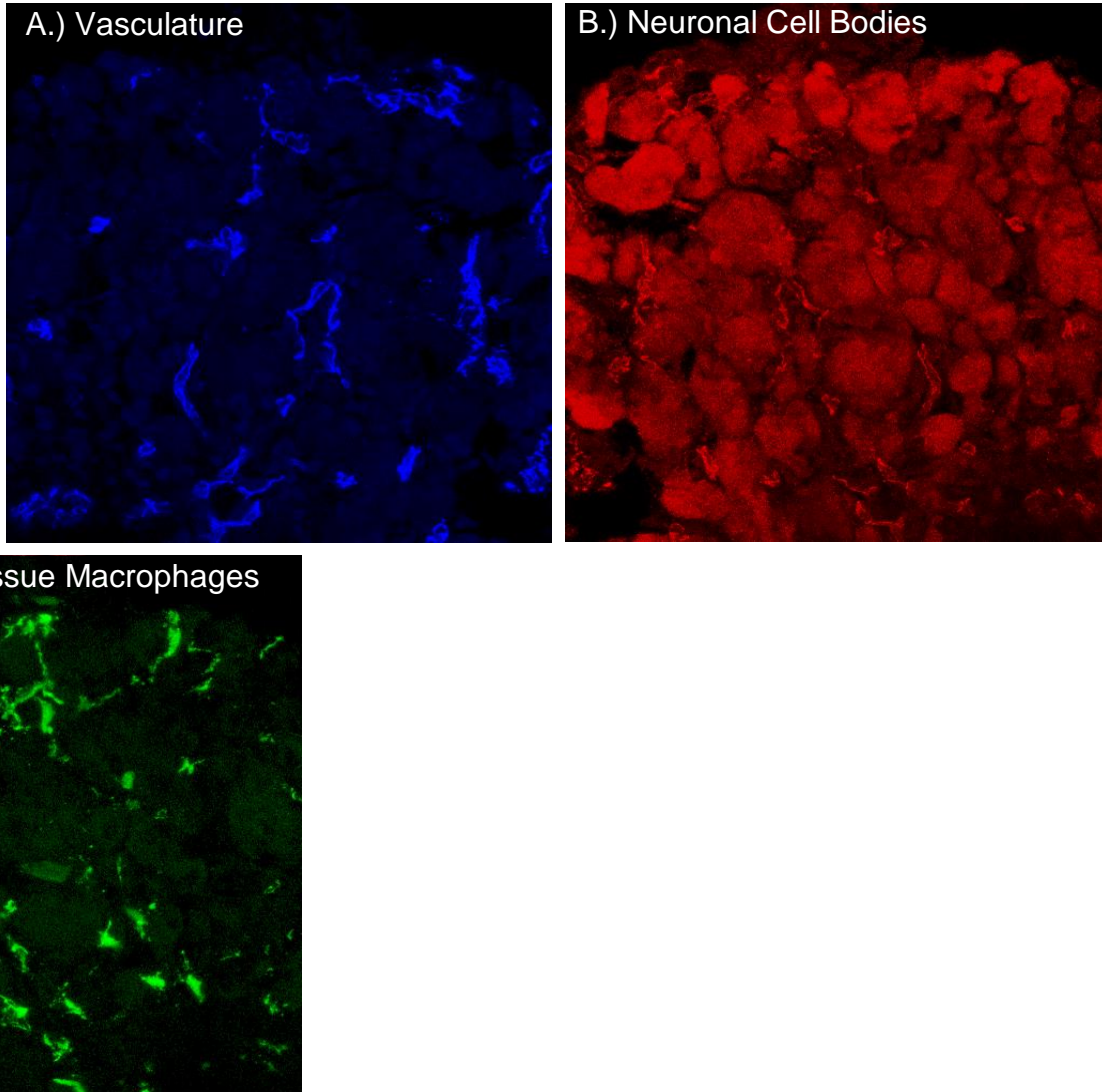
Figure 2.5
Immunostaining in L4 DRG with monoclonal antibodies against A. CD31 vasculature (PECAM) B. Neuronal specific enolase (NSE), C. CD115-EGFP (+). Localization of all three markers (D) indicate immune cells are exclusive of vasculature.
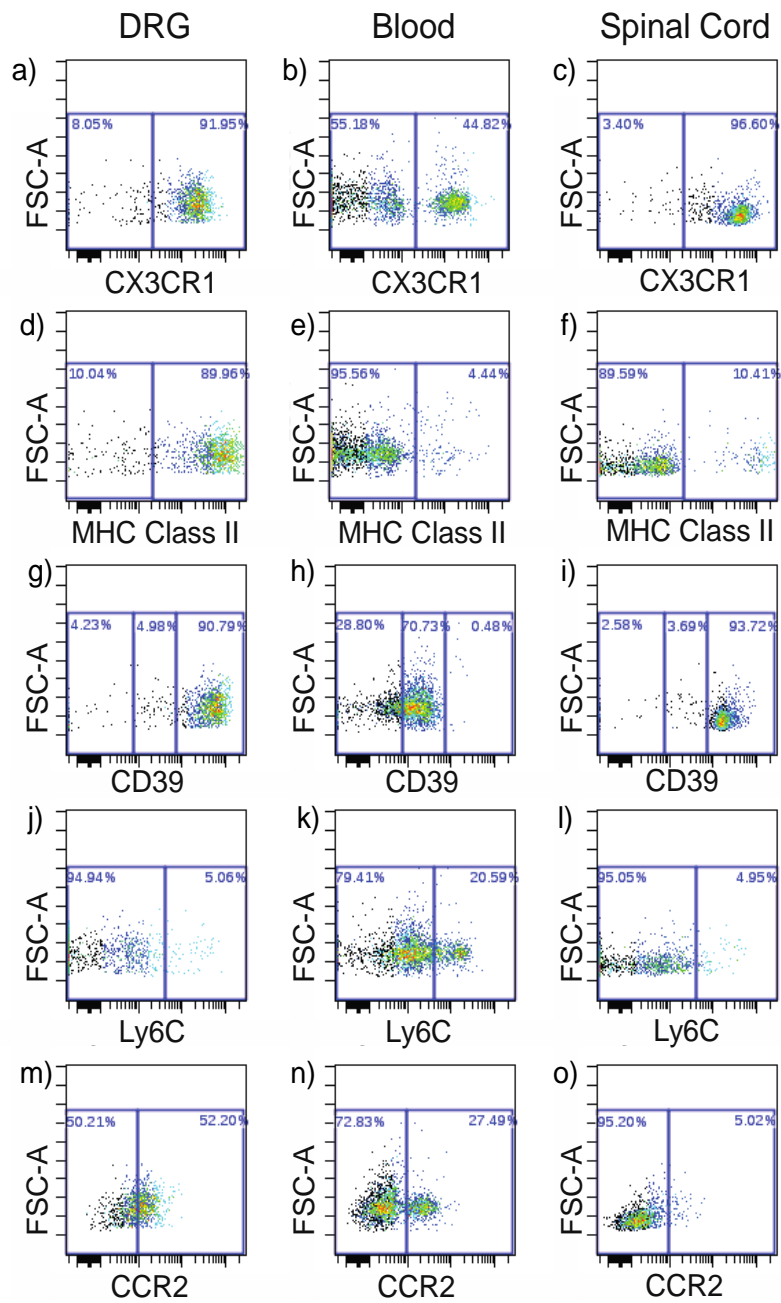
Figure 2.6
L2-L5 DRG, blood, and lumbar enlargement of spinal cord were isolated from a naïve female C57Bl/6J mouse and stained with indicated markers. Graphs represent Ly6G-CD11b+ macrophages stained for CX3CR1 (a-c), MHC Class II (d-f), CD39 (g-i), Ly6C (j-l), and CCR2 (m-o).
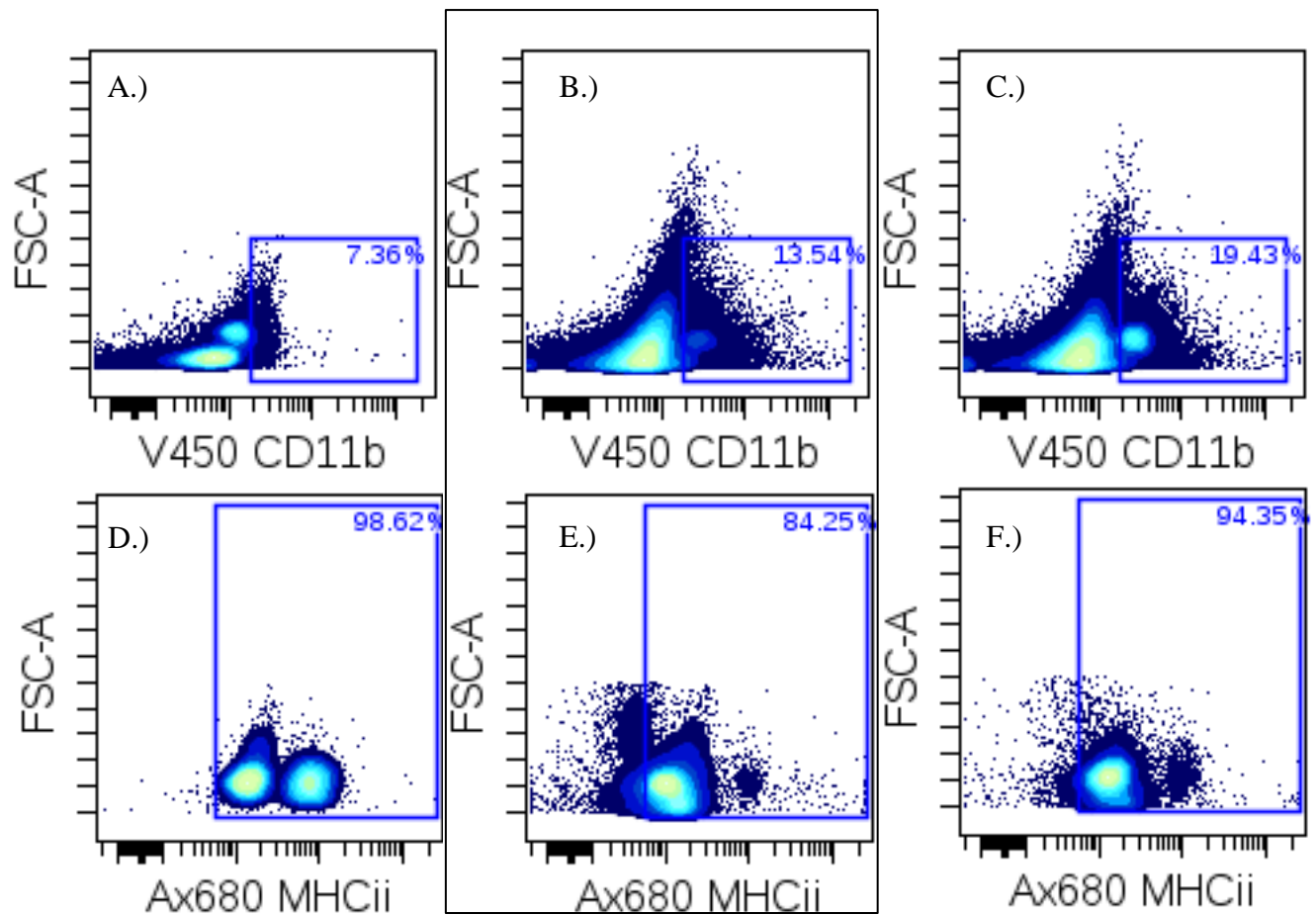
Figure 2.7
Blood, DRG and Spinal Cord were collected from naïve Sprague Dawley male rats by cardiac puncture and dissection, respectively. All tissues were stained with antibodies against CD11b, Ly6G, and MHC II. Graphs (A, B, C) were gated on live, singlet cells determined by forward and side scatter analysis. Graphs (D, E, and F) were gated on CD11b^{+}, Ly6G^{-} cells.
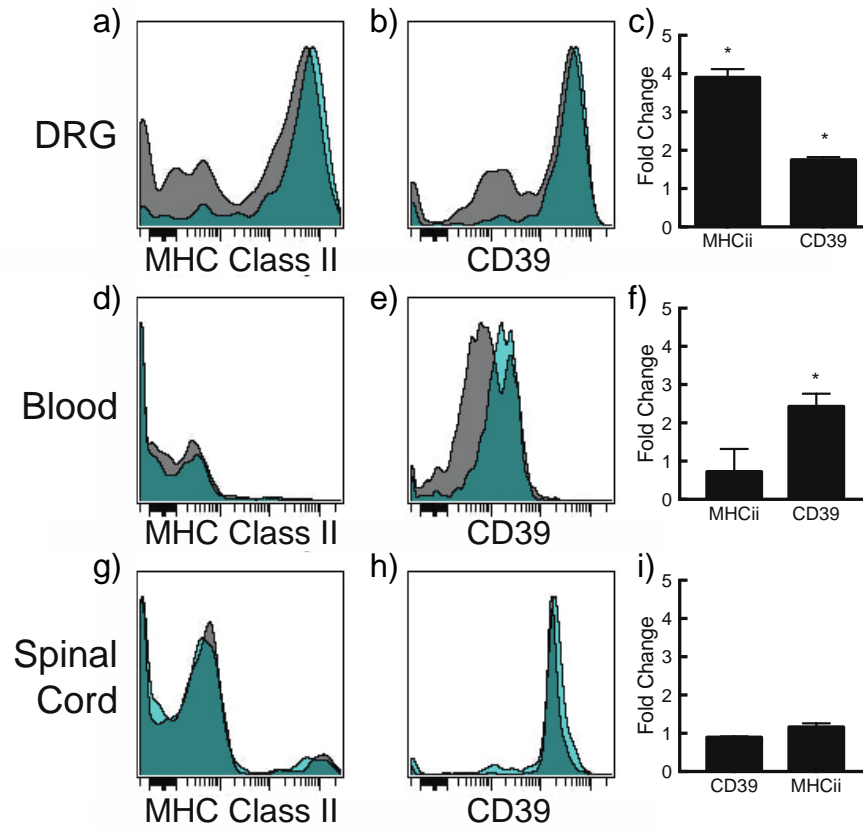
Figure 2.8
Up regulation of CD39 and MHC II on CD11b $^+$ Ly6G $^-$ mononuclear cells following CFA injection in L2-L5 murine DRG in male C57Bl/6J mice. Specific upregulation of MHC II on DRG resident tissue macrophages (c). No significant upregulation of MHC II on blood monocytes or microglia (f, i). Fold change calculated by percent of cells in gate, *, p <0.05.
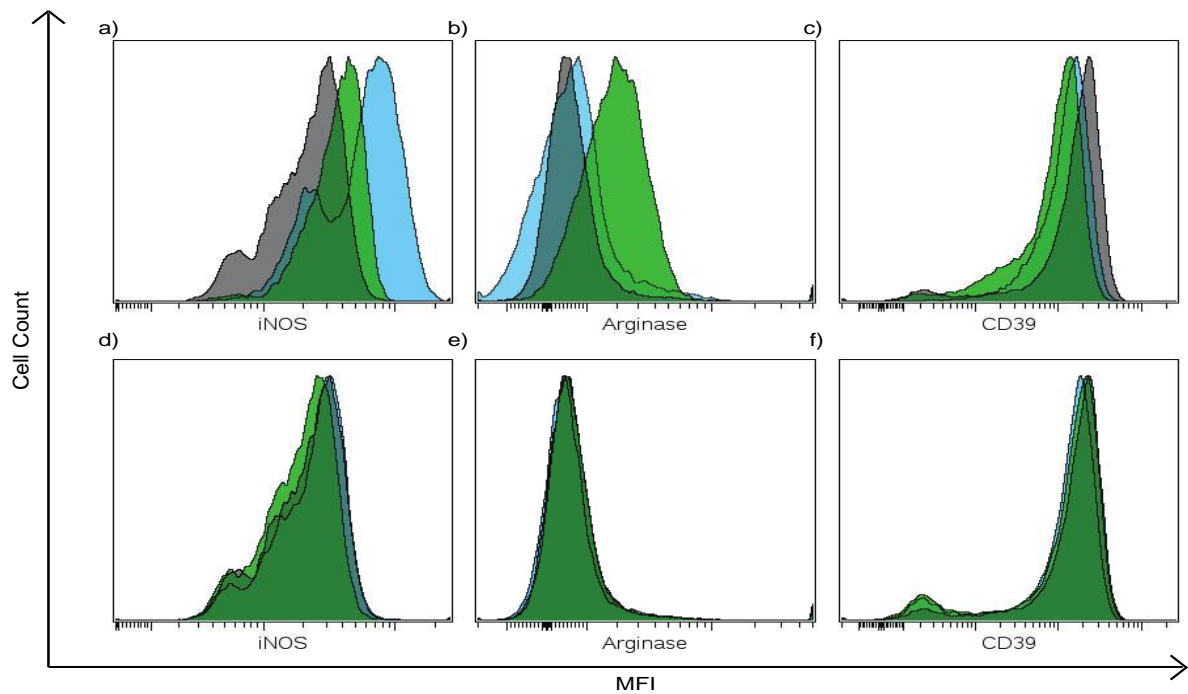
Figure 2.9
Bone marrow was isolated from femora and tibiae of female wildtype C57Bl/6J mice and cultured. A-C: Undifferentiated (grey), M1 (blue) or M2 (green) macrophages were induced. Control differentiation (a, b, c) expression changes in M1 marker iNOS and M2 marker arginase are shown for reference. D-F: Capsaicin 100nm (blue) or CFA 10ng/mL (green) was added to undifferentiated culture and levels of iNOS, arginase and CD39 were measured after 24 hours.

Female C57Bl/6J

Female C57Bl/6J

Figure 2.10

3 Days post either bilateral CFA injection or saline in the hind paws, C57Bl/6J female L2-L5 DRG were rapidly dissected and dissociated into culture media. Cells were cultured for 16 hours. Chemokine and cytokine production were evaluated from both saline(Grey) and CFA treated mice(Blue). n=4, * $p < 0.05$, two tailed student's t –test.

Figure 2.11
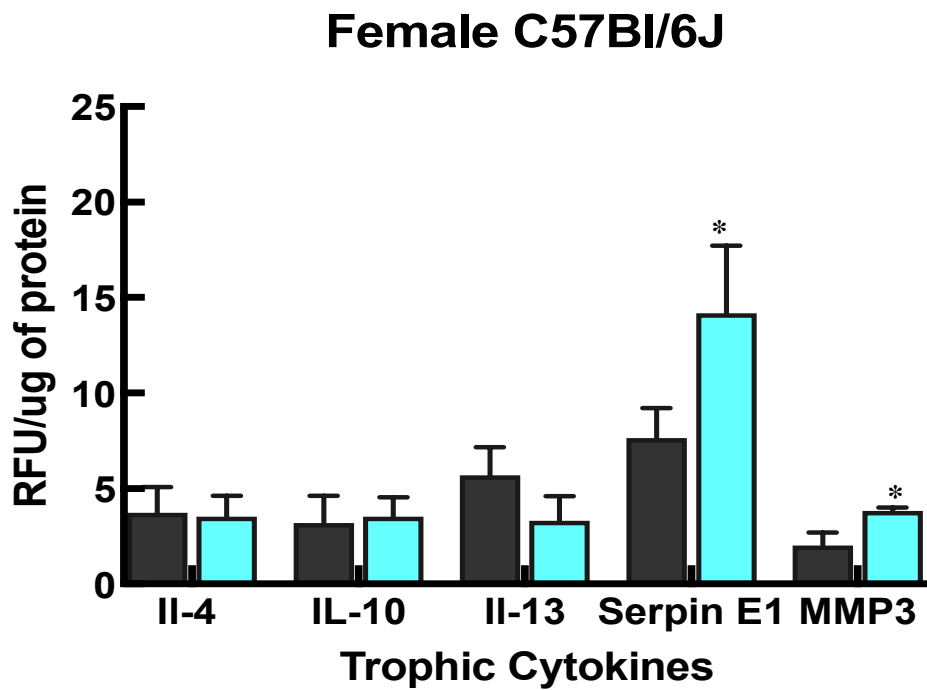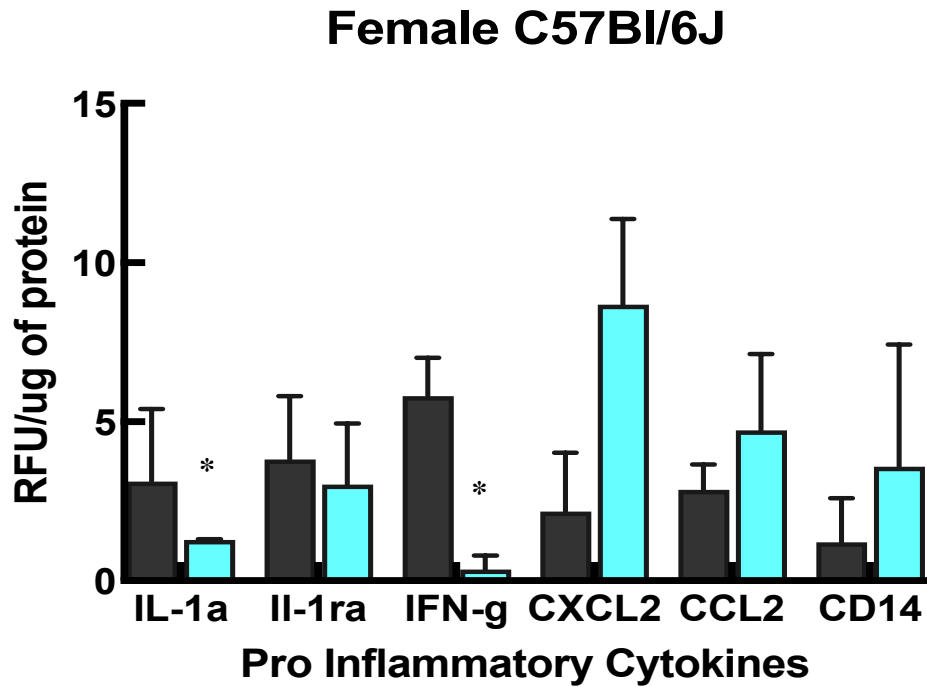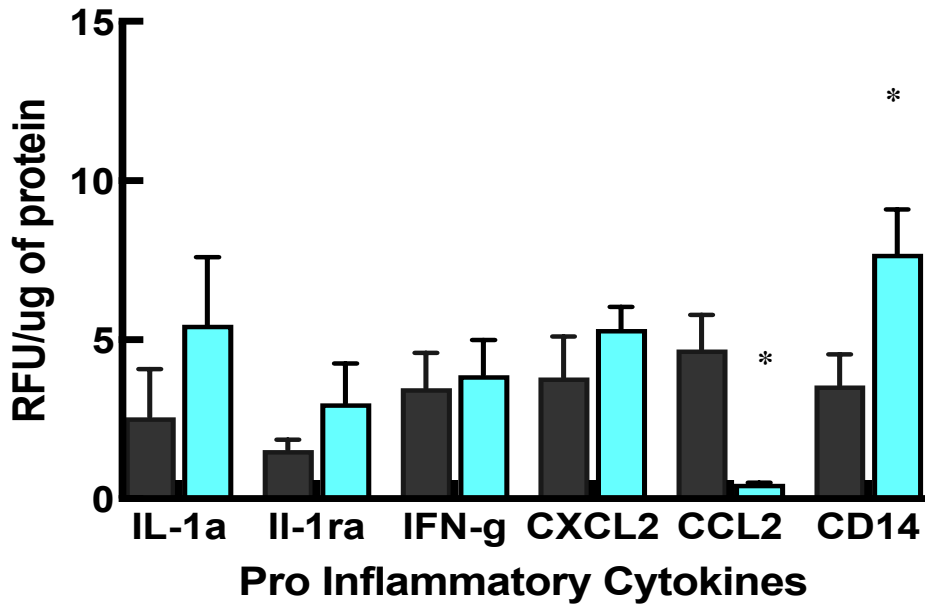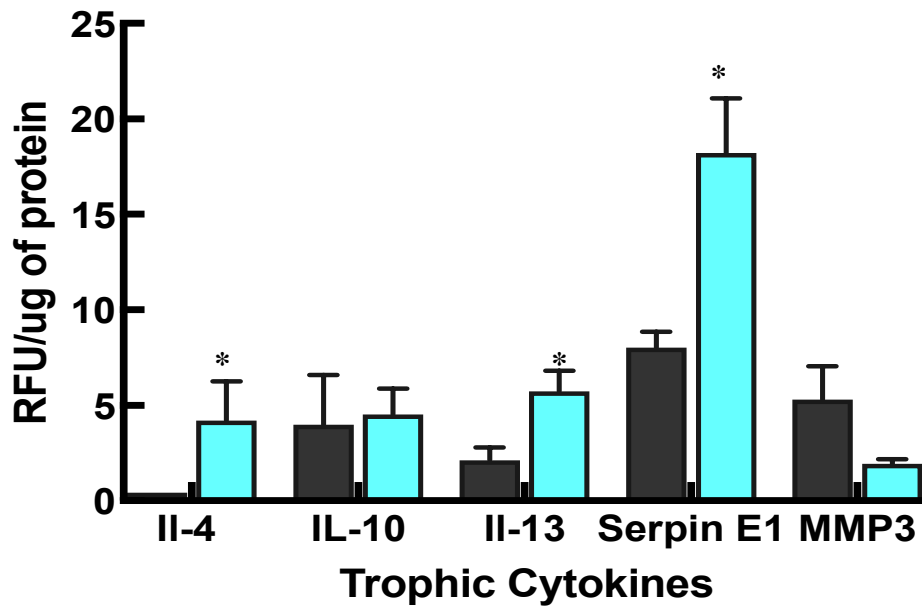3 Days post either bilateral CFA injection or saline in the hind paws, C57Bl/6J male L2-L5 DRG were rapidly dissected and dissociated into culture media. Cells were cultured for 16 hours. Chemokine and cytokine production were evaluated from both saline(Grey) and CFA treated mice (Blue). n=4, * p< 0.05, two tailed student's t –test.

CHAPTER 3

DISCUSSION

Studies show that nearly 100 million people suffer from chronic pain in the U.S. today [1]. Current therapies to treat pain, including opioids and NSAIDS, come with undesired side effects and often fail to provide lasting benefits. Understanding the basic mechanisms of pain in the ascending pathway and novel interactions between neurons and surrounding cells in the sensory ganglia is the first step to designing and implementing novel therapies.

Here we have shown that not only do significant macrophage populations exist in naïve sensory ganglia but they actively respond to neuronal stimulus. Naïve populations of resident tissue macrophages represent, phenotypically a hybrid of microglia and blood monocytes, expressing similar phenotypes distinct to both cell types. Thus, these cells may respond in similar but highly specialized manner to cellular changes in the dorsal root ganglia microenvironment.

The data presented here suggest that DRG macrophages have evolved to patrol and interact within the unique environment of the dorsal root ganglia. It is possible that the lack of blood brain barrier protection in DRG contributes to the phenotype of macrophages expressed in naïve and injured conditions. High expression of MHC Class II may provide ongoing antigen recognition ability required to protect neuronal cell bodies. Exposure of cells within DRG to pathogens and systemic cytokines is compounded by the highly vascular nature of the dorsal root ganglia, thus elevated patrolling and monitoring of pathogens may be warranted for neuronal protection. Furthermore, MHC Class II has been widely associated with pro inflammatory states of macrophages, suggesting that the primarily M2 characteristic macrophages we describe in naïve DRG may be primed for rapid conversion to M1 inflammatory macrophage activity under pathological conditions. The functional state of DRG macrophages may play a role in persistent pain development as evidenced by our data

showing rapid upregulation of MHC II and CD39 in the lumbar dorsal root ganglia following intraplantar injection.

Persistent pain may arise from aberrant continual inflammatory input, thus understanding the complex milieu of cytokine and chemokines in the dorsal root ganglia following peripheral injury may illuminate inflammatory mechanisms of persistent pain. Here we have shown distinct cytokine regulation in male and female mice following CFA injection in the hind paw. Interestingly, the cytokine milieu consists of both pro and trophic- inflammatory signals in both sexes indicating a complex immune response network. We hypothesize a biphasic response in which initial pro inflammatory cytokines are supplanted by trophic-inflammatory cytokines as normal inflammatory response dies down. To confirm, these experiments will need to be repeated in neuropathic pain injury models. Our data indicate that in the time course of resolution, 3 days following CFA injection mice exhibit peak pain behaviors. Thus, the 3-day time point may be a turning point where both pro- and trophic inflammatory functions coincide. A complete time course will need to be completed to resolve overlapping immune response questions.

A consistent upregulation of serepin E1 was noted in both male and female CFA treated animals. We hypothesis that this finding may be indicative of a neuronal protective adaption by macrophages in which they reduce scar tissue formation following injury. Serepin E1 inhibits urokinase-type plasminogen activator (uPA) and tissue-type PA (1, 2) which both play a role in angiogenesis and extracellular matrix remodeling [88]. Thus macrophages may initiate a neuronal protective mechanism, by inhibiting scar tissue formation with the production and release of serpin E1 following peripheral injury. Further studies such as investigation of fibrin and extra cellular matrix remodeling following peripheral injury may further evaluate the role this may play in neuronal plasticity and pain processing.

Communication between neuronal cell bodies and resident tissue macrophages may occur, especially under times of intense DRG neuronal stimulus, such as following distant injury or chronic pain states. To this end ectonucleotidase triphosphate diphosphohydrolase-1, CD39, hydrolyzes purinergic receptor ligands in specific P2 receptors ligands such as ATP. Neuronal expression of purinergic receptors able to respond to ATP and AMP the substrate and product of CD39 indicate a potential mechanism of communication between neurons and macrophages [62]. High naïve expression as well as upregulation following peripheral insult (CFA) indicate this potential communication mechanism is altered under pathological states and may provide an effect therapeutic target to further alleviate pain states.

Modulation of ATP to AMP by CD39, provides the substrate for subsequent enzymes such as CD73 which hydrolyze AMP to adenosine [62]. Adenosine has been implicated in numerous mechanisms of pain processing [86]. Receptors on sensory neurons such as $A_{2A}R$ have been shown to exhibit pro-nociceptive properties and may be activated by ligand production from resident tissue macrophages [87]. Thus regulation of purinergic ligands in the extracellular milieu by macrophages may impact pain processing and be a potential therapeutic target. Further pharmacological studies inhibiting CD39 will be needed to elucidate the therapeutic potential of anti-CD39 therapies.

Future studies involve macrophage response to purinergic receptor ligands, looking for calcium influx changes in the cell. Furthermore, identifying phosphorylation changes in macrophage following ATP exposure, will help elucidate the intracellular signaling cascade that may drive phenotypic as well as functional changes in resident tissue macrophages. Additionally, we hope to evaluate human DRG macrophages and acutely culture isolated macrophages form human tissue samples. Comparing human DRG macrophages to data collected in mice and rats will support or reveal limitations of these findings to human pain processing.

Our findings suggest not only is there a significant population of resident tissue macrophages in the dorsal root ganglia but they are able to respond to distant insult with rapid phenotypic changes. Given the severe lack of treatment modalities for persistent pain, these discoveries may aid in the identification of therapeutic targets as well as our general knowledge of the pathological mechanisms of persistent pain.

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CHAPTER 4

PREAMBLE- The following chapter will be written in a narrative as it outlines the struggles as well as other preliminary data collected during my time in the lab.

APPENDIX DATA

METHODS

von Frey- Tactile sensory thresholds were determined by applying von Frey filaments (North Coast Medical, CA) to the plantar surface of the left hind paw and recording the withdrawal response using the up-down method (Dixon, 1980). Mice were placed in a clear Plexiglas chamber with a wire mesh floor and allowed to habituate for 1 hour. Testing was conducted using filament sizes with respective bending gram forces of 0.04, 0.07, 0.16, 0.4, 1.0, 2.0 and 4.0, starting filament = 0.4 g. Filaments were pressed upward into the soft spot of the left hind paw until the filament was bent in half for a duration of 5 seconds. A response was recorded if the mouse expressed a lifting, shaking or licking behavior toward the hind paw during the application of the filament.

Hargreaves - Thermal sensory thresholds were determined by assessing the latency of each mouse to respond to a noxious radiant heat source (Plantar Test, Ugo Basile, Italy; IR set to 28) (Hargreaves et al., 1988). Following a 60-minute habituation period, the heat source was positioned directly beneath the left hind paw to obtain withdrawal latencies. A maximal cut-off of 30 seconds was utilized to prevent tissue damage.

Cell Fractionation – Cells were centrifuged at 150g for 5 minutes and supernatant removed. Cells were resuspended in 90 μl of HBSS (Ca^{2+}/Mg^{2+} free) pH 7.2 with 10 μl of CD11b (Miltenyi Biotec) microbeads and placed on ice for 15 minutes. Added 2 mL of buffer (HBSS- Ca^{2+}/Mg^{2+} free with 0.5% BSA) to cell suspension. Centrifuged cell suspension at 150Xg for 5 minutes. Aspirated supernatant

completely. Resuspended cell pellet in 500 uL of buffer (HBSS- Ca^{2+}/Mg^{2+} free with 0.5% BSA). Fractionated cells through large cell column (MACS) in HBSS- Ca^{2+}/Mg^{2+} free with 0.5% BSA. Unlabeled cells were further fractionated into neuronal and non-neuronal preparations.

Percoll Gradient- Rapidly dissociated dorsal root ganglia were placed in 37% Percoll in 1x PBS and layered in a 15 mL conical tube with 2mLs of 70% Percoll, layered with 2 mLs of 37% Percoll with cells, followed by 2 mLs of 30% Percoll solution then 2mLs of HBSS. Centrifuge at RT for 30 minutes at 1760 rpm with no break on centrifuge. Harvest the 37/70 interface (mononuclear immune cells). Transfer into fresh culture media.
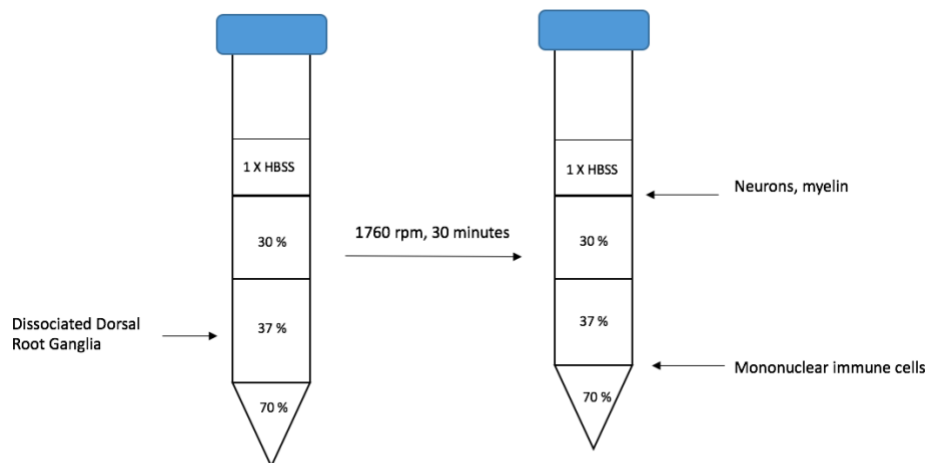
Culture of human blood monocytes- Healthy monocyte differentiation assays: Peripheral Blood Mononuclear Cells (PBMCs) were isolated from whole blood by Ficoll-Paque gradient (GE Healthcare). Cells were cultured in a 5% CO2 incubator at 37 C in RPMI 1640 media supplemented with Gentamicin (8ug/ml) Penicillin /Streptomycin (100 UI/ml), human macrophage colony stimulating factor (M-CSF 20ng/ml), and 2.5% human plasma (from the PBMC donor) or 10% FBS. 4 hrs. Post seeding all media was changed in all wells removing all floating cells. At 3 days following monocyte isolation 50% of the cell culture media will be replaced per day. On day 5, macrophage differentiation was initiated. Cultures will be incubated for 2 days with no stimulation to create

undifferentiated controls; or either lipopolysaccharide (LPS 10ng/ml) and interferon gamma (50 ng/ml) - to induce an M1 inflammatory macrophage differentiation - or transforming growth factor beta TGF-B (10 ng/ml) and interleukin 4 (IL-4 20 ng/ml) -for M2 trophic macrophage differentiation. Day 7, differentiated macrophages were detached from culture plates with 20 minutes of cold incubation and Versine buffer (0.02% EDTA) and gentle scraping.

RESULTS.

To investigate a means to remove neuronal cells from the dorsal root ganglia dissociation, we tried to use a Percoll gradient. Successful separation of mononuclear immune cells from myelin and most neuronal cells was achieved but due to the time and stress on the cells during the Percoll protocol many if not all macrophages exhibited autoflorescences (Figure 5.1). We subsequently tried to bead sort using Miltenyl Bead Sorting with pre conjugated magnetic beads against Cd11b (Cat#130-049-601) however cell yield was low and only ~50% of cells survive the separation.

In addition to the above data we also collected information about the infiltration of neutrophils and macrophages into the dorsal root ganglia following peripheral injury. To this end, we found a decreased number of neutrophils and macrophages in the DRG following CFA administration (Figure 4.2). Contrary elevated levels as expected were found in the blood of the animals following intraplantar injections of CFA.

Understanding the pathology of human dorsal root ganglia macrophages may be difficult as human samples require facilities and set ups that allow seamless transfer of the tissue in a timely fashion from the donor patient to the researcher. However, human isolated monocytes from leukopacs may give the first insight to possible interactions of resident tissue macrophages in the dorsal root ganglia of humans. Here we successfully isolated human monocytes from healthy donor blood and differentiated them into trophic and pro inflammatory types (Figure 5.3).

In an effort to explore novel therapeutics, CB2 agonist Gp1a was administered to mice following CFA injection. Behavioral testing indicated systemic (IP) injection of Gp1a during chronic pain states may attenuate pain more rapidly than saline treated counter parts (Figure 5.4).

The pain input of macrophages is still widely unknown; here we deplete resident tissue macrophages in ITGAM-DTR/EGFP female mice prior to CFA injection to understand the pain

behaviors of DRG resident tissue macrophage depleted mice. ITGAM-DTR-EGFP mice demonstrated

a resistance to thermal pain as seen by a lack of hypersensitivity to IR behavioral tests (Figure 5.5).
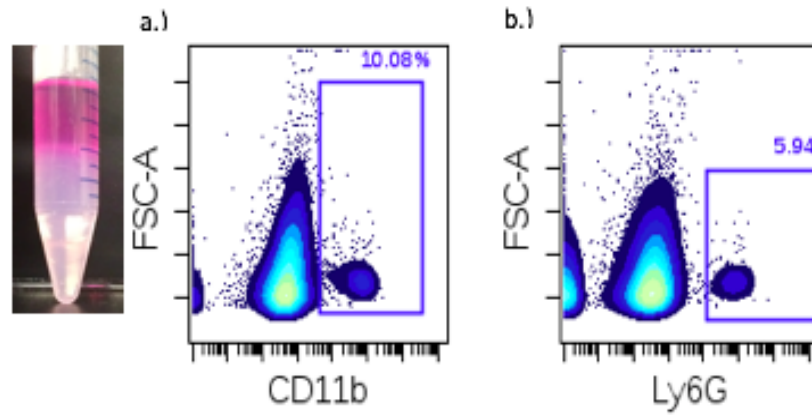
Figure 4.1
Flow Cytometry of naïve male CD115-Egfp, L2-L5 DRG following dissociation and Percoll gradient separation, all graphs gated on live singlet cells (a) Cd11b+ cells (b) Ly6G+ cells.
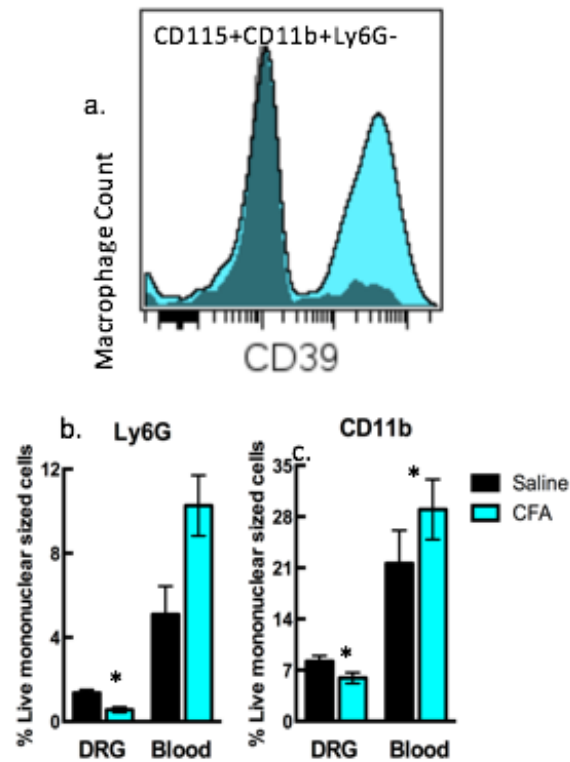
Figure 4.2
24 hours following intraplantar injection of either saline or CFA, female C57/B6N, L2-L5 DRG were rapidly dissected and dissociated and stained with various macrophage and neutrophil markers. CD115+CD11b+Ly6G- tissue resident macrophages unregulated CD39 (a) where grey histograms represent saline treated animals and teal CFA treated. Macrophage and Neutrophil percent of total CD115 cells reported in DRG.
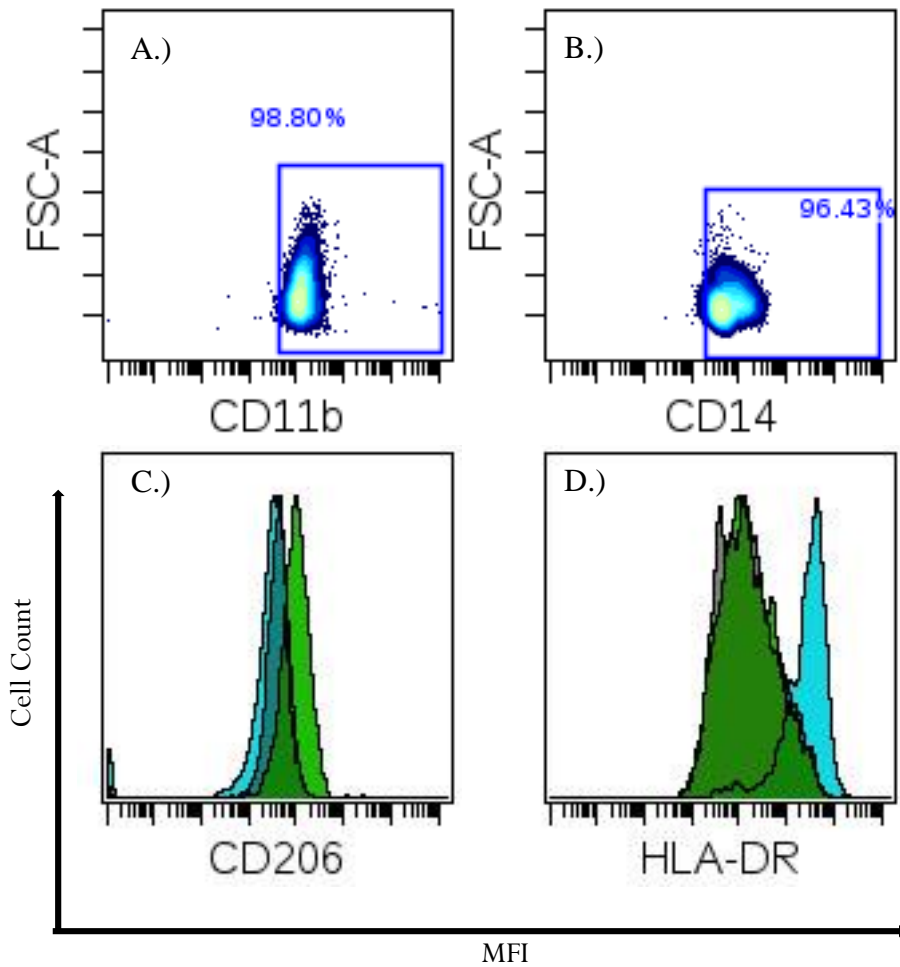
Figure 4.3
Human macrophages were acutely cultured from human leukopacs. Top Row: Acutely cultured macrophages express CD11b and CD14 traditional macrophage lineage markers (A, B). Undifferentiated (grey), M1 (blue) or M2 (green) macrophages were induced. Control differentiation (C, D) expression changes in M1 marker HLA-DR and M2 marker CD206 are shown for reference.
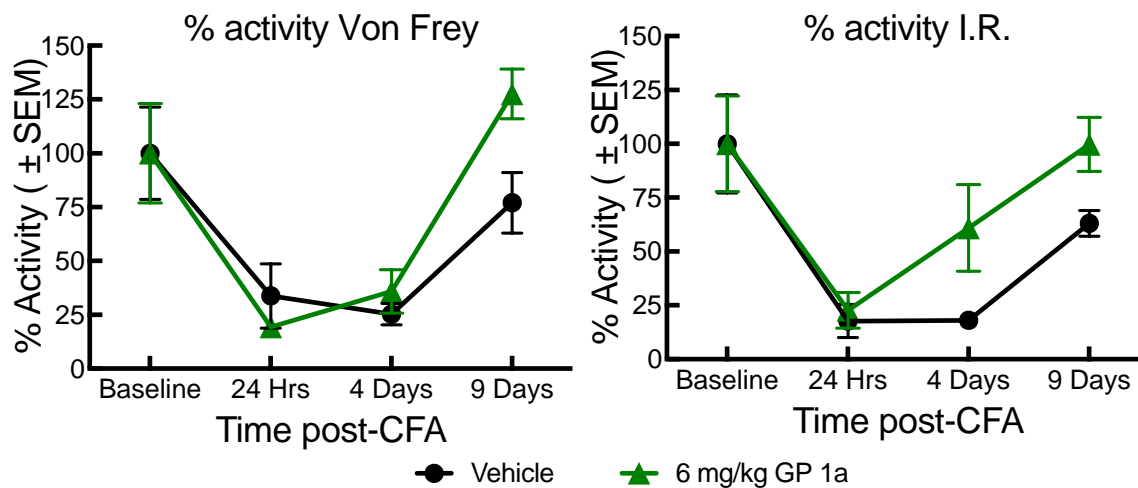
Figure 4.4
Von Frey and IR analysis of mice treated with GP 1a, a CB2 receptor agonist. Wild-type C57Bl6/J female mice were baselined prior to hind paw injection with Complete Freund's Adjuvant (CFA) and intraperitoneal injection of 6mg/kg GP 1a or vehicle. Analyses were repeated at various points throughout the inflammatory state.
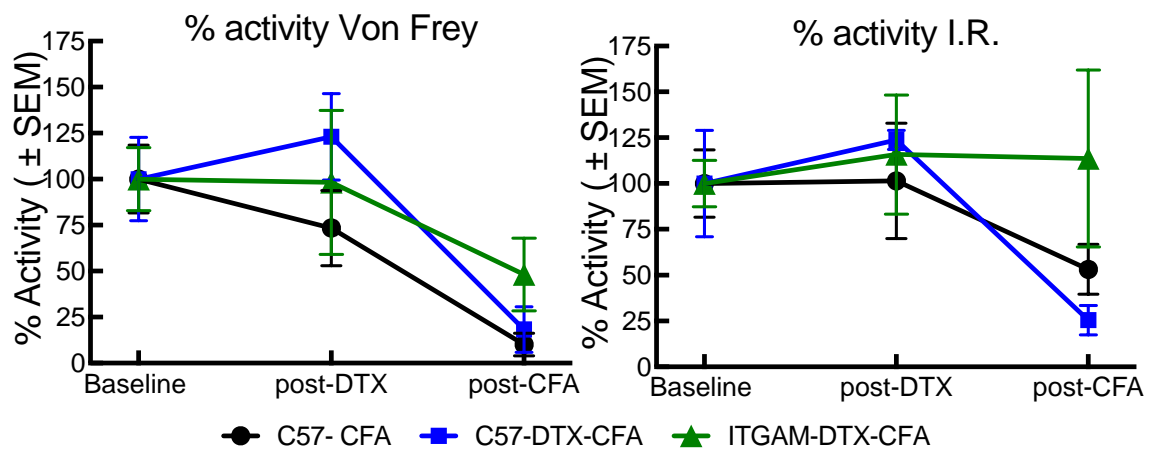
% activity Von Frey

% activity I.R.

Legend: ● C57- CFA   ■ C57-DTX-CFA   ▲ ITGAM-DTX-CFA

Figure 4.5

CD11b+ cells were depleted in ITGAM-DTR/EGFP female mice via DTx injection. 24 hours post-macrophage depletion, mice received hind paw injection with CFA and subjected to von Frey/IR analysis at several time points and compared to wild-type.

DISCUSSION OF APPENDIX DATA

Here our results show that Percoll separation does eliminate significant neuronal contamination as well as myelin from the DRG tissue preparation. However, the limited resident tissue macrophages isolated from the mouse lumbar ganglia limits the ability to successfully culture pure macrophage cultures, limiting the scope of our studies currently.

When evaluating the neutrophil and CD11b overall population in the dorsal root ganglia we saw a reduction of CD11b and Ly6G positive cells in the DRG whereas the blood saw a noticeable increase. This may be the result of activated immune cells tracking out of the dorsal root ganglia following injury, further supported by the high expression of the antigen presentation protein MHC Class II which may drive the cells to migrate to lymph nodes following injury to initiate an adaptive immune response. However further studies understanding the infiltration of blood monocytes and proliferation of resident tissue macrophages in response to injury is needed.

Human studies have also proven hard to conduct due to the limited access to fresh non fixed human dorsal root ganglia. Here we attempt to isolate blood monocytes from human leukopacs and differentiate them into pro or trophic states. Here we were able to establish an isolation and culture protocol of human blood monocytes as well as successfully differentiate these cells into a pro and trophic state as seen by marked upregulation of CD206 and HLA-DR (MHC Class II) on CD14+ human macrophages. Using human isolated macrophages, we can further begin to understand the potential response of DRG macrophages in human pain processing and their response to various purinergic agonist.

Presently, a small body of evidence suggests the existence of resident DRG macrophages and their phenotypic changes following insult. Supporting a link between macrophages and inflammatory pain, studies demonstrate that cannabinoid receptor type 2 (CB2) agonists attenuate pain in acute,

chronic, neuropathic, and inflammatory states and suppress pro-inflammatory cytokine release. Additional studies suggest macrophage activity is altered by CB2 activation.

Our findings suggest an analgesic effect following systemic GP 1a administration in a mouse model of inflammatory pain as well as diminished CFA-induced hypersensitivity in macrophage-depleted mice. More studies should be conducted to confirm these findings and determine if DRG macrophages undergo phenotypic changes in response to insult or CB2 agonists. These data will be critical for determining the role of DRG macrophages in nociceptor remodeling and sensitization. The results of future studies may lead to the development of novel therapeutic approaches for the management of inflammatory pain.

MATERIALS USED.

GENE	Forward Primer	Reverse Primer
CD11b	5'-TTACGTAGCGAATGACCCCT-3'	5'-GTTTCACAAAACCAATGGGG-3'
CD39	5'-TCAGTCCCACAGCAATCAAA-3'	5'-ACGAGGGAAGAGGAAGGAAA-3'
CD115	5'-AGGGCAACTGAGTAGGGTCA-3'	5'-CTCTGCTGGTGCTACTGCTG-3'
CD163	5'TGTATGCCCTTCCTGGAGTC-3'	5'-TGTGCAGTGTCCAAAAGGAG-3'
Arginase-1	5'TTTTCCAGCAGACCAGCTTT-3'	5'-GGAACCCAGAGAGAGCATGA-3'
CCL-2	5'- ATTGGGATCATCTTGCTGGT-3'	5'- CCTGCTGTTCACAGTTGCC-3'
CSF-1	5'GGTAGTGGTGGATGTTCCCA-3'	5'CCAGGATGAGGACAGACAGG-3'