UNIVERSITY OF CALIFORNIA SAN DIEGO

The Immune System's Role in Neuroma Progression

A Thesis submitted in partial satisfaction of the requirements
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in

Biology

by

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Professor Cressida Madigan

2022

The Thesis of Carmelina Maria Azar is approved, and it is acceptable in quality and form for publication on microfilm and electronically.

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ABSTRACT OF THE THESIS

The Immune System's Role in Neuroma Progression

by

Carmelina Maria Azar

Master of Science in Biology

University of California San Diego, 2022

Professor Sameer Bhugru Shah, Chair
Professor Cory Matthew Root, Co-Chair

Neuromas are a type of chronic peripheral nerve injury resulting from unsuccessful nerve regeneration. Neuromas contain improperly woven together axons and result in increased pain and sensitivity. In this study, I explored the role of complement proteins and inflammasomes in early neuroma formation using a mouse model. I conducted survival sciatic nerve transection surgeries on mice, severing one sciatic nerve in each mouse. The proximal stump of the transected nerve and the contralateral uninjured nerve of each mouse were harvested at either a

one- or two-week timepoint. I performed RT-qPCR on the proximal injured stumps and the contralateral uninjured nerves. I present data indicating a significant increase in the presence of inflammasome markers ASC and NLRP3 and complement protein C1qB in the injured compared to the control nerves of mice in the two-week timepoint. We also present preliminary findings that suggest an increase in the presence of C1qB, ASC and NLRP3 from the one- to the two-week timepoints. These findings pave the way for a clearer understanding of how the complement system and inflammasomes affect neuroma progression and whether they could be a target for clinical interventions in the future.

LITERATURE REVIEW: THE COMPLEMENT SYSTEM'S ROLE IN PERIPHERAL NERVE INJURY

The Peripheral Nervous System

The peripheral and central nervous systems are the two main branches of the nervous system, a command center carrying electrical signals throughout the body. The central nervous system is the brain and spinal cord, whereas the peripheral nervous system (PNS) is composed of nerves branching out of the spinal cord. The PNS allows for widespread communication between the central nervous system and the body [1].

Peripheral nerves are a composite tissue consisting of many cell types. The primary electrical cells of nerves -- neurons -- are encased by myelinating or non-myelinating Schwann cells, and are surrounded by endoneurial connective tissue. Many nerve fibers bundled together form a fascicle and are surrounded by perineurium, another connective tissue layer. A typical nerve is composed of several fascicles, which are surrounded by the epineurium, a third connective tissue layer. Given the architectural complexity of the extracellular matrix, fibroblasts are abundantly present between nerve fibers. Nerves are also highly vascular, with intrinsic vessels within the nerve trunk and a segmental nerve supply via the nerve bed [2].

The Acute Phase of Traumatic Nerve Injury in the PNS

When nerves are severed, an acute injury state is induced. This acute phase involves a strong inflammatory response, characterized by the quick and local production of inflammatory cytokines (i.e TNFα, IL-1α and IL-1β) and chemokines at the site of injury [3-4]. Additionally, biological and physical changes occur in the proximal and distal stumps. The proximal stump is the part of the nerve still attached to the cell body while the distal stump is attached to the end of the axon. Since the proximal stump is still attached to the cell body, it is considered "alive" and

is the point from which the nerve will regrow to connect with the distal stump. In the distal stump, the upregulation of TNFα and IL-1α drives Wallerian degeneration, a coordinated response that in mice starts 3-4 days and ends 12-14 days after injury [3]. During Wallerian degeneration, cytokines and chemokines recruit macrophages, which, along with resident Schwann cells, clear out degraded axons and myelin. Degraded myelin takes up space, contains anti-regenerative molecules, and can inhibit axon renewal [4-6]. The faster the degraded axons and myelin can be cleared, the faster regeneration is hypothesized to begin. Therefore, rapid and early Wallerian degeneration may lead to more effective nerve repair [7-8]. Concurrently, the basal lamina compacts into Bands of Bungner, along which injured neurons regrow [19]. Finally, resident cells upregulate and secrete neurotrophic factors, which also help neuronal survival and regenerative axonal growth [3].

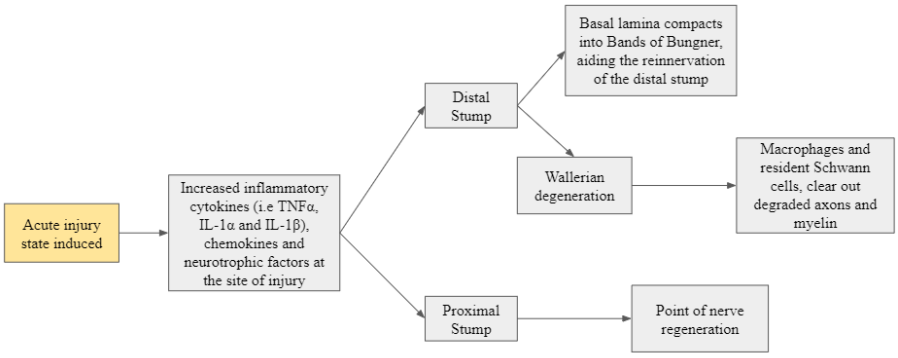

Figure 1: Acute Nerve Injury in the PNS

The Chronic Phase of Peripheral Nerve Injury in the PNS

Beyond the acute injury phase, over a time frame of weeks to months, nerves that remain unrepaired progressively enter a chronic injury state. This state is characterized by structural changes in the extracellular matrix of the distal stump, the downregulation of neurotrophic factors and the loss of macrophage and Schwann cell's ability to clear out degraded axons and

myelin. This may be a consequence of chronic inflammation, which can have major adverse effects, locally and systemically [9]. Long-term impacts include limitations on the number of axons that can grow through a distal stump as well as degenerative changes in targeted motor and sensory organs [10-11]. In the proximal stump, a number of changes also occur, including initial recoil of the nerve stump, a disorganized cytoskeleton at axonal termini, and impaired axonal transport [13-14]. A particularly adverse response of inadequately repaired or unrepaired nerves in a chronic injury state is the presence of neuromas at the injured proximal stump, which emerge as a result of uncontrolled neuronal tissue growth at the site of nerve injury [12].

The literature suggests that several of these processes may be strongly influenced by the immune response, and in particular, a component of the immune response known as the complement system.

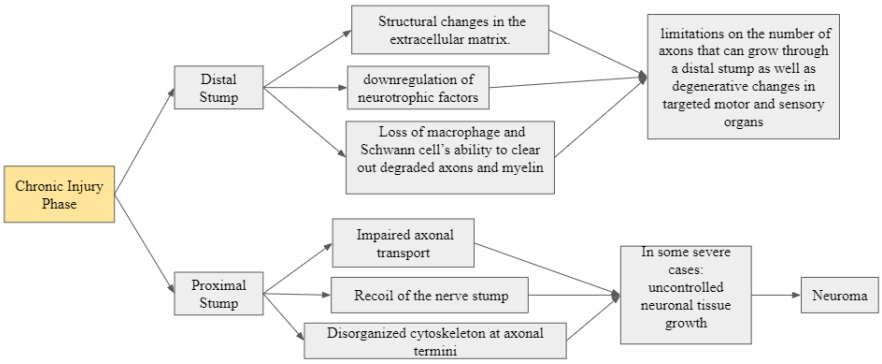

Figure 2: Chronic Nerve Injury in the PNS

The Complement System

The complement system, an integral component of the innate immune system, is composed of more than 40 proteins that are dissolved in the blood, body fluids and tissues. Playing a large role in inflammation, lysis, and opsonization, the complement system is a rapid

first line defense against invading pathogens and plays an important role in tissue injury response and repair. Its main activation pathways are generally classified as the classical, alternative and lectin pathways. While each pathway is differentially activated, they all converge in the creation of C3a and C5a, which are proinflammatory mediators (anaphylatoxins), and the C5b fragment, which forms the C5b-9 membrane attack complex (MAC). The MAC causes cell lysis by punching holes in the cell membrane of invading pathogens [15].

Although the complement system is a much needed defense against invading pathogens, it needs to be tightly regulated as it is not foreign pathogen/tissue specific; indeed, an unchecked complement response can damage healthy host cells and tissue. One component of autoregulation is a negative feedback loop where complement protein C3b binds to Factor H, inducing Factor I to feedback and inhibit C3b. In opposition, complement up-regulators like properdin will inhibit Factor I. Another auto-regulatory mechanism is the fact that C3b molecules have short half-lives, so they can only act on their site of activation before decaying. This prevents the spread to other sites and limits the spatial extent of healthy host cell destruction [16, 17-18]. Beyond defense against pathogens, a properly regulated complement system is also important for wound healing and the repair of tissues, including nerves. In the subsequent sections we will detail a number of findings associated with roles and regulators of complement activation in the context of peripheral nerve injury and regeneration.

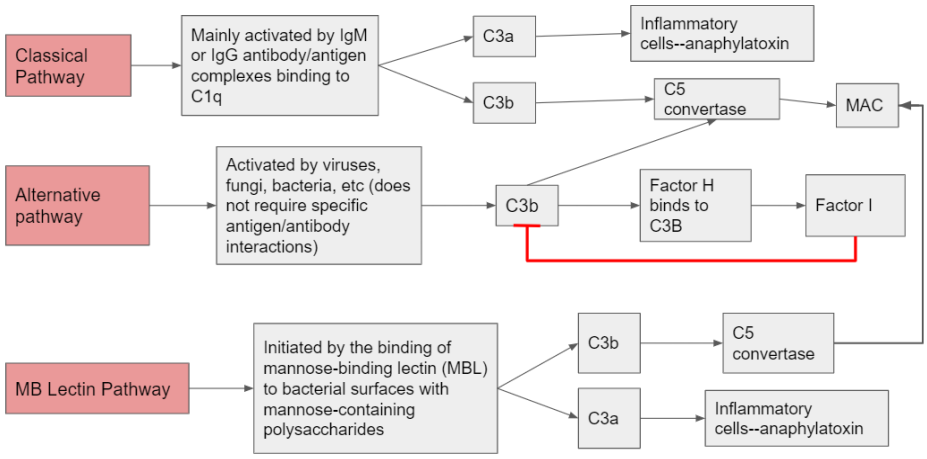

Figure 3: Complement Activation

Complement activation during peripheral nerve injury and nerve regeneration

Within nerves, complement mRNA is located in the peripheral nerve trunk and is most likely produced by Schwann cells [20]. The axons of a peripheral nerve contain complement proteins, however, they do not contain any complement regulatory elements. Rather, the regulatory proteins are found in the perineurium, the connective tissue that encases and protects a nerve fascicle [20]. In a non-injured peripheral nerve, the complement system is believed to serve as surveillance against invading pathogens [17].

In injured nerves, complement pathways are activated, and contribute to the regulation of Wallerian degeneration. Such regulation must be tightly controlled, because 1) unchecked Wallerian degeneration can damage nearby healthy or regrowing axons, but 2) lingering degenerative material will impede the nerve's ability to regenerate [3]. While the timing of regulation is not completely clear, it is known that complement activation and Wallerian degeneration are closely coupled following nerve injury. The primary influence on Wallerian degeneration is through MACs. Upon traumatic injury to the nerve, nerve stumps distal to the site of injury begin Wallerian degeneration. An hour after injury, degenerated myelin activates

resident complement proteins. These complement proteins create MACs that emerge within 12 hours and remain up to 3 days. MACs accelerate Wallerian degeneration by destroying axons and myelin nearby; without MACs, Wallerian degeneration, which typically will have been initiated by 3 days post-injury, would occur too slowly, impeding nerve regeneration [3, 21]. Degenerated myelin also induces the complement system to create C3bi, a complement protein that opsonizes myelin and targets it for removal. Complement C3 can then bind to opsonized myelin, causing a 2 to 3 fold increase in myelin phagocytosis. If the destroyed myelin is not removed fast enough, it will impair nerve regeneration. Therefore, the complement system accelerates the recruitment of professional phagocytes to the injury site [22].

Interactions between complement and Wallerian degeneration also affect axonal regeneration through the regulation of neurotrophic factors. For example, *Wld*^{s} mice, which exhibit slow and delayed Wallerian degeneration, do not exhibit upregulation of neurotrophic factors after peripheral nerve injury [3, 23]. Though complement cascades have not been studied in *Wld*^{s} mice, given the critical role of MAC in modulating Wallerian degeneration, [21] this suggests that the complement system may also influence neurotrophic factor expression. In addition, the complement system interacts with inflammasomes, which play a role in the inflammatory response in tissue and nerve injury [24-26] and are usually present at the site of injury. Inflammasomes are activated and promote the release of interleukin-1β (IL-1β) and IL-18. This inflammatory response contributes to regenerative processes in both the proximal and distal stumps.

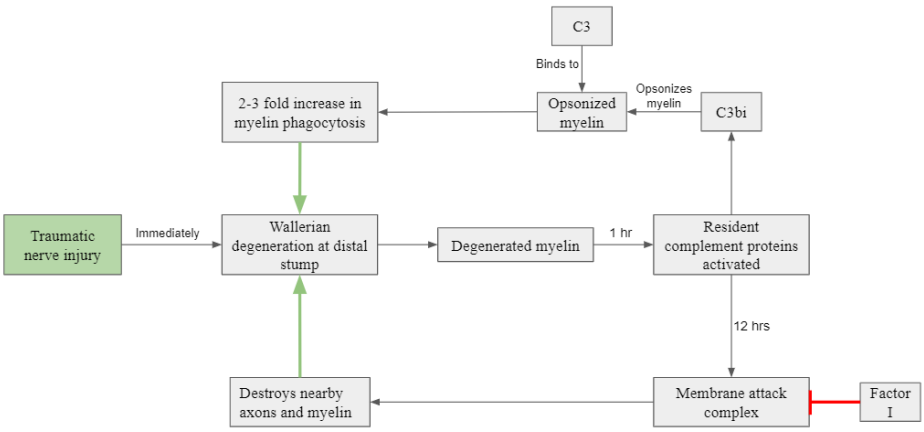

Figure 4: The Complement System and Wallerian Degeneration

Regulation of the complement system in peripheral nerve injury

The complement system must closely self-regulate in order to limit unnecessary damage to intact nerves. Immune cells (both innate and adaptive), cytokines, growth factors, and factors H and I are all components that can directly or indirectly result from complement activation, but can feed back to regulate it as well. The following sections discuss the complement system's interaction with a variety of different factors, including immune cells, in the context of nerve injury.

i) Neutrophils and monocytes/macrophages

While the interplay between the complement system and neutrophils has not been studied in the context of peripheral nerve injury, our general understanding of neutrophil interactions with complement provides some clues as to their relevance. The complement system can recruit neutrophils and in turn, neutrophils can create and regulate complement components [27]. For example, neutrophils contain complement factors like C3, factor B and properdin [28]. Factor C3 is located on neutrophil membranes and downregulates the complement system by binding to

factor H, which inhibits the alternative pathway. On the other hand, neutrophils can upregulate the complement system by producing properdin which stabilizes and contributes to the complement system [28-31]. The mechanisms underlying a neutrophil's decision to create C3 versus properdin are not well known, but there is assumed to be a balance between the creation of the two. It is speculated that the MAC may destroy neutrophils under certain conditions [32], so neutrophils may create factor H to avoid this fate.

Neutrophils are an early arrival, via the circulation, to the scene of a peripheral nerve injury, helping macrophages phagocytose degraded axons and myelin [33]. When neutrophils arrive at the injury site, they recruit circulating monocytes that 1) differentiate into macrophages in the nerve for increased debris clearance and 2) further activate the complement system by creating C5, C6, C7, C8 and C9. These complement proteins are all needed to create MACs, thus implying that neutrophils influence Wallerian degeneration via complement [33]. At the same time, such upregulation of complement proteins is likely to be transient, given the inhibition of complement by neutrophils via C3/Factor H noted above, thus reducing the likelihood of complement-induced damage.

The fact that monocytes are so integrated with the complement system suggests an additional indirect relationship between neutrophils and the complement system in peripheral nerve injury repair [28, 34-35]. This is especially evident as the tissue-specific form of monocytes, macrophages, can regulate the complement system through the creation of additional complement components and regulators, such as CD46, CD55 and CD59. Macrophages also contain complement receptors on their surface, including anaphylatoxin receptors, further bridging macrophages' role to the inflammatory response [34]. Collectively, these data suggest that the recruitment of monocytes to the site of an injured nerve, and their subsequent

differentiation into macrophages, can have complement-related impacts on peripheral nerve injury.

ii) Mast cells

Mast cells typically play a role in allergic and inflammatory responses of the innate immune system. Mast cells can also create complement proteins (C3 and C5) and contain complement protein receptors on their surface which allow for complement proteins to activate mast cells [34, 36-38]. Once activated, mast cells release tryptase and chymase and cause degranulation. These serine proteases reciprocally activate complement proteins, therefore contributing to a positive feedback loop [38]. It was additionally found that the secretion of complement proteins (i.e C3) from mast cells is upregulated in response to the cytokines TNFα and IL-4 or IL-13 [36, 38]. In nerves, mast cells release proinflammatory cytokines (TNFα, GM-CSF, and IL-8) and chemokines (CCL2, CCL3 and CCL4) [39]. Granulocyte-macrophage colony-stimulating factor (GM-CSF) initiates the differentiation and proliferation of monocytes, activation of macrophages, and recruitment of neutrophils [40]. IL-8 is commonly known to promote chemotaxis in neutrophils and stimulate phagocytosis upon neutrophils' arrival to target, while CCL2 and CCL3 are involved in recruiting monocytes [41]. The release of cytokines and chemokines from mast cells is suggestive of a relationship between the complement system, mast cells, and cytokines/ chemokines during peripheral nerve injury. Given that effective Wallerian degeneration depends on the release of cytokines (like TNF-α) and the formation of a MAC (of which C5 is a critical component) one might predict that during peripheral nerve injury, mast cells release cytokines, including (but not limited to) TNF-α. In turn, cytokines may stimulate mast cells to release complement proteins, such as C5, which may help create MACs needed for Wallerian degeneration. Although none of these hypotheses have been tested, the above concepts

represent a starting point to connect the roles of mast cells, the complement system and Wallerian degeneration in peripheral nerve injury.

iii) B-cells and T-cells

B-cells and T-cells are components of adaptive immunity. B-cells secrete the antibodies IgG and IgM, which can activate the classical pathway of the complement system. There are also some complement receptors on B-cells, though it is not clearly known whether B-cells themselves can produce and release complement proteins [34, 42]. T-cells on the other hand can produce complement factors and also regulate the complement system by activating CD46, a complement regulator [43].

To discover the role of B and T cells in peripheral nerve injury, a mouse mutant was examined that did not contain T and B cells [44]. Interestingly, following femoral nerve injury, these mutant models had better motor recovery and nerve regeneration up to eight weeks after the injury occurred. The authors' hypothesis was that differential macrophage activation in the mutant mice allowed for better regeneration. As cytokines secreted by B and T-cells target macrophages, the authors hypothesized that the lack of this lymphocyte-specific cytokine secretion may positively affect macrophage roles in the regenerative process [44].

While the role of the complement system was not considered in these studies, these findings may be interpreted in the context of complement system function. One possible explanation is that the antibodies released from B-cells induce the complement system without check; too much complement activation can cause more damage than is needed, therefore hindering regeneration. Additionally, it has been found that the complement system activates Th1 production but inhibits Th2 production [43, 45]. Generally, Th1 cells produce cytokines that

cause a pro-inflammatory response, which must be balanced by anti-inflammatory cytokines produced by Th2 cells [46]. Over-activation of the complement system may increase Th1 cytokines, leading to an increased pro-inflammatory response and impeded nerve regeneration. In contrast, without B and T cells, reduced complement activity could enhance nerve regeneration.

Another plausible explanation is related to T-cell activation of CD46, a complement regulator. Over-activation of CD46 may inhibit the complement system, impeding Wallerian degeneration and therefore impairing regeneration. However, this scenario may be less likely, considering that T-cells also produce several different complement proteins, including C3 and C5 which are needed for MAC. While it is lesser known to what extent B-cells can produce complement proteins, there is some evidence indicating that they create and secrete C5 as well as other complement proteins [34]. This would suggest that B and T-cells in fact aid in regeneration, in paradoxical contrast to the previous study. In order to better frame the study's conclusions through a complement system lens, we need to investigate what aspects of complement regulation (e.g., positive/negative, as well as timing) are more directly or sensitively influenced by B and T-cells.

iv) Dendritic Cells

Dendritic cells bridge innate and adaptive immunity and act as antigen presenting cells, which phagocytose and present an antigen to cells of the adaptive immune system. Dendritic cells are immature until they receive maturation signals, at which point they are then able to activate T-cells. In their immature state they can produce complement protein C1q, although there is debate on whether they continue to produce this protein once mature [34, 47]. Mature

dendritic cells produce many complement proteins such as C2-C9 [34] as well as the complement regulators CD46, CD55 and CD59 [48-49].

CD 55 inhibits all three pathways (classical, alternative and lectin) of the complement system, while C1q is part of the classical system. The possibility that C1q is unable to be created in a mature dendritic cell, whose job is to activate T-cells, might suggest that parts of the complement system cannot work in tandem with adaptive immunity (or at least need to be turned off in order for T-cells to become active). However, CD55 is downregulated during the interaction between dendritic cells and T-cells [41], suggesting that complement activation is important for this interaction. Therefore, there are conflicting results that simultaneously highlight the importance of complement activation and inhibition during T-cell and dendritic interaction.

v) Natural Killer cells

Part of the innate immune system, natural killer cells are known to contain many complement receptors on their surfaces, allowing them to interact with complement factors [34, 50-51]. However, it is not known whether they can produce and release complement factors themselves. In injured nerves, natural killer cells are found at and distal to the injury site, and trigger the degradation of axons in the PNS [52]. The specific relationship between natural killer cells and the complement system during peripheral nerve injury is unclear, but it can be assumed that there is some interaction. For example, the complement receptors located on NK cells' surface could allow for communication with the complement system during Wallerian degeneration and the clearance of damaged axons/myelin.

vi) Toll-like receptors

Toll-like receptors (TLRs) are pattern-recognition receptors that play a large role as infection sensors in the innate immune system. TLRs are quickly activated and highly specific to different pathogen structures [53]. TLRs and the complement system are closely intertwined. They can both be activated by the same microbial molecules [53-55] and they can regulate each other through the interaction of their various receptors. For example, the expression of complement components is upregulated when TLR4 is activated. Additionally, complement can inhibit TLR production of different cytokines, including the IL-12 family [55-57]**,** which causes the differentiation of Th1 from naive CD4+ T-cells [58]. Therefore, though the complement system does result in increased Th1 [43-45], it may auto-regulate by downregulating Th1 development via TLR pathways. Additionally, TLR2 can activate complement receptor 3 (CR3), which, when on the surface of macrophages, further upregulates TLR signaling [53, 59].

TLRs are important aspects of nerve injury and regeneration. TLR1, TLR2, TLR3, TLR4, and TLR7 are highly activated in peripheral nerves after axotomy and can help activate Schwann cells. Conversely, the activation of Schwann cells causes TLR expression. The expression of TLRs in Schwann cells causes higher expression of pro-inflammatory mediators that aid in Wallerian degeneration [60]. Additionally, after injury, macrophage recruitment to the sciatic nerve distal stump is significantly less in TLR deficient mice than in wild type mice. As a consequence, TLR deficient mice had less effective Wallerian degeneration and axonal regeneration [61]. TLRs also locally increase neurotrophin production, additionally enhancing regeneration [2, 60]. The crosstalk between the complement system and TLRs during peripheral nerve injury and regeneration has not been studied yet, but given roles in other tissue types, it is likely they work together to mediate macrophage recruitment and myelin debris clearance.

vii) Coagulation factors and wound healing

Wound healing in the skin is a well studied process which may have several similarities to what happens after nerve injury leading to nerve regeneration. Although coagulation pathways have not been well-studied in nerve injury, vascular and nerve injury usually occur concurrently and therefore likely trigger the activity of coagulation and wound healing factors.

Platelets, a major player in coagulation, have been implicated in activating the complement system [27, 62]. Additionally, thrombin and plasmin, both parts of the coagulation cascade, activate C3 and C5 [63-64**,** 62]. It was found that C3 and C5 help with wound healing, both accelerating the process and increasing the strength of the repair [65-67]. Experimentally, increasing C5 in skin wounds led to increased influx of inflammatory cells and fibroblasts as well as more deposition of collagen. When collagen is exposed within a wound, the coagulation (clotting) cascade and platelets are activated in wound healing pathways [63].

However, plasmin can also play a regulatory role and destroy parts of the complement system needed to create the MAC [27, 68]. The complement system in turn regulates parts of the coagulation cascade such as fibrin formation. Activated platelets secrete several cytokines like TNF-alpha, PDGF, TGF-beta, epidermal growth factor (EGF), fibrinogen, histamines, platelet derived growth factor (PDGF), fibronectins and more. These all help with clotting as well as recruit macrophages and fibroblasts to the wound [69-70].

The complement cascade in wound repair is further activated by platelet degranulation and proceeds to help recruit inflammatory leukocytes to the injury and lyse bacteria, if present [63]. After platelet and complement activation, neutrophils are recruited, becoming the first inflammatory cells arriving at the wound. Neutrophils will bind to P-selectins, which are adhesion molecules on endothelial cell surfaces, and clear debris, working with the complement

system to lyse bacteria [27, 63]**.** Monocytes (which convert to macrophages on site) and lymphocytes are also recruited to the wound. In addition to phagocytosing debris, macrophages release cytokines, interleukins, transforming growth factor (TGF), platelet-derived growth factor, and tumor necrosis factor (TNF) among other factors. These activate fibroblasts and aid in angiogenesis. Wound healing also consists of the fibroplasia phase, where fibroblasts enter the wound site to help stimulate capillary growth [27, 63, 71-72].

In comparing wound healing and the processes after nerve injury, both include the release of cytokines that signal for TNF-alpha and IL-1 [3, 69-70], two essential inflammatory regulators. Neutrophils are also some of the first inflammatory cells at the site of injury for both processes [27, 33]. Therefore, both have an inflammation phase where immune cells are recruited to the injury site. The fibroplasia phase in wound healing also exists in nerve regeneration, as fibroblasts in the epineurium and perineurium help stabilize the strength of the nerve cell by synthesizing collagen (or, when misregulated, creating components of scar) [2, 63]. Since exposed collagen triggers the coagulation cascade [63], this further ties concepts in wound healing and nerve repair together. The fact that wound healing and nerve repair share common major players indicates that these processes are likely meant to work side-by-side, with products from one process regulating aspects of the other and vice versa.

Therapeutic implications

The above review demonstrates a number of direct and indirect influences of the complement system on nerve degeneration and regeneration. These studies suggest that manipulation of the complement system has important translational implications for enhancing nerve repair or preventing/overcoming barriers to nerve repair. Such approaches have not been

formally introduced into the clinic. However, several preclinical pharmacological and genetic therapeutic strategies demonstrate both the potential feasibility of such approaches as well as some limitations and points of caution in clinical translation.

A particularly promising modulator of the complement system is recombinant human c1 esterase inhibitor (rhC1INH), which inhibits the classical complement pathway via the C1 protein. This therapeutic (e.g., Ruconest/Conestat alfa) has been deployed clinically for a number of indications, including angioedema and chronic kidney disease [74-75]. Compellingly, treatment of rats with rhC1INH reduces acute damage in the injured nerve, though the long-term effects of rhC1INH on nerve regeneration outcomes as well as any systemic effects are unknown [21].

While modulation of complement may be powerful, therapeutic interventions should not completely inhibit the complement system in the context of nerve regeneration. Indeed, complete knockout of the complement system results in fewer regenerating axons and delayed axonal regeneration than controls after sciatic crush injury in rats [73]. Additionally, rats with a depleted complement system had fewer macrophages recruited and activated. Macrophages are needed for axonal regeneration, as they clear cellular debris and promote the creation of trophic and tropic factors aiding in axonal growth [73]. Therefore, clinical interventions need to be done on select components of the complement system rather than knocking it out entirely.

While this review has largely characterized the MAC as a key node in complement regulation of nerve regeneration, we acknowledge that there is some controversy surrounding the extent to which MACs influence nerve regeneration. For example, MAC residue on nervous tissue can cause excessive calcium influx, which activates calpains, causing structural damage to

the nerve. In fact, calpain inhibitors have been found to prevent axonal cytoskeletal loss in models of complement-mediated CNS nerve injury [21], suggesting their potential as a supplementary treatment strategy in the PNS as well.

i) Caveats of pre-clinical models

It should be noted that many re-clinical studies have been performed in rodent models. However, the timing of complement system activation may differ in mice and rats versus other mammals, including primates. For example, axonal degeneration was detected as early as 36 to 44 hours in mice and rats, but after one week in baboons [3]. Thus, these temporal factors need to be included in dosing strategies.

Differences in gene regulation between humans and mice poses another challenge in using mice as a model for the complement system. For example, in humans, the complement system is partially controlled by genes within the Regulators of Complement Activation (RCA) cluster on chromosome 1. While mice also have the RCA cluster, they are missing the locus for Complement Receptor 1 (CR1/CD35). CR1/CD35 acts as a receptor for and a negative regulator of the complement system [76]. Despite the creation of mouse models with a modified CR1 to be more human-like, the nuances in complement cascade regulation are missed [76].

ii) Therapies for a specific chronic injury: neuroma

We largely constrained discussion of complement to the acute response to injury. However, the fact that chronic injury results in the downregulation of neurotrophic factors and reduced trophic support from Schwann cells -- two processes that interface with the complement

system -- suggests that the complement system is also likely to influence chronic injury. This has not been studied.

The impact of inadequate nerve repair or a “chronic-like” state may be seen in neuromas, which are the result of uncontrolled neuronal tissue growth at the site of nerve injury [12]. In addition to Schwann cells and fibroblasts, neuromas contain bundles of axons that are improperly woven together [77]. An image of a neuroma in the forefoot is shown in figure 5 [88]. It can be seen that the neuroma caused swelling and thickening of the nerve. Additionally, images from a study done on neuromas in rat sciatic nerves are shown in figure 6. The normal and neuroma-containing sciatic nerves were stained with H&E and imaged [86].

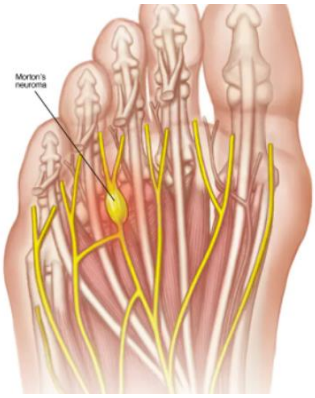

Figure 5: Forefoot Neuroma

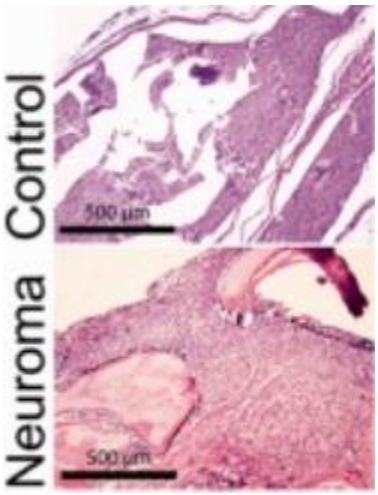

Figure 6: H&E Staining on Normal and Neuroma-Containing Rat Sciatic Nerves

Likely factors in neuroma formation are the pro-inflammatory cytokines TNF-α, IL-1 β and IL-6; these are upregulated in rats who underwent sciatic nerve transection that resulted in neuromas, as compared to the rats whose nerve transections did not result in neuromas [12]. As such cytokines stimulate mast cells to release complement proteins (which then form the MAC complex to kickstart Wallerian degeneration), it is possible that neuroma formation and progression may be influenced by complement activation.

Neuromas cause neuropathic pain due to increased sensitivity. They are hard to manage as normal treatment options have limited success, often leading to symptom relapse. There are non-operative and operative treatments; the latter is resorted to when non-surgical treatments don't work. One of the non-operative therapies is steroid injection. Steroids have been used to address pain following mastectomies, which are likely a consequence of sensory nerve neuromas [87]. Though the mechanism of action is unclear, it is possible that inflammatory pathways may be targeted by such a therapy. Other studies have used steroids to treat neuromas in the forefoot. These studies found that a third of patients had recurrence of their symptoms after one year, necessitating surgical interference [79]. It was concluded that if steroid injections were used within one year of the start of neuroma symptoms, the outcomes were better [77-79]. Additionally, even when surgery is performed, patients are not relieved of their symptoms 30% of the time [79]. This suggests that early intervention has benefit. Therefore, it would be valuable to understand how the complement system plays a role in neuroma progression, especially early on. This would potentially allow for early targeting of complement components to prevent neuroma progression, therefore leading to better outcomes.

Conclusion

There has been a considerable amount of research performed on inflammatory influences on nerve regeneration. However, far less has been studied about the complement system, and how this intersects/interacts with inflammatory and regenerative processes. These remain exciting areas of future research and possible avenues for developing therapeutic interventions.

1. INTRODUCTION

Based on the literature review I authored, I chose to focus on some particularly interesting and unexplored roles of the immune system in nerve regeneration; namely how these roles interact with inflammasomes and influence the formation of neuromas. Currently, neuroma treatment is inadequate and not universally agreed upon [80]. Exploring the role of inflammasomes and the immune system, specifically the complement cascade, in neuroma progression might open the door to more viable treatment options.

I created a neuroma mouse model by conducting a sciatic nerve transection and harvesting the proximal stump and contralateral nerve one- and two-weeks post-operation. Studies have shown that neuromas can form between 10 to 28 days after injury [77, 86]. Therefore, the two-week timepoint was chosen to represent early neuroma formation, while the one-week timepoint was used to get an idea of the injury site before neuroma formation, during neuroma progression. Obtaining a better understanding of the injury site before the complete formation of a neuroma might allow for the creation of preventative therapeutics. Early intervention is important because once a neuroma has fully formed, it may be difficult to treat or completely reverse the damage. As explained in my literature review, steroid injections have been used to treat neuromas as they decrease the surrounding inflammation. Importantly, steroid treatment works best when applied within 1 year of symptom onset [79]. Therefore, finding early neuroma markers may allow us to intervene with neuroma progression before it is too late.

I focused on three markers: one for the complement system, C1qB, and two for inflammasomes, ASC and NLRP3. C1qB is a polypeptide chain that is a part of C1q, which is involved in the classical pathway. C1q plays a large role in the activation of the complement system. Additionally, C1q's interaction with various proteases allow for the creation of the membrane attack complex (MAC), which is an integral player in nerve injury and can affect nerve regeneration [81]. Interestingly, the presence of C1q at a neuronal injury site has been found to increase the inflammatory response in the CNS [81]. Given that there might interaction between C1q and inflammatory processes, C1qB was chosen as our primary complement marker.

ASC was chosen as one of the inflammatory markers because forms a part of and helps activate most inflammasome complexes [82]. NLRP3 is a specific type of inflammasome that is well characterized and is known to be present in peripheral nerves [82-83]. Therefore, ASC and NLRP3 were determined to be appropriate markers for inflammasome expression and the inflammatory response in a sciatic nerve transection.

2. METHODS

Sciatic Nerve Transection Surgical Procedure

A sciatic nerve transection (complete severing of the nerve) was conducted on either the left or right sciatic nerve of 15 mice. The transection was a survival surgery, requiring the mice to be put under anesthesia and undergo recovery. They were euthanized at either one or two weeks. Their injured proximal stump as well as their uninjured contralateral nerves were harvested for analysis.

The mice were put in an induction chamber with 5% isoflurane for 2-5 minutes, until heavy, even breathing was observed. Then, they were transferred to a warmed water pad and a nose cone delivering oxygen and 2-5% isoflorane, as needed. Reflexes were checked by toe pinching and the isoflurane was adjusted according to the presence of reflexes. They were then given buprenorphine, cefazolin and saline. Nair, a hair remover, was used to clear the fur off the surgical site. A ¾ cm incision to either their right or left hind leg was made, cutting proximally, under and parallel to their femur. A depiction of the surgical site during surgery is shown in figure 7. The blade cut through the skin and fascia to expose the muscle and sciatic nerve underneath. Once the sciatic nerve was located, isoflurane was increased to 5% (if not already there) and the nerve was severed with surgical scissors. Once the nerve was cut, isoflurane was adjusted accordingly. Forceps were used to grab and orient the proximal and distal stumps away from each other to encourage neuroma formation and prevent re-connection. Two to three 4-0 Ethilon sutures were used to close the fascia. Staples were used to close the skin. Neosporin was applied to the wound and Vetbond glue was applied to the foot on the injured side in order to prevent self-mutilation of the toes. Mice were given buprenorphine twice a day for three consecutive days post-operation. Carprofen was given to manage pain symptoms as needed. The mice were euthanized after either one or two weeks. For those that were euthanized at two weeks, their skin staples were removed approximately 10 days after surgery.

Figure 7: Zoomed Out (Left) and Close Up (Right) of Surgical Site During Surgery

Euthanasia and Harvest

Mice were euthanized using carbon monoxide and cervical dislocation at either one or two weeks post-surgery. The proximal and distal stumps on the transected side were harvested, placed in separate 0.5 ml Eppendorf Safe-Lock tubes and flash-frozen in dry ice. They were stored in a -80 °C freezer. Each mouse yielded one proximal stump that was used as one sample for data collection. The whole sciatic nerve from the uninjured side was harvested to be used as a control. A single proximal stump or contralateral nerve from one mouse was never divided into multiple samples. The injured proximal stump and uninjured contralateral nerve that came from the same mouse were tracked as a pair through the entire data collection process.

RNA Extraction

QIAGEN's RNeasy Micro Kit was used for RNA extraction. Samples were removed from the -80 °C freezer and placed on dry ice. One empty round-bottom 2.0 mL Eppendorf Safe-Lock tube was obtained for each sample. One stainless-steel bead was placed in the empty tubes and the tubes, with the beads inside, were weighed. A sample was then placed in each tube and

the tubes were re-weighed. The mass of the tube, bead and sample was subtracted from that of the empty tube with only the metal bead to obtain the mass, in grams, of each sample. The micro kit requires the use of no more than 5 mg of tissue; all samples were confirmed to be under that limit. 350 uL of Buffer RLT was added to each tube. The samples were kept on dry ice until Buffer RLT was added.

The samples were then disrupted and homogenized using the TissueLyser II, which shakes the tubes at high speeds, using the friction between the bead and tube to break down the samples. The samples were placed in the tissue lyser 4 times, at 30 Hz for 3 minutes each time. The tubes were rearranged between runs to ensure even homogenization. The tubes were then centrifuged at 25 °C for 3 minutes at 14,000g. The supernatant was transferred into a new 2.0 mL Eppendorf Safe-Lock tube without a stainless-steel bead.

The rest of the RNA extraction was automated using the Qiacube, into which the RNeasy Microkit protocol was programmed. A variable amount of RDD dependent on the number of samples was added to a DNase I stock solution. The samples, empty rotor adaptors, DNase + RDD mixture and other necessary reagents (buffer RPE, 80% and 70% ethanol, buffer RW1, and RNase-free water) were positioned in the Qiacube according to the company handbook.

The protocol yields 15 uL of solution by default. 2 uL of the solution was analyzed for quality and yield of RNA. The remaining 13 uL were immediately stored in the -80 °C freezer until they were used for cDNA synthesis.

cDNA Synthesis

cDNA synthesis was performed using ThermoFisher's SuperScript III First-Strand Synthesis System. For each sample, 8 uL of total RNA, 1 uL of oligo(dT) and 1uL of a 10 mM dNTP mix were added to 0.2 mL flat cap PCR tubes, incubated at 65 °C for 5 minutes and placed on ice for 1 minute. 2 uL of 10X RT buffer, 4 uL of 25 mM MgCl2, 2 uL of 0.1 M DTT, 1 uL of RNaseOUT (40 U/uL), and 1 uL of SuperScript III RT (200 U/uL) were added to each sample in the listed order. The tubes were incubated for 50 minutes at 50 °C, terminated for 5 minutes at 85 °C and then placed on ice. 1 uL of RNaseH was added to each sample and the samples were incubated at 37 °C for 20 minutes. The resulting 21 uL solutions were stored in a -20 °C freezer until used for PCR.

PCR

PCR was performed with Bio-Rad's SsOAdvanced Universal SYBR Green Supermix. The injured proximal stump and uninjured contralateral nerve from each mouse were paired. Primers for C1qB, ASC, NLRP3 and GAPDH were used on each proximal stump and contralateral nerve. GAPDH, a common housekeeping gene, was chosen as the reference gene based on a similar experiment that utilized it [84]. One empty 0.5 ml Eppendorf Safe-Lock tube was obtained for each sample. In each tube, 12 uL of SYBR Green Supermix, 3 uL of each forward and reverse primer, and 6 uL of cDNA were added to create a 24 uL solution. The components were lightly vortexed and centrifuged. Each tube of 24 uL was split into two wells of a ThermoFisher Armadillo PCR plate. 10 uL of solution was pipetted into each well, creating duplicates, and leaving 4 microliters per tube to account for pipetting errors. The PCR plates were centrifuged and placed into the Bio-Rad PCR machine.

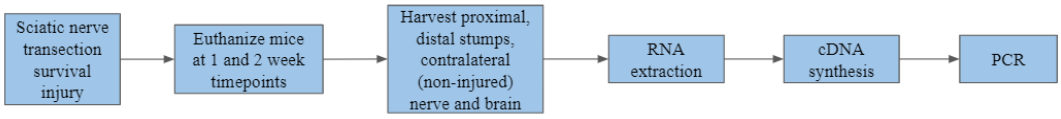

Figure 8: Overview of Methods

Data Analysis

Analysis of the data was based off methods used in a paper discussing how to best analyze CT values [85]. The delta CT (ΔCT) and delta delta CT (ΔΔCT) values for each sample were obtained by using the following equations:

$$\text{delta CT} = CT_{target} - CT_{GAPDH}$$

$$\text{delta delta CT} = (CT_{target} - CT_{GAPDH})_{proximal} - (CT_{target} - CT_{GAPDH})_{contralateral}$$

The 2^(-ΔΔCT) method was used to determine the relative changes in gene expression and these values were graphed for the 2 week timepoint alone (as shown in figure 14 in the results section) and for the 1 versus 2 week timepoints (as shown in figure 15 in the results section).

To analyze the two-week timepoint data, all delta CT values were put into the 2^(-ΔCT) formula, as recommended by another paper [85]. A paired T-test was run on the 2^(-ΔCT) values, pairing contralateral nerves and injured proximal stumps from the same mouse. No statistical analysis was conducted on the 2^(-ΔCT) values for the 1 week timepoint because the sample size of one was too small.

3. RESULTS AND DISCUSSION

The total number of mice that underwent surgery and the portion of those mice that survived are shown in table 1. The surviving mice produced 9 pairs of proximal stumps with contralateral nerves for the 2 week timepoint and 6 pairs for the1 week timepoint. After eliminating samples which had outlying CT values, undetectable RNA amounts or an RNA yield with high residue, I

was left with 6 pairs for the 2 week and 1 pair for the 1-week timepoint. An example of one such eliminated sample is shown in figure 9.

Table 1: Cohorts of All Mice That Underwent Surgery

	Cohort 1	Cohort 2	Cohort 3	Cohort 4
Initial number of mice	3	4	10	2
Number of mice surviving post-op	2	3	10	2
Used for	RT-qPCR	RT-qPCR	RT-qPCR	IHC
Timepoint	2 week	1 week	7 used for 2 week 3 used for 1 week	1 week

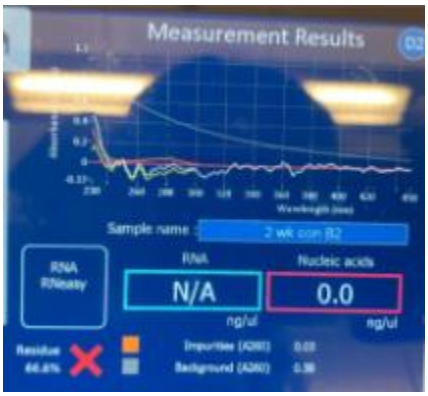

Figure 9: Discounted Contralateral Nerve with No Detected Nucleic Acids and High Residue

Images during the harvest of a control and injured nerve at 2 weeks are shown in figures 8 and 9 respectively. These images are representative of most of the injury and control sites and were taken after the mice were euthanized, right before harvesting the nerve. As can be seen in figure 11, the proximal and distal stumps have not grown back together. The proximal stump (on the left, circled in red) appears to have the characteristic bulbous end of a neuroma, indicating

that the neuroma model was successful. Figure 12 depicts the RNA yield for the proximal stump shown in figure 11. This is a successful example of RNA yield, one that is typical of the samples that I included in my statistical analysis.

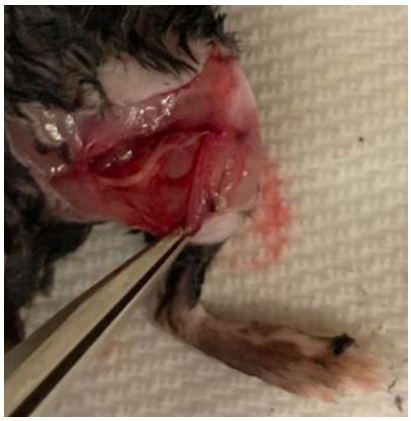

Figure 10: 2 Week Timepoint, Harvest Site of Control Nerve

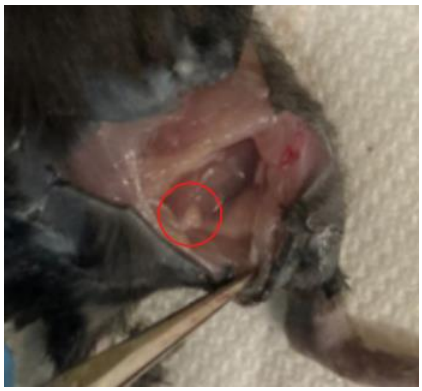

Figure 11: 2 Week Timepoint, Harvest Site of Injured Nerve, Proximal Stump Circled

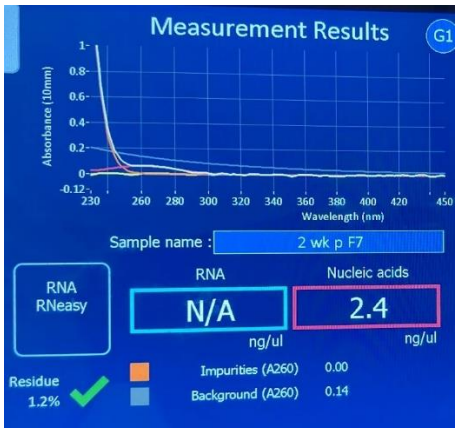

Figure 12: Successful RNA Yield for Proximal Stump Shown in Figure 11

Amplification curves and CT values were obtained for primers encoding ASC, NLRP3, C1qB and GAPDH on injured proximal stumps and uninjured contralateral nerves. The raw CT values are shown in tables 2 and 3 below; all amplifications were between 22 and 38 cycles. Only some of the samples were run in duplicates; the colors in the table are meant to group the samples into their duplicates (no color means that the sample was run as a single). An example of some of the amplification curves I obtained for the injured and control nerves of mice from the two-week timepoint are shown in figure 13 below. While these amplifications curves are an example of a successful trial, there were other trials which resulted in unsuccessful curves; these trials were removed.

Table 2: Raw CT Values for Control Nerves

ASC	NLRP3	C1qB	GAPDH
30.7	37.18	32.9	26.17
32.57	36.53	30.32	26.28
30.28	35.56	29.97	23.58
30.24	35.06	33.72	25.62
30.68	36.27	33.82	26.06
31.18	37.15	30.82	25.65
30.09	35.79	31.05	24.94
30.78	35.3	32.29	24.68
30.58	35.61	29.58	24.83
	36.12	31.84	24.78
			25.07
			24.73

Table 3: Raw CT Values for Injured Nerves

ASC	NLRP3	C1qB	GAPDH
27.4	31.85	28.57	22.06
27.2	32.53	27.34	25.23
29.76	34.59	29.4	25.03
29.48	34.4	29.79	26.5
30.53	35.96	30.6	26.07
27.6	33.17	31.18	24.29
27.67	33.31	28	24.01
28.67	32.96	29.35	24.46
28.33	32.69	28.74	23.76
29.92	33.45	28.94	22.82
			22.59

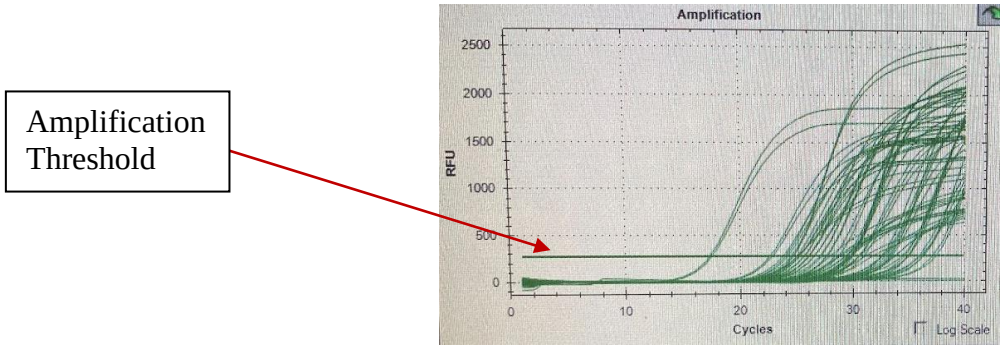

Figure 13: Amplification Curves Depicting Proximal Stumps and Contralateral Nerves of Mice from Two-Week Timepoint with Primers for ASC, NLRP3, C1qB and GAPDH.

Table 4 shows calculated ΔΔCT values for ASC, NLRP3 and C1qB at 2 weeks. A positive ΔΔCT value indicates an increase in relative gene expression, whereas a negative ΔΔCT value indicates a decrease. As can be seen in table 4, the ΔΔCT values indicated an increase in expression of all three genes (except for 1 of C1qB's ΔΔCT values, which was slightly positive: 0.31).

Table 4: ΔΔCT Values for Primers ASC, NLRP3 and C1qB

ddCT ASC	ddCT NLRP3	ddCT c1qb
-2.63	-0.46	-0.22
-2.48	-1.35	-1.08
-1.965	-1.96	-3.31
-1.735	-1.76	-2.415
-4.38	-2.15	-2.525
-1.37	-0.685	0.31

Figure 14 shows the 2^(-ΔΔCT) values for just the two week timepoint (note that statistical analysis was done on the 2^(-ΔCT), not 2^(-ΔΔCT) values). There is a statistically significant increase in ASC, NLRP3 and C1qB in injured nerves compared to contralateral nerves, suggesting increased inflammasome and complement activation. I next tested whether these genes were also increased at a 1 week time period in a pilot cohort of animals. Figure 15 shows gene expression for ASC, NLRP3 and C1qB for the one- and two- week timepoints side by side. Comparing the 2^(-ΔΔCT) values indicates an increase in ASC, NLRP3 and C1qB expression from one to two weeks. While an increase in gene expression seems to be the case, the 1 week cohort was under powered, so was no statistical analysis could be done. Therefore, the data presented in figure 15 could be elaborated upon in future studies.

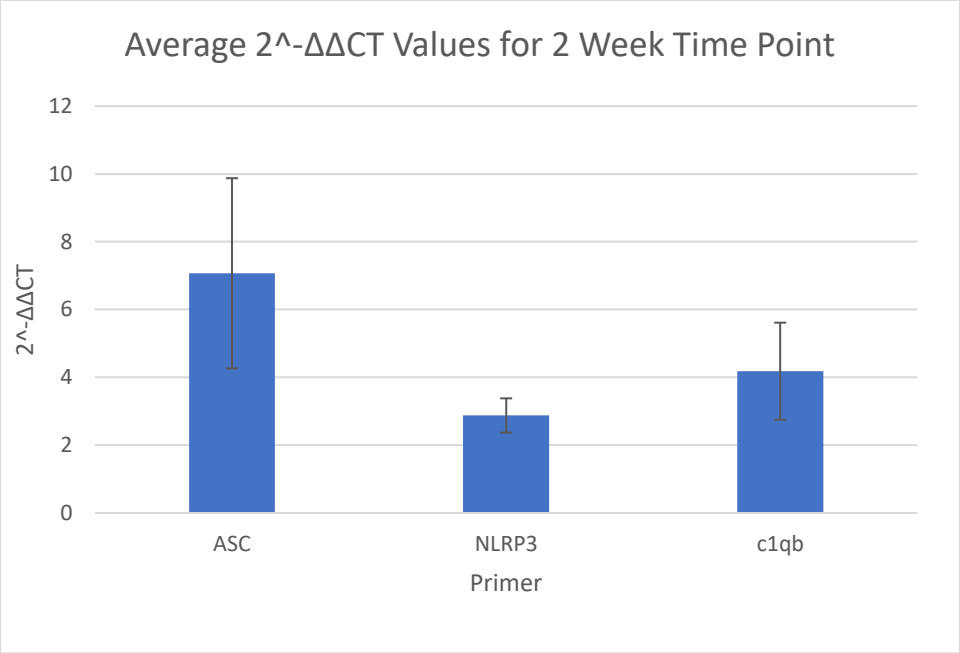

Figure 14: Average 2^-ΔΔCT Values for 2 Week Time Point

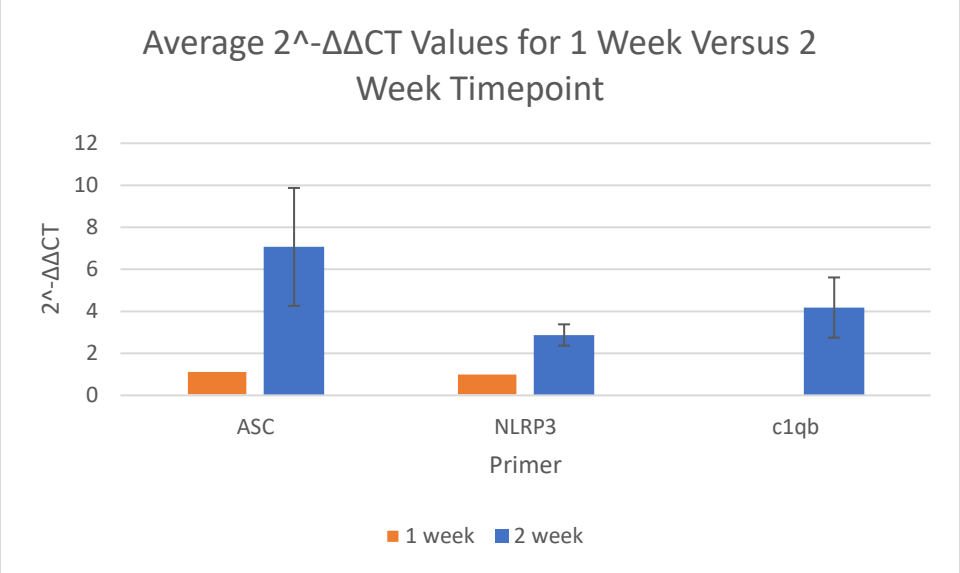

Figure 15: Average 2^-ΔΔCT Values for 1 Week Versus 2 Week Timepoints

Lastly, the paired t-test that was performed on the 2 week 2^(-ΔCT) values had statistically significant evidence for an increase in ASC and NLRP3 expressions in the proximal stump compared to the contralateral nerve (both $p < 0.01$) as well as for C1qB ($p < 0.05$), as

shown in table 5. My findings conclude that ASC, NLRP3 and C1qB are upregulated two-weeks post-transection, during early neuroma formation. This indicates that inflammasomes and the complement system play a role in neuroma progression and could be a potential target for future therapeutics.

Table 5: P-Values from T-Test Ran on 2 Week 2^(-ΔCT) Values

ASC	NLRP3	C1qb
0.0003241238837	0.004303939863	0.03523407638

4. METHODOLOGICAL REFINEMENT AND TROUBLESHOOTING

During surgery

There were three cohorts of sciatic nerve transection surgeries. The first cohort consisted of three mice and all three initially recovered from the surgery. One was found dead in the cage 6 days post-operation for unknown reasons; no open wounds or prior signs of pain had been present. The remaining two mice were euthanized at the 2 week timepoint. Their proximal stumps, distal stumps and non-injured contralateral nerves were harvested.

The second cohort contained 4 mice, 3 of which recovered normally after the procedure. The fourth mouse began displaying seizure-like behavior within 2-3 minutes of waking up from anesthesia. The cause was unknown, as there were no concerns leading up to or during the surgery. The remaining three mice were euthanized at 1 week timepoints and their proximal stumps, distal stumps and non-injured contralateral nerves were harvested.

The third cohort consisted of 10 mice; all 10 recovered normally from the surgery with no complications. This cohort required the isoflurane to be administered at 5% the entire surgery,

which is more than usual and created a cause for concern; high isoflurane concentrations could result in death. A possible reason for these mice needing higher concentrations of isoflurane could be due to their lower weights (all 20 grams or less) as compared to the previous cohorts in which no mouse weighed less than 25 grams. Of the 10 mice, 3 were euthanized at 1 week and 7 were euthanized at 2 weeks. Their proximal stumps and non-injured contralateral nerves were harvested.

Animal Care Post-Surgery

The first cohort had no post-surgery complications other than one of them being found dead in the cage 6-days post-operation. In the second cohort, 2 of the three mice displayed toe mutilation on the side of the nerve transection. This was not normally seen in mice by our lab, therefore it was an issue that we had not accounted for beforehand. They were given carprofen daily to alleviate any pain. We tried several different strategies to prevent further mutilation. First, we attempted to cone the mice, but the smallest available cones were too large. We tried wrapping bandages around the foot, but they came off overnight. Finally, we put 2-3 layers of Vetbond glue over the foot which was the most successful technique. Given Vet bond's success, it was then incorporated into the third cohort's surgical procedure, right before the mice were taken off anesthesia. The third cohort had no postoperative complications.

Nerve Harvest

Making sense of the injury environment for the 2 week time-point was difficult. High inflammation around the injury site made it unclear where the proximal and distal stumps were. For one of the mice in the first cohort, fat underneath the sciatic nerve was mistaken for an inflamed proximal stump and collected. This can be seen in f14 below. I collected part of the

white tissue directly above the forceps, which is not part of the proximal or distal stump. This resulted in an RNA yield of 37.7 ng/uL, as depicted in figure 17, whereas previous samples of full length nerves had a yield of less than 10 ng/uL. Given the abnormally high RNA yield, I deduced that I had harvested the surrounding fat rather than the proximal stump. Figuring out that the larger white tissue is fat and not part of the nerve allowed me to disregard it and pinpoint the proximal stump more easily in the third cohort. It required me to make a bigger incision proximally and look deeper into the muscle. Therefore, harvesting the proximal stumps in the third cohort was a much more smooth and accurate process.

Figure 16: Injury Site During Proximal Nerve Harvest for Mouse in Cohort 1

Figure 17: RNA Yield of Proximal Nerve Harvested from Mouse in Figure 16 from First Cohort

Data Collection

i. RNA Extraction

Many methods were researched preliminarily to find the most optimal total RNA yield from less than 5 mg of tissue. The proximal stumps, distal stumps and control nerves were around 2 to 3 mg on average. RNA extraction, especially on such small nerves, is not a standard procedure for our lab. Additionally, many RNA extraction kits did not contain specific information on nerves. Therefore, I had to trial different RNA extraction kits in order to determine which would provide the best RNA yield.

The first kit tried was Qiagen's RNeasy Lipid Tissue Minikit. This protocol involved using Qiazol as the lysis reagent and adding chloroform to help with phase separation. The results were promising, but since the lipid kit was made for tissues rich in fat and was not specifically advertised for tissue weighing less than 5 mg, I looked for other kits more specific to my conditions. Qiagen's RNeasy Micro Kit had a maximum weight limit of 5 mg, so that was the

next method attempted. This protocol uses Buffer RLT as the lysis reagent. Results from this kit were slightly more promising and on average had lower residues than those from lipid kit.

Lastly, I interchanged different parts of the micro and lipid kits to see whether a specific combination yielded better results. For example, I used qiazol instead of buffer RLT in the micro kit or tried adding a chloroform step to the buffer RLT.

The lipid and micro kits gave similar results, however, I determined that the microkit resulted in slightly lower residues for similar RNA yields. Therefore, I decided to use Qiagen's RNeasy Micro Kit with no modifications. However, even with this optimized protocol, the total RNA yield for the samples was very low, providing concern for the feasibility of PCR analysis. A summary of the trialed methods is shown in table 6.

Table 6: Summary of Preliminary Trials to Determine Most Optimal Method of RNA Extraction

Protocol used in Qiacube	**Buffer used**	**Any extra steps?**	**Sample Size**	**RNA Yield for each sample**	**Residue for each sample**
Qiagen's RNeasy Lipid Tissue Minikit	Qiazol, as stated in protocol	No, followed exact lipid kit protocol	6	5 samples' RNA yields were between 3-5 ng/uL, whereas 1 sample yielded 11.4 ng/uL.	3 of 6 samples had high residue (>3%). The rest had low residue (<2 %).

Table 6: Summary of Preliminary Trials to Determine Most Optimal Method of RNA Extraction (Cont)

Protocol used in Qiacube	**Buffer used**	**Any extra steps?**	**Sample Size**	**RNA Yield for each sample**	**Residue for each sample**
Qiagen's RNeasy Microkit	Qiazol, instead of protocol's buffer RLT	No, followed exact microkit protocol other than lysis buffer	2	N/A for both samples	N/A residue for both samples
Qiagen's RNeasy Micro Kit	Qiazol, instead of protocol's buffer RLT	Added chloroform and let sit for 30 minutes before inserting in Qiacube (this step is taken from the lipid kit)	1	0.3 ng/uL	High residue (100%)
Qiagen's RNeasy Micro Kit	Buffer RLT	No, followed exact microkit protocol	5	All 5 samples' RNA yields were between 1-4 ng/uL	All had low residue (<2%).

ii. PCR Analysis

Having a small amount of total RNA required using more cDNA in order to get reasonable CT values. After running several trials with practice nerves and varying the amount of cDNA for a duplicate pair from 3-6 uL, it was found that 6 uL obtained the best amplification curves. Each sample yielded 21 uL of cDNA. Therefore, only three primers could be used to run duplicates, with a fourth primer being used to run a single. One of the three duplicates had to be a reference gene, which I chose to be GAPDH. Therefore, the amount of primers I could test was limited.

Since PCR is not a standard procedure for our lab and our samples were particularly small, there was a learning curve when conducting PCR analysis. The PCR conducted on the samples from the first two cohorts of mice did not have an adequate reference gene used. Therefore, the PCR for the third cohort of samples was adjusted to contain GAPDH as a reference for each sample. This reduced the total amount of primers that could be tested but allowed for more accurate data analysis.

Acknowledgements

This thesis, in part is currently being prepared for submission for publication of the material. Azar, Carmelina; Elsa Sanchez-Lopez; Shah, Sameer. “The Immune System’s Role in Neuroma Progression.” The thesis author was the primary investigator and author of this material.

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