APOE Inhibition of Remyelination and Regulation of OPC differentiation

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Abstract:

Apolipoprotein E (APOE) interacts with the lipoprotein receptor to transport lipoproteins and facilitates ingestion of apoptotic cells, also known as efferocytosis. APOE-deficient macrophages ingest fewer apoptotic cells than wildtype cells. Because apoptotic cells are found in lesions of demyelination, we speculated that APOE and efferocytosis may be involved in remyelination. Our hypothesis is that APOE regulates OPC differentiation via the microglia secretome. To test this hypothesis, we assessed remyelination *in vivo* using *APOE*$^{-/-}$, *APOE2*, *APOE3*, and *APOE4* knock-in mice and *in vitro* using the OPC differentiation assay. *In vivo, w*e found *APOE3* mice showed delayed remyelination at the 5+2 week time point, but by 5+3 week the *APOE3* variant was similar to WT. This indicates that APOE3 partly inhibits the remyelination process. Furthermore, it was found that *APOE*$^{-/-}$ had significantly more remyelination than other genotypes at the 5+1 time point, which suggests murine APOE inhibits remyelination temporarily. *In vitro*, the OPC differentiation assay showed that *APOE3* and *APOE*$^{-/-}$ macrophage secretomes retained significantly more round and primary processes than the positive control indicating there were less factors driving initial cell differentiation. This suggests that murine APOE is required for efferocytosis and secretome content, but APOE3 may inhibit efferocytosis and alter the beneficial factors for OPC differentiation.

Introduction:

Multiple sclerosis (MS) is a demyelinationing disease of the central nervous system that affects 350,000 people in the United States. In higher risk regions of the world such as North America and Northern Europe, approximately 60-200 out every 100,000 people have MS, while in countries with relatively low risk, such as Japan, 6-20 people in 100,000 are affected (Sospedra, 2005). The disease causes a variety of disabilities ranging from problems with sensation and motor function to autonomic and neurocognitive issues. There are three general

presentations of MS: relapsing-remitting, secondary progressive stage, and primary-progressive MS (Sospedra, 2005).

MS results from the demyelination of axons, which impedes electrical impulse conduction and trophic and metabolic support. However, it has been shown that these effects can be reversed through axon remyelination (Lloyd, 2019). Microglia, the macrophages of the brain, have a central role in the remyelination process. They are involved in a variety of brain functions, including promoting development, maintaining homeostasis, and responding to brain infections and damage. While microglia both protect the brain and promote regeneration, they can also have damaging effects on the brain through the promotion of inflammation. Early in embryonic development, precursor myeloid cells migrate to the neuroectoderm, where they become microglia of the brain (Lannes, 2017). After birth, microglia support the differentiation of oligodendrocytes (the cells that myelinate axons) and myelination (Hamilton, 1994). Additionally, they help support regions of the brain that contain myelinated axons by “maintain[ing] oligodendrocyte progenitor numbers in the adult healthy brain” (Hagemeyer, 2017). In demyelinated lesions, the myelin debris inhibits remyelination (Kotter, 2006). How microglia support remyelination is partly unknown. However, it has been found that microglia support myelin repair by phagocytosing myelin debris (Triarhou, 1985), secreting various growth factors and cytokines (Dillenburg, 2018; Pasquini, 2011; Mason, 2001), and clearing cholesterol via the protein APOE (Cantuti-Castelvetri, 2018). Interestingly, it has been found that, in older mice, both myelin debris and cholesterol crystals accumulate in demyelinated lesions and this phenotype can be induced experimentally by knocking out the *apoe* genes (Cantuti-Castelvetri, 2018).

APOE is a 34-kDa glycoprotein with 299 amino acids (Shore, 1973). There are three human variants of this protein: APOE2, APOE3, and APOE4 (Mayley, 2008). APOE3 is the most common of these variants with an allele frequency of 65-70% (Zannis, 1982). Both APOE2 and APOE4 differ from APOE3 at one of two amino acid positions. APOE3 has Cys-112 and

Arg-158, while APOE4 has an amino substitution at position 112 for arginine and APOE2 has an amino acid substitution at 158 for cysteine (Mahley, 2008). The APOE4 protein has been associated with an increased risk for coronary heart disease as well as Alzheimer's disease (Utermann, 1984). Additionally, if an individual with HIV is homozygous for APOE4, their AIDs will progress at a faster rate (Burt, 2008). On the other hand, APOE2 has been found to be overrepresented in octogenarians, (i.e. people between the ages of 80-89), so it is thought to influence longevity (Schächter, 1994). Due to structural differences between APOE3 and APOE2, APOE2 has a defective LDL-receptor binding site (Mahley, 2008). These three variants illustrate how a small change in protein sequence can drastically affect the function of APOE.

APOE is involved in lipoprotein transport by clearing triglycerides and cholesterol-rich lipoproteins. It does this by interacting with the low-density lipoprotein receptor. It has been found that the deletion of the *apoe* gene in *apoe*$^{-/-}$ mice results in the failure to clear lipoproteins from the blood (Moghadasian, 2001). APOE also has an effect on macrophages' ability to efferocytose (Grainger, 2004). *In vitro*, it has been found that *apoe*$^{-/-}$ macrophages phagocytose fewer apoptotic cells than their wildtype (WT) control. However, when APOE is reintroduced to these macrophages, this problem is corrected. Additionally, WT macrophages ingest fewer apoptotic thymocyte cells when these thymocytes were *apoe*$^{-/-}$ mice (Grainger). And even fewer were ingested when both the apoptotic cells and macrophages were both derived from *apoe*$^{-/-}$ mice (Grainger, 2004). This suggest that APOE may play a role in facilitating efferocytosis. Grainger, et al. also investigated the effects of *apoe in vivo* and found that in *apoe*$^{-/-}$ mice, there were more dead macrophages than in WT mice. They believe this may be because more macrophages were recruited due to less apoptotic cells being cleared (Grainger, 2004). It is likely that fewer apoptotic cells were cleared due to decreased efferocytosis by *apoe*$^{-/-}$ macrophages (Grainger, 2004). They also found that the proinflammatory cytokine TNF-alpha and fibrinogen, a positive acute phase reactant, were elevated in the liver in *apoe*$^{-/-}$ mice. None of these effects were seen if the gene for the LDL receptor was deleted or if mice were fed a

high cholesterol diets (Grainger, 2004). This indicates that APOE's interaction with the LDL receptor in lipoprotein metabolism is independent of APOE's effects on the number of macrophages and apoptotic cell uptake (Grainger, 2004). Thus, APOE could be regulating local inflammation by suppressing the function of macrophages. Although it is not well understood how the lack of APOE in the brain affects the ability of macrophages to function efficiently, some suggestions have been made (Grainger, 2004). Grainger et al. posits that it could be indirectly affecting macrophage uptake levels by changing the expression of certain factors that are necessary for apoptotic cell clearance or by affecting Mertk expression, which is a key receptor for apoptotic cell clearance (Grainger, 2004). Thus, if APOE were to interact with Mertk, then efferocytosis may either be enhanced or it may be hindered.

I am studying how APOE may promote or interfere with the process of remyelination. Our hypothesis is that APOE regulates OPC differentiation via apoptotic cell-stimulated macrophage or microglia secretome. By regulating OPC differentiation, APOE would promote or inhibit the remyelination of axons. To test this hypothesis, we conducted both *in vivo* and *in vitro* assays with *apoe2*, *apoe3*, and a*poe4* knock-in and *apoe*$^{-/-}$ mice using OPC differentiation assays and the cuprizone model of demyelination and remyelination. The *in vivo* assay takes sections of the brain to investigate their level of remyelination at different weekly time points (See Figure 1 for cuprizone time course of demyelination and remyelination). We plan to assess remyelination in the *apoe* mice to determine whether repair is similar to or delayed when compared to the time course of our standard WT (C57BL/6J) mice.

The *in vitro* OPC differentiation assay quantifies the extent of morphological processes of isolated OPCs after exposure to various *apoe* macrophage supernatants. Data from the lab conducted previously indicated that macrophages from *apoe3* mice were deficient in efferocytosis whereas *apoe2* and *apoe4* macrophages ingests apoptotic cells similarly to WT macrophages. This suggests that the supernatants from *apoe3* macrophages may have a different composition of secreted factors and the altered secretome could have reduced effects

on OPC differentiation. Here, we plan to assess whether supernatants from apoptotic cell-stimulated *apoe3* macrophages can induce OPC differentiation. This may provide insights that could be correlated to the in vivo experiments.

Methods:

Animals:

C57BL/6J mice served as WT mice that were bred and maintained in our colony under IACUC-approved protocols. *apoe2*, *apoe3*, *apoe4*, and *apoe*$^{-/-}$ mice were previously bred onto the C57BL/6J background and obtained from Dr. Nobuyo Maeda. Mice were bred and maintained for 8 weeks before exposure to 0.2% cuprizone treatment and for *in vitro* experiments.

Sectioning/ Luxol fast blue (LFB) and periodic acid Schiff's base (PAS) staining:

Brains used for myelin staining were extracted from 4% paraformaldehyde (PFA) perfused animals, gross cut to isolate the midbrain, post-fixed in 4% PFA for 3-4 hours, and transferred to 30% sucrose for over-night cryoprotection. Brains were mounted in OCT the following day and stored in -80˚C freezer until sectioned. A cryostat was used to cut the brains into 5-µm coronal sections that displayed the corpus callosum and fornix and mounted onto glass slides. This analysis was conducted on three sections that were at least 30 micrometers away from each other to obtain a relative representation across the length of the corpus callosum above the fornix. To examine how much of the corpus callosum was myelinated, coronal brain sections were stained with LFB-PAS. The LFB stains myelin in the brain blue, while the PAS base stains demyelinated axons pink. On day one, the brain sections on slides were rinsed in PBS and dehydrated in 70%, 80%, and 90% ethanol. Then, the slides were put into LFB solution and baked overnight at 60˚C. On the second day, the slides were allowed to cool and were rinsed with 80% ethanol and dH_2O. The slides were dipped into 0.05 % $LiCO_3$,

70% ethanol, and rinsed in dH_2O to remove excess LFB. The decoloration of each slide was analyzed under the microscope. Once the brain sections were properly stained blue, the slides were oxidized in 1% periodic acid for 5 minutes and then rinsed in dH_2O. Next, the slides were placed into Schiff's reagent for 10 minutes and washed under running tap water. Finally, the slides were decolorized in 0.25% acid alcohol, rinsed with H_2O, and mounted in resin. The myelination of the corpus callosum was scored by a blind observer with a didymium filter using x200-600 magnification with an Olympus BX-40 microscope. The scores ranged from 0-3, where 0 is completely unmyelinated and 3 is completely myelinated. Increments of 0.25 were used for intermediate scores of partial myelination (Doan, 2012). The LFB staining protocol was developed by previous lab member Lorelei Taylor who modified it from Elise Cash.

OPC differentiation assay:

The protocol for culturing, enriching, and isolating the mice oligodendrocyte precursor cells (OPCs) was developed by the Matsushima lab prior to this project and were based on modified methods described by Yang et al, 2016 and Niu et. al, 2012. Two new media reagents were adopted: Modified Growth Media or mOGM (DMEM/F12 + 1% N2 Supplement + 1% B27 Supplement + 5μg/mL insulin + 10ng/mL PDGF + 10ng/mL FGF + 1% PEN STREP) and Modified Isolation Media or mOIM (DMEM/F12 + 0.004% EDTA + 0.5% DNase I + 5μg/mL insulin + 1% PEN STREP).

Mixed glia cells from 3-day old neonatal PLP-eGFP mice mouse pups were cultured in DMEM and 10% FBS media. After four days, the oligospheres were isolated and re-suspended in mOGM media, which was replaced every two days. After 2 weeks, the oligospheres were removed from the media and dissociated in mOIM. Afterwards, the oligospheres were mechanically dissociated and incubated for 20 minutes. This was repeated until a majority of the oligospheres were single cells and used as oligodendrocyte progenitor cells (OPCs).

The isolated OPCs were then re-suspended in DMEM media at a concentration of 2 x 10^5 cells/mL and seeded into a 96-well plate with 25 µL in each well. To this plate, 25 microliters of macrophage supernatants were added and the plate was then incubated at 37˚C. Then the wells are imaged after 48 and 72 hours. The macrophage supernatants are produced by exposing adherent macrophages to apoptotic cells for one hour and then thoroughly washing the undigested cells away. The macrophage cultures are then replenished with media without phenol red which may stimulate macrophages and without fetal bovine serum that would add unwanted cell support factors to the supernatant. After 24 hours, we harvest supernatants and clarify the cellular debris by centrifugation. Supernatants are frozen -80C until their use on OPC cultures.

Figure 1: Cuprizone model of demyelination and remyelination

This model (modified from Matsushima and Morell 2001) has strengths in the reliability, robustness, and reproducibility of the changes at a restricted location so that time-sensitive correlations can be made at the morphologic, cellular and molecular levels. There are four major cell types within the heavily myelinated fiber tract: mature oligodendrocytes, oligodendrocyte precursor cells during repair, microglia and astrocytes. In the adult, mature oligodendrocytes predominate; however, during cuprizone insult, mature oligodendrocytes die by apoptosis and this sets in motion a cascade of events including microglia, astrocyte and OPC accumulation

where most peak numbers are observed around week 5, full demyelination. Upon remyelination, OPCs differentiate and remyelinate the lesion over the next 3 weeks. Remyelination is typically complete by week 5+5. Microglia clear apoptotic cells and myelin debris that is thought to be inhibitory to remyelination and additionally provide cues for OPCs to mature (Matsushima, 2001).

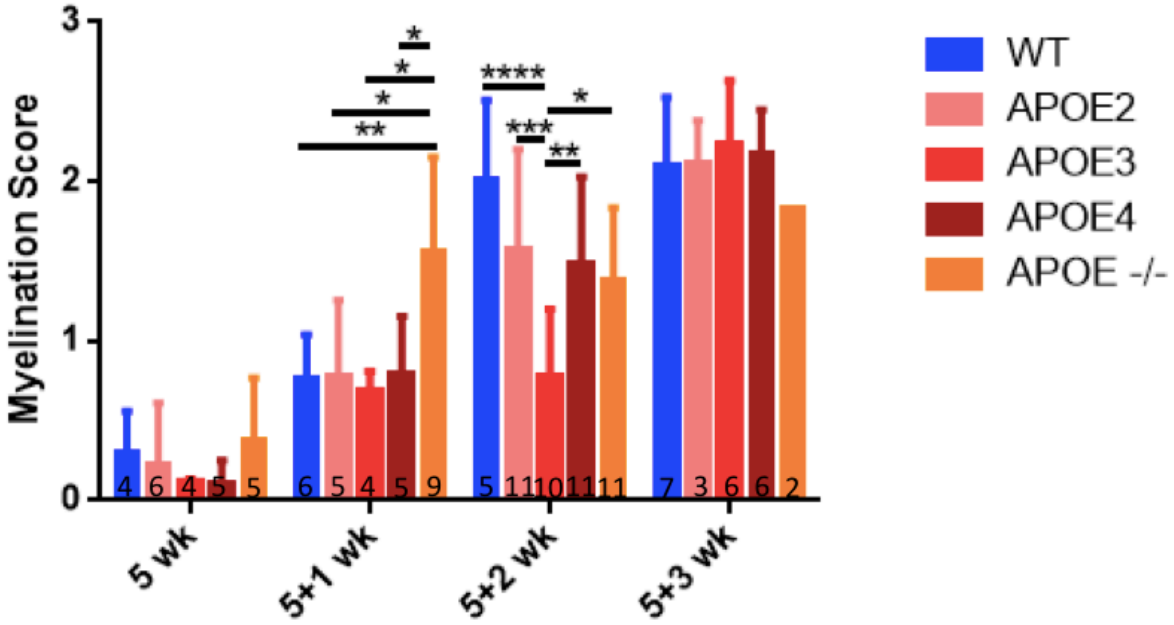
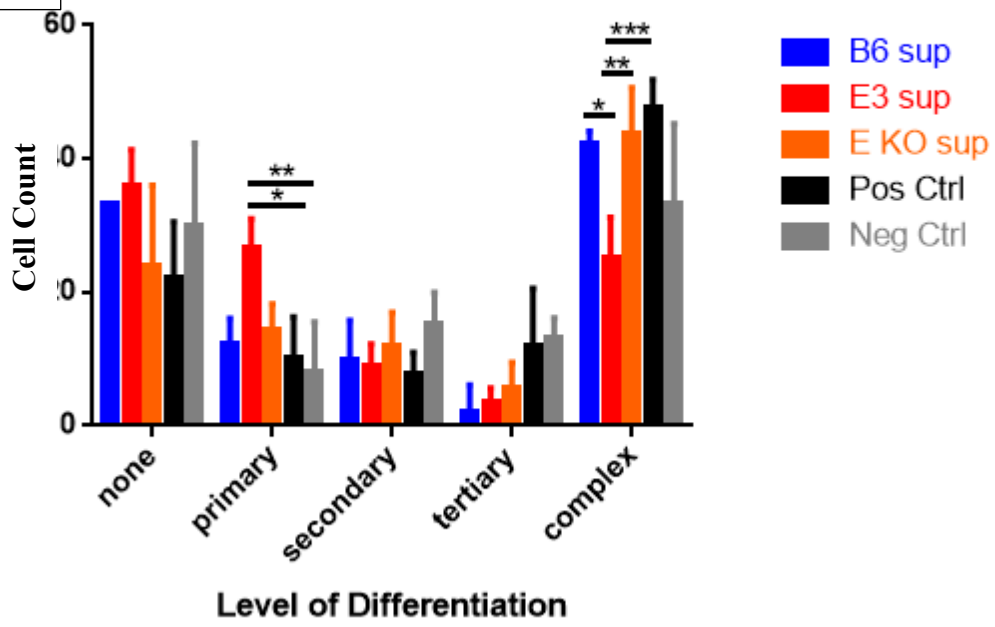


Figure 2: LFB-PAS staining suggests that apoe3 mice are delayed in remyelination

LFB-PAS staining of *apoe3* (APOE3 in the figure) mice brains at the 5+2 timepoint shows significantly less levels of myelin when compared to the WT and other *apoe* variants [*apoe2* (APOE2) and *apoe4* (APOE4)]. At the 5+1 time point, *apoe*$^{-/-}$ (APOE-/- in the figure) brains exhibited significantly more myelination. At the 5+3 week time point there is no significant difference between the levels of myelination. P<.05, **P<.01, ***P<.001, ****P<.0001. N-numbers displayed on graph. Statistical significance was obtained by two-way Anova, and Bonferroni's multiple comparison test. Data was collected by Leyla Ozelkan and Isabel Elssner.

A.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Cell Count
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primary
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Level of Differentiation
B6 sup
E3 sup
E KO sup
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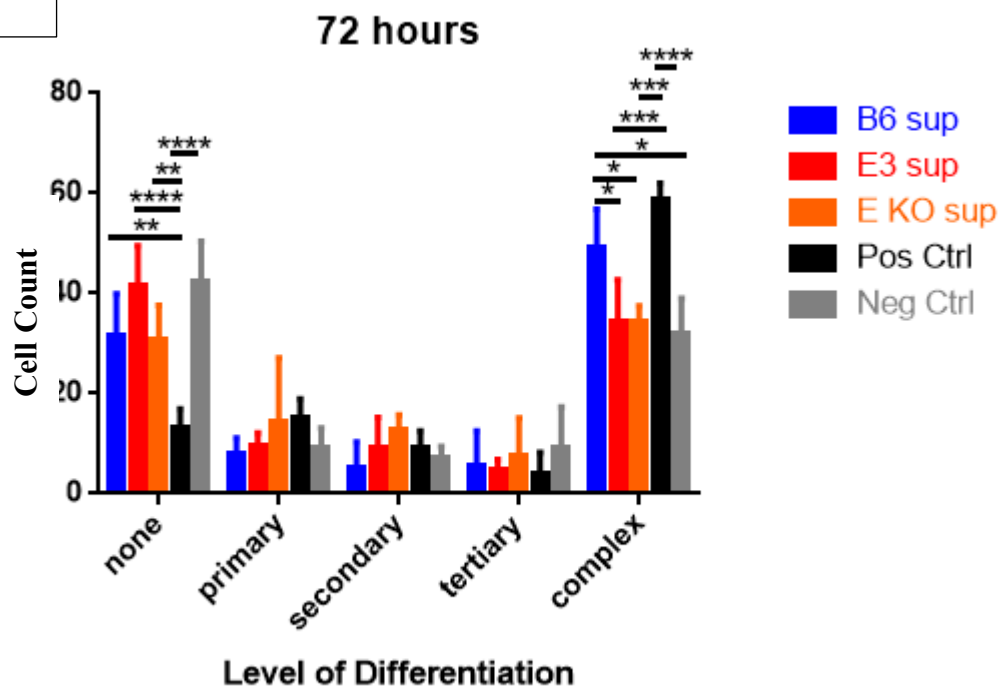
B.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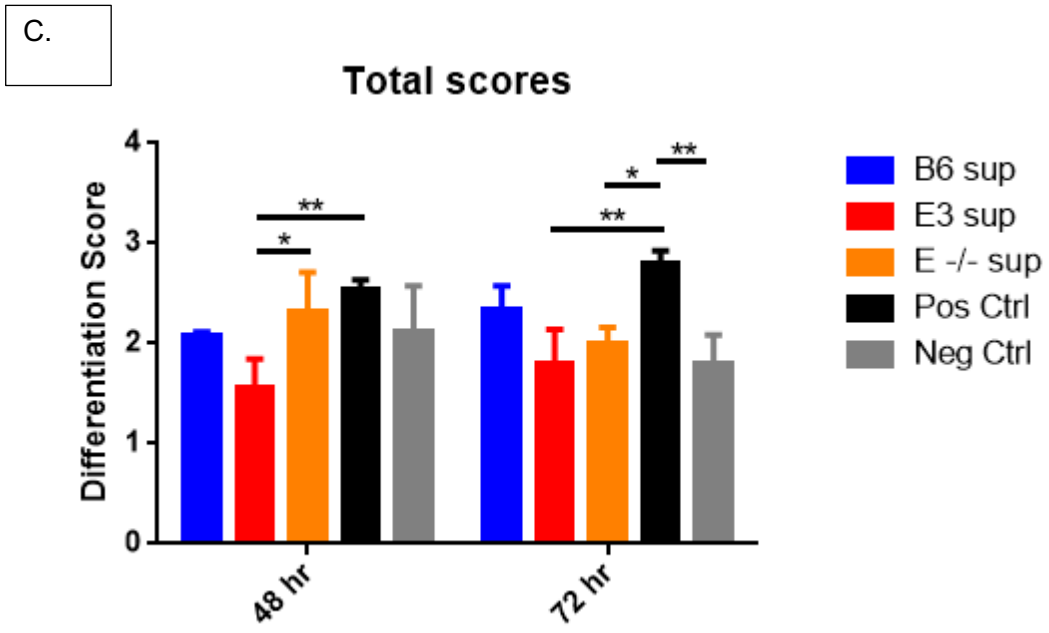

Figure 3: OPC differentiation is inhibited by APOE3 macrophage supernatants

Pictures are taken of the OPCs at 48 (A) and 72 hours (B) post the introduction of the macrophage supernatants. A. OPCs treated with *APOE3* (E3) macrophage supernatants at 48 hours had significantly more OPCs with primary processes than those treated with the positive and negative control supernatant. Additionally, OPCs treated with *APOE3* macrophage supernatants had significantly less complex OPCs than those treated with WT (B6), *APOE*$^{-/-}$ (E KO), and the positive control supernatant. B. OPCs treated with *APOE3* macrophage supernatants at 72 hours had significantly more OPCs without any differentiation than those treated with the positive control supernatant. Additionally, OPCs treated with *APOE3* macrophage supernatants had significantly less complex OPCs than those treated with WT (B6) and the positive control supernatant. C. At 48 hours, OPCs treated with *APOE3* macrophage supernatants show significantly less complex than *APOE*$^{-/-}$ supernatants and the positive control. At 72 hours, OPCs treated with *APOE3* and *APOE*$^{-/-}$ macrophage supernatants show significantly less complex than the positive control. WT is B6. Negative control: just media, Positive control: T3 and db-cAMP. P<.05, **P<.01, ***P<.001, ****P<.0001. Statistical

significance was obtained by two-way Anova, and Bonferroni's multiple comparison test. Data collected and analyzed by Isabel Elssner.

Results:

***In vivo* assessment using cuprizone model of demyelination and remyelination.**

This model uses cuprizone diet given over a 5-week period to induce full demyelination (Figure 1). At week 5, cuprizone feed is replaced with normal chow and spontaneous remyelination occurs. Harvesting brains at week 5+1, 5+2 and 5+3 typically allows for the robust change in remyelinating fibers. Mice were previously treated with cuprizone and harvested at the indicated time points during remyelination. I cut coronal sections of the corpus callosum above the fornix and kept them at -80C until their LFB-PAS staining. This region of the corpus callosum minimizes variables such as fiber direction and provides robust range in fiber myelination for scoring after LFB-PAS staining.

LFB-PAS staining suggests that *apoe3* mice are delayed in remyelination. (Figure 2).

We know that following full demyelination in mice brains after 5 weeks of cuprizone treatment, mice start to undergo remyelination with myelin levels increasing each week following the 5 week timepoint. We hypothesize that APOE may inhibit the remyelination process. To test this hypothesis, we conduct LFB-PAS staining of the corpus callosum in WT mice, *apoe2*, *apoe3*, *apoe4*, and *apoe*$^{-/-}$ mice. The myelin stains blue by the LFB and the rest of the brain stains pink with the PAS. After staining, the corpus callosum sections are scored blindly on a scale from 0 (no myelination) to 3 (full myelination). At the 5+1 week time point, *apoe*$^{-/-}$ brains exhibit significantly more myelination than the WT and other *apoe* variants. WT mice are just beginning to remyelinate and scores are relatively lower compared to the *apoe*$^{-/-}$ mice suggesting murine apoe may be inhibitory. There is no change in *apoe*$^{-/-}$ mice myelination in subsequent weeks and there is no difference compared to WT mice as remyelination increases

and matches remyelination extent with *apoe*$^{-/-}$ mice. The mechanism for the inhibitory effect of murine APOE is not clear.

For *apoe* knock-in mice, remyelination varies significantly. *apoe2, apoe3,* and *apoe4* mice show similar demyelination at week 5 compare to WT mice (Figure 2). In addition, early remyelination at week 5+1 is also similar among the three strains. In contrast, while *apoe2*, *apoe4* and WT mice continue to remyelinate, there is a significant delay in remyelination for the *apoe3* brains at the 5+2 week timepoint. *apoe3* mice are nearly half the score (0.75 compared to ~1.5) of their counterparts. This lower myelination score by *apoe3* mice increases by the 5+3 week timepoint as there no significant difference in remyelination levels. Therefore, it appears the presence of APOE3 may be inhibitory and causes a temporary delay in remyelination.

OPC differentiation is inhibited by *apoe3* macrophage supernatants (Figure 3).

Oligodendrocyte precursor cells (OPCs) differentiate into mature oligodendrocytes capable of myelinating axons. We know that APOE3 inhibits the remyelination process (Figure 3), so our hypothesis is that APOE3 has an inhibiting effect on oligodendrocyte precursor cells. To test this hypothesis *in vitro*, we isolate OPCs and expose them to supernatants from *apoe3* macrophages stimulated by apoptotic cells. The ability of macrophage supernatants to induce OPC differentiation are compared to WT supernatants and T3+db-cAMP positive control wells. Negative controls are OPCs with media alone. Pictures are taken of the OPCs at 48 and 72 hours post the introduction of the macrophage supernatants.

At 48 hours, *apoe3* macrophage supernatants (red bar) show more OPCs in the primary state with single processes (Figure 3A). The positive control T3+db-cAMP (black bar) show significantly fewer primary cells, nearly half the number, in comparison to *apoe3* supernatants indicating OPCs are moving towards differentiation. There is no difference among the OPCs in the secondary or tertiary phenotypes. Additionally, OPCs treated with *APOE3* macrophage supernatants had significantly less complex OPCs than those treated with WT (blue bar) *APOE*$^{-}$

$^{-/-}$ (orange bar), and the positive control supernatant. At 72 hours, *apoe3* macrophage supernatants show the most OPCs in the rounded state similar to negative control. (Figure 3B). This suggests apoe3 supernatants lack important factors to drive OPC differentiation. In contrast, positive control and WT (B6) supernatants show significantly more OPCs in complex states and suggests greater movement towards differentiation.

At 48 hours (Figure 3C), *apoe3* supernatant does not show substantial OPC differentiation whereas positive control and unexpectedly *apoe*$^{-/-}$ show maturation from rounded OPCs. When we assess the total scores for each timepoint, it appears the 72 hours may be more reliable. The *apoe3* supernatants perform poorly when comparing the positive control. Similarly, the *apoe*$^{-/-}$ also show less differentiation that is comparable to the negative control. These data suggest *apo3* and *apoe*$^{-/-}$ supernatants do not support late OPC differentiation as robustly as the positive control. Therefore, *apoe3* supernatants at 48 hours and 72 hours fail to support OPC differentiation. Interestingly, *apoe*$^{-/-}$ supernatants supported early OPC differentiation at 48 hours but not at 72 hours. Unfortunately, the WT (B6) macrophage supernatant is predicted to be similar to T3+db-cAMP; however, these supernatants did not behave as expected possibly due to prior freeze-thaws that may have lowered their active factors. The main conclusion is that at 72 hours, OPCs treated with *apoe3* and *apoe*$^{-/-}$ macrophage supernatants show significantly less complex processes than the positive control.

Discussion:

We speculate that APOE may bind apoptotic cells and either facilitate or hinder efferocytosis. We have shown that proper efferocytosis by macrophages may be important for secretion of oligodendrocyte precursor cell differentiation. This process may be intercepted by APOE and may have consequences to lesion repair. Here, we assessed both *in vivo* and *in vitro* whether APOE subtypes affect OPC differentiation and remyelination. Our data finds an accelerated remyelination in *apoe*$^{-/-}$ mice at week 5+1 week that is unexpected. It is not clear

why it is so high unless there was a technical error in staining. However, our OPC differentiation assay shows at 48 hours, *apoe*$^{-/-}$ macrophage supernatants that OPC differentiation was temporarily accelerated. It is plausible that initial factors produced early facilitates OPC differentiation in *apoe*$^{-/-}$ mice; however, we deduce in the presence of APOE, they are suppressed or inhibited.

In contrast, LFB-PAS staining of the corpus callosum of *apoe3* mice showed a significant delay in myelination at the 5+2 time point when compared to the WT and other *apoe* variant mice. LFB-PAS stain at week 5+3 suggests *apoe3* mice recover remyelination similar to WT mice. The OPC differentiation assay showed that OPCs treated with *apoe3* macrophage supernatants for 48 hours were significantly less complex than *apoe*$^{-/-}$ and the positive control. At 72 hours, OPCs treated with *apoe3* macrophage supernatants were also significantly less complex than the positive control. This suggests that APOE3 may interfere with efferocytosis and prevent proper secretome content. Indeed, previous lab experiments suggest macrophages from *apoe3* mice are defective in efferocytosis (data not shown). We believe that, after clearing apoptotic cells, macrophages produce factors that promote OPC differentiation, but APOE3 may change the production of these factors.

In summary, we believe that APOE3 could be interfering with the phagocytosis of apoptotic cells by microglia found in demyelinated regions of the brain by interacting with the Mertk receptor on microglia as a competitive inhibitor (Figure 4). Preliminary experiments conducted by others in the lab demonstrated that APOE can bind to Mertk in an ELISA. APOE2 and APOE4 were not able to bind to Mertk well; however, APOE3 significantly bound to Mertk. Thus, if apoptotic cells were prevented from binding to Mertk by APOE3, this would then inhibit efferocytosis and cause the microglia to secrete different factors that would inhibit OPC differentiation and thereby inhibit the remyelination of these regions.

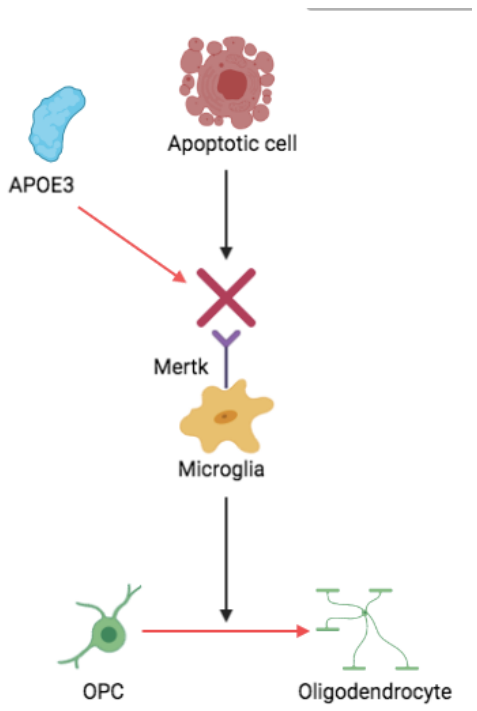

Figure 4: Proposed APOE inhibition of OPC differentiation mechanism

APOE3 acts as a competitive inhibitor to the phagocytosis of apoptotic cells in demyelinated regions by interacting with the Mertk receptor on microglia. APOE3 appears to cause the microglia to secrete factors that inhibit the differentiation of OPCs or prevent the production of differentiation factors.

So far only two OPC differentiation assays have been completed and while they show similar trends, additional assays will need to be conducted in the future to corroborate the trends and verify our findings. Additionally, repeat experiments need to be conducted to corroborate the trends of the LFB-PAS staining.

In the future, we would like to conduct phagocytosis assays to determine how effectively *apoe2, apoe3, apoe4 and apoe*$^{-/-}$ macrophages ingest apoptotic cells in comparison to WT macrophages. This would reveal any differences in macrophage ingestion of apoptotic cells across the APOE variants. Additionally, we have collected the supernatants of various *apoe*

mice and conducted proteomics on them. It would be interesting to determine which factors of the supernatants promote or inhibit OPC differentiation or the ability of macrophages to phagocytose apoptotic cells.

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