NEUROIMMUNE CONNECTIONS IN THE SPLEEN: A FIRST LOOK AT SPLEEN GLIA

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Abstract

Non-myelinating Schwann cells (NMSCs) are the most abundant cells in the peripheral nervous system. These cells ensheath the unmyelinated autonomic nerves innervating every visceral organ. However, outside of the enteric nervous system, very little is known about what they do. Glia are best studied in the brain, can support neurons and also modulate neuronal signaling and inflammation. In the peripheral nervous system, neuronal signaling itself may be a key immune modulator as immune organs are sympathetically innervated and stress modulates immune responses. Increased sympathetic signaling after stressful events results in profound changes in the spleen, the largest secondary lymphoid (immune) organ. For example, after a large stroke, sympathetic signaling within the spleen increases and leads to the death of B-cell populations. This thesis advances our knowledge of non-myelinating Schwann cells in the spleen (spleen glia), and how sympathetic innervation of the spleen may modulate inflammation. Chapter 1 provides an overview of what is known about the roles of the spleen and our sympathetic nervous system in inflammation. Moreover, we discuss what is known about how responses between the two systems are coordinated and what the possible key mechanisms are, particularly the importance of the splenic nerve. We also explore the role of glia in both the CNS, ENS, and PNS and mechanisms by which they alter neurotransmission and immunity. Chapter 2 contains a detailed immunohistochemical and transcriptomic characterization of spleen glia. Here I utilize immunohistochemistry to provide the first in depth characterization of the cells. This analysis reveals the expression of two common glial markers (GFAP and S100B), a close association with sympathetic nerves, vasculature and immune cells, as well as the density and location of the cells throughout the organ. I combine this with RNA-sequencing to further characterize spleen glia. I report that they express a host of glial genes, genes involved in neurotransmission as well as genes involved in immune responses. Lastly, I use the sequencing to provide a transcriptomic comparison of spleen glia to brain glia and enteric glia, determining that spleen glia are unique when compared to these different populations. In Chapter 3, I explore the relationship of spleen glia to the splenic nerve which they ensheath. Here, I utilize SARM1 knockout animals that are protected against Wallerian degeneration after nerve injury. The work here reveals robust physical changes to spleen glia indicating potential trophic factors involved in maintaining this relationship. Chapter 4 provides a summary of the findings reported in this dissertation and describes future studies. In total, this dissertation provides a deeper context to neuroimmune interactions in the spleen and the first characterization of a novel cell type, spleen glia.

Dedication

This dissertation is dedicated to my nieces and nephews, Zaila Lucas, Zyion Lucas, Noah Lucas, and Dawaun Lucas Jr. I hope one day you choose the path of higher education and look to me as an inspiration.

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Chapter 1

Introduction and Background

1.1 Introduction

Glia are a set of diverse cell types in both the central and peripheral nervous system (CNS and PNS, respectively). Glia help to maintain homeostasis, facilitate neurotransmission, and offer trophic support to neurons. There is also evidence that neurons and glia communicate with immune cells and modulate the response to injury and infection. This is called "neuroimmune" communication. Specifically, neuroimmune signals can come directly from neurons to immune cells, or indirectly through other cells that act as mediators, such as stromal or glia cells. Both glia and neuroimmune communication is best described in the CNS. In the PNS, there is less known about the role of glia, but we do know they ensheath and myelinate autonomic nerves that innervate nearly every organ. The role of the autonomic nervous system in immune function has received increasing attention over recent decades–peripheral neuroimmune interactions have been implicated in many diseases such as multiple

sclerosis (Heesen et al., 2007; Veiga-Fernandes and Artis, 2018), colitis (Tanaka et al., 2019; Delvalle et al., 2018a), and even cancer (Kuol et al., 2018b,a). Additionally, these interactions are also prominent after injury such as stroke and spinal cord injury (Ajmo Jr. et al., 2009; Brommer et al., 2016) and shown to be necessary for homeostatic regulation (Veiga-Fernandes and Artis, 2018). However, precise mechanisms are largely unexplored.

Most of what is known about glia and immune signaling in the PNS comes from studying enteric glia, which are non-myelinating Schwann cells in the gut. Enteric glia can detect local levels of cytokines and respond through gliosis, an activated proliferative state marked by increase expression of GFAP and S100B (Sofroniew, 2009; Fawcett and Asher, 1999; Delvalle et al., 2018a). Upon activation, enteric glia contribute to the inflammatory response through secretion of cytokines and immunomodulatory molecules such as TNF, ATP, and S100B (Gulbransen and Sharkey, 2012). Additionally, they are able to distinguish pathogenic from probiotic bacteria and modulate their expression of pattern recognition receptors, such as Toll-like receptors, a seemingly gut specific role of glia that allows them to properly respond to exogenous threats (Gulbransen, 2014). However, it is not clear how enteric glia are regulated during many of these processes. Specifically, it is unknown what signals enteric glia receive to tell them to respond during inflammation or upon encountering pathogens or what cells support their changes.

There are many things that make studying neuroimmune interactions in enteric glia and the gut challenging. First, the gut contains several types of neuronal innervation and may have substantial glial heterogeneity as well. It receives innervation from both parasympathetic and sympathetic fibers and specific contributions from each arm are not clear (see "The Autonomic Nervous system and inflammation" below). In addition, the gut also has its own autonomous network, the enteric nervous system. It also contains areas of immune tissue known as Peyer's patches which are lymphoid follicles made up of B and T lymphocytes. Peyer's patches are directly innervated by nerves ensheathed by enteric glia, however there is not yet any data on how (or whether) glia within patches are different from other enteric glia. In addition, enteric glia also reside in a very unique environment which subjects them to immune responses due to changes in the epithelial barrier. The epithelial barrier is regulated by neuronal input (Costantini et al., 2010, 2012) and when bacteria cross the mucosal barrier this stimulates immune responses that complicate analyses of neuroimmune interactions.

Here, I focused on the spleen to more directly study peripheral neuroimmune interactions. It is primarily innervated by the sympathetic nervous system, removing the complication of two arms of the autonomic nervous system. It is well-studied in immune responses, and is the largest secondary lymphoid organ in the body. In this chapter we introduce the role of the autonomic nervous system and its role in the

immune response with a specific focus on the sympathetic arm. We review studies of neuroimmune interactions in the spleen and what we know about how glia in both the central and peripheral nervous system are involved with a focus on peripheral glia.

1.2 The autonomic nervous system and inflammation

The autonomic nervous system is a division of our PNS that is not under our conscious control. It is traditionally split into two arms, parasympathetic and sympathetic which have complementary roles. The system functions through reflexes which originate in the brain stem, and propagate through the spinal cord and out to the peripheral organs (Waxenbaum and Varacallo, 2019). The autonomic nervous system utilizes two neurons to relay all signals; one preganglionic neuron synapses with one postganglionic neuron before innervating its target (Waxenbaum and Varacallo, 2019).

1.2.1 The parasympathetic division

The parasympathetic nervous system traditionally thought of as the “rest-and-digest” division, is active during times of rest, after eating during digestion, and is

involved in sexual arousal. The preganglionic neurons originate in cranial nerves III, VII, IX, and X or the sacral spinal cord (Tindle and Tadi, 2020; Neuhuber et al., 2017) and utilize acetylcholine as their primary neurotransmitter. Additionally, parasympathetic tracts contain both afferent nerve fibers (carrying sensory information to the brain), and efferent nerve fibers (carrying information to internal organs). The vagus nerve, cranial nerve X, contains afferent sensory fibers originating in the nodose ganglion and efferent motor fibers originating in dorsal motor nucleus. This nerve contains approximately 90% afferent fibers (Berthoud and Neuhuber, 2000) and stimulates the cholinergic anti-inflammatory pathway, the most studied parasympathetic neuroimmune response.

The efferent vagus nerve regulates systemic cytokine levels through the α-7 nicotinic acetylcholine receptor reported on T lymphocytes (Ren et al., 2018) . Stimulating the vagus nerve has been shown to increase the anti-inflammatory cytokine IL-10 systemically and attenuates the production of many proinflammatory cytokines such as IL-1β, IL-6 and TNF (Tsaava et al., 2020; Johnston and Webster, 2009). This effect is beneficial in animal models of diseases and disorders where overactive inflammation is harmful such as sepsis (Lei and Liu, 2016), rheumatoid arthritis (Koopman et al., 2016; Levine et al., 2014), and inflammatory bowel disorders (Meregnani et al., 2011). For example, rheumatoid arthritis (RA) is a chronic autoimmune inflammatory disease primarily affecting the joints. In a mouse model of RA, stimulating the

vagus nerve significantly decreases swelling of the ankle joint and serum levels of proinflammatory cytokines IL-1β, IL-6 and TNF (Levine et al., 2014). While there are many parasympathetic fibers throughout the body not originating from the vagus nerve, the vagus nerve is the most extensive and most heavily studied

1.2.2 The sympathetic division

The sympathetic nervous system (SNS) is colloquially known as the “fight-or-flight” division of the autonomic nervous system and is activated during times of stress. The short preganglionic neurons are cholinergic and originate in the thoracolumbar division of the spinal cord. They synapse in various ganglia within the sympathetic trunk and release acetylcholine. The long post-ganglionic nerves originate in ganglia and travel far distances to their target tissue and release various catecholamines (Alshak and M Das, 2019), and other molecules including ATP, a source of cellular energy but also serves a potent neurotransmitter in activating immune cells (Westfall et al., 2002), and NPY, the most abundant peptide in our nervous system and mostly serves as a vasoconstrictor (Tan et al., 2018). The sympathetic nervous system is extremely important for immune homeostasis (Bellinger et al., 2008). For example, sympathetic denervation of mice using 6-hydroxydopamine (6-OHDA), a neurotoxin selective to noradrenergic fibers, decreases proliferation of lymphocytes in the spleen and lymph nodes (Bai et al., 2011). Furthermore, co-administering

6-OHDA with desmethylimipramine blocks the uptake of 6-OHDA, and subsequent destruction of nerves. Doing so also mitigates the decrease in lymphocyte proliferation, indicating the necessity of sympathetic nerve communication for lymphocyte functioning.

While early studies identified the importance of the sympathetic nervous system, it is unclear if these effects were due to direct stimulation of sympathetic fibers to organs or via the sympathetically-mediated release of circulating catecholamines from the adrenal glands. Sympathetically-mediated immune suppression can be seen in many clinical examples including trauma to the nervous system, as in stroke and spinal cord injury (SCI). After both stroke and SCI, serum and plasma levels of epinephrine and norepinephrine are elevated as early as 6 hours and persists out to 2 weeks (Zhang, 2014; Faaborg et al., 2014) leaving both patients and animals susceptible to infections as immune cells begin to die (Pruss et al., 2017; Dirnagl et al., 2007). Treating animals with adrenergic blockers such as carvedilol or propranolol, two non-specific beta-adrenergic blockers, not only decreases the size of strokes (Ajmo Jr. et al., 2009), but also improves mortality and reduces their susceptibility to infection (McCulloch et al., 2017). This can also be seen in autoimmune diseases like multiple sclerosis (MS). In MS the immune system destroys myelin in the CNS resulting in destruction of nerves and subsequent loss of function. In a rodent model of MS called experimental autoimmune encephalomyelitis (EAE), ablating adrenergic

nerves using the neurotoxin DSP4 prior to induction of disease significantly increases inflammation as well as the onset and severity of disease (Simonini et al., 2010). This indicates that sympathetic signaling is necessary for dampening inflammation and controlling disease progression as in the same animals stimulating them with the synthetic epinephrine precursor L-threo-3,4- dihydroxyphenylserine (L-DOPS) prevented further worsening.

There is thus significant research on the sympathetic nervous system and immune responses, yet much is still to be elucidated. It is still unclear how much direct innervation is involved in controlling inflammation locally and systemically. There is some evidence however that direct innervation may play a role in the spleen's response to infection and injury (see "Immunomodulatory functions of the splenic nerve" below). However, these studies do not exclude influence from circulating catecholamines. Nor do they examine the role of glia in mediating and modulating inflammatory effects and how they may influence duration of neuro-modulatory effect. Indeed, with the notable exception of enteric glia, visceral glia have largely been excluded from studies. Here, I set out to begin to understand the interaction between nerves, immune cells and glia in the spleen.

1.3 Anatomy and function of the spleen

Found in all vertebrates, the spleen is the largest secondary lymphatic organ in the body. It is responsible for stimulating both humoral (secretion of antibodies and antimicrobial molecules) and cell mediated (cytokine release, phagocyte activation, and immune response) pathways. The spleen is compartmentalized into two main divisions, the red pulp and white pulp. The red pulp contains the majority of monocytes in the body (Den Haan and Kraal, 2012) and contains sinusoids filled with blood and macrophages that phagocytose old red blood cells.

1.3.1 The white pulp

The white pulp represents the immunological portion of the organ, where immune responses take place, and is further divided into T (periarterial lymphoid sheath) and B (follicle) lymphocyte zones. White pulp blood vessels are porous, facilitating antigen-lymphocyte interactions that initiate immune responses. All immune cells enter the white pulp through the marginal zone (MZ) (Bajénoff et al., 2008), a region considered to be a main responder for innate immune responses due to the large population of macrophages. Many of the macrophages housed in the MZ express specific receptors unique to their location which are known as pattern recognition receptors. Innate immune defense involves interactions between microbes and these

specific receptors. Within the marginal zone of the white pulp is the B-cell corona (follicle). This space contains many of the B lymphocytes, macrophages and dendritic cells within the spleen, and contains the germinal center in which the B lymphocytes migrate to proliferate, differentiate and undergo somatic hypermutation. Adjacent to the marginal zone is the periarteriolar lymphoid sheath (PALS) region. This region is predominately home to T lymphocytes and other antigen presenting cells such as dendritic cells and macrophages and surrounds a central arteriole (Cesta, 2006). Interestingly, the white pulp is densely packed with lymphocytes, but there are small conduits, <1 μm channels, that allow small molecules from the blood to interact with lymphocytes that are not directly adjacent to the blood vessels (Nolte et al., 2003). In addition to antigens, conduits are ideally situated to carry sympathetic neurotransmitters (norepinephrine and epinephrine, but also substance P and ATP) to lymphocytes. Therefore, they may be critical to facilitating neuroimmune communication as this conduit system provides a framework for directing lymphocytes through communication from locally produced and bloodborne molecules in the spleen.

1.3.2 The splenic nerve

The spleen is innervated by the splenic nerve, which contains 98% sympathetic fibers (Bellinger et al., 1989; Nance and Burns, 1989). First order preganglionic neurons originate from the intermediolateral gray column of the thoracic spinal cord

synapse on second order neurons with cell bodies in the superior mesenteric ganglion that project into the spleen, forming the splenic nerve (Felten and Olschowka, 1987; Nance and Burns, 1989; Heusermann and Stutte, 1977). Although the extent of splenic nerve branching it is not completely clear , electron microscope studies have shown that branches of this nerve terminate in in the white pulp and come into close contact with immune cells (Murray et al., 2017; Felten and Olschowka, 1987; Heusermann and Stutte, 1977). The close proximity of nerve fibers to immune cells and the fact that immune cells express receptors for neurotransmitters released by the nerve suggests some role for sympathetics in the spleen. This is evident as large sympathetic events such as stroke, trauma or spinal cord injury induce profound splenic responses. One such response to these traumatic events is a splenic contraction in which increased sympathetic tone causes the spleen to dramatically shrink in size and dump its housed immune cells into circulation. This can be seen after stroke (Seifert et al., 2012; Offner et al., 2006), spinal cord injury (Brommer et al., 2016) and traumatic brain injury (Ritzel et al., 2018). Not only is this evident in rodent models, but humans as well, and has been characterized directly through injection of epinephrine (Bakovic et al., 2013). The immune response to increased sympathetic tone appears to be occurring in two phases. Generally, sympathetic stimulation appears to be proinflammatory in the acute phase, after which there is a depletion of spleen immune cells (splenocytes) that subacutely leads to increased risk of infection

of the host (immunosuppression). Another example from stroke is sympathetically-mediated death of B lymphocytes, that also increases the susceptibility to infection (McCulloch et al., 2017). The immunosuppressive effects of sympathetic activation after stroke has led to a tremendous amount of attention in the past with clinical studies often showing conflicting results. This adds to the need for further dissection of neuroimmune responses, as infections after CNS trauma increase morbidity and mortality. Several aspects of this response are not well defined. As alluded to earlier, it is not clear how sympathetic tone interacts with immune cell state in the spleen (i.e. is sympathetic signaling alone enough to activate immune cells?), and the contribution of direct innervation by the splenic nerve versus indirect influences of sympathetic innervation to the adrenal glands are not known.

1.4 Immunomodulatory functions of the splenic nerve

As mentioned earlier, there is some research that hints that the nerve itself may be important. One example can be seen from studies injecting lipopolysaccharide (LPS) into animals. LPS is the major component of the cell wall of gram-negative bacteria. It binds toll-like receptors on immune cells which initiates an innate immune response, even without an active infection. After LPS is injected into mice, splenic nerve activity greatly increases and persists well over an hour after injection (MacNeil et al., 1996). However, it is not clear if this increase in nerve activity directly leads to immune cell

activation. The result of LPS injections is subsequent systemic inflammation which can be measured by increases in both blood and splenic levels of TNF, IL-6 and IL-1β. This effect can also be seen in models of "sterile inflammation" such as after stroke (Zhang, 2014; Pennypacker and Offner, 2015) and spinal cord injury (Pruss et al., 2017) where the sympathetic and inflammatory trigger is not exogenous.

The importance of the splenic nerve can also be seen in splenic responses after the nerve is transected (neurectomy). Injection of LPS into rats who have undergone splenic neurectomy leads to decreased inflammation (Rosas-Ballina et al., 2008; Vida et al., 2011) and decreased proliferation of lymphocytes (Bai et al., 2011; Madden et al., 1994). In a foot-shock stress paradigm that suppresses the function of immune cells in the spleen, splenic neurectomized rats are protected from stress induced loss of plaque-forming B-cells (MacNeil et al., 1996; Laukova et al., 2018). A similar regulatory effect can be seen in primary lymphoid organs such as bone marrow. Animals with denervated bone marrow have impaired hematopoiesis and regeneration Hanoun et al. (2015). This establishes that direct innervation to immune organs may play a crucial role in inflammation and immune responses.

However, there is also some research that suggests the splenic nerve is not important for splenic immune responses. One study seems to suggest that the sympathetic nervous system has no real effect on inflammatory outcomes after LPS. Rats who

underwent splenic neurectomy, adrenalectomy or both, show no differences in proinflammatory cytokines TNF, IL-1β and IL-6 after intravenous LPS injections (Meltzer et al., 2003) . This suggests that some part of the spleen-mediated immune response may be removed from direct neural regulation. It may be that both hypotheses are true and that variations in experimental design tip the balance between the two influences. For instance, when comparing LPS studies, various LPS injection protocols (intraperitoneal vs intravenous), timing (1-12hr), species of LPS (*E .coli* strains vs *S. enterica*), animal model (mouse vs. rat) and genetic background all may contribute to varying results. Moreover, these studies do not acknowledge the potential role of glia in the spleen which likely influence interactions between the sympathetic nervous system and immune cells.

1.5 Glia in health and disease

1.5.1 CNS glia

Glia exist in both the central and peripheral nervous system. Not only do they provide support and nutrition to neurons and nerves, but they have a host of other capabilities including maintenance of the blood brain barrier, pathogen defense, neurotransmission, aiding digestion and myelination. Glia are best studied in the brain, where there are three main cell types: microglia, oligodendrocytes, and astrocytes.

Developmentally, microglia are immune cells resembling macrophages in many ways (Nayak et al., 2014). The primary function of oligodendrocytes is to wrap their cell membranes around axons of neurons to produce an insulation called myelin that aides in the propagation of neural electrical signals (Bradl and Lassmann, 2010). Finally, astrocytes are the most abundant glial cell type in the CNS (M Nedergaard, 2003). They are important for maintaining the blood-brain-barrier (Abbott et al., 2006; Davson and Segal, 1995), regulating vasoconstriction (Macvicar and Newman, 2015), and modifying neurotransmission via tripartite synapses (Perea et al., 2009). They can also produce calcium waves through neuronal release of neurotransmitters such as ATP and glutamate (Fujii et al., 2017; Scemes and Giaume, 2006; McCarthy and Salm, 1991). In addition, astrocytes can also participate in immune responses (Drögemüller et al., 2008; Lian et al., 2016). They have been shown to phagocytose unwanted debris (Jung and Chung, 2018; Wakida et al., 2018), secrete cytokines and chemokines to recruit immune cells to sites of injury (Choi et al., 2014; Xie and Yang, 2015), and express complement proteins implicating their role in innate immune responses (Lian et al., 2016; Pekny et al., 2007; Hartmann et al., 2019).

1.5.2 PNS glia

The peripheral nervous system (PNS) contains several distinct glial cell types. The main glial cell of the PNS, Schwann cells, exist in two forms: myelinating and non-myelinating (Fallon and Tadi, 2019; Harty and Monk, 2017). Both types of Schwann cells have been described in many different tissues including olfactory ensheathing cells in the olfactory nerve, terminal glia (teloglia) located at axon terminals at the neuromuscular junction and sensory nerve endings in the skin, and satellite glia in the dorsal root ganglia. Myelinating Schwann cells are analogous to oligodendrocytes of the CNS, wrapping around nerves and producing myelin. While much work has been done defining their role in the periphery, much of what is known about myelinating Schwann cells came from initial studies on oligodendrocytes.

Non-myelinating Schwann cells are the most abundant cells in the peripheral nervous system (Griffin and Thompson, 2008). These cells ensheath the predominantly unmyelinated autonomic nerves that innervate visceral organs. However, non-myelinating Schwann cells in visceral organs are not well studied. The exception to this is enteric glia.

1.5.2.1 Enteric glia

Enteric glia are the principal glia in the enteric nervous system. The enteric nervous system is a neural network in the gut wall that coordinates the reflex behaviors of the intestine. Enteric glia and astrocytes are thought to be analogous in many ways. They express similar proteins and perform many of the same functions, such as facilitating neurotransmission (Gulbransen, 2014) and regulating organ barrier (Savidge et al., 2007). Although enteric glia and astrocytes have many similarities, research has revealed specific transcriptional differences and morphologic heterogeneity that indicate enteric glia as a fundamentally unique glial type (Rao et al., 2015). It is of importance to note that in addition to neuronal heterogeneity in the gut, there are multiple subpopulations of glial cells throughout the gut that are all referred to as enteric glia. There are four subpopulations, differentiated by location and morphology (Gulbransen, 2014). Intraganglionic glia reside in the myenteric and submucosal ganglia and are star-shaped with short, irregularly branched processes that morphologically resemble astrocytes of the CNS. Interganglionic glia, those within interganglionic nerve tracts, have elongated processes resembling fibrous astrocytes of the white matter tracts. Mucosal glia reside below mucosal epithelial cells and have several long-branched processes. Lastly, intramuscular glia are associated with nerves and smooth muscle cells, and are elongated run along nerve fibers in muscles. Each of the subtypes reside in very unique microenvironments contributing the

heterogeneity of morphology but their molecular differences are not known (Grubišić and Gulbransen, 2017). Enteric glia are known to play a role in gut pathophysiology. They interact with both innate and adaptive arms of the immune system to contribute to local inflammation. They express toll-like receptors (Turco et al., 2014) and up-regulate MHCII mRNAs during disease states, such as in Chagas disease (Von Boyen and Steinkamp, 2010). They also have the ability to release nitric oxide, an inhibitory neurotransmitter (Cerantola et al., 2020). This data suggests a complex interaction between nerves, immune cells and enteric glia.

A similar interaction is likely in other visceral organs but not studied. Because of our interest in sympathetically-mediated immune responses we chose to study the glia that are in the spleen. Due to uniform innervation, we hypothesized that glia in the spleen may be more homogeneous than in the gut. Outside of early electron microscope studies identifying their presence in the spleen (Heusermann and Stutte, 1977; Barlow-Anacker et al., 2017), spleen glia were fairly uncharacterized at the start of my work.

1.6 Overview of dissertation

This dissertation thus focuses on improving our understanding of spleen glia, as well as their relationship to the nerve. In chapter 2, I use immunohistochemistry to

define the location of spleen glia in relation to the splenic nerve, vasculature, and immune cells. In addition, I validate a transgenic reporter mouse expressing GFP under the GFAP promotor which may be used to visualize spleen glia, along with two antibodies that label spleen glia. I also for the first time sequence their transcriptomes and describe mRNAs they express that are involved in communication with the nerve fibers and immune cells. Lastly, I compare spleen glia transcriptomes to various brain and peripheral glia defining that they are a unique glial population which may be more heavily involved in immune responses. The work here is a comprehensive description of these cells and is an essential first step to elucidating their role in neuroimmune responses in the spleen.

In chapter 3, I develop a surgical model to injure the splenic nerve in order to define the relationship between spleen glia and the splenic nerve. Utilizing transgenic *Sarm1 -/-* mice, whose nerves are protected from Wallerian degeneration, I subject animals to splenic nerve cut (splenic neurectomy) and see the loss of nerves in wild-type animals. I report preliminary studies that in wild-type animals but not *Sarm1* knockouts there is also significant injury to the ensheathing spleen glia. This chapter reveals robust physical changes to spleen glia indicating potential trophic factors involved in maintaining this relationship between spleen glia and peripheral nerves.

The work provided in this dissertation lays a foundation for understanding neuroimmune interactions in the spleen. The advancements presented here will hopefully inform the ultimate goal of identifying a cellular target which might be manipulated in order to gain control of peripheral inflammation during disease and injury.

Chapter 2

Spleen glia are a transcriptionally unique glial subtype interposed between immune cells and sympathetic axons

The following chapter includes all text and figures from the following publication, of which I am the first author.

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TAL performed tissue collection, immunohistochemistry, qPCR and bioinfromatic analysis. TAL and LZ performed cell dissociation. TAL and MSB conceived experimental plan and wrote manuscript.

2.1 Summary

Glia are known to play important roles in the brain, the gut, and around the sciatic nerve. While the gut has its own specialized nervous system, other viscera are innervated solely by autonomic nerves. The functions of glia that accompany autonomic innervation are not well known, even though they are one of the most abundant cell types in the peripheral nervous system. Here, we focused on non-myelinating Schwann cells in the spleen, spleen glia. The spleen is a major immune organ innervated by the sympathetic nervous system, which modulates immune function. This interaction is known as neuroimmune communication. We establish that spleen glia can be visualized using both immunohistochemistry for S100B and GFAP and with a reporter mouse. Spleen glia ensheath sympathetic axons and are localized to the lymphocyte-rich white pulp areas of the spleen. We sequenced the spleen glia transcriptome and identified genes that are likely involved in axonal ensheathment and communication with both nerves and immune cells. Spleen glia express receptors for neurotransmitters made by sympathetic axons (adrenergic, purinergic, and Neuropeptide Y), and also cytokines, chemokines, and their receptors that may communicate with immune cells in the spleen. We also established similarities and differences between spleen glia and other glial types. While all glia share many genes in common, spleen glia deferentially express genes associated with immune responses,

including genes involved in cytokine-cytokine receptor interactions, phagocytosis, and the complement cascade. Thus, spleen glia are a unique glial type, physically and transcriptionally poised to participate in neuroimmune communication in the spleen.

2.2 Introduction

Glia play important and complex functional roles in both the central (CNS) and peripheral (PNS) nervous systems. In the brain, astrocytes integrate and process synaptic information and control synaptic transmission and plasticity (Perea et al., 2009). On the other hand, enteric glia in the gut modulate gut motility via bidirectional signaling with neurons, altering smooth muscle cell contraction (Grubišić et al., 2018). While the gut has its own specialized nervous system, a division of the autonomic nervous system, other visceral organs are innervated solely by autonomic (sympathetic and parasympathetic) and sensory nerves. The functions of non-myelinating glia that accompany autonomic innervation are not well known, despite being one of the most abundant cell types in the PNS (Griffin and Thompson, 2008). Although the structure and function of glia in each visceral organ is relatively unknown, they are likely specialized to the nerves they accompany and the organs in which they reside. This may be particularly important for glia in immune organs such as the spleen, in which the nerves ensheathed by glia come in close contact with immune cells (Felten and Olschowka, 1987), potentially positioning them to contribute

to neuroimmune crosstalk.

Non-myelinating Schwann cells in the spleen (spleen glia) associate with axons and are derived from the neural crest (Barlow-Anacker et al., 2017; Felten and Olschowka, 1987; Heusermann and Stutte, 1977). The spleen is the largest secondary lymphoid organ and plays a vital role in detecting bloodborne antigens and conferring immunologic memory. It is innervated by the splenic nerve which is predominantly sympathetic. The sympathetic nervous system modulates immunity (Bellinger et al., 2008; Bucsek et al., 2018; Deng et al., 2004; Miller et al., 2019), and epinephrine and norepinephrine released from the adrenal glands and sympathetic nerves within organs act on adrenergic receptors expressed by immune cells (Miller et al., 2019; Klehmet et al., 2009; Pruss et al., 2017). An example of this in the spleen occurs after stroke, when the sympathetic nervous system mediates the death of B-lymphocytes, thus increasing the susceptibility to infection (McCulloch et al., 2017). Given the well-described functional roles of other glia such as altering neurotransmission, releasing cytokines and chemokines, and phagocytosis, spleen glia may serve to modulate this and other neuroimmune interactions in the spleen. However, a complete characterization of their localization within the organ and their anatomical interactions with other cell types is lacking. Additionally, the genomic identity of spleen glia is unknown. A comprehensive anatomical and transcriptomic description of spleen glia is therefore essential to determine if they are localized near immune cells, whether they express

receptors and other genes that might be used for neuroimmune crosstalk in the spleen, determine what other processes they may mediate, and to begin to understand how similar they are to other glial types.

Thus, in our research we utilized immunohistochemistry and spleen glia reporter mice to characterize the anatomy and morphology of spleen glia, including their anatomical interactions with sympathetic axons and immune cells. In addition, we generated a transcriptome of spleen glia to define which mRNAs they express and infer possible functions. Lastly, we compared the transcriptome of spleen glia to that of other CNS and PNS glia to determine similarities and differences between different glial cell types.

2.3 Results

2.3.1 Spleen glia express S100B and GFAP and ensheath sympathetic axons

We began by defining the relationship between spleen glia, nerves, blood vessels and immune cells in the spleen. To achieve this, we utilized a peripheral glia reporter mouse (C57BL/6J *Gfap*-cre, *Rosa26-eGFP*) in which pulmonary glia were labelled with GFP (Suarez-Mier and Buckwalter, 2015). We first wanted to define where GFP

expressing cells were in relation to the greater spleen architecture. To determine this we stained spleen sections for DAPI to mark all cell bodies and performed low-power tile-scan imaging. We see that GFP^{+} cells primarily reside in white pulp regions and around arteries (Figure 2.1 A). To verify that this reporter mouse also expressed GFP in spleen glia, we immunostained spleen sections for two glial markers, GFAP and S100B. We observed GFP-expressing cells with an elongated morphology that co-immunostain for both glial markers in the spleen (Figure 2.1 B&C). The GFP reporter was sensitive and specific: $92 \pm 1.5\%$ of GFAP expressing cells expressed GFP, and $99 \pm 0.47\%$ of GFP expressing cells co-immunolabeled for GFAP. Additionally, we found that S100B strongly labels spleen glia with high sensitivity, labelling 100% of GFP-expressing cells. The antibody also very weakly labels a subset of macrophages in the spleen, likely due to its known cross-reactivity with S100A6.

The spleen is innervated by the splenic nerve, which itself is comprised of sympathetic axons that originate from cell bodies in the superior mesenteric ganglion. In general, unmyelinated peripheral nerves can be uniformly associated with glia or have naked terminals (Forbes et al., 1977; Novi, 1968). To assess the association of spleen glia and axons, we immunostained spleen sections for two nerve markers: tyrosine hydroxylase (TH) and protein gene product 9.5 (PGP 9.5). The former is unique to sympathetic and dopaminergic nerves, and the latter is a marker specific to neural and neuroendocrine cells (Figure 2.2). We observed elongated GFP-expressing spleen glia

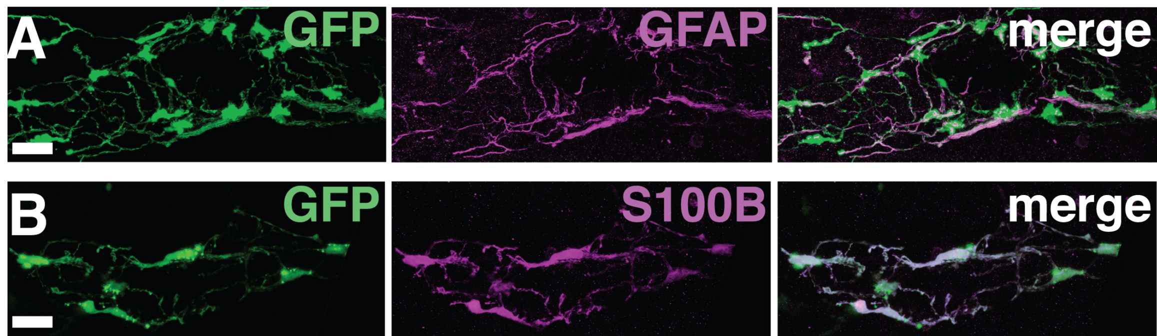

Figure 2.1: *Gfap*-cre, Rosa-eGFP reporter mice express GFP in GFAP- and S100B-expressing cells in the spleen.
(A) Low power tile scan of *Gfap*-GFP reporter mouse counterstained with DAPI. Circles and stars represent white pulp regions. Spleen glia can be seen to mostly reside in white pulp regions. Scale bar, 100 μm. Representative photomicrographs of 40 μm thick spleen sections from *Gfap*-GFP reporter mice immunostained for **(B)** GFAP and **(C)** S100B. Scale bars, 20 μm. WP, white pulp; RP, red pulp.

tightly associated with axons (within 1 µm) expressing both TH (Figure 2.2 A&B) and PGP 9.5 (Figure 2.2 C&D), with the two existing <1um away from each other. Glia tightly intertwined around axons, and we also observed occasional slight axonal protrusions that appear to come to the surface of the glia. These may represent varicosities described in EM experiments where neurotransmitters are postulated to be released through glial fenestrations. Additionally, upon measuring concordance between glia and axons we observed that all glia (100%) were associated both TH^+ and PGP 9.5^+ immunolabeled axons in those respective separate stains, while 97.45 $\pm$ 0.85% of TH^+ axons and 98.0 $\pm$ 0.66% of PGP 9.5^+ axons were associated with glia. This result reveals that glia inside the spleen are exclusively associated with sympathetic axons, and that nearly all splenic nerve axons are physically associated

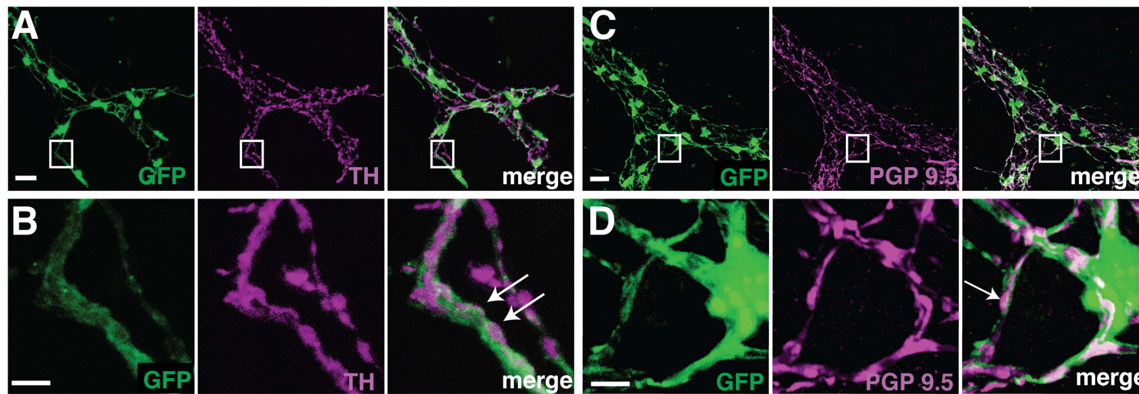

Figure 2.2: Spleen glia ensheath sympathetic nerves in the spleen. Representative photomicrographs of 40 μm thick sections of spleen. GFP expression is closely associated with nerves expressing tyrosine hydroxylase (TH)**(A&B)** This close relationship can also be seen using a pan-neuronal marker protein gene product 9.5 (PGP 9.5) **(C&D)** Both panels show the tight association between nerve and glia. The morphology suggests that axonal protrusions containing neurotransmitter vesicles (TH stain) may protrude out of a fenestrated glial sheath (arrows). Scale bars = 20 μm (A&C), 5 μm (B&D).

with glia.

2.3.2 Spleen glia form intricate networks around arterioles that are closely apposed to lymphocytes

The splenic nerve enters the spleen at the hilum, traveling on the outside of the splenic artery. Sympathetic fibers accompany the artery as it branches into fenestrated arterioles that course through the white pulp of the spleen. Fenestrated arterioles have a discontinuous endothelial lining (Aird, 2007), which, where present, can

be visualized with the endothelial marker CD31. We observed glia and nerves forming intricate networks around arterioles which were discontinuously lined by CD31-expressing endothelial cells (Figure 2.3).To verify that the glia and nerves were located around the splenic arterioles in the white pulp we immunostained spleen sections for T and B lymphocytes (Figure 2.4). Interestingly, we observed that glia favor T lymphocyte regions as staining reveal prominent residence in these locations followed by branching towards B cell zones (Figure 2.4).Spleen glia are in white pulp regions ¿ 90% of the time, and 100% (63/63) of white pulp regions contain glia. Glia that were not in white pulp are around larger arteries in the red pulp at the edge of white pulp regions (Figure 2.4 A). Arterioles are surrounded by a meshwork of glia as they travel in the peri-arteriolar lymphoid sheath, or PALS region, where $CD3^{+}$ T lymphocytes are housed, and continue to be ensheathed as they course into $B220^{+}$ B lymphocyte follicles (Figure 2.4 A&B). Thus, there is a close physical relationship between the glia, nerves, and lymphocytes. However, we did not observe a similar close association with IgM^{+} marginal zone B-cells,we instead saw that the glia are primarily in adjacent regions (Figure 2.4 F). Also, the red pulp, which receives its blood supply after arterioles have become capillaries and contains primarily monocytes, macrophages, and red blood cells, exhibited very sparse staining for nerves or glia.

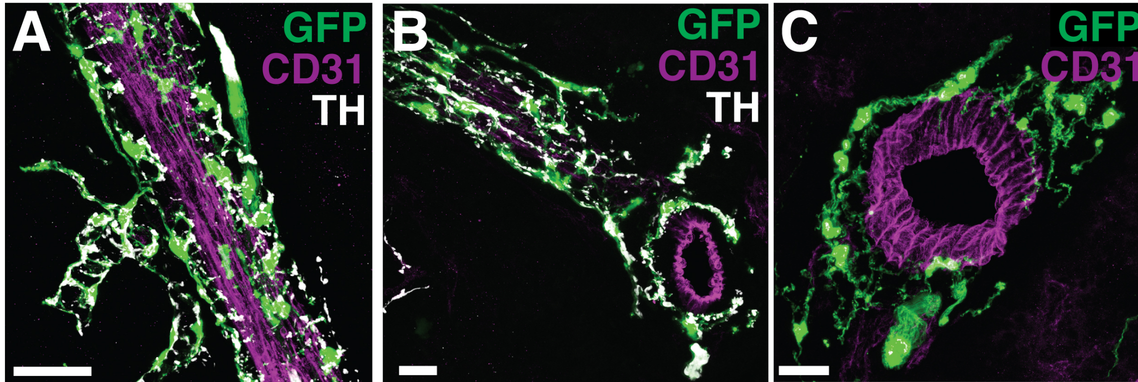

Figure 2.3: Spleen glia form networks that associate with vasculature. Representative photomicrographs of 40 μm sections of spleen depicting thickened processes of spleen glia wrapping around CD31$^+$ vessels. **(A)** Network of glia and nerves closely associate with a continuously lined artery in the spleen. These networks course through white pulp regions and wrap around smaller arteries within PALS regions **(B)** and can also be seen with thickened processes around larger vessels **(C)**. Scale bars, 20 μm

2.3.3 Transcriptional profiling of spleen glia

Our anatomical characterization of spleen glia demonstrates that they are physically juxtaposed between axons and lymphocytes poised to receive molecular signals emanating from the bloodstream (e.g. antigens). This close physical relationship means it is possible that glia participate in neuroimmune signaling and are also likely to provide physical and potentially nutritional support to axons. To determine whether they express genes consistent with these or other functions, we performed RNA sequencing. As described in Materials and Methods, spleen glia were size-selected from spleens of C57BL/6J mice, and total RNA was obtained (Figure 2.5 A)

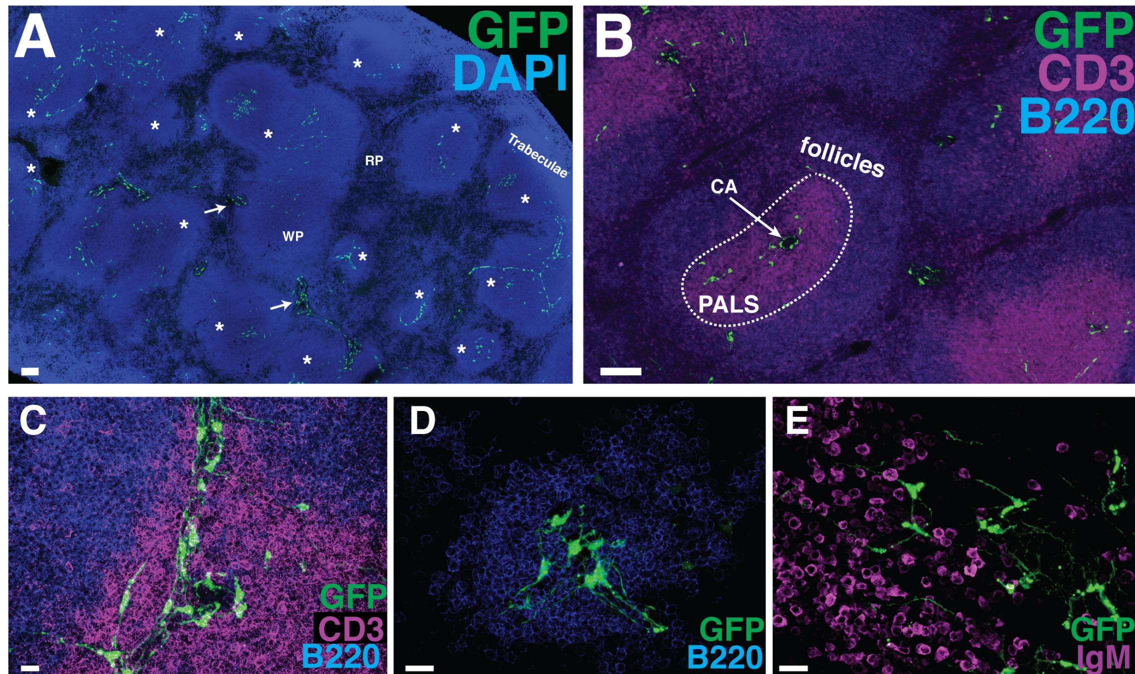

Figure 2.4: Spleen glia course through lymphocyte-containing white pulp.
Representative photomicrographs of 40 μm sections of spleen depicting spleen glia throughout PALS and B-cell follicles. Low power tile scan spleen section showing spleen glia within white pulp regions throughout the spleen. Spleen glia can be seen in close proximity to T-cells in the PALS regions and around the central arteriole **(A&C)** and within B-cell follicles **(B&C)**. The tubular networks of glia branch within B-cell follicles **(B)**, and course through the T-cell-rich PALS regions **(D)**. Scale bars, 100 μm (A), 20 μm (B-D); PALS, periarterial lymphoid sheath; CA, central arteriole.

from size-selected spleen glia. Prior to sequencing, RT-qPCR was carried out to confirm expression of known markers of peripheral glia (*S100b* and *Sox10*). Both markers were enriched in the size-selected spleen glia RNA vs. whole spleen RNA, with $\log_2$ fold increases of 5.4 ± 0.88, and 5.3 ± 1.2, respectively (Figure 2.5 B). In addition, we observed depletion of protein tyrosine phosphatase, receptor type, C (*Ptprc*) with $\log_2$ fold decrease of -1.5 ± -0.65. Ptprc is a type I transmembrane protein that is present on all differentiated hematopoietic cells and encodes the immune cell marker CD45. These results indicate that our size selected cells were strongly enriched for glia and de-enriched for immune cells. We selected 8 samples (4 male & 4 female) aged 8-12 weeks that were highly enriched for glial markers *S100b* and *Sox10* ($\log_2$ fold enrichment >5) for RNA sequencing analysis.

We next utilized cellular deconvolution to further increase specificity for spleen glia genes in our dataset. Deconvolution is a bioinformatic approach that allows researchers to determine the fraction of a cell-type in bulk sequencing data (Kang et al., 2019; Newman et al., 2019). Deconvolution analysis revealed that our sequenced samples contained majority glial cells (mean = 73% ± 4%). All white blood cell types combined accounted for <2% of all genes, while endothelial cells represented the bulk of the contaminating cells, approximately 18% (Figure 2.5 C). Signature gene sets from contaminating cells were then subtracted in proportion to each cell type's contribution in their corresponding bulk sample, and the newly generated purified

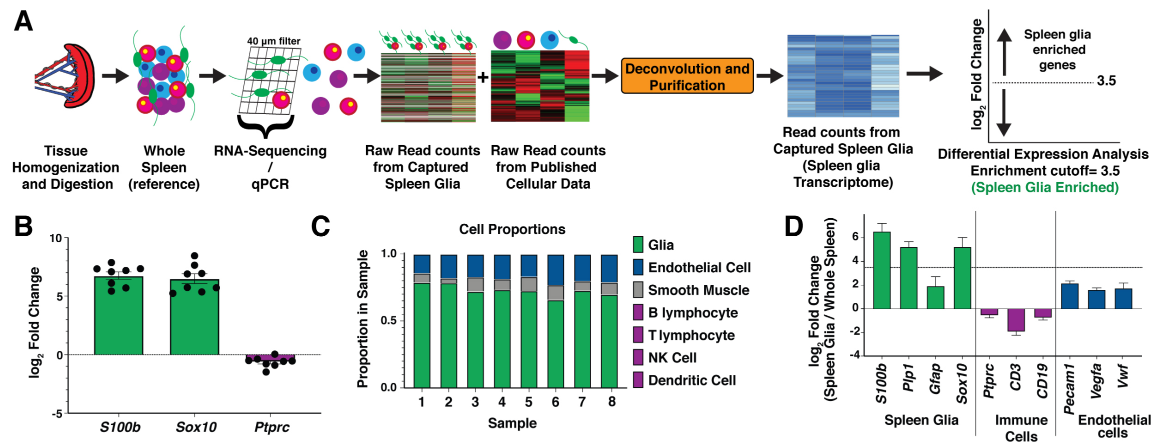

Figure 2.5: Generating the spleen glia transcriptome.
(A) Strategy for isolating and obtaining spleen glia transcriptome and spleen glia enriched gene set. **(B)** Quantitative real-time PCR of reverse-transcribed genes from RNA selected from size-selected spleen glia prior to RNA sequencing. We observed enrichment of two peripheral glial markers (*S100b*, *Sox10*) and de-enrichment of *Ptprc*, which encodes the cell surface marker CD45 that is expressed on all immune cells. **(C)** Cell-type deconvolution results after RNA sequencing demonstrate cell-type proportions in bulk samples prior to purification. **(D)** Enrichment of glial, immune cell, and endothelial cell genes in the spleen glia transcriptome after post-deconvolution purification. Error bars, SEM.

spleen glia transcriptomes were used for downstream differential expression analysis.

To define spleen glia-specific genes even more stringently within the purified spleen glia transcriptome, we used an enrichment cutoff of $\log_2$ fold >3.5 (FDR <0.01). We refer to this set of stringently selected genes as "spleen glia enriched genes". Expression cutoff was tested through sensitivity as described in the methods and was determined to be optimal. The $\log_2$ fold >3.5 enrichment compared to whole spleen was chosen after we examined fold enrichment of genes common to glia, immune cells and endothelial cells. Differential expression analysis reveals $\log_2$ fold enrichment >5 of peripheral glial genes *Sox10* and *S100b* over whole spleen (Figure 2.5 D). Furthermore, there is similar enrichment of other known glial genes including *Atp1b2*, *Gja1* (Connexin 43), and *Plp1.* We then checked for contamination of known markers of immune cells and endothelial cells. Analysis demonstrated that immune cell genes Ptprc, T-cell specific (*Cd3*) and B-cell specific (*Cd19*) genes were de-enriched, with $\log_2$ fold $<$ -1. Furthermore, we saw only marginal enrichment of endothelial cell specific genes, with $\log_2$ fold increases of about 2. We thus chose $\log_2$ fold > 3.5 for further downstream analysis.

Using the expression cutoffs, we established a "spleen glia enriched genes" list containing 2,202 genes. (Table 2.1) lists the top 25 enriched genes. Several genes

Gene Name	Description	Log_2 FC	FDR	Spleen Glia TPM	Whole Spleen TPM
Pde2a	phosphodiesterase 2A, cGMP-stimulated	20.83	1.73E-11	1.165	0.000
Tnc	tenascin C	9.78	2.08E-24	6.005	0.009
Gabrq	GABA A receptor, subunit theta	9.51	6.37E-32	4.743	0.004
Tmem88b	transmembrane protein 88B	9.47	5.23E-25	3.361	0.000
Nlgn3	neuroligin 3	9.24	9.55E-29	1.900	0.000
Fzd2	frizzled class receptor 2	9.06	1.87E-29	2.378	0.000
Sbspon	somatomedin B and thrombospondin, type 1	8.75	2.40E-28	5.591	0.010
Flnc	filamin C, gamma	8.75	9.53E-20	1.206	0.000
Tnnt2	troponin T2, cardiac	8.70	3.59E-15	8.041	0.013
Col6a3	collagen, type VI, alpha 3	8.69	2.70E-61	9.384	0.014
Csmd1	CUB and Sushi multiple domains 1	8.68	3.46E-26	1.760	0.001
Sfrp5	secreted frizzled-related sequence protein 5	8.66	2.78E-16	15.894	0.030
Pi15	peptidase inhibitor 15	8.54	1.57E-27	2.565	0.006
Kcne4	potassium voltage-gated channel subfamily E, gene 4	8.51	4.65E-28	20.511	0.050
Gpr20	G protein-coupled receptor 20	8.47	8.94E-23	4.655	0.011
Fam151a	family with sequence similarity 151, member A	8.43	1.29E-22	5.106	0.011
Bmp3	bone morphogenetic protein 3	8.40	7.09E-20	1.015	0.000
Grem1	gremlin 1, DAN family BMP antagonist	8.32	2.09E-20	1.640	0.003
Capn6	calpain 6	8.30	1.06E-20	1.243	0.000
Pcdhb11	protocadherin beta 11	8.30	1.94E-24	1.160	0.000
Epha8	Eph receptor A8	8.29	1.78E-22	1.064	0.000
Cadm2	cell adhesion molecule 2	8.29	3.12E-14	3.300	0.019
Thbs2	thrombospondin 2	8.28	3.54E-24	14.551	0.038
Kcna5	potassium voltage-gated channel, 5	8.25	2.41E-20	2.851	0.008
Ppp1r3c	protein phosphatase 1, regulatory subunit 3C	8.23	6.93E-25	2.366	0.004

Table 2.1: Top 25 most differentially expressed genes in spleen glia. Log2 fold change is relative to whole spleen. FC, fold change; FDR, false discovery rate; TPM, transcripts per million reads.

on this list are expressed in both PNS and CNS glia, including potassium voltage-gated channels *Kcne4* and *Kcna5*, indicating that spleen glia, like enteric glia and astrocytes, may be involved in homeostatic regulation of extracellular potassium. Moreover, we see robust expression of Neuroligin 3, a cell adhesion molecule involved in maintaining the glial sheath around peripheral nerves (Gilbert et al., 2001) , and Tenascin 3, a glycoprotein necessary for gliogenesis (Wiese et al., 2012). Additionally, we examined this gene set for sex differences. However, there were few significant differences (data not shown), so we pooled all samples for the remainder of analysis.

2.3.4 Spleen glia express various neurotransmitter receptors

Our immunohistochemical characterization demonstrated that spleen glia were always associated with sympathetic axons. Considering this, we next investigated how the nerve and glia might communicate. We searched for genes encoding receptors of molecules known to be released by sympathetic axons including norepinephrine (NE), and to a lesser extent epinephrine (EPI), purines, and neuropeptide Y. We examined all receptors for these three neurotransmitters that were present in the whole spleen and the spleen glia transcriptome (Figure 2.6 A). We indeed observed significant expression and enrichment of various adrenergic receptors in spleen glia compared to whole spleen. Of the adrenergic receptor subtypes, the β2-adrenergic receptor was most highly expressed by spleen glia. The β2-adrenergic receptor is also expressed by astrocytes, enteric glia, and Schwann cells. In addition, our data showed that spleen glia highly express α-adrenergic receptors, three of which are highly enriched compared to whole spleen (*Adra1b*, *Adra1d*, and *Adra2c*). This is notable because the α1-adrenergic receptor has a higher affinity for NE than EPI (Rang et al., 2016) and the splenic nerve predominantly releases NE (Kirpekar and Misu, 1967).

Spleen glia were also more enriched for Neuropeptide Y receptor 1 expression than whole spleen RNA. Neuropeptide Y is primarily released by sympathetic nerves in the PNS (Tan et al., 2018). Expression of its receptor in Schwann cells has already been

validated (Park et al., 2015). Its exact role, however, has not been fully elucidated.

Spleen glia also enrich for several purinergic receptors (Figure 2.6 A). ATP has long been recognized as an intracellular energy source, but it is also a potent extracellular neurotransmitter co-released by sympathetic nerves (Westfall et al., 2002). Purinergic signaling in glia has been linked to glial proliferation, motility, and survival as well as cytokine signaling and injury responses in Schwann cells and astrocytes. *P2ry2* was the most highly enriched purinergic receptor subtype. This subtype has known functions in astrocytes inducing activation and release of inflammatory molecules (Erb and Weisman, 2012).

2.3.5 Pathway analysis reveals expected and potentially novel roles for spleen glia

Spleen glia likely serve similar functions as other peripheral glia, such as ensheathing and supporting nerves, guiding axons during axon outgrowth and response to injury and disease. However, they may serve other functions as well. To assess their likely functions in an unbiased fashion, we carried out pathway analysis on the spleen glia enriched gene list (TPM>1; $\log_2$FC >3.5; FDR < 0.01) (Figure 2.7).

Interestingly, one of the most highly enriched pathways was the focal adhesion pathway (Figure 2.7 A). Spleen glia highly expressed several known focal adhesion

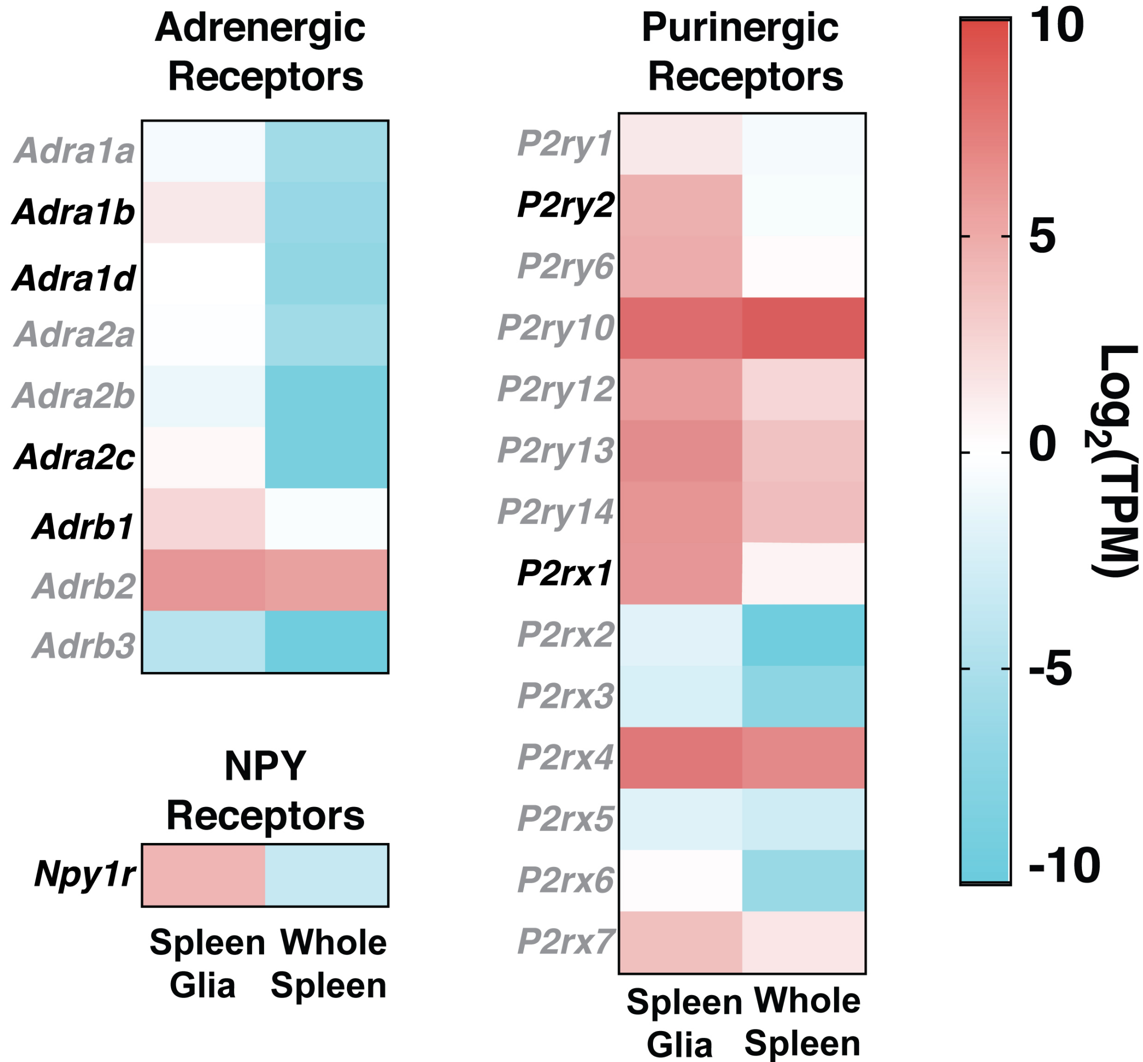

Figure 2.6: Heatmaps of neurotransmitter receptors genes expressed by spleen glia.
Boxes to the right of each gene are colored according to expression level in spleen glia (left) and whole spleen (right). Bolded gene names indicate genes that are highly enriched in spleen glia compared to whole spleen ($\log_2$FC > 3.5, TPM $>$ 1, FDR < 0.01). Neurotransmitter receptor genes known to encode receptors for sympathetic nerve messengers. Spleen glia express neurotransmitter receptors for norepinephrine (adrenergic receptors), neuropeptide Y, and ATP. TPM, transcripts per million; FDR, false discovery rate.

genes such as thrombospondins and laminins (Figure 2.7 B). Both molecules have known functions in other glial cell types and activate downstream Pi3k-Akt signaling, which is also enriched in spleen glia. For example, thrombospondin-2 expression in astrocytes is necessary to regulate synaptic adhesion (Christopherson et al., 2005). In addition, spleen glia express ErbB receptors, which suggests they are active participants in NRG1-ErbB2/3 signaling, a fundamental cell adhesion signaling pathway between Schwann cells and axons (Newbern and Birchmeier, 2010). Another known function of Schwann cells is facilitation of axon guidance, and pathway analysis also determined that this process is significantly enriched in spleen glia. Genes for ephrins, semaphorins, and plexins were extensively represented (Figure 2.7 C). Members of these gene families are described in so-called "repair" Schwann cells that promote axonal regrowth after injury (Koncina et al., 2007).

Glia may have organ specific functions, and spleen glia reside in an organ that is heavily involved in immunity. Accordingly, we hypothesized that spleen glia may be active partners in mediating immune responses. Indeed, AGE-RAGE signaling, cytokine-cytokine receptor interaction and complement cascade signaling were among the top 10 enriched pathways (Figure 2.7 D-F). Each of these pathways are driven by expression of immune relevant genes including cytokines and chemokines, interleukin's and their receptors, and complement proteins. Interestingly, although some cytokines and chemokines are enriched in spleen glia, many of the most enriched

genes are receptors. For example, the gene for Gp130 (*Il6st*) is enriched, and critical for astrocyte activation and ability to control infection in the brain (Drögemüller et al., 2008). Gp130 is a receptor that associates with a number of cytokine receptors to transmit signals to JAK/STAT3 or 5. Spleen glia express many of these gp130 co-receptors, including *Osmr, Cntfr, Il6ra*, and *Lifr*, implying that spleen glia likely respond to oncostatin and/or IL31, Cntfr, IL6, and LIF, respectively. Several transcripts for cytokines and chemokines including *Il34, Il7*, and *Cxcl13* are also enriched in spleen glia, as are the TBGβ family members *Tgfb2, Gdf6* and *Gdf10*.

We next selected genes from our dataset to validate that they are expressed in spleen glia. We assessed two genes that are glial-specific, Connexin 43 (*Gja1*) and proteolipid protein (*Plp1*). Connexin 43 is a gap junction protein present in astrocytes and enteric glia (Bhave et al., 2017; Brown et al., 2016). Immunostained sections demonstrate that an anti-connexin 43 antibody labels cells with a glial location and characteristic morphology throughout the spleen (Figure 2.8 A). PLP1 has been shown to be highly expressed in enteric glia and was highly enriched in spleen glia. We utilized *Plp1*-eGFP animals and counterstained spleen sections with both GFAP (Figure 2.8 B) and S100B (Figure 2.8 C) antibodies. As with our *Gfap*-GFP reporter mouse, we observe high-colocalization of both GFAP and S100B with PLP-GFP (99% 1.7, 99.7% .577 respectively).

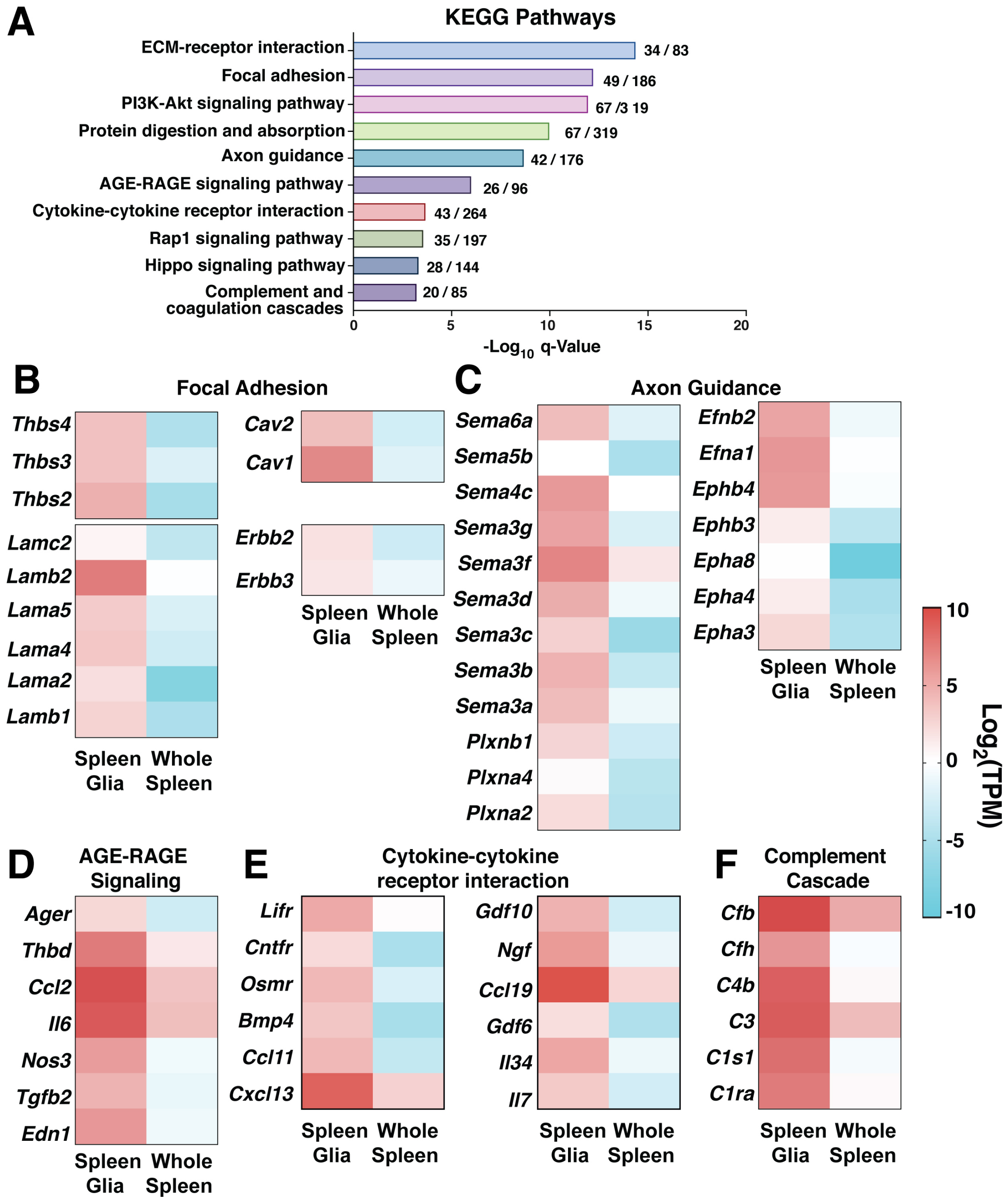

Figure 2.7: Pathway analysis of significantly enriched spleen glia genes reveals roles in focal adhesion, axon guidance, and immune responses.

Figure 2.7: (A) Bar graph representing the top 10 KEGG Pathways in spleen glia. Numbers to the right of bars indicate the number of genes enriched in glia / total gene number in the pathway.**(B-F)** Heatmaps of genes in these KEGG pathways that are enriched in spleen glia (left) with expression in whole spleen (right), colored by expression level in transcripts per million (TPM, see scale). **(B)** The Focal Adhesion KEGG pathway contains thrombospondins, laminins, and Erb-B receptors.**(C)** Selected genes from the Axon Guidance KEGG pathway, many with known roles in axonal support by other peripheral glia cell types. **(D-F)** Selected genes from the immune-related KEGG pathways–AGE-RAGE signaling, Cytokine-cytokine receptor interaction, and Complement cascade. All genes used in KEGG analysis are from the spleen glia enriched gene set ($\log_2$FC $>$ 3.5, TPM$>$1, FDR$<$0.01 compared to whole spleen).

To verify enrichment of immune related genes in spleen glia we focused on *Cxcl13* (Figure 2.8 D). *Cxcl13* encodes a B lymphocyte chemoattractant and was previously shown to be expressed by follicular dendritic cells and a subset of T lymphocytes. Using RNAscope, we labeled *Cxcl13* and *Gja1* RNA in spleen sections. We observed *Cxcl13*$^+$ puncta in various locations throughout the organ as expected, and interestingly some glia did co-localize while others did not . Overall, 10% of *Cxcl13*$^+$ cells colabelled with *Gja1*$^+$ puncta and 30% of *Gja1* puncta were associated with *Cxcl13* puncta. Thus, *Cxcl13* is enriched in spleen glia even though it is also expressed by other rare cell types in the spleen.

To confirm expression of neurotransmitter related genes we focused on *Npy1r* (Figure 2.8 E). Neuropeptide Y(NPY) is primarily produced by sympathetic neurons but has been shown to be produced by macrophages. Signaling through the *Npy1r* receptor can attenuate inflammation. We hypothesized that signaling in spleen glia

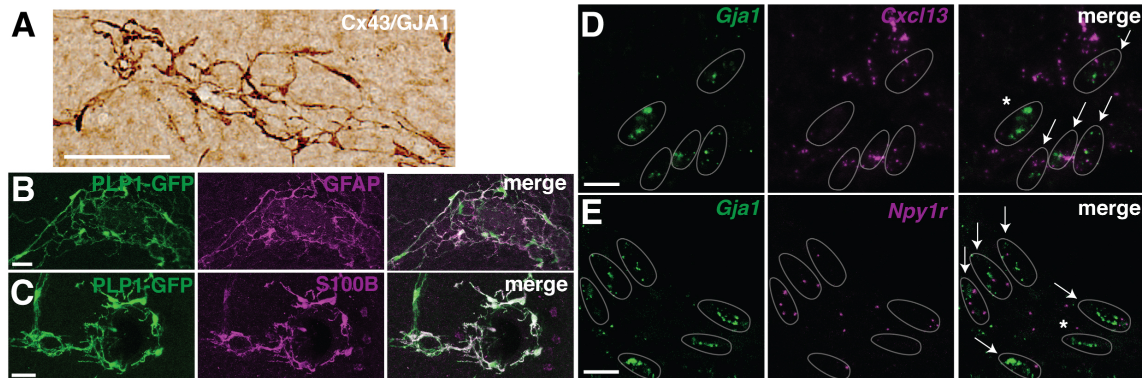

Figure 2.8: Confirmation of protein and RNA expression of selected glial, immune and neurotransmitter genes from the spleen glia transcriptome.
(A-C) Representative photomicrographs of 40 μm thick spleen sections stained for **(A)** connexin 43 (Cx43, or *Gja1*) demonstrating characteristic spleen glia morphology and anatomy. **(B-C)** *Plp1*-GFP reporter mice immunostained for **(B)** GFAP and **(C)** S100B. **(D&E)** Representative *in situ* hybridization images of 12 μm thick spleen sections labelled with *Gja1* (Cx43) and **(D)** Chemokine (C-X-C motif) ligand 13 (*Cxcl13*) and **(E)** Neuropeptide Y receptor type 1(*Npy1r*). Circles represent outline of cell bodies, arrows denote cells that are double positive while stars represent cells that are *Gja1* positive only. Scale bars, 20 μm.

may be linked to regulation of inflammation. We observed expression of *Npy1r* puncta throughout the organ and upon quantification we observe that 20% of $Npy1r^+$ cells colabel with *Gja1* and 29.2% of *Gja1* puncta colabel for *Npy1r* puncta.

2.3.6 Spleen glia exhibit similarities and differences compared to other glia

To elucidate differences between glial cell types, we compared the spleen glia transcriptome to publicly available transcriptomes of astrocytes, oligodendrocyte precursor cells (OPCs), oligodendrocytes, and enteric glia outlined in (Table 2.2). Z scores were calculated for each gene, plotted on a heatmap, and cell types were arranged after unbiased hierarchal clustering analysis (Figure 2.9 A). Spleen glia shared many genes with all these glial cell types and were most similar to enteric glia. Clustering analysis identified five unique gene groupings across all the cell types. We performed pathway analysis on the genes in each cluster (Figure 2.9 B).

Cluster 1 was highly expressed in CNS glia compared to PNS glia. The pathways enriched in this cluster represent GABAergic, glutamatergic and dopaminergic synapse signaling, all present in the CNS but perhaps not dominant in the PNS. Cluster 2 represented genes enriched in OPCs and astrocytes but not oligodendrocytes. Hedgehog signaling was the most abundant pathway of this cluster and has known roles in both cell types. It is involved in the formation of mature oligodendrocytes from OPCs, and the regulation of inflammation by astrocytes (Allahyari et al., 2019; Laouarem and Traiffort, 2018).

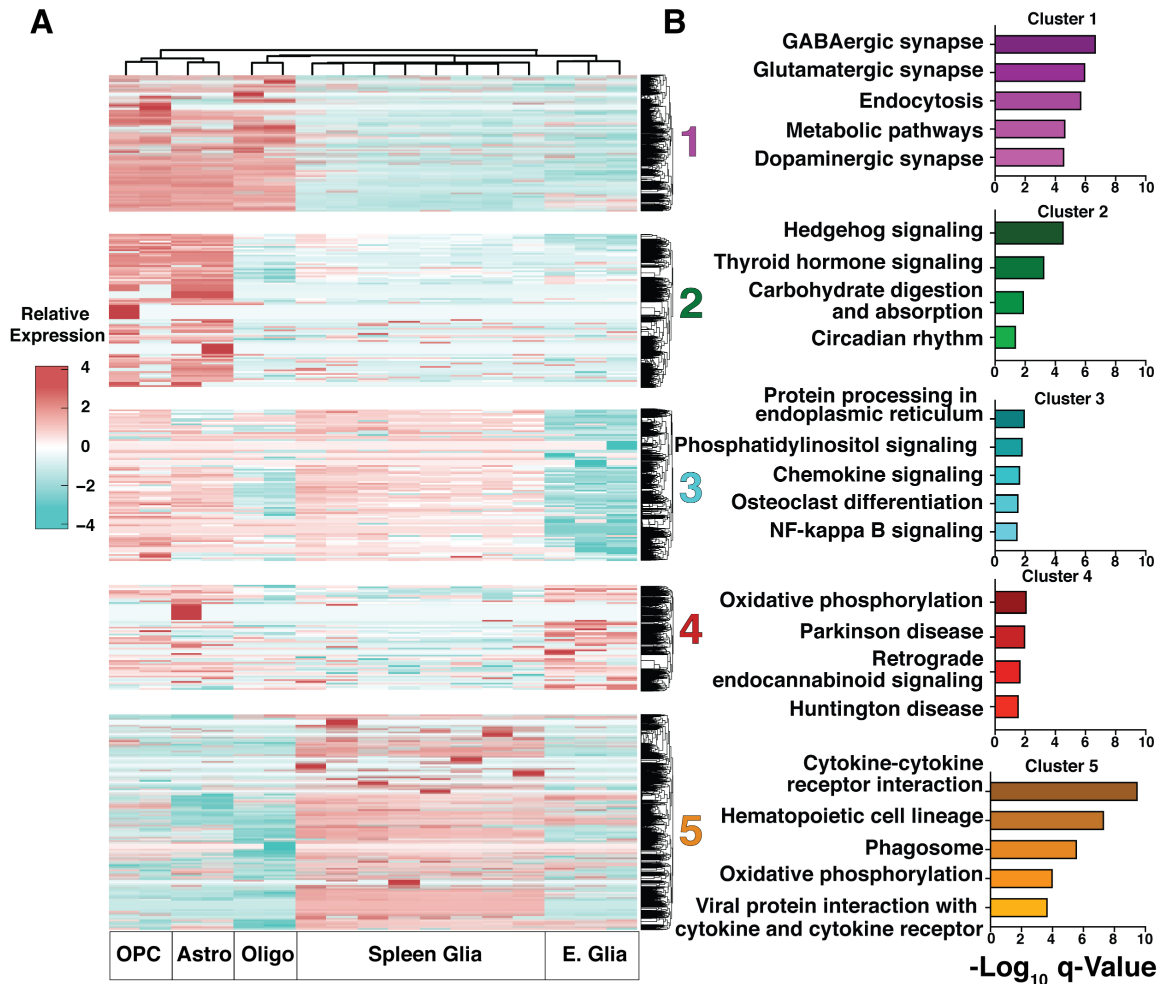

Figure 2.9: Transcriptome comparison between spleen glia and other glial types.
(A) Z-score Heatmap of transcriptomic data from five glial types after hierarchical clustering analysis identified five main clusters. Each row is one gene, and color represents relative expression of that gene in the cell type of its column compared to its expression levels in all other cell types. **(B)** Bar graphs representing top KEGG Pathways from each cluster.OPC, oligodendrocyte precursor cell; Astro, Astrocyte; Oligo, Oligodendrocyte; E. Glia, Enteric Glia.

Cluster 3 represented genes that are downregulated in enteric glia compared to spleen glia and Cluster 4 was upregulated in enteric glia compared to spleen glia. Pathways were least significant for these two clusters, possibly due to more shared genes and functions between all cell types. However, Cluster 3, composed of the genes with the lowest expression in enteric glia, contained immune genes found in spleen glia as well as other glial cell types, including those in the "chemokine signaling" and "NF-κb signaling" pathways. Both pathways suggest a role for spleen glia in immune regulation. There is also extensive evidence that OPCs and astrocytes use chemokines to regulate myelination and inflammation in the CNS. Genes in Cluster 4 were generally lower in spleen glia and predominately enriched in enteric glia. The top pathway here was "oxidative phosphorylation," a process which ultimately leads to the production of ATP.

Cluster 5 contained genes that were uniquely enriched in spleen glia over other glial cell types. The top enriched pathway within this cluster was "cytokine-cytokine receptor interactions," which included genes encoding cytokine and chemokine receptors and their respective ligands. This finding suggests that spleen glia may be more involved in initiating and responding to inflammation than other glial types. Additionally, Cluster 5 contains genes in the phagosome pathway, which is notable because phagocytosis is a central process in inflammation and defense against virus and bacteria. Expression of genes for the toll-like receptor gene *Tlr2* and antigen

processing gene *Tap1* specifically seem to suggest the utilization of toll-like receptor promotion of ER-mediated phagocytosis (Desjardins, 2003).

This is consistent with pathway analysis on the enriched spleen glial genes alone and implies that spleen glia may be directly involved in both innate and adaptive immune responses. Although further research needs to be done to determine exact mechanisms, we have demonstrated that spleen glia are anatomically and genetically poised to participate in immune responses in the spleen. The processed spleen glia transcriptome, after deconvolution, is available as a resource for future study (`https://buckwalterlab.shinyapps.io/SpleenGlia/`).

2.4 Discussion

Here we provide an in-depth anatomical and transcriptional characterization of spleen glia, while identifying a mouse reporter as a tool to study them. Spleen glia are elongated cells visualized using immunostaining for four common glial markers GFAP, S100B, PLP1, and Cx43/*Gja1*. They maintain a tight association with nerves, are predominantly in white pulp regions, and come into close contact with lymphocytes. We identified genes involved in the ensheathment of axons and communication with both nerves and immune cells. Spleen glia express many genes in common with other glial types but express immune genes more highly. This transcriptome is a first step

towards better understanding spleen glia and implies that spleen glia are unlikely to function solely as support cells for axons. Rather, they are most likely active players in neuroimmune communication and function.

Spleen glia have a uniform morphology forming dense networks that ensheath sympathetic axons. Another study describing immunohistochemical localization of spleen glia (Ma et al., 2018) used a GFAP antibody that exhibited more widespread staining than we observe. In addition to the cells we observe, they observe extensive staining throughout the red pulp and marginal zone that is not present in our reporter mice, or our GFAP or S100B stains, and is not adjacent to axons. Because we don't see this staining with any of our methods, we believe that their immunostaining reflects some background contamination.

Interestingly, the axonal morphology we observe at higher power appears to show protruding varicosities from axons with less glial coverage at these sites. This is similar in appearance to axon varicosities described in literature, where glia form fenestrations at sites of neurotransmitter release (Burnstock, 2008; Douglas and Ritchie, 1962). In this case, the target cells for sympathetic neurotransmitters are likely immune cells, glia, and vascular smooth muscle cells.

Glial ensheathment of axons is likely maintained through several molecular signaling pathways. For instance, spleen glia express ErbB receptors, important for the

Neuregulin 1 (Nrg1)-ErbB2/3 receptor cascade that facilitates Schwann cell-axon interactions. Neurons produce Nrg1 which interacts with ErbB2/ErbB3 receptors on glia. This is critical for Remak cells, a type of non-myelinating Schwann cell ensheathing small axons such as C-fibers (Harty and Monk, 2017). Loss of Nrg1 in sensory axons results in more axons per Remak bundle and reduced Remak cell ensheathment. This pathway likely functions similarly in spleen glia, determining which and how many axons become ensheathed. Spleen glia also express Neuroligins, a family of cell adhesion proteins expressed in other glial types. Neuroligin 3 is a vertebrate gliotactin necessary for the development of the glial sheath in the PNS of Drosophila melanogaster (Gilbert et al., 2001). Furthermore, loss of astrocytic Neuroligin 2 leads to a decrease in both astrocyte size and cortical excitatory synapses (Stogsdill et al., 2017). Neuroligins likely also function similarly in spleen glia to tether and ensheath axons.

Spleen glia and sympathetic axons course around arteries and arterioles in the spleen, typical for sympathetic innervation throughout the body. In the spleen, lymphocyte-rich white pulp regions surround arteries. The anatomical location of spleen glia suggests involvement in immune responses. Pathway analysis supports this as well. We carefully considered whether our spleen glia transcriptome is contaminated by genes from immune cells in the spleen. Although we cannot eliminate the possibility of any contamination by immune cells, our initial results on size-selected

cells prior to deconvolution and purification indicates low contamination of immune cells (<2%). There is also de-enrichment of cognate immune cell genes for lymphocytes, white blood cells, MHC class II, and immunoglobulin genes. We also considered whether there was bleed-through of cytokine and chemokine gene expression from the whole spleen. Of the top five expressed chemokines and cytokines in whole spleen, four are de-enriched in spleen glia (*Ccl5*, *Ccl4*, *Tgfb1*, and *Ccl6*). The remaining one, *Cxcl1*, didn't meet our cutoff of enrichment ($\log_2$fold = 2.6) in spleen glia but is also expressed by both astrocytes and Schwann cells (Ni et al., 2019; Ntogwa et al., 2020).

More work will be needed to elucidate the true role of spleen glia in immune responses. However, immune gene expression is not unique to spleen glia; for example, astrocytes and Schwann cells also express complement proteins (de Jonge et al., 2004; Lian et al., 2016). The complement cascade functions to initiate and propagate inflammation. Spleen glia may participate in the alternative complement pathway, as they differentially express complement factors Factor B and H (Hoffman and O'Shea, 1999). Moreover, spleen glia express pro-inflammatory cytokines that are upregulated during complement activation, such as *Nos* and *Il6*, both expressed in astrocytes (Hamby et al., 2006; Van Wagoner et al., 1999). Since we generated our spleen glia transcriptome in the absence of immune stimulation, the expression of these cytokines suggests they serve homeostatic immune functions. Our data also demonstrate high expression of *Il15* by spleen glia (TPM=11.8), which promotes long-term proliferation

of activated T-cells (Sprent et al., 2000).

A limitation of this study is our isolation protocol. Due to the size, shape and density of spleen glia, common RNA isolation protocols produce RNA at lower quality and quantity than what is needed for next-generation sequencing. In developing our isolation protocol, we found that the cells are rare, that RNAses are high in the spleen (limiting the usefulness of RiboTag ribosomal pulldown methods), and that FACS sorting pulverizes the cells. However, we took advantage of the large size of spleen glia to develop a size-selection method that yielded sufficient quantity and purity of glial RNA. We utilized a stringent bioinformatic approach including cellular deconvolution and expression cutoffs to maximize the specificity of our gene lists.

A notable casualty of this stringent approach was *Gfap*. Although the *Gfap* promotor was effective at labeling spleen glia for anatomical characterization, we discovered that its RNA expression level was too low to make it a reliable genetic marker. It was undetectable in 3 of 8 whole spleen samples with average TPM of 0.24, and expression level of 1.2 in spleen glia. This is consistent with enteric glia literature that describes *Gfap* expression to be lower than other glial markers such as *S100b* and *Plp1* (Rao et al., 2015). The low level of *Gfap* RNA may indicate that, as in the brain, GFAP is a highly stable protein that remains for long periods of time without robust gene expression (Rolland et al., 1990).

There are likely other glia-specific proteins that are excluded from our final "highly enriched" transcriptome as our methods were designed to err on the side of specificity so that we would be confident that this set of genes is indeed enriched in spleen glia. The cutoff of $\log_2$ fold-enrichment >3.5 means genes in our final dataset are at least 11-fold more highly expressed in spleen glia than in whole spleen. We validated the expression in spleen glia of three glial-specific genes that are in this "highly enriched" dataset using immunohistochemistry; S100B, Plp1, and Cx43. Cx43 and Cx47 are also known to be expressed in enteric glia higher than Gfap, (Grubišić and Gulbransen, 2017; McClain et al., 2014)], while myelinating glia express Cx32 and Cx47 (Freidin et al., 2009; Nualart-Marti et al., 2013).

Spleen glia expressed many genes in common with other glial cell types, including receptors for neurotransmitters that facilitate communication with neurons and nerves, including purinergic and adrenergic receptors (Lecca et al., 2012). In sympathetic nerves, ATP is released as a co-transmitter alongside NE. Signaling through purinergic receptors induces various responses in glial cells depending on receptor subtype and context. For example, signaling through P2X ion channels induces a pro-inflammatory response in enteric glia and astrocytes (Bhave et al., 2017; Gandelman et al., 2010), while signaling through P2Y G-protein coupled receptors induces proliferation of astrocytes (Quintas et al., 2011) and calcium responses in enteric glia (Brown et al., 2016). Spleen glia express both subtypes and the necessary machinery

for calcium signaling, including the gap junction gene connexin 43, suggesting that they may similarly have calcium waves induced by purinergic signaling.

Adrenergic signaling in glia affects inflammation, behavior, and disease, most studied in astrocytes. For example, $\beta 2$ adrenergic signaling in astrocytes initiates TNF induced inflammation (Laureys et al., 2014), while $\alpha 2$ receptors stimulate GABA release to suppress neuronal activity (Gaidin et al., 2020). Spleen glia highly express both subtypes, with $\alpha 1$ receptors being the most differentially expressed. Adrenergic signaling in the spleen is important in immune cell activation and proliferation (Bai et al., 2011; Madden et al., 1994). As in astrocytes, adrenergic signaling in spleen glia likely plays a role in altering transmission to target cells such as immune cells. Since many immune cells also express adrenergic receptors, cell-specific knockout experiments will help us understand their role in spleen glia.

This work is an essential first step towards understanding neuroimmune communication in the spleen and uncovering organ-specific differences amongst non-myelinating Schwann cells. We demonstrated that spleen glia form extensive networks throughout the white pulp of the spleen, are transcriptionally unique, and are likely involved in neuroimmune communication. Our data also provides a starting point to explore what markers are distinct in spleen glia. In addition, we can now ask what

changes occur in spleen glia during active immune responses, and whether they regulate neuroimmune communication. One question of interest for the future is whether spleen glia are like non-myelinating Schwann cells in other secondary lymphoid organs such as lymph nodes and Peyer's Patches, and another is how they resemble non-myelinating Schwann cell in other visceral organs, and other unmyelinated peripheral nerves. However, due to the lack of data on non-myelinating glia in visceral organs this is yet unknown. We speculate that there is likely to be heterogeneity. For example, peripheral glia ensheathing parasympathetic versus sympathetic nerve fibers may express different neurotransmitter receptor genes. Large differences may exist depending on the local environment of glia. Enteric glia associated with intrinsic neurons of the gut in different layers may be somewhat different while being more clearly distinct from those in Peyer's patches, bone marrow, thymus and lymph nodes, or those ensheathing unmyelinated nerves in the sciatic nerve. Future work will shed light on this and define any molecular and functional differences. The reporter mouse used in this study and the web application we created for exploring the transcriptome data provide critical tools to aid these future studies.

2.5 Materials and Methods

2.5.1 Animals

All animal procedures were performed in accordance with the protocol approved by the Institutional Animal Care and Use Committee at Stanford University. C57BL/6J mice aged 8 to 12-weeks were purchased from The Jackson Laboratory, Bar Harbor, ME, and utilized for all studies: *Gfap*-Cre (JAX#12886) and Rosa26 eGFP (JAX#4077).*Plp1*-GFP animals were graciously provided to us by Meenakshi Rao at Boston's Children's Hospital.

2.5.2 Perfusion and tissue preparation

Mice were anesthetized with 6% chloral hydrate in PBS and terminally perfused through the left ventricle with 10–20ml of cold 0.9% heparinized saline (10 units/ml). Spleens were collected and fixed in 4% paraformaldehyde in phosphate buffer overnight then transferred to 30% sucrose in phosphate buffer until sunken, usually overnight. PFA-fixed spleens were sectioned in both sagittal and coronal planes using a freezing sliding microtome to generate 40 μm thick sections (Microm HM430).

2.5.3 Immunohistochemistry and antibodies

Immunohistochemistry was performed on PFA-fixed free-floating tissue sections using standard protocols. Sections were washed in Tris Buffered Saline (TBS) and blocked with 5% serum for 1 hour. Primary antibodies were diluted in 0.1% Triton X-100 and 3% serum and applied overnight at room temperature. We used the following primary antibodies: rabbit anti-GFAP at 1:3,000 (Dako), rabbit anti-S100B at 1:500 (Dako), rabbit anti-TH at 1:500 (Millipore), rabbit anti-Pgp9.5 at 1:500 (Cedarlane), hamster anti-Cd3 at 1:500, rat anti-B220 at 1:500, rat anti-Cd31 at 1:100 (BD), biotinalayted goat anti-IgM 1:500 (ThermoFisher). The following day, the sections were rinsed extensively with TBS and incubated with a fluorescent secondary antibody for 5 hours. Secondary antibodies were all used at 1:500 and include donkey anti-rabbit and rat anti-hamster.To ensure that there was no non-specific binding of secondary antibodies, control sections were incubated with secondary antibody alone. With the exception of the S100B antibody, all primary antibodies yielded the expected morphology and no significant background staining. The S100B antibody exhibits known weak cross-reactivity with S100A in macrophages, which is easily distinguished in the spleen by weaker staining and a macrophage morphology. Sections were washed in TBS then wet-mounted with Vectashield hard set mounting media with DAPI (Vector Labs) and coverslipped.

2.5.4 Image acquisition

Sections were imaged using 40x, 1.15 numerical aperture and 63x, 1.30 numerical aperture oil objectives on a Leica TCS SPE confocal microscope using Leica Application Suite Advanced Fluorescence software. Stacked images of fluorescent spleen sections were reconstructed using ImageJ (NIH), and Photoshop (Adobe) software was used to change brightness and contrast of the images. In each case all settings were applied equally to each color channel. To quantify double labeling, we used an unbiased approach. For example, to examine whether GFP expression in GFAPcre-GFP mice was present in GFAP^{+} cells, spleen sections from three animals were immunostained for GFAP with an Alexa Fluor 555-conjugated secondary antibody. When a GFP-expressing cell was identified in the green channel, the channel was switched to red to determine whether it also stained for GFAP. Conversely, to evaluate which percent of GFAP immunostained cells express GFP, GFAP^{+} cells were identified in the red channel then the channel switched to green to score GFP expression. Each mouse contained three replicates and 100 cells were counted for each of these analyses.

2.5.5 Dissociation and isolation of spleen glia

Spleens from mice aged 8-12 weeks were removed, minced, resuspended in 4ml of dissociation buffer (HBSS, 10 mM HEPES, 1.4 mg/ml Collagenase A, 0.4 mg/ml

DNase I, 5% FBS) and incubated with constant agitation at 37°C for 1 hour. After the hour, Dispase II (2 mg/ml) was added and the samples were incubated for an additional 30 min. Upon completion, 5 mM EDTA was added to the sample and incubated at room temperature for 5 min and subsequently centrifuged at 1000 rpm for 5 min at 4°C. Red blood cells were lysed with 1 ml of ACK buffer (Giboco) for 5 min at room temperature and diluted with 13 ml of cold PBS + 5% FBS before another centrifugation at 1000 rpm for 5 min at 4°C. The cell pellets were resuspended in 5 ml of PBS + 5% FBS and passed through a 40-micron cell strainer. After washing with 5 ml of PBS + 5% FBS, the remaining cells on the cell-strainer ("size-selected"), were lysed using 500 ul of RLT buffer (Qiagen). RNA from size-selected and whole spleen samples from each animal were purified using the Qiagen RNeasy Micro Plus kit.

2.5.6 Quantitative RT-PCR

RNA was reverse transcribed using the Invitrogen SuperScript® III First-Strand Synthesis System and used for qPCR using SYBR on the Applied Biosystems™ QuantStudio™ 6 Flex Real-Time PCR System. The standard protocol was used, and all reactions were performed in triplicate with *Gapdh* (F: ACTCTTCCACCTTCGATGCC, R: TGGGATAGGGCCTCTCTTGC) as a housekeeping gene. Primers used were: *S100b* (F: GGTTGCCCTCATTGATGTCTT, R: TTCGTCCAGCGTCTCCAT), *Sox10* (F:

CAAGCTCTGGAGGTTGCTG, R: TGTAGTCCGGATGGTCCTTT), and *Ptprc* (F: GGGTTGTTCTGTGCCTTGTT, R: GGATAGATGCTGGCGATGAT). All primers were validated in house using a combination of literature, BLAST and control samples. The $\Delta\Delta$Ct method was used to quantify fold enrichment of size-selected cells over whole spleen samples (Livak and Schmittgen, 2001).

2.5.7 RNA isolation and sequencing

Cell homogenates from whole spleen and size-selected samples were sequenced from 8 animals (4 male, 4 female) approximately aged 8-12 weeks of age. Quality and quantity of RNA was measured using the Agilent Bioanalyzer PicoChip, and only samples that yielded a RIN number > 9.0 were used. Total RNA (5ng) was then reverse transcribed using the SMART-Seq v4 Ultra Low Input RNA Kit for Sequencing (Clontech), which generates high-quality cDNA from ultra-low amounts of total RNA. Libraries were then constructed from synthesized cDNA using the Nextera XT DNA Library Preparation Kit (Illumina) for RNA-Sequencing. Libraries were sequenced on the Illumina HiSeq 4000 platform to obtain 150bp paired-end reads.

2.5.8 Mapping and analysis of RNA-sequencing data

Approximately 20-30 million 150bp reads were obtained from all 16 (8 size-selected, 8 whole spleen) samples. These reads were trimmed of adapter sequences, low quality bases, and very short reads using trimGalore! (v0.4.5), a wrapper script which utilizes cutadapt (Martin, 2011) and FastQC (v0.11.7). Remaining reads were aligned to the mouse mm10 genome (GRCm38.p6) using the STAR aligner (Dobin et al., 2013) (v2.7.1), which utilizes an algorithm that minimizes alignment time, mapping errors, and alignment biases. Transcript abundances were then annotated and quantified using the RSEM software package (Li and Dewey, 2011) (v1.3.1). Differential gene expression analysis was then carried out using the DESeq2 (Love et al., 2014) (v1.26) package within the R environment. Transcripts with low abundances (<10 counts for any of the samples) were excluded from analysis. Plots were generated using ggplot2 (v3.2.1).

2.5.9 Deconvolution and Expression Purification

To determine the proportion of contaminating cells in our size-selected samples, we obtained reference sequencing data from cells likely to be contaminating our samples. Cell type comparison data was obtained from publicly available RNA-sequencing datasets on Gene Expression Omnibus (GEO; `https://www.ncbi.nlm.`

nih.gov/geo/). Cell types and sources can be found in (Table 2.2). Samples were chosen based on data generated from non-transgenic animals who had not undergone any experimental manipulations beyond saline or PBS injections. Gene counts from collected data were used as a reference sample in CiberSortX (Newman et al., 2019), a digital cytometry program which deconvolves cell types based on signature genes generated from reference samples. Signature genes and proportions generated by CiberSortX were used to scale and purify expression in size-selected samples by imputing cell expression from glia samples. The newly generated expression files were used for downstream differential expression analysis.

To define spleen-glia specific genes we used a fold-change cutoff of >3.5 to assure that only genes highly enriched in glia were considered. This cutoff was tested by sensitivity analysis through changing the cutoff ±25% and seeing no changes in the data interpretation.

2.5.10 *In situ* Hybridization

In situ hybridization was carried out using commercially available fluorescence detection assays and probes (RNAScope) according to the manufacturer's specification. Spleens were perfused, drop fixed in 4% PFA for 24 hours, sunk in 30% sucrose, and then embedded in OCT at -80°C until sectioning. Spleens were sectioned at 10um and tissue was used within 2 months.

Quantification was carried out to determine concordance between gene probes of interest. Each probe was imaged on at least three different animals. Three images were taken per section and cells were counted as positively stained if there were three or more puncta associated with a DAPI positive nucleus. For each quantification, at least 1000 cells were counted in each animal to determine the percentage of positive cells.

2.5.11 Gene expression comparisons

To compare the gene expression of spleen glia to that of other glial cells, gene counts were generated for astrocytes, oligodendrocyte precursor cells (OPCs), oligodendrocytes, enteric glia and spleen glia following the analysis outlined above. Counts were normalized and z-scores were then calculated for each gene and plotted using the ComplexHeatmap Package with default settings in the R environment. Gene clusters were generated using k-means clustering after silhouette computation determined that five clusters were optimal. This partitioning was then used in plotting as a split variable.

Cell Type	Organ source	# of Replicates	Experimental Manipulation	Reference
Astrocyte	Brain	2	At rest	(Zhang et al., 2014)
B-cells	Spleen	50	At rest	(Schaum et al., 2018)
Dendritic Cells	Spleen	50	At rest	(Schaum et al., 2018)
Endothelial	Brain	2	At rest	(Zhang et al., 2014)
Endothelial	Lymph Node	6	At rest	(Berendam et al., 2019)
Enteric Glia	Gut	2	At rest	(Rao et al., 2015)
Enteric Glia	Gut	3	Saline injection	(Delvalle et al., 2018b)
Macrophages	Spleen	50	At rest	(Schaum et al., 2018)
Neurons	Brain	2	At rest	(Zhang et al., 2014)
NK cells	Spleen	50	At rest	(Schaum et al., 2018)
Oligodendrocyte	Brain	2	At rest	(Zhang et al., 2014)
OPC	Brain	2	At rest	(Zhang et al., 2014)
Smooth Muscle	Heart	6	At rest	(Dobnikar et al., 2018)
T-cells	Spleen	50	At rest	(Schaum et al., 2018)

Table 2.2: Cell transcriptomes used for deconvolution comparison to spleen glia.

2.5.12 Experimental design and statistical analysis

The aim of this study was to establish a complete characterization of spleen glia, and identify similarities and differences between them and other glia. Immunohistochemistry utilized three sections from three animals for a total of nine replicates per stain. Transcriptional profiling was done on eight total animals with four replicates for each sex of both spleen glia and whole spleen samples. Statistical details can be found in the section entitled “Mapping and Analysis of RNA-sequencing”, above. Raw transcriptome datasets generated from size-selected cells and whole spleen were uploaded to GEO (Accession # GSE151856) as both read counts and differential expression tables. To improve visualization of the complete processed

data set after deconvolution, a web application was developed; this can be found at `https://buckwalterlab.shinyapps.io/SpleenGlia/`.

Chapter 3

Investigating the relationsip between spleen glia and the splenic nerve

Tawaun A. Lucas & Marion S. Buckwalter

TAL performed all surgeries, tissue collection, and immunostaining. TAL and MSB conceived of the study, designed the experiments and wrote the manuscript.

3.1 Summary

The spleen is innervated by a single sympathetic nerve. While the sympathetic nervous system is generally known to alter immune responses, the contribution from direct innervation versus circulating catecholamines is not well understood as past studies have been variable. There are many factors that contribute to variability in research, and one of them includes the lack of a consistent model to study the effect of direct innervation to the spleen. Furthermore, many past studies have failed to investigate the role of glia in neuroimmune communication within the spleen. In this chapter we set out develop a surgical methodology to perturb direct sympathetic signalling in the spleen and investigate the subsequent glial response. We show that we can successfully transect the splenic nerve in a mouse model. Upon investigating the changes to glia, we observed that the cells appear to die by day 10 post nerve transection. Demonstrating an effective technique to block direct sympathetic signalling in the spleen, this work will be pivotal for allowing us to uncover interactions between glia, nerve and immune cells in the spleen.

3.2 Introduction

The spleen is innervated by a single nerve, splenic nerve, that is comprised of 98% sympathetic fibers (Bellinger et al., 1989; Heusermann and Stutte, 1977). The nerve

originates in the superior mesenteric ganglion and travels to the spleen wrapped around the splenic artery (Bellinger et al., 1989). Upon entering the organ, the nerve remains around the artery and arterioles, which branch extensively after entering the spleen. While sympathetic signaling globally is thought to be largely anti-inflammatory, the effect due to direct innervation of the spleen is not well understood.

Some studies suggest that the nerve is active during inflammation and may be contributing to the subsequent cellular and humoral responses. For example, a dose dependent increase in splenic nerve activity can be seen after intravenous LPS injections into rats (MacNeil et al., 1996). However, studies transecting the nerve (neurectomy) during inflammatory responses have shown variable results. LPS-stimulation of pro-inflammatory TNF by the spleen is attenuated by splenic neurectomy (Rosas-Ballina et al., 2008), but splenic neurectomy after intermittent foot shock also protects animals from sympathetically meditated immunosuppression, measured by loss of plaque forming B lymphocytes and immune cell proliferation (Laukova et al., 2018). Finally, some studies have shown no effect of neurectomy. Rats injected i.v. with LPS exhibited no measurable differences in TNF, IL-1β or IL-6 after splenic neurectomy, adrenalectomy or a combination of the two (Meltzer et al., 2003). Additionally, splenic neurectomy does not affect sympathetically-mediated splenic contraction after stroke (Ajmo Jr. et al., 2009).

One source of variability may be due to the animal models used for experimentation. Small animal surgery is extremely difficult so many studies utilizing neurectomy opt to utilize larger rodents (i.e. rats or guinea pigs), allowing for better visualization and easier transection of the nerve. However, many of the experiments measuring splenic inflammatory responses after LPS, injury and disease have been in mice. And although rats are bigger than mice, mice offer far more superior genetic models. The first recombinant mouse model was identified in 1987, compared to 2010 for the first recombinant rat model. The use of transgenic models allows researchers far more precise manipulations of protein expression to test their effects on clinical outcomes. This leaves a need for the development of a splenic neurectomy surgery in a mouse model. In addition, many of the studies exclusively focus on nerve-immune cell interactions in the spleen, excluding the presence and influence of glia during these responses.

As we reported in Chapter 2, non-myelinating glia potentially contribute greatly to the local inflammatory milieu in the spleen. However, little to nothing is known about spleen glia and their relationship to the nerve. Our data revealed a close physical relationship and several potential nerve-glia signaling mechanisms that may be involved in maintaining their close relationship. Typically, Schwann cells around peripheral nerves respond to denervation through a complex de-differentiation process and cell-type switch which allows the cells to support axon regeneration (Jessen

and Arthur-Farraj, 2019). This is best studied in the sciatic nerve. However, enteric glia may also support nerve re-growth. Like astrocytes, enteric glia are active participants at enteric synapses and respond to neuronal activity (Rühl et al., 2004; Gulbransen and Sharkey, 2012). Upon denervation glia respond through the release of neurotrophic factors, including nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) and glial cell line-derived neurotrophic factor (GDNF) (Turco et al., 2014; Gulbransen, 2014). We hypothesized the spleen glia likely would take on a similar neurotrophic role after neurectomy.

The goal of this study was thus to first develop and validate a splenic neurectomy surgery in mice. Secondly, we wanted to investigate the effects of splenic neurectomy on spleen glia. In the future we want to utilize this model to study nerve-glia-immune cell interactions during inflammatory disease states. This thesis chapter contains a description of the new mouse splenic neurectomy surgery. Also, utilizing immunohistochemistry and a time course analysis we were able to track the changes of both nerve fibers and spleen glia over a 1-month time period. We also describe a pilot experiment to measure the response of nerve fibers in animals in which are protected from Wallerian degeneration.

3.3 Results

We began by first developing a laparotomy surgery in which we transect the splenic nerve (neurectomy) to assess the extent of axonal loss and how spleen glia may respond. In developing the technique, we discovered that surgical technique is very important. The spleen has to be carefully exteriorized by moving intestines and pancreas. The pancreas is very delicate and needs to be gently moved as to not induce injury. The large and small intestines are removed using blunt dissection to detach connective tissue holding them in place. The nerve is identified and transected at the most lateral portion using a dissecting scope to avoid transection of the nerve supplying the pancreas. Visualization of the splenic nerve is extremely difficult in mice, and transection requires careful and through dissection as close to the artery as possible without puncturing the vessel.

To visualize spleen glia and assess the success of the transection, we utilized a peripheral glia reporter mouse (C57BL/6J GFAP-cre, ROSA-eGFP) in which pulmonary and spleen glia are labelled with GFP (Suarez-Mier and Buckwalter, 2015). We immunostained spleen sections for the sympathetic nerve marker Tyrosine hydroxylase (TH) to identify axons and verify neurectomy. Upon qualitative observation, we observed a noticeable reduction in TH fibers throughout the organ at 14 days post neurectomy surgery when compared to laparotomy only sections (Figure 3.1 A).

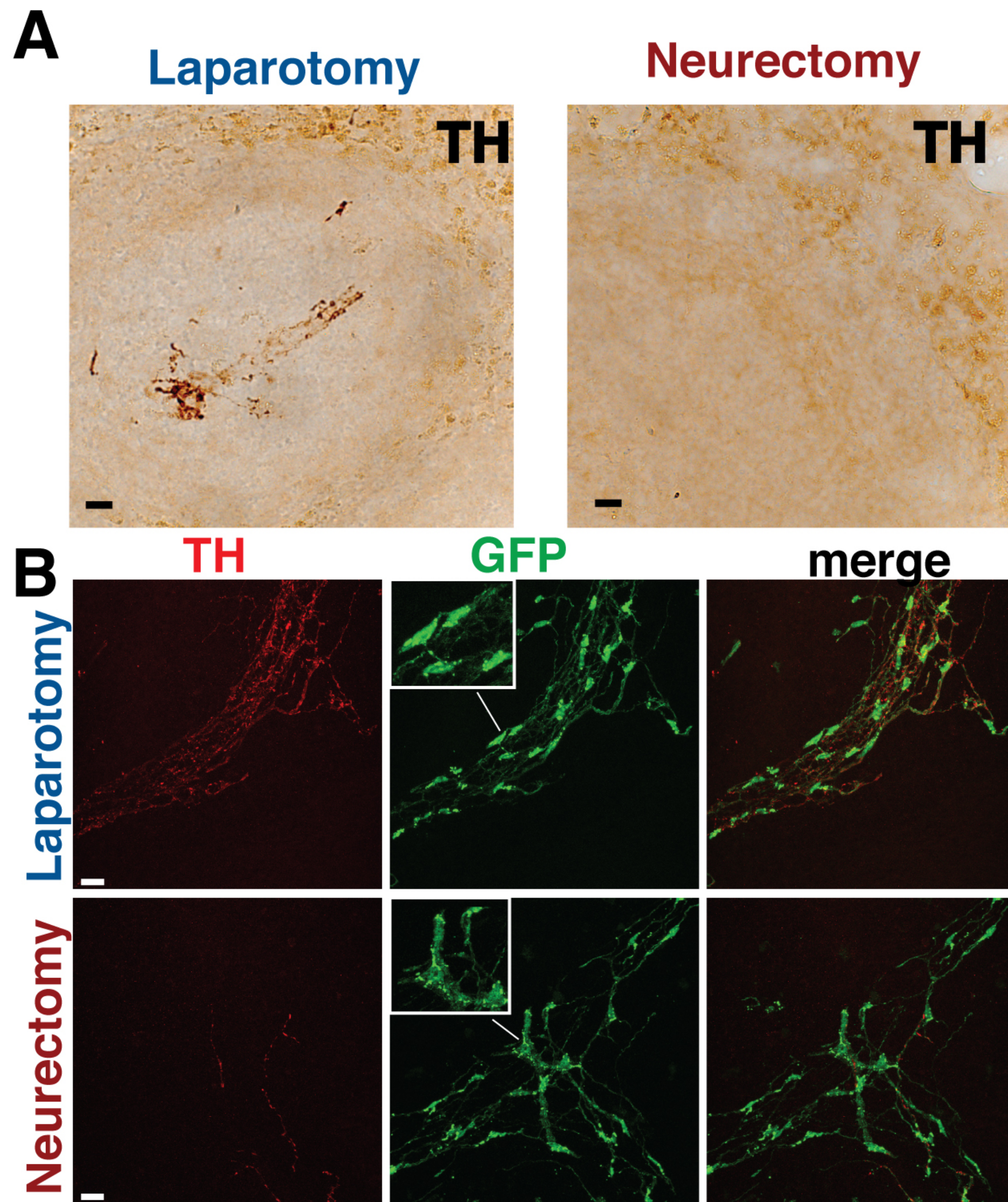

Figure 3.1: Evaluation of Neurectomy Surgery.

Figure 3.1: Representative images of 40 um thick spleen sections from normal Gfap-cre::Rosa-GFP C57BL/6J mice who underwent either laparotomy (control) or splenic neurectomy surgery, immunostained for tyrosine hydroxylase (TH) to mark sympathetic axons. All animals were sacrificed 14 days after surgery.**(A)** The left panel shows characteristic TH staining in a white pulp region of an animal who underwent laparotomy alone. Right panel shows a similar white pulp region in an animal who underwent splenic neurectomy. **(B)**Representative photomicrographs 1 week after neurectomy surgery. Laparotomy animals (top) show typical density of both TH nerve fibers and GFP^+ glia. TH nerve density is greatly diminished in neurectomy animals (bottom, left). The appearance of GFP^+ spleen glia (bottom, middle) are also affected by neurectomy. Arrows highlight aggregation of GFP in cell bodies and retracted processes compared to animals who underwent laparotomy surgery alone. Scale bars = 20 μ m.

To confirm axonal loss and determine if spleen glia were altered after nerve injury, we double-stained GFAP-GFP reporter animals with TH after a 7-day recovery (Figure 3.1 B). On visual inspection, we observed a clear reduction in TH nerve fibers throughout the spleen in neurectomized animals compared to laparotomy only animals. We also observed that spleen glia, which are visualized by GFP, began to exhibit signs of distress. At this 7-day time point, the GFP expressing cells appear to form less dense networks and exhibit an increased amount of GFP aggregated within the cell body, such as might be seen if they are retracting their processes (Figure 3.1 B, arrows).

After nerve transection, Wallerian degeneration leads to degeneration of axons (Fawcettt and Keynes, 1990). The onset of Wallerian degeneration in peripheral nerves have been described to begin as early as 24-hours after injury. There is little research about this process in peripheral unmyelinated autonomic nerves, but we

assume it is similar. The expected rate of Wallerian degeneration is ∼1-3 mm /day, varying depending on type of injury and size of nerve. Our neurectomy surgery transects the nerve ∼3 mm from entry into the spleen. We thus expected to see loss of axons on approximately day 2-3. To assess this, and quantify the extent of axonal loss over time, we conducted laparotomy or neurectomy surgery and immunostained spleen sections for TH at 24 hours, 7 days, 10 days, 14 days and 28 days. Our analysis revealed a significant reduction in the numbers of TH fibers throughout the spleen beginning at 7 days after neurectomy (Figure 3.2). However, there is a trend that is evident as early as 24hr after neurectomy surgery (p=0.1). By seven days we see near complete loss of TH fibers which persists 14 days after surgery, and stays reduced until day 28, when there is a slight recovery. This recovery is expected as the cell body is intact and autonomic nerves are known to re-grow (Fawcettt and Keynes, 1990; Verdú and Navarro, 1997).

Peripheral Schwann cells respond to nerve injury by cellular reprogramming that generates cells specialized for promoting regeneration and repair. These repair cells clear redundant myelin, attract macrophages, support survival of damaged neurons, encourage axonal growth, and guide axons back to their targets (Jessen and Arthur-Farraj, 2019). A key component of this reprogramming is de-differentiation and activation in which the cells re-express genes that are suppressed during the immature Schwann cell state, including GFAP (Jessen and Arthur-Farraj, 2019; Jessen

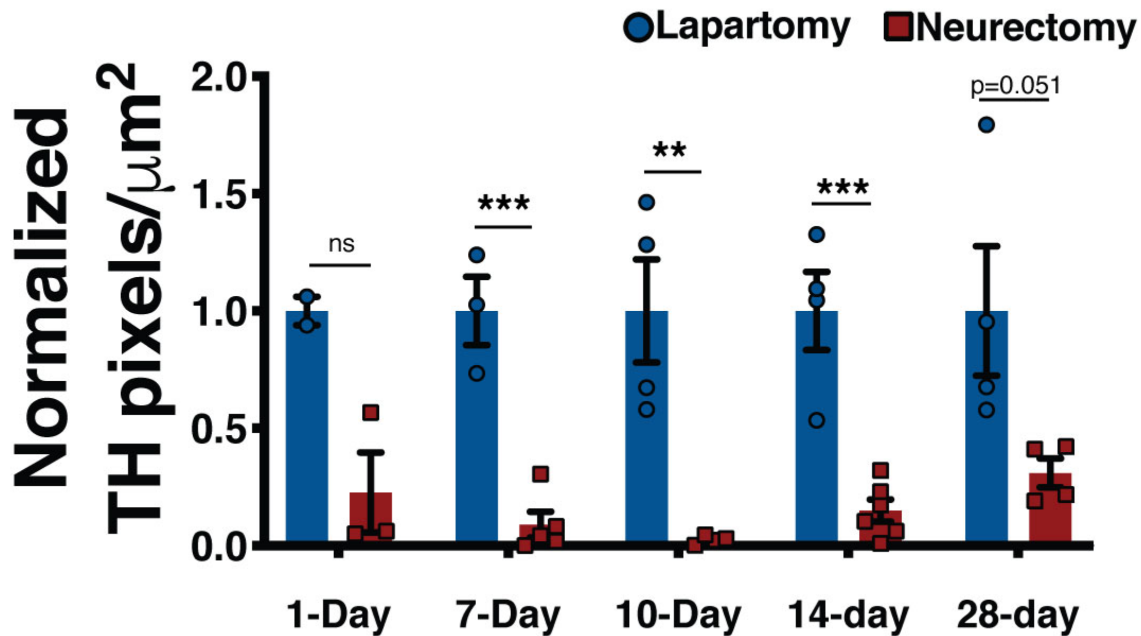

Figure 3.2: Neurectomy surgery reveals progressive loss of sympathetic axons with recovery at 28 days.
Bar graph depicting time course quantification of TH immunostaining throughout the spleen after splenic nerve injury as normalized to laparotomy only animal. Values are given as mean ± SEM. Multiple t-tests as each time point is an independent experiment. **, $p<0.01$; ***, $p<0.001$

and Mirsky, 2019). Since it was clear that GFP expressing glia in the spleen begin to exhibit physical change at seven days we hypothesized that spleen glia might undergo similar changes. To assess how spleen glia might change over time we next investigated their physical changes over the same time course. Immunohistochemical analysis demonstrated nearly healthy appearing glia at the earliest timepoint (24 hours). At this early time point glia exhibit typical hallmarks of naïve and sham surgery animals. They have irregularly shaped, yet discernible cell bodies boldly filled with GFP throughout the cytoplasm forming extensive networks of processes that travel along nerve fibers . However, by day 7, as previously described, GFP expressing glia begin to show signs of distress. They appear to have retracted some of their processes as their cell bodies and processes to appear thickened with more aggregated GFP (Figure 3.3A). Glia at this time are also in less dense networks and do not have uniform GFP. By 10 days spleen glia appear to be trending towards death or dead, with no identifiable cell bodies or processes labelled with GFP, instead round specks of GFP are visible. This phenotype persists out to 28 days where some recovery is visible (Figure 3.3 B). To assess if this death was due to apoptosis, we labelled the same sections using an activated caspase-3 antibody. Interestingly, spleen glia do not double stain for caspase-3 at any time point outlined above (data not shown), indicating that the atrophy or death of spleen glia may be happening through different mechanisms.

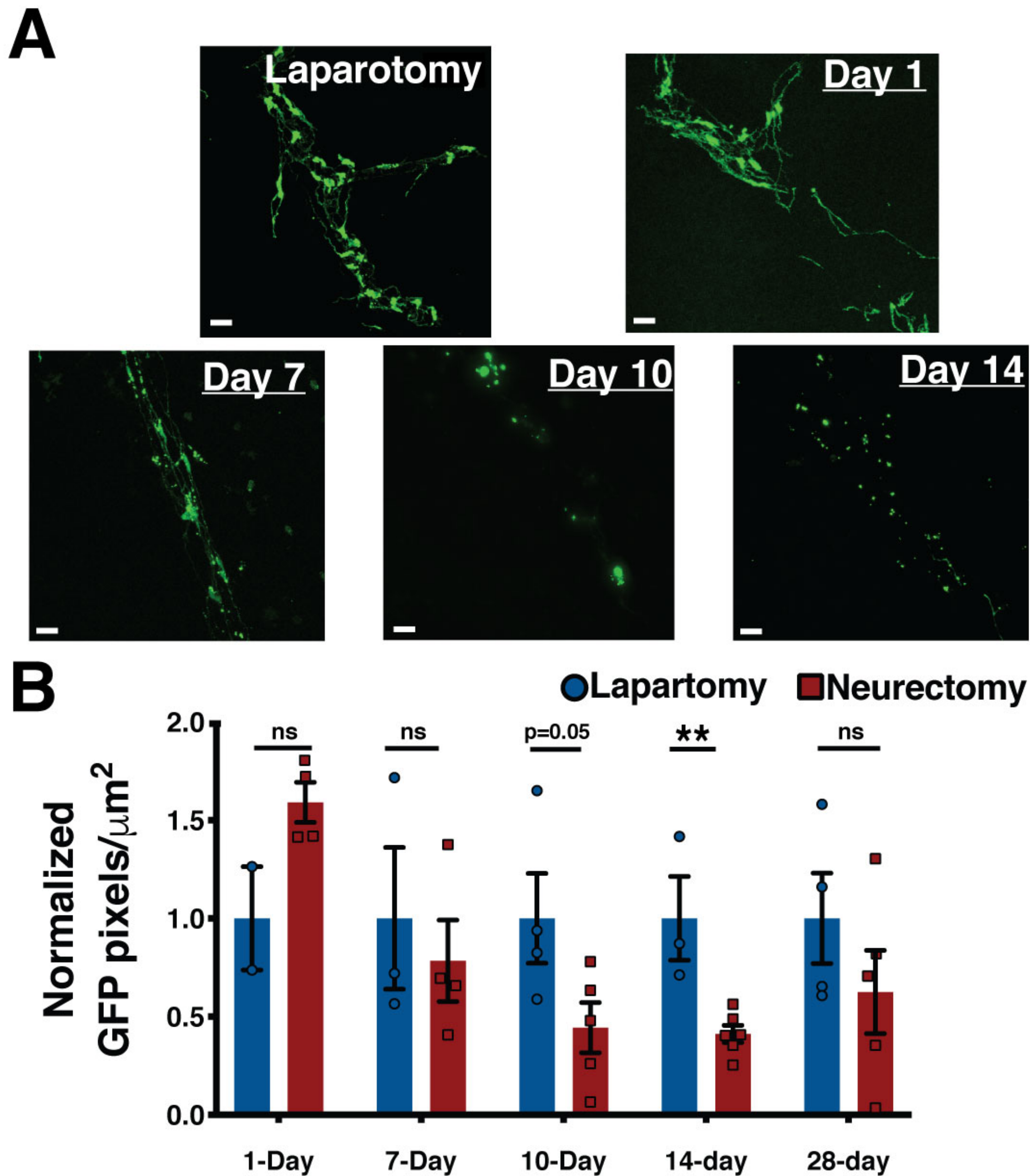

Figure 3.3: Time course analysis reveals progressive glial cell injury after splenic neurectomy.
(A) Representative photomicrographs of 40 μm thick spleen sections from animals who underwent splenic neurectomy surgery. Time course analysis reveals that by 7 days post splenic neurectomy surgery glia begin to exhibit morphological changes and by 10 days are likely dead with no visible GFP$^+$ cell bodies or processes on days 10 and 14, and some re-appearance of glia on day 28. **(B)** Time course quantification of GFP staining in spleen sections. Scale bar= 20 μm. Values are given as mean $\pm$ SEM. Multiple t-tests as each time point is an independent experiment. **, $p < 0.01$.

Considering that the loss of sympathetic nerve fibers in the spleen produced a substantial physical change in spleen glia, we considered that it is likely the nerve delivers trophic support to the cells. This support could likely come from humoral means or through physical contact. To determine which of the two might be the case we utilized transgenic *Sarm1*$^{-/-}$ mice. Sarm1 knockout mice are resistant to Wallerian degeneration in injury models, including TBI (Marion et al., 2019; Maynard et al., 2020) and several neuropathies (Gilley et al., 2017; Ziogas and Koliatsos, 2018; Cheng et al., 2019). To test whether glia survival is affected after nerve injury, we conducted a pilot experiment in which *Sarm1*$^{-/-}$ animals underwent laparotomy or neurectomy surgery using their wild-type littermates as controls. To begin, we first wanted to validate nerve protection in our neurectomy model. Spleen sections were immunostained for TH after 4 and 7 days of recovery. Our analysis shows no difference in nerve fibers at 4 days. This effect may be due to the experiment being underpowered. However, as expected we see complete loss of TH fibers in the organ at 7 days (p¡0.05; compared to laparotomy wild-type). As expected, *Sarm1*$^{-/-}$ animals who received neurectomy surgery did not have a decrease in sympathetic axons compared to laparotomy at either time point (Figure 3.4). Although small, this experiment reveals that Wallerian degeneration of unmyelinated splenic nerve fibers is likely blocked in *Sarm1*$^{-/-}$ animals, and that they will be a useful tool to help understand the relationship between sympathetic axons and spleen glia.

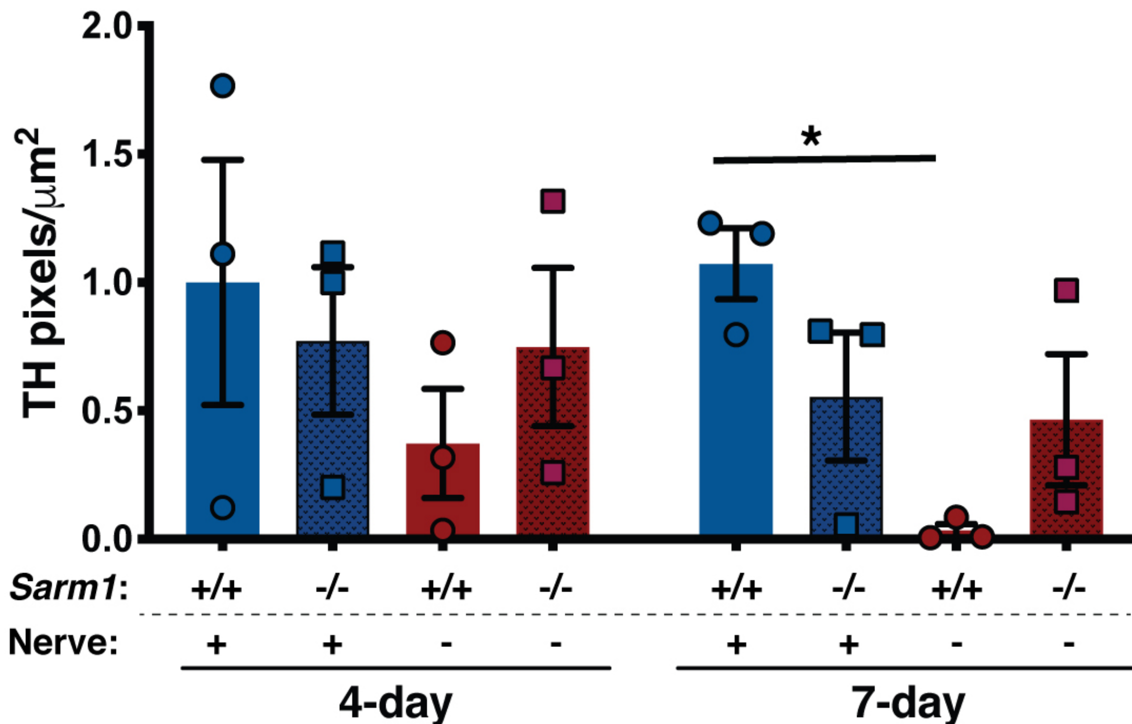

Figure 3.4: Wallerian degeneration of splenic nerve is protected after nerve transection in *Sarm1*$^{-/-}$ animals.
Bar graph quantification of TH fibers in Sarm1 knockout animals vs. wildtype C57BL/6J. The graph depicts a trend of decrease of TH fibers at both 4 and 7 days when compared to laparotomy animals from pilot experiments. This trend is not seen in wildtype controls. Values are given as mean ± SEM. Two-way ANOVA followed by Dunnett's multiple comparisons test. *, $p < 0.05$.

3.4 Discussion

Here we describe and validate a splenic neurectomy model and provide a characterization of the relationship between spleen glia and the splenic nerve. Surgical transection of the splenic nerve causes degeneration of fibers as early as 24 hours post-surgery and they are nearly all gone by day 7. We. begin to see recovery by 28 days. Upon damage to the nerve spleen glia become distressed, with physical changes appearing by day 7. Spleen glia retract their processes and exhibit signs of atrophy, and by day 14 the majority of cells appear to be dead. This process is not through apoptosis as spleen glia do not express caspase-3, a central molecule in the execution of apoptosis at any of our time points. In *Sarm1*$^{-/-}$ animals, nerve fibers are protected from degeneration after injury at 7 days when compared to wild-type animals. The data presented here is a first step in understanding the trophic factors involved in spleen glia-nerve interactions. Future studies will examine the glia in *Sarm1*$^{-/-}$ mice to determine whether physical contact helps spleen glia maintain their integrity and examine early transcriptomic changes after neurectomy. Additionally, we provide a surgical technique to perturb direct innervation to the spleen, a first step necessary to understand the role of the splenic nerve in splenic functioning.

This neurectomy technique provides a means to investigate the role of direct innervation to the spleen on glia and on sympathetically-mediated immune responses.

Splenic neurectomy directly injures the splenic nerve without damage to the rest of the sympathetic nervous system. This is important because the sympathetic nervous system is important in many other homeostatic functions, so perturbations that act globally not only affect the targeted organ but also a host of other tissues and physiologic functions. For example, animals treated with 6-OHDA, a neurotoxin specific to adrenergic nerves, exhibit decreased heart rate (Forbes et al., 1977), decreased locomotor activity (Sgroi et al., 2014) and even death (Koski et al., 2019). In our model, we are able to directly injure the nerve resulting in decreased nerve fibers in the spleen beginning as early as 24 hours after injury which persists to 28 days. Our model of complete transection seems to be similar in recovery time to nerve crush injury of the sciatic nerve, which takes a similar amount of time to demonstrate recovery (Hoffman and O'Shea, 1999). We however do not yet know how long the nerve would take to completely reinnervate the spleen or whether it would reach pre-injury levels.

In marked contrast to glia in the sciatic nerve after injury, we find that spleen glia exhibit signs of death after splenic neurectomy. Typically, after injury to a peripheral nerve, Schwann cells undergo profound changes in their identity, converting to a denervated Schwann cell type, which provides support for axon regeneration, this resulting change in identity is referred to as the repair Schwann cell and termed adaptive cellular reprogramming (Jessen and Arthur-Farraj, 2019; Jessen and Mirsky, 2016). This cell type switch has been shown in several tissues including the pancreas

(Eberhard and Tosh, 2008), liver (Yanger et al., 2013) and skin (Verdú and Navarro, 1997; Davis et al., 2012). It is likely that a similar resulting cell occurs from denervated spleen glia, however further research needs to be done to elucidate how spleen glia change and what characteristics they may have. It may also very well be that glia do not convert into repair cells during the time points observed but rather take on an entirely new identity, another response from peripheral glia. This can be seen in the ear in which damage to hair cells causes glial cells to convert into new hair cells (Jessen and Arthur-Farraj, 2019). This is not unique to the ear as enteric glia have been shown to be multipotent and have the ability to convert to neurons after injury (Laranjeira et al., 2011; Gershon, 2011). Perhaps, as there is dieback of both axon and glia , glia remaining on the nerve increase their rates of proliferation and and provide support for axons to renter the spleen.

Changes in spleen glia after nerve transection are not completely unexpected. It is well known that one regulator of Schwann cell gene expression is the axon, as different axons differ markedly in their ability to instruct Schwann cells (Jessen and Mirsky, 2019). Axons direct whether Schwann cells will simply ensheathe the normally unmyelinated nerve fibers or myelinate the larger fibers. The ability of Schwann cells to substantially influence regeneration in the peripheral nervous systems derives in part from their ability to produce a variety of trophic factors, as well as from expressing a number of cell adhesion molecules that are known to influence neurite growth

on their surfaces. Among these are nerve growth factor (Liu et al., 2007; Kimpinski et al., 1997), brain derived neurotrophic factor (*Bdnf*; (Kimpinski et al., 1997; Kellner et al., 2014), glial-derived nerve growth factor (*Gdnf*); (Schäfer and Mestres, 1999), and ciliary neurotrophic factor (Liu et al., 2014). Our transcriptomic data (Chapter 2) show that at baseline spleen glia are also high expressors of many of these well characterized factors influencing nerve-glia relationships. One example is neuroligins, which are a family of cell adhesion proteins important for neuronal development and synapse formation. Spleen glia have high expression of two neuroligins, NL2 and NL3. NL3 has been shown to be necessary for peripheral ensheathment of nerves in the fly (Gilbert et al., 2001). In the CNS, knockout of NL3 causes severely stunted branching of oligodendrocytes and astrocytes in culture (Stogsdill et al., 2017; Linneberg et al., 2015). Similarly, knockdown of neuronal neurexins, the binding partner of neuroligins, produces the same stunted branching patterns (Stogsdill et al., 2017). Spleen glia also express GDNF and BDNF. GDNF is a soluble glial factor that increases neurite extension and promotes survival of motor and sensory neurons both *in vitro* and *in vivo* (Marquardt et al., 2015). BDNF has been shown to promote neuronal regeneration and functional recovery in models of spinal cord injury (Lin et al., 2016). Another example of likely axon-glia trophic interactions are those between the type III neuregulins and Erb/b receptors on axons and Schwann cells / spleen glia respectively. Spleen glia express *Erbb2*.Type III neuregulin binds to Erb/b2 receptors on

Schwann cells and activates PI-kinase in Schwann cells, a key signaling pathway found to be essential for ensheathment and myelination (Newbern and Birchmeier, 2010). Thus, spleen glia likely maintain their relationship to sympathetic axons using some or all of these mechanisms. However future research is needed to dissect these mechanisms and understand how spleen glia change and what state the cells are in after injury. We hypothesize that spleen glia may be more dependent on trophic factors from the nerve and/or physical contact with the axons than sciatic nerve Schwann cells. The spleen is quite unique as it has only sympathetic fibers innervating the organ, and is it possible that their dependence on the nerve is uniformly seen in non-myelinating Schwann cells that ensheath sympathetic axons. Deletion experiments of the several trophic factors expressed by spleen glia or the nerve may prove to be useful in understanding their role in spleen glia and interaction with the nerve

As expected, nerve fiber degeneration seems to be protected in pilot experiments using *Sarm1*$^{-/-}$ mice. One of the interesting findings was that there was actually some damage that occurred to the nerve fibers in these animals. This is not unique to the splenic nerve, however, *Sarm1*$^{-/-}$ animals who receive traumatic brain injury still exhibit 30% loss of injured axons when compared to that of wild-type controls (Marion et al., 2019). This suggests that although most nerve fibers are protected there are a subset that are still susceptible to injury. On another note, we observed that the

overall density of nerve fibers in the spleens of knockout animals only receiving laparotomy surgery seemed to be less than their wild-type counterparts only receiving laparotomy surgery, yet, statistically unsignificant ($p= 0.4$) (Figure 3.4). However, since the number of animals we had in each group were fairly, this trend is likely a result from being underpowered. Nonetheless, at day 7 *Sarm1*$^{-/-}$ animals exhibit no statistically significant differences from either laparotomy group, in the amount of TH^{+} nerve fibers throughout the organ. Due to the small size of the experiment and variability observed we are planning to increase the sample sizes in future experiments, as well as to study the effect on the glia. Nevertheless, the trends observed are exciting first step to measuring changes in spleen glia after neurectomy while axons are still present.

One limitation of this study is that the method we used to measure the presence of nerve fibers relies on immunostaining for Tyrosine Hydroxylase (TH), the rate limiting enzyme in norepinephrine production. We don't know the effect of proximal nerve transection on TH vesicles. Additional immunostains for other nerve markers, such as protein gene product 9.5 (Pgp 9.5) or neurofilament (NF) are needed to verify that the axons are gone. NF is an intermediate filament found in the cytoplasm and thus should be present in intact axons regardless of proximal nerve conditions. Pgp 9.5 is a ubiquitin hydrolase and thus abundantly present throughout the entire fiber and also should not be affected by signals upstream.

The work described here with the *Sarm1*$^{-/-}$ mice is very exciting pilot data. Future experiments with higher sample sizes will follow. Combined with further analysis of different glial markers we will be able to elucidate the extent of nerve damage in both wild type and *Sarm1*$^{-/-}$ animals as well as the response of spleen glia in both genotypes.

This work will also be pivotal for allowing us to uncover interactions between glia, nerve and immune cells in the spleen. We have demonstrated an effective technique to block direct sympathetic signaling in the spleen in a mouse model and shown that glia undergo substantial physical alterations and likely death. One question still of interest to us is what transcriptional changes occur in glia that lead to atrophy of the majority of cells. Our lab has described an effective method to isolate and sequence spleen glia and we plan on characterizing this response and designing experiments to test what factors in the nerve are necessary for glial survival. Lastly, there are many experimental gaps on the importance of the nerve in splenic immune responses. Splenic neurectomy will be an important tool to study the underlying molecular mechanisms in mice.

3.5 Materials and Methods

3.5.1 Animals

All animal procedures were performed in accordance with the protocol approved by the Institutional Animal Care and Use Committee at Stanford University. Breeding pairs of Gfap-Cre (JAX#12886), Rosa26 eGFP (JAX#4077), and *Sarm1*$^{-/-}$ (JAX#018069) were purchased from The Jackson Laboratories (Bar Harbor, ME). Mice were housed in a temperature-controlled 12-hour light-dark alternating facility, with ad libitum access to food and water. All experiments were performed with 8-12-week-old male. To generate mice that express GFP in glia, we bred homozygous female GFAP-cre animals with homozygous male Rosa-eGFP animals. Animals used in experiments were heterozygous for both transgenes. To generate Sarm1 knockout animals, we either purchased knockouts or bred *Sarm1*$^{+/-}$ animals to each other. Experimental animals were homozygous Sarm1 knockout mice (*Sarm1*$^{-/-}$) or wildtype littermate controls. Both groups were subjected to neurectomy or laparotomy surgery and allowed to recover for specified timepoints ranging from 4 -10 days.

3.5.2 Neurectomy Surgery

Neurectomy surgeries were developed in-house and carried out in accordance to APLAC guidelines for major surgery. Animals were anesthetized with 2% Isoflurane in 2 L/min 100% oxygen and maintained at 37°C both during surgery and recovery using a feedback-controlled heating blanket. Mice were shaved, anesthetized and placed in a sterile surgical field with their ventral side up. After an abdominal incision, skin was separated from the peritoneal wall by blunt dissection. Using curved surgical scissors another incision was made along the linea alba to expose the abdominal organs. Intestines were then exteriorized by leading with the ileum and placing the bulk of the small intestine on a sterile surgical drape. The spleen was then exteriorized, and nerve-vessel bundles identified. Using blunt dissection, the nerve was carefully dissected away from the vessels until transected. Laparotomy animals had intestines and spleens exteriorized but did not receive any blunt dissection of the nerve, and had their abdomen open for similar time periods. Mice were concurrently injected with 25 mg/kg cefazolin (VWR #89149-888) and 1 mg/kg of buprenorphine SR (Zoopharm, Windsor, CO) to prevent infection and for pain management, respectively. The peritoneal wall was closed using a continuous suture technique with absorbable suture and skin was closed using Reflex 7mm Wound closure system (Braintree Scientific #RF7 KIT). Validation of surgery can be found in results section.

3.5.3 Perfusion and tissue preparation

Mice were terminally sedated with 6% chloral hydrate in PBS and perfused through the left ventricle with 10–20ml of cold 0.9% heparinized saline (10 units/ml). Spleens were collected and fixed in 4% paraformaldehyde in phosphate buffer overnight then transferred to 30% sucrose in phosphate buffer until sunken, usually overnight. PFA-fixed spleens were sectioned in both sagittal and coronal planes using a freezing sliding microtome to generate 40 μm thick sections (Microm HM430). Spleen sections were sections were stored in cryoprotectant medium (30% glycerin, 30% ethylene glycol, and 40% 0.5 M sodium phosphate buffer) at 20°C until processing.

3.5.4 Immunohistochemistry

Immunohistochemistry was performed on PFA-fixed free-floating tissue sections using standard protocols. Sections were washed in Tris Buffered Saline (TBS) and blocked with 5% serum for 1 hour. Primary antibodies were diluted in 0.1% Triton X-100 and 5% serum and applied overnight at room temperature. We used the following primary antibodies: rabbit anti-TH at 1:500 (Millipore), rabbit anti-S100b (Novus). The following day, the sections were rinsed extensively with TBS and incubated with a fluorescent donkey anti-rabbit secondary antibody for 5 hours. Sections were washed in TBS then wet-mounted with Vectashield hard set mounting media

with DAPI (Vector Labs) and coverslipped. In some instances, Diaminobenzidine tetrahydrochloride (DAB) immunohistochemistry was performed. In these stains a similar protocol was followed with a pretreatment in 30% hydrogen peroxide (1hr) prior to blocking and incubation with avidin–biotin peroxidase complex (Vectastain Elite ABC kit, Vector Laboratories) for 1 hr after a biotinylated secondary antibody was applied.

3.5.5 Image acquisition and Quantification

Sections were imaged using 40x, 1.15 numerical aperture oil objectives on a Leica TCS SPE confocal microscope using Leica Application Suite Advanced Fluorescence software. Stacked images of fluorescent spleen sections were reconstructed using ImageJ (NIH), and Photoshop (Adobe) software was used to change brightness and contrast of the images. In each case all settings were applied equally to each color channel. To quantify normalized TH pixels mark and find protocols were used in the Leica Application suite to image 12 semi-random white pulp and red pulp locations in a given section. White vs. red pulp was identified based on the pattern of DAPI staining in the blue channel, then confocal pictures were taken in the red channel to capture TH immunostaining. 3 sections per animal were utilized to generate a total of 36 images. Florescent pixels were then quantified using thresholding in ImageJ.

Quantification and thresholding were carried out in accordance to previously published protocols outlined in Inman et al. (2005). Fluorescent pixels were calculated as percent area covered and normalized to laparotomy animals. The experimenter was blind to experimental group until after quantification was completed.

3.5.6 Statistical analyses

Data graphs are presented as mean ± SEM. The differences between experimental groups were analyzed by t-test or Two-way ANOVA, followed by Dunnet's multiple comparison test. MS Excel, ImageJ, Origin 2016 (OriginLab, Northampton, MA, USA), and Prism GraphPad 8 (GraphPad Software, San Diego, CA, USA) software was used for data and statistical analysis.

Chapter 4

Conclusions

4.1 Summary

In this dissertation I have reported the first in-depth characterization of spleen glia. First, using immunohistochemistry, I characterized the morphology and anatomy of spleen glia. I followed up by isolating the cells and sequencing their transcriptomes, and then used that data to compare them to other glial types. I identified several molecules expressed in spleen glia that are implicated in signaling to nerves and immune cells, including the neuropeptide Y receptor *Npy1r* and the chemokine *Cxcl13*. Secondly, I developed a new surgical methodology to cut the splenic nerve and described how spleen glia die upon nerve cut. The work outlined in this dissertation lays an important foundation for future work examining the functions of spleen glia. Below I discuss the implication of my overall findings and summarize exciting directions for future research.

4.2 Discussion

I discovered that spleen glia are a unique glial type that likely have important functional roles beyond just supporting nerves within the organ. My analysis of spleen glia gene expression established that they express key neural and immune genes at baseline. This is strongly suggestive that spleen glia are involved in homeostatic functions of the sympathetic nervous system and immune system locally. This is consistent with the role of enteric glia, which play a role in maintaining normal gut function. For instance, deletion of enteric glia leads to injury to the intestinal epithelium (Savidge et al., 2007) and reduction in gut motility (Rao et al., 2017). Deletion of spleen glia is a future experiment. However, the transcriptomic dataset I generated demonstrates that they likely communicate with immune cells and receive signals from sympathetic axons.

Since the spleen is heavily involved in both the innate and adaptive immune response, it is likely that spleen glia serve to influence both arms of the immune system. They express complement proteins, macrophage stimulating proteins (*Csf1*, *Csf3*) and pattern recognition receptors (*Ager*, *Tlr4*) which are all involved in innate immune responses. Additionally, spleen glia express chemoattractants for T (*Ccl19*, *Ccl21*) and B (*Cxl13*, *Tnfsf13b*) lymphocytes, and lymphocyte co-stimulatory molecules (*Icam*, *C40*) that are involved in activation of lymphocytes for adaptive immune responses.

This would not be unique to spleen glia as astrocytes have also been shown to affect both arms of the immune system. My hypothesis is that spleen glia work in conjunction with the nerve to regulate immune responses and to both limit and exacerbate inflammation. The direction of their influence likely depends on conditions and the immune state of the spleen.

Indeed, both astrocytes and enteric glia can exert both pro- and anti-inflammatory effects in a context dependent manner. For example, enteric glia can detect local levels of the proinflammatory cytokines IL-1, IL-4, and TNF (Von Boyen et al., 2004). Binding of these cytokines elicits glial cell activation similar to reactive gliosis seen in CNS astrocytes marked by increased expression and secretion of S100B and GFAP, and this contributes to inflammation and GI dysfunction (Von Boyen and Steinkamp, 2010; Von Boyen et al., 2004). Similarly, astrocytes excrete the proinflammatory cytokine IL-6 in response to LPS through activation of toll-like receptors on their cell surface (El-Hage et al., 2011; Wiese et al., 2012). Astrocytes are also a major source of T and B lymphocyte chemoattractants. During active infection, reactive astrocytes express CCL5 as well as CXCL10, both chemoattractants for T cells (Sauder et al., 2000; Babcock et al., 2003)). Furthermore, in brain samples from patients with multiple sclerosis, astrocytes have been shown to express the B cell chemoattractant B-cell activating factor (BAFF) (Krumbholz et al., 2005). Spleen glia also express many of these genes and may produce pro-inflammatory responses through similar

mechanisms.

In addition to their ability to stimulate immune responses, both astrocytes and enteric glia serve to keep immune responses in check and to maintain homeostasis through immunosuppression. These anti-inflammatory effects are controlled, in part, by the release of soluble glial compounds such as glial-derived neurotrophic factor (GDNF) and ciliary neurotrophic factor (CNTF). In the gut, enteric glia release GDNF that acts on type 3 innate lymphoid cells (ILC3), leading to release of anti-inflammatory IL-22 and increased expression of repair genes in the gut epithelium (Ibiza et al., 2016; Chow and Gulbransen, 2017). In addition, GDNF can signal to immune cells in the gut to decrease expression of pro-inflammatory cytokines such as TNF and IL-1 (Von Boyen et al., 2011). In the CNS, astrocytes can also limit inflammation by forming a physical barrier, known as a scar, that helps restrict not only leukocyte access but also access of inflammatory molecules into the parenchyma (Fawcett and Asher, 1999; Sofroniew, 2009). This glial constraint on neuroinflammation is critical in a host of diseases and after injury, including EAE (Bribian et al., 2018; Voskuhl et al., 2009), Alzheimer's disease models (Serrano-Pozo et al., 2013; Chun et al., 2018), Parkinson's disease models (Tomov, 2020; Cabezas et al., 2013), infection (Adams and Gallo, 2018), spinal cord injury (Yuan and He, 2013; Bradbury and Burnside, 2019), and stroke (Becerra-Calixto and Cardona-Gómez, 2017;

Huang et al., 2014). In addition to the physical barrier of the glial scar, astrocytes release anti-inflammatory molecules in response to injury and inflammation, e.g. TGFβ (Wiese et al., 2012; Cekanaviciute et al., 2014b,a) and IL-10 (Recasens et al., 2019).

Our transcriptomic analysis reveals that, similar to enteric glia and astrocytes, spleen glia express molecules that can up- or down-regulate inflammatory responses. For example, tenascin C (*Tnc*) was the second most differentially expressed gene in spleen glia. Tenascin-C is a matrix protein induced upon injury (ref) that binds and activates toll-like receptor four (*Tlr4*), which among other mediators releases IL-6 (Zuliani-Alvarez et al., 2017; John et al., 2003). Spleen glia significantly express all three genes and therefore may use this pathway as a mechanism to produce inflammation in the spleen. In addition, spleen glia express anti-inflammatory glial compounds. One example is the expression of thrombospondin-2 (*Thbs2*). Thrombospondin 2 is a matricellular protein that has been implicated in tissue repair (Adams and Lawler, 2011). When it is overexpressed, it suppresses the production of the proinflammatory cytokines, IFNγ and TNF, and induces the depletion of tissue-resident T lymphocytes (Bornstein et al., 2000). Thbs2 may be working similarly in spleen glia and the spleen. An exciting future research direction will be to determine in which context spleen glia promote pro- versus anti-inflammatory responses.

Complement pathway components were highly enriched and expressed in spleen

glia. Complement is a component of the innate humoral immune response, is involved in inflammation, opsonization, and cytolysis. It requires the coordination of many proteins for activation and regulation. While it is unclear what the role of complement is in peripheral glia, in the CNS, complement proteins are upregulated by astrocytes as well as other resident cells following brain infection (Pekny et al., 2007; Hartmann et al., 2019) and stroke (Hersh and Yang, 2018). There has not been much focus on the astrocytic specific effects of complement, but when it is systemically depleted, rats exhibit smaller infarct sizes after ischemia (Ma et al., 2019), suggesting that complement may be contributing to detrimental inflammatory responses. Complement may also have a dual role in stroke. Animals deficient in C3, an integral part of both complement pathways, show decreased neurogenesis after stroke (Alawieh et al., 2015). In the PNS, complement is highly expressed in lymphatic tissues, including the spleen and lymph nodes, due to the large presence of macrophages and neutrophils.

There are also some experimental findings that implicate complement in Wallerian degeneration. Wallerian degeneration occurs when transection or injury to nerves results in degradation of distal nerve fibers and proliferation and activation of Schwann cells. During this process macrophages are recruited to the site of injury to help clear debris, and recruitment is thought to happen through complement proteins that produced from Schwann cells (de Jonge et al., 2004). The function of complement signaling in spleen glia may be similar. Additionally, the spleen lacks

lymphatics so antigen and bloodborne pathogens are brought into the organ through the vasculature. Our data revealed that spleen glia are located around arteries and arterioles, and thus are in a prime position to be one of the first cells exposed to antigens in the spleen. Complement expression in spleen glia may play a role in initiating the innate immune response in this instance.

Glia have been implicated in many aspects of neuroimmune communication throughout the nervous system. We hypothesize that spleen glia participate in neuroimmune responses through bidirectional communication with both nerves and immune cells (Figure 4.1). The splenic nerve likely directly communicates with immune cells to modulate immune responses, and this communication is likely modulated by spleen glia that are in close proximity. This idea is similar to the concept of the tripartite synapse in the CNS (Perea et al., 2009), in which astrocytes can alter neurotransmission between two neurons. In the case of the spleen, the three players would be glia, the splenic nerve, and immune cells. One example of possible mediators of this communication that I found in my transcriptomic analysis is purinergic receptors. Spleen glia highly express two purinergic receptors, *P2ry2* and *P2rx1*, and also genes necessary for calcium signaling through the phospholipase C pathway known to be activated by purinergic G-protein coupled receptors (*Plcg1*, *Itpr3*, *Dag1*, *Prkca*). Purinergic signaling in glia has been shown to alter neurotransmission and inflammation in both astrocytes and enteric glia. Specifically, in enteric glia purinergic signaling produce

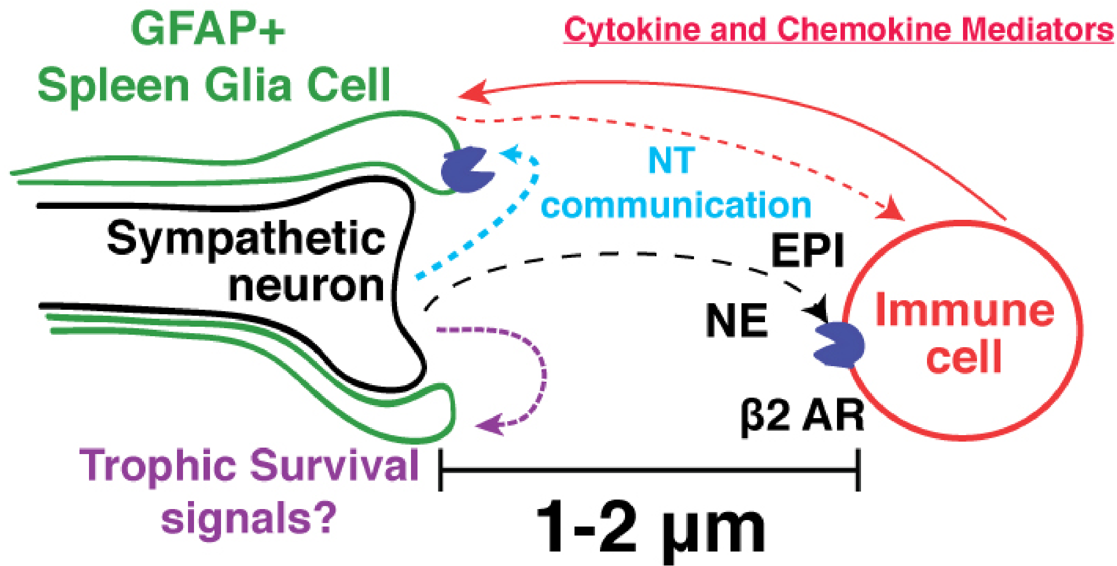

Figure 4.1: Proposed hypothesis of neuroimmune connections in the spleen

calcium waves in glia leading to subsequent proliferation of the cells and release of ATP (Lecca et al., 2012; Quintas et al., 2011). Purinergic signaling in astrocytes also induces calcium waves and leads to the release of proinflammatory molecules and promotes synaptic inhibition (Junger, 2011).

There is also evidence for mechanisms that could carry out bidirectional communication between immune cells and spleen glia. Spleen glia express multiple cytokines and chemokines that can promote both pro- and anti-inflammatory responses, as well as specific receptors for cytokines including interleukins 1 and 17. For example they express the type 1 interleukin receptor (*Il1r1*) which is primarily responsible for transmitting the inflammatory effects of interleukin-1 (Zenobia and Hajishengallis, 2015).

In addition, spleen glia highly express genes required to form the interleukin 17 receptor *(Il17ra/c)*. Activation of the receptor has been shown to promote expression and production of inflammatory cytokines (TNF or IL-1β) and chemokines (MCP-1, IL-8) driving activation of macrophages and release of antimicrobial products (Aggarwal and Gurney, 2002).

A key unknown about sympathetically-mediated immune changes is how much the splenic nerve itself versus circulating catecholamines produced from sympathetic stimulation of the adrenal glands contribute to sympathetically-mediated immune changes. Contradictory conclusions reported in literature after LPS injections and splenic neurectomy made me re-evaluate the rigor and validity of the experiments that have been published. Future experiments could use the neurectomy surgery I developed that inhibits direct sympathetic signaling in the lab to elucidate the role of the nerve during LPS responses in a time dependent manner. I found evidence that the splenic nerve extensively innervates immune cell regions in the spleen and comes into very close contact with lymphocytes (Felten and Olschowka, 1987). The lymphocytes themselves are also very high expressors of adrenergic receptors, specifically the beta-2 adrenergic receptor (Wu et al., 2018). Future experiments should however also include analysis of the glial response with a focus on early timepoints after infection and injury. Whatever the role of the splenic nerve may be, the work I present in this dissertation now makes it possible to precisely test the hypothesis that glia, nerves

and immune cells work together in producing immune responses.

Together the transcriptional data and physical changes I present here demonstrate the uniqueness of spleen glia. However, it is important to note that the spleen is not the only secondary lymphoid organ. Lymph nodes, Peyer's patches of the gut, mucosal and nasal associated lymphoid tissue, adenoids and tonsils all are secondary lymphoid organs, and all receive some innervation from the autonomic nervous system and presumably contain glia. We observe expression of GFAP$^+$ cells and sympathetic axons in the lymph node (data not shown). However, like spleen glia, glia in all of these locations have not been well characterized or implicated in organ functions. It will be important to not only further characterize glia in the spleen but also determine their similarity and differences with glia in the other secondary lymphoid organs.

4.3 Future Directions

When I began this work I had many pre-existing hypotheses and while I learned a lot, there still remains a significant amount to be discovered (outlined above). The work also led to new hypotheses and questions.

One surprising result from our analysis was expression of immune cell homing chemokines in spleen glia. While we expected to observe evidence of molecules that might communicate with immune cells to activate or suppress them, we did not expect

expression of molecules known to organize lymphocytes within the spleen. Immune cell homing and lymphocyte organization is important for a proper immune response from the organ. In particular, spleen glia highly express molecules that organize the two major lymphocyte populations, T (*Ccl19*) and B (*Cxcl13*) lymphocytes, causing their homing to the spleen. Specifically, CXCL13 and its receptor CXCR5 control the organization of B cells within follicles of lymphoid tissues (Ansel et al., 2000; Förster et al., 1996) and mice deficient in this chemokine have impaired trafficking of B1 cell subsets (Ansel et al., 2002). Our transcriptional data suggests that spleen glia are high expressors of the chemokine and expression was confirmed in spleen glia using in situ hybridization and immunohistochemistry (data not shown). However, there are likely many contributors to the homing of immune cells to the spleen, in particular follicular dendritic cells also express this chemokine as do T cells (Workel et al., 2019; Vermi et al., 2008). Considering the intimate relationship glia have with these cells, experiments deleting these molecules specifically from glia would determine their role in homing and trafficking during infections. More intriguingly, *Cxcl13* systemically is considered a biomarker for germinal center activity and thus antibody production from plasma cells (Havenar-Daughton et al., 2016). It would be interesting if spleen glia had a role in signaling to B lymphocytes during infection models and eliciting their efficient differentiation into plasma cells. Another phenomenon in which this may be important is during splenic contraction after stroke in which disorganization

of immune cell populations and disruption of splenic architecture occurs (Offner et al., 2006). Considering the large sympathetic surge after stroke, perhaps the release of norepinephrine from the splenic nerve shifts the balance of these homing molecules in spleen glia leading to disorganization. Some preliminary studies I conducted 3 days after stroke, a time of maximal disorganization, show no differences in splenic mRNA levels of either chemokine. However, it is likely that the response occurs more acutely than this and at day 3 it could be that expression is already normalized. Future work will explore the intriguing possibility that spleen glia may contribute to developmental or disease-mediated physical organization of the spleen.

In chapter 3 I developed a surgical methodology that allows me to directly injure the splenic nerve. Considering the intimate relationship between spleen glia and the splenic nerve we became interested in how this relationship was maintained. Upon investigating our transcriptional data, we found high expression of several neurotrophic factors and their receptors including *Bdnf*, *Gdnf*, *GFRa* and *Ctnfr*. Since we are primarily interested in glial changes, we opted to develop a surgery that injured the nerve (neurectomy). We hypothesized that like Schwann cells of the sciatic nerve, spleen glia would de-differentiate and similarly aid in nerve regrowth. Surprisingly, our analysis revealed that spleen glia are dependent on the nerve for survival–after neurectomy, glia begin to change by 7 days and appear dead by 10 days. As presented in Chapter 3, I am exploring this by using transcriptomics and mice with inhibited

Wallerian degeneration. These sets of experiments are aimed at describing how glia change after nerve injury in a time dependent manner and identifying molecular markers that maintain the nerve-glia relationship using transcriptomics. My hypothesis is that changes to the many neurotrophic factors outlined above play a role and loss of their signaling results in atrophy. This will be an important future direction.

4.4 Final Remarks

Spleen glia offer an exciting new cell type to be explored in the context of neuroimmune communication in the spleen. By characterizing the location and expression patterns of spleen glia at baseline this dissertation has laid a foundation. This work is an essential first step to understanding spleen glia and providing tools to study them in neuroimmune communication. Indeed, my results show substantial expression of both immune and neurotransmitter genes that support the hypothesis that spleen glia can communicate with both nerves and immune cells. However, spleen glia are only one facet of the neuroimmune response that is occurring in the spleen. Contributions from the many myeloid, endothelial and stromal cells are also critical for inflammation as shown in various other tissues. In addition, the spleen is only one of many other secondary lymphoid organs that all combine to elicit the entirety of the immune response seen throughout the body. Understanding all of the cellular players involved including glia within other secondary lymphoid organs will provide the most

accurate understanding of the peripheral neuroimmune response during infection and after injury.

Chapter 5

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