Age-associated ROS production during remyelination in Multiple Sclerosis (MS)

by

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Abstract

Multiple sclerosis (MS) is a chronic inflammatory disease characterized by central nervous system (CNS) lesions, resulting in axonal loss and physical and cognitive disability. Loss of myelin sheath known as demyelination, is followed by its regeneration in a process, known as remyelination, that protects axons from degeneration, thereby slowing the permanent disability related to axonal loss. Acute demyelination is followed by myelin debris removal which is crucial for remyelination. The process of remyelination is then characterized by the recruitment and proliferation of oligodendrocyte progenitor cells (OPCs) and their subsequent differentiation into myelinating oligodendrocytes. Microglia and monocyte-derived macrophages (MDMs) play multiple roles during remyelination. Microglia can stimulate OPC proliferation in vitro, and microglia/MDMs can secrete factors, such as activin, that promote oligodendrocyte differentiation. Both microglia and MDMs also phagocytose debris from injured myelin, which is a known remyelination inhibitor.

Remyelination declines during aging. However, it is still unknown what causes this age-dependent decline. Previous studies have shown that microglia and MDMs produce reactive oxygen species(ROS) up to toxic levels in middle-aged mice. Given that aging is associated with increased ROS in the CNS, we hypothesized that a population(s) of aged microglia/macrophages in the CNS produce excessive ROS contributing to age-associated remyelination decline. I used the LPC (lysophosphatidylcholine) model of demyelination and examined the presence of age-associated ROS production during remyelination in young (2-3months) and middle-aged (8-10 months) mice receiving intraspinal LPC injections. I first characterized the accumulation of microglia and MDMs using lineage tracing with young and middle-aged male and female $CX3CR1^{CreEr}$; $ROSA26^{tdT}$ mice. This mouse line expresses tdTomato (tdTom) in microglia under

the ROSA26 promotor. The benefit of this method is that microglia and their progeny are permanently labelled using a reporter that does not change during inflammation. I found a delayed accumulation of microglia but not monocyte-derived macrophages in middle-aged CX3CR1^{CreEr}; ROSA26^{tdT} mice. To evaluate ROS production, I quantified oxidative damage to lipids by immunostaining against different lipid peroxidation markers within remyelinating lesions. Based on MDA and E06 immunoreactivity, I found that lipid peroxidation peaks in middle-aged mice at early to later stages of remyelination, i.e. 7 and 21DPL. In young mice, based on MDA immunostaining, lipid peroxidation increases from 3 to 21 DPL.

I next inhibited elevated ROS production by orally administering Setanaxib (GKT137831), an inhibitor of ROS-producing enzyme, NADPH oxidase (NOX1/4 isoform), to middle-aged female C57BL/6 mice after LPC injection, to see if reduced ROS production can boost remyelination. I found that Setanaxib reduced excessive ROS production marked by decreased E06 immunoreactivity in the lesion at 7DPL. I then immunostained against OPC marker PDGFRα and proliferation marker Ki67 to quantify proliferating OPCs and found no difference in proliferating OPC densities in Setanaxib and Vehicle groups. Similarly, upon immunostaining against transcription factor Myrf^{+}, there were no differences in Myrf^{+} oligodendrocytes in Setanaxib and Vehicle groups. Also, immunostaining against pan-leukocyte marker CD45, microglia/MDM marker IBA1 and myelin basic protein (MBP), revealed that Setanaxib did not boost MBP^{+}myelin debris clearance and accumulation of microglia/MDMs in the lesion. Taken together, Setanaxib did not boost remyelination in middle-aged female C57BL/6 mice and therefore, reducing excessive ROS production alone, cannot rescue age-associated delayed remyelination.

Preface

This thesis is an original work by Sharmistha Panda. This research project was conducted at the University of Alberta and was supervised by Dr Jason Plemel. The data collection and analysis were primarily done by me with the invaluable contribution from my undergraduate student Sowmya Challa (Figure 3-2, 3-3), visiting scholar Reza Naeimi (Figure 3-3) and PhD candidate Charbel Baaklini(Figure 3-1,3-4(IMARIS analyses)). The intraspinal lysophosphatidylcholine injections were performed by Charbel Baaklini, with the assistance of Madelene Ho, Kelly V Lee and me. Animal perfusions (with 4% PFA), tissue collection and processing were performed by Kelly V Lee, Madelene Ho, Abhisha Patel and Charbel Baaklini.

This research project, of which the thesis is a part, received research ethics approval from the University of Alberta Research Ethics Board. All the animal experiments and procedures were conducted complying with the Canadian Council on Animal Care's Guidelines and policies and approved by the University of Alberta Health Sciences Animal Care and Use Committee (AUP00002605). This project received funding from the Department of Defense, United States of America.

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helping me in my experiments, he has always been there during my tough days and made me laugh to my fullest. He has been my pillar of support and his empathy towards my challenging periods has made all this possible. His honest feedback and suggestions have helped me improve my presentations, experiment planning and image analysis skills. He has played a crucial role in this journey, and I will be always indebted to him for his unwavering support. Next, I would like to thank Sowmya Challa, my undergraduate student and Reza, a visiting scholar from Iran for their immense contributions to this research. Their great organizational skills and ability to learn things quickly helped us collect a vast amount of good data in a short time. I would then thank Kelly V Lee, our lab technician for helping me get on board in the lab when I first joined. She also helped me in my experiments and provided constructive feedback during lab presentations. I am grateful to Madelene Ho, Olivia La Caprara, Andre Faria, Sameera Zia and Brady Hammond for always making the lab environment positive for me to thrive. They have always supported me, offered useful suggestions and helped me foster great social connections in the lab. I would thank all the members of the Plemel/Kerr lab for their time-to-time guidance and support during my journey.

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And in the end, I would convey my most heartfelt gratitude to my family- my dad, mom and little brother. Their blessings and emotional support helped me navigate through difficulties

and they have always had my back when I needed them. This research would not have reached its completion without their trust and confidence in me. Being an ardent believer in Hinduism, I would also thank God for their constant support and belief in me during my research journey.

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List of Abbreviations

MDA	Malondialdehyde
MS	Multiple Sclerosis
LPC	Lysophosphatidylcholine/Lysolecithin
ROS	Reactive Oxygen Species
MDM	Monocyte-derived macrophages
MBP	Myelin basic protein
OxPC	Oxidised phosphatidylcholine
CNS	Central nervous system
OPC	Oligodendrocyte progenitor cells
TNFα	Tissue necrosis factor-alpha
PDGFRα	Platelet-derived growth factor alpha
Myrf	Myelin regulatory factor
tdT	tdTomato
Nrp1	Neuropilin
TG2	Transglutaminase
ADGRG1	Adhesion G Protein-Coupled Receptor G1
PDGFR AA	Platelet-derived growth factor alpha AA
NOX	NADPH oxidase
RRMS	Relapsing-Remitting Multiple Sclerosis
OL	Oligodendrocyte

CHAPTER 1: Introduction

1.1 General overview

Multiple Sclerosis (MS) is an autoimmune and inflammatory condition affecting the central nervous system (CNS). It is characterized by demyelination of myelinated axons within the CNS, resulting in varying degrees of destruction of myelin and axons. Common symptoms of MS include numbness, weakness, visual impairment, loss of balance, dizziness, urinary bladder urgency and fatigue(Calabresi, 2004; Goldenberg, 2012).

Damage to the myelin sheath, known as demyelination, can be followed by a robust regenerative process called remyelination. It regenerates the lost myelin sheath and restores normal saltatory conduction. Failure of remyelination can lead to axonal degeneration and more disability(Bodini et al., 2016; Franklin & Ffrench-Constant, 2017; Greg et al., 2023; Irvine & Blakemore, 2008) . Current research focuses on finding ways to boost remyelination.

Remyelination in the central nervous system requires the proliferation of oligodendrocyte progenitor cells (OPCs) and their subsequent differentiation into myelinating oligodendrocytes(Baxi et al., 2017; Franklin & Ffrench-Constant, 2008; Zawadzka et al., 2010). Remyelination by mature oligodendrocytes is limited(Bacmeister et al., 2020; Mezydlo et al., 2023) and highly regulated by cells in the lesion environment, with a critical role by phagocytic microglia and monocyte-derived macrophages (MDMs)(Baaklini et al., 2023).

Microglia and MDMs play a dual role in the disease progression of MS(Baaklini et al., 2019; Kamma et al., 2022). They release cytokines, including TNF-α(Gardner et al., 2013; Mendiola et al., 2020; Plemel et al., 2020), and IL-1β(Lévesque et al., 2016; Mendiola et al., 2020; Plemel et al., 2020) and reactive oxygen species(ROS)(Michaels et al., 2020) which may contribute to neurodegeneration. However, in the context of remyelination, microglia and MDMs regulate multiple stages of myelin repair.(Baaklini et al., 2023; Kotter et al., 2006; Kotter et al., 2001; Miron et al., 2013; Ruckh et al., 2012). For example, microglia stimulate oligodendrocyte

progenitor cell (OPC) proliferation in vitro, and microglia/MDMs can secrete factors, such as activin, that promote oligodendrocyte differentiation(Miron et al., 2013; Plemel et al., 2017). They also phagocytose myelin debris, which is a known remyelination inhibitor(Baaklini et al., 2023; Kotter et al., 2006; Plemel et al., 2013). However, clearance of myelin debris is slow in middle-aged animals and this, likely contributes to age-dependent remyelination decline(Ruckh et al., 2012). As aging is associated with exacerbated reactive oxygen species (ROS) production after white matter injury (Michaels et al., 2020), we hypothesized that aged microglia or macrophages release ROS that potentially interfere with remyelination. In the following sections, I highlight various aspects of remyelination- its significance, the role of microglia and macrophages and the impact of aging. Finally, I discuss current findings on age-associated ROS production in MS models and how it forms the basis of our hypothesis.

1.2 Remyelination- Process and significance

Remyelination is associated with lesser disability in MS(Bodini et al., 2016; Stephan Bramow et al., 2010). It restores lost myelin, accelerates axonal conduction, lessens axonal degeneration, and may even promote functional recovery(Duncan et al., 2009; Franklin & Ffrench-Constant, 2017; Irvine & Blakemore, 2008; Mei et al., 2016).

The process of remyelination is characterized by two distinct stages based on the dynamics of oligodendrocyte lineage cells: 1) oligodendrocyte progenitor cells (OPCs) proliferate and migrate towards demyelinated axons, and 2) OPCs start to differentiate into myelinating oligodendrocytes, contact denuded axons and form a new myelin sheath(Franklin & Ffrench-Constant, 2017; Plemel et al., 2017). A more elaborate description of the process is illustrated in Figure 1-1(Franklin & Ffrench-Constant, 2017). Various extracellular factors and intracellular signals regulate these stages.

Alternatively, remyelination may also occur via surviving oligodendrocytes (Yeung et al.,2019). In the shadow plaques—a biomarker of remyelination in MS(Patrikios et al., 2006)—surviving oligodendrocytes were found to contribute to remyelination(Yeung et al.,2019). However, in several follow-up studies in animal models surviving oligodendrocytes were found to produce only a few at most internodes (myelinated portion of the axon) that were short in length (Bacmeister et al., 2020; Franklin et al., 2020; Mezydlo et al., 2023). Therefore, overall, the major contribution to remyelination is from the production and differentiation of OPCs. The goal of current clinical approaches is to enhance the remyelinating capacity of OPCs, which may have tremendous benefits for people living with MS.

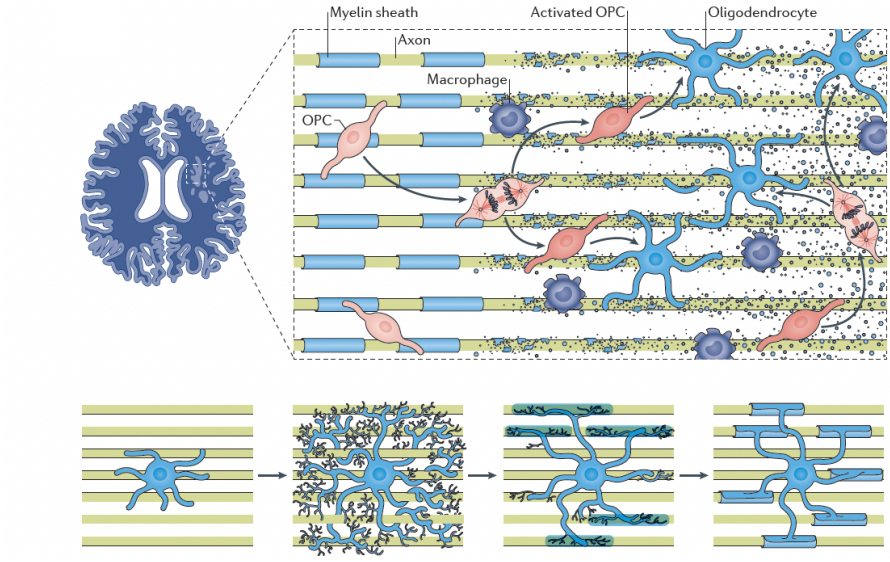

Figure 1-1: **Process of remyelination**
Damage to myelinated axons in the CNS triggers a regenerative response initiated by activation of OPCs. OPCs proliferate and mature into becoming myelinating oligodendrocytes. Macrophages (including microglia and MDMs) play a crucial role in remyelination by clearing myelin debris and secreting factors required for proliferation and maturation of oligodendrocyte lineage cells.(Franklin & Ffrench-Constant, 2017)

1.3 Role of Microglia and MDMs in Remyelination

Microglia and MDMs are important regulators of remyelination.(Baaklini et al., 2019; Lloyd & Miron, 2019). Depleting microglia and MDMs by clodronate-liposomes, which kill phagocytic cells, reduces remyelination efficiency in a spontaneously remyelinating experimental rat model(Kotter et al., 2001; Kotter et al., 2005). Microglia and MDM clear myelin debris, secrete regenerative factors to promote remyelination and modulate the extracellular matrix, which all support remyelination(Baaklini et al., 2019; Lloyd & Miron, 2019; Voet et al., 2019). Recently, we found that following LPC-induced demyelination microglia and MDMs both phagocytose myelin debris(Baaklini et al., 2023). However, microglia proliferate to monopolize a demyelinated lesion(Baaklini et al., 2023; Plemel et al., 2020) and limit MDM accumulation and proliferation(Plemel et al., 2020). Surprisingly, microglia depletion did not slow myelin debris clearance, because the loss of microglia resulted in more MDM phagocytosis(Baaklini et al., 2023). However, microglial depletion reduces OPC recruitment, differentiation, and remyelination demonstrating that they have non-redundant roles during remyelination(Baaklini et al., 2023).

One potential alternative role of microglia is the scavenging of free radicals. Microglia also scavenge end products of oxidative stress such as oxidized phosphatidylcholine (OxPC) that are capable of neurodegeneration(Dong et al., 2021). Whether there is meaningful OxPC accumulation that impairs remyelination is currently unclear. Therefore, the innate immune response is necessary for remyelination. Both microglia and MDMs contribute to remyelination with overlapping and non-overlapping roles. In the following sections, we discuss the roles of microglia and MDMs in detail.

a) Microglia and MDMs support remyelination by clearing myelin debris.

As mentioned earlier, microglia and MDMs phagocytose myelin debris following demyelination which is crucial for OPC recruitment and their differentiation into remyelinating oligodendrocytes(Neumann et al., 2009). Myelin debris inhibits remyelination(Kotter et al., 2006; Plemel et al., 2013; Syed et al., 2016). Recently, we found that microglia and MDM engulf approximately equal amounts of myelin debris after acute demyelination in the LPC model (Figure 1-2)(Baaklini et al., 2023). Microglia and MDMs express genes related to phagocytosis and lysosomal pathways post-myelin damage(Hammond et al., 2019; Plemel et al., 2020), suggesting that they adopt a phenotype characterized by phagocytosis. Myelin debris is likely a contributor to delayed remyelination for people with MS, given that remyelination is slower in myelin-rich white matter (WM) relative to grey matter (GM) (Chang et al., 2012). These WM microglia become enriched with lipid metabolism pathways, consistent with debris clearance, while GM microglia during remyelination express enhanced glycolytic pathways (van der Poel et al., 2019), suggesting a region-specific difference in the microglial response during remyelination.

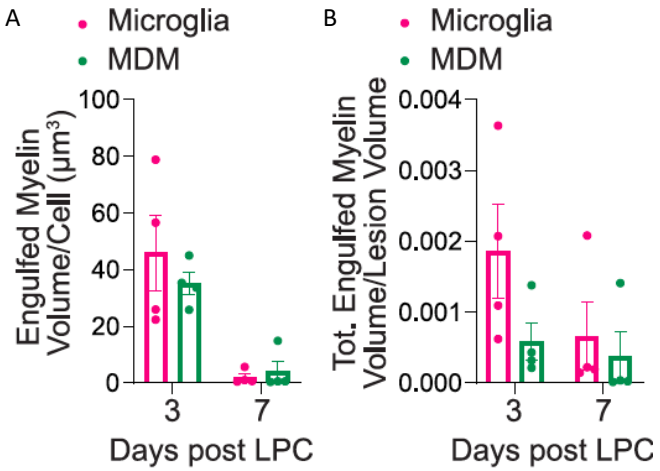

Figure 1-2: **Microglia and monocyte-derived macrophages (MDMs) engulf equal amounts of myelin debris** (Baaklini et al., 2023)
(A)We quantified microglial and MDM intracellular myelin basic protein (MBP) volume on a per-cell basis in LPC-induced spinal cord lesions of 6- to 8-week-old CX3CR1^{CreEr}; ROSA26^{tdT} mice. Total microglial (tdT+) intracellular MBP volume was divided by the DAPI count inside tdT+ 3D surface. Total MDM (tdT CD45+) intracellular MBP volume was normalized to DAPI count inside tdT CD45+ 3D surface.
(B)We also quantified microglial and MDM intracellular MBP volume and divided it by total lesion volume.

Myelin debris clearance is regulated by receptor systems expressed by microglia and MDM. The lipid-sensing triggering receptor expressed on myeloid cell 2 (TREM2) is critical for myelin debris phagocytosis by microglia and MDMs(Cantoni et al., 2015; Nugent et al., 2020; Poliani et al., 2015). Microglia lacking TREM2 fail to increase critical cholesterol metabolism and lipid trafficking genes, leading to the accumulation of lipids within microglia eventually increasing endoplasmic reticulum (ER) stress(Gouna et al., 2021; Nugent et al., 2020). TREM2 is highly expressed in microglia that phagocytose myelin in active MS lesions(Cignarella et al., 2020; Nugent et al., 2020; Voss et al., 2012). Loss of TREM2 reduces myelin debris clearance in the cuprizone model of

demyelination administration(Cantoni et al., 2015; Poliani et al., 2015). Studies have found that TREM2 agonism *in vivo* elevates the efficiency of myelin clearance, as depicted by increased myelin phagocytosis and intracellular degradation after demyelination(Bosch-Queralt et al., 2021; Cantoni et al., 2015). Treatment with TREM2 agonists increases microglia expression of phagocytic and lysosomal proteins and promotes oligodendrocyte differentiation and remyelination(Cignarella et al., 2020). CX3CR1 is another receptor critical for myelin debris phagocytosis. It is a chemokine receptor expressed on microglia that binds to fractalkine (CX3CL1) and recruits microglia to sites of inflammation(Hickman et al., 2019). CX3CR1 knockout mice show failure in myelin debris clearance following cuprizone-induced demyelination(Lampron et al., 2015).

b) Microglia secrete pro-remyelinating factors

Microglia release various cytokines, chemokines and growth factors, that signal onto oligodendrocyte lineage cells(Baaklini et al., 2019). Tumour necrosis factor-α(TNF-α) a proinflammatory cytokine is secreted by microglia following cuprizone-induced demyelination in mice to promote OPC expansion and remyelination via TNF receptor 2 (TNFR2)(Arnett et al., 2001; Voss et al., 2012). Transglutaminase 2(TG2), which interacts with OPC-specific Adhesion G Protein-Coupled Receptor G1 (ADGRG1), is also secreted by microglia to promote OPC proliferation(Giera et al., 2018). OPC proliferation requires platelet-derived growth factor AA (PDGF AA) and its receptor (PDGFRα)(Murtie et al., 2005; Richardson et al., 1988) during development and remyelination. Sherafat and colleagues found Neuropilin-1 (Nrp1), a type-I transmembrane molecule, is expressed on ameboid and reactive microglia in the

demyelinated corpus callosum and promotes OPC proliferation by activating PDGFRα on OPCs(Sherafat et al., 2021). Taken together, these findings indicate that microglia secrete several factors that boost the expansion of OPCs in demyelinating conditions.

Following OPC proliferation, microglia also promote their differentiation into mature oligodendrocytes by secreting factors such as insulin-like growth factor 1 (IGF-1) (Suh et al., 2013)and activin-A(Dillenburg et al., 2018; Miron et al., 2013). Microglia also secrete interleukin-1β (IL-1β), which upregulates the expression of IGF-1 during remyelination in cuprizone-treated mice. IGF-1 supports oligodendrocyte lineage cell survival(Mason et al., 2001). The removal of the activin-A receptor from OPCs during development impairs both OPC differentiation and myelination, suggesting that activin-A receptor signalling is necessary for myelination(Dillenburg et al., 2018). Taken together, microglia secrete several factors that facilitate remyelination, either by promoting the proliferation of OPCs or by promoting differentiation of OPCs into myelinating oligodendrocytes. Recently, the node of Ranvier was identified as a major site for microglia-neuron interaction during remyelination(Ronzano et al., 2021). Thus, microglia play a crucial role in remyelination by releasing factors that enhance microglia-oligodendrocyte lineage cells and microglia-neuron interactions. However, in the studies mentioned above, the markers used to characterize microglia are also expressed by MDMs and thus, there is no clear distinction between microglia and MDM populations (Figure 1-3, adapted from a book chapter that I had contributed to(Brady et al., 2022)).

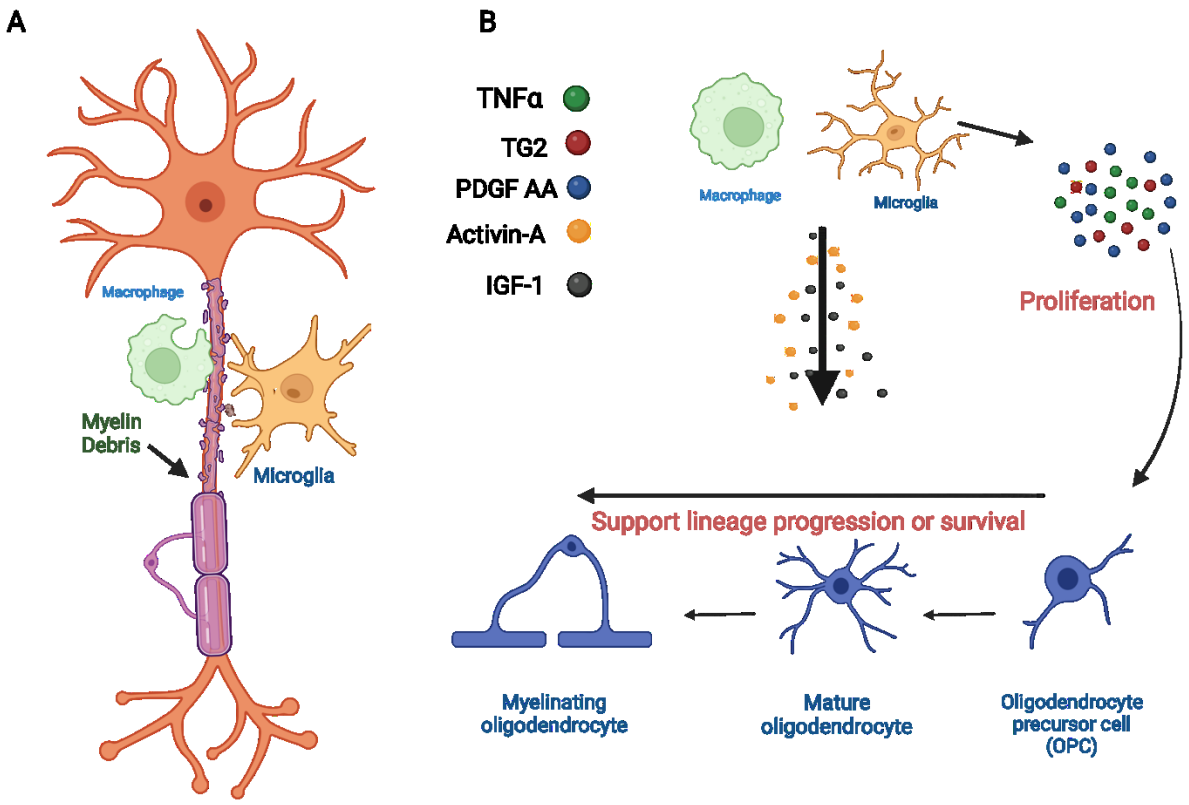

Figure 1-3: Role of microglia/MDMs in remyelination
(A)Microglia and monocyte-derived macrophages (MDM) clear myelin debris which is a known remyelination inhibitor.
(B)Microglia/MDMs secrete factors required for OPC proliferation and their maturation to myelinating oligodendrocytes.
Made with BioRender (Adapted from the section: Microglia. Physiology, pathophysiology and therapeutic potential, from the book: Microglia and Multiple Sclerosis (2022)).

1.4 Aging delays remyelination

Myelin debris clearance by microglia in demyelinating lesions decreases with age and this impaired debris clearance is associated with slower remyelination(Rawji et al., 2018; Rawji et al., 2020a; Ruckh et al., 2012). Rejuvenation of remyelination by heterochronic parabiosis enhances debris clearance together with remyelination(Ruckh et al., 2012). In this study, heterochronic (young and old pairs) were compared to isochronic old and young pairs. They found that young macrophages are more efficient in clearing

myelin debris as compared to aged macrophages. Within the heterochronic pairs, the MDM of young mice enhanced the clearance of myelin debris in aged mice and this debris clearance partially rejuvenated oligodendrocyte differentiation in aged mice. Thus, aged MDMs are impaired in their capacity to clear myelin debris, contributing to age-associated delayed remyelination.

Live imaging of spinal cord explants from demyelinated lesions in young and middle-aged mice demonstrates that reduced myelin debris clearance with aging coincides with a reduction in the recruitment, surveillance, and phagocytosis by microglia (Rawji et al., 2018). These alterations in microglial dynamics may be associated with reduced expression of microglia genes related to motility and phagocytosis with age (Galatro et al., 2017) or accumulation of myelin debris within microglia that may lead to saturation(Safaiyan et al., 2016).

Several studies indicate the involvement of NLRP3 (NOD-like receptor protein 3) inflammasome activity in the aging process and inhibition of inflammasome activity mitigates inflammation and slows down age-associated degeneration in various disease models(Liang et al., 2024). The activation of the NLRP3 inflammasome occurs not only in reaction to tissue damage and infection but also in conjunction with the aging process(Holbrook et al., 2021). The NLRP3 inflammasome is a multiprotein complex that is activated by a two-step mechanism. Initially, cells detect signals from pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs) using receptors such as Toll-like receptor 4 (TLR4), which triggers the synthesis of sensors, primarily NLRP3. In the subsequent phase, various cellular processes, like membrane damage or lysosomal rupture, facilitate the assembly of the NLRP3 inflammasome complex. Throughout this sequence, NLRP3 undergoes de-ubiquitination

and binds with ASC, which then interacts with pro-caspase-1, leading to the formation of the large multimeric protein complex known as the NLRP3 inflammasome. Active caspase-1, generated by this complex, cleaves pro-IL-1β to produce its mature and secreted form, IL-1β, thereby triggering a robust inflammatory reaction(Liang et al., 2024; Meyers & Zhu, 2020).

In aged mice, microglia and MDMs accumulate excessive cholesterol-laden myelin debris following acute demyelination, much like a foamy macrophage within the atherosclerotic plaque, which ultimately results in cholesterol crystal formation, phagolysosomal membrane rupture, and subsequent activation of the NLRP3 inflammasome which leads to caspase-1 dependent release of the pro-inflammatory cytokine IL-1β and pyroptotic cell death(Cantuti-Castelvetri et al., 2018). Aged NLRP3 deficient mice, however, show improved remyelination. Impaired debris clearance, therefore, triggers heightened inflammation via NLRP3 activation and impairs remyelination(Cantuti-Castelvetri et al., 2018). Taken together, clearing myelin debris is a critical step towards remyelination, which becomes more inefficient with age resulting in unresolved inflammation.

Pharmacological strategies that enhance myelin debris clearance rejuvenate remyelination in middle-aged mice. For example, liver X receptor agonists promote reverse cholesterol transport (Cantuti-Castelvetri et al., 2018) and retinoid X receptor agonists stimulate myelin engulfment (Natrajan et al., 2015), both of which accelerate remyelination in aged mice(Cantuti-Castelvetri et al., 2018; Huang et al., 2011). Therefore, boosting myelin debris clearance is a therapeutic strategy to improve remyelination.

The aged environment plays a vital role in regulating the remyelination process(Ruckh et al., 2012). With slower myelin debris clearance and accumulation of oligodendrocyte lineage

cells(Sim et al., 2002), studies have also indicated a slower immune response in the lesion area in middle-aged animals. In an earlier study, Chao Zhao, and colleagues (Zhao et al., 2006) characterized microglia and macrophage response in LPC-induced lesions in young and old rats. They found a delayed microglia and macrophage response in old rats during remyelination as compared to young rats. This was expanded upon more recently by Rawji and colleagues(2020b) who found that middle-aged mice have significantly fewer MDMs 3 days after LPC injection (DPL). Taken together, aging is associated with inefficient myelin debris clearance, and delayed accumulation of microglia/MDMs followed by slower accumulation of oligodendrocyte lineage cells. With these processes being crucial during remyelination, we considered the possibility of the age-related environmental factors that interfere with these processes.

1.5 ROS- is it an inhibitor of remyelination?

Reactive Oxygen Species (ROS) refers to small oxygen-derived molecules which include oxygen radicals such as superoxide and hydroxide and non-radicals such as hydrogen peroxide, hydrochlorous acid and ozone(Panday et al., 2015). Major endogenous sources of ROS include enzymes such as NADPH oxidases (NOX), and mitochondrial electron transport chain (ETC)(de Almeida et al., 2022). Other endogenous sources of ROS include uncoupled nitric oxide (NO)synthase, cytochrome p450, xanthine oxidase (XO), the endoplasmic reticulum (ER), peroxidases, lipoxygenases (LOX)and cyclooxygenases(COX)(Cervantes Gracia et al., 2017; de Almeida et al., 2022; Förstermann et al., 2017). Xanthine oxidase (XO)facilitates the last two steps of purine metabolism which converts hypoxanthine to xanthine and then xanthine to uric acid

through oxidative hydroxylation(Schmidt et al., 2019). Oxygen rapidly accepts electrons produced during the oxidation of hypoxanthine and xanthine by XO, leading to the formation of superoxide radical and hydrogen peroxide(George & Struthers, 2009). With aging, XO levels increase, contributing to oxidative stress, immunosenescence, and inflammation(Battelli et al., 2020).Cyclooxygenase (COX) metabolizes arachidonic acid to produce prostaglandin precursors that play roles in inflammation, ROS production, and lipid oxygenation(Tada & Suzuki, 2016). There are two forms of COX: COX-1 and COX-2. COX-1 is mostly present in all tissues and is involved in normal physiological functions. COX-2, on the other hand, is found in several tissues such as the kidney, gastrointestinal tract, brain, lungs, and thymus, and its production can be stimulated in various tissues during inflammation(Mitchell & Kirkby, 2019). Lipoxygenases (LOX) are a group of enzymes with central iron atoms that oxidize polyunsaturated fatty acids, primarily arachidonic acid, to produce various hydroperoxides, which are by-products of the ROS-mediated lipid peroxidation(Zhong et al., 2019). The formation of ROS happens when the Fe^{3+} in activated LOX is reduced upon interaction with its substrate (Watanabe et al., 2018).ROS production is also triggered by exogenous sources such as pollution, UV radiation, smoking and drugs(Sies & Jones, 2020).

Mitochondrial ROS production mainly occurs at the electron transport chain (ETC) located on the inner mitochondrial membrane during oxidative phosphorylation. Leakage of electrons from Complex I and III leads to the superoxide radical(Li et al., 2013).In 1954, Denham Harman proposed the free radical theory of aging, which linked the mitochondrial production of ROS with aging(HARMAN, 1956). In 1972, this theory was revised and was known as the mitochondrial free radical theory of aging, linking

age-related mitochondrial dysfunction to heightened ROS production that possibly reduced the lifespan of an individual(Harman, 1972). However, this theory was discarded when it was found that increasing ROS inhibition via overexpression of antioxidant enzymes in *Drosophila melanogaster* does not affect the lifespan of the organism(Mockett et al., 2010).

However, elevated ROS production with aging causes oxidative damage to DNA genomes, proteins, and ultimately cellular senescence or death(Bokov et al., 2004). In the context of MS, oxidative damage caused by ROS was detected in the form of lipid peroxidation; oxidized phospholipids (OxPC) marked by E06 immunoreactivity and malondialdehyde (MDA) in neurons, and damaged DNA(8-OHdG) in oligodendrocytes in active lesions of human MS tissue(Haider et al., 2011).

Lipid peroxidation is an oxidative chain reaction wherein ROS attack lipids containing carbon-to-carbon double bonds, such as polyunsaturated fatty acids (PUFA), that leads to the formation of lipid peroxyl radicals and hydroperoxides (Yin et al., 2011). Increased lipid peroxidation signifies elevated ROS levels, thus middle-aged mice exhibit heightened ROS production during remyelination. Malondialdehyde is a byproduct of lipid peroxidation and has been widely used in various studies as a measure to evaluate ROS production(Michaels et al., 2020).

In MS autopsy specimens, another major endogenous source of ROS-NADPH oxidase (NOX) is found enriched in areas of lipid peroxidation(Fischer et al., 2013). NADPH oxidase is a multiunit enzyme complex consisting of gp91phox, p22phox, p40phox, p47phox and p67 phox. Based on various homologs of the gp91phox subunit, the NADPH oxidase (NOX) “family” consists of different isoforms of the enzyme: NOX1, NOX2, NOX3, NOX4, NOX5, Dual

oxidase Duox proteins (Duox1 and Duox2) (Panday et al., 2015). NOX2 was the first identified NADPH oxidase and NOX1 was the first identified homolog. NOX1 shares a 60% amino acid sequence with NOX2(Bánfi et al., 2000; Panday et al., 2015; Suh et al., 1999).NOX1, NOX2 and NOX3 are typically localized in the plasma membrane, whereas NOX4 can be found in the endoplasmic reticulum and nucleus in addition to the plasma membrane(Sies & Jones, 2020). NOX5 is found in the endoplasmic reticulum and Duox1 and Duox 2 are found in the plasma membrane(Sies & Jones, 2020). In the CNS, NOX1 and NOX2 isoforms can be found in microglia(Chéret et al., 2008; Hu et al., 2021; Michaels et al., 2020) and NOX4 can be found in neurons(Gao et al., 2012; Vallet et al., 2005) and microglia(Li et al., 2009).

NOX2 subunits such as gp91phox and p22phox are membrane and p40phox, p47phox and p67phox exist in the cytosol as a complex. Upon stimulation p47phox undergoes phosphorylation and p40phox, p47phox and p67phox translocate to the membrane, thereby forming the entire activated NOX2 complex(Panday et al., 2015). Activation of NOX1 involves a similar mechanism as NOX2 whereas NOX4 activation relies solely on p22phox and not cytosolic components(Panday et al., 2015).

In human MS tissue, immunohistochemistry revealed the colocalization of NOX1 and NOX2 subunits with Iba1+/CD68+ microglia(M. T. Fischer et al., 2012). Like MS tissue, heightened lipid peroxidation and NOX2 subunit gp91phox (NOX 2 isoform) expression are found after LPC-induced demyelination in middle-aged mice (Michaels et al., 2020).

Michaels and colleagues found the upregulation of *Cybb*—the gene encoding gp91phox— and gp91phox was localized to middle-aged microglia (Figure 1-4)(Michaels et al., 2020). Therefore, the convergence of white matter injury and age causes an increased expression of this ROS-producing enzyme.

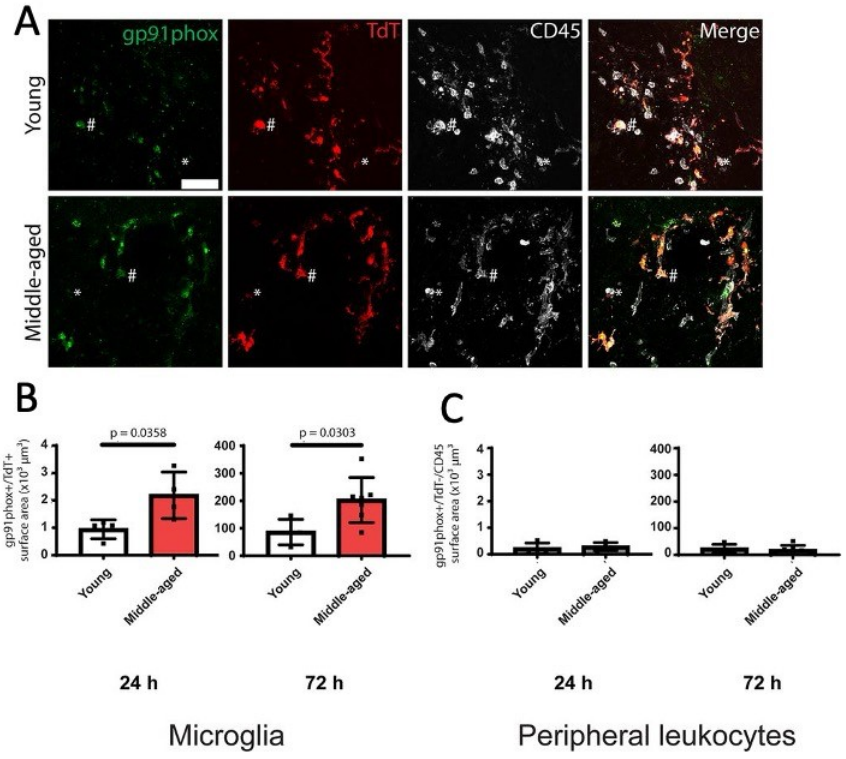

Figure 1-4: Upregulation of gp91phox is associated with aged microglia in the LPC-induced demyelination model.

(A)Representative images of CX3CR1^{CreEr}; ROSA26^{tdT} mice stained with antibodies against gp91phox (NOX2) and pan leucocyte marker CD45. After 4-6 weeks of tamoxifen injection, microglia are labelled with tdTom. However, this lineage tracing method also labels border-associated macrophages (BAMs), discussed later in the chapter-Material and Methods

(B)Quantification of gp91phox+tdt+ cells per unit area of the lesion. Microglia predominantly express gp91phox indicating that microglia are the primary ROS-producing cells at acute demyelination(3DPL)

(C) Quantification of gp91phox+tdt- cells per unit area of lesion. Upregulation of gp91phox is minimal in peripheral leukocytes and shows no difference in young and old mice. (Michaels et al., 2020)

Heightened ROS production could be a potential roadblock to remyelination as it impedes the transition of OPCs into myelinating oligodendrocytes(Spaas et al., 2021). ROS released by microglia/MDMs damages surrounding oligodendrocyte lineage cells (OLs)(Li et al., 2005). Various studies have elucidated the vulnerability of OLs and OPCs to oxidative injury caused by elevated ROS levels(Back et al., 2002; Bernardo et al., 2003; Giacci & Fitzgerald, 2018; Haider et al., 2011; Miyamoto et al., 2013; van Horssen et al., 2008). Suppressing ROS through radical scavenging rescued OPC proliferation and differentiation, thus promoting white matter repair in an animal model of cerebral hypoperfusion(Miyamoto et al., 2013). In the cuprizone model of demyelination, NOX4 deficient mice show improved myelin debris clearance by microglia/MDMs, OPC proliferation and locomotor function(Yamanaka et al., 2023). Thus, ROS negatively impacts OPC proliferation and differentiation to mature OLs and in the context of age-associated delayed myelin repair, it may be a novel remyelination inhibitor since middle-aged animals show higher ROS production in the LPC model of demyelination(Michaels et al., 2020).

1.6 Hypothesis and Research Aims

As described in previous sections, remyelination is crucial for myelin repair, lesser axonal degeneration, and functional recovery(Franklin & Ffrench-Constant, 2017). However, the onset of remyelination is delayed in middle-aged animal models of MS(Rawji et al., 2020b; Shields et al., 1999; Sim et al., 2002). ROS affects remyelination by blocking the transition of OPCs into myelinating OLs(Spaas et al., 2021) and aged animal models of MS show exacerbated ROS production(Michaels et al., 2020). Taken together, we hypothesize that ROS delays

remyelination in middle-aged mice and that by inhibiting ROS production with the NOX1/4 isoform inhibitor, Setanaxib (Aoyama et al., 2012), we will boost remyelination in aged mice.

To test our hypothesis, I examined ROS production in both young and middle-aged $CX3CR1^{CreEr}$ $ROSA26^{tdT}$ mice at different time points post-LPC injection, that represent the remyelination timeline. Microglia and MDMs play a crucial role in ROS production to toxic levels via ROS-producing enzyme NADPH oxidase (NOX)(Qin et al., 2013). In the context of MS, autopsy samples from people with MS demonstrate microglia/MDMs as common sources of ROS (Marie T. Fischer et al., 2012). In middle-aged animal models of MS, microglia/MDMs exaggerate ROS production following LPC-induced demyelination(Michaels et al., 2020). Given that these cells are highly reactive during remyelination and may also contribute to ROS production, I also evaluated their contribution in our animal model by quantifying microglia and MDM cell densities in the lesion area at 3,7 and 21DPL.

To reduce ROS production to toxic levels in aged mice, I orally administered NOX 1/4 isoform inhibitor, Setanaxib(GKT137831), to middle-aged C57BL/6 mice for 6 consecutive days following intraspinal LPC injection. As mentioned earlier, the NOX1 isoform is expressed by microglia/macrophages in human MS tissue(M. T. Fischer et al., 2012) and NOX4 knockout mice show enhanced myelin debris clearance and OPC proliferation that is crucial for remyelination(Yamanaka et al., 2023). Therefore, I used NOX1/4 inhibition as a strategy to evaluate its impact on remyelination.

The animals were then euthanized at 7DPL and spinal cord tissues were collected for further tissue processing and immunohistochemical analyses (explained in Chapter 2 Methods) to assess the impact of Setanaxib on ROS production and remyelination. I investigated

microglia/MDM cell densities, myelin debris clearance and OPC and OLs densities to see if inhibiting ROS by Setanaxib boosts remyelination.

CHAPTER 2: Materials and Methods

2.1 LPC model of demyelination

We used the LPC (lysophosphatidylcholine /lysolecithin) model as described in the methods of various studies using the toxin model of demyelination(Baaklini et al., 2023; Keough et al., 2015; Michaels et al., 2020; Plemel et al., 2020). Since the 1970s(Hall, 1972), it has been widely used to study remyelination following toxin-induced demyelination, due to its precise temporal regulation and defined anatomical location(Plemel et al., 2018). LPC injection into the spinal cords of old rats displays inefficient remyelination as compared to their younger counterparts(Gilson & Blakemore, 1993). In this study, we target the spinal cord white matter as they are virtually all myelinated, unlike the corpus callosum which consists of 50-70% unmyelinated axons and can interfere with the interpretation of remyelination(Lai & Lewis, 1980).

Intra-spinal LPC injection induces a primary myelinopathy within hours which spares many axons(Plemel et al., 2018). Acute demyelination is observed within 1-3 days post LPC injection (DPL) in young mice followed by OPC recruitment that occurs by 7-10 DPL. The process of remyelination is then completed by 21DPLin young mice(Jeffery & Blakemore, 1995; Keough et al., 2015). LPC-induced demyelination triggers rapid MDM/microglial response. However, microglia monopolize the lesion environment in the later stages of injury(Baaklini et al., 2023; Plemel et al., 2020). Various studies have investigated the lesion microenvironment in middle-aged mice after LPC induced demyelination and it was found that middle-aged mice display fewer phagocytic myeloid cells, greater myelin disruption volume and axonal loss at 3DPL(Michaels et al., 2020; Rawji et al., 2018). At 7DPL, young mice show greater microglia/MDM density and enhanced myelin debris clearance as compared to middle-aged

mice(Rawji et al., 2020b), highlighting age-associated decline in immune response and phagocytosis.

In the heterochronic parabiosis study by Julia Ruckh and colleagues, exposure of the aged lesion environment to young macrophages enhances debris clearance together with remyelination in aged mice following LPC induced demyelination(Ruckh et al., 2012). In this study, heterochronic (young and old) pairs show enhanced OPC proliferation at 7DPL, OPC differentiation at 14 and 21 DPL and improved remyelination at 21DPL as compared to isochronic old (old and old) pairs. Isochronic old pairs show reduced OPC proliferation at 7DPL, OPC differentiation at 14 and 21DPL and remyelination as compared to isochronic young(young and young) pairs. Thus, in the LPC model of demyelination, aging is associated with delayed microglia/MDM recruitment in the lesion, inefficient phagocytosis of myelin debris, delayed OPC recruitment and differentiation and reduced remyelination as compared to young controls.

2.2 Lineage tracing strategy to distinguish microglia from MDMs

As microglia predominantly drive immune response in LPC-induced lesions at later stages of injury(Baaklini et al., 2023; Plemel et al., 2020), we used the CX3CR1^{CreEr} transgenic line crossed with ROSA26^{tdT} (Baaklini et al., 2023; Michaels et al., 2020; Plemel et al., 2020) to distinguish microglia from MDMs and elucidate age-dependent microglial response in the context of remyelination. ROSA26 is a genomic mouse locus commonly used for ubiquitous or conditional gene expression of cDNA constructs in transgenic animals(Hohenstein et al., 2008).

In this lineage tracing strategy, after 4-6 weeks of postnatal administration of tamoxifen(P13-P15), microglia and their progeny are permanently labelled with tdTomato that does not change during stages of injury (Figure2-1)(Baaklini et al., 2023). However, a limitation of this strategy is that it also labels border-associated macrophages (BAMs)(Baaklini et al., 2023;

Goldmann et al., 2016; Kierdorf et al., 2019; Prinz et al., 2017; Yona et al., 2013). However, in the context of LPC-induced lesions, the cells expressing tdTomato fluorescence are primarily microglia and there is less involvement of BAMs in the lesions(Baaklini et al., 2023; Plemel et al., 2020).

For microglia/MDM density analyses, I evaluated microglia based on tdTomato expression in these CX3CR1^{CreEr} ROSA26^{tdT} mice. Microglia were defined based on the expression of tdTomato (tdT) and the pan-leukocyte marker CD45, while monocyte-derived macrophages (MDM) were defined as CD45-positive and tdTomato negative. This is further described in detail in the 'Image acquisition and quantification' section.

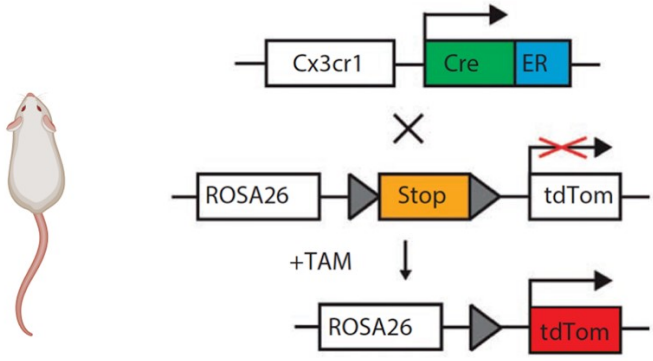

Figure 2-1: ***Schematic 1***
We used the CX3CR1^{CreEr} transgenic line crossed with ROSA26^{tdT} to lineage trace and visualize microglia and to distinguish them MDMs. Upon tamoxifen (TAM)injection (P13-15) it expresses tdTomato (tdT) in all CX3CR1 expressing cells under the ROSA26 promotor. However, after 4-6 weeks of tamoxifen injection only microglia and BAMs retain tdT flouroscence due to their capacity to self-renew. Therefore, this lineage tracing strategy is efficient in differentiating microglia from MDMs.

2.3 Experimental methods

1. Animals

For microglial lineage tracing experiments, we purchased the following mice from The Jackson Laboratory: CX3CR1^{CreEr}MGI: J:190965 and ROSA26^{tdT}. We used mice 6–8 weeks of age for breeding. We bred ROSA26^{tdT} with CX3CR1^{CreEr} to get, CX3CR1^{CreEr} +/-; ROSA26^{tdT}/-. We housed animals in the University of Alberta Health Sciences Lab Animal Services (HSLAS) Facility under a 12-h light/dark light cycle, 20ºC–24ºC temperature range, 40–70% humidity range, and pathogen-free conditions. We used both male and female CX3CR1^{CreEr}; ROSA26^{tdT} mice for our lineage tracing experiments as we wanted to focus on age-dependent microglia response and ROS production post-LPC induced demyelination irrespective of the sex of the animals.

For Setanaxib experiments, we purchased middle-aged female C57BL/6 mice from Charles River Canada and housed them in the University of Alberta Health Sciences Lab Animal Services (HSLAS) Facility under a 12-h light/dark light cycle, 20ºC–24ºC temperature range, 40–70% humidity range, and pathogen-free conditions. We used female mice for all the Setanaxib experiments as women are three times more likely to be affected by RRMS and SPMS (Cottrell et al., 1999; Kamma et al., 2022)

2. Tamoxifen dosage

For microglial lineage tracing experiments, we dissolved tamoxifen in corn oil to a concentration of 20 mg mL^{-1}. We injected all pups intraperitoneally at postnatal days 13–15 (P13-P15) with 0.05 mL tamoxifen for three consecutive days.

3. Setanaxib dosage

To make a stock solution, we dissolved 200mg of Setanaxib (GKT137831, MedChemExpress(Catalogue no: HY-12298))in 1ml DMSO and sonicated the solution for 5-10 minutes to be fully dissolved. To make a 25 ml working solution, 10 ml PEG300, 1.25 ml Tween80, and 13.125 ml Saline were added to 0.625 ml stock solution. The working solution of Setanaxib and vehicle(control) was administered daily via oral gavage to 10-month-old middle-aged female C57BL/6 mice for 6 consecutive days post LPC injection.

4. Lysophosphatidylcholine (LPC) injection into the ventral spinal cord

As described in our previous work(Baaklini et al., 2023), we anesthetized 6–8-week-old and 8-10-month-old mice by intraperitoneally injecting a cocktail of NMDA receptor agonist ketamine (100 mg kg^{-1}), a2 adrenergic receptor agonist xylazine (5 mg kg^{-1}) dissolved in sterile saline (0.9%). The mice were allowed to reach the surgical plane of anesthesia before the surgical area was trimmed. We disinfected the skin with chlorhexidine digluconate solution (diluted to 10%) and applied eye lubricant (Optixcare Eye Lube) to cover both mouse eyes. On a stereotaxic frame, the mice were placed and the T3-T4 spinal cord region was surgically exposed. We then injected LPC (0.5 mL, 1%) at a rate of 0.25 mL per minute into the ventral white matter of the spinal cord. The incision was sutured using (Vicryl type IV curved, tapered), and the mice were subcutaneously administered slow-release buprenorphine (0.5 mg kg^{-1}), sterile saline (0.9%), and atipamezole (1.67 mg kg^{-1}) for ethical recovery. The mice were monitored daily for 7-10 days (or till an earlier experimental endpoint). With this model, 1-3 days post LPC injection (DPL) is characterized by acute demyelination followed by the onset of remyelination via OPC proliferation and differentiation that occurs by 7-10 DPL in young mice.

This is followed by myelin ensheathment and completed remyelination by 21DPL in young mice(Jeffery & Blakemore, 1995; Keough et al., 2015).

5. Tissue processing

Following our standard lab protocol as described in(Baaklini et al., 2023), at defined experimental time points post-LPC injection, we euthanized mice by injecting sodium pentobarbital (Euthanyl) intraperitoneally. In the surgical plane of anesthesia, the mice were transcardially perfused with 20 mL PBS (0.01M) followed by 20 mL of ice-cold 4% paraformaldehyde (PFA). We collected the spinal cords and fixed these tissues overnight in an excess of PFA at 4ºC. The tissue was then cryoprotected by submerging it in a sucrose solution (30% in 0.01M PBS) for a minimum of 24 hours at 4ºC. We embedded the tissue in an optimum cutting temperature (O.C.T.) solution followed by flash freezing using dry ice and 2 - methylbutane and stored at 80ºC. We then sectioned the tissue at 20 μm thickness with a cryostat (Leica #CM1950) and mounted it on microscope slides (Fisherbrand Superfrost #22-037-246).

6. Immunohistochemistry

Slides were stained using our lab's standard IHC protocol. At first, they were rehydrated in 1X PBS for 10 mins. Then, we added donkey serum to block non-specific binding for 30-45 minutes. Primary antibodies were added to the slides at optimum concentration and incubated overnight at 4ºC. The slides were washed thrice in PBS-Tween20, followed by incubation with a secondary antibody added. It was incubated for 2 hours and then washed and mounted using Flouromount-G. We used the following primary antibodies as markers for leukocytes(rat-CD45,1:100,BD Pharmingen), microglia/MDMs(rabbit-IBA1,1:500,Wako;goat-IBA1,1:500,Novus Bio),MBP(rabbit-MBP,1:400,Abcam),OPCs (goat- PDGFRα,1:400,Novus Bio), proliferation (rat-Ki67,1:200,e-Bioscience), oligodendrocyte lineage cells (rabbit-Myrf,

1:1000,Abclonal), E06(rabbit-E06,1:100, Absolute Antibody), Malondialdehyde(rabbit-MDA,1:100,Abcam). The following secondary antibodies of matching species were used at 1:400 dilution (purchased from Jackson ImmunoResearch): 647 Alexa Fluor Anti-Rabbit, 488 Alexa Fluor Anti-Rat, 488 Alexa Fluor Anti-Goat, 594 Alexa Fluor Anti-rat. We added DAPI in 1:1000 along with other secondary antibodies.

7. Image acquisition and quantification

Following immunostaining, slides were imaged on a Zeiss LSM710 20x and Leica Falcon SP8 laser scanning confocal microscope 25x. Images were saved as .lsm files for analysis using the image processing software Fiji(Schindelin et al., 2012). All image acquisition and analyses were done blind to experimental conditions. For microglial and MDM density analysis, we used a semi-automated approach as described in the methods of our previous work(Baaklini et al., 2023). We first converted images to tiff format, followed by background noise reduction using noise2noise (Varun et al., 2022)and rolling ball algorithms. We then thresholded the nuclear signal (DAPI) and marked the centre of the nucleus using the Ultimate Points feature in Fiji. For microglia counts ($DAPI^{+}tdT^{+}$), we thresholded the tdT signal and a tdT threshold ROI(region of interest) was created. The DAPI points that fell outside the tdT ROI were deleted. We used the Analyze Particles feature in Fiji to count the DAPI points that were within the tdT ROI.

For MDM counts, we used the tdT ROI and the DAPI points that fell inside the tdT ROI were deleted. We then thresholded the CD45 signal followed by creating a CD45 threshold ROI and deleted the DAPI points that fell outside the CD45 ROI. We then used the Analyze Particles feature in Fiji to count the DAPI points remaining within the CD45 ROI. The microglial

and MDM cell densities were calculated based on the cell counts and lesion areas. For MDA and OxPC analyses, we used a manual thresholding approach in Fiji(Schindelin et al., 2012) to estimate the percentage area of the lesion that had malondialdehyde or OxPC. For counting MDMs/microglia that had E06 deposition, we used the manual cell counting approach in the Cell counter feature in Fiji. The total cell counts of each type were divided by lesion area to get respective cell densities. We used the same approach in Fiji to quantify proliferating OPCs ($(DAPI^{+}PDGFR\alpha^{+}Ki67^{+})$ and oligodendrocyte lineage cells ($DAPI^{+}Myrf^{+}$).

For the myelin debris engulfment analyses, as described in the methods of our previous work(Baaklini et al., 2023), we used the 3D rendering software IMARIS. We applied image processing algorithms for each channel to reduce background noise-DAPI (median filter#1(3*3*3), median filter#2(5*5*5), median filter#3(5*5*5), MBP (background subtraction, threshold cutoff), IBA1(Gaussian filter, median filter, background subtraction, threshold cutoff) and CD45 (Gaussian filter, median filter, background subtraction, threshold cutoff). Each image used for our analysis was cropped to extract the lesion and a 3D surface was made. We then created colocalization channels of DAPI and CD45; DAPI and IBA1, followed by using a surface creation tool to generate 3D regions of interest (surfaces) from z-stacked fluorescent signals via thresholding. $DAPI^{+}CD45^{+}$ and $DAPI^{+}IBA1^{+}$ 3D surfaces were created. Similarly, an MBP surface was created. Using these 3D surfaces, we then created $CD45^{+}$ and $IBA1^{+}$ cells with DAPI as their nucleus marker. We then calculated the total number of $IBA1^{+}$ and $CD45^{+}$ cells. To estimate phagocytosis by $CD45^{+}/IBA1^{+}$ cells (microglia/MDMs), we evaluated the volume of MBP inside the cells.

8. Statistical analyses

All statistical analyses were performed using GraphPad Prism software (version 10.0.3). The threshold for significance was set at $P<0.05$ for all our experiments. All data have been shown as mean ± standard error. Please refer to figure legends in Chapter 3: Results for specific details. A summary of all experimental methods has been illustrated in Figures 2-2 and 2-3.

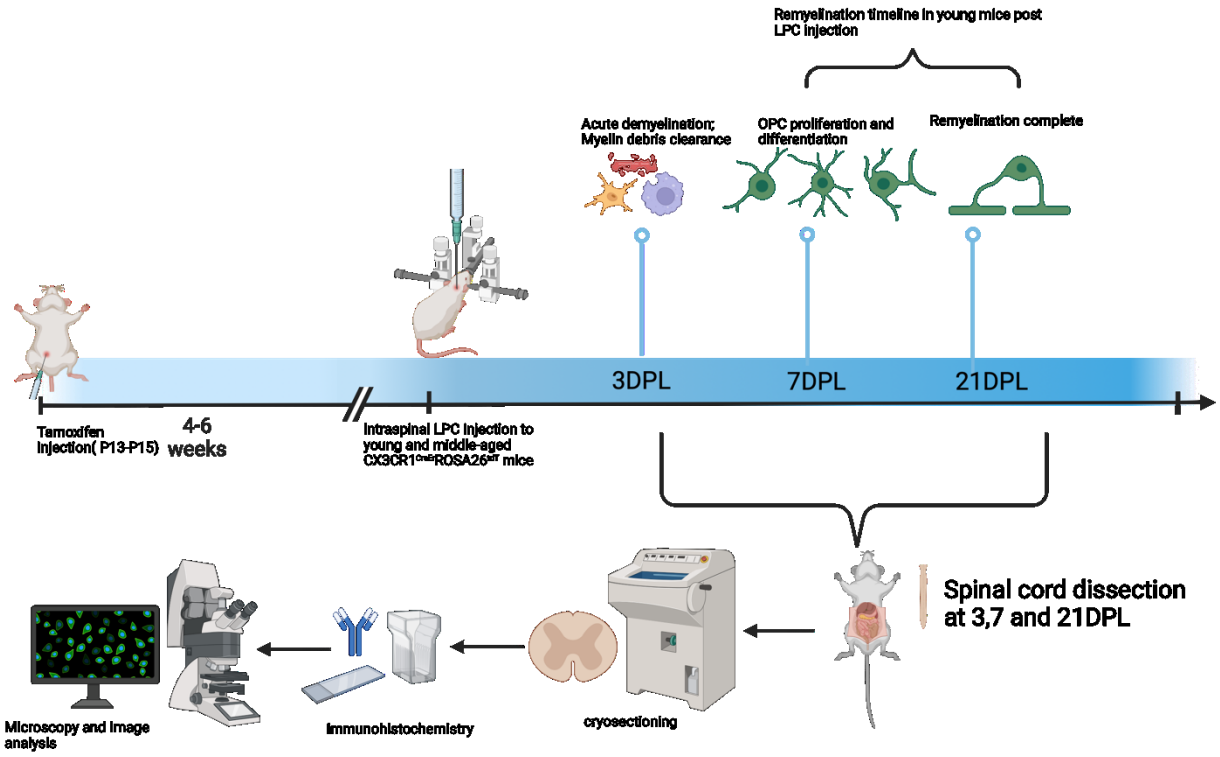

Figure 2-2: ***Schematic 2***
Young (2-3 month old) and middle-aged (8-10 month old) male and female CX3CR1^{CreEr};ROSA26^{tdT} mice were injected with LPC at ventral spinal cord to induce focal demyelination. Spinal cord tissues were then isolated at 3(acute demyelination), 7(early remyelination) and 21DPL, immunostained against lipid peroxidation marker MDA and confocal microscopy was performed to investigate oxidative stress during remyelination in young vs middle-aged mice. Further, immunostaining against suitable markers were performed to assess changes in immune response (if any) in aged mice (details in Chapter 3 Results).

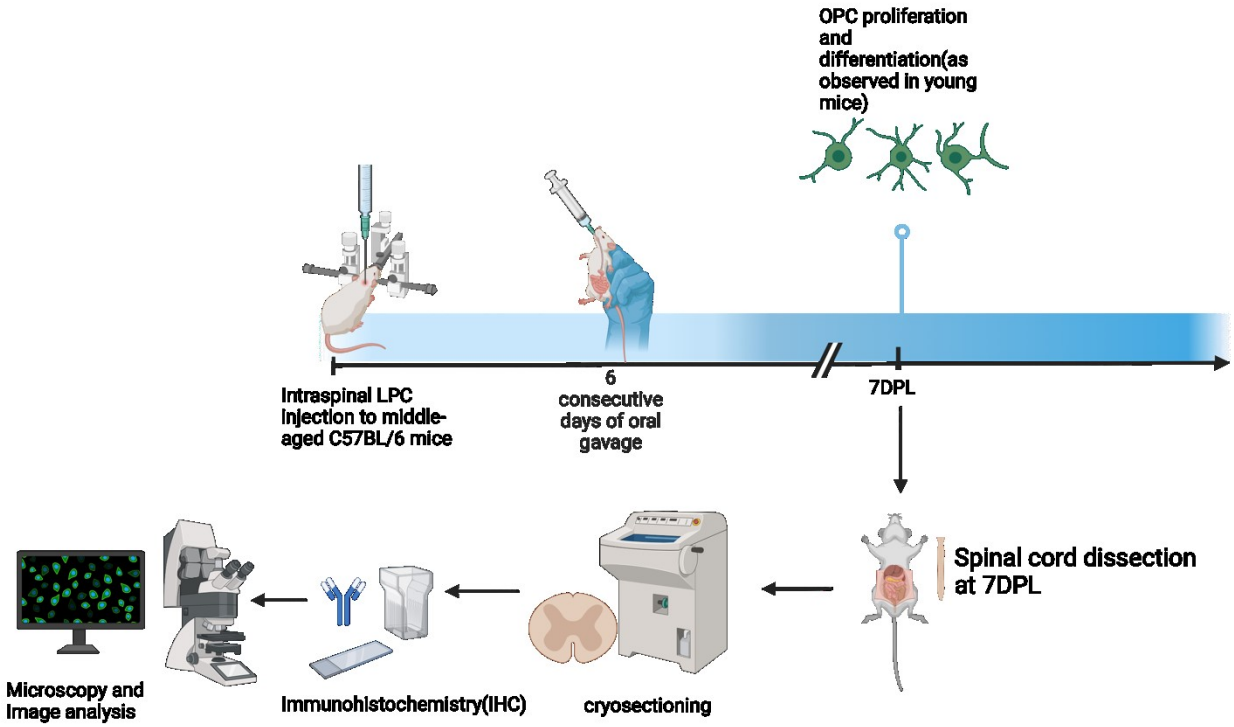

Figure 2-3: ***Schematic 3***
Middle-aged (10-month-old) C57BL/6 mice were injected with LPC intraspinally to induce focal demyelination. Setanaxib(60mg/kg) or Vehicle was administered orally for 6 consecutive days. Spinal cord tissues were then isolated at 7DPL (early remyelination), immune-stained against oxidized phospholipid marker E06, and confocal microscopy was performed to investigate changes in oxidative stress during remyelination in Setanaxib or Vehicle treated mice. Further, immunostaining against suitable markers were performed to evaluate immune response, myelin debris clearance, OPC proliferation, accumulation of mature oligodendrocytes in Setanaxib or Vehicle treated mice (details in Chapter 3 Results), to see if Setanaxib boosts remyelination.

CHAPTER 3: Results

3.1 Results

1. **Lipid peroxidation is exacerbated during remyelination in middle-aged mice**

Remyelination protects axons from degeneration, slowing the permanent disability related to axonal loss(Irvine & Blakemore, 2008). For this reason, strategies to promote remyelination are hoped to mitigate neurodegeneration associated with MS(Plemel et al., 2017)**.** Indeed, people exhibiting more remyelination have less disability(Bodini et al., 2016; S. Bramow et al., 2010). As MS spans decades of life, remyelination in MS must also be considered in the aging context. Age is also directly tied to disability progression: irrespective of disease course, age is the best predictor of MS progression(Confavreux & Vukusic, 2006). Overcoming the age-associated delay in remyelination may, therefore, slow MS progression. One such potential age-associated remyelination inhibitor is reactive oxygen species (ROS). In response to perturbation, microglia and macrophages escalate ROS production to toxic levels with the ROS-producing NADPH oxidase (NOX)(Qin et al., 2013). As part of the aging process, ROS production is enhanced in aged microglia/ macrophages after demyelination(Michaels et al., 2020). Excessive ROS may be a novel remyelination inhibitor as ROS prevents the transition of OPCs into oligodendrocytes(French et al., 2009).

Autopsy samples from people with MS demonstrate that one common source of ROS is microglia and macrophages(Marie T. Fischer et al., 2012) . Given that these cells are highly reactive during remyelination and may also contribute to ROS production, I wanted to evaluate their contribution to our animal model. To differentiate microglia and macrophages I evaluated microglia based on tdTomato expression in CX3CR1^{CreEr} ROSA26^{tdT} mice. Microglia were defined based on the expression of tdTomato (tdT) and the pan-leukocyte marker CD45, while monocyte-derived macrophages (MDM) were defined as CD45-positive and tdTomato negative

(Figure 3-1A). Following LPC injury, the vast majority of leukocytes are microglia and macrophages(Ousman & David, 2000, 2001), and therefore, other non-MDM leukocytes contribute minimally to these assessments. In young mice, MDM density declined between 7 and 21 DPL (Figure 3-1B), whereas in middle-aged mice MDM density did not change between 3 and 21 DPL. These data are consistent with microglial proliferation during remyelination that prevents the further accumulation of MDM(Baaklini et al., 2023; Plemel et al., 2020). MDMs are surrounded by microglia and undergo apoptosis and therefore, microglia monopolize the lesion environment following LPC-induced demyelination(Plemel et al., 2020).The presence of fewer MDMs around the 21DPL time point possibly accounts for the huge variance in MDM densities (Figure 3-1B).

Microglia proliferate after acute demyelination(Plemel et al., 2020). Similarly, I found that in young mice microglia density increases throughout remyelination (Figure 3-1 C). By comparison, in middle-aged mice, the accumulation of microglia was delayed with significantly fewer microglia at 7 and 21 DPL compared to controls. At all-time points, microglia were more numerous than MDM. I also compared the area of LPC-induced lesions in young and middle-aged mice at 3,7 and 21DPL and found no significant difference between young and middle-aged groups(Figure 3-1 D). Taken together, during remyelination in middle-aged mice microglia accumulate more slowly than they do in young mice.

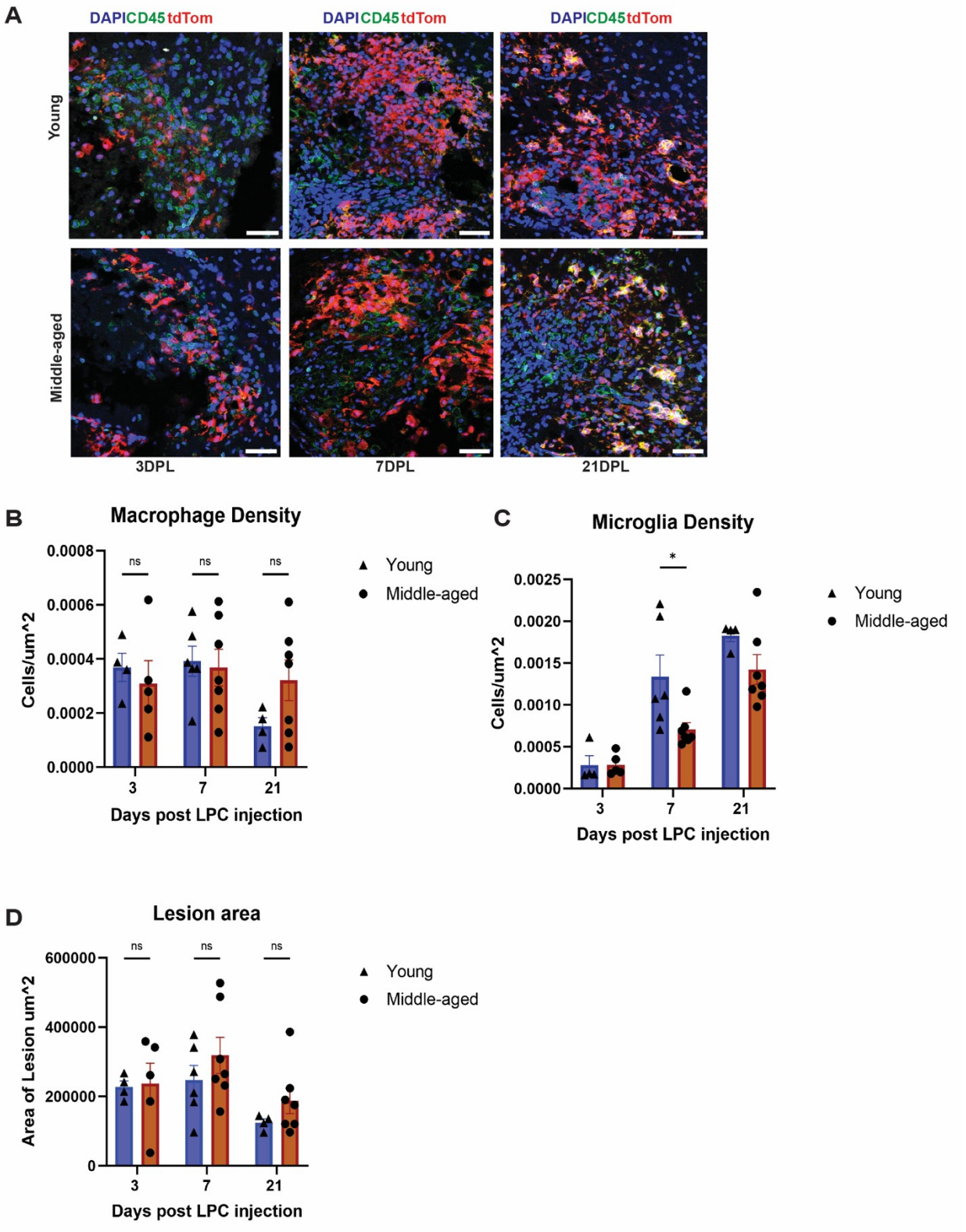
A
DAPICD45tdTom
DAPICD45tdTom
DAPICD45tdTom
Young
Middle-aged
3DPL
7DPL
21DPL
B
Macrophage Density
Cells/um^2
0.0008
0.0006
0.0004
0.0002
0.0000
ns
ns
ns
3
7
21
Days post LPC injection
Young
Middle-aged
C
Microglia Density
Cells/um^2
0.0025
0.0020
0.0015
0.0010
0.0005
0.0000
*
3
7
21
Days post LPC injection
Young
Middle-aged
D
Lesion area
Area of Lesion um^2
600000
400000
200000
0
ns
ns
ns
3
7
21
Days post LPC injection
Young
Middle-aged

Figure 3-1: Microglia response is delayed during remyelination in middle-aged mice.
(A)Immuno-staining of young and middle-aged CX3CR1^{CreEr}; ROSA26^{tdT} mice against pan leukocyte marker CD45; Microglia are labelled with tdTomato under the ROSA26 promoter. Scale bar:50μm
(B)Quantification of CD45^{+}tdT^{-}MDMs; There is no significant difference (adjusted p values>0.05) between MDM densities in young and middle-aged mice; 2-way ANOVA with Sidak's multiple comparison test.
(C) Quantification of CD45^{+}tdT^{+}microglial cells; At 7DPL, microglia density is higher in young mice than middle-aged mice; Microglia accumulation in middle-aged mice is delayed. 2-way ANOVA with Sidak's multiple comparison test (Adjusted p value=0.0184)
(D) Comparison of lesion areas of young and middle-aged mice at 3, 7 and 21DPL; 2-way ANOVA with Sidak's multiple comparison test.

To understand whether ROS is present during remyelination and increased in middle-aged mice we injected LPC into the ventral spinal cord of mice and euthanized mice at different stages of remyelination. We use middle-aged mice because they have remyelination decline(Rawji et al., 2020a)**,** and for people with MS, this age is associated with progression risk(Confavreux & Vukusic, 2006). We injected young and middle-aged CX3CR1^{CreEr} ;ROSA26^{tdT} mice with LPC because these mice express tdTom within microglia and border-associated macrophages, but not monocytes(Plemel et al., 2020; Prinz et al., 2017). To canvas the various stages of remyelination mice were euthanized at an acute demyelination time point (3DPL), during ongoing OPC proliferation and differentiation (7 DPL) and ongoing myelin ensheathment in young mice (21 DPL)(Jeffery & Blakemore, 1995; Keough et al., 2015). To evaluate lipid peroxidation, I conducted immunohistochemistry on spinal cord sections with a primary antibody raised against malondialdehyde (MDA) (Figure 3-2A) and oxidized phosphatidylcholine(E06) (Figure 3-2C).

Malondialdehyde is a final product of fatty acid peroxidation. MDA immunoreactivity increased throughout remyelination, peaking at 21 DPL. Middle-aged mice contained more MDA immunoreactivity within the lesion at 21 DPL as compared to young mice at the same time point (Figure 3-2 A, B). The antigen for E06 antibody is oxidized phosphatidylcholine

(OxPC)(Dong et al., 2021). E06 immunoreactivity did not increase at 21 DPL, but instead heightened E06 immunoreactivity was identified at 7D7 DPLn middle-aged mice compared to young mice (Figure 3-2 C, D). Taken together, I found increased immunoreactivity for different oxidized lipid species with age and remyelination that varied depending on the antibody (E06 antibody or anti-MDA) used for the assessment.

I next wanted to define the accumulation of microglia and MDM with lipid peroxidation. Given that E06 is a monoclonal antibody against OxPC that is well validated in other studies(Dong et al., 2021; Mohammadi et al., 2018), I chose this antibody for all subsequent studies in this thesis. I quantified the density of microglia ($CD45^{+}tdT^{+}$) and MDM ($CD45^{+}tdT^{-}$) that co-labelled with E06 immunoreactivity. Microglia with E06 immunoreactivity was higher in middle age at 3 and 21 DPL, whereas MDM expressing E06 immunoreactivity was higher in middle-aged mice at 3 DPL compared to young mice. Overall E06 immunoreactivity in myeloid cells was higher in middle-aged mice.

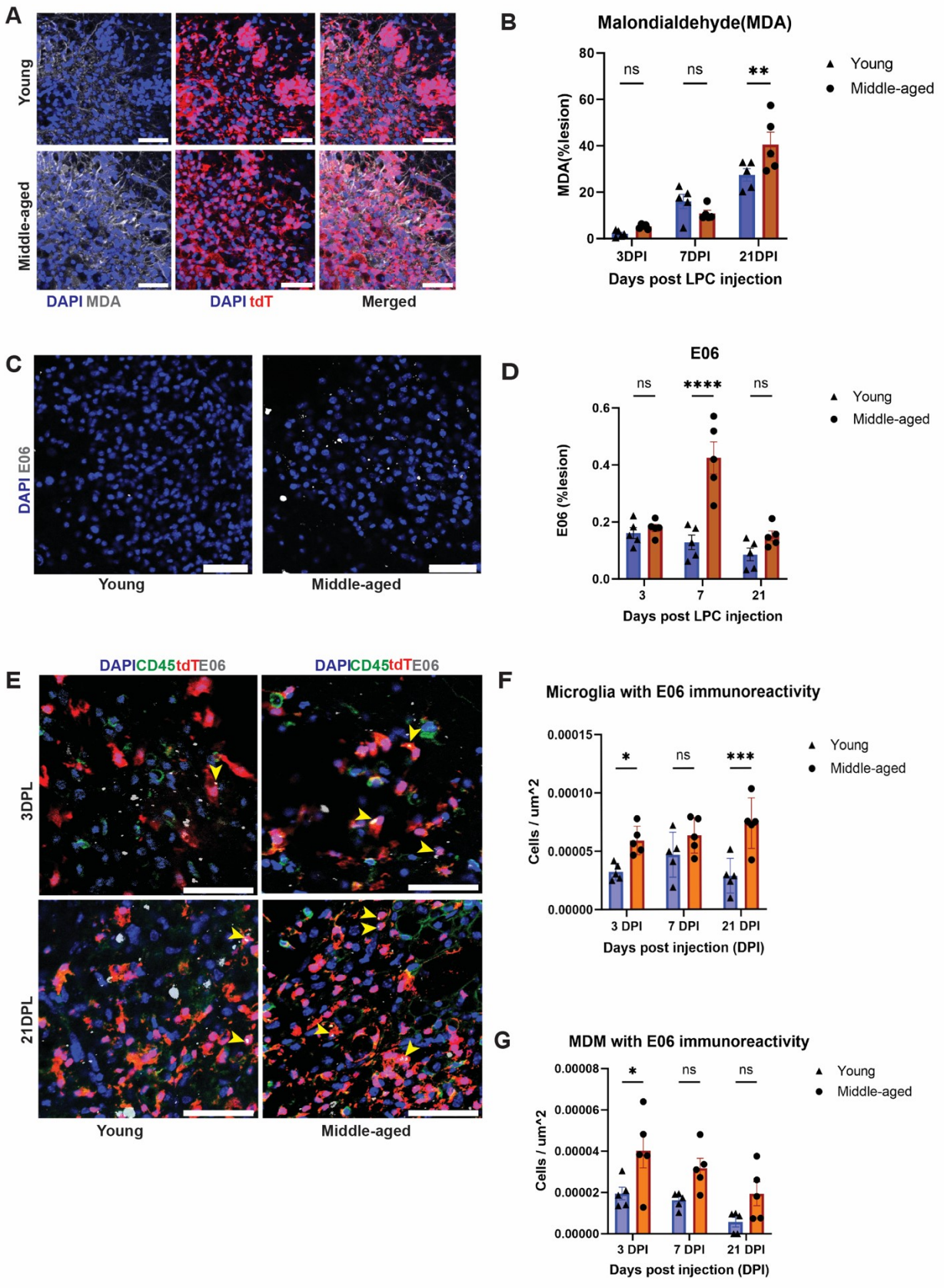
A
Young
Middle-aged
DAPI MDA
DAPI tdT
Merged
B
Malondialdehyde(MDA)
MDA(%lesion)
ns
ns
**
3DPI
7DPI
21DPI
Days post LPC injection
Young
Middle-aged
C
DAPI E06
Young
Middle-aged
D
E06
E06 (%lesion)
ns
****
ns
3
7
21
Days post LPC injection
Young
Middle-aged
E
DAPICD45tdTE06
DAPICD45tdTE06
3DPL
21DPL
Young
Middle-aged
F
Microglia with E06 immunoreactivity
Cells / um^2
*
ns
***
3 DPI
7 DPI
21 DPI
Days post injection (DPI)
Young
Middle-aged
G
MDM with E06 immunoreactivity
Cells / um^2
*
ns
ns
3 DPI
7 DPI
21 DPI
Days post injection (DPI)
Young
Middle-aged

Figure 3-2: Lipid peroxidation is exacerbated during remyelination in middle-aged mice.
(A)Immuno-staining of young and middle-aged CX3CR1^{CreEr}; ROSA26^{tdT} mice against lipid peroxidation marker MDA;Microglia are labelled with tdTomato under the ROSA26 promoter. Malondialdehyde spatially colocalizes with microglia at 21DPL. Scale bar: 50μm
(B) Quantification of %MDA area; Middle-aged mice show heightened lipid peroxidation at 21DPL; 2-way ANOVA with Sidak's multiple comparison test (Adjusted p value:0.0092)
(C) Immuno-staining of young and middle-aged CX3CR1^{CreEr}; ROSA26^{tdT} mice against oxidized phospholipid marker E06. Scale bar: 50μm
(D) Quantification of %E06 area; Middle-aged mice show increased presence of oxidised phospholipids at 7DPL; 2-way ANOVA with Sidak's multiple comparison test (Adjusted p value<0.0001).
(E) Immuno-staining of young and middle-aged CX3CR1^{CreEr};ROSA26^{tdT} mice against pan leukocyte marker CD45 and oxidised phospholipid marker E06; Microglia are labelled with tdTomato under the ROSA26 promoter. Yellow arrows indicate microglia with E06 immunoreactivity. Scale bar: 50μm
(F) Quantification of CD45^{+}tdT^{+} E06^{+} microglial cells; At 3 and 21DPL, microglia in middle-aged mice have increased levels of oxidized phospholipids; 2-way ANOVA with Sidak's multiple comparison test (Adjusted p values=0.0379 (3DPL), 0.0004(21DPL))
(G) Quantification of CD45^{+}tdT^{-}E06^{+} MDMs; At 3DPL, MDMs in middle-aged mice have increased levels of oxidized phospholipids; 2-way ANOVA with Sidak's multiple comparison test (Adjusted p value=0.0187)

2. Setanaxib decreases excessive lipid peroxidation, however, does not promote remyelination in middle-aged mice.

Given that we found elevated signs of ROS in middle-aged mice during remyelination, and ROS prevents the transition of OPCs into oligodendrocytes(French et al., 2009), we wanted to test whether ROS impacts remyelination. NADPH oxidase (NOX) is a significant producer of ROS in microglia that impairs oligodendrocyte production(Li et al., 2005). Its expression is increased within microglia in MS(Marie T. Fischer et al., 2012) and is heightened in aged microglia in response to pathological stimuli(Qin et al., 2013). We hypothesize that heightened ROS via NOX impairs remyelination in middle-aged mice. Therefore, we treated middle-aged mice daily after LPC injection with 60 mg/kg of the NOX1/4 isoform inhibitor Setanaxib.

To evaluate whether Setanaxib impacted lipid peroxidation, I conducted immunohistochemistry on Setanaxib and vehicle-treated mice at 7DPL, where OPC differentiation and proliferation typically take place in young mice(Jeffery & Blakemore, 1995;

Keough et al., 2015). I found that Setanaxib decreased E06 immunoreactivity, and consistently reduced lipid peroxidation within the demyelinated lesion (Figure 3-3A, B). Based on this finding, I further investigated if Setanaxib could alter remyelination. A critical step during remyelination is the recruitment of OPCs. As OPC proliferation and differentiation is present at 7DPL(Baaklini et al., 2023; Keough et al., 2015; Rawji et al., 2020a), I conducted immunohistochemistry against the OPC marker PDGFRα and proliferation marker Ki67 in spinal cord tissues isolated from Setanaxib or Vehicle treated middle-aged C57BL/6 mice (Figure 3-3C). Despite reducing OxPC immunoreactivity, Setanaxib failed to increase the density of OPCs (PDGFRα $^{+}$) or proliferating OPCs ($Ki67^{+}PDGFR\alpha^{+}$) within the lesion compared to vehicle-treated mice (Figure 3-3D, E). OPC differentiation is a rate-limiting step during remyelination (Franklin, 2002; Franklin & ffrench-Constant, 2008) and ongoing at 7DPL in middle-aged mice(Rawji et al., 2020a). I, therefore, conducted immunohistochemistry against the oligodendrocyte-specific marker Myrf+ and used this marker to quantify oligodendrocyte cell density. Like OPC density, Setanaxib failed to promote increased oligodendrocyte density at 7DPL (Figure 3-3 F, G). Given that OPC recruitment and differentiation are rate-limiting during remyelination, we conclude that Setanaxib is unlikely to enhance remyelination.

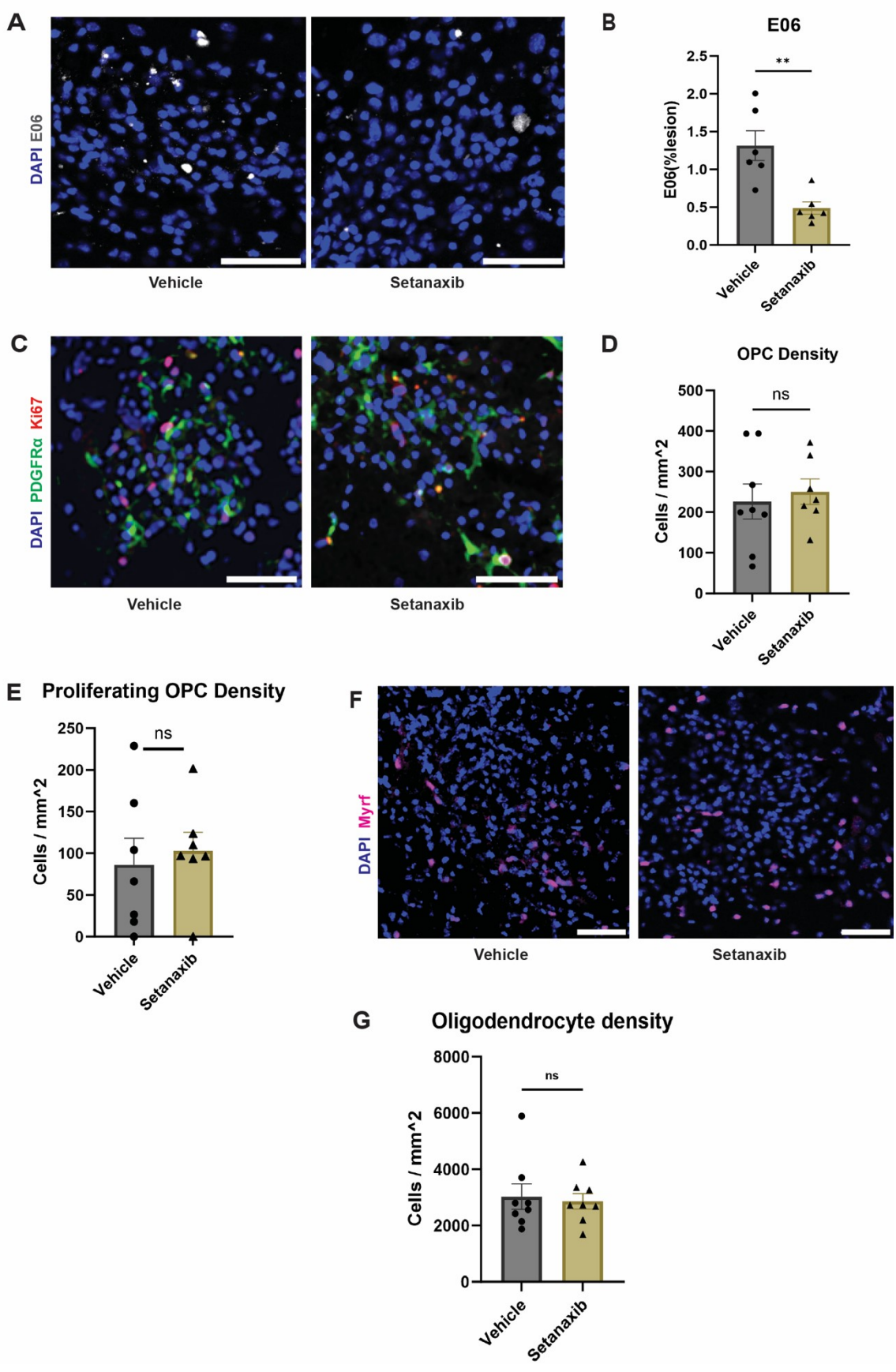
A
DAPI E06
Vehicle
Setanaxib
B
E06
E06(%lesion)
**
Vehicle
Setanaxib
C
DAPI PDGFRα Ki67
Vehicle
Setanaxib
D
OPC Density
Cells / mm^2
ns
Vehicle
Setanaxib
E
Proliferating OPC Density
Cells / mm^2
ns
Vehicle
Setanaxib
F
DAPI Myrf
Vehicle
Setanaxib
G
Oligodendrocyte density
Cells / mm^2
ns
Vehicle
Setanaxib

Figure 3-3: Setanaxib decreases excessive lipid peroxidation, however, does not promote remyelination in middle-aged mice
(A)Immuno-staining against oxidized phospholipid marker E06 shows reduced E06 accumulation in Setanaxib treated middle-aged C57BL/6 mice against Vehicle treated group (Scale bar:50μm).
(B) Quantification of %E06 area in Setanaxib and Vehicle groups; Unpaired t test, p value=0.0031
(C) Immuno-staining against OPC marker PDGFRα and proliferation marker Ki67 shows no difference in accumulation and proliferation of OPCs in Setanaxib and Vehicle treated middle-aged C57BL/6 mice (Scale bar:50μm).
(D) Quantification of $PDGFR\alpha^+$ and (E) $PDGFR\alpha^+$ $Ki67^+$ cell densities; Unpaired t test, p values= 0.6643,0.6752
(F)Immuno-staining against oligodendrocyte nuclear marker $Myrf^+$ (transcription factor) shows no difference in accumulation of oligodendrocytes in Setanaxib and Vehicle treated middle-aged C57BL/6 mice (Scale bar: 50μm)
(G)Quantification of $Myrf^+$ oligodendrocyte cell density; Unpaired t test, p value=0.1387

As myelin debris clearance is required for remyelination to proceed(Cantuti-Castelvetri et al., 2018; Natrajan et al., 2015; Neumann et al., 2009; Plemel et al., 2013; Ruckh et al., 2012), I next investigated if Setanaxib boosts myelin debris clearance in microglia or MDMs. To evaluate myelin debris engulfment, I immuno-stained myelin debris using anti-MBP, conducted confocal microscopy of remyelinating lesions, and evaluated the volume of myelin debris inside of microglia and macrophages using a 3D rendering software named IMARIS (Figure 3-4A), similar to our recent work(Baaklini et al., 2023). As this work was conducted in C57/BL6 mice I am not able to differentiate microglia and MDM and refer to this whole population as microglia/macrophages. I quantified the density of microglia/macrophage based on immunoreactivity against the macrophage marker IBA1 and found that Setanaxib did not alter microglia/macrophage accumulation at 7DPL (Figure 3-4 A, B). Similarly, Setanaxib did not change the amount of myelin debris phagocytosed by microglia/MDMS (Figure 3-4 C). Also, there was no difference in myelin debris outside of microglia/MDMs (Figure 3-4 D). I also calculated the percentage of myelin debris inside microglia/macrophages by dividing the volume of myelin debris inside microglia/macrophages by the total amount of myelin debris in the lesion

(phagocytosed+ non-phagocytosed myelin debris) and multiplied by 100. There is however no difference between Setanaxib and Vehicle groups (Figure 3-4 E). Figure 3-4F shows a percentage distribution of phagocytosed and non-phagocytosed debris. Inhibition of NOX with Setanaxib, therefore, did not interfere with myelin debris clearance. Taken together, Setanaxib reduces age-associated lipid peroxidation but fails to boost OPC recruitment, proliferation, or their subsequent differentiation into oligodendrocytes.

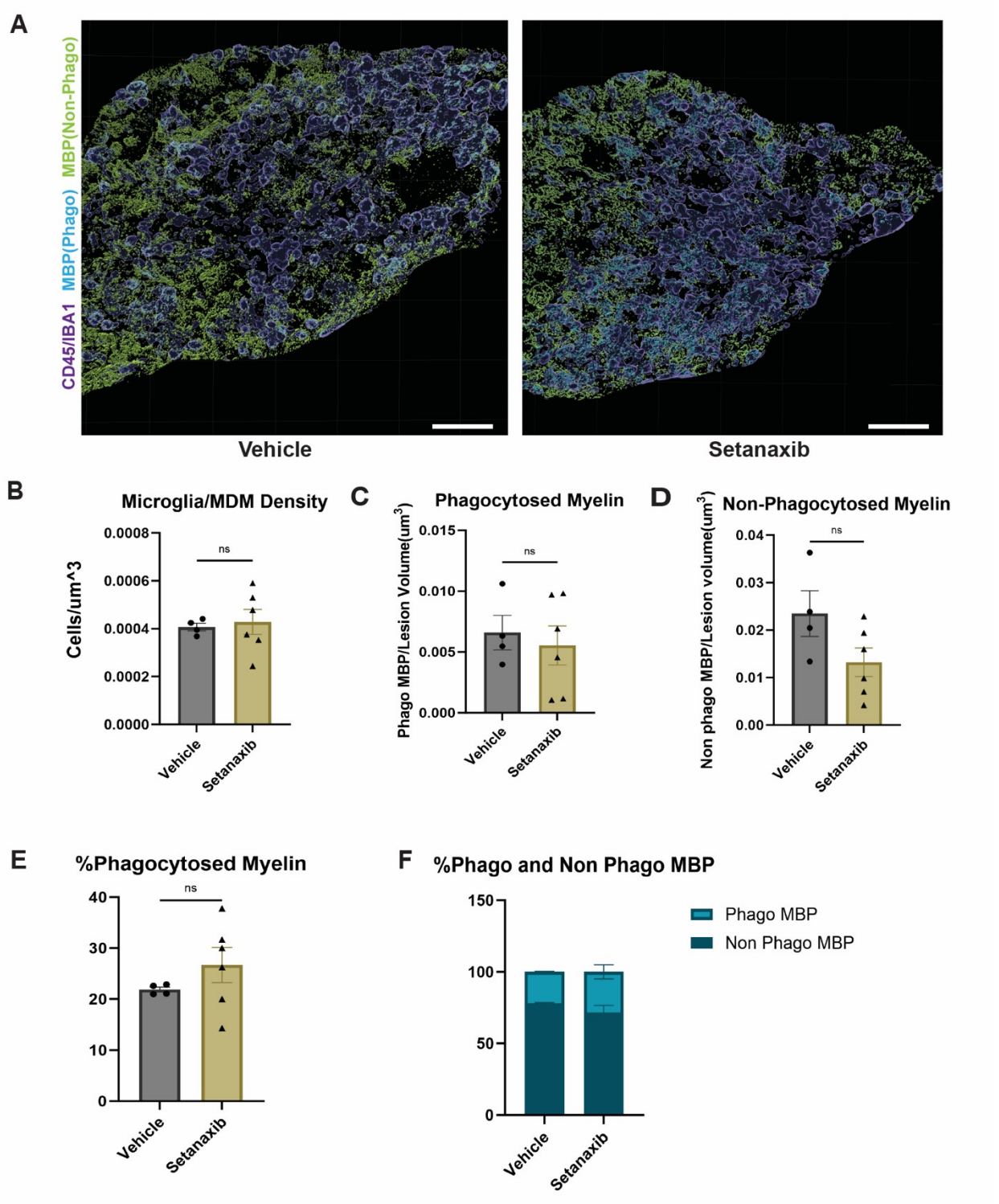
A
CD45/IBA1 MBP(Phago) MBP(Non-Phago)
Vehicle
Setanaxib
B
Microglia/MDM Density
Cells/um^3
0.0008
0.0006
0.0004
0.0002
0.0000
ns
Vehicle
Setanaxib
C
Phagocytosed Myelin
Phago MBP/Lesion Volume(um3)
0.015
0.010
0.005
0.000
ns
Vehicle
Setanaxib
D
Non-Phagocytosed Myelin
Non phago MBP/Lesion volume(um3)
0.04
0.03
0.02
0.01
0.00
ns
Vehicle
Setanaxib
E
%Phagocytosed Myelin
40
30
20
10
0
ns
Vehicle
Setanaxib
F
%Phago and Non Phago MBP
150
100
50
0
Phago MBP
Non Phago MBP
Vehicle
Setanaxib

Figure 3-4: Setanaxib does not boost microglia/macrophage response and myelin debris clearance in middle-aged mice
(A) Immuno-staining against pan leukocyte marker CD45, macrophage/microglia marker IBA1 and myelin basic protein marker MBP shows no difference in myelin debris clearance by microglia/MDMs in Setanaxib and Vehicle treated middle-aged C57BL/6 mice(Scale bar: 50 μm).
(B) Quantification of $CD45^+/IBA1^+$ cell densities;Unpaired t test,p value=0.7541
(C) Quantification of phagocytosed myelin debris;MBP^+ myelin debris inside $CD45^+$ or $IBA1^+$ or $CD45^+$ and $IBA1^+$co-labelled cells; Normalized to lesion volume;Cells were identified based on DAPI; Unpaired t-test,p value=0.6613
(D)Quantification of non-phagocytosed myelin debris;MBP^+ myelin debris outside of $CD45^+$ or $IBA1^+$ or $CD45^+$ and $IBA1^+$co-labelled cells; Normalized to lesion volume;Cells were identified based on DAPI; Unpaired t-test,p value=0.0907
(E) Quantification of % phagocytosed myelin based on amount of MBP^+ myelin debris inside $CD45^+/IBA1^+$ cells; Unpaired t test, p value=0.2994
(F) Percentage of phagocytosed and non-phagocytosed myelin debris in Setanaxib and Vehicle groups

3.2 Conclusions

Aging delays microglia ($CD45^+tdT^+$) response in LPC-induced lesions, based on quantification of microglia cell densities in young and middle-aged male and female $CX3CR1^{CreEr}$;ROSA26 ^{tdT} mice. MDM densities however do not significantly vary between young and middle-aged groups. Microglia depletion in young animals reduces OPC recruitment and differentiation(Baaklini et al., 2023). However, in the context of delayed microglia response in middle-aged animals, it remains unclear what causes this and how it could potentially affect OPC recruitment and differentiation into myelinating oligodendrocytes.

In the LPC model of demyelination, middle-aged animals show more ROS production in the lesion during remyelination based on immunoreactivity against different oxidized lipid species i.e. MDA and E06. Excessive ROS production could contribute to delayed remyelination in aged animals as it impedes the transition of OPCs into myelinating oligodendrocytes(Spaas et al., 2021).

At acute demyelination (3DPL), both aged microglia and MDMs show more lipid peroxidation based on E06 immunoreactivity. During remyelination at 21DPL, E06

immunoreactivity is higher in aged microglia, however, there is no difference in levels of lipid peroxidation in young and aged MDMs. Therefore, microglia primarily accumulate oxidative damage in the form of lipid peroxidation.

Oral administration of Setanaxib to middle-aged female C57BL/6 mice reduces lipid peroxidation based on E06 immunoreactivity. However, it does not rescue delayed remyelination in middle-aged C57BL/6 mice based on its inability to boost phagocytosis of myelin debris by microglia/MDMs or promote OPC proliferation. It also did not show any effect on the accumulation of $Myrf^{+}$ myelinating oligodendrocytes.

Taken together, middle-aged animals show delayed microglia response and heightened ROS production during remyelination. The presence of oxidative damage in the form of lipid peroxidation is observed mainly in aged microglia during acute demyelination and complete remyelination timelines. Decreasing ROS production by a NOX inhibitor Setanaxib, however, fails to boost remyelination in middle-aged C57BL/6 mice. My data thus, remains consistent with the conclusion that reducing ROS production alone does not rescue the age-associated delay in remyelination.

CHAPTER 4: Discussion

4.1 Overview

Multiple sclerosis (MS) is a persistent condition characterized by inflammation, demyelination, and neurodegeneration within the central nervous system (CNS). It is a complex, immune-mediated disease resulting from a combination of genetic and environmental factors. The primary pathological feature of MS is the buildup of demyelinated lesions affecting both the white and grey matter in the brain and spinal cord(Filippi et al., 2018). Regeneration of myelin sheath, known as remyelination, protects axons from degeneration, thereby slowing the permanent disability related to axonal loss(Franklin & Ffrench-Constant, 2008). Remyelination is driven by OPC accumulation, proliferation and differentiation into myelinating oligodendrocytes(Franklin & Ffrench-Constant, 2017).

Remyelination is delayed during aging(Shields et al., 1999) with delayed accumulation and differentiation of OPCs into mature oligodendrocytes(Sim et al., 2002). Various studies have investigated intrinsic molecular/epigenetic changes in aged OPCs to elucidate molecular mechanisms that potentially halt the transition of OPCs into myelinating oligodendrocytes. Aged OPCs fail to respond to differentiation signals and show hallmarks of cellular aging(Neumann et al., 2019).Xiao Ru Ma and colleagues demonstrate that aging in mouse OPCs results in impaired nuclear entry of SIRT2 and reduced NAD+ levels and supplementing β-nicotinamide mononucleotide (β-NMN), an NAD+ precursor, could restore nuclear entry of SIRT2, enhance OPC differentiation, and promote remyelination in older mice(Ma et al., 2022). In another study, activation of the Apelin-APJ signalling pathway restores remyelination in aged mice(Ito et al., 2021).

Age-dependent decline in remyelination also depends on extrinsic factors such as the extracellular matrix(ECM)(Zhang et al., 2021). Increasing stiffness of the extracellular matrix

with age is now recognized as a significant factor affecting OPC differentiation(Gharagozloo et al., 2022). Piezo1, a mechanosensitive ion channel, is a key mediator of OPC mechanical signalling (Segel et al., 2019; Zhang et al., 2021) and inhibiting Piezo1 recovers impaired OPC differentiation in aged brains (Neumann et al., 2019b; Segel et al., 2019). Molecules in the ECM, such as hyaluronan and chondroitin sulfate proteoglycans (CSPGs), negatively regulate effective remyelination in multiple sclerosis (MS) patients or in experimental autoimmune encephalomyelitis (EAE) brains(Keough et al., 2016; Pu et al., 2018). CSPGs accumulate with age (Macke et al., 2020; Richard et al., 2018),therefore, could contribute to inefficient remyelination with aging.

Microglia and MDMs play a crucial role in remyelination(Baaklini et al., 2019), however, microglia/MDM response is delayed in aged animals (Rawji et al., 2018; Rawji et al., 2020b; Zhao et al., 2006) with impaired phagocytosis of myelin debris(Cantuti-Castelvetri, et al., 2018; Rawji et al., 2020b). Myelin debris impedes OPC differentiation(Kotter et al., 2006; Plemel et al., 2013). Myelin debris clearance by microglia/MDMs in demyelinating lesions decreases with age, which is linked to slower remyelination (Rawji et al., 2018; Rawji et al., 2020a; Ruckh et al., 2012). Based on a heterochronic parabiosis study by Julia Ruckh and colleagues, in which young and old mice were paired, it was shown that exposure to the young systemic environment rejuvenated remyelination in old mice by enhancing myelin debris clearance (Ruckh et al., 2012). Young macrophages could clear myelin debris more efficiently than older ones, partially rejuvenating oligodendrocyte differentiation in the older mice. Consequently, aged macrophages are less capable of clearing myelin debris, contributing to delayed remyelination associated with aging. Taken together, efficiency of myelin debris clearance declines with age and contributes to delayed remyelination.

Aging exacerbates reactive oxygen species (ROS) production(Michaels et al., 2020) and excessive ROS production could delay remyelination as it impedes the transition of OPCs into myelinating oligodendrocytes(Spaas et al., 2021). ROS produced by microglia/MDMs damages surrounding oligodendrocyte lineage cells (OLs)(Li et al., 2005). OLs and OPCs are particularly vulnerable to oxidative injury caused by elevated ROS levels(Back et al., 2002; Bernardo et al., 2003; Giacci & Fitzgerald, 2018; Haider et al., 2011; Miyamoto et al., 2013; van Horssen et al., 2008). Suppressing ROS through radical scavenging rescued OPC proliferation and differentiation (Miyamoto et al., 2013). Therefore, we hypothesized that ROS delays remyelination and inhibiting excessive ROS levels by using an NADPH oxidase (NOX1/4) inhibitor Setanaxib (Aoyama et al., 2012) could rescue age-associated delay in remyelination by promoting myelin debris clearance and OPC proliferation and differentiation, which are crucial for remyelination. We found that NOX1/4 inhibition via Setanaxib neither boosts myelin debris clearance nor promotes OPC proliferation and differentiation.

4.2 Delayed microglia/MDM response during remyelination in middle-aged mice

Microglia and MDMs play various roles during remyelination (as discussed in Chapter 1 Introduction) and in the context of ROS production, autopsy samples from people with MS and spinal cord tissues from middle-aged mice following LPC-induced demyelination, demonstrate microglia and macrophages as common producers of ROS(Marie T. Fischer et al., 2012; Michaels et al., 2020) . Based on the CX3CR1^{CreEr};ROSA26^{tdT} microglial lineage-tracing approach (Baaklini et al., 2023; Michaels et al., 2020; Plemel et al., 2020), we found that microglia response is delayed in middle-aged mice during remyelination. We also found a decline in MDM density in young mice between 7 and 21 DPL which is consistent with microglial proliferation during remyelination that prevents the further accumulation of

MDM(Baaklini et al., 2023; Plemel et al., 2020). MDMs are surrounded by microglia and undergo apoptosis and therefore, microglia monopolize the lesion environment following LPC-induced demyelination(Plemel et al., 2020).

Various studies have also found a delayed immune response in aged animals, however, there is no distinction between microglia and macrophages in these findings(Rawji et al., 2020b; Zhao et al., 2006). Therefore, microglia density calculated based on tdTomato fluorescence in CX3CR1^{CreEr};ROSA26^{tdT} mice eliminates the inclusion of MDMs. However, one disadvantage of using this lineage-tracing approach is that it also labels border-associated macrophages (BAMs)(Prinz et al., 2017; Yona et al., 2013). An alternative approach is using the Tmem119 ^{CreEr} ROSA26^{tdT} mouse model to exclude BAMS (Bennett et al., 2016; Kaiser & Feng, 2019) and thus remains a part of future goals for this project.

Delay in microglia response during remyelination could contribute to delay in remyelination, as microglia ablation reduces OPC recruitment and differentiation (Baaklini et al., 2023). However, it still needs to be elucidated as to how delay in microglia response affects OPC proliferation into myelinating oligodendrocytes in aging animals. One possible mechanism could be that delayed microglia/MDM response leads to delayed phagocytosis of myelin debris in middle-aged mice during remyelination (Rawji et al., 2020b) and in the presence of myelin debris, OPCs fail to transform into mature oligodendrocytes(Plemel et al., 2013).

4.3 ROS production during remyelination in middle-aged mice

Based on immunostaining against different oxidized lipid species, we found that the aged lesion environment has higher lipid peroxidation during early and late remyelination stages respectively and not during acute demyelination. Lipid peroxidation is an oxidative chain reaction that involves free radicals (ROS) attacking lipids containing carbon-to-carbon double

bonds such as polyunsaturated fatty acids (PUFA) resulting in lipid peroxyl radicals and hydroperoxides(Yin et al., 2011). Higher lipid peroxidation indicates elevated ROS levels and hence, middle-aged mice show heightened ROS production during remyelination.

Based on various studies, the E06 antibody used in this study is commonly used to label oxidized phosphatidylcholines (OxPCs)(Dong et al., 2021; Dong & Yong, 2022; Hörkkö et al., 1999; Palinski et al., 1996). However, it cannot distinguish between different OxPC components(Dong & Yong, 2022). OxPCs remain stable(Bochkov et al., 2010) however, could affect the physiochemical properties of the plasma membrane via micelle formation(Dong & Yong, 2022). This could lead to altered lipid asymmetry, membrane flipping, and increased membrane permeability(Bach et al., 2009; Dong & Yong, 2022; Pande et al., 2010; Volinsky et al., 2011). OxPCs could also promote macrophage death by inducing ceramide accumulation through ceramide synthase activity(Halasiddappa et al., 2013). OxPCs have also been used to induce toxin-based focal demyelination in mouse spinal cords(Dong & Yong, 2022). Taken together, lipid peroxidation in the form of OxPC deposition could accelerate disease progression in MS.

In young mice, toxic levels of ROS production may be regulated by the expression of antioxidant enzymes by $CD68^{+}$ microglia/macrophages during early remyelination at 7DPL(Moezzi et al., 2022). However, the expression of antioxidant enzymes during remyelination in middle-aged mice remains to be elucidated.

In this study, we found no significant difference in MDA immunoreactivity between young and middle-aged mice during acute demyelination at 3DPL. This, however, contradicts the study conducted by Nathan Michaels and colleagues(Michaels et al., 2020), where exacerbated lipid peroxidation (based on MDA immunoreactivity), indicative of excessive ROS

production was shown in middle-aged mice at 3DPL. A possible reason could be the use of a different anti-MDA antibody in this project that differs from the one used in the study by Nathan Michaels and colleagues (Michaels et al., 2020) as the latter has been discontinued by the manufacturing company (Abcam).

With respect to delayed microglia response in our study, one potential mechanism behind this could be age-associated microglial senescence (Shahidehpour et al., 2021; Streit et al., 2009)in white matter(Matsudaira et al., 2023) which is possibly driven by excessive ROS production(Passos et al., 2010) during remyelination in middle-aged mice. Increased ROS production hinders the differentiation of OPCs into mature myelinating oligodendrocytes(Spaas et al., 2021). ROS produced by microglia/MDMs inflicts damage on surrounding oligodendrocyte lineage cells(Li et al., 2005). In autopsy samples from people with MS, oxidative damage caused by ROS is found in oligodendrocytes in the form of oxidized DNA and products of lipid peroxidation(Haider et al., 2011). Taken together, in the context of remyelination, excessive ROS production may delay necessary microglial response, impede the transformation of OPCs into mature oligodendrocytes and inflict DNA damage and lipid peroxidation in oligodendrocytes. Therefore, heightened ROS production in middle-aged mice could be a possible reason behind age-associated remyelination decline.

4.4 Lipid Peroxidation in microglia during remyelination

Although there were no differences in the overall levels of lipid peroxidation at 3DPL based on E06 and MDA immunoreactivity between young and middle-aged animals, I found that the number of microglia and MDMs with lipid peroxidation based on E06 immunostaining was more in middle-aged mice as compared to young mice. At 21DPL, microglia in middle-aged mice show more lipid peroxidation based on E06 immunoreactivity. Taken together, microglia

primarily accumulate oxidative damage in the form of lipid peroxidation during acute demyelination and remyelination.

Ferroptosis, a recently discovered regulated cell death(RCD) pathway(Dixon et al., 2012) is triggered when excessive intracellular iron (labile iron) accumulation leads to more ROS production via the Fenton reaction(Papanikolaou & Pantopoulos, 2005). This further leads to lipid peroxidation and loss of membrane integrity(Sun et al., 2022) which initiates cell death by ferroptosis (Yang & Stockwell, 2016; Ye et al., 2020). Active and chronic human MS lesions and cerebrospinal fluid (CSF) of MS patients show markers of ferroptosis, such as the presence of elevated levels of (labile) iron and products of lipid peroxidation such as OxPC (Van San et al., 2023). In the same study, it was found that treatment with a ferroptosis inhibitor, UAMC-3203, alleviated disease progression in relapsing-remitting MS(RRMS). In the EAE and cuprizone animal models of MS, excessive lipid peroxidation and ferroptosis contribute to oligodendrocyte loss and demyelination(Jhelum et al., 2020; Li et al., 2022). Taken together, excessive iron accumulation and consequent lipid peroxidation could amplify disease progression in MS.

With age, iron accumulation increases in microglia, along with enhanced deposition of OxPCs and markers of cellular degeneration(Hametner et al., 2013), indicative of microglial ferroptosis. However, in the context of this study, it is unknown if the presence of lipid peroxidation in aged microglia at 3 and 21DPL comes from ROS generation through labile iron deposition and Fenton reaction. Also, we did not investigate morphological hallmarks of ferroptosis in microglia such as the presence of smaller mitochondria with reduced membrane densities, the absence of mitochondria crista, and outer mitochondrial membrane rupture(Xie et

al., 2016). Further investigation is required to assess age-associated iron deposition and ferroptosis in microglia and how it could potentially impact remyelination.

4.5 Setanaxib as a potential drug to boost remyelination in middle-aged animals

Setanaxib has been used as a NADPH oxidase (NOX1/4) inhibitor in an animal model of liver fibrosis(Aoyama et al., 2012). Setanaxib is also in clinical trials for Type 2 Diabetes and Idiopathic Pulmonary Fibrosis (NCT03865927) and is protective following brain ischemia(Wang et al., 2020). I administered 60 mg/kg Setanaxib or Vehicle orally to middle-aged female C57BL/6 mice for 6 consecutive days post-LPC injection to see if Setanaxib reduces ROS production and if this has any effect on remyelination. I collected spinal cord tissues at the 7DPL time point when we expect OPC proliferation and differentiation in young mice(Keough et al., 2015). Setanaxib reduced lipid peroxidation based on E06 immunoreactivity, indicating its efficiency in reducing ROS production via NOX1/4.

Setanaxib efficiently inhibits NOX1 and NOX 4 isoforms of the NADPH oxidase enzyme but does not show efficacy in the inhibition of NOX2 isoform(Aoyama et al., 2012). In the context of demyelinated lesions, microglia/MDMs express NOX1 and NOX2(M. T. Fischer et al., 2012; Michaels et al., 2020). This indicates that Setanaxib potentially inhibits NOX1-mediated ROS production from microglia/MDMs. NOX1 expression is also found in astrocytes and endothelial cells in autopsy samples from people with MS (M. T. Fischer et al., 2012) , indicating a possible inhibition of astrocytic NOX1 by Setanaxib. However, in the context of remyelination, the role of astrocytic ROS production and its inhibition by Setanaxib still needs to be investigated. Taken together, Setanaxib potentially reduces ROS production by microglia, MDMs, astrocytes and endothelial cells via NOX1 inhibition.

At 7DPL, middle-aged mice show delayed engulfment of myelin debris(Rawji et al., 2020b). I further investigated if Setanaxib boosts myelin debris clearance as it is an essential step toward remyelination (Plemel et al., 2013). We found no effect of Setanaxib on myelin debris clearance by MDM /microglia, based on the 3D rendering software IMARIS. Further, I looked into the effects of Setanaxib on OPC accumulation, proliferation and differentiation into $Myrf^{+}$ oligodendrocytes. Setanaxib did not promote any of these processes based on immunostaining against OPC marker PDGFRα, proliferation marker Ki67 and transcription factor Myrf that is expressed in oligodendrocytes and drives myelination(Huang et al., 2021) This suggests that inhibiting ROS production alone by Setanaxib may not boost key steps towards remyelination which involve efficient myelin debris clearance and transformation of OPCs into myelinating oligodendrocytes. However, in the study by Nathan Michaels and colleagues(Michaels et al., 2020), reducing ROS production by indapamide post-LPC injection reduced lipid peroxidation, demyelination and axon loss in middle-aged mice. Taken together, reducing ROS production by antioxidant therapies may not completely rescue the age-associated delay in remyelination even though it may alleviate the initial stages of injury.

4.6 Significance

In the context of MS, we found that the aged environment is associated with excessive ROS production which could be a potential inhibitor of remyelination based on previous studies(Li et al., 2005; Spaas et al., 2021). However, based on our data from the Setanaxib experiments, I conclude that reducing ROS alone does not completely alleviate age-associated remyelination failure in MS and therefore, calls for more investigation into other inhibitory molecules that could be playing a crucial role in delaying myelin repair in aged people with MS.

4.7 Limitations and Future Directions

In this study, I used CX3CR1 ^{CreEr} ROSA26 ^{tdT} mice to distinguish microglia from MDMs. In this transgenic line, CX3CR1^{CreEr} is heterozygous for the CX3CR1 gene. Since this lineage tracing approach also labels BAMs as mentioned earlier, a future direction for this study would be using Tmem119 ^{CreEr} ROSA26^{tdT} mice to exclude BAMS and investigate microglia populations specifically, although previous studies indicate minimal involvement of BAMs following LPC-induced demyelination(Baaklini et al., 2023; Plemel et al., 2020).In the context of ROS production and lipid peroxidation, more studies could be performed to investigate if lipid peroxidation i.e. MDA and OxPC has any effect on OPC proliferation and maturation during remyelination. Also, it remains to be elucidated if lipid peroxidation during remyelination comes from age-dependent iron accumulation. Setanaxib inhibits NOX1/4 isoforms however, does not inhibit NOX2 isoform(Aoyama et al., 2012). As NOX2 is upregulated in microglia following LPC demyelination(Michaels et al., 2020), there is a possibility of ROS production by NOX2 which potentially impedes remyelination. Another future goal of this project would be to use NOX2 inhibition along with NOX1/4 inhibition to examine the effects of ROS inhibition on remyelination.

Microglia response following demyelination is heterogenous(Plemel et al., 2020) and as microglia are ROS producers following demyelination (Michaels et al., 2020), a single-cell RNA sequencing approach could be used to define specific ROS-producing microglia populations (Mendiola et al., 2020). This may provide more insight into microglia dynamics in the aged environment following demyelination.

For Setanaxib experiments, I looked into the early remyelination time point i.e. 7DPL. Further, studies can be conducted at the acute demyelination and complete remyelination time

points i.e. 3 and 21 DPL respectively, to investigate the effects of Setanaxib on myelin debris clearance or myelination if any.

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