THE INFLUENCE OF BACE1 EXPRESSION ON THE RECRUITMENT OF MACROPHAGES TO THE INJURED PERIPHERAL NERVE

by

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Abstract

Macrophages play a critical role in regeneration following peripheral nerve injury. Hematogenous macrophages are recruited to the distal nerve segment and shape the injured nerve microenvironment to be more conducive to regeneration through the clearance of cellular debris and the production of neurotrophic factors. Enhanced macrophage recruitment and debris clearance has been observed in BACE1 KO mice. This phenotype could be the result of BACE1 activity in macrophages, other nerve resident cells (neurons, Schwann cells), or it could also be the result of the hypomyelination phenotype that is also observed in BACE1 KO mice. To date it is uncertain what potential mechanisms may be driving this enhanced recruitment and debris clearance phenotype. Further, it is unknown which cell types in the peripheral microenvironment contribute most towards this phenotype when BACE1 is deleted. BACE1 is a promiscuous enzyme and has many substrates, thus may play a role in a variety pathways that could result in this enhanced macrophage recruitment and debris clearance phenotype. This thesis discusses several known BACE1 substrates and how they could impact macrophages in the context of peripheral nerve regeneration. Previous work done in our lab suggests that elimination of expression of BACE1 in cells derived from bone marrow may recapitulate the enhanced macrophage recruitment and activity phenotype. This thesis utilizes a mouse model with macrophage specific deletion of BACE1 to investigate whether BACE1 expression by macrophages mediates the immumodulatory phenotype observed in global BACE1 KO mice. This thesis demonstrates that there is not enhanced recruitment of macrophages to the injured peripheral nerve following injury. This suggests that the elimination of BACE1

expression by macrophages is not essential for the increased recruitment phenotype observed in global BACE1 KO mice.

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Chapter 1: Introduction

Introduction

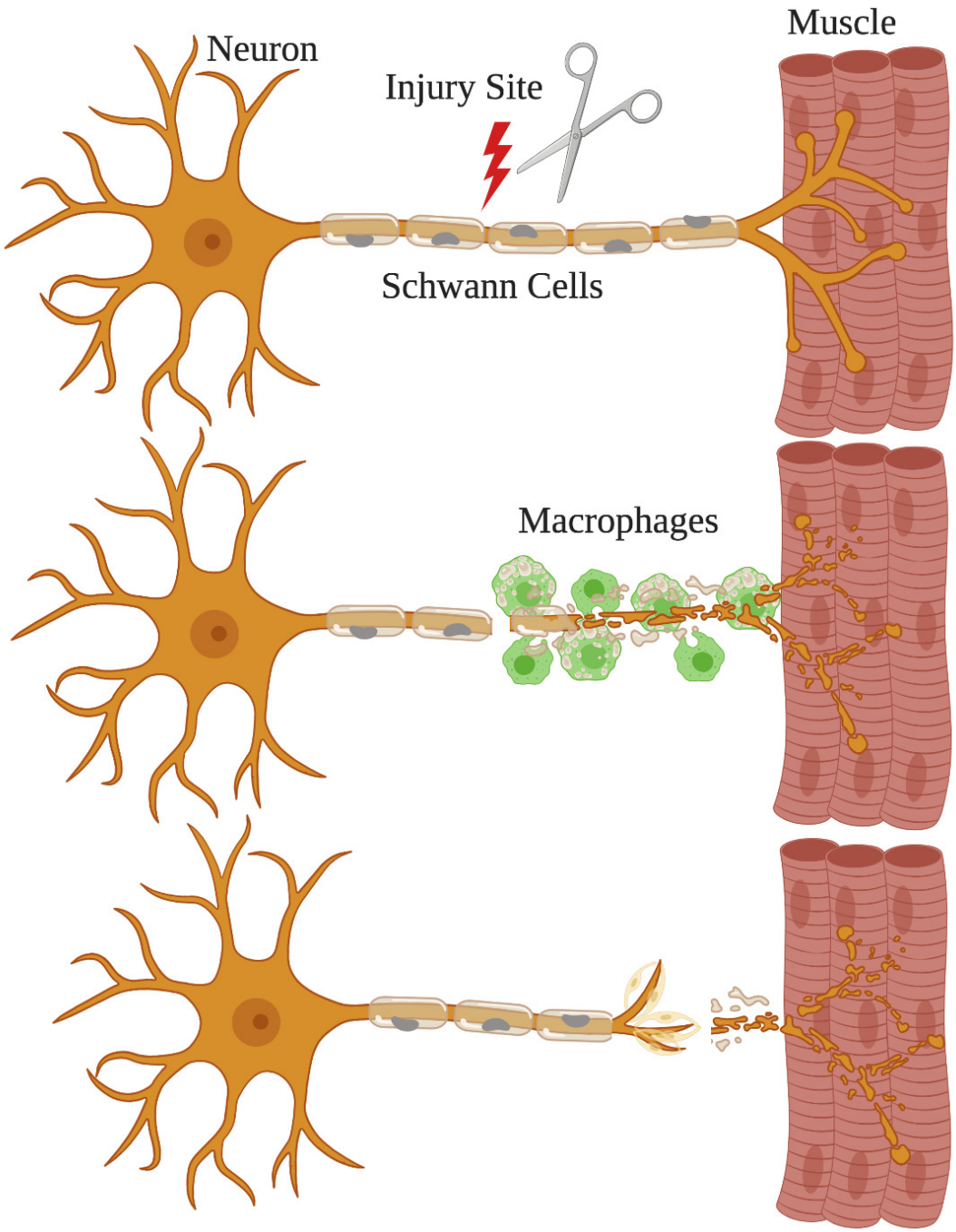
The peripheral nervous system has limited capacity for regeneration in response to injury. However, this level of regeneration is still insufficient to restore full function and mitigate morbidity and disability in humans. Following peripheral nerve injury, extensive coordination of several different cell types is required for the degeneration and subsequent regeneration of the peripheral nerve, beginning with the breakdown of axons and myelin sheaths into amorphous debris. Next, in order to create a microenvironment conducive to axonal regeneration, macrophages are recruited from the circulation to the distal nerve segment to clear this cellular debris and promote axonal regeneration through the release of neurotrophic factors. While neurons and Schwann cells play a critical role in the regeneration process, the focus will be on the role of macrophages, how they interact with the other cells of the injured nerve microenvironment and how inhibition of BACE1 may play a role in modulating these interactions.

Beta-Site Amyloid Precursor Protein (APP) Cleaving Enzyme 1, also known as β-Secretase (BACE1) has been extensively studied for its role in Alzheimer's disease. Cleavage of APP by BACE1 and subsequent cleavage by γ-secretase leads to the generation of Aβ peptides that form Aβ plaques that are a pathological hallmark of Alzheimer's disease. In addition to this role in disease, BACE1 activity is involved in other biological processes. The genetic deletion or pharmacological inhibition of BACE1 results in increased recruitment of macrophages and subsequent cellular debris clearance in the distal nerve segment (*1*). While this phenotype has shown to promote axonal regeneration,

little is known about the underlying mechanism of this enhanced recruitment and debris clearance. The major cell types that comprise the microenvironment of the injured peripheral nerve and could be mediating this phenotype are neurons, Schwann cells, and hematogenous infiltrating macrophages but the relative contributions of each remain unknown. In order to elucidate a potential mechanism, it would be most prudent to start with possible signaling pathways that contain currently known BACE1 substrates that have immunomodulatory capacity. The focus of this review will be the role of macrophages in peripheral nerve injury, how BACE1 modulates macrophage behavior, and discuss known BACE1 substrates that have immunomodulatory capacity.

Fig 1. Sequence of events post-nerve injury

A motor neuron (left) innervates muscle tissue (right) via axon myelinated by Schwann cells (center). After sustaining an injury, the distal nerve segment will degenerate into amorphous debris. Hematogenous macrophages (green) are recruited from circulation and enter the distal stump. Macrophages make the microenvironment more permissive for axonal regeneration by clearing cellular debris and releasing cytokines and neurotrophic factors. Axons regenerate with the guidance of Schwann cells and the aid of macrophages via neurotrophic factors and the elimination of debris which poses a physical barrier and inhibitory signaling to the regenerating axon.


Muscle
Neuron
Injury Site
Schwann Cells
Macrophages

Degeneration following injury

Upon injury of a nerve there is initially a lag period before the segment of nerve distal to the site of injury begins to degenerate (*2*). The initial step of this process is a flux of extracellular Ca^{2+} to the inside of the axon (*3*). The depletion of extracellular calcium or blockage of Ca^{2+} ion channels can attenuate the degeneration of axons for up to 4 days (*2*). The effectiveness of channel blockage to delay degeneration illustrates that this process is related to the activity of calcium channels as opposed to Ca^{2+} flux that is the result of a compromised axonal membrane (*2*). Further, the proximal stump undergoes the same disruption during injury but this segment will not degenerate as the distal segment does (*2*). A consequence of this calcium flux is the activation of calpains, a family of proteases that are known to cleave cytoskeletal proteins such as neurofilament (*2*, *4–6*).

Great advancement into the mechanisms of Wallerian degeneration have emerged from the discovery of the C57BL/*Wld*^{s} mouse, a spontaneous mouse mutant that exhibits very slow Wallerian degeneration (*7*). Wallerian degeneration refers to the breakdown and granular disintegration of the distal nerve stump following axonal injury (*8*, *9*). *Wld*^{s} is a fusion protein that has the enzymatic activity of nicotinamide mononucleotide adenylyltransferase (NMNAT) and a modified N-terminal sequence that results in the relocation of the protein to the cytoplasm of axons (*10–12*). NMNAT2 is actively transported from the cell body and catalyzes the formation of nicotinamide adenine dinucleotide (NAD+) from nicotinamide mononucleotide (NMN) (*13*, *14*). Once an axon is severed this transport is blocked and due it it's short half-life NMNAT2 rapidly degrades, resulting in decreasing NAD+ levels and rising NMN levels. This leads to axonal disintegration that can be prevented by the addition of exogenous NAD+ (*15*). It has been

proposed that *Wld*^{s} is able to protect axons due to its axonal localization and increased stability compared to NMNAT2, allowing the continued synthesis of NAD+, delaying Wallerian degeneration (*13*, *16*). Interestingly, there is evidence that degeneration can be promoted by the accumulation NMN as opposed to depletion of NAD+ as illustrated by the axonal protection when NMN-synthesizing enzyme inhibitors are used despite the downstream reduction in NAD+ (*14*).

Further advancement came through the discovery that an equivalently robust blockage of Wallerian degeneration could be achieved through the deletion of Toll-like receptor adaptor protein Sterile alpha and TIR motif containing 1 (SARM1)(*17*). This provided evidence of a mechanism of Wallerian degeneration that was driven by normal biology rather than the result of a mutated fusion protein. SARM1 deletion can also rescue the axonal degeneration resulting from loss of NMNAT2 without reducing NMN (*18*), suggesting SARM1 plays a role in previously known pathways discovered in *Wld*^{s} mice (*19*). SARM1 and NMN appear to be required for generating a flux of extracellular Ca^{2+} in response to injury, which could drive calcium dependent effectors suggested by (*2*).

The process of axonal degeneration likely plays a role on the modulation of Schwann cells following injury through a potential injury signal or loss of axonal signaling (*20*). In response to injury Schwann cells will rapidly switch their transcriptional program, differentiate from a myelinating state to a repair state, and produce cytokines for the mobilization of cells of the immune system for regeneration (*21–24*).

Recruitment of Macrophages to the site of Injury

Monocyte differentiation and extravasation begins when inflammatory signals induce gene expression programs that allow increase expression of adhesion molecules to promote the binding of endothelial cells by monocytes to begin rolling and extravasation (*25*). Correlating with the arrival of macrophages, the blood nerve barrier will become permeabilized to allow greater influx of hematogenous leukocytes into the distal nerve segment (*26*, *27*). Macrophages begin to infiltrate into the distal segment after 3 days, peak around 6 days, and persist in large numbers for at least 14 days (*28*).

To date several cytokines and chemokines have demonstrated a role in macrophage recruitment to the injured peripheral nerve, including Tumor Necrosis Factor α (TNFα), interleukin-1α (Il-1α), interleukin-1β (Il-1β), C-C motif chemokine ligand 2 (CCL2), leukemia inhibitory factor (LIF), and pancreatitis-associated protein III (PAP-III) (*21–24*, *29–31*). In experiments where these cytokines are disabled, macrophage recruitment is attenuated to varying degrees but the loss of any single molecule does not completely block recruitment (*22*). While the reduction of any cytokine or chemokine individually will not completely abrogate the recruitment of macrophages, it does illustrate that this is a robust response that is the result of mobilization through multiple pathways (*21*).

In addition to chemoattractant proteins produced by constituent cells in the nerve for the purpose of recruitment of hematogenous leukocytes, there is also evidence of humoral components of the immune system taking part in recruitment. Complement is a humoral component of the innate immune system that is able to opsonize and begin a cascade of proteolytic cleavage of complement proteins to act as chemoattractants and mediators of inflammation. Complement is able to recognize external and internal danger

signals in three ways. The cleavage cascade can start with the recognition of bound antibodies, recognition of mannose-binding lectins, and spontaneous binding and activation in the absence of inhibitory signals (*32*). In co-culture experiments with macrophages and degenerating nerves there is a reduction in the ability for macrophages to invade the nerve when complement is depleted (*33*). This reduced recruitment effect is also observed in vivo in mice that have been depleted of complement by cobra venom factor (*34*). There is also evidence that some of these receptors have functional overlap, playing a role in recruitment and phagocytosis. In addition to complement, the scavenger receptor may also play a role in the recruitment of macrophages as demonstrated when an anti-scavenger receptor antibody blocked recruitment of macrophages in a nerve explant co-culture experiment (*35*).

Observations like these highlight the interconnectedness of the inflammatory response, the various pathways involved, and the versatile nature of macrophages in this process. Macrophages are critical to the process of regeneration because when recruitment is attenuated there is reduced myelin debris clearance, axonal regeneration and functional recovery (*22*, *36*)**.**

Role of macrophages in cellular debris clearance

A critical component of peripheral nerve regeneration is the removal of myelin and axonal debris in the distal nerve segment. Not only is this debris a physical barrier to the regenerating nerve but the debris also contains inhibitory signaling molecules that further attenuate the regenerating axon (*37–40*). The bulk of this process takes within 20 days following injury in mouse models (*22*, *28*, *41*). Debris clearance largely occurs in two

phases, initially driven by endogenous Schwann cells and resident macrophages in the first 6 days and later by infiltrating hematogenous macrophages (*28*, *42–46*).

Upon arrival into the distal nerve stump, macrophages begin phagocytosis of axonal and myelin debris. This process is, in part, driven by traditional opsonizing elements of the humoral immune system. In a study using a mouse model that lacks mature B-cells and thus cannot produce antibodies, saw attenuated clearance of myelin debris. This effect was rescued by serum or purified IgG/IgM from a donor mouse. These antibody deficient mice were also observed to have less lysozyme expression and diminished phagocytic morphology (*28*). Complement has also been shown to be an important opsonin for macrophage phagocytosis in the peripheral nerve (*33*, *34*). In the absence of complement component 3 (C3), a protein critical for complement activation, macrophages that have already invaded degenerating nerves in co-culture, display a significant reduction in phagocytosis of myelin debris (*33*).

Beyond opsonin-dependent phagocytosis of myelin debris there is also evidence of the involvement of the versatile scavenger receptor in the mediation of phagocytosis (*35*, *47*). The scavenger receptor is functionally versatile as it plays a role phagocytosis through broad extracellular milieu ligand specificity and also plays a role in the recruitment of macrophages (*35*, *48*). In a sciatic nerve explant and peritoneal macrophage co-culture experiment in which an anti-scavenger receptor antibody was used, there was a significant decrease in myelin phagocytosis. Morphological data also corroborated this effect as significantly less pseudopodia and post-phagocytic myelin was observed in these macrophages (*35*). It is not clear if any other opsonin-independent pathways are involved in the phagocytosis of cellular debris by macrophages but TAM receptor mediated

phagocytosis has been observed in Schwann cells so similar mechanisms may be relevant to macrophages (*41*).

Neurotrophic functionality of Macrophages

Macrophages can contribute neurotrophic signaling to the regenerating nerve in a variety of ways. Conditioned media produced by macrophages that have phagocytosed myelin debris can increase survival and neurite quantity of dorsal root ganglia neurons in culture (*49*). Schwann cells have a reduced capacity for phagocytosis when macrophages are depleted, this may be evidence that macrophages provide important signaling to Schwann cells in order to get them to ramp up their debris clearance mechanisms (*41*).

Macrophages play a critical role in sensing the hypoxic environment cause by the disruption of blood vessels. Macrophages produce vascular endothelial growth factor (VEGF) in response to hypoxia, stimulating polarized angiogenesis of blood vessels that form a scaffold for Schwann cells to follow in order to reach the distal nerve segment following nerve transection (*50*).

Another cohort of macrophages will persist in the distal nerve segment for at least 6 weeks following injury and provide pro-regenerative signaling molecules to the regenerating axons and Schwann cells (*50*, *51*). These persisting macrophages aid in regulating the return of Schwann cells to a myelinating differentiation state to myelinate regenerating axons. Ablation of macrophages after a week post injury led to increased Schwann cell proliferation, a reduction in myelinated axons, and reduced recovery of compound motor action potential (CMAP) but had no effect on CMAP amplitude or axonal regrowth (*51*). An unbiased sequencing approach led to investigation of Gas6 as a potential

mediator of this phenotype and found that in a bone marrow transplant experiment with Gas6 KO mice that there was altered remyelination, specifically in regard to internodal length (*51*).

Macrophages are involved in a heavily regulated remodeling process of the extracellular matrix (ECM). This process involves delicate balance of enzymes involved in digestion of ECM components, known as matrix metalloproteinases (MMPs), and their inhibitory counterparts (*52*). The degenerating nerve basement membrane must be permeable enough to allow the influx of hematogenous macrophages but must remain intact to provide benefits to the regenerating axon. The basement membrane provides a niche in which the Schwann cells can proliferate and await the regenerating axon to remyelinate it (*52*, *53*).

Following injury, macrophages will not only recruit to the site of injury but they will also recruit and associate with injured neuronal cell bodies in dorsal root ganglia (DRG) (*54*). The DRG infiltrating macrophages will arrive after 48 hours and will persist for at least 28 days (*54–56*). In Ccr2 KO mice this infiltration is abolished which suggests that Ccl2 is critical for the recruitment of macrophages to DRGs containing cell bodies of injured axons (*55*). These macrophages seem to have neurotrophic effect, demonstrated when their recruitment to DRGs is blocked, there is attenuation the enhanced regenerative capacity of axons that had sustained a conditioning lesion in several models (*56*). A conditioning lesion will normally activate neuronal regeneration associated gene programs that will reduce the latency time for axonal regeneration if there is a second insult. Macrophages localized here also express CD206 which is an anti-inflammatory marker in

macrophages which may suggest that these DRG interfacing macrophages exhibit a pro-regeneration polarization state (*55*).

Beta Secretase

BACE1 is a well-studied enzyme in the field of Alzheimer's disease. However, this enzyme's promiscuous substrate specificity results in potential roles in multiple processes beyond the pathology of Alzheimer's disease. This enzyme is a transmembrane aspartyl protease that is a critical participant in the cleavage of Amyloid Precursor Protein (APP) and generation of Aβ plaques, one of the pathological hallmarks of Alzheimer's disease. Initially BACE1 will cleave APP on the luminal side of the cell membrane, releasing a large soluble ectodomain and leave behind the remaining truncated transmembrane and intracellular domains. This cleavage event is the rate limiting step in the generation of Aβ fragments. Subsequent cleavage by gamma secretase in the transmembrane domain will generate the Aβ fragment and APP intracellular domain (*57*). BACE1 substrates are mostly type 1 transmembrane proteins however, there are exceptions like St6gal, which is a type 2 transmembrane protein (*58*). Its broad substrate specificity has led to the discovery of over 60 known substrates of BACE1. Thus far BACE1 has been found to have the highest expression in brain and the pancreas, although they have high and low enzymatic activity in each, respectively (*59*). Expression is present in other peripheral tissues but only very low amounts. Genetic and pharmacological manipulation of BACE1 has revealed several phenotypes that are of particular interest to the process of peripheral nerve regeneration. Investigation of these phenotypes thus far has centered on the same principal components

of nerve regeneration, intrinsic neuronal regeneration capacity, inhibitory debris clearance, and pro-regeneration effects of Schwann cells.

BACE1 KO neurons have a greater capacity for regeneration than their wild type counterparts. While BACE1 KO mice do not experience shortened latency before axonal regeneration begins, they do grow more rapidly once regeneration begins after 2 days. This latency can be shortened with a conditioning lesion in BACE1 KO mice but it does not improve upon the conditioning lesion response seen in WT mice. By day 3 following a crush injury, BACE1 KO axons grow significantly further distally than WT and this difference increases over longer time points. Not only do the axons grow more rapidly, but there is also a greater number of regenerating axons in the BACE1 KO. This improvement in regeneration results in greater reinnevation of neuromuscular junctions (NMJs) as early as 10 days post injury (*1*). BACE1 also plays a role in the regulation of Schwann cells. In BACE1 KO mice, peripheral nerves are hypomyelinated which is likely due to dysregulation of neuregulin 1 type III, a known BACE1 substrate that plays a role in the myelination of axons (*60*, *61*). While reduced BACE1 activity has a pro-regenerative effect, the opposite is true of BACE1 overexpression. Overexpression of human BACE1 in neurons of transgenic mice results in decreased axonal regeneration, reduced NMJ reinnervation, and impaired functional recovery following crush injury (*62*). There was no effect on myelination observed when BACE1 is overexpressed in neurons (*62*).

In BACE1 KO mice there is an increase in macrophages that are recruited to the distal nerve segment following nerve injury. While these macrophages do not appear to arrive any earlier than expected, they do so in greater populations. In vivo morphological data suggests that BACE1 KO macrophages are more phagocytically active, as evidenced

by an increase in the number of macrophages with a myelin-laden foamy appearance. This increased phagocytosis is also observed in vitro when primary mouse intraperitoneal macrophages are exposed to mouse gamma globulin opsonized polystyrene beads. This increased in vitro phagocytosis may lead to increased axonal debris clearance in vivo, observed as diminished YFP fluorescence following nerve injury in a mouse line that expresses YFP in a subset of axons (*1*). It is unclear if these macrophages are able to clear cellular debris more efficiently than WT counterparts because BACE1 KO mouse axons are hypomyelinated, making direct comparison more challenging (*63*, *64*). The improved clearance of axonal debris may be the result of the hypomyelination phenotype leading to less debris to be cleared by macrophages overall. At this time it is unclear of whether BACE1 activity modulates macrophages directly or indirectly.

In order to begin to determine the mechanisms of this phenotype, a critical step is to determine what the consequences are of reduced BACE1 activity in the various cell types of the regenerating nerve microenvironment. A nerve grafting experiment was done where BACE1 KO or WT sciatic nerves were grafted into a recipient sciatic nerve, essentially flanking the donor graft both proximally and distally with host nerve tissue. Interestingly there was increased axonal growth when WT graft was placed into BACE1 KO host but not vice versa (*1*). This suggests that the BACE1 activity status of the recipient is more important than that of the donor. The likely cell types mediating this recipient effect are the neurons and/or macrophages. Neuronal cell bodies are located proximal to the graft tissue and macrophages are recruited from recipient circulation. The graft tissue illustrates the role of BACE1 activity on Schwann cells in this phenotype. This experiment suggests that BACE1 activity doesn't have a strong effect on Schwann cells in promoting a

permissive environment for regeneration (*1*). After 7 days post sciatic nerve transection Tumor Necrosis Factor Receptor 1 (TNFR1) expression was nearly doubled in sciatic nerves of BACE1 KO mice. This increased expression was also observed in mice that received BACE1 KO donor marrow in a bone marrow transplant experiment. At this time it is unclear whether this is an upregulation of TNFR1, the result of increased macrophage recruitment, or a combination of both due to the loss of BACE1 activity (*65*).

BACE1 Substrates with immunomodulatory potential

Interleukin 1 receptor 2 (Il1-R2)

Interleukin 1 is a potent and highly regulated pro-inflammatory cytokine. It exists as a family of ligands with agonist activity and receptor antagonists that bind to a family of receptors and accessory proteins as reviewd by (*66*). For the purposes of this review the focus will be on IL-1α and IL-1β ligands and their receptors. Both ligands are synthesized as pro-cytokines and must be cleaved in order to become more active and bind the Interleukin-1 receptor. However, the way each ligand is activated through cleavage and released from cells is markedly different (*67*). Il-1α and Il-1β both bind Il1-R1 which provides route of common regulation between the two ligands. Regulation of Il1-R1 occurs through the decoy receptor, Interleukin 1 receptor 2 (Il-1R2), and soluble receptor antagonist, Interleukin 1 receptor antagonist (Il-1RA). Despite the commonality of their regulatory components evidence suggests that Il-1α and Il-1β have different activities and effects (*67*, *68*).

Il-1α is a dual function cytokine with functionality in both its pro-form as a regulator of transcription and its cleaved form which is an inflammatory mediator (*69*). It

has intracellular activity as a transcriptional regulator in addition to being released from necrotic cells to act as a damage associated molecular pattern (DAMP) to cells bearing the Il-1 receptor (Il-1R1) and Interleukin-1 receptor accessory protein (Il-1RAcP) (*67*, *70*, *71*). However, Il-1α is sequestered in nuclear foci under apoptotic conditions instead of being released into the milieu where it can induce an inflammatory response. When apoptotic cells are placed in matrigel, there is no recruitment of myeloid cells. In contrast, where robust infiltration of myeloid cells is observed when necrotic cells were embedded in matrigel (*68*, *69*). Il-1α can induce sterile inflammation while in the pro-form but its potency is increased by proteolytic processing. Its affinity for Il-1R1 can be increased by proteolytic cleavage into its mature form by leukocyte derived enzymes in the extracellular milieu such as granzyme B, chymase and neutrophil elastase (*72*, *73*). If there is sufficient Ca^{2+} flux in a cell as a result of signaling processes or cell membrane damage, Il-1α can be cleaved by intracellular calpain into its active form (*67*, *74*, *75*). Mature Il-1α can induce a range of responses in different cells types including leukocyte recruitment, maturation, differentiation, proliferation, and inflammasome priming (*66*).

Unlike Il-1α, Il-1β is not constitutively expressed and requires a priming signal for the cell to begin producing it to further the inflammatory response. Pro-Il-1β is expressed in macrophages in response to a priming signal in the form of Il-1α and other NF-κβ activating pathways, such as TLRs or TNFR (*76*, *77*). Once the cell receives a second activating signal, pro-Il-1β is cleaved into its active form by either Caspase-1 or Caspase-8. Caspase-1 dependent pro-Il-1β cleavage is the result of a signal that triggers the assembly of the inflammasome, which cleaves pro-Caspase-1 into its active form that results in cleavage, maturation, and release of pro-Il-1β from the cell (*67*, *68*, *78*).

Il-1R2 is a decoy receptor for both Il-1α and Il-1β that can negatively regulate Il-1 signaling in both cells expressing this decoy receptor and cells exposed to the secreted (sIl-1R2) or shed shIl-1R2) forms of the receptor. When located on the plasma membrane Il1-R2 will complex with Il-1RAcP and act as a non-signaling binding partner for Il-1 at the cell surface. This relationship with Il-1RAcP also allows Il-1R2 to act as a sink for the necessary accessory protein for Il-1R1 and further negatively regulate Il-1 signaling (*79*, *80*). A cytosolic form of Il-1R2 (icIl1-R2) can also bind Il-1α intracellularly and prevent maturation of Il-1α (*81*). In addition to these forms of Il-1 cis-regulation, there is also trans-regulation mediated by the shed and secreted soluble forms, that can bind Il-1 before it can bind Il-1R1. Il-1R2 is primarily expressed on leukocytes but can be induced on other cell types such as keratinocytes and endothelial cells (*80*, *82*, *83*).

Although Il-1 is a heavily regulated signaling pathway, cells have ways to get around Il-R2 in certain circumstances. The soluble form of Il-1R2 can be cleaved by caspase-1 after activation of the inflammasome (*81*). This cleavage by caspase-1 attenuates the ability of icIl-1R2 to bind Il-1α, which can restore its ability to act as a DAMP. Membrane bound Il-1R2 can be cleaved into shIl-1R2 by different "sheddases" such as ADAM17 or BACE1 (*58*, *84*, *85*). This makes sheddases very important components to this regulatory system as they not only serve to restore cell membrane Il-1R/Il-1RAcP function, but also may be important for generating shIl-1R2 which can bind extracellular Il-1α and Il-β attenuate signaling. This opens the possibility of a context dependent pleotropic role for sheddases, like BACE1, in the regulation of the Il-1 signaling pathway could have implications for the immunomodulatory phenotype observed in the injured peripheral nerve of BACE1 KO mice.

In the context of sterile inflammation both Il-1 ligands play significant roles. Il-1α is most influential as a DAMP at the initiation of inflammation. Il-1β is more important in its role of macrophage recruitment and phagocytic activity (*22*). Further, in the context of hypoxic sterile inflammation Il-1α is released from dying cells, recruits neutrophils to the site, and Il-1β recruits and retains macrophages (*68*). Similarly, following nerve injury the blood vessels are disrupted and cells are capable of sensing the hypoxic environment and altering their expression profile accordingly (*50*). The nature of macrophages in peripheral nerve regeneration appears to be pleotrophic, perhaps reflecting the waves of cytokines and cell types associated with injury. An acute highly regulated inflammatory response followed by a shift to an anti-inflammatory pro-regenerative phenotype (*86*).

Il-1 plays an important role in peripheral nerve regeneration. Following an injury of the peripheral nerve there is rapid and robust induction of IL-1β expression in a small population of cells near the site of injury (*22*). This change occurs after one hour which is nearly 12 hours prior to arrival of neutrophils and later hematogenous macrophages. Given the timing of this expression change it is unlikely to be hematogenous leukocytes, although there is a second peak of Il-1β expression at 14 days that is likely due to influx of hematogenous macrophages (*22*). It is possible that these Il-1β generating cells could be a subset of Schwann cells or fibroblasts, however, macrophages are known to express Il-1β in response to inflammatory stimuli such as Il-1α or other endogenous molecules released into extracellular space. Resident macrophages account for approximately 10% of all nucleated cells in uninjured nerves (*45*, *87*, *88*) so the sparsity of cells that are heavily expressing Il-1β would make these cells a likely source, although expression of Il-1β has also been observed in Schwann cells following injury (*89*). Il-1α is constitutively expressed

in the nerve so it would be present to serve as a DAMP to prime Il-1β expression following injury. Mature Il-1β levels begin to increase after 6 hours, a two fold increase, which increases further to 10 fold by 24 hours (*23*, *89*). This maturation mirrors the processing of pro-caspase, which suggests that this is mediated by activation of the inflammasome. Mice that have defects of Il-1 signaling have impaired functional recovery compared to WT, which suggests that there is benefit to this inflammatory signaling (*23*). On the other hand, Il-1β also plays a role in neuropathic pain as the result of inflammation. Il-1β/TNFα double knockout mice have reduced mechanical allodynia but also display evidence of impaired functional recovery (*23*). However, depletion of neutrophils, as opposed to blockage of cytokine signaling, improved neuropathic pain thresholds and restored functional recovery to WT levels (*23*).

Il-1R2 has been extensively studied in the context of the immune system and inflammation. BACE1 cleavage of IL1-R2 could influence many parts of this signaling pathway. BACE1 activity could boost the trans regulatory effect of Il1-R2 on adjacent cells through shedding of Il1-R2 into the extracellular milieu. The opposite could be true of its cis regulatory activity. Reduced BACE1 expression could lead to more Il-R2 remaining on the cell surface to block that cell's ability to respond to Il-1 and sinking Il-1RAcP. It is not fully understood how icIL1-R2 is generated so perhaps BACE1 may play a role, given its ability to generate shIL1-R2. Increased levels of icIL-1R2 could interfere with the calpain mediated maturation of Il-1α. This would reduce the ability of cells experiencing calcium flux to be able to use Il-1α to act as a DAMP and have downstream consequences for the inflammatory process as a whole. Decreasing BACE1 expression could reduce the amount of available icIl-1R2 and promote a more robust inflammatory response to injury. This

pathway is heavily regulated and has many redundancies that would limit the ability of BACE1 to have a dramatic impact on any single regulatory site, but perhaps it can influence the pathway in several different regulatory sites and drive the overall response in a pro-regenerative direction by leveraging the benefits of the inflammatory response, while minimizing its detrimental effects.

Optimal regeneration seems to be heavily reliant on very tight regulation of the inflammatory response following injury. If there is not enough of an inflammatory response then there is attenuated immune cell recruitment for trophic signaling and debris clearance. However, if the inflammation response is too great then there can be deleterious effects of this such as the development of neuropathic pain (*23*). Any alterations of the immune response for therapies will require a breadth of knowledge of how the various cell types and pathways are interconnected and influence one another to maximize the balance of repair and pain mitigation.

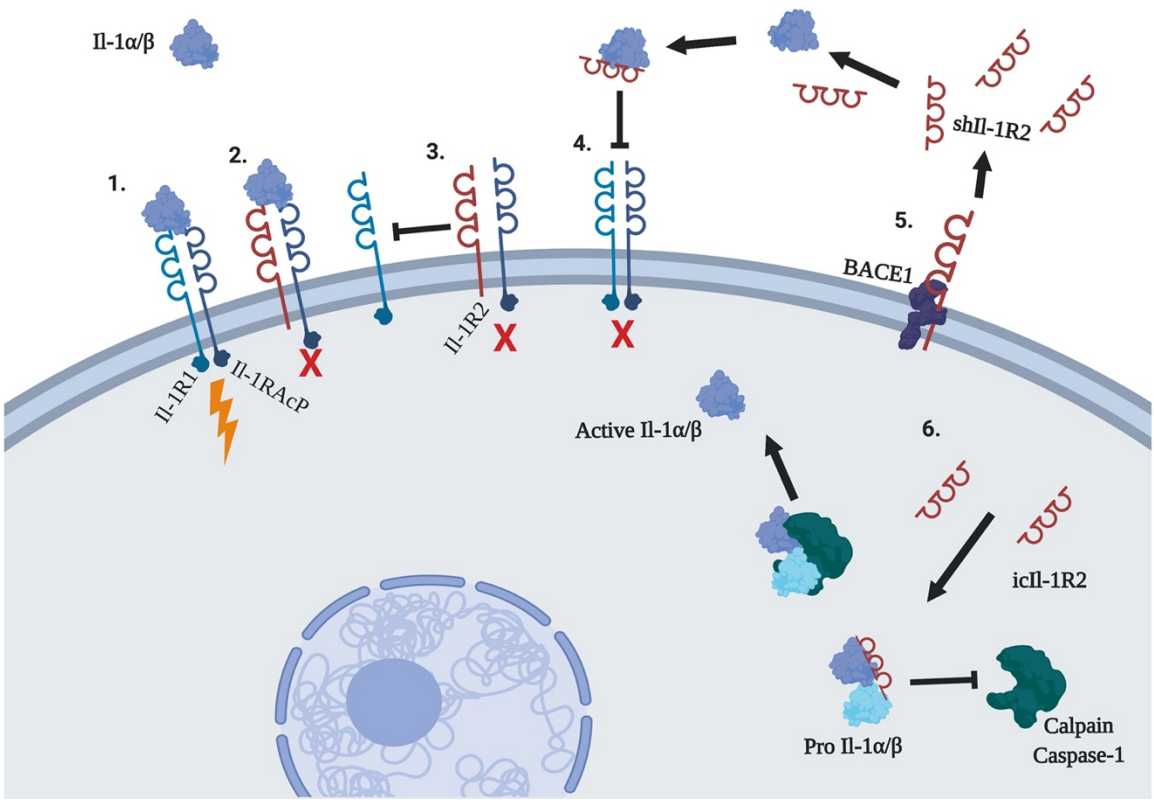


Fig 2. Possible regulatory mechanisms of IL-1 by BACE1 activity

Il1-R2 regulation of Il-1 signaling. (1) Il-1α or Il-1β binds Il1-R1 and Il-RAcP resulting in normal signaling. (2) Il1-R2 binds Il-1RAcp and either Il-1α or Il-1β but does not result in signaling. (3) Il1-R2 can regulate Il-1 signaling by binding Il-1RAcP and which leaves it unable to complex with Il-1R1 for signaling. (4) Il1-R2 can be secreted or shed by cells and can bind Il-1α and Il-1β extracellularly and prevent them from binding Il1-R1/Il-1RAcP. (5) BACE1 is one of a few enzymes capable of cleaving Il-1R2. BACE1 activity could influence the relative amounts of Il-R2 present in the cell membrane or shed into extracellular space. (6) Intracellular Il-1R2 can bind either pro-Il-1α and pro-Il-1β and prevent cleavage by calpain or caspase-1, respectively, for activation.

Jagged1 (Jag1)

Notch is a transmembrane protein that can be activated through ligand binding of an adjacent cell. This binding results in a conformational change in Notch that makes it more vulnerable to a series of proteolytic cleavages that release the Notch intracellular domain (NICD) into the cytoplasm where it subsequently relocates to the nucleus and activates target genes for transcription with the help of other coactivating proteins (*90*). This pathway does not have a mechanism for amplification so the strength of the signal is the result of the abundance of Notch receptors on the receiving cell and the amount of ligand on the sending cell. The ratio of Notch to ligand can also be an important part of the pathway's regulation. Cis inhibition of Notch occurs when a cell is expressing sufficient amounts of the ligand on its surface and that ligand binds to Notch instead the ligand from an adjacent cell (*90*, *91*). As a result of cis inhibition the sending/receiving state of the cell can be largely determined by this receptor to ligand ratio. For example if the amount of Notch receptor is greater than the amount of ligand then that cell is likely to be a receiver, the opposite is true of cells with more ligand. Ligand binding efficiency can also provide another layer of regulation to the Notch pathway (*92*). Jag1 has a relatively low binding affinity Notch, yet can render a cell unresponsive to other Notch ligands with greater binding affinity, adding an additional layer of complexity to this pathway (*93*).

BACE1 has also been shown to have a role in regulation of the Notch signaling pathway via cleavage and shedding of Jag1 (*94*). In BACE1 KO mice there is an abundance of full length Jag1 in the hippocampus. This abundant Jag1 ligand results in more activated Notch that is detected via increased NCID in this region of the brain. In vitro overexpression of BACE1 causes a decrease in the amount of full length Jag1 and this

effect is reversed when cells are treated with a BACE inhibitor. BACE1 demonstrates specificity for Jag1 as it is unable to efficiently cleave its homolog Jag2. These studies suggest that BACE1 is a regulator of Notch signaling via cleavage of its substrate Jag1 (*94*, *95*). The increased Jag1 in BACE1 KO mice causes a shift in the balance of notch and ligand resulting in a shift towards astrogenesis and away from neurogenesis in the developing brain. There is also evidence of BACE1 regulation of Notch signaling playing a role in the peripheral nerve. As mentioned previously, BACE1 KO mice have hypomyelinated axons due to the loss of BACE1 dependent neuregulin 1 signaling. Notch has been shown to enhance the sensitivity of Schwann cells to NRG-1 (*96*). In addition Schwann cells show increased proliferation in BACE1 KO mice (*97*) which suggests that it could be the result of increased Notch signaling due to the loss of BACE1 regulation of Jag1. BACE1 KO mice have increased full length Jag1 and Delta1 and downstream NICD in sciatic nerves compared to WT. The addition of another Notch ligand, Delta1, as a possible BACE1 substrate adds to the impact that BACE1 can have on the Notch pathway (*97*).

In addition to development and cell fate decisions, Notch signaling plays a role in the activity of macrophages (*98–101*). Macrophages typically express the Notch receptor relying on cells they interface with to provide ligand stimulation. Inflammatory cytokines and several TLR agonists have been shown to increase the expression of Notch in macrophages (*98*). In the context of wound healing the expression of Notch by infiltrating macrophages is dynamic and can increase 5 fold 3 days after sustaining a skin wound (*99*). This increase in Notch expression is accompanied by an increase in inflammatory markers and this inflammation is beneficial for wound healing early on in the healing process.

Diabetic mice exhibit poor wound healing in the later stages and have sustained high expression of inflammatory markers. Reducing Notch signaling in these diabetic mice improves their late wound healing, suggesting that inflammatory response is important early in the healing process but can be deleterious if it persists (*99*). This paradigm is similar between wound healing and nerve regeneration. Both are marked by an early inflammatory phase where debris is cleared and more inflammatory cells are recruited, followed by a later phase characterized by growth factor release and the resolution of inflammation.

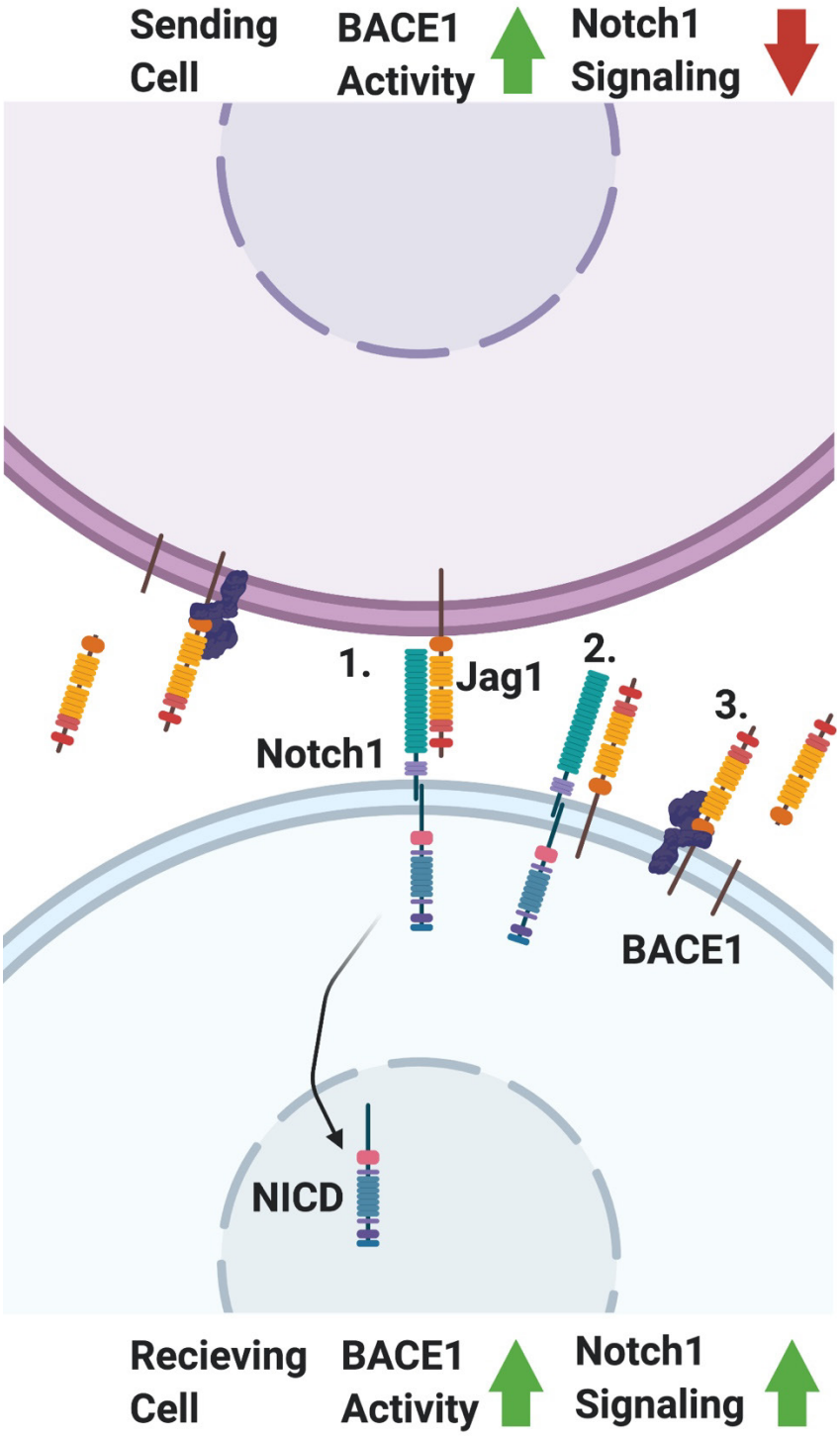
Notch signaling plays a role in modulating the inflammatory response of macrophages in a variety of ways. Notch signaling has been shown to increase the translocation of NFκβ to the nucleus where it can interact with NICD to boost its ability to act a transcriptional cofactor and boost the inflammatory response (*98*). Cells that are transfected with NICD show increased NFκβ activation, particularly when in conjunction of an inflammatory stimulus such as LPS, which is an agonist of TLR4 which is one of the receptors upstream in the pathway. In an in vitro Notch activation assay, extracellular domains of Notch ligands Delta4, Jag1, and Delta1 were adhered to culture plates and macrophages were then introduced. These Notch ligands were shown to increase the expression of a downstream reporter gene in these macrophages. In another experiment macrophages that were transfected with a robust repressor of NFκβ signaling, SR-Iκβα, were unable to activate the pathway, even in the presence of NCID. This suggest that Notch signaling is not activating a parallel pathway but impacting NFκβ and facilitating crosstalk between the two pathways (*98*). Notch also influences the phagocytic activity of macrophages by reducing the expression of SIRPα. SIRPα expressed by macrophages,

dendritic cells, and some neurons and binds CD47 to block phagocytosis. This serves as a "don't eat me" signal from host cells. The reduction of SIRPα results in loss of this signal and increases the phagocytic activity of macrophages. SIRPα activity also can attenuate the ability of macrophages to respond to inflammatory stimuli involved in polarization of macrophages toward an inflammatory phenotype. This suggests that SIRPα activity is not only important for phagocytosis but also in more broad mediation of the inflammatory response (*100*).

Given the abundance of Notch receptors and Notch ligands, it is unlikely that BACE1 would be able to have absolute regulatory control of any of these effects, but rather it could be a crucial mediator that helps tip the balance of signaling in the direction towards a pro-regenerative phenotype in the peripheral nerve.

Fig 3. Possible regulatory mechanisms of Notch signaling by BACE1 cleavage of Jag1

Jagged-1/Notch signaling and regulation in the context of BACE1 activity. A cell that is sending (Top) a Notch signal to a receiving cell (Bottom) via notch ligand Jag1. (1) Notch/Jag1 binding causes a conformational change in Notch1 which results in subsequent cleavages resulting in the release of the intracellular domain of Notch (NCID) to relocate to the nucleus and activate downstream signaling. (2) Notch signaling can be regulated by binding Jag1 on the surface of the receiving cell. (3) BACE1 can regulate Notch1 signaling via the cleavage of Jag1. This cleavage can have different effects depending on whether it is taking place on a receiving cell or sending cell. In the sending cell, BACE1 activity results in reduced Jag1 available for signaling. In the receiving cell BACE1 activity results in increased Notch1 signaling by reducing the amount of cis-regulating Jag1.


Sending Cell
BACE1 Activity
Notch1 Signaling
1.
Jag1
2.
3.
Notch1
BACE1
NICD
Recieving Cell
BACE1 Activity
Notch1 Signaling

ST6 Beta-Galactoside Alpha-2,6-Sialyltransferase 1 (ST6Gal-1)

ST6gal-1 is a sialyltransferase enzyme that plays a role in the post translational modification of proteins and modification of glycolipids by catalyzing α2,6 sialylation of N-glycans (*102–104*). While this enzyme is expressed in detectable amounts in nearly every tissue at a basal level, liver cells can rapidly upregulate expression of ST6gal-1 during the inflammatory acute phase response (*102*). In addition to the increased expression driven by the inflammatory response there is also a proteolytic cleavage and release of ST6gal-1 into the serum (*102*). While this cleavage is likely a form of regulation, there is also evidence that certain cleaved forms of ST6gal-1 retain enzymatic activity. In a study of the sialylation of IgG in B cells (*105*) showed that when B cell ST6gal-1 was selectively ablated, there was still ST6gal-1 dependent sialylation of IgG in serum. (*103*) characterized an E41 cleaved form of secreted ST6gal-1 that was able to bind to an affinity column used to purify sialyltransferases, suggesting that this form retains its activity.

BACE1 has been shown to cleave ST6gal-1 in vitro and in a study of BACE1 KO mice there was a decrease in serum ST6gal-1 compared to WT. The opposite effect was observed in mice that overexpress human BACE1, which showed an increase in serum ST6gal-1 (*103*). BACE1 expression and serum ST6gal-1 levels were also shown to be increased in a rat model of hepatitis, supporting earlier studies regarding hepatic inflammation playing a role (*102*). BACE1 cleavage of ST6gal-1 results in the generation of an enzymatically active form of ST6gal-1, E41 (*103*).

BACE1 appears to play a role in the regulation of sialylation through its cleavage of ST6gal-1. This can have a range of immunomodulatory outcomes relevant to the differentiation and recruitment of macrophages to the site of injury (*25*, *106*). The current

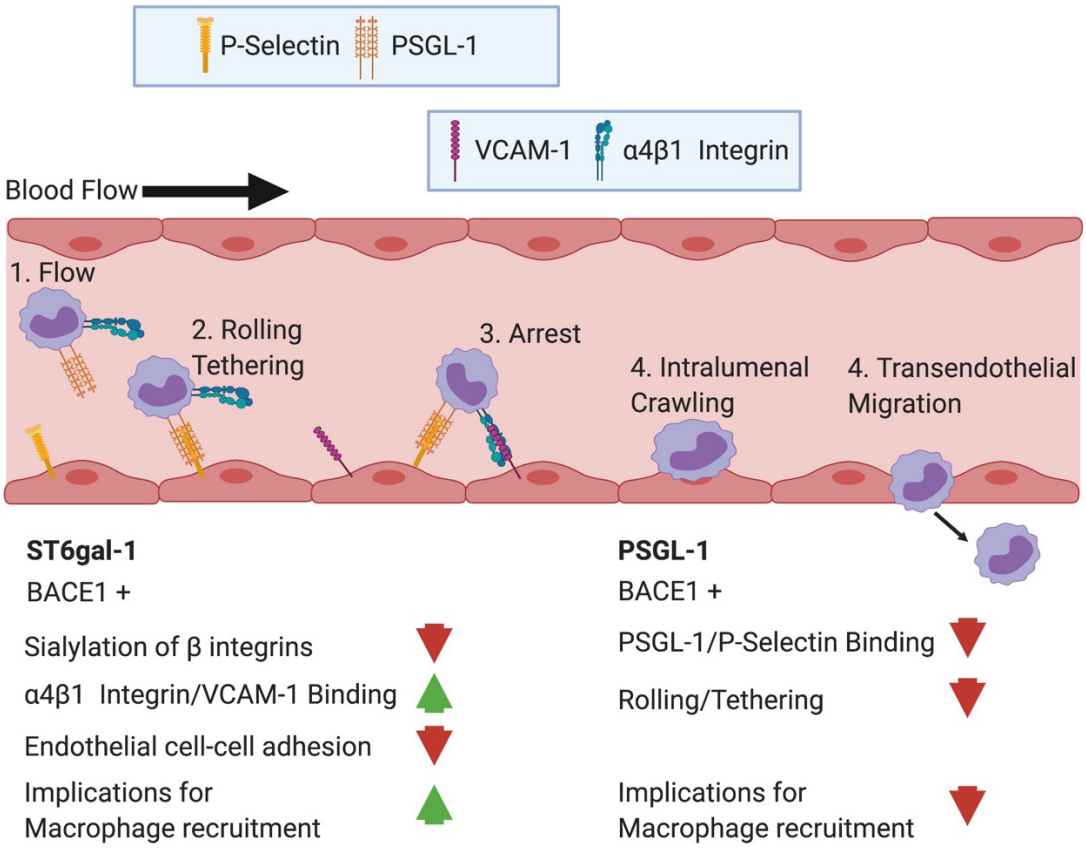
literature suggests that in the context of ST6gal-1, BACE1 activity is beneficial for monocyte recruitment through enhancing macrophage/endothelial cell adhesion and by promoting extravasation by increasing endothelial cell-cell adhesion (*25*, *106*). In a study of the monocyte cell line, U937, cells showed increased binding of VCAM-1 coated plates in a monocyte α4β1 integrin dependent manner following PMA mediated differentiation. This binding interaction of α4β1 on monocytes to endothelial VCAM-1 is important for the trafficking of monocytes to tissues following an inflammatory response. Sialylation of β1 integrins has been shown to be key regulator of the adhesion reaction between these adhesion molecules and VCAM-1 and fibronectin. Hyposialylated α4β1 shows increased binding affinity for VCAM-1. Upon differentiation of monocytes by PMA there is an increased expression of BACE1 associated with macrophage activation via protein kinase C (PKC) pathway. This increased expression of BACE1 leads to increased cleavage of ST6gal-1 and hyposialylation of α4β1. This hyposialylation and subsequent increased binding affinity correlates temporally with the activation of PKC and can be reversed when cells are treated with a BACE inhibitor (*25*). A different study by (*106*) highlighted the connection between the inflammatory response, BACE1 expression, and the modulation of cell adhesion. Treating a human monocyte cell line, THP-1 cells, with exogenous TNFα resulted in increased cell adhesion with endothelial cells in co-culture experiments. TNFα also caused a reduction in tight junction stability that was observed morphologically via electron microscopy in addition to reduced immunofluorescent VE-Cadherin staining. BACE1 expression increased in response to TNFα which in turn cause a global reduction in 2, 6 sialylation levels as observed by lectin staining and flow cytometry. This reduction

in sialylation compromised endothelial cell-cell adhesion and enhanced monocyte adhesion to endothelial cells (*106*).

Evidence for BACE1 expression being upregulated by the inflammatory response is building as both of these experiments suggest this to be the case. While macrophage trafficking seems to be enhanced by BACE1 cleavage and downregulation ST6gal-1, another component of monocyte trafficking may be enhanced by deletion of BACE1.

Fig 4. Possible mechanisms of BACE1 regulation of ST6gal-1 and PSGL-1

Potential implications of BACE1 cleavage of ST6gal-1 and PSGL-1 on macrophage recruitment. The binding cascade leading to transendothelial migration and recruitment of leukocytes, in this case monocytes, begins with binding interactions between PSGL-1 and P-selection. These adhesion molecules disrupt flow of monocytes through the blood stream (1) by initiating the rolling and tethering to endothelial cells (2). This binding progresses until the arrest of the monocyte (3) with the addition of VCAM-1 and Integrin binding. At this point VCAM-1/Integrin binding takes over and plays a critical role through transendothelial migration (4). BACE1 cleaves ST6gal-1 resulting in decreased sialyation of $\alpha 4\beta 1$ integrin, which increases it's binding affinity to VCAM-1. Further, this hyposialyation leads to decreased endothelial cell-cell adhesion. This combination of these effects would like provide conditions more permissive to macrophage recruitment. Conversely, BACE1 cleavage of PSGL-1 would reduce the amount PSGL-1 available on the cell surface to bind with P-selectin. This would likely reduce the frequency of the initial binding reaction at the start of this cascade and thus BACE1 activity would likely attenuate macrophage recruitment.


P-Selectin
PSGL-1
VCAM-1
α4β1 Integrin
Blood Flow
1. Flow
2. Rolling Tethering
3. Arrest
4. Intralumenal Crawling
4. Transendothelial Migration
ST6gal-1
BACE1 +
Sialylation of β integrins
α4β1 Integrin/VCAM-1 Binding
Endothelial cell-cell adhesion
Implications for Macrophage recruitment
PSGL-1
BACE1 +
PSGL-1/P-Selectin Binding
Rolling/Tethering
Implications for Macrophage recruitment

P-Selectin Glycoprotein Ligand-1 (PSGL-1)

P-selectin is constitutively expressed by endothelial cells and leukocytes, including monocytes, express PSGL-1. Transient binding interactions between p-selectin and its ligand PSGL-1 are critical for the initial rolling and tethering of monocytes to endothelial cells during recruitment to the injured nerve (*107*). While these binding interactions are short in duration, they result in the reduced velocities of leukocytes in the blood stream. This reduced velocity facilitates increased opportunity for increasing interactions between leukocytes and endothelial cells and chemokine signaling. During the rolling phase there is increasing integrin binding between endothelial cells and monocytes, which progressively slows the monocyte leads to arrest and extravasation. This cascade of adhesion and signaling interactions results in the activation, maturation, and trafficking of macrophages (*107*).

P-selectin has been shown to play a role in the recruitment of neutrophils and macrophages to the distal segments of nerves that had sustained partial sciatic nerve ligation (*108*). P-selectin KO mice had diminished recruitment to the injury site and a reduction of inflammatory cytokines TNFα, IL-1β, and IL-6 (*108*). This could be the result of downstream effects of inflammatory cytokines produced by cells populating the injury site, as it has been shown that IL-1 signaling results in increased expression of P-selectin by endothelial cells in mice. However, this effect has not been observed in humans.

BACE1 has been shown to cleave PSGL-1 in cell lines expressing endogenous or transfected BACE1 and no cleavage was observed in primary cells derived from BACE1 KO mice (*109*). The increased macrophage recruitment in BACE1 KO mice could be the

result of reduced cleavage of PSGL-1 on the surfaces of endothelial cells and monocytes, increasing the number of binding interactions to begin rolling and tethering.

Concluding remarks of the introduction

Regeneration following peripheral nerve injury is slow but given the critical role macrophages play in regeneration, immunomodulation of these cells may present an opportunity improve recovery and reduce morbidity associated with nerve injury. The substrate promiscuity of BACE1 could promote robust immunomodulation by acting on many pathways to alter the interactions of the various cell types in the peripheral nerve microenvironment. Further study is necessary to determine the relative contributions of each cell type in the peripheral nerve microenvironment for mediating the enhanced macrophage recruitment, particularly the phenotype observed in global BACE1 KO mice.

Chapter 2: Macrophage-specific deletion of BACE1 does not enhance macrophage recruitment to the injured peripheral nerve

Introduction to chapter 2

The following was published in J.A. Fissel and M.H. Farah, Macrophage-specific deletion of BACE1 does not enhance macrophage recruitment to the injured peripheral nerve. J. Neuroimmunol 349, 577423 (2020).

Following peripheral nerve injury, an orchestration of several different cell types contributes to the regeneration of the peripheral nerve. Initially, axons in the distal nerve segment degrade into amorphous debris (*110*). Next, myelin begins to break down and Schwann cells undergo a rapid transcriptional overhaul, modify their differentiation from a myelinating state to a repair state (*111–113*), and begin to release cytokines and chemokines to mobilize cells of the immune system for regeneration (*21*, *22*, *23*, *24*, *31*). Hematogenous macrophages are recruited to the distal nerve segment by cytokine and chemokine signaling to clear cellular debris (*21–24*, *29–31*). This clearance is critical to remove a physical barrier to regenerating axons (*114*) and to eliminate the inhibitory effects of this debris (*37–40*). In addition to debris clearance, macrophages produce neurotrophic factors that further contribute to shaping a microenvironment conducive to axonal regeneration (*41*, *49–51*).

Increased recruitment of macrophages and subsequent cellular debris clearance has been observed in the injured nerves of mice with genetic deletion or pharmacological inhibition of β-secretase (BACE1) (*1*). Currently, little is known about the underlying cellular mechanism of this enhanced recruitment and debris clearance. Neurons, Schwann cells, fibroblasts, and hematogenous infiltrating macrophages are the major cell types that comprise the microenvironment of the injured peripheral nerve and could be mediating this phenotype, but the role BACE1 expression plays in each cell type is unknown. Assessing the role of macrophages in the global BACE1 knockout (KO) mouse model has presented challenges due to the hypomyelination phenotype in these mice (*1*, *63*, *64*). This reduced myelination not only obfuscates analysis of myelin debris clearance in vivo, but also presents an alternative explanation that the enhanced recruitment and debris clearance observed in global BACE1 KO macrophages could be attributed to less myelin debris at baseline. Given that the hypomyelination phenotype is the result of signaling between axons and Schwann cells (*60*, *61*), macrophage-specific deletion of BACE1 is unlikely to result in hypomyelination and presents the opportunity to assess the relative contribution of macrophages towards the enhanced recruitment and debris clearance observed in global BACE1 KO mice. Here, we crossed a floxed *Bace1* mouse and a mouse possessing Cre recombinase under control of *Lyz2* promoter/enhancer elements to obtain a conditional knockout (cKO) mouse with a macrophage-specific deletion of Bace1 in order to investigate the potential role of macrophage-derived BACE1 in mediating these phenotypes.

Results

The following was published in J.A. Fissel and M.H. Farah, Macrophage-specific deletion of BACE1 does not enhance macrophage recruitment to the injured peripheral nerve. J Neuroimmunol 349, 577423 (2020).

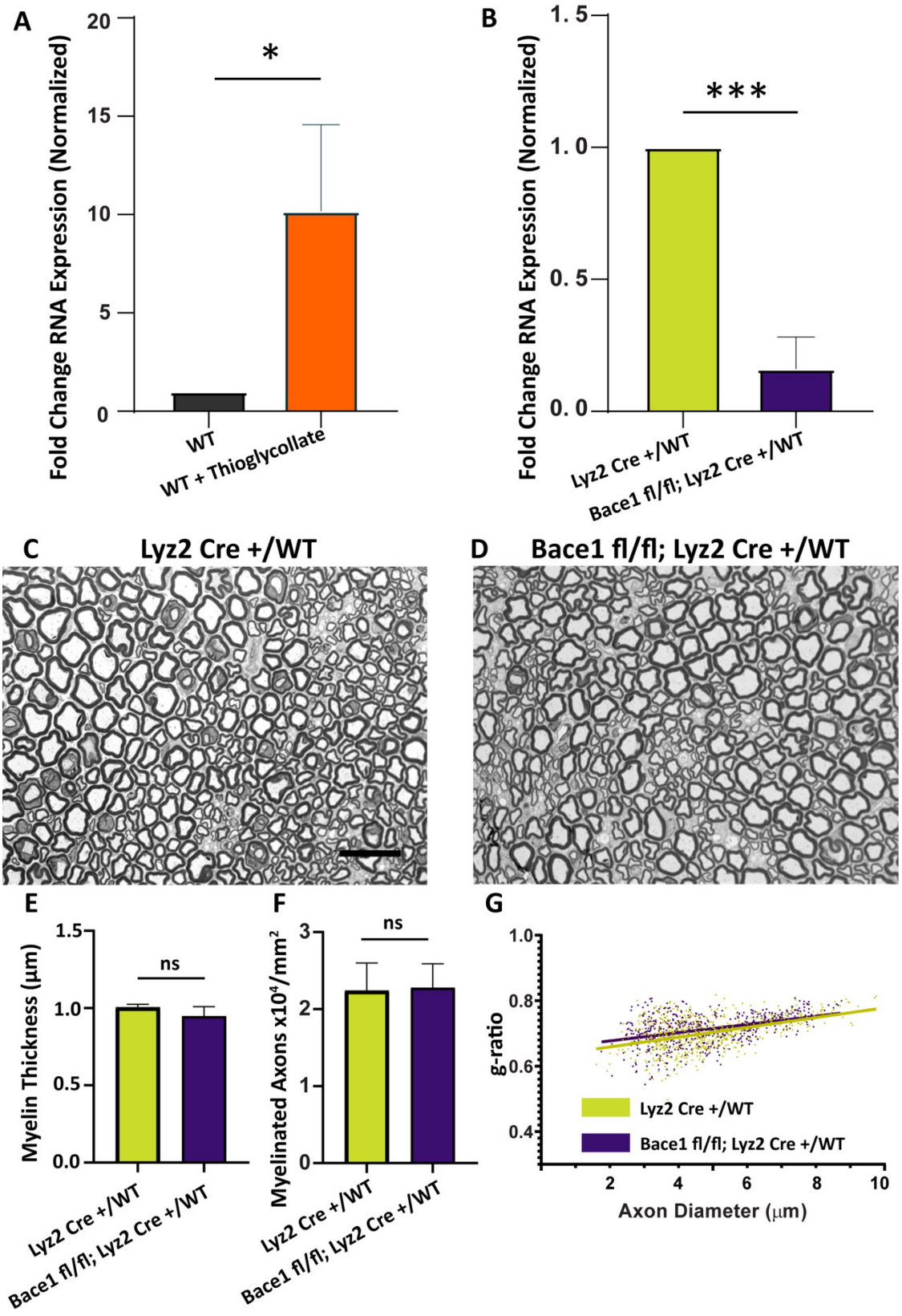
BACE1 expression and activity is relatively low outside of the nervous system (*115–117*). However, considering previously published observations of improved recruitment and debris clearance by macrophages in injured peripheral nerves of global BACE1 KO mice, we sought to determine if BACE1 expression by macrophages increases in inflammatory contexts. To that end, we measured BACE1 expression in thioglycollate-activated murine peritoneal macrophages and observed an approximately ten-fold increase of BACE1 expression in macrophages that were activated by thioglycollate compared to non-thioglycollate treated controls ($p = 0.0225$) **(Figure 1A**). Considering that BACE1 expression increases in activated macrophages, we moved to assess potential effects of macrophage-specific deletion of BACE1 on macrophage recruitment following peripheral nerve injury.

In order to ascertain the relative contribution of BACE1 expression by macrophages on enhanced macrophage recruitment and debris clearance phenotype, we sought to determine if macrophage-specific deletion of BACE1 is sufficient to recapitulate the modulation of macrophages observed in global BACE1 KO mice. The initial step in the characterization of cKO mice was to determine if there was successful deletion of BACE1 in macrophages by assessing the expression of BACE1 in peritoneal

macrophages harvested from macrophage-specific BACE1 cKO mice. Using RT-PCR we measured the expression of BACE1 in peritoneal macrophages from both cKO and heterozygous Cre expressing control mice and observed a significant reduction of approximately 80% in the cKO macrophages ($p = 0.0010$) **(Figure 1B)**. We further characterized the nerves of cKO mice to determine if normal myelination of axons occurs. Given that the hypomyelination phenotype observed in global BACE1 is the result of signaling between axons and Schwann cells, we anticipated normal levels of myelination, and as expected, there was no difference in myelination between our cKO mice and Cre expressing controls as assessed by myelin thickness ($p = 0.1962$), myelinated axon density ($p = 0.8970$), and g-ratio ($p = 0.2985$) **(Figure 1C-G)**.

Fig. 5. BACE1 expression in peritoneal macrophages increases under inflammatory conditions, is significantly reduced in Bace1$^{fl/fl}$ Lyz2 Cre $^{+/WT}$ macrophages, and has no effect on myelination in cKO mice.

Peritoneal macrophages that are activated with thioglycollate have significantly higher expression of BACE1 mRNA compared to peritoneal macrophages that were not treated with thioglycollate (A). BACE1 mRNA expression is significantly reduced in peritoneal macrophages harvested from Bace1$^{fl/fl}$;Lyz2 Cre $^{+/WT}$ mice relative to Lyz2 Cre $^{+/WT}$ control mice (B). N = 3 independent experiments with 3 mice for each condition/genotype, *p = 0.0225 (A), ***p = 0.0010 (B). Cross sections of uninjured sciatic nerves of Lyz2 Cre$^{+/WT}$ (C) and Bace1$^{fl/fl}$;Lyz2 Cre $^{+/-}$ (D) have comparable levels of myelinated axons. There is no significant difference in myelin thickness, p = 0.1962, (E), myelinated axon density, p = 0.8970, (F), or g-ratio, p = 0.2985, (G), (n=3). Scale bar = 20μm for (C) and (D). *J.A. Fissel and M.H. Farah, Macrophage-specific deletion of BACE1 does not enhance macrophage recruitment to the injured peripheral nerve. J Neuroimmunol 349, 577423 (2020).*

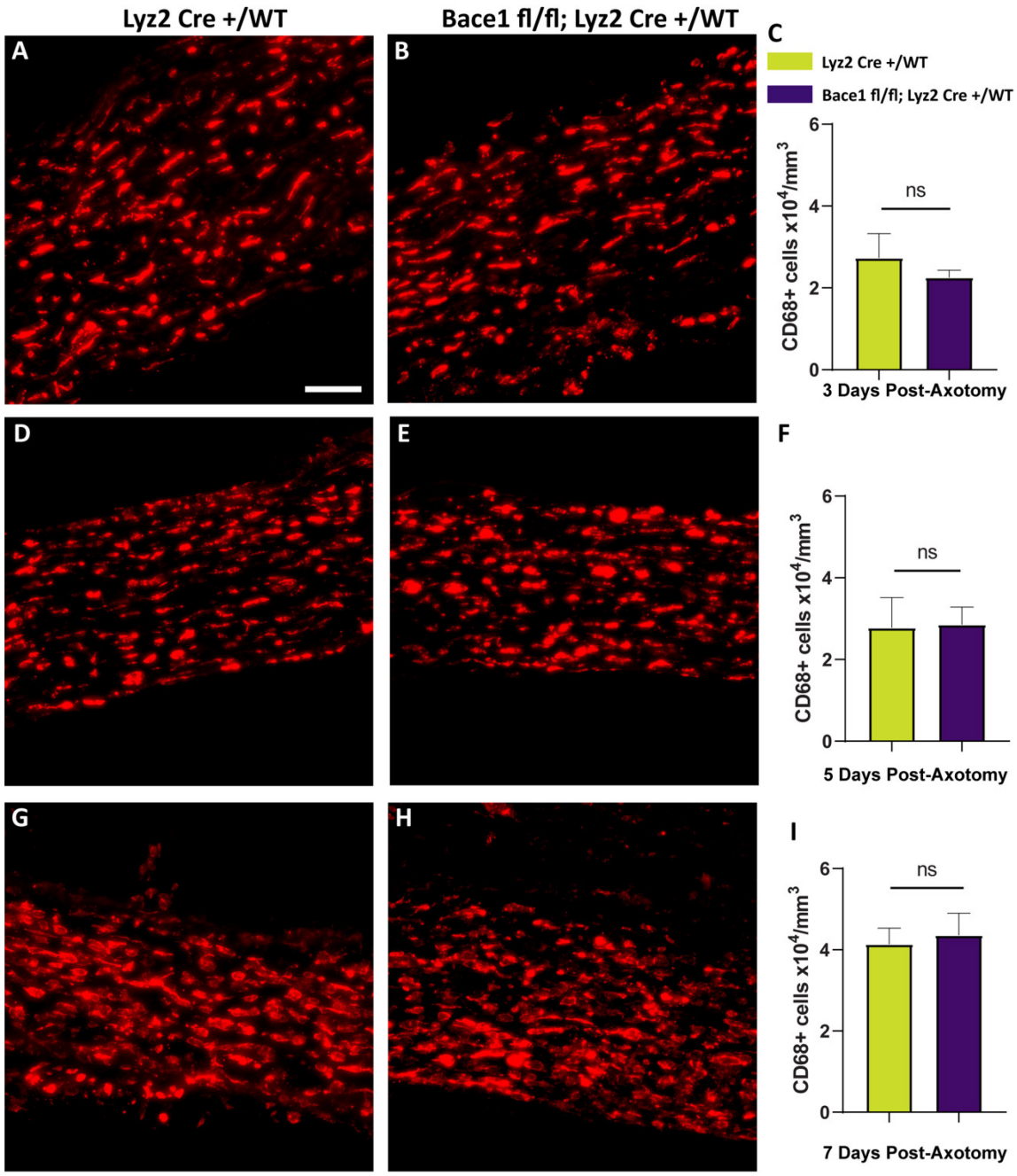

A
Fold Change RNA Expression (Normalized)
20
15
10
5
0
*
WT
WT + Thioglycollate
B
Fold Change RNA Expression (Normalized)
1. 5
1. 0
0. 5
0. 0
***
Lyz2 Cre +/WT
Bace1 fl/fl; Lyz2 Cre +/WT
C
Lyz2 Cre +/WT
D
Bace1 fl/fl; Lyz2 Cre +/WT
E
Myelin Thickness (µm)
1.5
1.0
0.5
0.0
ns
Lyz2 Cre +/WT
Bace1 fl/fl; Lyz2 Cre +/WT
F
Myelinated Axons x10⁴/mm²
3
2
1
0
ns
Lyz2 Cre +/WT
Bace1 fl/fl; Lyz2 Cre +/WT
G
g-ratio
1.0
0.8
0.6
0.4
Lyz2 Cre +/WT
Bace1 fl/fl; Lyz2 Cre +/WT
2
4
6
8
10
Axon Diameter (µm)

After validation of macrophage-specific deletion of BACE1 in peritoneal macrophages, we proceeded on to assess the impact of BACE1 deletion on macrophage recruitment following peripheral nerve injury in vivo. Initial in vivo assessments were to determine if these mice have the same enhanced recruitment to distal nerve segments following sciatic nerve transection as observed in previous studies (*1*, *65*). We measured recruitment in these mice by harvesting distal nerve segments and staining for CD68, a marker of macrophages, at 3 time points throughout the period of macrophage infiltration to the injured peripheral nerve. This dynamic period for recruitment begins at around day 3, peaks near day 7, and then persists through day 14 (*28*). At day 3 post-transection we observed no visible difference in the number of macrophages at an early stage of recruitment to the injured nerve **(Figure 2A,B**). Upon quantification of the density of macrophages in the distal nerve segments, we confirmed there was no significant difference in between the genotypes (p=0.2557) **(Figure 2C)**. Next, at day 5 post-sciatic nerve transection we observed no difference in recruitment of macrophages to the distal nerve segment (p = 0.8511) **(Figure 2D-F**). Finally, we measured the number of macrophages at day 7 to see if there was increased recruitment near the peak of the response, but again there was no difference (p=0.5371) **(Figure 2G-I**). Additionally, we considered that there may be an immunomodulatory effect driven by the Cre recombinase itself that could be masking the recruitment phenotype in these cKO mice (*118*), therefore we assessed recruitment of macrophages at day 7 post-sciatic nerve transection in WT and WT $Cre^{+/WT}$ mice. We observed no difference in the density of macrophages in the distal nerve segment, which suggests that Cre recombinase did not impact the recruitment of macrophages (p=0.5371) **(Figure 3 A-C**). Together, these data suggest that

macrophage-specific deletion of BACE1 does not play a role in enhancing recruitment of macrophages to the distal nerve segment following peripheral nerve injury.

Fig. 6. Macrophage-specific deletion of BACE1 does not alter recruitment of macrophages to the distal nerve segment post-sciatic nerve transection.

Nerve sections, 20 μm thick, stained for CD68, a marker for macrophages (A,B,D,E,G,H). Macrophages recruited to the distal nerve segments of Bace1$^{fl/fl}$;Lyz2 Cre $^{+/WT}$ mice (B,E,H) and Lyz2 Cre $^{+/WT}$ control mice (A,D,G) at 3 (A,B), 5 (D,E), and 7 (G,H) days post-sciatic nerve transection. Quantification of macrophage density in the distal nerve segment post-sciatic nerve transection (C,F,I). There were no significant differences between genotypes at 3 days (C), $p = 0.2557$, 5 days (F), $p = 0.8511$, and 7 days (I), $p = 0.5371$. Values are mean ± SD (C,F,I). For comparisons at 3 days (n=3) per genotype, at 5 days (n=5) for Bace1$^{fl/fl}$;Lyz2 Cre$^{+/WT}$ and (n=4) for Lyz2 Cre$^{+/WT}$, and at 7 days (n=4) per genotype. Scale bar = 100μm. *J.A. Fissel and M.H. Farah, Macrophage-specific deletion of BACE1 does not enhance macrophage recruitment to the injured peripheral nerve. J Neuroimmunol 349, 577423 (2020).*


Lyz2 Cre +/WT
Bace1 fl/fl; Lyz2 Cre +/WT
A
B
C
Lyz2 Cre +/WT
Bace1 fl/fl; Lyz2 Cre +/WT
CD68+ cells x10^4/mm^3
ns
3 Days Post-Axotomy
D
E
F
5 Days Post-Axotomy
G
H
I
7 Days Post-Axotomy

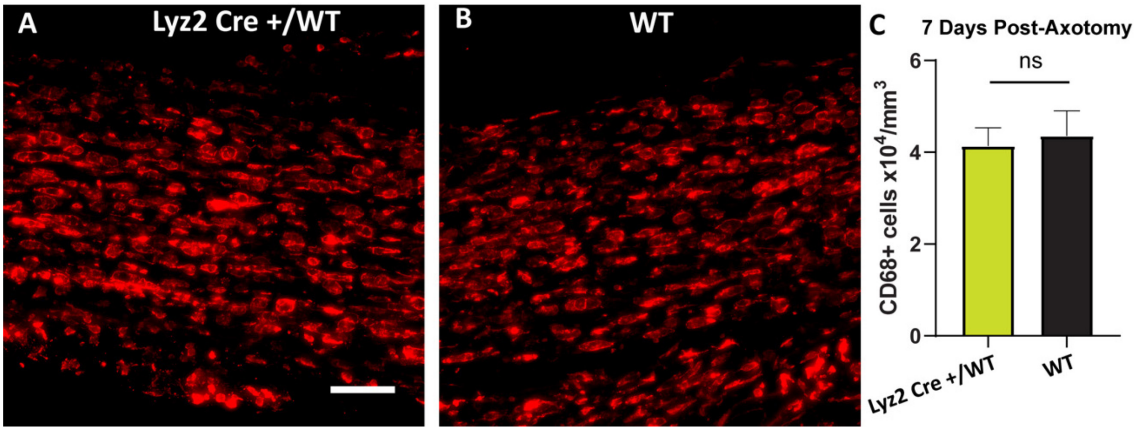


Fig. 7. Lyz2 promoter-driven expression of Cre recombinase does not alter recruitment of macrophages to the distal nerve segment 7 days post-sciatic nerve transection.

Nerve sections, 20µm thick, stained for CD68, a marker for macrophages (A,B). Macrophages recruited to the distal nerve segments of WT Lyz2 $Cre^{+/WT}$ (A) and WT (B) mice at 7 days post-sciatic nerve transection. Quantification of macrophage density in the distal nerve segment 7 days post-sciatic nerve transection (C). There was no significant difference between genotypes (n = 4), p = 0.5371, values are mean ± SD. Scale bar = 100µm. *J.A. Fissel and M.H. Farah, Macrophage-specific deletion of BACE1 does not enhance macrophage recruitment to the injured peripheral nerve. J Neuroimmunol 349, 577423 (2020).*

Next, we sought to determine if reduction of BACE1 could modulate the behavior of infiltrating macrophages. Over the course of the nerve injury, the polarization state of macrophages transitions from a pro-inflammatory M1 polarization to a pro-regeneration M2 polarization (*89*, *119*, *120*). This transition is beneficial as M2 macrophages have been associated with improved peripheral nerve regeneration (*121–123*). With this in mind, we assessed whether cKO mice have an increased abundance of macrophages that have transitioned to an anti-inflammatory/pro-repair M2 phenotype. To gauge the level of M2 polarization, we determined what percentage of CD68+ cells in the peripheral nerve were also positive for CD206, a marker of M2 polarization. There was no difference in the percentage of macrophages that were CD206+ between cKO mice and Cre expressing controls at day 5 post-transection ($p = 0.3196$) **(Figure 4A-C)**.

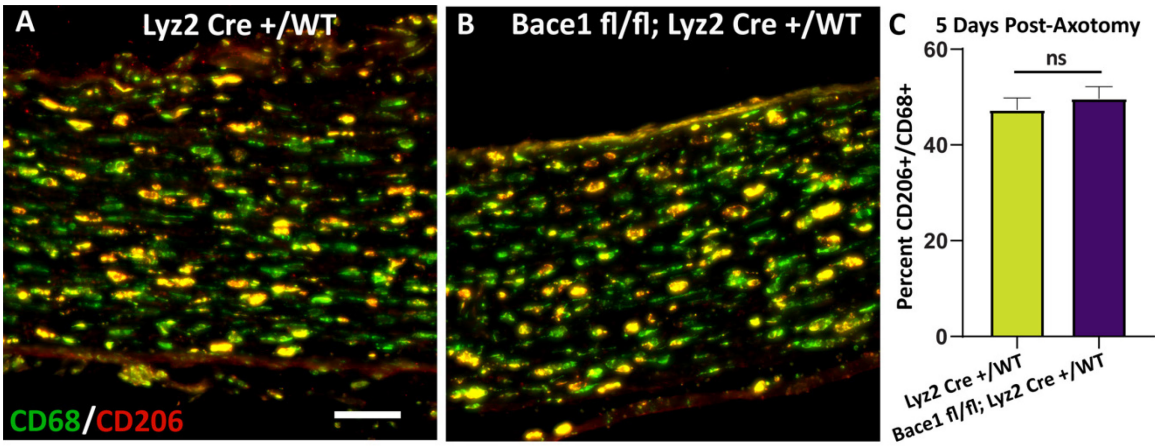


Fig. 8. Macrophage-specific deletion of BACE1 does not alter the polarization state of macrophages in the distal nerve segment 5 days post-sciatic nerve transection.

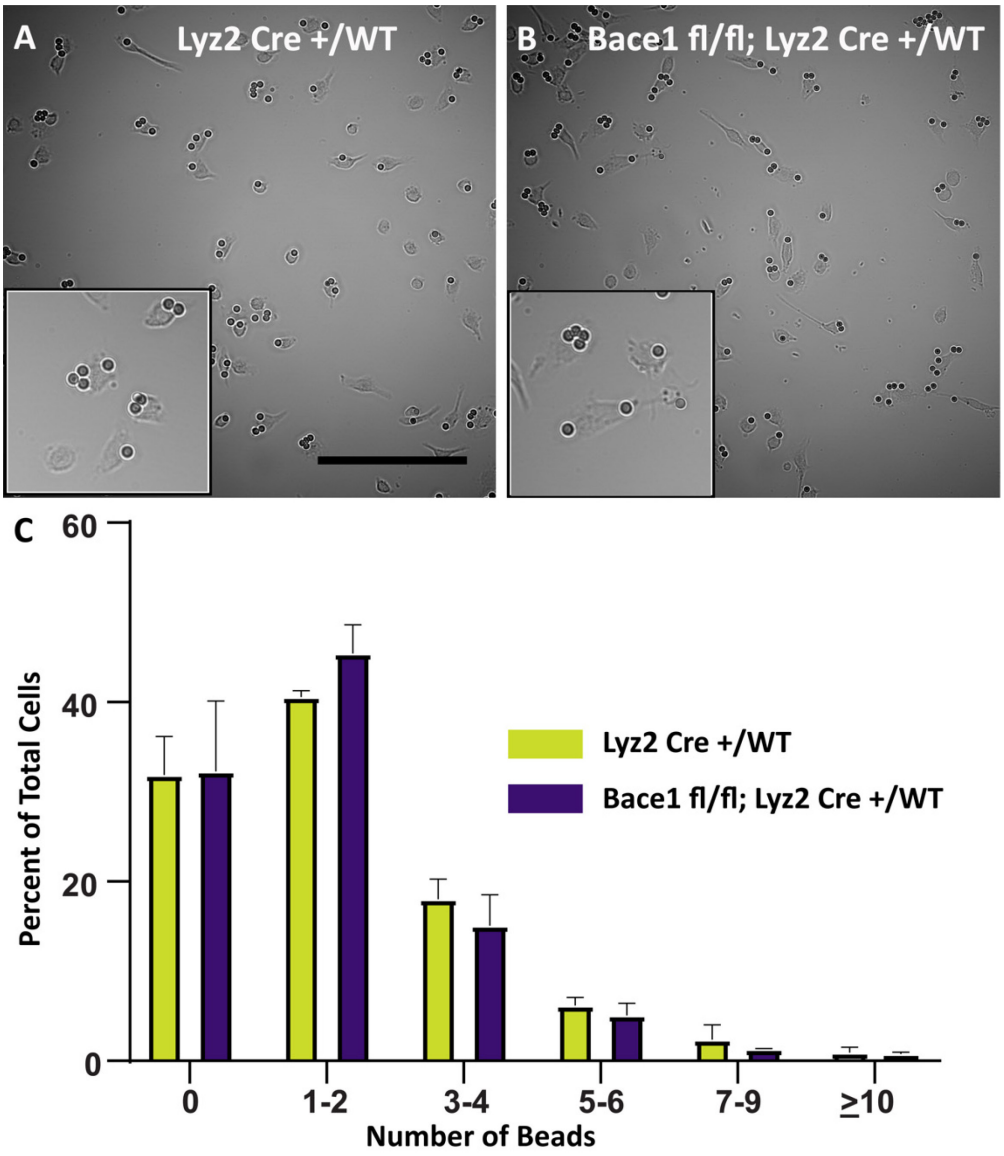
Merged images of 20µm thick nerve sections stained for a marker for macrophages (CD68, green) and a marker for M2 macrophage polarization (CD206, red) (A,B). Double positive cells appear as yellow. Populations of CD68+ macrophages that are positive for CD206 in the distal nerve segments of Bace1$^{fl/fl}$;Lyz2 Cre$^{+/WT}$ mice (B) and Lyz2 Cre$^{+/WT}$ control mice at 5 days post-sciatic nerve transection (A). Quantification of the percentage of CD68+ macrophages that are also CD206+ 5 days post-sciatic nerve transection (C). There was no significant difference between genotypes (n = 3), p = 0.3196, values are mean ± SD. Scale bar = 100µm. *J.A. Fissel and M.H. Farah, Macrophage-specific deletion of BACE1 does not enhance macrophage recruitment to the injured peripheral nerve. J Neuroimmunol 349, 577423 (2020).*

After observing no differences in the recruitment and polarization of macrophages in cKO mice, we investigated if there was an enhanced capacity for phagocytosis in

macrophages harvested from these mice. In previous work we have observed enhanced debris clearance in global BACE1 KO mice as demonstrated by increased phagocytosis of antibody opsonized polystyrene beads and by the observation of reduced axonal debris 7 days post-axotomy (*1*). To assess if BACE1 cKO mice have the same enhanced phagocytic activity of global BACE1 KO mice, we used an opsonized polystyrene bead phagocytosis assay similar to a previous study (*1*). Using this assay we did not see any difference between the number of beads phagocytosed by cKO mice and WT Cre expressing control mice **(Figure 5A-C).**

Fig. 9. Phagocytosis of opsonized beads remains unaffected in peritoneal macrophages with macrophage-specific deletion of BACE1 in vitro.

Bright field images of cultured Lyz2 $Cre^{+/WT}$ control (A) and $Bace1^{fl/fl}$;Lyz2 $Cre^{+/WT}$ (B) peritoneal macrophages incubated with mouse gamma globulin opsonized, 3μm diameter polystyrene beads. Quantification of the number of beads phagocytosed per macrophage harvested from $Bace1^{fl/fl}$;Lyz2 $Cre^{+/WT}$ mice and Lyz2 $Cre^{+/WT}$ control mice after 5 min incubation with opsonized beads (C). N = 3 independent experiments with 3 mice per genotype, $p > .1$ for all pairs. Values are mean ± SD. Scale bar = 100μm. *J.A. Fissel and M.H. Farah, Macrophage-specific deletion of BACE1 does not enhance macrophage recruitment to the injured peripheral nerve. J Neuroimmunol 349, 577423 (2020).*


A
Lyz2 Cre +/WT
B
Bace1 fl/fl; Lyz2 Cre +/WT
C
60
40
20
0
Percent of Total Cells
Lyz2 Cre +/WT
Bace1 fl/fl; Lyz2 Cre +/WT
0
1-2
3-4
5-6
7-9
≥10
Number of Beads

Summary of chapter 2

In this chapter, we determined that there was no difference in macrophage recruitment to the distal nerve segments in mice at 3, 5, and 7 days post-transection between the macrophage-specific BACE1 cKO mice and Cre expressing controls in the injured peripheral nerve. Further, there was no difference in the polarization state of macrophages in the injured peripheral nerve or phagocytic activity of macrophages in vitro (*124*).

Chapter 3: Conclusions

Macrophage-specific deletion of BACE1 does not enhance macrophage recruitment to the injured peripheral nerve

The following was published in J.A. Fissel and M.H. Farah, Macrophage-specific deletion of BACE1 does not enhance macrophage recruitment to the injured peripheral nerve. J Neuroimmunol 349, 577423 (2020).

A previous bone marrow transplantation experiment provided some evidence that the immunomodulatory phenotype of macrophages in global BACE1 KO mice may be driven by BACE1 expressed by macrophages (*65*); however, it did not directly address the level of recruitment of macrophages nor whether other nerve resident cells could be contributing to macrophage recruitment. Additionally, there could also be a developmental effect of BACE1 expression on the phenotype of macrophages. Many of the phenotypes observed in global BACE1 KO mice (*63*, *64*) are reduced or eliminated when BACE1 deletion is induced in adult mice (*125*). This could explain why we did not observe an increase in recruitment despite deletion of BACE1 in macrophages.

Although we observed no differences in macrophage recruitment to the injured distal nerve segment in a cKO model, these data provide insight into the relative contribution of BACE1 expression by macrophages towards the enhanced macrophage recruitment seen in global BACE1 KO mice. This study investigated the role of endogenous BACE1 expression by macrophages; however, neurons and Schwann cells

are known to play a large role in the peripheral nerve microenvironment and express high levels of BACE1 (*64*, *116*, *126*). This suggests that they could be driving the enhanced macrophage recruitment and debris clearance phenotype. To date, a robust array of cytokines produced in the distal nerve segment have been implicated in recruitment of macrophages to the injured peripheral nerve (*21–24*, *29–31*). Perhaps BACE1 expression by Schwann cells in the injured nerve microenvironment plays a role in the production of these cytokines, and thus recruitment and activity of macrophages.

This study also highlights the need for further exploration into the alternative hypothesis that the enhanced recruitment and debris clearance by macrophages in global BACE1 KO mice may be driven by hypomyelination, rather than enhanced mobilization and activation of macrophages. While there is evidence of enhanced myelin and axonal debris clearance in global BACE1 KO mice, this effect being driven by hypomyelination cannot be ruled out. The observed increased debris clearance could very well be the result of reduced myelin debris at baseline. Neuregulin-1 (NRG1) KO mice display similar levels of hypomyelination as BACE1 KO mice. NRG1 is a BACE1 substrate and signaling molecule critical for myelination. The absence of NRG1 during development results in hypomyelination; however, mice with an inducible axon-specific deletion of NRG1 show no myelination abnormalities when NRG1 is ablated post-development (*127*, *128*). Following peripheral nerve injury, inducible NRG1 KO mice show attenuated remyelination of axons but no difference in axonal regeneration, myelin debris clearance, or macrophage recruitment (*128*).

In summary, this study suggests that expression of BACE1 by adult macrophages may not be essential in mediating the increased macrophage recruitment to the injured distal nerve segment observed in global BACE1 KO mice. These data is limited to the role of BACE1 expression by macrophages in the context of peripheral nerve injury, but not broader immunomodulatory states. Further investigation into the expression of BACE1 by other cell types in the regenerating peripheral nerve microenvironment may answer questions posed by this study. Alternatively, future studies could be directed at testing the hypothesis that this observed phenotype is the result of the hypomyelination observed in these mice, rather than immunomodulatory effects on macrophages driven by BACE1 expression in the injured nerve microenvironment.

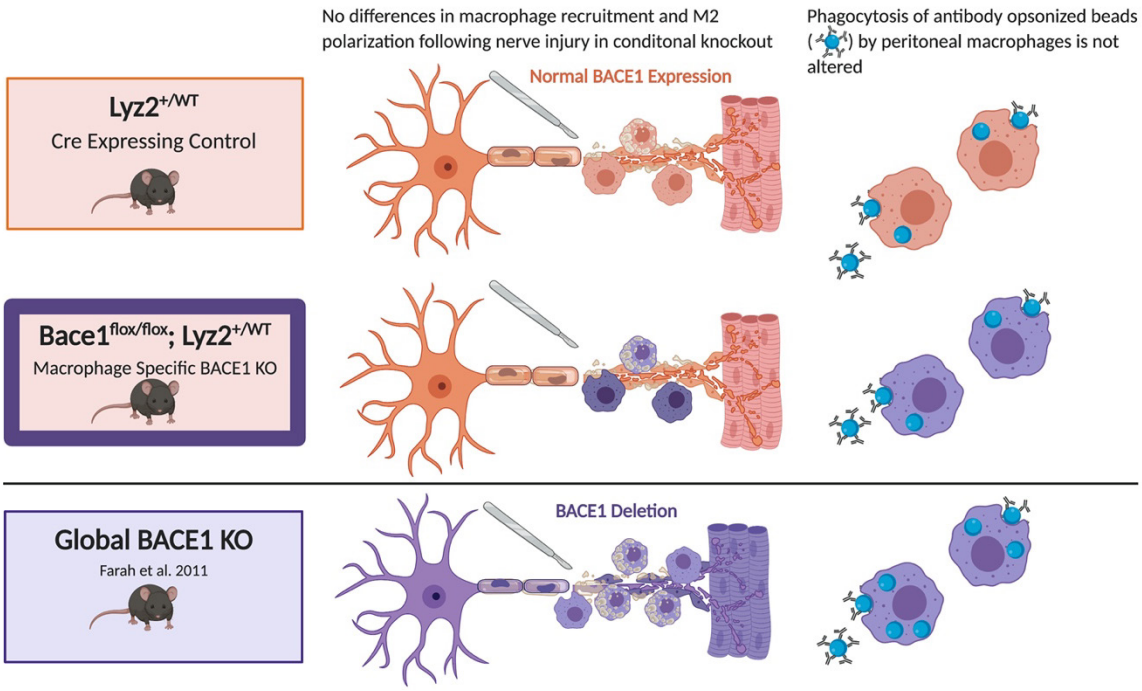


Fig. 10. Summary of results of macrophage-specific deletion of BACE1 in injured nerves of adult mice and in vitro phagocytosis assay.

A representation of the results of chapter 2. Mouse genotypes are listed (left) along with results of in vivo (center) and in vitro (right) data. BACE1 genotype by cell type is indicated by color, orange cells have WT BACE1, purple cells have deletion of BACE1. There is no difference in macrophage recruitment to the distal nerve segment between Bace1$^{fl/fl}$;Lyz2 Cre$^{+/WT}$ mice and Lyz2 Cre$^{+/WT}$ control mice following nerve injury. Bottom row summarizes previously published enhanced recruitment phenotype in global BACE1 and enhanced in vitro phagocytosis of global BACE1 KO macrophages. *J.A. Fissel and M.H. Farah, Macrophage-specific deletion of BACE1 does not enhance macrophage recruitment to the injured peripheral nerve. J Neuroimmunol 349, 577423 (2020).*

Results of macrophage-specific deletion of BACE1 and the implications for potential immunomodulatory mechanisms of BACE1 substrates

Mice with macrophage-specific deletion of BACE1 do not share the same levels of enhanced macrophage recruitment as global BACE1 KO mice (*124*). This suggests that the enhanced recruitment phenotype is mediated by other cell type(s) in the injured nerve microenvironment. As discussed in chapter 1 there are several BACE1 substrates that are known to have immunomodulatory effects, and some have been documented in the context of peripheral nerve injury. In light of the results of chapter 2 further discussion of how each of these substrates may or may not play a role is warranted.

IL-1 signaling can impact macrophages in a variety of ways. However, given the results of chapter 2, reduced BACE1 cleavage of IL-1R2 by macrophages is unlikely to play a role in enhancing macrophage recruitment to the injured nerve. Loss of BACE1 cleavage IL-1R2 could still be impacting macrophage recruitment through neurons and Schwann cells during the initial damage associated inflammatory signaling following nerve injury(*22*, *23*, *89*). Reduced BACE1 activity in these cells could reduce the amount of shed IL-1R2 (*58*, *84*, *85*) released into the extracellular milieu that, in the presence of BACE1 activity, could bind Schwann cell derived IL-1 in this space and prevent IL-1 signaling, thus loss of BACE1 could increase inflammatory signaling. While it is understood that BACE1 activity likely plays a role in the extracellular shedding of IL-1R2, it is not well understood if it plays a role in the generation of intracellular IL-1R2. If BACE1 can alter levels of intracellular IL-1R2 then this would provide another potential avenue for reduced BACE1 activity to play a role in DAMP signaling in neurons and Schwann cells.

The results of chapter 2 may also change how Notch signaling could be playing a role in global BACE1 mice. BACE1 is known to be expressed by neurons and Schwann cells and there is evidence that it plays a role in the regulation of Notch signaling through cleavage of Jag1(*94*). In the absence of BACE1 cleavage there would be an excess of Jag1 on the cell surface of neurons and Schwann cells that may result in increased signaling with macrophage derived Notch (*98*, *99*). There is evidence that this results in an increase of inflammatory markers in macrophages . This increased inflammation could result in the enhanced recruitment and debris clearance seen in global BACE1 KO mice.

BACE1 activity has been shown to enhance binding of macrophages to adhesion molecules associated with endothelial cells in vitro and could potentially benefit trafficking and recruitment through cleavage of ST6gal-1 (*25*, *106*). Under inflammatory conditions macrophages upregulate BACE1 cleavage of ST6gal-1 and increase the binding affinity for VCAM-1 in vitro (*25*). There is also evidence that BACE1 activity decreases endothelial cell-cell binding affinity through cleavage of ST6gal-1 (*25*). Given that the effects of BACE1 activity seem to be beneficial in the context of ST6gal-1 perhaps the role of this substrate would be buoyed by further studies into the possibility that the enhanced recruitment and debris clearance in BACE1 global KO mice is the result of hypomyelination.

Finally, PSGL-1's possibilities for playing a role in the immunomodulatory phenotype of global BACE1 KO mice become increasingly unlikely. PSGL-1 is expressed by macrophages and binds p-selectin on endothelial cells to initiate the adhesion cascade resulting in rolling, tethering, and arrest during trafficking from the bloodstream (*107*). With loss of BACE1 activity there should be a greater abundance of

PSGL-1 on the surface of macrophages that would be able to bind p-selectin and increase trafficking from the bloodstream and recruitment to the injured nerve (*108*). While any role for PSGL-1 cannot be ruled out, it is unlikely to be a primary driver of this phenotype.

While none of these substrates can be ruled out by the results of chapter 2, it does provide some insight into which mechanisms may be more important to the BACE1 KO macrophage phenotype. This phenotype is likely a combination of many different mechanisms, in different cell types, working together to produce this response to nerve injury.

Final Conclusion

This thesis demonstrates that macrophage-specific deletion of BACE1 has no effect on the recruitment of macrophages to the injured peripheral nerve of adult mice following injury. Further research into the potential effects of cell type specific deletion of other constituent cells of the injured nerve microenvironment is warranted. Alternatively, further study into the hypothesis that this enhanced recruitment and activity of macrophages may be due to hypomyelination in global BACE1 KO mice is also merited. Further assessment of potential roles of BACE1 substrates IL-1R2, Jag-1, ST6gal-1, and PSGL-1 in the injured nerve would also provide insight into potential mechanisms of this phenotype in global BACE1 KO mice. Further, interrogating each cell type in the peripheral nerve micro environment with cell type specific deletion of BACE1 could also help guide future inquiries into which BACE1 substrates are likely to be mediating this phenotype.

Chapter 4: Methods

Animals

Conditional knockout mice were on a C57BL/6 background and bred by crossing floxed *Bace1* mice, C57BL/6-*Bace1*$^{tm1.1mrl}$ (Taconic Biosciences, Rensselaer, NY, USA) (*129*), with mice expressing Cre recombinase under control of *Lyz2* promoter/enhancer elements, B6.129P2-*Lyz2*$^{tm1(cre)Ifo}$ (The Jackson Laboratory, Bar Harbor, ME, USA). Macrophage-specific cKO mice were homozygous for the floxed *Bace1* allele and heterozygous for Cre recombinase (Bace1$^{fl/fl}$;Lyz2 Cre$^{+/WT}$). Control mice were C57BL/6 wild type (WT) mice that were heterozygous for Cre recombinase (*Lyz2* Cre$^{+/WT}$). A total of 79 mice between 10-16 weeks old were used in this study. Roughly equal amounts of male and female mice were used in this study as there was no significant difference between male and female in BACE1 expression, macrophage recruitment, or macrophage activity. The animal surgeries and experimental protocols were approved by the Animal Care and Use Committee of the Johns Hopkins University School of Medicine.

Peritoneal macrophage harvest and culture

Mice were killed by CO_2 asphyxiation, and a small incision in the skin was made to expose the peritoneum. 5 mL of cold Gibco® RPMI 1640 media (Thermo Fisher Scientific, Waltham, MA, USA) with 10% Gibco® fetal bovine serum (FBS) (Thermo Fisher Scientific, Waltham, MA, USA) and 1% penicillin-streptomycin was injected into the peritoneal cavity and gently massaged for 15 seconds. RPMI was aspirated with a syringe until approximately 2 mL of media was recovered. To collect the remaining

media, a small incision was then made in the peritoneal membrane and media was aspirated by transfer pipet (*130*). Cells were then spun at 250 X *g* for 10 min, resuspended and counted. Approximately 60,000 cells/well were seeded onto glass coverslips in 24 well plates for phagocytosis assays, and $2x10^6$ cells/well were seeded into 6 well plates for expression analysis. These cells were then incubated at 37°C and 5% CO_2 for 1 h to allow peritoneal macrophages to adhere to the cell culture dish. Following incubation, wells were washed to remove contaminating cells, leaving behind adhered macrophages. Cells were then either immediately used for expression analysis or incubated 24 h prior to phagocytosis assays. Mice used for macrophage BACE1 expression analysis in an inflammatory context were treated by injection of 1 mL of 3% Brewer thioglycollate medium (Millipore Sigma, Burlington, MA, USA) into the peritoneal cavity 4 days prior to harvest.

RT-PCR

Total RNA was extracted from peritoneal macrophages from each mouse with *Quick*-RNA Miniprep Kit (Zymo Research, Irvine, CA, USA) then cleaned and concentrated (RNA Clean & Concentrator-25, Zymo Research, Irvine, CA, USA). Quality and concentration of RNA was determined by Nanodrop. One microgram of RNA was added to each reaction, and a cDNA library was reverse transcribed for each of the samples using Invitrogen SuperScript III first-strand synthesis system kit (Thermo Fisher Scientific, Waltham, MA, USA). The cDNA libraries were then combined with Quantitect® SYBR® Green PCR Kit (Qiagen, Germantown, MD, USA). For each mouse, BACE1 and β_2 microglobulin (B2M) primers were added to separate reactions to

amplify BACE1 and B2M transcripts in the peritoneal macrophages. B2M was selected as a reference gene based on reports of improved stability in macrophages in an inflammatory context (*131*). BACE1 primers were selected to have one primer binding site in exon 2, which is deleted in the presence of Cre recombinase, and another primer binding site in exon 3. The following primers were used: BACE1 primers, 5'-CCTTTCCTGCATCGCTACTAC-3' (forward); 5'-GGGCACATACACACCCTTT-3' (reverse) and B2M primers, 5'-GGGTGGAACTGTGTTACGTAG-3' (forward); 5'-TGGTCTTTCTGGTGCTTGTC-3' (reverse). Each sample was run in triplicate and analyzed by 7900HT Fast Real-Time PCR System (Applied Biosystems, Waltham, MA, USA). All values were normalized to B2M mRNA and reported as fold change from WT levels.

Sciatic nerve transection

Mice were anesthetized with 2% isoflurane (Baxter, Deerfield, IL, USA), and fur covering the skin on the left thigh was shaved with an electric hair trimmer. Mice were continuously supplied with isoflurane, and surgeries were performed under sterile conditions. A small incision was made in the skin along the thigh to expose underlying muscle and connective tissue. With surgical microscope, a small incision was made in the connective tissue at midthigh, and a surgical retractor was used to expose the nerve. Small surgical scissors were then used to transect the nerve, and the distal end was tucked away from the proximal end to prevent regeneration of the axons. The wound was then sutured closed. Distal nerve segments and uncut contralateral controls were harvested at stated time points post-surgery.

Mouse perfusion

Mice were deeply anesthetized with 10% chloral hydrate (Millipore Sigma, Burlington, MA), briefly exsanguinated with Gibco® 1x phosphate-buffered saline (PBS) solution (Thermo Fisher Scientific, Waltham, MA, USA), then perfused with 2% paraformaldehyde (PFA) in 1x PBS solution for immunofluorescence and 2% PFA/2% glutaraldehyde in 1x PBS solution for light microscopy.

Myelinated axon quantification

Following perfusion, nerves were dissected out, post-fixed in 2% PFA/2% glutaraldehyde in 1x PBS solution overnight at room temperature (RT), and embedded in plastic. Nerves were then cross-sectioned at 1 μm thickness and stained for toluidine blue. Images of the nerve sections were taken at 100x magnification. Myelinated axon density was determined by counting the number of myelinated axons in a measured area ($0.01mm^2$) using Zeiss Zen image analysis software. For the quantification of myelin thickness and g-ratio, 200 myelinated axons were analyzed for each mouse, at random, from 3 fields of view from each of 3 different cross sections. For each myelinated axon, the axon diameter and the nerve fiber diameter were determined by ImageJ 1.52a. Myelin thickness was calculated as (fiber diameter – axon diameter)/2, and g-ratio was calculated as (axon diameter/fiber diameter)(*132*).

Immunofluorescence

Following perfusion, nerves were dissected out, and 3 mm of nerve tissue at the cut end was discarded before further processing of distal nerve segment. The remaining nerve was post-fixed in 2% PFA solution overnight at RT. Nerves were then washed with 1x PBS and cryoprotected in 30% sucrose in 1x PBS overnight at 4°C. Nerve tissue was then immersed in Tissue-Tek® O.C.T. Compound, flash frozen, and sectioned longitudinally at 20 μm thickness by cryostat (Microm HM 505E). Sectioned nerves were washed in 1x PBS for 10 min at 37°C, and washed again 2 times for 10 min at RT in 1x PBS with .3% Triton X-100 (PBST) to remove O.C.T. compound and permeabilize the tissue. Sections were then blocked with PBST with 5% normal goat serum (NGS) for 1h at RT. Next, sections were incubated with PBST 1% NGS with primary antibody overnight at 4°C. Anti-CD68 (1:1000 BioLegend) antibodies were used as a marker for macrophages and anti-CD206 (1:1000 Santa Cruz) was used as a marker of M2 polarization. Nerves were then washed with 1x PBST 2 times for 15 min before incubation with appropriate secondary antibodies (Alexa Fluor 488 and Alexa Fluor 546 (Invitrogen)) diluted at 1:1000 in PBST with 1% NGS for 1h at RT. Slides were again washed twice for 15 min in PBST before mounted with Life Technologies Prolong Gold Anti-fade with DAPI (Thermo Fisher Scientific, Waltham, MA, USA) and coverslipped. Sciatic nerves were then imaged using a Axio Imager Fluorescence Microscope (Zeiss, Oberkochen, Germany). Images used in macrophage recruitment analysis were acquired in 3D z-stacks in 2μm intervals at 20x magnification in 3 fields of view.

Quantification of immunofluorescence images

Macrophages were quantified by counting CD68+ cells using Zeiss Zen image analysis software. Individual cells were determined by DAPI staining of cell nuclei and analyzing multiple focal planes. The area of each nerve was then traced and quantified for volume calculations (*62*). Density of macrophages was calculated using the total number of macrophages and the volume of the image. M2 macrophage polarization was determined by counting all CD68+ cells and CD206+ cells in the same manner and reported as a percentage of CD68+ cells that were also positive for CD206.

Phagocytosis assay

To prepare opsonized bead solution, 3 μm diameter polystyrene beads (Millipore Sigma, Burlington, MA, USA) were opsonized with mouse IgG (Thermo Fisher Scientific, Waltham, MA, USA) during 1h incubation at 37°C. Beads were then diluted to a 10:1 ratio with peritoneal macrophages. Following 24h incubation post cell harvest, peritoneal macrophages (harvested as stated in section 2.2) were centrifuged with the opsonized bead solution for 1 min at 250 X *g* and incubated at 37°C for an additional 5 min. Following incubation, uningested beads were washed away with 1x PBS and the remaining macrophages were fixed with 3% PFA solution. Coverslips were then mounted and imaged with at 40x magnification in z-stacks in in 2 μm intervals at 3 fields of view. Total cells and beads phagocytosed by each cell were counted. This was performed in 3 replicates per mouse.

Statistical analysis

Each mouse was randomly assigned a number and neither the thioglycollate treatment status nor genotype was known throughout data collection and analysis to ensure blinded analysis of the data. All statistical analysis was performed using a Student's t-test. All data analysis was performed using GraphPad Prism 8, and any value of $p < 0.05$ was scored as statistically significant. All graphed data was generated using GraphPad Prism 8 and presented as mean ± standard deviation.

Figure Art

Artistic figures in chapter 1 were created with BioRender.com.

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CAREER OBJECTIVE

I intend to pursue a career as a clinical microbiology laboratory director. I will soon be training for ABMM certification as the 2021-2023 CPEP Medical and Public Health Microbiology Fellow at Children's Hospital Los Angeles.

EDUCATION

Ph.D. Pathobiology 2014 - 2020
Johns Hopkins University School of Medicine, Baltimore, MD

B.S. Microbiology, Minor in Biochemistry 2013
New Mexico State University, Las Cruces, NM

RESEARCH EXPERIENCE

Ph.D. Candidate
Johns Hopkins University School of Medicine; Baltimore, MD August 2014 – October 2020
Mentor: Dr. Mohamed H. Farah

Researching the potential therapeutic effects of BACE1 inhibitor for peripheral nerve regeneration, with emphasis on researching the mechanisms underlying the enhanced recruitment and activity phenotype of macrophages in mice that have reduced BACE1 activity through genetic deletion or pharmacological inhibition. The contribution of endogenous BACE1 expression in macrophages is being assessed in mice with macrophage specific deletion of BACE1.

Laboratory Research Assistant
Sapphire Energy; Las Cruces, NM May 2013 - May 2014

Worked in Quality Assurance/Quality Control and Crop Protection groups for the research team of an algae biofuel company. Analyzed samples from algae ponds to detect pathogens, tested susceptibility of pathogens to fungicide panel, reported findings, and made recommendations for pond fungicide treatments.

Laboratory Research Assistant
New Mexico State University; Las Cruces, NM October 2011 - May 2013

Independent research project to determine if programmed cell death observed during the vegetative incompatibility response of the plant pathogen *Cryphonectria parasitica* is

autophagic. The main tools of this research were a gene knockout and a GFP gene fusion construct of the gene of interest, *atg-8*.

TRANSLATIONAL RESEARCH ROTATIONS

Clinical Microbiology Rotation – Antimicrobial Susceptibility Testing
Johns Hopkins University School of Medicine; Baltimore, MD December 2019 – June 2020
Preceptor: Dr. Patricia (Trish) J. Simner

Clinical Microbiology Rotation – Metagenomic Next Generation Sequencing
Johns Hopkins University School of Medicine; Baltimore, MD April 2020 – June 2020
Preceptor: Dr. Patricia (Trish) J. Simner

TEACHING EXPERIENCE

Johns Hopkins University Teaching Institute Collaborative Teaching Fellowship
Goucher College; Baltimore, MD August 2019 – December 2019

Delivered lessons, assisted with in class activities, and trained students in basic laboratory techniques in Principles of Chemistry I.

Teaching Assistant - Pathology for Graduate Students: Neuropathology
Johns Hopkins University School of Medicine; Baltimore, MD May 2017 & 2018

Coordinated guest lecturers, graded exams, and helped lead journal club discussions.

Teaching Assistant - Pathology for Graduate Students: Basic Mechanisms
Johns Hopkins University School of Medicine; Baltimore, MD September 2015 & 2016

Prepared and delivered lecture on basic anatomy/physiology of the endocrine system.

HHMI BioCat Peer Facilitator
New Mexico State University; Las Cruces, NM August 2011- May 2013

Served as a peer facilitator for Biology 211 Cellular and Organismal Biology. Guided students during in-class activities. Held office hours for students who sought extra help, tutoring, or clarification of course topics.

COMMITTEE SERVICE

Graduate Program in Pathobiology Admissions Committee
Johns Hopkins University School of Medicine; Baltimore, MD December 2018

Graduate Program in Pathobiology Recruitment Committee
Johns Hopkins University School of Medicine; Baltimore, MD January 2016

PUBLICATIONS

H.H. Mostafa*, **J.A. Fissel***, B. Fanelli, Y. Bergman, V. Gniazdowski, M. Dadlani, K.C. Carroll, R. Colwell, P.J. Simner. (Accepted) Metagenomic Next-Generation Sequencing of Nasopharyngeal Specimens Collected from Confirmed and Suspect COVID-19 Patients. *mBio*

C.P. Morris, Y. Bergman, T. Tekle, **J.A. Fissel**, P.D. Tamma, P.J. Simner. (In Press) Cefiderocol Antimicrobial Susceptibility Testing against Multidrug-Resistant Gram-negative Bacilli: A Comparison of Disk Diffusion to Broth Microdilution. *Journal of Clinical Microbiology*

J.A. Fissel, M.H. Farah. (2020) Macrophage specific deletion of BACE1 does not enhance macrophage recruitment to the injured peripheral nerve. *Journal of Neuroimmunology. 349, 577423*

J.A. Fissel, M.L. Yarbrough, T. Tekle, C.A.D. Burnham, P.J. Simner. (2020) Reporting Considerations for Cefepime Susceptible and Susceptible-Dose Dependent Results for Carbapenemase-Producing *Enterobacterales*. *Journal of Clinical Microbiology. 58*(9).

Y. Liu, B. Sebastian, B. Liu, Y. Zhang, **J.A. Fissel**, B. Pan, M. Polydefkis, M.H. Farah. (2017) Sensory and autonomic function and structure in footpads of a diabetic mouse model. *Scientific Reports*, *7*, 41401.

L. Liu, **J.A. Fissel**, A. Tasnim, J. Borzan, A. Gocke, P.A. Calabresi, M.H. Farah. (2016) Increased TNFR1 expression and signaling in injured peripheral nerves of mice with reduced BACE1 activity. *Neurobiology of Disease,* 93, 21-27.

CONFERENCES

American Society for Microbiology Microbe, Chicago, IL
Frequency of Cefepime Susceptible and Susceptible-Dose Dependent Results for Carbapenemase-Producing Enterobacterales, June 2020 (Accepted Abstract, meeting canceled)

Society for Neuroscience Annual Meeting, Nanosymposium, San Diego, CA
Increased TNFα/TNFR1 signaling on macrophages in the injured peripheral nerve of BACE1 KO mice, November 2016

6th Molecular Mechanisms of Axon Degeneration Meeting, Bar Harbor, ME
Increased TNFα/TNFR1 signaling on macrophages in the injured peripheral nerve of BACE1 KO mice, September 2016

16th International Neural Regeneration Symposium, Pacific Grove, CA
Potential Therapeutic Effect of BACE1 Inhibitor in Treating Diabetic Neuropathy in a Mouse Model, November 2015