Author

Nicole Phan began working under Dr. Green as a lab assistant and developed a tremendous interest in their research. She discussed the possibility of conducting an independent project within the lab and began this work investigating cerebral amyloid angiopathy. Nicole particularly enjoyed the unpredictability of research, and the excitement of knowing that a final outcome can be completely different than original directions might have suggested. After graduation, Nicole plans to apply to medical school.

Investigation into Cerebral Amyloid Angiopathy Induced by Microglial Elimination

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Abstract

Alzheimer's disease (AD) is an age-related, progressive neurodegenerative disorder characterized by the presence of β-amyloid (Aβ) plaques and neurofibrillary tangles. Over 90% of AD patients with confirmed plaques and tangles also exhibit cerebral amyloid angiopathy (CAA). Microglia are believed to regulate Aβ accumulation and deposition in AD, with failures in immune clearance mechanisms believed to underlie the onset of AD. Our lab has demonstrated that microglia in healthy adult mice depend on colony-stimulating factor 1 receptor (CSF1R) signaling for survival, and inhibition of CSF1R results in rapid global elimination of microglia. We found that administration of the CSF1R inhibitor, PLX5622, in pre-pathological 5xfAD animals results in the appearance of CAA. To extend these findings, we sought to characterize the consequences of CAA induction on the local brain environment. If microglia protect against CAA onset and blood vessel damage to the cortex in 5xfAD animals, then the elimination of microglia should worsen the blood-brain barrier (BBB) integrity and blood vessel-associated tissue necrosis. Cohorts of animals treated for 3 and 6 months underwent immunohistochemical analysis for endothelial components and markers of brain bleeding. The findings indicate that microglia play a protective role against CAA-induced damage, specifically within the thalamus.

Faculty Mentor

Nicole's work highlights the importance of microglia in protecting the aged brain from the development of cerebral amyloid angiopathy (CAA). CAA is the accumulation of the amyloid-beta peptide in the blood vessels of the brain and is associated with the development of Alzheimer's disease as well as with increased risk of stroke. Nicole worked independently to assess the consequences of CAA that accumulated due to the pharmacological depletion of these microglia in mouse models of Alzheimer's disease. She learnt several advanced laboratory techniques, and was able to show that CAA disrupts the integrity of the blood vessels. Projects like this highlight the importance of research-intensive Schools such as UCI, and the high caliber of our undergraduate students, and provide unique training and experiences that will serve Nicole and others well in their future careers.

Kim Green
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Key Terms

- Alzheimer's disease
- Amyloid
- Cerebral Amyloid Angiopathy
- Microglia

Introduction

Alzheimer's disease (AD) is an irreversible, progressive neurodegenerative disease identified by the presence of amyloid plaques and neurofibrillary tangles. Notably, neuro-pathological studies have established that over 90% of AD patients with confirmed plaques and tangles also have cerebral amyloid angiopathy (CAA), which involves infiltration of the brain's blood vessels with the protein amyloid-β (Aβ). The accumulation of Aβ deposits along the walls of the vessels in the brain weakens their overall integrity, making them more prone to breaking. The exact mechanisms driving the onset of CAA remain unknown; however, increased production of Aβ or impairments in its clearance from the central nervous system (CNS) are potential mechanistic candidates. One of the ways in which Aβ is cleared from the brain is through microglia phagocytosis, in which microglia engulf Aβ aggregates and engage internal lysosomal machinery to break down and dispose of them. These microglia undergo activation to move to areas with toxic materials to clear and offer protection, but their clearance mechanisms are compromised (Kettenmann et al., 2011; Sarlus and Heneka, 2017). Indeed, attempts to treat AD via Aβ immunotherapy, which stimulates microglial uptake and removal of Aβ, appeared to be successful in removing parenchymal Aβ deposits, seen in AD, in clinical trials; however, vascular Aβ pathology, seen in CAA, was exacerbated (Oddo et al., 2004; Wilcock and Colton, 2009). Importantly, these studies highlight a relationship between microglia and CAA onset, but whether microglia directly contribute to the development of CAA remains unanswered.

We previously demonstrated the dependence of microglia on colony-stimulating factor 1 receptor (CSF1R) signaling for their survival in the healthy (Elmore et al., 2014) and diseased (Spangenberg et al., 2016; Rice et al., 2015) adult brain. In this study, wild type and 5xfAD animals were treated with control or PLX5622-formulated chow (1200 ppm) for 3 and 6 months (n=4–9/group). 5xfAD mice have five familial Alzheimer's disease (fAD) mutations leading to rapid accumulation of Aβ starting at 2 months of age, and were thus used to visualize severe amyloid pathology (Oakley et al., 2006). Recently, we found that the ablation of microglia from pre-pathological 5xfAD for 3 months reduces the deposition of parenchymal Aβ; instead, Aβ accumulates within blood vessels, consistent with Aβ immunotherapy studies (Wilcock et al., 2004). Here, we sought to further explore these findings by characterizing the consequences of CAA induction on the local brain environment. We hypothesize that if microglia normally protect against cortical CAA onset and blood vessel damage in 5xfAD

animals, then the elimination of microglia should worsen the blood brain barrier integrity and blood vessel-associated tissue necrosis. These findings will further our understanding of the role of microglia in the development of CAA, potentially aiding the creation of new and effective therapies to alleviate this condition.

Materials and Methods

All experiments were carried out in accordance with the Institutional Animal Care and Use Committee (IACUC) at the University of California, Irvine, and were consistent with Federal guidelines. To evaluate the role of microglia in mediating Aβ deposition, we generated tissue from wild type and 5xfAD animals treated with control or PLX5622-formulated chow (1200 ppm) for 3 months (wild-type control n=5, wild-type + PLX5622 n=4, 5xfAD control n=8, 5xfAD + PLX5622 n=10) and 6 months (wild-type control n=5, wild-type + PLX5622 n=4, 5xfAD control n=7, 5xfAD + PLX5622 n=6), and sacrificed at 4 and 7 months. As animals in the cohort of ~6 month PLX5622 treatment exhibited more robust vascular pathology, we used these animals for most analyses. Mice in the ~3 month PLX5622 treatment groups were used to compare the size of vascular Aβ deposits. Aβ plaques were visualized using Thioflavin-S (ThioS) staining, which binds to fibrillar Aβ found in dense core deposits. Brain hemispheres were sliced coronally at 40 μm using a microtome and stored in 1x phosphate buffered saline (PBS) + 0.05% NaN$_3$. Vascular amyloid pathology was quantified using co-localized staining of Aβ and collagen IV in blood vessels. Blood brain barrier integrity was examined by staining intermediate filaments with Vimentin, endothelial cells with GLUT-1, astrocytes with glial fibrillary acidic protein (GFAP), astrocyte end-feet with water channel aquaporin 4 (Aqp4), and tight junctions with Claudin-5, in addition to Prussian blue staining to observe micro-hemorrhages. These stains allow us to observe the relationship between microglia, CAA, and the surrounding vasculature.

Confocal Microscopy

Tissue from the 4- and 7-month cohort of wild type, microglia-intact, and microglia- eliminated 5xfAD animals were used (i.e., 5xfAD and 5xfAD+PLX5622 groups). The retrosplenial (RS) cortex and thalamus were imaged due to the robust and consistent presence of vascular pathology. The Thio-S staining method followed previously established protocols by our lab (Rice et al., 2014). Immuno-fluorescent labeling was performed following a standard indirect technique as previously described, followed by staining with Alexa fluorescent secondary antibody (Neely et al., 2011). The following primary antibodies and dilutions were used:

Claudin-5 1:400 (Invitrogen 35-2500), Collagen IV 1:300 (abcam ab658), GLUT-1 1:400 (Millipore Sigma 07-1401), Aqp4 1:400 (Sigma A5971), GFAP 1:1000 (abcam ab4674), and Vimentin 1:1000 (abcam ab92547). Gross measurement of staining of the blood vessels and CAA were obtained by imaging comparable sections of tissue on a confocal microscope (Leica TCS SPE) at the 10x objective with a 2x digital zoom and 2 micron step size to generate three dimensional Z-stacks. Protein expression was quantified by calculating percent area and mean gray value using ImageJ software (NIH). Bitplane Imaris 7.5 was used for automated analyses using surface modules.

Prussian Blue staining was employed to assess blood-brain barrier (BBB) integrity in the cohort of animals treated with control or PLX5622-treated chow, using spleen tissue as the positive control. Tissues were placed onto slides to dry overnight prior to staining. Slides were hydrated in DI water for 5 minutes, then transferred from DI water to Iron Stain solution (equal volume of Potassium Ferrocyanide solution and Hydrochloric Acid solution) for 5 minutes, taking care not to use metal forceps for transfer during the staining procedure because this would contaminate observed sections. Slides were rinsed thoroughly with DI water and transferred into Nuclear Fast Red solution for 3 minutes then rinsed in DI water four times. Finally, the slides were dehydrated in 95% ethanol followed by 100% ethanol for 2 minutes each, then cover- slipped using DPX (VWR).

Results

CSF1R Inhibition Eliminates Microglia

We have previously studied the effects of CSF1R inhibition leading to the elimination of microglia (Elmore et al.,
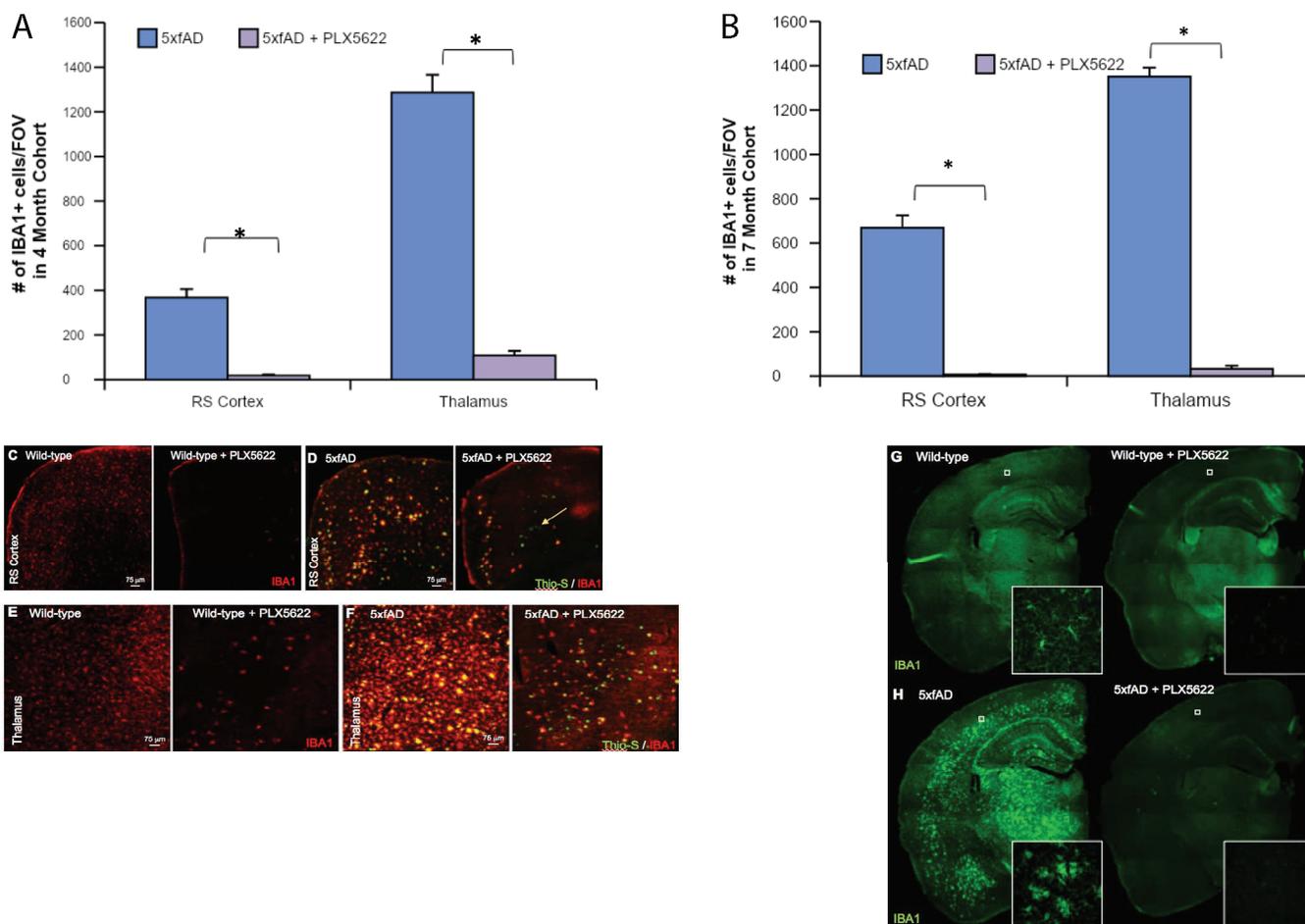

Figure 1

CSF1R inhibition eliminates microglia long term. (A and B) Microglia are significantly decreased (p<0.001 for both, significance denoted by *). (C and D) Immunolabelling for microglia (IBA1 in red) and staining for dense-core plaques (Thio-S in green) in RS cortex in the 4-month cohort. Decrease in microglia can be visualized by the lack of labeled microglia, in red, in each of the right images. (E and F) 4-month cohort immunolabelling for microglia (IBA1 in red) and staining for dense core plaques (Thio-S in green) in the thalamus. Decrease in microglia can be visualized by the lack of labeled microglia, in red, in each of the right images. (G and H) Immunolabelling for dense-core plaques (Thio-S in green) in the 7-month cohort. Elimination of dense-core plaques seen in the right image of figure H.
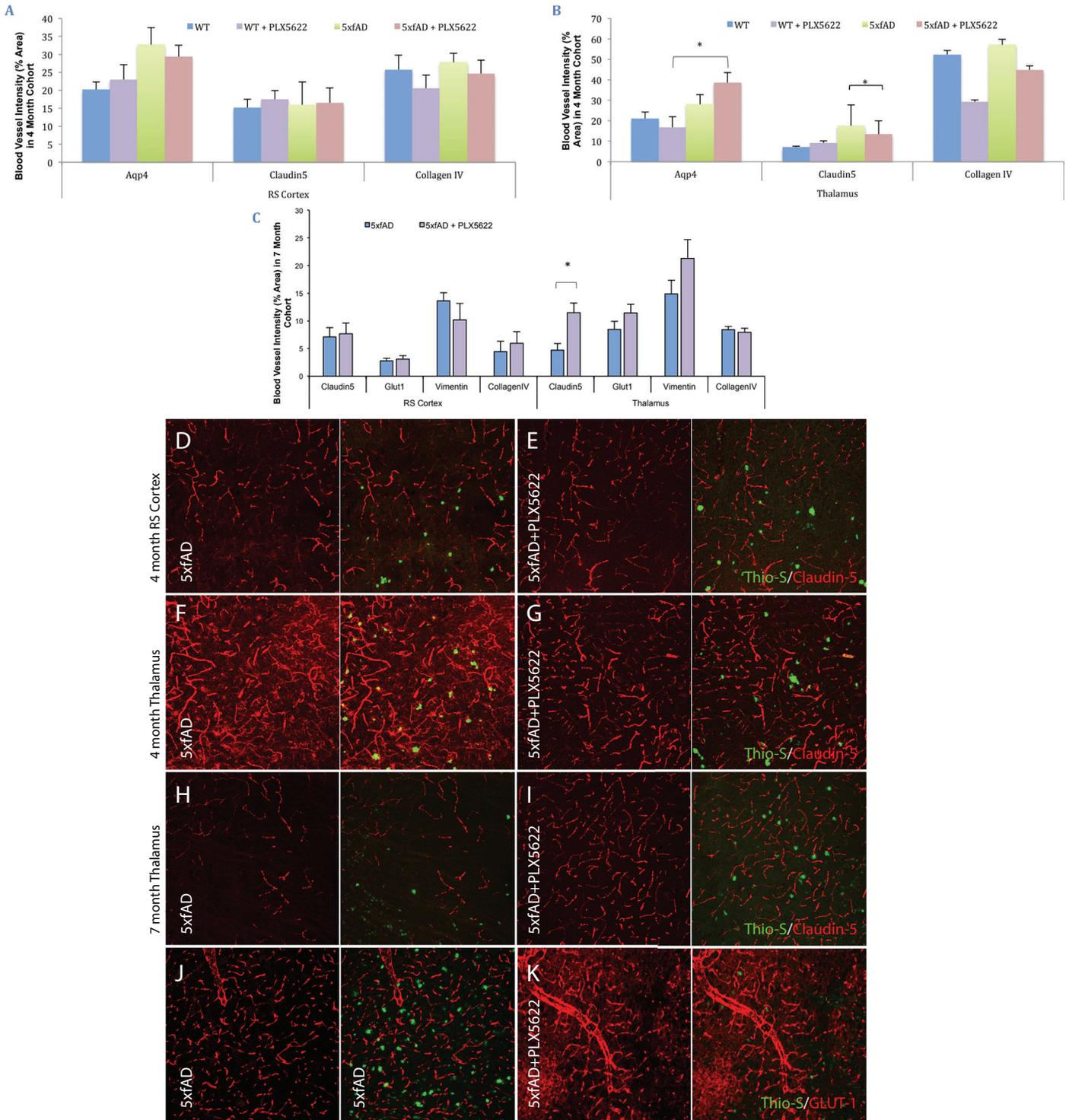
Figure 2

Microglia regulate vascular integrity in an age- and regional-dependent manner. (A) Blood vessel intensity of Aqp4, Claudin-5, and Collagen IV in the 4-month cohort in the RS cortex. (B) Blood vessel intensity in the 4-month cohort in the thalamus. There is a significant increase in Aqp4 between the treated groups (P=0.0330) and a significant decrease in Claudin-5 in the thalamus of the 5xfAD groups with microglia elimination (5xfAD and 5xfAD+PLX5622) (P=0.0246). (C) Blood vessel intensity in the 7-month cohort RS cortex and thalamus. There is a significant increase in 7-month Claudin-5 in the thalamus (P=0.0106). (D, E, F and G) Immunolabelling in 4-month cohort for tight junctions (Claudin-5 in red), dense core plaques (Thio-S in green) in RS Cortex and thalamus. Decrease in intensity between F and G. (H and I) 7-month immunolabelling for tight junctions (Claudin-5 in red), dense core plaques (Thio-S in green) in the thalamus. Increase in intensity between H and I. (J and K) Immunolabelling in the 7-month cohort for endothelial cells (GLUT-1 in red), dense core plaques (Thio-S in green) (Statistical significance denoted by *).

2014). PLX5622 is a potent CSF1R inhibitor, sufficient for elimination of microglia, without inhibition of c-kit, a stem-cell factor receptor (Dagher et al., 2015). Microglia were immunolabeled with IBA1 and dense core plaques were stained using Thio-S. Treatment with PLX5622 for 3 and 6 months demonstrated significant decrease ($P < 0.001$) in both cohorts within the RS cortex and thalamus, where the most robust vascular pathology is seen (Figure 1A and 1B). Imaging for the RS cortex in the 4-month cohort demonstrates the decrease of microglia, stained in red, in both wild type and 5xfAD animals (Figure 1C and 1D). The same can be visualized in imaging for the thalamus in the 4-month cohort (Figure 1E and 1F). Dense core plaques, found in 5xfAD animals, decrease when treated with PLX5622
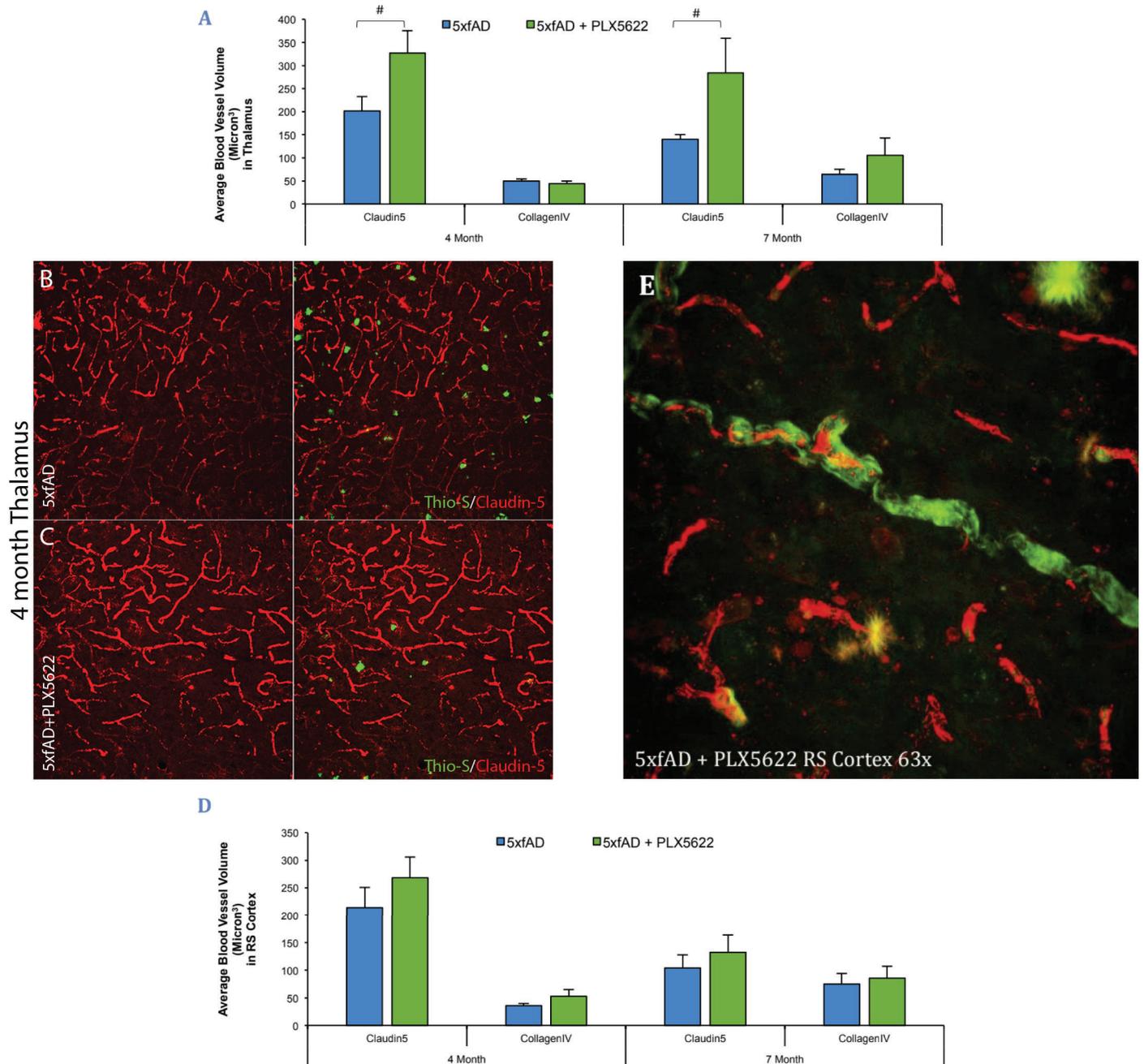

Figure 3

Average blood vessel volumes. (A) Average blood vessel volume in the thalamus in 4- and 7-month cohorts. There is a trending increase in Claudin-5 tight junctions in both 4- and 7-month cohorts ($P=0.0738$ and $P=0.0675$, respectively. Statistical trends denoted by #). (B and C) Immunolabelling for tight junctions (Claudin-5 in red) and dense-core plaques (Thio-S in green) in 4-month cohort thalamus for 5xfAD (B) and 5xfAD + PLX5622 (C). (D) Average blood vessel volume in RS cortex of both 4- and 7-month cohorts. (E) Immunolabeled 63x image (Thio-S in green and Claudin-5 in red) of 7-month 5xfAD + PLX5622 RS cortex. Dense-core plaques enwrap the blood vessel.

(Figure 1G and 1H). Thus, treatment with PLX5622 is sufficient to eliminate microglia within the adult mouse brain.

Microglia Regulate Vascular Integrity in an Age- and Regional-Dependent Manner

To measure blood vessel intensity (percent area), Claudin-5 and Collagen IV were used for 4- and 7-month cohorts, and Vimentin and GLUT-1 were used in the 7-month cohort only. Staining for tight junctions with Claudin-5 demonstrated a significant decrease (P=0.0272) in fluorescent intensity with microglia elimination in the thalamus of the 4-month cohort (Figure 2B) but a significant increase in intensity within the 7-month cohort (P=0.0106) (Figure 2C), relative to untreated controls. Aqp4 staining for water channels within the 4-month cohort demonstrates a significant increase (P=0.0330) between the wild type + PLX5622 group and the 5xfAD + PLX5622 group. Quantification of Vimentin and GLUT-1 did not show significant changes (data not shown). No significant changes were found in the RS cortex of both the 4- and 7-month cohort (Figure 2A and 2C). Importantly, this highlights age-dependent effects of microglia elimination on vascular integrity and importantly points to the vasculature within the thalamus as being particularly susceptible to changes with the absence of microglia.

Blood Vessel Volume

Gross observations suggested that blood vessel volume may be altered with aging, prompting further investigation into the average blood vessel volume between the two time points. Blood vessel volume was quantified using Bitplane Imaris of the 4- and 7-month tissue stained with Collagen IV and Claudin-5. Upon analysis, we observed a trending increase in blood vessel volumes in both the 4- (P=0.0738) and 7-month (P=0.0675) cohorts within the thalamus, labeled for tight junctions by Claudin-5 (Figure 3A), but there were no significant changes observed within the RS cortex of the 4- and 7-month cohorts (Figure 3D). However, microglia elimination did not significantly alter blood vessel volume in the 4- or 7-month cohorts of animals, indicating that microglia do not regulate the adhesion or accumulation of Aβ to or within blood vessels.

Changes in Vasculature with Microglia Elimination Coincides with Increased Iron Staining

Observed changes of the blood vessels within the thalamic region prompted assessment of BBB integrity in the 4- and 7-month cohorts using Prussian blue iron staining. Spleen tissue was used as a positive control for the Prussian blue stain (Figure 4A and 4D). Observation and analysis of the Prussian blue iron stain revealed no detection of iron

staining in the 4-month cohort of animals in any group (Figure 4B and 4C). Iron staining in the 7-month cohort of animals revealed no detection of iron staining in the 5xfAD group in the RS cortex and thalamus (Figure 4D and 4E). However, there is an increase in iron staining within the thalamus in microglia-depleted animals, indicative of compromised BBB integrity within this region (Figure 4F).
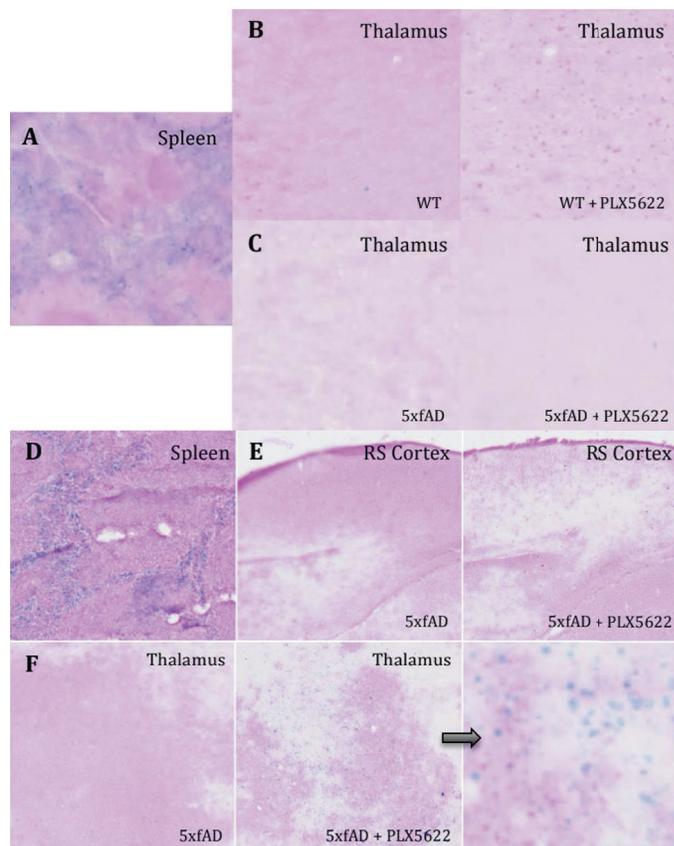

Figure 4

Changes in vasculature with microglia elimination coincides with increased iron staining. (A) Prussian blue images of positive control (spleen). (B) Prussian blue images of 4-month wild type and wild type + PLX 5622 within the thalamus. (C) Prussian blue images of 4-month 5xfAD and 5xfAD + PLX5622 within the thalamus. (D) Prussian blue images of positive control (spleen). (E) Prussian blue images of 7-month 5xfAD and 5xfAD + PLX5622 within RS cortex. (F) Prussian blue image of 7-month 5xfAD, 5xfAD + PLX5622 and enhanced close-up image within the thalamus. The 5xfAD + PLX5622 enhanced image demonstrates presence of hemosiderin (iron), labeled in blue.

Discussion

We have previously found that the ablation of microglia from pre-pathological 5xfAD leads to Aβ accumulation within blood vessels and sought to further investigate these findings by characterizing the consequences of CAA induc-

tion on the local brain environment. Microglia are the main immune cells in the brain, removing debris or dying cells to maintain homeostasis. However, the chronic activation or elimination of these microglia can compromise microglial phagocytosis, leading to accumulation of Aβ within the blood vessels and the progression of AD (Sarlus and Heneka, 2017). AD is an irreversible, progressive neurodegenerative disease identified by the presence of amyloid plaques and neurofibrillary tangles and is typically accompanied by CAA. CAA is characterized by the accumulation of Aβ within the blood vessels and studies have shown a decrease in blood vessel size due to CAA in AD subjects (Bouras, et al., 2006). Moreover, Aβ deposition in the vasculature occurs in close proximity to smooth muscle cells, causing a loss of cell function leading to stroke (Biffi and Greenberg, 2011; Aldea et al., 2019; Hill et al., 2015). Aβ deposits with replacement of smooth muscle cells can lead to lobar hemorrhages that damage the vessel wall resulting in decreased adhesion to basement membranes and vascular rupture (Smith & Greenberg, 2009).

Thio-S staining, blood vessel immunohistochemistry, and iron staining were used to observe blood vessel pathology. Blood vessel staining of the 4- and 7-month cohorts of 5xfAD groups demonstrated a significant decrease in Claudin-5$^+$ tight junctions in the thalamus of the microglia eliminated 4-month cohort but a significant increase within the 7-month cohort. Claudin-5$^+$ is a tight junction protein within the BBB that provides a protective barrier in which its dysfunction can lead to AD and increased permeability of BBB (Greene et al., 2019). An increase of Claudin-5$^+$ tight junctions within the 7-month cohort with elimination of microglia could be compensatory and time-dependent due to the early pathology of the developing mice. Studies have shown that remodeling appears with an initial decrease followed by up regulation of Claudin-5$^+$ expression (Greene et al., 2019). We see the Thio-S enwrap the blood vessel, reducing the expression of tight junctions within the 4-month cohort, possibly leading to a response of increased tight junction expression over time, as seen in the 7-month cohort.

Observation of the Aqp4 stain for water channels demonstrated a significant increase between the wild type treated with PLX5622 and 5xfAD treated with PLX5622 groups within the thalamus of the 4-month cohort. These findings are consistent with previous reports demonstrating an increase in aquaporin 4 (Aqp4) staining in AD, indicative of worsening vessel integrity (Moftakhar et al., 2010). Aqp4 is a protein channel involved in transportation of water by being in close proximity to capillaries through astrocyte

end feet, with an important function of BBB regulation (Moftakhar et al., 2010). Additionally, decreased Aqp4 expression can lead to ineffective clearance of Aβ within the vasculature (Xu et al., 2015). Studies have found that patients with AD have more pronounced vascular CAA in the thalamus (Burwinkel et al., 2018 & Thal et al., 2008). Moreover, we find robust and consistent changes in the thalamus, highlighting regional effects of microglia elimination on blood vessel maintenance.

Aβ is shuttled from the neurons to the blood vessels by macrophages, but it is unclear if microglia assist as well (Zaghi et al., 2009). The findings of the blood vessel intensity led us to further investigate if the average blood vessel volumes were changed with aging and/or in the absence of microglia in 5xfAD animals. The expression of Claudin-5$^+$ tight junctions within the thalamus trended toward an increase with microglia elimination in both the 4- and 7-month cohorts. Due to the observed alterations in thalamic blood vessel volume and intensity, we decided to assess bleeding within the brain by observing the presence of hemosiderin, an iron storage complex that produces a ferric oxide material during hemoglobin breakdown (Liu et al., 2014). Hemosiderin is labeled by using Prussian blue staining for the detection of iron within the brain, which is indicative of compromised BBB integrity. We found no significant difference within the RS cortex of both wild type 4- and 7-month cohorts in iron staining, as well as the 5xfAD groups, consistent with our data showing that microglia elimination did not significantly alter blood vessel staining in this region. However, in the thalamus of the 7-month of the 5xfAD treated with PLX5622, we found evidence of bleeding in the absence of microglia, consistent with our findings of altered blood vessel staining in this region. The difference in hemosiderin levels between the 4- and 7-month cohorts may be due to minimal alterations in BBB integrity with three months of microglia depletion that intensifies and is further exacerbated with an additional three months of microglia depletion (i.e., 6 months of total PLX5622 treatment), leading to detectable micro-hemorrhages. In the future, we plan to analyze RNA-seq data from the thalamus to investigate blood vessel-related gene expression changes between this region and the cortex, both in the presence and absence of microglia. Thus, these findings indicate that microglia play a protective role against CAA-induced damage in the thalamus. Understanding how microglia exert these protective effects may allow for the development of targeted therapeutics for this condition.

Acknowledgments

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