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Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Purpose: The purpose of this research is to evaluate whether reactive oxygen species are an age-associated inhibitor of remyelination. We will evaluate ROS deposition during remyelination, evaluate the sources of ROS, and use ROS lowering medications to evaluate how impacts remyelination

Scope: We will use animal models of remyelination, microglial fate mapping, ROS measuring techniques and single cell RNA sequencing of ROS enriched cells to evaluate the source of ROS production. We will use medications to lower ROS and evaluate how ROS impacts remyelination

Major findings: Currently we have conducted tissue measurements of ROS and found changes in age and time during remyelination with heightened ROS. We have also found the medication setanaxib reduces ROS after injury and boost OPC production

15. SUBJECT TERMS

NONE LISTED

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1. Introduction

Background: Multiple sclerosis (MS) is associated with ongoing neurodegeneration driven by the loss of neurons and axons. Remyelination protects axons from degeneration, slowing the permanent disability related to axonal loss. For these reasons, boosting remyelination for people with MS is an active area for clinical trials as remyelination therapies could benefit most people with MS. However, remyelination naturally declines with age, potentially limiting how long any remyelination therapy may help people with MS. We currently understand that much of the age-related remyelination decline relates to the impaired macrophages and microglia immune response. In this proposal, we will investigate a new potential, age-related, remyelination inhibitor: the production of reactive oxygen species (ROS) from microglia and macrophages. **Hypothesis:** We find more ROS production by microglia/macrophage and lipid peroxidation in middle-aged mice. ROS is a known toxin that may either kill or stall oligodendrocyte lineage cells during remyelination. **We, therefore, hypothesize that a population of aged, but not young, microglia /macrophages exaggerate ROS production that inhibits remyelination.**

Aim1: Study design: We will differentiate microglia and macrophage using transgenic models and dissect ROS production in young and middle-aged mice following focal demyelination produced by intraspinal injection of LPC (lysolecithin). We will assess essential ROS producing enzymes, NADPH oxidase and examine the extent of lipid peroxidation in young and middle-aged mice.

Aim 2. Study design: To probe ROS production after demyelination in middle-aged and young mice, we will isolate microglia and macrophage, stratify them based on the production of ROS and conduct single-cell RNA sequencing. This approach, called Tox-seq, will identify the age and ROS-associated microglial and macrophage phenotypes that likely contribute to age-dependent remyelination decline. We will use ligand-receptor interaction to define potential candidates that impair oligodendrocyte differentiation or promote microglia/macrophage ROS production. We will screen candidates in culture.

Aim 3. Study design: We propose that ROS may be a remyelination obstacle enriched during aging. We find more lipid peroxidation and NADPH oxidase in middle-aged mice, and ROS is known to impair oligodendrogenesis. We will test a NOX inhibitor in middle-aged mice to determine if lowering ROS production improves remyelination in these mice.

From this work, we will also lay the groundwork for the expanded hypothesis that an age-associated population of microglia or macrophage actively inhibits remyelination. From the data collected here, we will define such phenotypes and identify new targets that we will examine in future years.

2. Keywords

Multiple Sclerosis, Remyelination, Myelin, Microglia, Macrophages, Reactive Oxygen species

3. Accomplishments

What were the major goals of the project?

In the second 12 months our goals were to:

Note: Subtask 1-4 are completed

- Measure ROS and oxidative stress during remyelination in young and middle-aged mice (Subtask 5)
- Conduct single cell RNA sequencing in young and middle aged mice (Subtask 6)

- Conduct single cell RNA sequencing bioinformatics (Subtask 7)
- Target screen in culture (Subtask 8)
- Test Setanaxib following LPC induced demyelination (Subtask 9)
- Determine changes in OPC density, oligodendrocyte density and remyelination status after setanaxib (Subtask 10)

What was accomplished under these goals?

Subtask 5- Measure ROS and oxidative stress during remyelination in young and middle-aged mice

Here we have nearly completed this task. With we have measured ROS based on malenaldhyde (MDA), which is the final product of polyunsaturated fatty acid peroxidation. MDA provides us an idea of how much lipid peroxidation remains using an antibody based approach. We find that after LPC induced demyelination there is more MDA signal in middle age mice at 21 days after LPC injection (DPI) (**Fig 1**). This timepoint corresponds to late stages of remyelination in young mice and early stages of remyelination in middle-aged mice because remyelination is delayed with age.

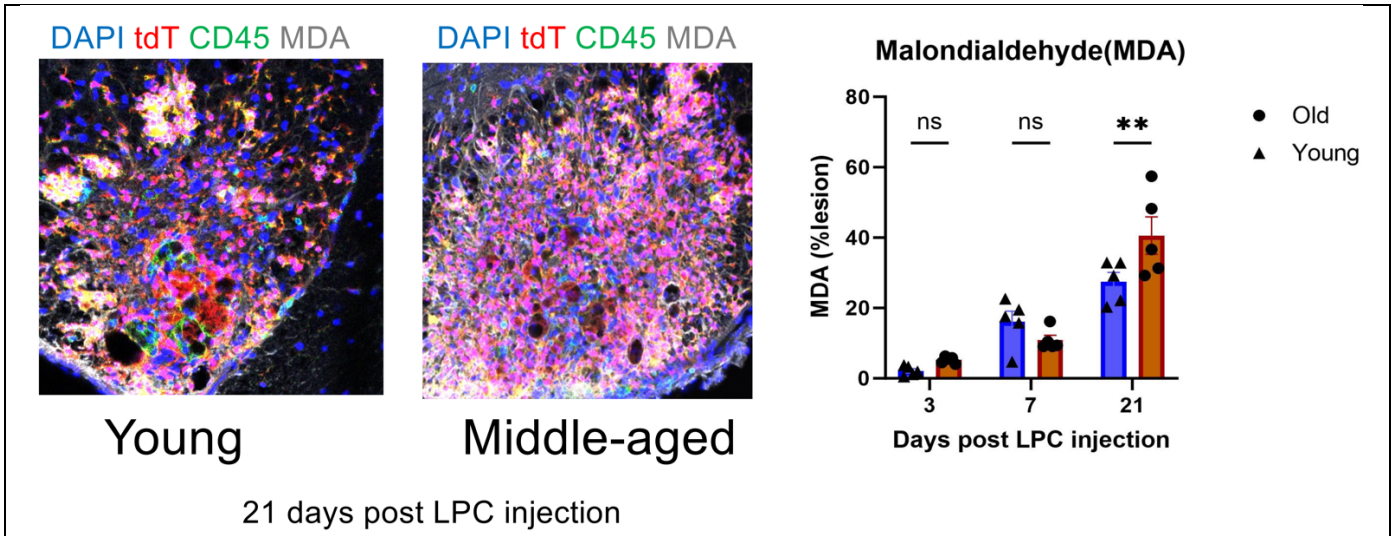

Figure 1. Lipid peroxidation is enriched within middle aged (old) mice at 21 days after LPC induced demyelination. We measured MDA as a proportion of the lesion and found MDA increases with time and is higher in middle-aged vs young mice.

We also measured another antibody specific to ROS, E06, a monoclonal antibody that binds to oxidized phospholipids and lipophospholipids (like LDL). Different than MDA, we find that there is an increase in E06 only at 7 DPI middle-aged mice, with no increasing levels of ROS with time in young or middle-aged mice (**Fig. 2**). At 7 DPI this would correspond to early (middle-aged mice) or late (young mice) stages of oligodendrocyte production, an intermediate stage of remyelination

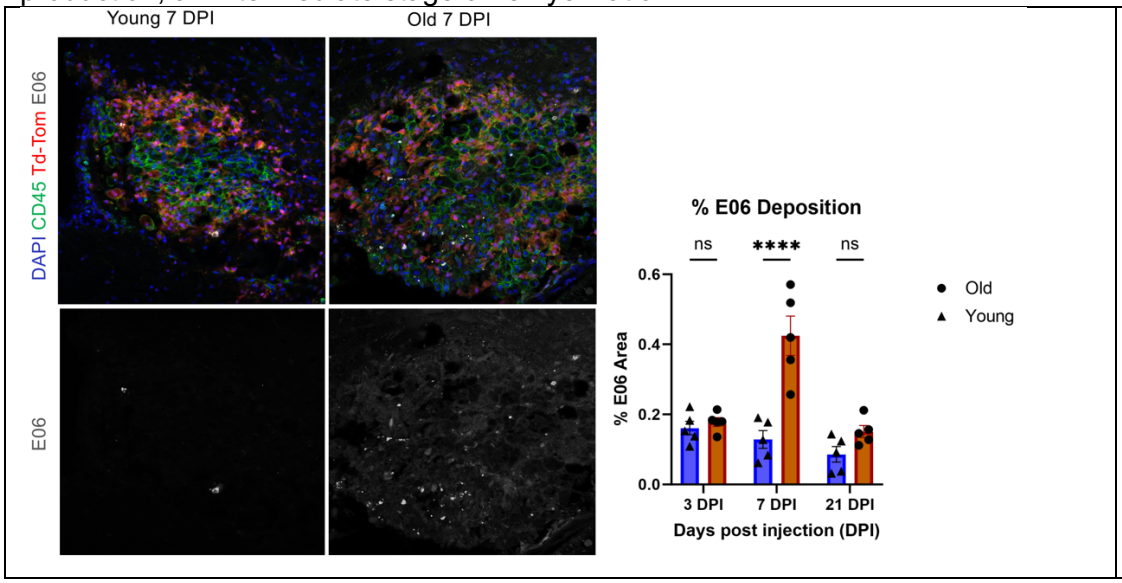

Figure 2. Oxidation of phospholipids (E06) is higher in the lesion area in middle-aged mice at 7 days post LPC injection (DPI). The antigen of E06 is specific to oxidized phospholipids and oxidized lipoproteins, which I different than MDA.

We also measured E06 to determine if this was found within phagocytic cells microglia and macrophages. As Microglia in our transgenic mice express tdTomato (Td-Tom) and the monocyte derived macrophages do not express td-Tom, we can use this to differentiate microglia and macrophage. In doing so we find that EO6 is found within microglia and MDM, often more so in middle-aged mice (**Fig. 3**)

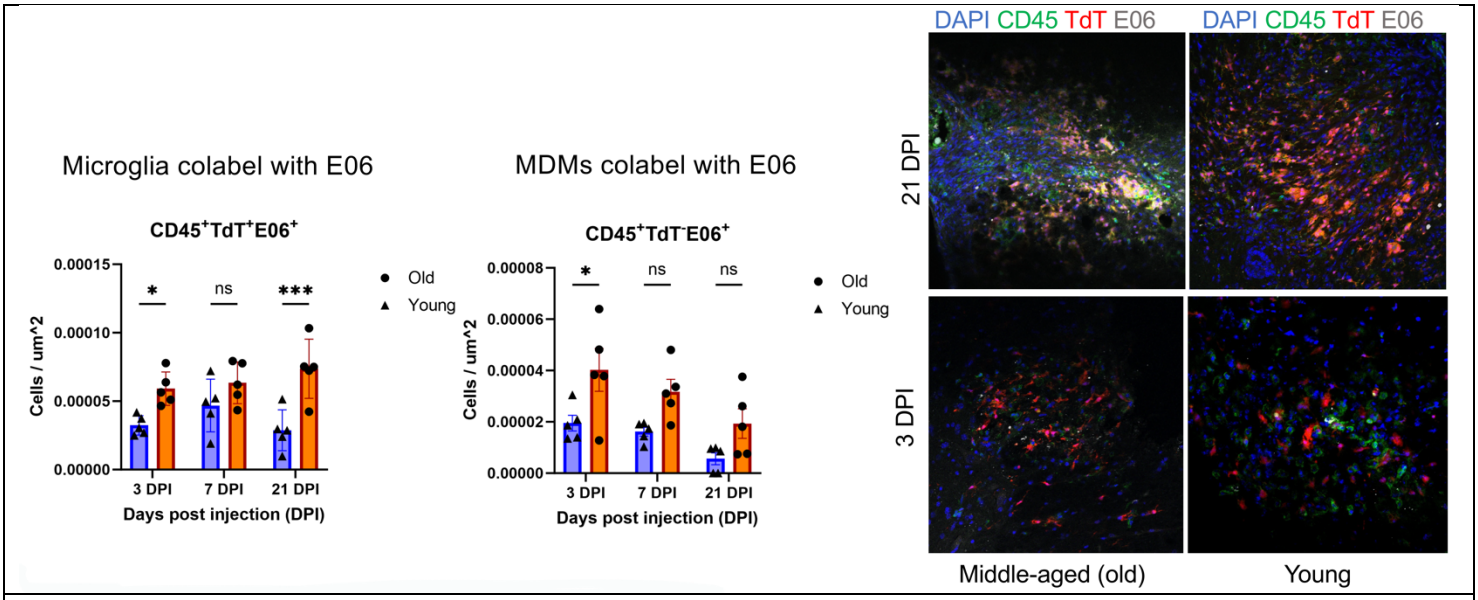

Figure 3. We find that microglia and monocyte-derived macrophages (MDM) colabel with E06, suggestive of phagocytosis. We find that in middle-age mice undergoing remyelination, the microglia have more E06 signal at 3 and 21 DPI and MDM have more E06 signal at 3DPI monocyte, as compared to young mice. We conclude that more oxidized phagocytic debris is found in middle-aged mice.

Due to differences in MDA and EO6 measurements of ROS we also measured lipid hydroperoxide formation using an LPO assay. Lipid hydroperoxide formation is an intermediary and would answer whether there is more production or deposition of ROS. This work is ongoing, but preliminary data suggests that middle-aged mice have no more lipid hydroperoxides, suggesting that more ROS in middle aged mice is likely related to slower clearance of oxidized lipids and MDA, as compared to more production of these molecules.

Subtask 6/7-Single cell RNA sequencing and bioinformatics in middle-age and young mice

Our initial plan, outlined in our statement of work (SOW), was to conduct tox-sequencing. Tox-seq is where we isolate microglia and macrophages with flow cytometry, then sort those ROS positive and ROS negative cells before conducting scRNA sequencing. In this regard, we have not made progress. However, we have measured microglia at different time points with single-cell RNA sequencing in middle-age and young mice to further our understanding of microglial ROS production during remyelination. Using scRNAseq, we characterized the microglial response from de- to remyelination in young (2-3 month) and middle-aged (~10-12 months) mice (Zia et al., in preparation) to assess efficient (young) and inefficient (middle-age) remyelination. Middle-age is also a risk for progression for people with MS. We dissected the LPC injection site using neutral red staining to define the lesion site, dissociated cells under cold conditions to minimize *ex vivo* artifacts[62], isolated microglia via fluorescence-activated cell sorting (FACS), and conducted scRNAseq with bioinformatic post processing to ensure cells are healthy microglia (**Fig. 4a-b**). We use gene expression patterns to annotate and define these microglial states based on genes that are enriched within a particular cluster. Microglial clusters not present in naïve animals we refer to as remyelination-associated microglial (ReAM) states (**Fig. 4c)**. At 7 days post LPC injection (DPI) in young mice, we found a multifaceted microglial response with several distinct microglial states (**Fig. 41d)**; this is when demyelination is complete and oligodendrogenesis has begun, but before remyelination. Specifically, we found interferon-responsive microglia (IRM), and proliferative microglia as well as two new remyelination associated microglia (ReAM) states that we defined based on a prominent differentially expressed gene, *Igf1*-ReAM and *Ccl3*-ReAM. Igf1 is a growth factor that promotes oligodendrocyte differentiation, suggesting microglia secrete factors like Igf1 to regulate remyelination. During active remyelination (21 DPI) in young mice, these microglia coalesced into *Plp1*-ReAMs. *Plp1* is a myelin gene, not expressed by microglia. As *Plp1*-ReAM are sparce during debris clearance (7 DPI), this state likely reflects uptake of *Plp1* transcript from myelin and oligodendrocyte pruning as a normal process during remyelination, similar to during development.

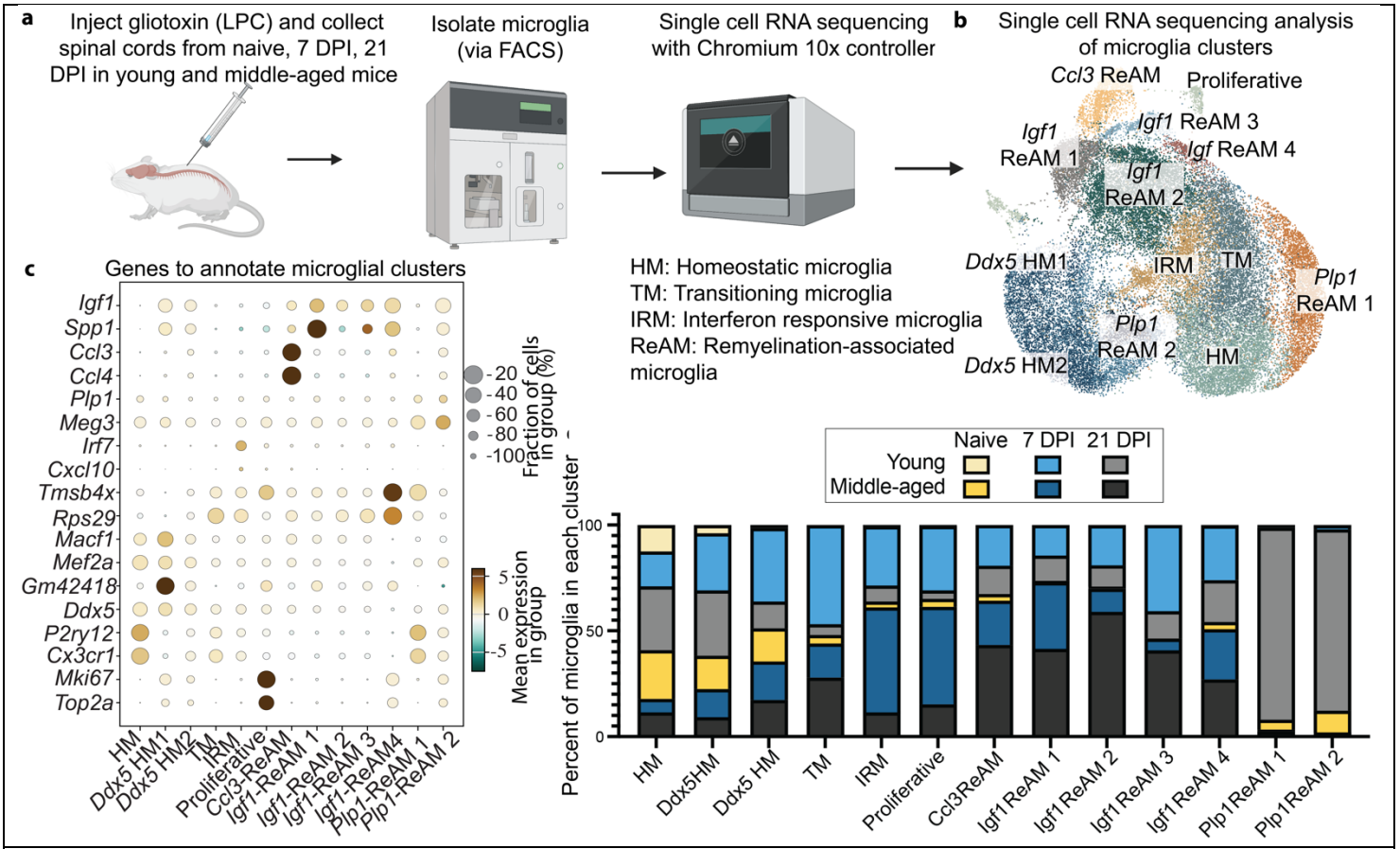

Figure 4. Multifaceted microglial response drives remyelination. (**a)** We conduct scRNAseq of microglia isolated from young and middle-aged mice throughout remyelination. Microglia were projected onto a uniform manifold approximation and projection (UMAP) (**b**). To annotate these clusters, we identified genes that were differentially regulated (**c**). HM were enriched in microglial homeostasis genes (*P2ry12, Cx3cr1*), proliferative microglia expressed proliferative genes (*Mki67, Top2a*), IRM expressed genes downstream from interferon stimulation (*Irf7, Cxcl10),* TM expressed homeostatic genes and reactive microglia genes. *Igf1*-ReAM, *Ccl3*-ReAM, and *Plp1*-ReAM were enriched for *Igf1, Ccl3,* and *Plp1,* respectively. (**d)** Young microglial states were diverse at 7DPI and terminate in *Plp1*-ReAM, whereas middle-aged microglia had delayed *Igf1*- and *Ccl3*-ReAM.

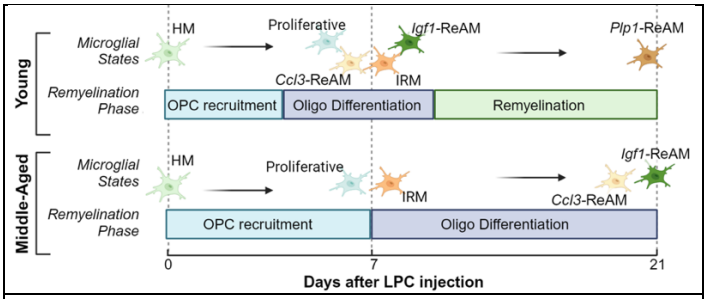

Figure 5: Middle-aged mice have a dysregulated temporal sequence of ReAM states. Namely, delayed *Igf1*-ReAM and *Ccl3*-ReAM states occur during remyelination of middle-aged mice.

As a summary we find that there is not just a difference between young and middle-aged animals with regards to remyelination, but there is certain microglial states that are delayed in middle-aged mice. Microglial proliferative sates arrive similarly in young and middle-aged mice, but the Igf1-ReAM and the CCl3-ReAM arrive weeks later in middle-aged vs young mice (**Fig 5**).

Using this information we were able to identify the ROS producing enzymes and how they change in microglia at different timepoints during remyelination (**Fig. 6**). We identified a comprehensive list of ROS producing enzymes and used this to develop a gene expression score that we can map onto our single cell RNA sequencing data. In doing so, we find that the greatest ROS producing microglial cluster is the Igf1-ReAM1, which was the Igf1-ReAM cluster with the greatest proportion of cells from aged animals (~70% were from aged mice). From this, we conclude that the Igf1-ReAM, which was enriched with cells from middle-aged mice, contained more ROS producing enzyme transcripts than other microglial clusters. This data suggests that middle-age Igf1-ReAM are not only delayed, but also potentially enriched with ROS producing enzymes. We find several ROS producing enzymes enriched within Igf1-ReAM

(Ncf2, Sdhd, Cyc1, Ogdh, Paox, Duoxa1, Xdh, Romo1, Ulbp1, mt-Co2, Cybb, Cyba, Atpsckmt, Etfa). ROS production is likely more complicated than any one enzyme and involves many enyzmes.

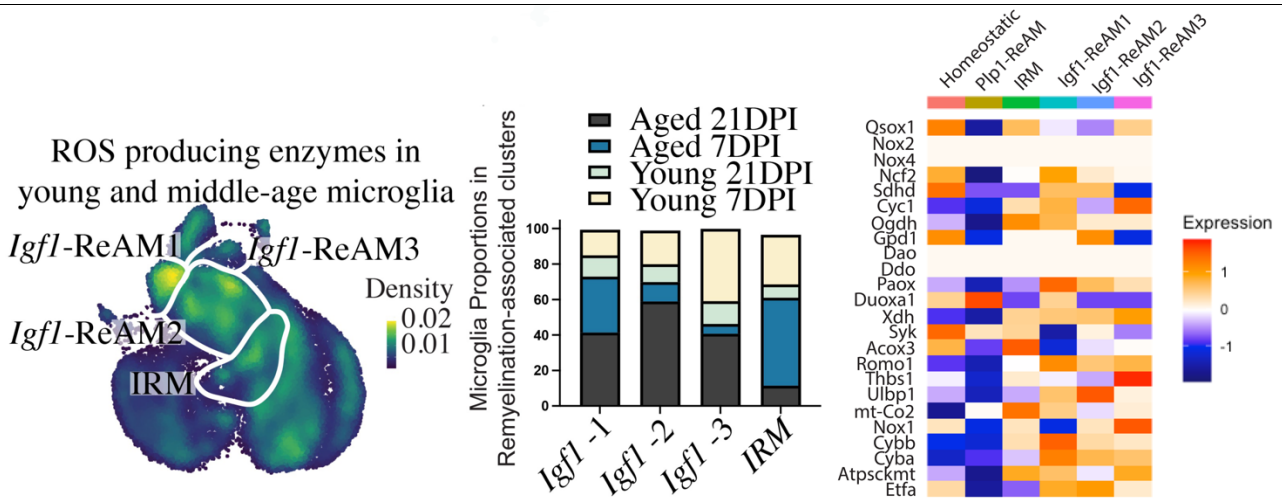

Figure 6. We defined a list of all known ROS producing enzymes and then emalgimated these genes into a single ROS producing enzyme Score, which we projected onto a UMAP of young and middle-aged single cell RNA sequencing data. Outlined is the major ROS producing clusters, Igf1-ReAM and IRM. Of these clusters, Igf1-ReAM expresses the most ROS producing enzymes, which is enriched with cells from middle aged mice. The major enzymes involved in the ROS score is presented as a heat map on the right, showing many ROS producing enzymes involved in ROS production that differ between these microglial clusters.

Subtask 8: Target screen in culture

We were able to used nicheNet, a tool to examine microglial ligands and that are expressed in conjunction with oligodendrocyte progenitor cell (OPC) receptors (**Fig 7**). We identified using bioinformatics 5 potential hits: Csf1, Jam2, Sema4d, Tgfb2, and Gas6, which were upregulated in microglia states found during different stages of remyelination. Based on NicheNet, these ligands expressed by microglia all bind to receptors expressed on OPCs. We then added these ligands to OPC cultures and evaluated the proportion of them that differentiate into MBP+ oligodendrocytes. Overall, we found that none of these factors enhanced oligodendrocyte production or altered OPC survival.

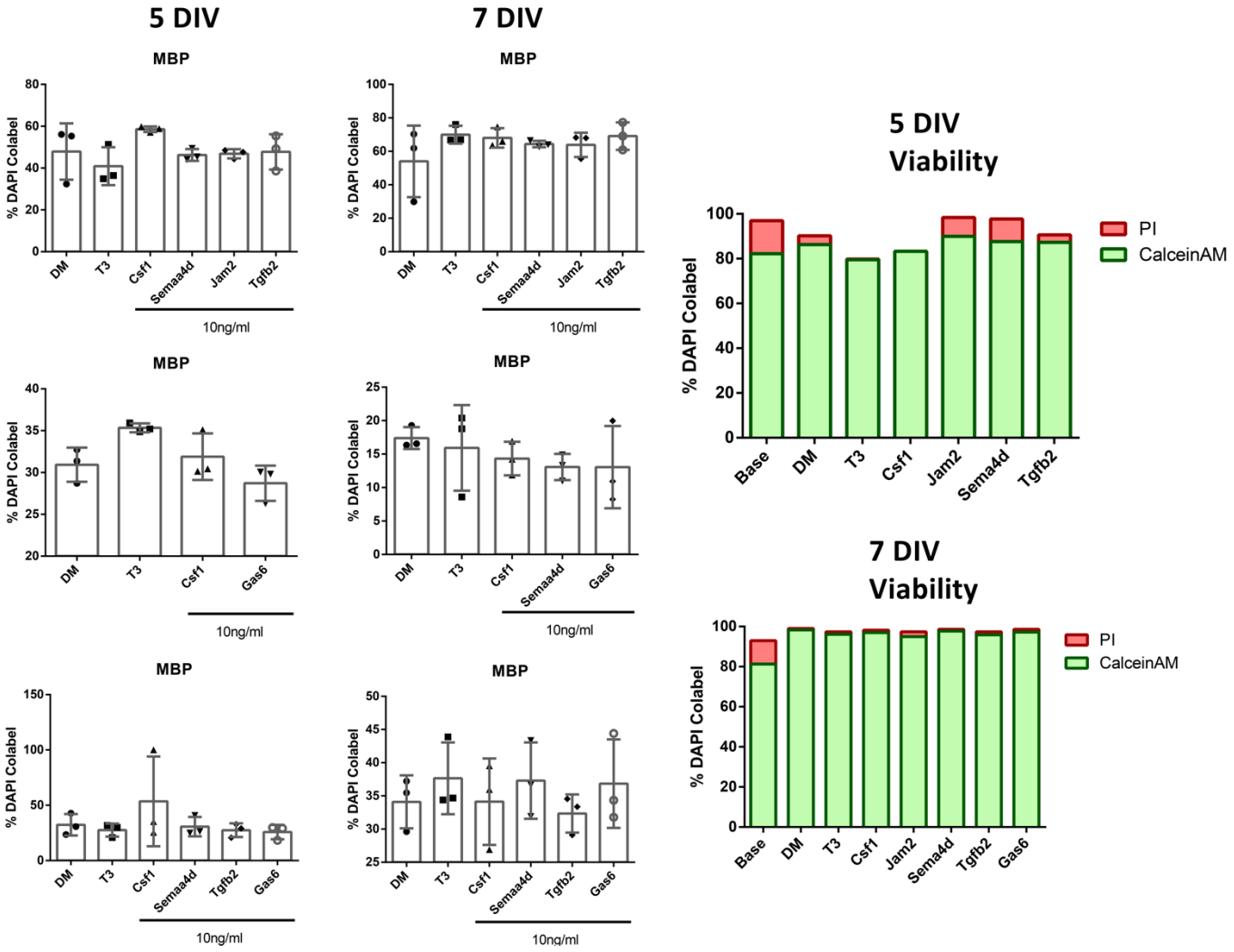

Figure 7. Oligodendrocyte progenitor cells were cultured in vitro for 5 or 7 days (DIV) with various microglia expressed ligands. OPCs were plated in a base differentiation media (DM) used to maintain viable oligodendrocyte lineage cells in the Plemel lab. From this base media, cells were treated with either T3 or microglia expressed ligands (Csf1, Jam2, Sema4d, Tgfb2, and Gas6). We found none of these cell altered oligodendrogenesis (measured as proportion of MBP postitive cells) or survival (measured with Calcein AM).

Subtask 9: Test Setanaxib following LPC induced demyelination (Subtask 9)
Subtask 10: Determine changes in OPC density, oligodendrocyte density and remyelination status after setanaxib

We tested whether Setanaxib, an antagonist of the ROS producing enzyme NADPH oxidase (gene: Cybb, Cyba) in mice following LPC injection in middle aged animals. Overall this may be a promising approach and we have made good progress. We have initially tested one time point (7 DPI) to determine whether Setanaxib can reduce ROS deposition using the E06 antibody. We find that 60mg/kg Setanaxib (orally) for 7 days was sufficient to reduce EO6 signal (**Fig 8**), suggesting that this medication can reduce ROS deposition by targeting NADPH oxidase.

We also measured OPC recruitment into the demyelinated lesion (PDGFRa positive cells) and the production of oligodendrocytes (Myrf positive cells) in the remyelinating lesion at 7 DPI with and without Setanaxib (**Fig 9**). We found that Setanaxib did increase OPC recruitment, but not the production of oligodendrocytes. Potentially we are measuring oligodendrocyte production too early and plan to incorporate more time points.

In addition to the data generated, another important accomplishment was using some of this data and putting it into another grant (Canadian Institutes of Health Research Project Grant), which was submitted Sept 2023. This funding would help support this projects as funds come to an end.

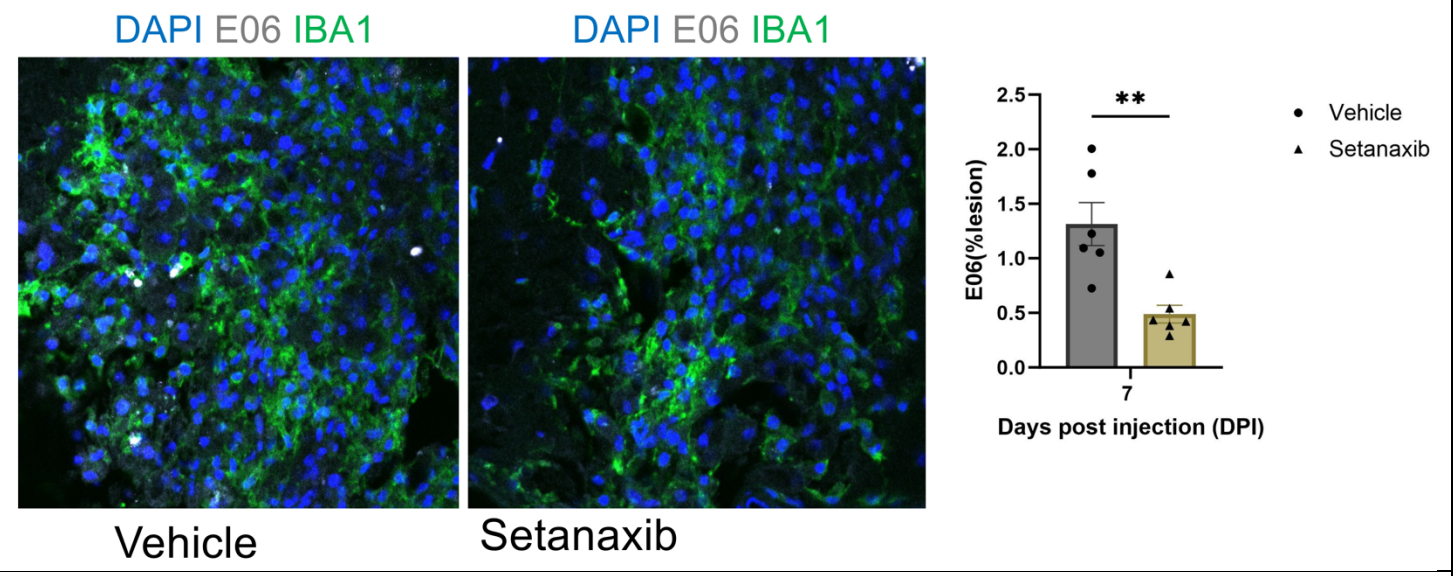

Figure 8. Middle-aged (8-12 months old) C57BL/6 mice were injected with LPC and provided with 60mg/kg Setanaxib (orally) for 7 days.Setanaxib is a known inhibitor for ROS-producing enzyme NADPH oxidase(NOX). We then quantified the immunoreactivity of lesion oxidised lipoproteins (E06+) and found that Setanaxib treatment reduced E06 signal.

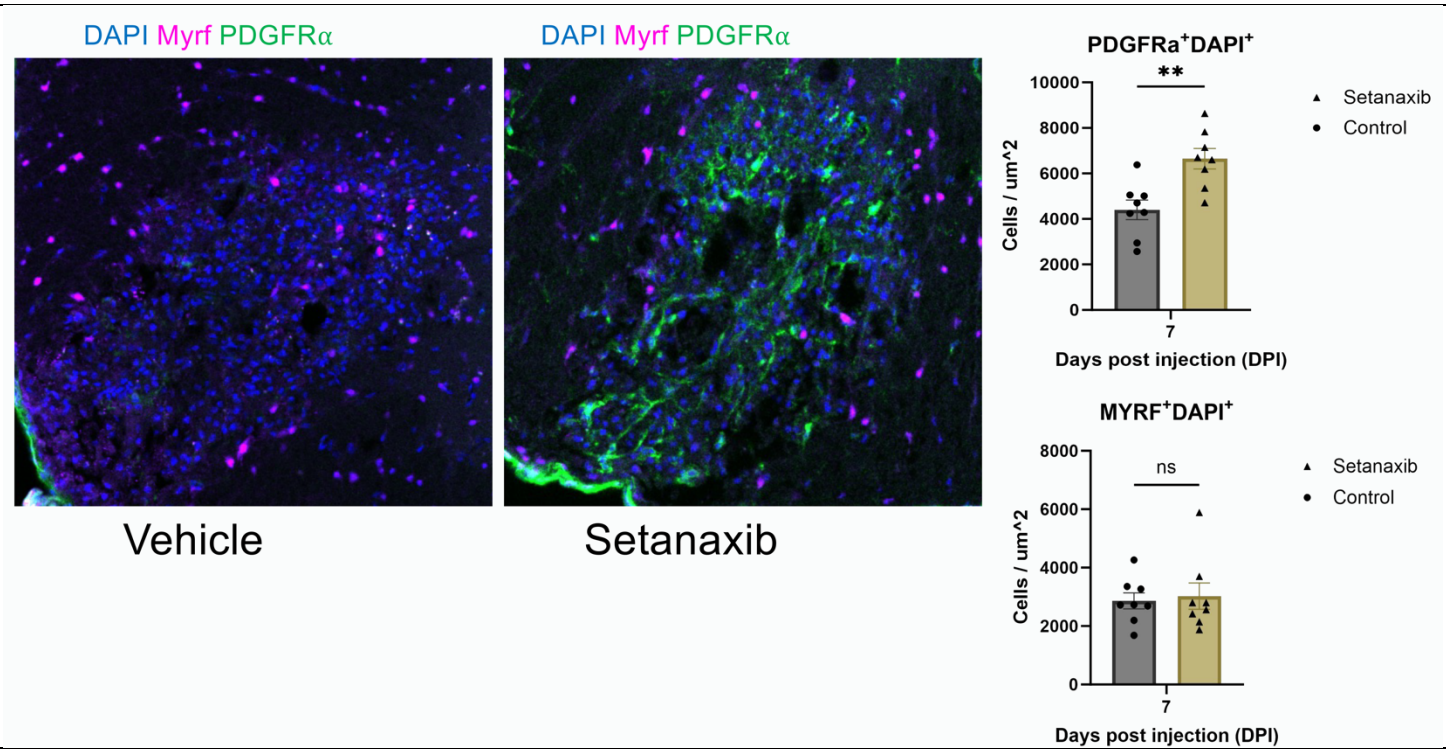

Figure 9. Middle-aged (8-12 months old) C57BL/6 mice were injected with LPC and provided with 60mg/kg Setanaxib (orally) for 7 days. **B-C**: We quantified OPCs (PDGFRα$^+$) and oligodendrocyte (Myrf$^+$) density. We found that Setanaxib treatment increased OPC density at 7 days post LPC, however, it did not increase oligodendrocyte density at this same timepoint.

What opportunities for training and professional development has the project provided?

The DOD funding did support the project of one visiting scholar and one summer student in the Plemel lab. These people were funded by other sources for their salary. The visiting scholar by a MS international foundation award (Reza Naeimi) and the summer student by a local summer student award (Sowmya Challa). However these DOD funds were used to pay for reagents, microscope time, and other disposables that these individuals used to gain experience in a lab setting, which will help both of their careers.

How were the results disseminated to communities of interest?

This work was presented by the first author on this project, Sharmistha Panda, at the International Society of Neuroimmunology Congress, held in Quebec Canada August 2023. Sharmistha presented the work as part of the poster presentation with the title of the poster “Age-associate ROS deposition during remyelination in MS”

What do you plan to do during the next reporting period to accomplish the goals?

With the addition of LPO assay to verify the findings of MDA and E06 staining, along with the testing of many antibodies in this project due to several key antibodies being discontinued, we experienced a level of effort increase with Subtask 5 and no longer have funds to complete the tox-seq experiments. It took longer than anticipated to identify antibodies and there were many new animals generated for the LPO assay. However, we will include the ROS data outlined in Subtask 6/7 into a manuscript we hope to submit in Jan 2024.

Our goal for the remainder of this project is to complete the testing of Setanaxib at addional time points so that the ROS deposition and Setanaxib treatment can be compiled into a manuscript for publication. Our goal is to complete a 21 DPI timepoint with Setanaxib or control treatments. This would enable us to better assess oligodendrocyte production and remyelination. We also hope to measure OPC proliferation given the difference in OPC recruitment. These should be completed within the next year

4. Impact

Fundamental to the research at hand is understanding why remyelination slows with age. It is an important question because current medications being tested in early clinical trials are finding improved remyelination (based on biomarkers) in younger, but not older, people with MS. Given MS is a disease of decades duration, age is a critical concern to develop new remyelination approaches. A large body of literature finds that microglia function is impaired in aging animals during remyelination. Often the microglial response is described as reduced. We used new tools and find that there is more ROS within middle-age animals than young animals. When we assess microglia using single cell RNA sequencing, there is microglial states that preferentially express ROS producing enzymes, which are enriched in middle-aged animals. If we prevent one of these enzymes, NADPH oxidase, then we find there is less oxidized lipids and improved OPC recruitment. These data suggest that ROS may be a target to improve remyelination in middle-aged animals. In terms of MS, microglia should be an important target to accelerate remyelination, especially when considering how age impairs microglia function.

What was the impact on other disciplines?

For other neurodegenerative diseases the heightened ROS deposition with age could be consistend across many conditions. Given that these diseases also have white matter damage and oligodendrocyte production, preventing NADPH oxidase is likely benefitial for many diseases. As ROS can be damaging, targeting this pathway could provide a way to improve neuroprotection and stimulate myelin regeneration.

What was the impact on technology transfer?

Nothing to report

What was the impact on society beyond science and technology?

Nothing to report

5. Changes/Problems

Changes in approach and reasons for change
Actual or anticipated problems or delays and actions or plans to resolve them
Changes that had a signifi cant impact on expenditures

Nothing to report

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents
Nothing to report

Significant changes in use or care of human subjects
Nothing to report

6. Products

Publications, conference papers, and presentations
There was one presentation
Panda SP, Baaklini CS, Naeimi R, Challa S, Lee KV, Hammond BP, Ho MFS, Maguir A, Andrade DV, Tonorio G, Ralha I, Kerr BK, Ioannou MS, Plemel JR. (Aug 2023) Age-associated ROS deposition during remyelination in MS, ISNI congress, Quebec, Quebec, Canada

Website(s) or other Internet site(s),
Nothing to report

Technologies or techniques
Nothing to report

Inventions, patent applications, and/or licenses
Nothing to report

Other Products
Nothing to report

7. Participants & Other Collaborating Organizations

What individuals have worked on the project?

Name:	**Sharmistha Panda**
Project Role	**Graduate Student**
Researcher identifier	**n/a**
Nearest person worked months	**8 months**
Contribution to project	**Is leading the assessment of ROS in tissue, the single cell RNA seq, and will conduct drug testing**
Funding Support	**100% from this CDMRP funding**

Name:	**Kelly Lee**
Project Role	**Research technician**
Researcher identifier	**n/a**
Nearest person worked months	**4months**

Contribution to project	**Support flow cytometry, animal surgeries, animal breeding, animal ethics, and research training for this project**
Funding Support	**40% from this from this CDMRP funding and 60% from a CIHR grant held in my name**

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
Nothing to report

What other organizations were involved as partners?
Nothing to report

8. Special Reporting Requirements

Nothing to report

9. Appendices

Nothing to report