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Blood-Derived Plasminogen Modulates the Neuroimmune Response in Both Alzheimer's Disease and Systemic Infection Models

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BLOOD-DERIVED PLASMINOGEN MODULATES THE NEUROIMMUNE RESPONSE IN BOTH ALZHEIMER'S DISEASE AND SYSTEMIC INFECTION MODELS

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The Rockefeller University 2020

Alzheimer's disease (AD) is a progressive neurodegenerative disease that affects 44 million people worldwide. To date, there are no effective treatments that prevent progression or death from the disease. It is clear that AD development is multifactorial and can arise from genetic or lifestyle factors and that vascular dysfunction and inflammation play a role in the progression of the disease. Many cardiovascular factors associated with an increase in systemic infection have been linked to a risk of AD development and progression. However, the link between vascular risk factors, systemic inflammation, and the neuroinflammation characteristic of AD are not fully understood.

Plasminogen is primarily a blood protein synthesized in the liver, which when cleaved into its active form, plasmin, plays roles in fibrinolysis, wound healing, cell signaling, and inflammatory regulation. Increasing evidence links plasminogen to the regulation of inflammatory responses in many organ systems of the body and implicates plasminogen in the progression of a diverse range of pathologies.

I aimed to determine whether plasminogen plays a role in the regulation of neuroinflammation during AD progression. I showed that blood-derived plasmin is a regulator of brain inflammatory activation. Depletion of peripheral plasminogen in an AD mouse model through antisense oligonucleotide (ASO) technology lessened AD pathology and decreased glial cell activation in the brain, whereas an increase in plasmin activity through α_2-antiplasmin ASO treatment exacerbated AD mouse pathology. Moreover, blood-derived plasminogen modulated the wild-type mouse brain's neuroinflammatory response to peripheral lipopolysaccharide injection and this was mediated by the p11 receptor, suggesting a more global role for plasminogen in regulating inflammatory communication between the periphery and brain. These studies suggest a crucial role for peripheral plasmin in mediating neuroimmune cell activation and could provide a link to systemic inflammatory risk factors that are known to be associated with the development of AD and other neurological disorders.

To Mom and Dad

for unrivaled encouragement and love

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List of Abbreviations

A2AP ASO	antisense oligonucleotide against α_2-antiplasmin
AD	Alzheimer's disease
ADAD	autosomal dominant Alzheimer's disease
aHUS	atypical hemolytic uremic syndrome
ANOVA	analysis of variance
AP	anterior-posterior
Apo(a)	apolipoprotein a
APP	amyloid precursor protein
ASO	antisense oligonucleotide
Aβ	beta-amyloid
BBB	blood brain barrier
CNS	central nervous system
CPM	choroid plexus and circumventricular macrophage
CTRL ASO	scrambled control antisense oligonucleotide
DAMP	damage-associated molecular pattern
DV	dorsal-ventral
EAE	experimental autoimmune encephalomyelitis
ECM	extracellular matrix
EOAD	early onset Alzheimer's diease
Fab	fragment antigen binding
FXII	factor XII
GFAP	glial fibrillary acidic protein
H2B	nuclear protein histone H2B
HFIP	hexafluoroisopropanol
ICD	intracellular domain
IL-1β	interleukin 1β
IP	intraperitoneal

KO	knockout
LAMP-1	lysosomal associated membrane protein 1
LDL	low density lipoprotein
LOAD	late onset Alzheimer’s disease
Lp(a)	lipoprotein a
LPS	lipopolysaccharide
LRP	low density lipoprotein receptor related protein
mAb	monoclonal antibody
ML	medial-lateral
MM	meningeal macrophage
MMP	matrix metalloproteinase
MS	multiple sclerosis
NFT	neurofibrillary tangle
PAI-1/2	plasminogen activator inhibitor 1/2
PAMP	pathogen-associated molecular pattern
PBS	phosphate-buffered saline
PLG ASO	antisense oligonucleotide against plasminogen
PSEN	presenilin
PVM	perivascular macrophage
ROS	reactive oxygen species
rtPA	recombinant human tissue plasminogen activator
sAPP	soluble amyloid precursor protein
SEM	standard error of the mean
TF	transferrin
TLR-4	toll-like receptor 4
tPA	tissue plasminogen activator
TXA	tranexamic acid
uPA	urokinase plasminogen activator

uPAR	urokinase plasminogen activator receptor
VEGF	vascular endothelial growth factor
WT	wild-type

CHAPTER 1: INTRODUCTION

1.1 Alzheimer's Disease Clinical Features

Alzheimer's disease (AD), is a neurodegenerative disorder characterized by memory decline and death and is the most common form of dementia (Lane et al., 2018, Long and Holtzman, 2019). Dementia effects 44 million people worldwide and the cost of care is a huge financial burden (Lane et al., 2018). The greatest risk factor for AD is age, and the prevalence doubles every five years after the age of 65. Most cases (~95%) are defined by cognitive decline after the age of 65 and are considered late-onset AD (LOAD). Less than 5% of AD cases are considered early-onset AD (EOAD). About 1-2% of AD is autosomal dominantly inherited (ADAD). Mutations in the beta-amyloid (Aβ) protein and its processing machinery lead to ADAD that presents earlier in life than sporadic AD and oftentimes with a more rapid progression (Long and Holtzman, 2019).

AD is discriminated from other forms of dementia by a specific progression of neuropathology which includes deposition of Aβ plaques throughout the brain, the presence of neurofibrillary tangles (NFTs), and dystrophic neurites filled with hyperphosphorylated tau protein. Other prominent features of AD include neuroinflammation indicated by reactive glia in the brain, as well as oxidative stress, synaptic and neuronal loss, and cerebrovascular disease (Serrano-Pozo et al., 2011, Long and Holtzman, 2019, Lane et al., 2018).

1.2 Aβ Hypothesis of Alzheimer's Disease

Aβ is a protein that forms when amyloid precursor protein (APP) is cleaved by β-secretase and γ-secretase (Figure 1.1). An initial cleavage by β-secretase leads to release of soluble APP (sAPP), whereas a secondary cleavage by γ-secretase leads to release the release of Aβ and the N-terminal intracellular domain (ICD). The beta-amyloid (Aβ) protein can be produced by cleavage at several sites to create a peptide that is usually 39 to 43 amino acids in length. The most common form of Aβ is 40 amino acids in length (Aβ40), whereas the Aβ42 cleavage is considered to be the most pathogenic form and has been implicated in AD (Bekris et al., 2010).

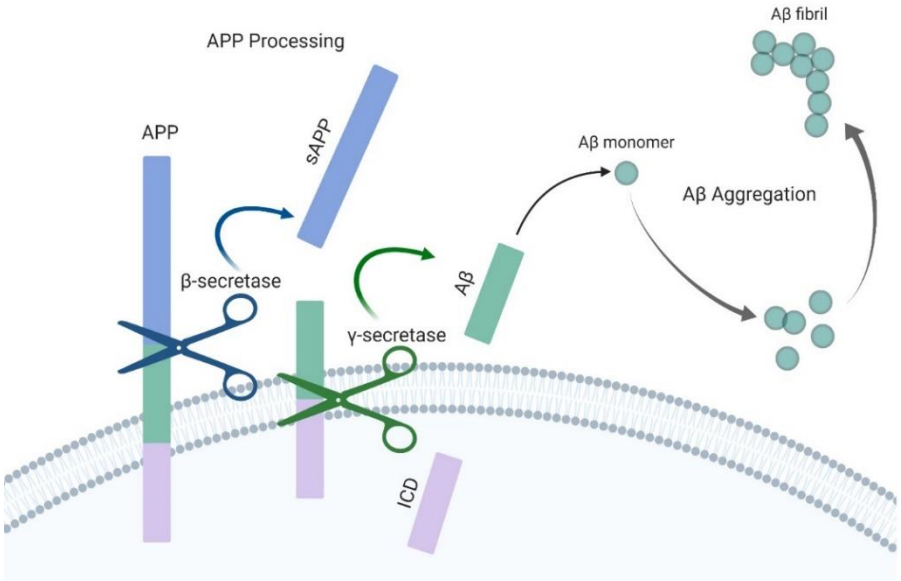


Figure 1.1. APP is processed to form Aβ. Transmembrane protein APP is cleaved by β-secretase to release sAPP and by γ-secretase to release Aβ and ICD.

One of the most striking features of AD, seen initially by Alois Alzheimer in 1907 when he diagnosed the first patient with what would later become known as AD, is the presence of plaques in the brains of AD patients. These plaques can be tightly aggregated into fibrillar species or exist as more diffuse aggregates, and are deposited throughout the brain of AD patients with a common progression. Amyloid plaques are primarily found in the neocortex and, to a lesser extent, in the allocortex (Serrano-Pozo et al., 2011).

Once the plaques characteristic of AD patients were sequenced in patient brain tissue (Glenner and Wong, 1984, Masters et al., 1985), it was hypothesized that the protein in these plaques, Aβ, was a primary driver of disease pathogenesis (Hardy and Selkoe, 2002, Hardy and Higgins, 1992). Genetic studies of patients with AD have indicated that overproduction of Aβ is one way, but not the only way, that AD can develop. Patients with genetic mutations in APP, Aβ, or presenilin 1 and 2 (PSEN1, PSEN2) which compose part of the γ-secretase complex tend to develop AD early and with rapid progression. These mutations lead to abnormal and increased Aβ production throughout life (Long and Holtzman, 2019, Serrano-Pozo et al., 2011).

In addition, mice do not produce Aβ normally, but if they are genetically altered with human Aβ mutations, they will produce Aβ and develop some of the cognitive deficits seen in human patients with AD. Common mouse models have mutations in APP and PSEN1 that have been found in patients with ADAD and these mutations lead to Aβ overproduction in the brains of these animals (Alzforum, n.d.-a).

While genetic studies and mouse models show that Aβ can be one cause of AD, amyloid burden does not correlate with degree of cognitive impairment (Serrano-Pozo et al., 2011) and there are people with a high burden of amyloid that do not develop AD and also lack tau and neuroinflammatory pathology (Nelson et al., 2009). Thus, other downstream effectors such as tau pathology, inflammation, neuronal dysfunction, and neurodegeneration must play a critical role in disease progression and cognitive decline.

1.3 Vascular Hypothesis of Alzheimer's Disease

Cardiovascular abnormalities associated with AD include coronary artery disease, atrial fibrillation, heart failure, vasculopathy, macro and microinfarcts, white matter hyperintensities, atherosclerosis, and hypertension (Tublin et al., 2019). Interestingly, all of these risk factors could lead to decreased cerebral blood flow. While AD has traditionally been considered a brain-specific disease due to the obvious brain pathologies and cognitive deficits characteristic of the disease, this view is likely limited, and it is now understood that cerebrovascular dysfunction is a key feature of AD (Strickland, 2018). In fact, virtually all AD patient brains show cerebrovascular pathologies upon autopsy (de la Torre, 2002, Hunter et al., 2012). Blood-brain barrier (BBB) dysfunction and breakdown occur early in disease, even before signs of neurodegeneration or cognitive impairment are evident (Montagne et al., 2017).

The brain requires adequate blood flow to function properly and when the vasculature becomes unhealthy, this affects neuronal survival and cognitive function. There is a large overlap between vascular dementia and AD and over half of patients with vascular cognitive impairment will later advance to dementia. Both decreased blood flow and Aβ aggregation leads to oxidative stress that can drive AD progression and neuronal death. Hypoperfusion can accelerate Aβ deposition, as well as increase other AD pathologies including hyperphosphorylated tau, BBB impairment, neuronal loss, and cognitive dysfunction (Tublin et al., 2019).

Scientists and physicians have noted a link between AD and cardiovascular abnormalities for many years and the vascular hypothesis of AD suggests that cerebral hypoperfusion may lead to AD pathology and neuronal degradation. More than 45% of early stage AD patients exhibit increased cerebral microbleeds by magnetic resonance imaging. Furthermore, postmortem analyses of AD patient brains show brain capillary leakages and perivascular accumulation of blood-derived macromolecules such as fibrinogen, thrombin, albumin, immunoglobulin G, and hemosiderin. These findings suggest significant loss of BBB integrity in AD (Montagne et al., 2017). However, it is not clear how accumulation of blood-derived macromolecules induces cognitive impairment in AD patients.

Fibrinogen leakage into the brain is one driver of neurodegeneration. Since fibrin is highly proinflammatory and can interact with receptors on macrophages and microglia, fibrin deposits in the AD brain can lead to chronic inflammation (Merlini et al., 2019). Moreover, fibrinogen interacts with Aβ, leading to structurally altered fibrin clots that are degradation-resistant and can elicit a stronger inflammatory response in AD patient brains (Cortes-Canteli et al., 2010, Ahn et al., 2010). Fibrinogen-induced CD11b/CD18 receptor-mediated microglial activation may be a major pathway in AD pathogenesis. The CD11b/CD18 integrin receptor is expressed on monocytes, macrophages, and microglia, and when fibrinogen binds to this receptor, it elicits many cell signaling responses, including cytoskeletal rearrangements, phagocytosis, adhesion, migration, and chemotaxis (Petersen et al., 2018). Fibrin deposits in the AD brain can activate microglia via binding to the CD11b/CD18 integrin receptor in areas devoid of Aβ plaques and genetic elimination of the fibrinogen binding motif to CD11b/CD18 reduces neuroinflammatory activity, synaptic deficits, and cognitive decline in a mouse model of AD. A summary of the ways that fibrin may lead to AD pathology via microglial binding is given in Figure 1.2.

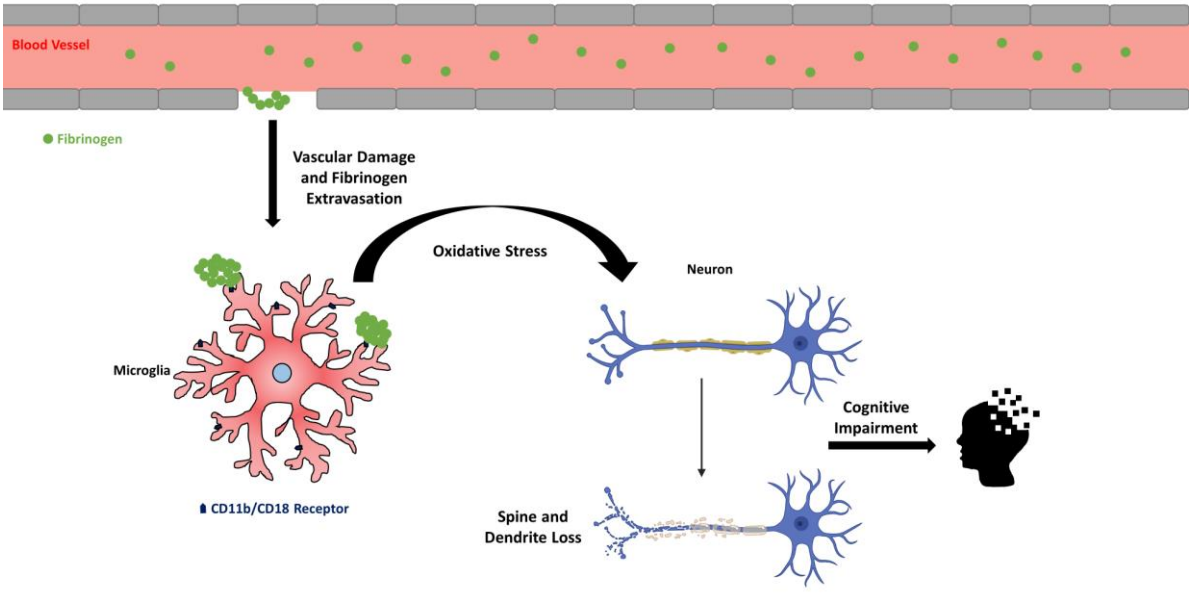


Figure 1.2. When there is damage to blood vessels in the brain, fibrinogen can leak into the parenchyma. Fibrinogen can activate microglia via the CD11b/CD18 receptor, contributing to oxidative stress, dendritic spine loss, and cognitive deficits.

1.4 Proinflammatory State in Alzheimer's Disease

AD, classically identified by the presence of extracellular Aβ plaques and intracellular tau tangles in the brain parenchyma, is a fatal cognitive disorder associated with neuronal loss and inflammation. The innate immune system is important to the progression of AD, and microglial and macrophage activation play a role in AD pathology (Heneka et al., 2015b). This neuroimmune response is activated in order to clear Aβ or pathogens from the brain and to tag impaired neurons for destruction (Mandrekar and Landreth, 2010).

The brain's immune system consists of several different types of cells, including astrocytes, microglia, and other resident macrophages that play a role in maintaining homeostasis and reacting to injury or disease. The primary immune cells of the brain are microglia, the resident macrophages of the brain. These cells are derived from the yolk sac early during brain development and maintained by local proliferation (Perry and Teeling, 2013). Microglia are involved in pruning synapses and phagocytosing cellular debris or taking up aggregates in the brain (Herz et al., 2017). Astrocytes also play a role in immune regulation in the brain and often become activated at times when microglia are activated or when monocytes enter the brain in response to injury (Priego and Valiente, 2019). Furthermore, astrocytes can expresses antigens critical to T-cell activation and produce cytokines and chemokines, modulating neuroimmune responses (Dong and Benveniste, 2001). There are three resident macrophage populations of the central nervous system (CNS) besides microglia that include perivascular macrophages (PVMs), meningeal macrophages (MMs), and macrophages of the choroid plexus and circumventricular organs (CPMs) (Perry and Teeling, 2013, Koizumi et al., 2019). These macrophages play roles in sampling debris, phagocytosing dying cells, and communicating with other cells in their local environment (Herz et al., 2017). PVMs, MMs, and CPMs express similar markers to one another, but can be distinguished by location within the brain. PVMs sit between the parenchymal basement membrane and endothelial vessel basement membrane. Like microglia, PVMs have been suggested to primarily originate from the yolk sac during development. However, studies using bone marrow chimeras have shown that this traditional view is wrong and PVMs are regularly replaced by bone marrow-derived myeloid progenitors circulating in the blood (Soulas et al., 2009, Lambertsen et al., 2011).

Immune dysfunction in the brain is notable in AD. Aβ plaques are often associated with microglial and astrocytic activation nearby, suggesting that Aβ may be the trigger of this innate immune activation. However, in later disease, microgliosis and astrogliosis continue to increase despite a plateau in the amount of Aβ deposition in the brain (Serrano-Pozo et al., 2011). Although Aβ can be a trigger of innate immune cell activation in the brain, it is not the only driver of this pathology in AD. Repeated insults and chronic immune activation can lead to the production of toxic cytokines, chemokines, and reactive oxygen species (ROS), which over time can result in neuronal death (Ramesh et al., 2013). Furthermore, PVMs play a role in phagocytosing Aβ (Hawkes and McLaurin, 2009), but this clearance of Aβ leads to upregulation of oxidative stress and ROS production (Park et al., 2017), suggesting that PVMs may contribute to the pathogenesis of AD in a vicious cycle. A neuroimmune response designed to be protective can become chronically activated during disease, damaging the integrity of neurons and leading to cell death.

1.5 Plasminogen and the Plasminogen Activator System

Plasminogen is primarily a blood-protein that is produced in the liver and then released into the circulation (Raum et al., 1980). Activation of plasminogen leads to the generation of

plasmin, a broad-spectrum serine protease. There are two major plasminogen activators which are also serine proteases, tissue-type plasminogen activator (tPA) and urokinase plasminogen activator (uPA). The two activators act via different mechanisms in differing compartments to activate plasminogen. tPA has a primary role in fibrinolysis, acting on polymerized fibrin, whereas uPA primarily localizes to the surfaces of cells such as neutrophils and macrophages for functions like ECM remodeling or monocytic cell migration. (Castellino and Ploplis, 2005). uPA usually binds to cell surfaces using the high affinity uPA receptor (uPAR). Both tPA and uPA are inactivated by the serine protease inhibitor (serpin) PAI-1. PAI-2 and nexin are also regulators of tPA and uPA activity (Castellino and Ploplis, 2005). Plasmin is inhibited by α_2-antiplasmin (Hudson, 2017). This network is summarized in Figure 1.3.

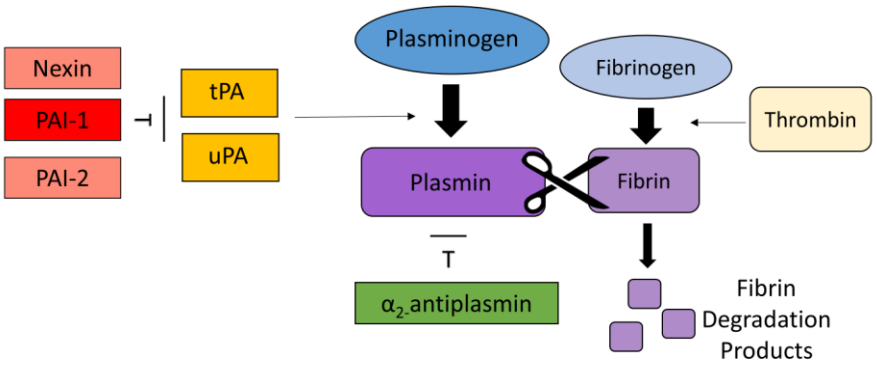


Figure 1.3. Overview of the plasminogen activator system. PAI-1, PAI-2, and nexin serve as inhibitors for the plasminogen activators tPA and uPA. Plasminogen is activated by tPA or uPA to form plasmin, which can then be inactivated by α_2-antiplasmin. The major substrate for plasmin is fibrin which is formed via the coagulation cascade following cleavage of fibrinogen by thrombin. Plasmin degrades fibrin into fibrin degradation products.

A recent article reviewed the evolutionary origins of the plasminogen activator system (Chana-Muñoz et al., 2019), suggesting that plasminogen, tPA, uPA, and PAI-1 emerged from a single common gene which then duplicated and further diverged into the plasminogen activator system proteins. The authors show that hepatocyte growth factor, lipoprotein a, and macrophage stimulating factor 1 are evolutionarily similar to plasminogen, whereas hyaluronan binding protein and hepatocyte growth factor activator are evolutionarily similar to tPA and uPA, and all diverged from a common ancestor gene. While these related proteins have diverse roles, they are generally involved with vascular functions and inflammation, similar to plasminogen (Chana-Muñoz et al., 2019). Inflammatory proteins are mediators of coagulation processes and these data suggest that coagulation and innate immune proteins evolved together to modulate these physiological processes.

Plasminogen deficiency in humans is rare and leads to several well-characterized pathologies. Low levels of plasminogen commonly lead to ligneous conjunctivitis characterized by thick, woody, fibrinous deposits on the body's mucosal membranes (Mingers et al., 1997). These deposits are commonly found on the inside of the eyelids but can also affect other mucosal membranes such as the larynx, vocal cords, nose, genitourinary tract, peritoneum, and gingiva (Mehta and Shapiro, 2008).

Plasminogen deficiency can be quantitative (Type I, decreased plasminogen production) or qualitative (Type II, normal protein levels with decreased activity) (Mehta and Shapiro, 2008). The first known patient with plasminogen deficiency was reported in 1978. This patient suffered from recurring thrombosis and was found to have normal levels of plasminogen protein with a reduced activity of about half the values of normal subjects. Further investigation of the family indicated that other relatives had reduced plasminogen activity, passed on as an autosomal characteristic, with heterozygotes for the abnormal plasminogen having half the normal plasmin activity, and one homozygote having basically no plasmin activity (Aoki et al., 1978). This deficiency, along with other deficiencies in plasmin activity, was later characterized as being due to a mutation in the active site of plasminogen, leading to Type II plasminogen deficiency (Robbins, 1992). While most plasminogen deficiencies come from genetic alterations, ligneous conjunctivitis has also been reported following treatment with the plasmin inhibitor tranexamic acid and resolved once treatment was discontinued (Diamond et al., 1991).

1.6 Plasminogen Contributions to Inflammation

As a broad-spectrum protease, plasminogen has many functions which contribute to the regulation of an inflammatory response (Figure 1.4). These include fibrinolysis, complement interaction, extracellular matrix (ECM) degradation, cell migration, and resolution of inflammation.

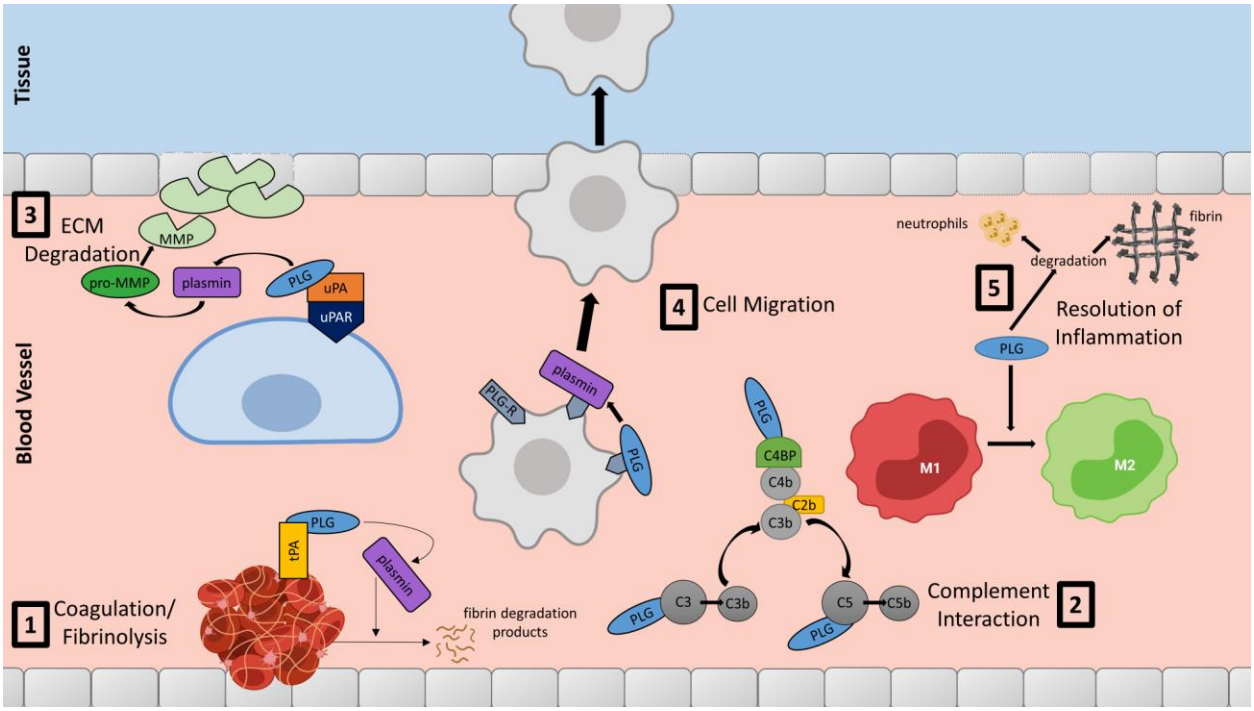


Figure 1.4. Overview of the ways plasminogen contributes to inflammation. 1). The major substrate for plasmin is fibrin. Following fibrin clot formation through the coagulation cascade, plasminogen participates in fibrinolysis by binding to the fibrin clot along with tPA. It becomes activated to form plasmin which will then degrade the clot, resulting in the formation of fibrin degradation products. 2). Plasminogen interacts with several proteins of the complement cascade including C3, C3b, C5, and C4BP which may aid in the modulation of the complement inflammatory response. 3). Cell-surface-bound plasmin aids in ECM degradation by activating MMPs that can degrade collagen and other proteins in the blood vessel wall. 4). Plasmin binds to cells via plasminogen receptors (PLG-Rs) which can lead to migration of leukocytes, neutrophils, monocytes, and macrophages. Note this function is often fibrin-dependent and thus fibrinolysis is an important part of plasmin-mediated cell-migration. 5). Plasminogen participates in wound healing by clearing fibrin and neutrophils once they are no longer needed at the injured site. Plasminogen also aids in polarization of M1 (proinflammatory) macrophages into M2 (anti-inflammatory) macrophages during this phase.

Fibrinolysis

Fibrin is a strong promoter of plasminogen activation, serving as a surface for both plasminogen and tPA binding, which allows for accelerated tPA-mediated cleavage of plasminogen into plasmin. In addition, the fibrin scaffold protects plasmin from being inactivated by α_2-antiplasmin (Hudson, 2017).

Fibrin acts as a framework for platelets, leukocytes, and fibroblasts to bind, release inflammatory signals, and participate in wound healing (Weisel, 2005). Fibrin can induce production of proinflammatory signaling molecules such as IL-8, TNF-α, IL-1β, IL-6, chemokines, and ROS (Altieri, 1999, Qi et al., 1997, Jennewein et al., 2011, Perez and Roman, 1995, Smiley et al., 2001). The $\alpha_M\beta_2$ binding motif of fibrinogen is specific to fibrinogen's

inflammatory functions and is responsible for fibrin binding to leukocytes to modulate leukocyte adhesion to the endothelium (Flick et al., 2004, Languino et al., 1993). Removal of the $\alpha_M\beta_2$ binding motif of fibrinogen protects from inflammatory disease including arthritis and neuroinflammatory disease (Flick et al., 2007, Adams et al., 2007). Because fibrin is proinflammatory, plasmin mediated-fibrinolysis can lead to the reduction of inflammation by removing fibrin.

However, fibrin degradation products also have inflammatory roles. Fibrin proteolysis by plasmin leads to the formation of various fibrin degradation products such as fragment E, fragment D, D-dimer, Bβ15-42, and α chain fragments (Jennewein et al., 2011). These fragments are known to have pro- and anti-inflammatory effects. For example, fragment E stimulates the production of proinflammatory cytokines IL-6 and IL-1β by peritoneal macrophages (Lee et al., 1999). D-dimer is also proinflammatory and stimulates neutrophil and monocyte activation and IL-6 production (Rao et al., 1994, Robson et al., 1994). In contrast fibrin fragment Bβ15-42 has an anti-inflammatory effect, leading to reduced leukocyte accumulation in response to myocardial infarct by binding to vascular endothelial cadherin to prevent leukocyte transmigration across endothelial cell monolayers (Petzelbauer et al., 2005).

In some cases, plasminogen depletion in mouse models of inflammation leads to a reduction in inflammatory signals. However, in many instances, this function of plasminogen is dependent on fibrin and fibrinolysis, with plasminogen only regulating inflammatory signals when fibrin is present (Berri et al., 2013, Silva et al., 2019, Raghu et al., 2014, De Nardo et al., 2010, Jennewein et al., 2011). It is crucial to note that fibrin is the major substrate for plasminogen, and importantly, loss of fibrinogen rescues the pleiotropic phenotypic effects that genetic plasminogen depletion has on mice. *Plg*$^{-/-}$ mice have increased mortality, thrombosis, rectal prolapse, and delayed wound healing, but *Plg*$^{-/-}$*/Fbg*$^{-/-}$ rescues these phenotypes and these mice are indistinguishable from *Fbg*$^{-/-}$ mice suggesting that fibrinolysis is the central function of plasminogen (Bugge et al., 1996).

Complement Interaction

The complement system consists of about 20 proteins that circulate in the blood and tissue fluids that become activated in a cascade to play a critical role in inflammation. This system enhances the ability of antibodies to attack and clear pathogens. There is evidence that plasminogen is a complement inhibitor, and it binds to C5, as well as C3 and its cleavage products, C3b and C3d, via lysine residues (Barthel et al., 2012). Plasmin on the surface of the bacteria *Moraxella catarrhalis* degrades C3b and C5b, which may contribute to this bacteria's resistance to being killed by the host (Singh et al., 2015). In addition, complement inhibitor C4b-binding protein binds plasminogen to create a complex that is present in serum and plasma. Activation of plasminogen by uPA leads to an increase in C4b-binding protein, suggesting that these proteins may interact during acute inflammation (Agarwal et al., 2015).

In about two-thirds of patients with atypical hemolytic uremic syndrome (aHUS), a thrombotic microangiopathy, the complement system gets over-activated and destroys healthy cells. A genetic screen indicated that plasminogen deficiency is common among aHUS patients (Bu et al., 2014). However, a more recent publication argues that plasminogen does not have a direct effect on complement proteins in aHUS and plasmin only inhibits complement activation at concentrations much higher than normal blood concentrations of plasminogen. Plasminogen did not inhibit complement-mediated lysis of red blood cells or endothelial cells, but did prevent

platelet aggregation and this proteolytic activity on thrombi may explain why plasminogen deficiency is seen in aHUS (Hyvärinen and Jokiranta, 2015).

Extracellular Matrix Degradation

ECM degradation is important to the immune response because the ECM is a barrier that prevents cells and pathogens from migrating out of the blood into other tissues. When proteins in the ECM are broken down, this allows for cell migration and invasion. ECM breakdown is essential to normal physiological processes such as reproduction, development, and tissue remodeling, and is also implicated in a variety of diseases. Plasminogen binds to the ECM and can degrade many different ECM proteins, either directly or indirectly, once activated into plasmin. Through ECM degradation, plasminogen can allow pathogens such as bacteria and parasites or immune cells like macrophages or activated T or B cells to migrate into other tissues of the body (Liu and Shih, 2007).

Degradation of human mesangial cells cultured on thin films of Matrigel, imitating the ECM, is concentration-dependent on exogenous plasminogen and is blocked by the presence of α_2-antiplasmin and aprotinin. In addition, a monoclonal antibody (mAb) against tPA or uPA leads to decreased ECM degradation, whereas a mAb against PAI-1 increases ECM degradation up to four-fold (Baricos et al., 1995).

Plasmin can also degrade the ECM indirectly through activation of matrix metalloproteinases (MMPs) (Castellino and Ploplis, 2005). MMP-2 and MMP-9 are both important for ECM degradation and are crucial enzymes for collagen degradation (Huang et al., 2014). An inhibitor against MMPs, TIMP-1, partially inhibits ECM degradation in the presence of plasmin (Baricos et al., 1995).

In the brain, plasmin may play a role in neuronal death via ECM interactions. Neurons and the ECM closely interact, and disruption of this interaction by plasminogen-mediated laminin degradation induces hippocampal neuronal death. tPA-knockout (KO) mice show decreased laminin degradation and a decrease in neuronal death in response to kainate (an excitotoxin) injection into the hippocampus. A similar effect is seen when α_2-antiplasmin is infused into the brain prior to kainate injection (Chen and Strickland, 1997). Thus, plasminogen mediates ECM disruption via laminin degradation that may contribute to neuronal death in the presence of excess excitatory amino acids.

Intraperitoneal thioglycollate injection is an experimental model for peritonitis. Thioglycollate-induced peritonitis elicits an influx of neutrophils and other immune cells into the peritoneal cavity and is thus a good model to study immune cell migration. *Plg*$^{-/-}$ mice show decreased leukocyte migration into the peritoneal cavity due to limited trans-ECM migration and decreased MMP-9 activation, and this deficit can be corrected in *Plg*$^{-/-}$ mice with the administration of activated MMP-9 (Gong et al., 2008). This deficit can also be restored in *Fib*$^{-/-}$ mice or by deletion of the major myeloid integrin $\alpha_M\beta_2$-binding motif on the γ chain of fibrin(ogen) (Silva et al., 2019).

Cell Migration

Leukocyte Recruitment

Plasminogen also has a role in cell migration and recruitment of immune cells to the sites of injury. White blood cells, or leukocytes, are the immune cells of the blood and lymphatic system and are important for an immune response to pathogens. There are several types of

leukocytes, including neutrophils, eosinophils, basophils, lymphocytes, and monocytes, which all have different targets in the blood related to the prevention of bacteria, fungi, parasites, and viruses from harming the body. There is evidence that plasminogen is important for full recruitment of these cells into areas of trauma or disease.

Plasminogen can also aid directly in chemotactic cell migration. Rabbit mononuclear cells and neutrophils isolated from peritoneal exudates respond chemotactically to plasminogen to migrate through pores in a chamber (Ward, 1968). In this study, different types of mononuclear cells were not identified and could have included T cells, B cells, NK cells, and monocytes.

Lipoprotein(a) [Lp(a)], a risk factor for cardiovascular disease, is associated with apolipoprotein a [apo(a)], a plasminogen-mimic due to kringle domains homologous to plasminogen. Apo(a) competes with plasminogen and interferes with leukocyte recruitment in a model of inflammation. The Lp(a)-apo(a) pathway impedes plasminogen activation and macrophage recruitment, as well as neutrophil recruitment and neutrophil cytokine release in a model of abdominal aortic aneurism (Huang et al., 1996).

Monocyte and Monocyte-Derived Cell Migration

Monocytes are leukocytes important to adaptive immunity. These cells patrol the endothelial cell wall, release pro-inflammatory cytokines in response to pathogens, and differentiate into macrophages to migrate into other tissues (Kratofil et al., 2017). The lungs of WT mice inoculated with two influenza A viruses have a large degree of cellular infiltration and alveolar damage which is reduced in $Plg^{-/-}$ mice. This decrease in immune cell infiltration is accompanied by a decrease in proinflammatory cytokine levels in $Plg^{-/-}$ lungs. This function of plasminogen seems dependent on fibrinolysis, as treatment with Ancrod, which promotes fibrin degradation, leads to increased lung inflammation and elevated cytokine levels. Ancrod treatment in $Plg^{-/-}$ mice reversed the immune cell recruitment protection and cytokine decrease seen with plasminogen deficiency. Likewise, inhibiting plasminogen-binding to fibrinogen using 6-aminohexanoic acid decreased inflammation by blocking plasmin-mediated fibrinolysis. Taken together these data suggest that plasminogen-mediated fibrinolysis is important for the pathogenesis and inflammation associated with influenza A infections (Berri et al., 2013).

Many of plasminogen's roles in inflammation can also be mechanistically linked to fibrin, which has its own role driving an inflammatory response (Davalos and Akassoglou, 2012, Flick et al., 2004). For example, in $Plg^{-/-}$ mice there is reduced monocyte, macrophage, and dendritic cell recruitment into the peritoneal cavity in a thioglycollate-induced peritonitis model (Silva et al., 2019). Although a previous study (Gong et al., 2008) suggested that this was due to macrophage retention in the peritoneal wall, this more recent study noticed that fibrin accumulated in the peritoneal wall of $Plg^{-/-}$ mice and hypothesized that decreased fibrinolysis by plasminogen contributed to the decreased monocytic cell migration. Interestingly, a double KO of plasminogen and fibrinogen ($Plg^{-/-}/Fbg^{-/-}$) rescued recruitment of macrophages into the peritoneal cavity in response to thioglycollate. These results are bolstered by the fact that experiments were run independently in two laboratories at separate institutions and they had the same experimental findings. In addition, an *in vitro* transwell migration assay that looked at cell migration across a fibrin matrix in the presence or absence of plasminogen further validated the idea that fibrin is necessary for plasminogen-dependent macrophage migration. Cells migrated through the membrane easily in the presence of plasminogen, but failed to migrate across a fibrin matrix in the absence of plasminogen. Furthermore, the requirement for plasminogen for

migration through fibrin matrices is dependent on the $\alpha_M\beta_2$-binding motif of fibrin, which sequesters macrophages in the absence of fibrinolysis (Silva et al., 2019).

Receptors for Cell Migration

As plasminogen has versatile functions, it is no surprise that there are many different plasminogen receptors. Plasminogen can bind to proteins with a C-terminal lysine (Miles and Plow, 1985) which includes membrane-bound proteins, intracellular proteins, nuclear proteins, and integrins (Flick and Bugge, 2017). Cell surface binding is crucial for many of plasminogen's functions except fibrinolysis when fibrin serves as the scaffold for plasminogen binding and activation by tPA. However, until recently, the plasminogen receptors that play a role in cell migration were not well defined. During inflammatory processes, cellular migration is important because inflammatory cells must get to the desired tissue in order to resolve inflammation effectively.

Nuclear protein histone H2B (H2B) was identified as a prominent macrophage-binding plasminogen receptor in a peritoneal thioglycollate model. Fragment antigen binding (Fab) fragments for H2B and several other plasminogen-binding receptors including α-enolase, annexin 2, and p11 (S100A10) can be used to block plasminogen binding on macrophages. Fab fragments directed against H2B have the greatest impact on plasminogen binding to macrophages, but Fab fragments against the other receptors also have some effect on plasminogen binding. Mice treated intravenously with anti-H2B Fab had about 50% less macrophage recruitment in response to peritoneal thioglycollate without affecting circulating monocyte levels (Das et al., 2007). In addition, $p11^{-/-}$ mice had ~50% decreased macrophage migration into the peritoneal cavity when stimulated with thioglycollate and p11-deficient macrophages show an eightfold decrease in migration through Matrigel (O'Connell et al., 2010). Interestingly, treatment with L-type calcium channel blockers which block expression of plasminogen receptors on the surfaces of macrophages, including H2B, α-enolase, annexin 2, and p11, blocks recruitment of macrophages into the peritoneal cavity in response to thioglycollate (Das et al., 2009).

In 2010, a novel cell-surface plasminogen receptor, PLG-R_{KT}, was discovered and found to be colocalized with uPA and uPAR on migratory cells including leukocytes. PLG-R_{KT} enhances plasminogen activation and can also bind tPA to further enhance plasminogen activation (Andronicos et al., 2010). Macrophages from $Plg\text{-}R_{KT}^{-/-}$ mice have a decreased ability to bind plasminogen (Miles et al., 2017) and in the intraperitoneal thioglycolate model, $Plg\text{-}R_{KT}^{-/-}$ mice show about 80% reduced macrophage recruitment into the peritoneal cavity (Andronicos et al., 2010). In addition, PLG-R_{KT} seems to modulate M2 macrophages specifically; in a pleurisy model, $Plg\text{-}R_{KT}^{-/-}$ mice show impaired recruitment of mononuclear cells into the pleural cavity in response to lipopolysaccharide (LPS). They have an increase in M1 macrophages in the pleural cavity and no change in M2 macrophage levels. This suggests that there is an impairment in polarization of macrophages from a M1 pro-inflammatory phenotype to a M2 anti-inflammatory phenotype which causes a defect in ability to resolve inflammation (Vago et al., 2019).

Resolution of Inflammation and Wound Healing

It is crucial that inflammation is resolved in a timely manner after an insult to avoid chronic inflammation which may have detrimental effects. Resolution is controlled by a decrease in chemokine concentration and inhibition of neutrophils in the injured tissue, and resolution pathways are activated shortly after the initial inflammatory response (Sugimoto et al., 2016). Plasminogen is transferred to wound sites by inflammatory cells early during healing (Shen et

al., 2012), where it plays a role in clearing fibrin and neutrophils and forming new connective tissue and blood vessels during the wound healing process (Sulniute et al., 2016). Plasminogen-deficient mice have impaired wound healing (Romer et al., 1996, Vago et al., 2019).

Plg$^{-/-}$ mice, as well as *Plg-R*$_{KT}^{-/-}$ mice, show deficits in ability to resolve inflammation due to a lack of ability to polarize M1 macrophages into M2 macrophages (Vago et al., 2019). Plasminogen induces macrophage reprogramming from M1 to M2 polarization in order to promote resolution of inflammation. In the resolution phase of LPS-induced inflammation, plasminogen and plasmin are upregulated in order to polarize macrophages into anti-inflammatory subtypes. In addition, if plasminogen or plasmin are given when inflammation is peaking, this leads to increased neutrophil apoptosis and efferocytosis via annexin A1 (Sugimoto et al., 2017). Furthermore, α-enolase, a plasminogen receptor, is necessary for inflammation resolution, and inhibitors of α-enolase-plasminogen binding lead to inadequate restoration of homeostasis to injured muscle tissue (Diaz-Ramos et al., 2012).

1.7 Plasminogen in Neurodegenerative Disease

Plasminogen Activator System in the Central Nervous System

While the liver is the primary source of plasminogen in the body, neuroendocrine tissues, as well as neurons in the cortex, hippocampus, and cerebellum express plasminogen (Mehra et al., 2016). In the CNS, plasminogen activation is primarily dependent on regulation by tPA and PAI-1. The major source of tPA in the brain is the endothelial cells of microvessels (Levin et al., 1997). However, tPA can be expressed by a variety of cells in the CNS, including neurons, microglia, astrocytes, oligodendrocytes, perivascular mast cells, pericytes, and infiltrating leukocytes, suggesting that tPA may have a variety of roles within the brain (Yepes et al., 2009, Mehra et al., 2016), including neuronal migration, synaptic outgrowth, neurotransmission, and synaptic plasticity (Huang et al., 1996, Madani et al., 1999, Zhuo et al., 2000). tPA protein and mRNA are localized at the synapse of neurons, and when released into the extracellular space, tPA can act on plasminogen and can be inhibited by PAI-1 (Qian et al., 1993). uPA is normally expressed at low levels in the brain by some neurons and astrocytes, but can be upregulated in some pathological conditions including multiple sclerosis (MS) and epilepsy (Mehra et al., 2016). In addition to PAI-1, neuroserpin is a tPA and uPA inhibitor that is expressed by neurons of the CNS. Neuroserpin forms unstable complexes with tPA, dissociating quickly, indicating that it is a transient inhibitor in the nervous system (Osterwalder et al., 1998).

Microglia and Innate Brain Inflammation

Microglia are the specialized resident macrophages of the brain that mediate the neuroimmune response by releasing cytokines and signaling molecules to clear cellular debris and by phagocytosing dead neurons. Many neurological diseases, especially neurodegenerative diseases like AD, MS, and stroke, are associated with activation of the glial cells of the brain that can induce neuronal death and harm the brain.

tPA can regulate microglial activation in response to excitotoxin-mediated neurodegeneration, independent of plasminogen activation (Tsirka et al., 1997b). Furthermore, plasminogen-deficient mice are resistant to neurodegeneration induced by acute kainate injection (Tsirka et al., 1997b, Tsirka et al., 1997a), and this function is independent of fibrin (Tsirka et al., 1997a). Plasminogen and plasmin both induce microglial activation and expression of IL-1β, TNF-α, and reactive oxygen species (Min et al., 2003). As I have shown in my thesis work, when blood-derived plasminogen is knocked down at the mRNA level, microglial and astrocytic

activation are decreased significantly following injection with LPS, possibly due to less migration of perivascular macrophages into the brain during LPS challenge (Baker et al., 2019). Fibrinogen can also induce microglial activation through its $\alpha_M\beta_2$ binding motif and blocking fibrinogen-$\alpha_M\beta_2$ interactions reduces proinflammatory microglial activation (Adams et al., 2007).

In addition, tPA enhances recruitment of microglia following stroke in a mouse model. Recombinant human tPA (rtPA) is often used following acute ischemic stroke as the standard of care. However, rtPA increases inflammation significantly when administered to mice following stroke by upregulating chemokines, cytokines, and microglial recruitment, leading to increased mortality in mice treated with rtPA (Lenglet et al., 2014). tPA can promote microglial activation independent of its proteolytic functions, by binding to annexin II and galectin 1 on microglia (Siao and Tsirka, 2002).

Multiple Sclerosis

MS is a progressive demyelinating disease of the brain and spinal cord that leads to issues with muscle control and basic body functions. MS patients have altered expression of tPA and PAI-1 in white matter regions of brain and spinal cord (Cuzner et al., 1996), and increased PAI-1 levels in the plasma (Onodera et al., 1999). A commonly used mouse model of MS is experimental autoimmune encephalomyelitis (EAE) where an antigen is injected that induces progressive demyelination.

Plasminogen deficiency protects from demyelination in an EAE model. In this mouse model, *Plg*$^{-/-}$ mice with induced EAE have delayed disease onset and reduced disease severity compared to WT animals, corresponding to much less macrophage/microglial accumulation in the spinal cords, as well as decreased demyelination and paralysis. Loss of plasminogen impeded macrophage migration during disease progression, leading to decreased neuroinflammation that was dependent on plasmin-mediated fibrinolysis, as supported by data from *Fbg*$^{-/-}$ and *Plg*$^{-/-}$/*Fbg*$^{-/-}$ mice. Both *Fbg*$^{-/-}$ and *Plg*$^{-/-}$/*Fbg*$^{-/-}$ mice have a decreased severity in EAE, but no delay in disease onset, suggesting that plasmin-mediated fibrinolysis is a key mediator of neuroinflammation in the spinal cords in this EAE model (Shaw et al., 2017).

tPA is implicated in MS development, and is upregulated 4-fold when EAE is induced. It seems to play a protective role in EAE, as tPA-deficient mice have an earlier onset and increased severity of demyelination (East et al., 2005, Dahl et al., 2016), likely because tPA is necessary to clear fibrin deposits on axons damaged during EAE progression. Axonal damage is a crucial contributor to MS progression and fibrinogen has been shown to be a critical regulator of microglial activation and axonal damage in EAE (Davalos and Akassoglou, 2012).

Alzheimer's Disease

There is conflicting evidence about the role plasminogen may play in the pathology of AD. On one hand, plasmin mediates an proinflammatory response. On the other, plasmin participates in fibrinolysis to break down fibrin, which itself is proinflammatory. In addition, there is some evidence that plasmin can degrade β-amyloid (Aβ), a protein that aggregates in the parenchyma of AD patient brains, which is also a proinflammatory protein (Tucker et al., 2000b, Saido and Leissring, 2012). In human brain tissue from AD patients, tPA, uPA, PAI-1, and α_2-antiplasmin levels are increased compared to age-matched controls suggesting that the plasminogen activator system may be upregulated in order to try to clear Aβ during the disease process (Barker et al., 2012).

Fibrin has been implicated in the pathogenesis of AD (Cortes-Canteli et al., 2010, Cortes-Canteli et al., 2012). Accumulation of fibrin in the vasculature of the AD brain leads to neurovascular damage and inflammation in the AD brain. AD patient brains seem to have reduced tPA activity, plasmin levels, and reduced fibrin clearance (Ledesma et al., 2000, Melchor et al., 2003). AD mice heterozygous for a plasminogen-knockout allele *($Plg^{+/-}$)* have increased neurovascular damage compared to AD mice wild type for plasminogen, and AD mice heterozygous for a fibrinogen α-chain knockout allele (*$Fbg^{+/-}$*), have decreased BBB damage. In addition, pharmacologic clearance of fibrin using the protease Ancrod reduces a neuroinflammatory response and microvascular injury, whereas plasmin-inhibition using tranexamic acid exacerbates neuroinflammation and neurovascular damage in an AD mouse model (Paul et al., 2007).

However, in some cases, although plasmin deficiency causes increased fibrin accumulation, this does not necessarily lead to an increased immune response, indicating the important role that plasminogen plays in inflammation independent of fibrinolysis. *$Plg^{-/-}$* mice injected intrahippocampally with LPS show increased parenchymal fibrin deposits but a decreased neuroinflammatory response to LPS, suggesting that plasmin may be important to a normal neuroinflammatory response, at least in response to an acute inflammatory challenge (Hultman et al., 2014). In addition, as I show with my thesis work plasmin may help drive the neuroinflammatory response in AD, as knockdown of blood plasminogen using an antisense oligonucleotide directed against liver-produced plasminogen leads to decreased activation of microglia and astrocytes, decreased migration of perivascular macrophages into the brain, and decreased AD pathology in a mouse model of AD (Baker et al., 2018). It is likely that plasminogen may play different roles in AD progression depending on the stage of disease and degree of BBB damage.

1.8 Links between Systemic and Central Nervous System Inflammation

Systemic inflammation may play a role in the development of a vast array of neurological disorders and diseases. CNS involvement has been noted in response to a variety of acute and chronic systemic inflammatory cues (Fung et al., 2012). Communication of the peripheral immune system with the brain is evidenced by the fact that individuals are at increased risk for dementia following systemic infections such as influenza or ulcerative colitis (Cunningham, 2013). In addition, AD patients have a decline in cognitive function following systemic proinflammatory events (Holmes, 2013). Furthermore, recent results from the Atherosclerosis Risk in Communities study, which included over 12,000 participants, suggest that systemic inflammation in midlife is predictive of 20-year cognitive decline. Individuals with the highest levels of systemic inflammation in midlife had the steepest cognitive decline in the 20-year period analyzed, especially in terms of memory, suggesting that inflammation may be a driver of cognitive decline years later (Walker et al., 2019).

Exactly how systemic inflammatory signals influence the neuroimmune response, either directly or indirectly, is not well understood. The idea that systemic inflammatory signals can influence the brain opposes the idea that the brain is an immune privileged site because of the BBB, the barrier that separated the parenchymal cells of the CNS from the rest of the body. The BBB is a complex and dynamic barrier composed of closely aligned endothelial cells, astrocyte endfeet which wrap around the capillaries of the brain, and pericytes in the basement membrane. Endothelial tight junction complexes in the BBB have major role in keeping neurotoxic plasma proteins, cells, and pathogens like bacteria and viruses out of the brain. The BBB must also allow

for the exchange of oxygen and carbon dioxide between the vasculature and parenchyma via free diffusion and the crossing of energy metabolites, nutrients, and regulatory molecules via receptor-mediated transport (Montagne et al., 2017).

The brain itself can amount its own immune response using microglia and astrocytes and peripheral immune cells are normally restricted from going into the brain by the BBB. Through local interactions, CNS macrophages and microglia play a major role in relaying information between the systemic immune system and the brain (Teeling et al., 2010, Perry and Teeling, 2013). There are indirect ways in which systemic immune cells can affect the CNS, including through signaling across the BBB via cytokines, ROS, and other enzymes (Tracey, 2009), diffusion by way of the circumventricular organs of the brain which are not surrounded by the BBB, and signaling through peripheral nerves such as the vagus nerve (Rosas-Ballina and Tracey, 2009a, Rosas-Ballina and Tracey, 2009b). In addition, peripheral immune cells like macrophages and T cells can migrate through the BBB, the choroid plexus, or via the gateway reflex (Czirr and Wyss-Coray, 2012, Ransohoff et al., 2003, Tanaka et al., 2017).

Chronic inflammation leads to persistent oxidative stress, as well as activation of pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs). PAMPs and DAMPs are known to be increased in response to aging as well and inflammation is increased overtime with age (Wang et al., 2018). Both systemic inflammation and CNS inflammation increase with age, and microglia may be primed over time to have an exacerbated response following an inflammatory stimulus (Fung et al., 2012). Persistent activation of PAMPs and DAMPs can stimulate microglial activation and microglia in turn can release ROS, a contributor to neuronal death (Wang et al., 2018). In addition, peripheral infection and surgery can induce a neuroinflammatory response, mediated by PAMPs and DAMPs (Fung et al., 2012).

Pathways linking peripheral inflammatory action and neuroinflammation are not yet fully understood, but there is evidence for both BBB-dependent and BBB-independent pathways of communication. Thus, targeting systemic inflammation may be beneficial in targeting neuroinflammatory diseases, and a better understanding of key mechanisms in these processes are warranted in order to develop targeted therapeutics.

1.9 Objectives

AD is a large public health problem, and no effective therapies yet exist. Upon searching the Alzforum Therapeutics Database for discontinued Alzheimer's disease therapies, 82 results appear. Another 105 therapeutics are in phases 1-3 of clinical trials. In recent years, several small molecules and immunotherapies targeting Aβ have failed in clinical trials, underscoring the need for new approaches to the understanding and treatment of AD (Alzforum, n.d.-b).

A large body of evidence links the vasculature, inflammation, and Aβ to AD progression, but the etiology and progression of these factors is poorly understood. While the brain was originally thought of as an immune privileged site, this notion is being overturned. There is an increasing understanding that peripheral and brain inflammatory signals communicate with one another in a variety of ways. Thus, targeting inflammation systemically may prove crucial to limiting the neuroinflammation characteristic of AD, and further downstream effects of neuroinflammation including oxidative stress and neuronal death which ultimately lead to cognitive decline and death.

Although plasminogen plays a major role in degradation of fibrin, a proinflammatory blood protein known to be deposited in the brains of patients with AD, it also plays a role in migration of inflammatory cells to a site of injury or disease. I aimed to better understand the role that blood plasminogen could be playing in modulating the immune response throughout early disease progression prior to BBB breakdown and fibrin deposition in the brain using an AD mouse model.

Throughout my thesis work, I have focused on gaining a better understanding of the role that the blood protein plasminogen may play in regulating neuroinflammatory progression in AD. This work led me to the hypothesis that plasminogen may play a more global role in regulating communication between systemic inflammatory signals and brain inflammatory cells throughout a variety of conditions including systemic infection. I have shown that plasminogen does play a major role in relaying information between the blood and the brain. However, plasminogen itself may not be a good therapeutic target due to its role in pleotropic functions within the body. Instead, targeting plasminogen receptors that are specific to plasminogen-mediated inflammatory cell migration may pave the way for novel treatment strategies for AD and other neuroinflammatory conditions.

CHAPTER 2: MATERIALS AND METHODS

2.1 Animals

All animal experiments were conducted in accordance with the guidelines of the United States National Institutes of Health Guide for the Care and Use of Laboratory Animals and with approval from the Animal Care and Use Committee of The Rockefeller University.

Tg6799 (5XFAD) transgenic mice (referred to as AD mice) are used where described. Tg6799 mice are double transgenic for human APP/Presenilin 1 and express 5 early-onset familial AD mutations in these genes on a B6SJLF1/J background. Both male and female mice were used and were always analyzed separately to ensure no differences due to sex. Wild type (WT) littermates were used as controls in all experiments.

C57Bl/6 mice (Jackson Laboratories) are used for some experiments where described. Mice were treated with CTRL or PLG ASO for 2 weeks prior to stereotactic LPS and Aβ injections or intraperitoneal LPS injections.

p11-KO mice which lack the p11 (S100A10) gene are used where described and were obtained from the Greengard Lab at The Rockefeller University. These mice have a targeted knockout of p11 on a 129S/SvEv * C57BL/6 background. Heterozygous knockouts and WT littermates were used as controls for these experiments.

2.2 Antisense Oligonucleotide Treatment in AD Mice

Antisense oligonucleotides (ASO) were prepared against plasminogen (PLG ASO) and α_2-antiplasmin (A2AP ASO) along with a relevant scrambled control (CTRL ASO) (Ionis Pharmaceuticals). The PLG and A2AP ASOs are 20 nucleotides long and chemically modified with a phosphorothioate backbone and 2-O-methoxyethyl wings in order to stabilize the molecule and optimize efficiency of knockdown. ASOs were designed by Ionis Pharmaceuticals to avoid any off-target effects, including both genomic off-target effects and non-genomic proinflammatory changes. Six cohorts of mice were used in this experiment: WT-CTRL ASO, WT-PLG ASO, WT-A2AP ASO, AD-CTRL ASO, AD-PLG ASO, AD-A2AP ASO (n = 7-18 mice/group). Mice were treated for 2 weeks at a dose of 150 mg/kg/week starting at 3 months-of-age, followed by 8 weeks of treatment at a dose of 100 mg/kg/wk. The weekly ASO dose was divided into 2 injections per week. Plasma was collected by tail bleed after 2 and 4 weeks of treatment and again by retroorbital bleed on the day of sacrifice to determine the extent of plasminogen or α_2-antiplasmin depletion.

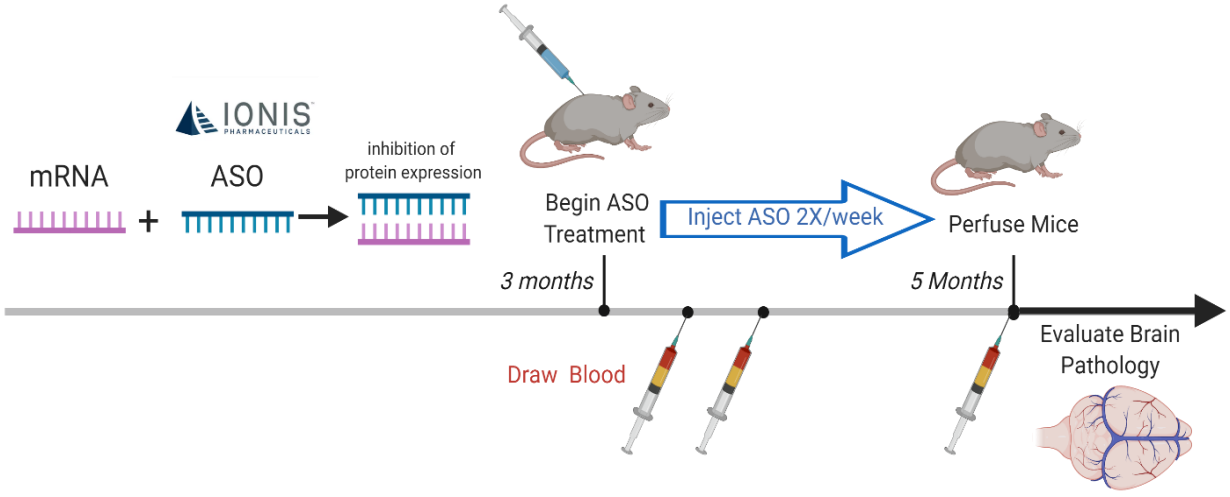


Figure 2.1. ASO treatment plan in AD mice. Mice are treated with an ASO against plasminogen or α_2-antiplasmin 2 times per week for 10 weeks starting at 3 months of age. Blood is collected after 2 and 4 weeks of treatment and at time of perfusion to determine effectiveness of ASO injections. At the end of ASO treatment, mice were perfused and brains were collected to evaluate pathology.

2.3 Stereotactic LPS and Aβ Injections

To examine the effect that peripheral plasminogen has on brain inflammation driven by direct injection, mice that had been treated with CTRL or PLG ASO were stereotactically injected with a solution of purified Aβ, LPS, or phosphate buffered saline (PBS) such that there were six cohorts of mice used in this experiment: CTRL ASO-PBS, PLG ASO-PBS, CTRL ASO-Aβ, PLG ASO-Aβ, CTRL ASO-LPS, and PLG ASO-LPS (n = 4-5 mice/group). Mice were treated at a dose of 150 mg/kg/week for 2 weeks starting at 10 weeks of age.

LPS Preparation

LPS (Sigma L2880) was prepared in PBS at a final concentration of 1.33 µg/mL.

Aβ preparation

Aβ42 (Anaspec) was monomerized by treatment with hexafluoroisopropanol (HFIP, Sigma-Aldrich). HFIP was evaporated off Aβ by leaving in a hood overnight, leaving Aβ films that were frozen at -80°C prior to use. To create oligomeric Aβ, Aβ was dissolved to 0.5 µg/µL in 0.08% NH3OH in PBS and incubated at 4°C with rotation overnight.

Intracerebral Injections

Mice were anesthetized intraperitoneally with atropine (0.6 mg/kg, 0.02 mg/mL, 2 µL/g) and their heads were shaved. Mice were placed in a stereotactic frame and a scalp incision was made to expose the skull. Burr holes were made in the skull at 3 locations (coordinates are indicated as anterior-posterior (AP), medial-lateral (ML), and dorsal-ventral (DV) from the bregma): **1)** AP, -0.74; ML, -1.8; DV, -1.5; **2)** AP, +1.82; ML, -1.5; DV, -1.5; and **3)** AP, +3.16; ML, -2.5; DV, -1.5. PBS, Aβ, or LPS (3 µL total volume) were injected to the depth indicated and the needle was left in place for 1 min following injection to allow diffusion of each reagent

into the brain. The skulls were closed with sutures and mice were allowed to recover. After one week, mice were perfused and their brains were collected for further analysis (Figure 2.2).

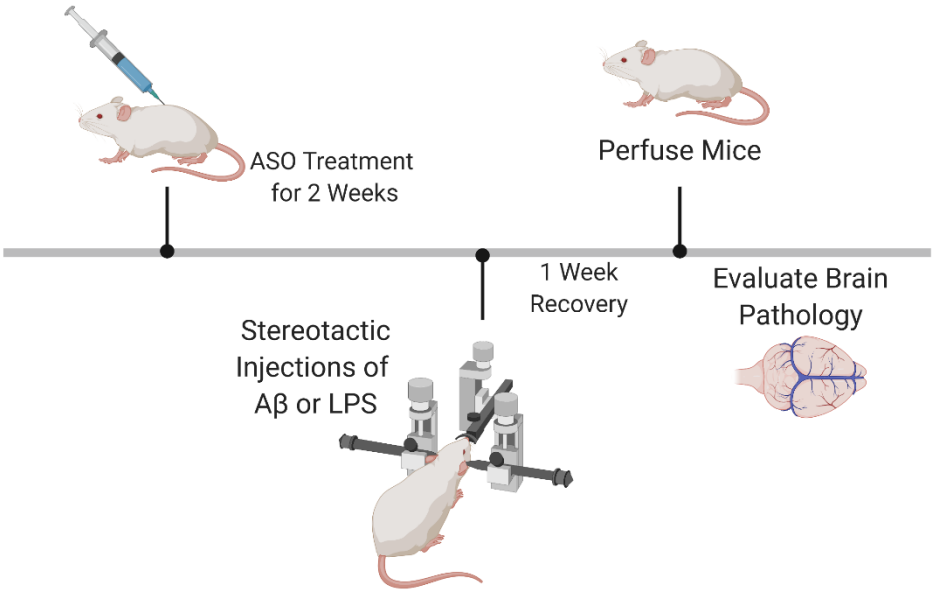


Figure 2.2. Intracerebral injection plan of LPS and Aβ in mice. Mice were treated with an ASO against plasminogen or a scrambled control 3 times per week for 2 weeks. After 2 weeks of ASO treatment, mice were anesthetized, placed in a stereotactic apparatus, and injected with LPS or Aβ. Following 1 week of recovery, mice were perfused and brain pathology was examined.

2.4 Peripheral LPS Injections

WT Mice Treated with PLG ASO

LPS from Escherichia coli O111:B4 (Sigma) was injected into the intraperitoneal (IP) cavity of WT (C57Bl/6) mice at a dose of 1 mg/kg for 3 consecutive days to robustly activate microglia. Mice were anesthetized and perfused with saline 4 hours following the final LPS injection. CTRL or PLG ASO-treated mice were administered either LPS or PBS such that there were four cohorts of mice used in this experiment: CTRL ASO-PBS, PLG ASO-PBS, CTRL ASO-LPS, and PLG ASO-LPS (n = 5-7 mice/group). Mice were treated at a dose of 150 mg/kg/week for 2 weeks starting at 10 weeks of age (Figure 2.3).

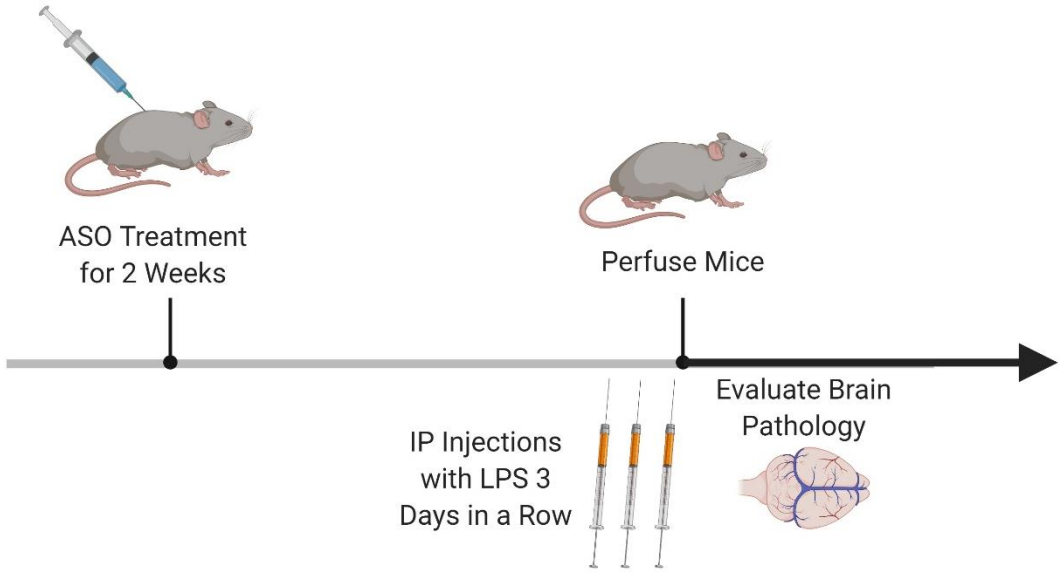


Figure 2.3. LPS treatment plan in WT mice. Mice were treated with an ASO against plasminogen 3 times per week for 2 weeks. After 2 weeks of ASO treatment, mice were injected with PBS or LPS at a dose of 1 mg/kg on 3 consecutive days. On the third day, mice were perfused and brains were collected to evaluate pathology.

P11 knockout mice

LPS (0.75 mg/kg) from Escherichia coli O111:B4 (Sigma) was injected into the IP cavity of homozygous or heterozygous p11 knockout mice for 3 consecutive days starting at 10 weeks of age. Mice were anesthetized and perfused with saline 4 hours following the final LPS injection. There were six cohorts of mice used in this experiment: $p11^{+/+}$-PBS, $p11^{+/-}$-PBS, $p11^{/-}$-PBS, $p11^{+/+}$-LPS, $p11^{+/-}$-LPS, $p11^{/-}$-LPS (n = 5-7 mice/group) (Figure 2.4).

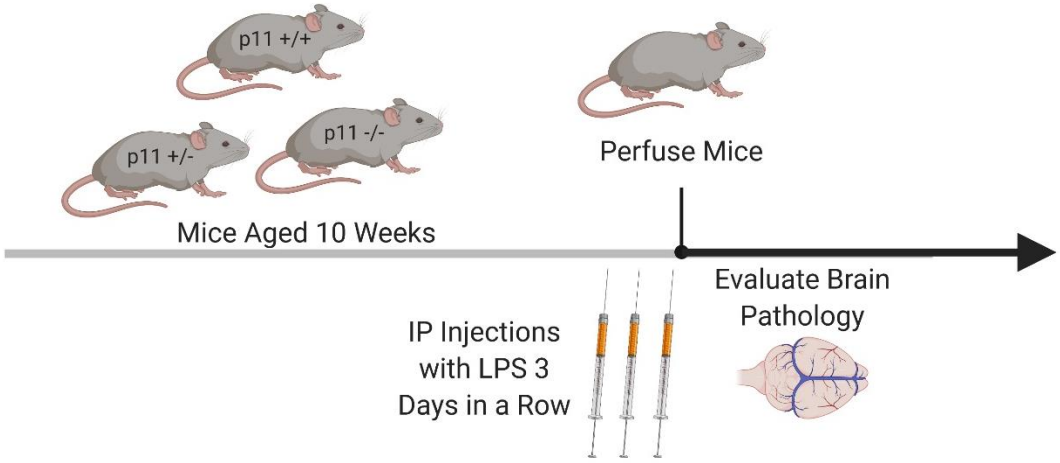


Figure 2.4. LPS treatment plan in p11 transgenic mice. Mice lacking p11, with partial levels of p11, or with full levels of p11 were injected with LPS at a dose of 0.75 mg/kg on 3 consecutive days starting at 10 weeks of age. On the third day, mice were perfused and brains were collected to evaluate pathology.

2.5 Intraperitoneal Macrophage Collection and Staining

IP macrophages were collected from CTRL ASO- and PLG ASO-treated mice by injecting 15 mL PBS into the peritoneal cavity and aspirating the fluid using a 31-gauge needle. Cells were spun down, collected, and plated. After growing for 1 week, cells were fixed in 4% paraformaldehyde and stained with PLG-R_{KT} (Epigentek), CD11b (DSHB), and DAPI (Vector Laboratories), and imaged on an Echo Revolve Upright Epifluorescent microscope at 20X.

2.6 Plasma Preparation

Blood was collected by either tail-clipping or retro-orbital plexus bleeding. For tail clipping, a small piece of soft tissue at the end of the tail was clipped, and ~30 uL of blood was collected into EDTA-coated tubes (BD Microtainer). For retro-orbital plexus bleeding, blood was collected into capillary tubes coated with Gel Repel (Z719951, Sigma) and 2.5 mg/ml polybrene (Santa Cruz, SC-134220). Plasma from both bleeding methods was prepared by centrifugation of whole blood and was frozen prior to Western blot analysis.

2.7 Whole Blood Staining

At the time of anesthesia, prior to perfusion, blood was collected by retroorbital plexus bleeding. Blood smears from each animal were created on slides and immediately frozen prior to staining. Slides were fixed in 4% paraformaldehyde and stained with PLG-R_{KT} (Epigentek), CD11b (DSHB), and DAPI (Vector Laboratories) to detect PLG-R_{KT} expression of leukocytes (CD11b+ cells).

2.8 Immunostaining

Mice were deeply anesthetized and perfused with saline prior to brain collection. Brain hemispheres were fixed in 2% paraformaldehyde or immediately frozen for brain sectioning and immunohistochemical analysis. Primary antibodies used were against: CD11b (microglia and macrophages; DSHB), glial fibrillary acidic protein (GFAP, astrocytes; Dako), NeuN (neurons; Millipore), lysosomal associated membrane protein 1 (LAMP-1; DSHB), 6E10 (Aβ; BioLegend), TMEM119 (microglia; Abcam), CD68 (microglia/macrophages; Bio-Rad), CD206 (perivascular macrophages; Invitrogen), PLG-R_{KT} (Epigentek), fibrinogen (Dako), S100A10 (p11; RD Systems), collagen IV (blood vessels; Millipore), laminin (blood vessels; Sigma), and CD31 (blood vessels; Abcam). Brain sections were incubated with primary antibodies for 3 hours at room temperature, rinsed in PBS, and then incubated with the appropriate fluorescent dye-conjugated secondary antibody. Congo Red dye was also used for detection of β-pleated sheets of Aβ plaques. Briefly, brain sections were incubated in Congo Red stain for 30 minutes, followed by 70% isopropanol for 15 minutes. All brain sections were washed and a coverslip was applied along with fluorescence mounting media (Vectashield) prior to visualization on a microscope.

2.9 Western Blotting

Mice were deeply anesthetized and perfused with saline prior to brain collection. Brain hemispheres were homogenized on ice in 2% SDS, 95 mM NaCl, 25 mM Tris, pH 7.4, 10 mM EDTA, and protease inhibitor cocktail (Roche). After centrifugation, extracts were used for Western blot. Both brain extracts and plasma samples (collection method described above) were run on reducing SDS-PAGE gels, transferred to PVDF membrane (EMD Millipore or BioRad), incubated overnight at 4°C in primary antibody (rabbit anti-plasminogen, Abcam; rabbit anti-tPA, Molecular Innovations; rabbit anti-PAI-1, Abcam; mouse anti-actin, Sigma-Aldrich; rabbit anti-α_2-antiplasmin, Abcam; mouse anti-6E10, Biolegend; rabbit anti-CD31, Abcam; rabbit anti-

fibrinogen, Dako; rabbit anti-VEGF, Cell Signaling; rabbit anti-occludin, Invitrogen; rabbit anti-transferrin, Abcam), and then incubated with an appropriate HRP-conjugated secondary antibody. Blots were developed with enhanced chemoluminescent substrate (Perkin-Elmer). Protein levels were quantified using densitometry with NIH Image J. Western blot results for brain protein extracts were normalized to actin.

2.10 Imaging Analysis

Following immunostaining, brain sections were imaged with a microscope (Axiovert 200, Carl Zeiss) equipped with Plan-Neofluar (10X NA 0.3, 20X NA 0.5, and 40X NA 0.75) objective lenses or on a Nikon Eclipse Ti2-U microscope equipped with Appo Fluor (4X NA 0.2, 20X NA 0.75, 40X NA 0.6) objectives at room temperature using air as the imaging medium. AxioVision software was used to collect images from an AxioCam color camera (Carl Zeiss) on the Zeiss microscope and Nikon Elements software and a Qi2 monochrome camera were used to acquire images from the Nikon microscope. Images of areas with positive staining were thresholded using NIH Image J. A researcher blind to the genotype and treatment of each mouse analyzed the total area of positive staining as a percentage of total image area (n = 3-4 sections from 4-18 mice per group depending on the experiment).

2.11 Plasmin Activity

Plasma was diluted 40-fold in Tris-imidazole buffer and activity was measured spectroscopically using Pefachrome® PL (Pefa-5264, Pentapharm), a highly sensitive chromogenic peptide substrate for plasmin. The change in OD/min at 405 nM was measured for each plasma sample as a determination of activity.

2.12 Contextual Fear Conditioning

Contextual fear conditioning was performed in 2 chambers with floors consisting of rods connected to a shock generator (Med Associates). During the training day, mice were placed into a chamber and received a shock (0.7 mA). Another shock was received at 5 minutes. After 24 hours, mice were tested in the same chamber for 5 minutes, where a video camera and automatic analysis software analyzed their freezing behavior. Contextual fear conditioning is used to test for hippocampal impairment, a brain region involved in remembering location and highly affected in AD.

2.13 Barnes Maze

The Barnes maze is a white circular platform with 20 equally spaced holes. Of the holes, 19 are covered with black plastic and 1 instead has a black escape box that allows the mouse to enter below the surface of the platform (the target hole). Visual cues were placed around the room to allow the animals to orient themselves while on the platform. Lighting is keep high at 1500 lux to motivate the animals to find the escape box. To remove any lingering scent on the maze from the previous animal, the platform and escape box were cleaned with 20% ethanol between mice. During training, mice were placed in the center of the maze in a black box for 30 seconds. After 30 seconds, the box was removed, and mice were allowed to freely explore and find the target hole for 4 minutes. After 4 minutes, if the mouse had not found the target hole, they were gently guided to the hole. Training was done once a day for 9 days. On the 11^{th} day, a 24-hr probe trial was run to assess spatial memory of the mice. All trials were recorded and scored for latency to find the target hole (first nose poke into the hole), latency to enter the target hole, and the number of errors visiting other holes. The experiment was recorded and analyzed using the Ethovision video tracking system.

2.14 Statistical Analysis

Statistical analyses were conducted using GraphPad Prism software for two-way analysis of variance (ANOVA) or t-test, as indicated in each figure legend. All values presented in graphs are mean ± SEM.

CHAPTER 3. BLOOD-DERIVED PLASMINOGEN DRIVES BRAIN INFLAMMATION AND PLAQUE DEPOSITION IN A MOUSE MODEL OF ALZHEIMER'S DISEASE

To explore whether peripheral plasminogen depletion affects AD pathology, we used an ASO-mediated gene knockdown strategy to deplete plasminogen in the blood of AD and WT mice and analyzed brain pathology following 10 weeks of ASO treatment.

3.1 Long-term treatment with PLG ASO is associated with weight loss in mice.

Plasminogen deficiency is associated with negative health consequences in humans (Mingers et al., 1997, Mehta and Shapiro, 2008). In addition, plasminogen knockout mice are viable but suffer from spontaneous fibrous deposits, delayed growth, and reduced fertility and survival (Ploplis et al., 1995). To assess for any negative health effects from chronic depletion of peripheral plasminogen using an ASO-mediated strategy, we recorded mouse weights throughout treatment (Figure 1.1). Throughout the first 5 weeks of treatment with ASOs all mice gained similar amounts of weight compared to their baseline at the start of treatment. However, after 5 weeks of treatment, PLG ASO-treated AD mice started to level out in weight, remaining relatively constant throughout the next 6 weeks of treatment. PLG ASO-treated WT mice lost weight throughout the remaining 6 weeks of treatment, ultimately dropping below their baseline weight. All other treatment groups (CTRL and A2AP ASO-treated WT and AD mice) continued to gain weight throughout all 10 weeks of treatment, suggesting that plasminogen-deficiency specifically was having an effect on the mice's overall health. No other obvious health differences between treatment groups were noted.

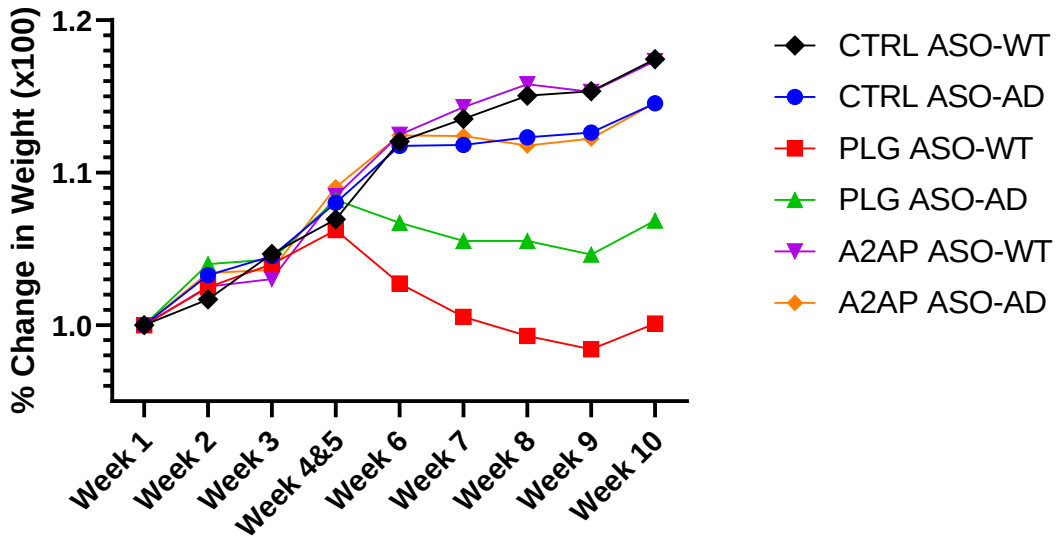


Figure 3.1. Long-term plasminogen depletion is associated with stunted weight gain in ASO-treated mice. The weights of mice were recorded throughout treatment with ASOs. While CTRL and A2AP ASO-treated mice continued to gain weight from baseline throughout all 10 weeks of baseline, AD and WT animals treated with PLG ASO stopped gaining weight around week 5 of treatment, and WT PLG ASO-treated mice lost weight throughout the remaining 5 weeks of ASO treatment. n = 15-18 mice per group.

3.2 The plasminogen system is altered in the peripheral blood of PLG ASO-treated mice, but not in the brain.

In order to analyze the extent of plasminogen depletion, we collected plasma after 2, 4, and 10 weeks of PLG ASO injections. Plasma plasminogen was efficiently knocked-down by 90-95% from CTRL ASO-treated plasma levels throughout the course of treatment in both AD and WT animals (plasma levels after 10 weeks of treatment shown in Figure 3.2A-B). Plasma plasminogen was not significantly different between WT CTRL ASO- and AD CTRL ASO-treated animals (Figure 3.2A-B). tPA and PAI-expression levels were examined in the plasma after PLG ASO treatment and were found to be unchanged compared to controls (Figure 3.2A, 3.2C-D). We also analyzed levels of plasminogen, tPA, and PAI-1 in the brains of AD and WT mice treated with either CTRL or PLG ASO. We found that expression of these plasminogen system activator proteins was not altered in response to PLG ASO treatment (Figure 3.2E-H). Therefore, although PLG ASO is effective at specifically reducing plasminogen levels in the blood of these mice, plasma tPA and PAI-1 are unaltered in response to plasminogen depletion, and brain levels of plasminogen, tPA, and PAI-1 are unaffected by ASO administration.

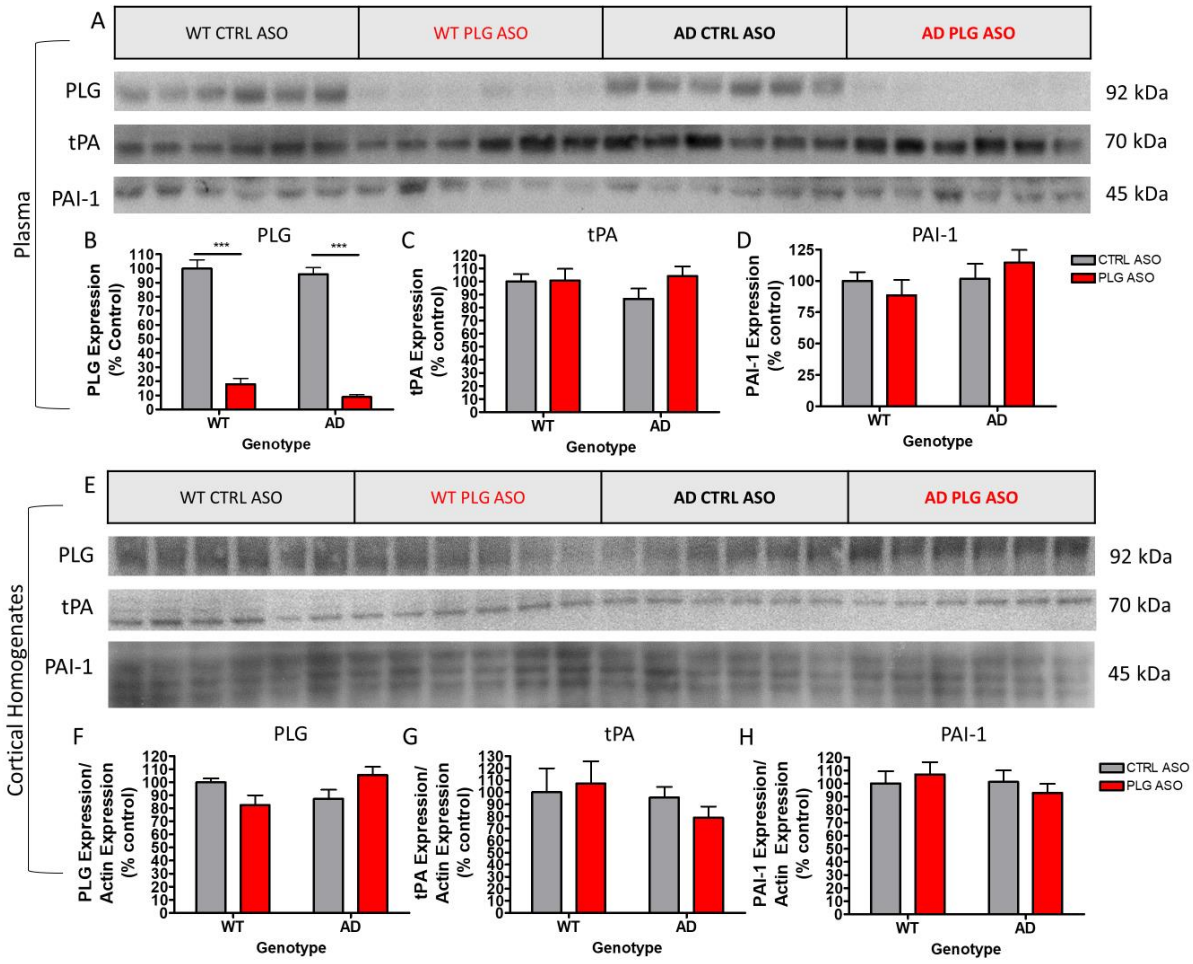


Figure 3.2. Plasminogen levels, but not all components of the plasminogen activator system, are depleted in the plasma of ASO-treated mice, but are unaffected in the brain. (A) Representative Western blots of plasminogen, tPA, and PAI-1 levels in the plasma of WT CTRL ASO, WT PLG ASO, AD CTRL ASO, and AD PLG ASO mouse groups. (B) Plasminogen levels decrease in plasma of PLG ASO-treated AD and WT mice compared to CTRL ASO-treated mice ($p < 0.0001$). (C) Plasma tPA levels are unchanged between genotype ($p = 0.28$) or ASO treatment ($p = 0.24$). (D) Plasma PAI-1 levels do not differ between genotype ($p = 0.26$) or ASO treatment ($p = 0.95$). € Representative Western blots of plasminogen, tPA, and PAI-1 levels in cortical homogenates normalized to actin across treatment groups. (F-H) Genotype and ASO treatment do not affect expression level of plasminogen ($p = 0.42$ for genotype, $p = 0.95$ for ASO treatment), tPA ($p = 0.29$ for genotype, $p = 0.76$ for ASO treatment), or PAI-1 ($p = 0.38$ for genotype, $p = 0.94$ for ASO treatment) in the brains of these animals. Two-way analysis of variance (ANOVA), n = 15-18 mice per group; all values presented as mean ± SEM. Results are from 3 independent experiments.

3.3 Depletion of plasma plasminogen reduces the brain's innate immune response in AD mice.

We analyzed whether plasma plasminogen, a protein involved in the inflammatory response, contributes to brain inflammation. We compared CD11b and GFAP expression levels between CTRL ASO- and PLG ASO-treated AD and WT mice to determine microglial/macrophage and astrocyte activation, respectively, in both the cortex (Figure 3.3) and hippocampus (Figure 3.4). In WT mice, the expression levels of CD11b (Figure 3.3A-B) and GFAP (Figure 3.3C-D) were similar after CTRL ASO and PLG ASO treatment. In AD mice treated with CTRL ASO, both CD11b and GFAP levels were increased in the cortex (Figure 3.3B, 3.3D) and hippocampus (Figure 3.4B, 3.4D) when compared to WT mice, but levels of microglial/macrophage are decreased to WT levels in both the hippocampus and cortex in AD animals treated with PLG ASO (Figure 3.3A-B, 3.4A-B). In addition, astrocyte activation in AD mice decreased significantly by about half in the cortex and returned to WT levels in the hippocampus with PLG ASO treatment (Figure 3.3C-D, Figure 3.4C-D). This decrease in inflammatory activity in plasminogen-depleted AD animals further corresponds with a two-thirds reduction in 6E10 staining, indicating reduced Aβ deposition (Figure 3.3E-F), suggesting that recruitment of inflammatory markers may be important to the formation of stable plaques in this mouse model. Importantly, while the same trend is seen in both the cortex and hippocampus, stronger differences are found in the cortex (Figure 3.3B, 3.3D, 3.3F) than the hippocampus (Figure 3.4B, 3.4D, 3.4F), likely due to the fact that there is more plaque deposition in the cortex than the hippocampus in this AD mouse line at this age.

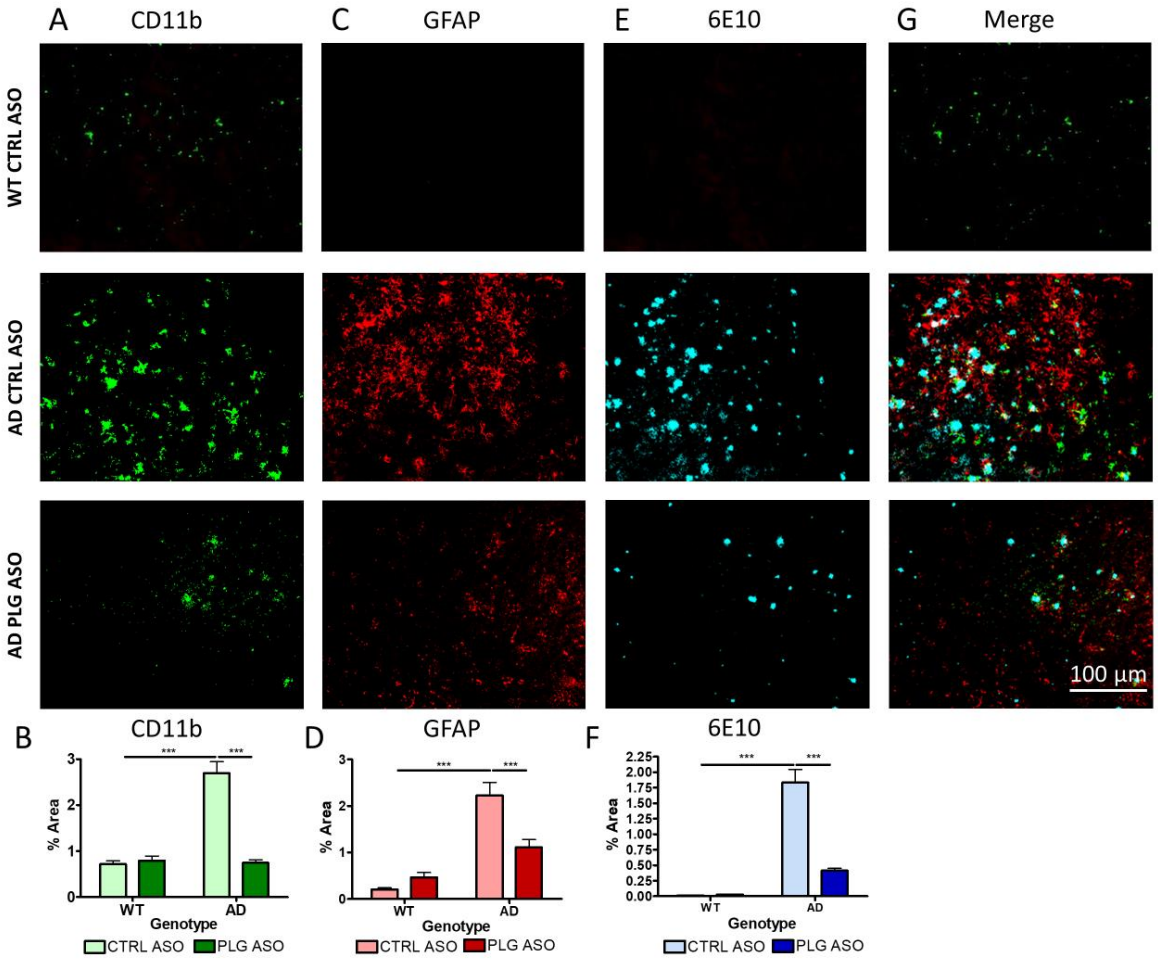


Figure 3.3. Microglia/macrophage activation, astrocyte activation, and plaque load are reduced in the cortex of PLG ASO-treated AD mice compared to CTRL ASO-treated AD mice. (A) Representative images of CD11b staining in the cortex of WT CTRL ASO-treated mice and AD mice with CTRL or PLG ASO treatment. (B) Microglial/macrophage activation in the cortex is significantly increased in AD CTRL ASO-treated animals compared to WT groups ($p < 0.0001$). CD11b staining is significantly reduced in AD PLG ASO-treated animals compared to the AD CTRL ASO group ($p < 0.0001$), comparable to WT animals. (C) Representative images of GFAP staining in the cortex of WT CTRL ASO-treated mice and AD mice with CTRL or PLG ASO treatment. (D) Astrocyte activation in the cortex is increased in AD CTRL ASO-treated animals compared to WT CTRL ASO mice ($p < 0.0001$). However, GFAP staining is significantly reduced in AD PLG ASO-treated animals compared to AD CTRL ASO counterparts ($p < 0.0001$). (E) Representative images of Aβ staining in cortex of WT CTRL ASO-treated mice and AD mice with CTRL or PLG ASO treatment. (F) Aβ deposition in the cortex is decreased in PLG ASO-treated AD animals compared to CTRL ASO-treated AD animals ($p < 0.0001$). (J) Merged images comparing microglial/macrophage and astrocyte activation surrounding plaques in the cortex of WT CTRL ASO-treated mice and AD mice treated with either CTRL or PLG ASO. Two-way ANOVA, n = 15-18 mice per group; all values presented as mean ± SEM. Results are from 3 independent experiments.

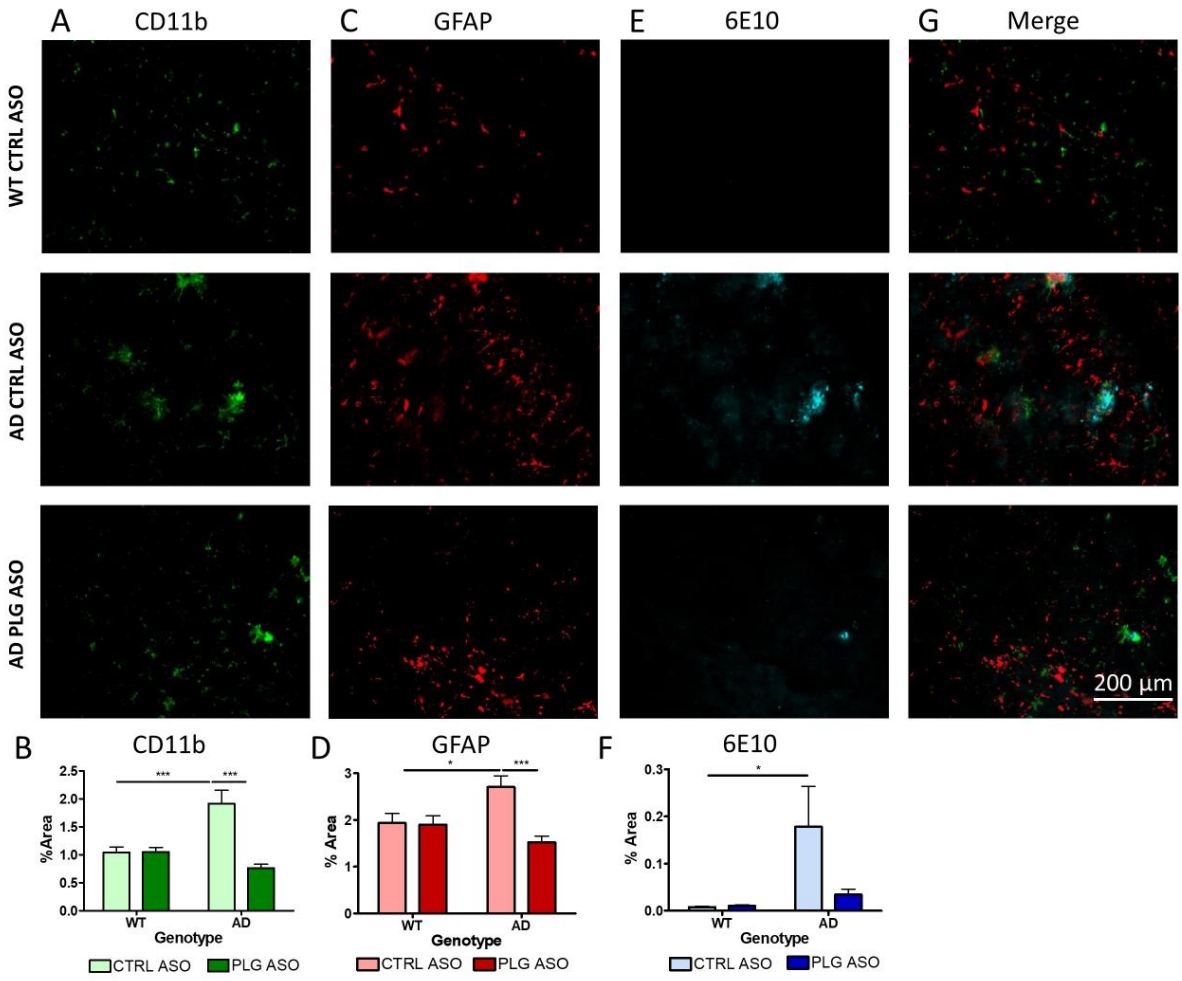


Figure 3.4. Microglia/macrophage activation, astrocyte activation, and Aβ plaque load are reduced in the hippocampus of PLG ASO-treated AD mice compared to CTRL ASO-treated AD mice. (A) Representative images of CD11b staining in the hippocampus of WT CTRL ASO-treated mice and AD mice after CTRL or PLG ASO treatment. (B) Microglial/macrophage activation in the hippocampus is significantly increased in AD CTRL ASO-treated animals compared to WT animals ($p = 0.03$), but CD11b staining is significantly reduced in AD PLG ASO-treated animals compared to the AD CTRL ASO group ($p < 0.0001$). (C) Representative images of GFAP staining in the hippocampus of WT CTRL ASO-treated mice and AD mice with CTRL or PLG ASO treatment. (D) Hippocampal astrocyte activation is increased in AD CTRL ASO-treated animals compared to WT groups ($p = 0.03$), but GFAP staining is significantly reduced in AD PLG ASO mice compared to AD CTRL ASO ($p = 0.0043$). (E) Representative images of Aβ using 6E10 antibody in the hippocampus of WT CTRL ASO-treated mice and AD mice with CTRL or PLG ASO treatment. (F) Plaque load in the hippocampus is increased in the brains of AD animals compared to WT ($p = 0.036$), but not in PLG ASO-treated AD animals. The difference in plaque load between AD CTRL ASO- and AD PLG ASO-treated animals is not significant ($p = 0.11$). (G) Merged images comparing microglial/macrophage and astrocyte activation surrounding plaques in the hippocampus of AD mice treated with either CTRL or PLG ASO. Two-way ANOVA, n = 15-18 mice per group; all values presented as mean ± SEM. Results are from 3 independent experiments.

3.4 Depletion of plasma plasminogen reduces AD pathology in AD mice.

AD pathology includes fibrillary plaque deposition and neuronal degeneration. In addition, increased autophagy and clearance mechanisms are upregulated in response to Aβ plaque deposition in the AD brain. To investigate whether decreased neuroinflammatory activation was associated with less AD pathology, we examined fibrillar Aβ plaque deposition (Congo Red), neuronal staining (NeuN), and presence of lysosomes (LAMP-1) and apoE in the cortex of AD animals treated with CTRL or PLG ASO.

PLG ASO reduced fibrillar plaque deposition (Figure 3.5), as indicated by Congo Red staining. While 6E10 is reactive to amino acids 1-16 of the Aβ sequence and is able to detect soluble and insoluble Aβ, as well as APP, Congo Red specifically detects fibrillar amyloid protein. Although insoluble Aβ plaque deposition is decreased in PLG ASO-treated AD mice, APP expression in the brain and insoluble Aβ levels are unaffected (Figure 3.6) indicating that plaque deposition differences may be a result of differential APP processing or clearance of Aβ.

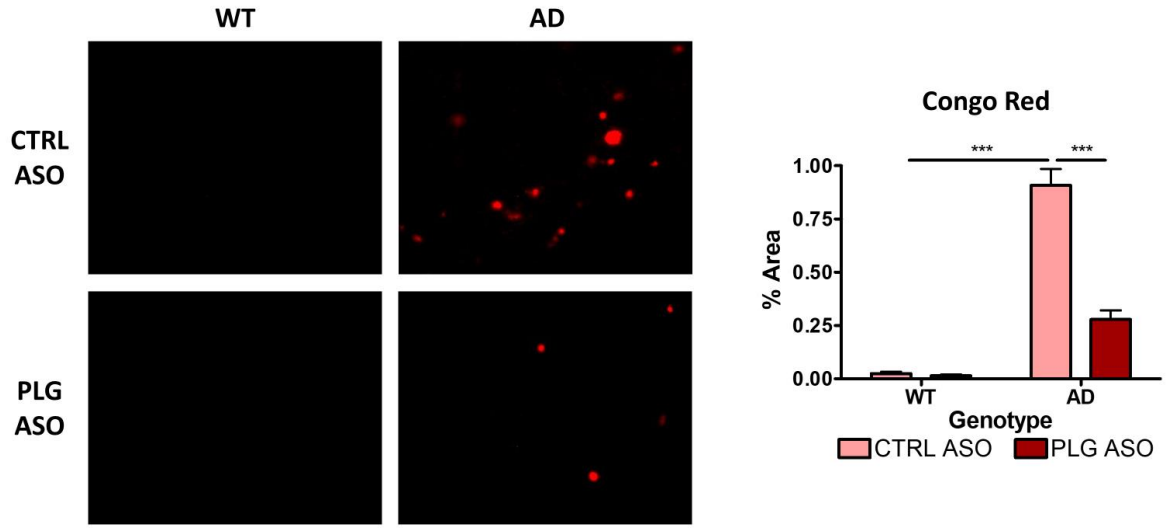


Figure 3.5. PLG ASO treatment attenuates fibrillary plaque deposition in the cortex of AD mice. Representative images of Congo Red staining show fibrillar amyloid plaque deposition in CTRL- or PLG ASO-treated WT and AD mice. Fibrillar plaque deposition is decreased in the cortex of PLG ASO-treated AD animals compared to CTRL ASO-treated AD animals ($p < 0.0001$). Two-way ANOVA, n = 15-18 mice per group; all values presented as mean ± SEM. Results are from 3 independent experiments.

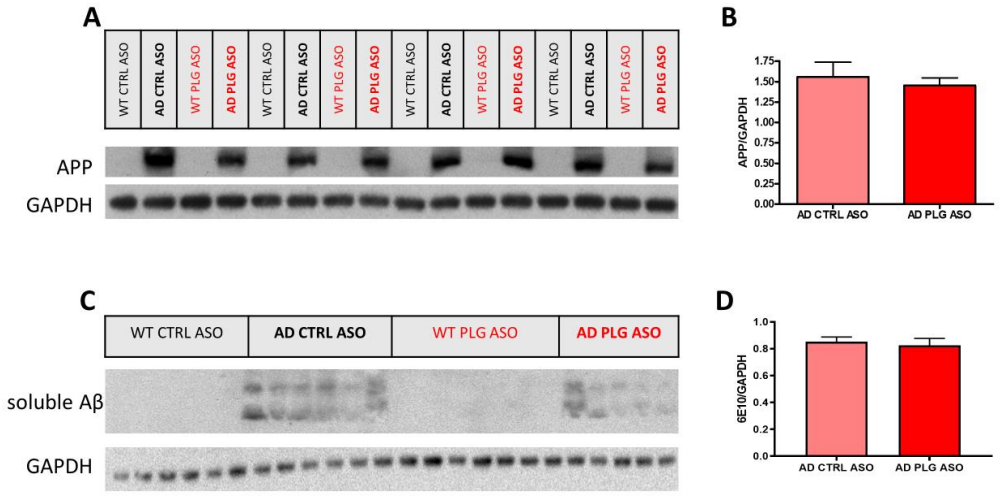


Figure 3.6. APP expression level in the brain is not changed in AD mice with PLG ASO treatment. (A) Representative Western blot showing APP and GAPDH expression in the cortex of WT and AD mice treated with CTRL or PLG ASO. (B) There is no difference in APP expression, normalized to GAPDH expression, in the cortex of AD mice treated with CTRL or PLG ASO ($p = 0.17$). (C) Representative Western blot showing soluble Aβ expression (4-12 kD) and GAPDH expression in the cortex of WT and AD mice treated with CTRL or PLG ASO. (D) There is no difference in soluble Aβ expression, normalized to GAPDH expression, in the cortex of AD mice treated with CTRL or PLG ASO ($p = 0.67$). T-test, n = 15-18 mice per group; all values presented as mean ± SEM. Results are from 3 independent experiments.

We quantified expression of neuronal marker NeuN and found that staining was increased in PLG ASO-treated AD animals compared to CTRL ASO-treated AD mice (Figure 3.7), suggesting reduced neuronal damage with plasminogen depletion.

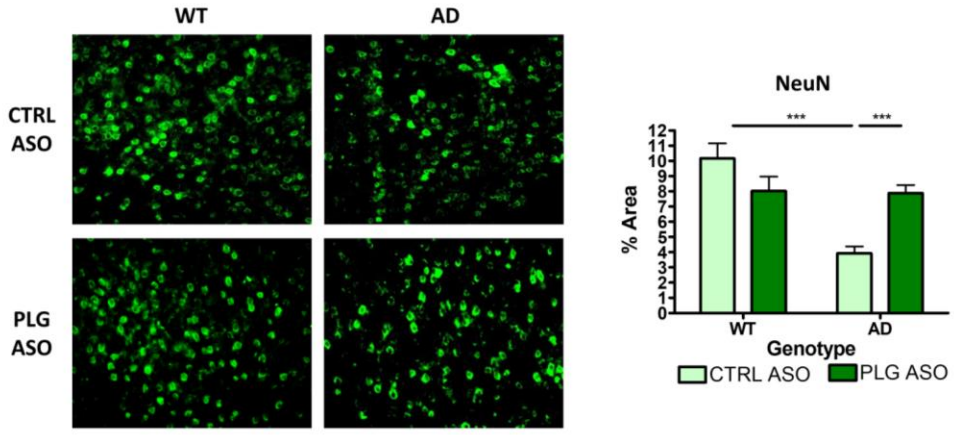


Figure 3.7. PLG ASO treatment attenuates neuronal damage in the cortex of AD mice. Representative images of NeuN staining in the cortex of WT and AD mice treated with either CTRL or PLG ASO. Neuronal staining in the cortex is decreased ($p < 0.0001$) in CTRL ASO-treated AD animals, but returns to WT levels in AD animals with PLG ASO treatment ($p < 0.0001$). Two-way ANOVA, n = 15-18 mice per group; all values presented as mean ± SEM. Results are from 3 independent experiments.

Furthermore, LAMP-1 staining was decreased in AD animals with PLG ASO-treatment, indicating these animals may have less autophagy through lysosome recruitment in the brain (Figure 3.8). Together, these results suggest that depletion of plasminogen in the plasma not only reduced neuroinflammation, but also improved AD pathology in these mice.

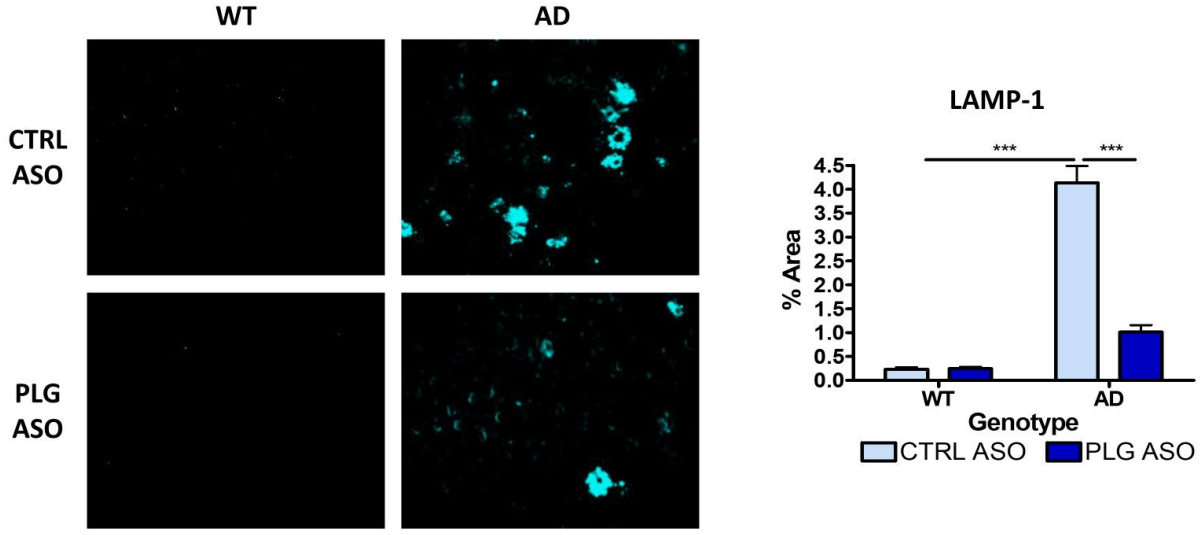


Figure 3.8. PLG ASO treatment attenuates autophagy signaling in the cortex of AD mice. Representative images of LAMP-1 staining, a marker of autophagy, in the cortex of WT CTRL ASO-treated mice and CTRL- or PLG ASO-treated AD animals. LAMP-1 staining is increased ($p < 0.0001$) in AD animals compared to WT, but decreased in PLG ASO-treated animals compared to CTRL ASO-treated AD mice ($p < 0.0001$). Two-way ANOVA, n = 15-18 mice per group; all values presented as mean ± SEM. Results are from 3 independent experiments.

Apolipoprotein E (apoE) plays a critical role in lipoprotein metabolism in both the brain and the periphery and has been implicated in Alzheimer's disease and cardiovascular disease. Peripherally, apoE is primarily produced by the liver, binds triglyceride-rich lipoproteins in the blood, and is the main ligand for the low density lipoprotein (LDL) receptor and the LDL receptor related protein (LRP). In the brain, astrocytes are the main producer of apoE which plays a role in cholesterol metabolism and Aβ clearance. ApoE has been shown to effect APP processing, as well as Aβ production and deposition. In addition, apoE is involved in the clearance of Aβ and this apoE-mediated clearance is impaired in AD (Rasmussen, 2016). We looked at apoE expression in the brain to see whether apoE is effected by AD genotype or plasminogen level (Figure 3.9). In the cortex of AD animals, apoE is significantly increased, likely around plaques where it is aiding in clearance. With PLG ASO treatment, apoE level drops significantly.

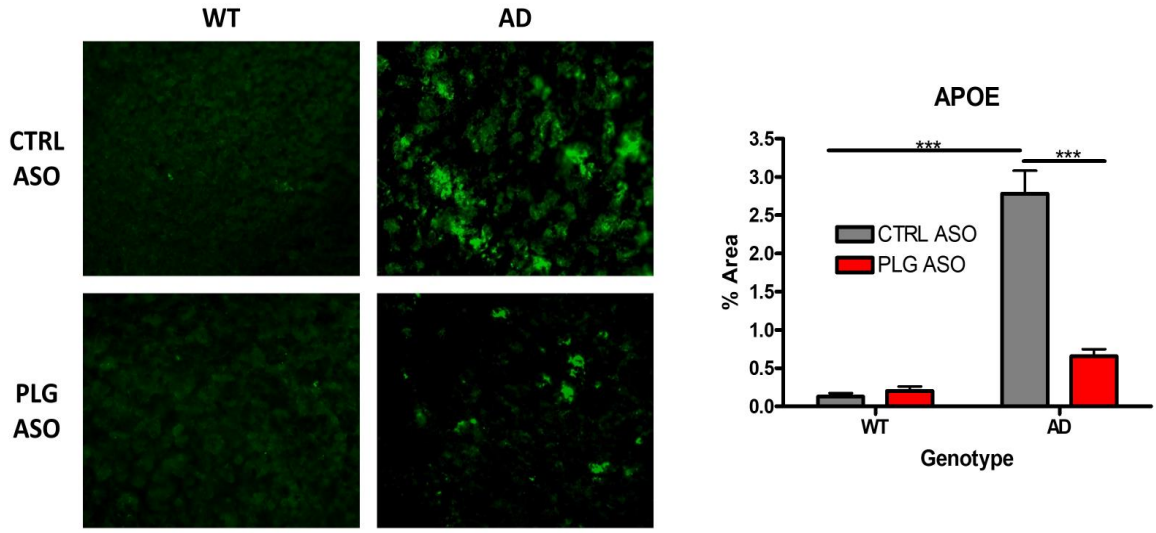


Figure 3.9. PLG ASO treatment attenuates apoE expression in the cortex of AD mice. Representative images of apoE staining, in the cortex of WT CTRL ASO-treated mice and CTRL- or PLG ASO-treated AD animals. ApoE staining is increased ($p < 0.0001$) in AD animals compared to WT, but decreased in PLG ASO-treated animals compared to CTRL ASO-treated AD mice ($p < 0.0001$). Two-way ANOVA, n = 15-18 mice per group; all values presented as mean ± SEM. Results are from 3 independent experiments.

3.5 At 5 months of age, there is no sign of vascular damage in the cortex of AD mice.

AD CTRL mice showed signs of AD pathology by 5 months of age when we sacrificed these animals for this experiment. Because peripheral plasminogen depletion significantly reduced AD pathology and neuroinflammation, we looked to see if vascular damage was responsible for this effect. The BBB is composed of pericytes, astrocytic endfeet, and endothelial cells packed tightly together with tight junction proteins to form a semi-permeable, selective barrier against systemic infection (Govindpani et al., 2019). In AD, the BBB gets leaky, allowing blood proteins such as fibrinogen and immunoglobulin to be deposited into the brain (van de Haar et al., 2016, Ryu and McLarnon, 2009, Sengillo et al., 2013).

To assess whether AD mice had BBB damage and whether this was modulated by peripheral plasminogen, we looked at a variety of vascular molecules in cortical homogenates (Figure 3.10). We looked at CD31 as an endothelial cell marker to see whether there were any differences in overall expression of endothelial cells, indicating blood vessel density changes (Figure 3.10A). In addition, we used vascular endothelial growth factor (VEGF) as an indicator of vascular injury (Figure 3.10B) since VEGF is upregulated during angiogenesis in response to vessel injury (Shibuya, 2011). Furthermore, we examined whether fibrinogen expression levels were different in the brains of AD mice (Figure 3.10C), indicating leakage into the parenchyma and at expression of occludin (Figure 3.10D), a tight junction protein that may be altered in response to BBB damage. There is some fibrinogen expressed in the brains of these mice by Western blot, likely due to difficulties during perfusion to clear away all fibrinogen inside the vessel wall. However, there were no expression changes in any of these markers by either genotype or ASO injection, suggesting that at 5 months of age, AD mice do not yet show signs of BBB damage. Thus, changes in BBB damage do not explain how peripheral plasminogen depletion affected AD pathology and the neuroimmune response.

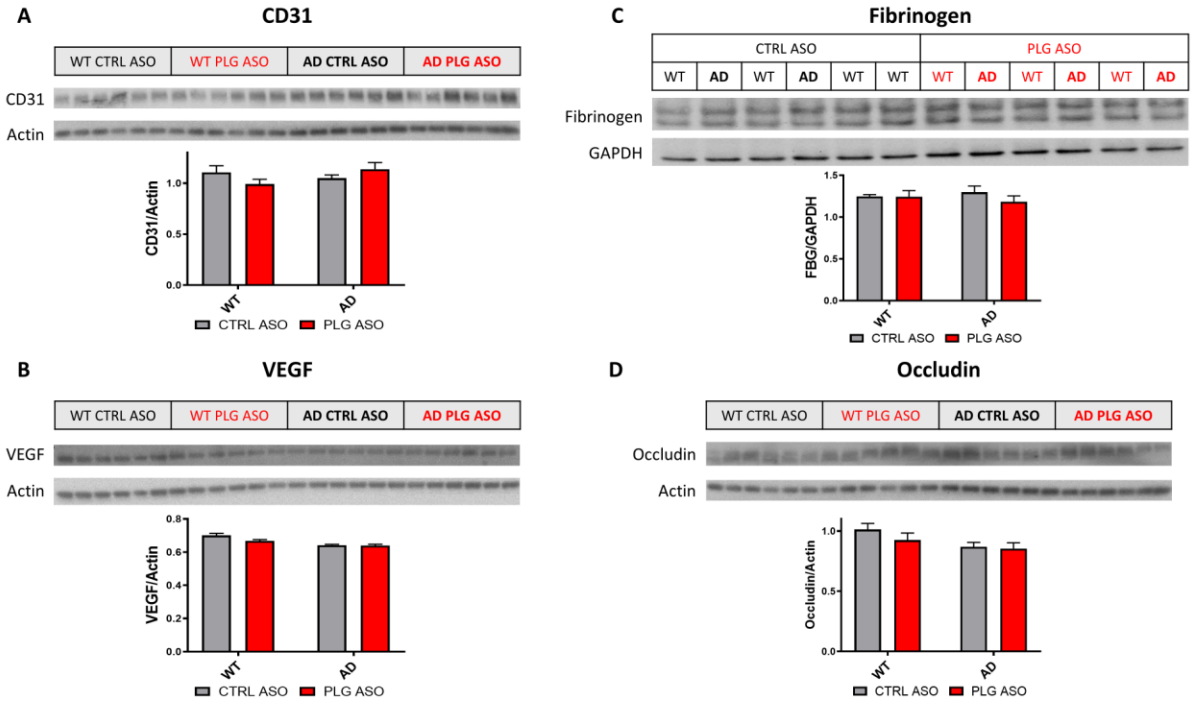


Figure 3.10. Vascular integrity remains intact in AD mice at 5 months of age. Representative Western blots of cortical homogenates indicating (A) endothelial cell expression (CD31), (B) vascular damage and angiogenesis (VEGF), (C) fibrinogen leakage into the BBB, and (D) tight junction protein changes (occludin). No significant changes in expression were seen by genotype or ASO injection. Two-way ANOVA, n = 15-18 mice per group; all values presented as mean ± SEM.

3.6 A2AP ASO treatment effectively depletes the protein in the plasma, but does not alter other plasma plasminogen system proteins.

Since we had evidence that plasma plasminogen contributed to the neuroinflammatory response in AD, we explored whether this inflammatory action was due to plasminogen's cleavage into the active serine protease plasmin. To do this, we investigated whether depleting α_2-antiplasmin in the plasma, and thus increasing plasmin through reduced inhibition, would increase the inflammatory response. We used an ASO-mediated gene knockdown strategy to deplete α_2-antiplasmin in AD and WT mice. To assess α_2-antiplasmin depletion in the circulation, plasma was collected after 2 weeks and 10 weeks of subcutaneous A2AP ASO injections. Plasma α_2-antiplasmin was knocked down efficiently in AD and WT animals to less than 5-10% of CTRL plasma levels (levels after 10 weeks of treatment are shown in Figure 3.11A-B), while plasma plasminogen and tPA levels were not affected (Figure 3.11A, 3.11C-D). We also analyzed plasmin activity in the plasma of ASO-treated animals (Figure 3.11E) and observed that A2AP ASO injections increased plasmin activity in both WT and AD animals. We believe this result is a measure of free plasmin in the plasma, rather than plasmin bound to an inhibitor as a PAI-1 complex would be unlikely to efficiently cleave the assay's chromogenic substrate. The increase in plasmin activity is more drastic in AD A2AP ASO-treated animals compared to their WT counterparts, perhaps due to the fact that plasmin activity is increased in the presence of Aβ (Van Nostrand and Porter, 1999, Exley and Korchazhkina, 2001). However,

if this is the case, it is surprising that there is no difference in plasmin activity between AD and WT animals treated with CTRL ASO.

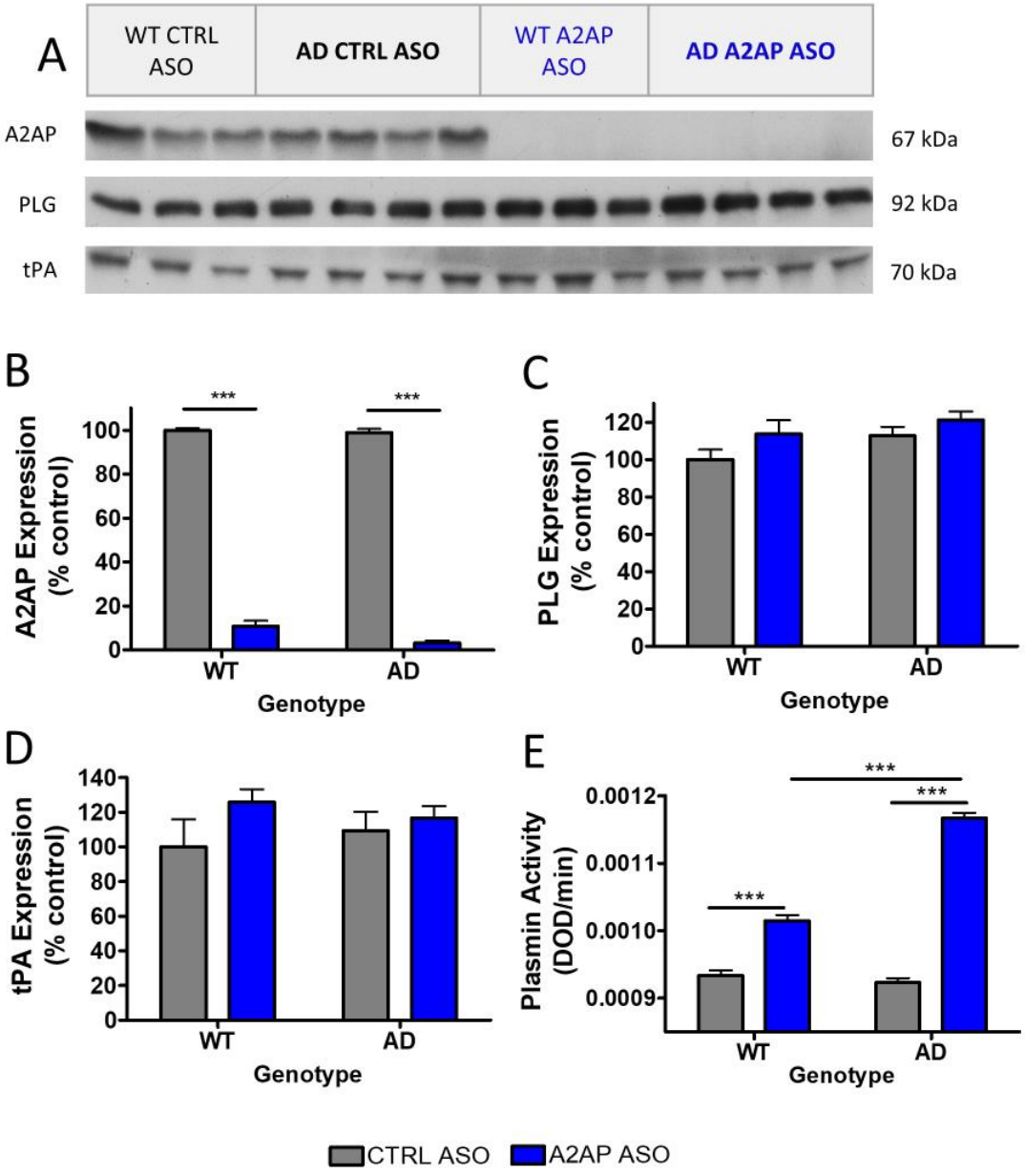


Figure 3.11. α_2-antiplasmin levels are depleted in the plasma of A2AP ASO-treated mice, without affecting plasma levels of plasminogen activator system proteins. (A) Representative Western blot of α_2-antiplasmin, plasminogen, and tPA levels in the plasma of WT CTRL ASO, WT A2AP ASO, AD CTRL ASO, and AD A2AP ASO-treated mice. (B) α_2-antiplasmin levels are significantly depleted in the plasma of A2AP ASO-treated animals compared to controls ($p < 0.0001$). Plasminogen (C) and tPA (D) expression levels in the plasma are unaffected by A2AP ASO treatment ($p = 0.65$, $p = 0.40$, respectively). (E) Plasmin activity in the plasma is increased by A2AP ASO treatment ($p < 0.0001$), and this increase is exacerbated by the AD genotype ($p < 0.0001$). Two-way ANOVA, n = 7 mice per group; all values presented as mean ± SEM.

3.7 Depletion of plasma α_2-antiplasmin increases microglial/macrophage activation and fibrillar plaque deposition, but not neuronal damage, lysosome presence, and astrocyte activation in AD mice.

We compared CD11b expression levels between CTRL ASO- and PLG ASO-treated AD and WT mice to determine microglial/macrophage activation in the cortex (Figure 3.12). In AD mice treated with CTRL ASO, CD11b levels were increased in the cortex when compared to WT mice, and increased further in AD animals with A2AP ASO treatment (Figure 3.12A-B), suggesting that monocytic cells have enhanced activation due to increased PL activity. The expression levels of CD11b (Figure 3.12B) were similar between CTRL ASO- and A2AP ASO-treated WT mice where an immune response to Aβ was not present. The increase in immune response in A2AP ASO-treated AD animals was also accompanied by increased Congo Red staining (Figure 3.12C-D), indicating an exacerbated fibrillar plaque load. We also examined GFAP, NeuN, and LAMP-1 levels and did not see any difference in levels of these proteins between AD CTRL ASO and AD A2AP ASO-treated mice (Figure 3.13). This may be due to the fact that astrocyte activation is saturated in these animals and that the disease has not yet progressed in mice of this age to see exacerbated neuronal death compared to AD controls.

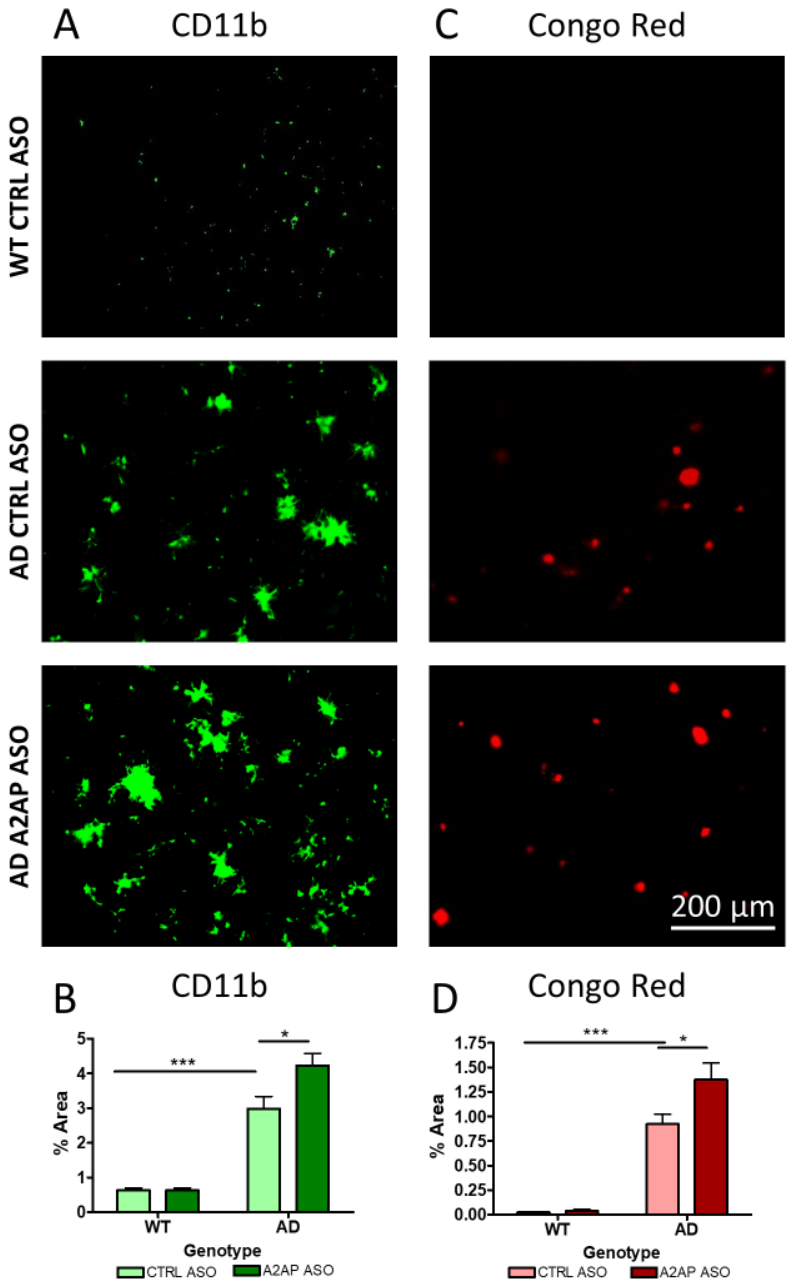


Figure 3.12. Microglia/macrophage activation and fibrillar plaque load are increased in the brains of A2AP ASO-treated AD mice. (A) Representative images of CD11b staining in the cortex of WT CTRL ASO-treated mice and AD mice with CTRL or A2AP ASO treatment. (B) Microglial/macrophage activation in the cortex is significantly increased in AD animals ($p < 0.0001$), and this increase is exacerbated in mice with depleted plasma α_2-antiplasmin ($p = 0.02$). (C) Representative images of Congo Red staining in the cortex of WT CTRL ASO-treated mice and AD mice with CTRL or A2AP ASO treatment. (D) Fibrillar plaque deposition is increased in AD animals treated with A2AP ASO compared to CTRL ASO ($p = 0.03$). Two-way ANOVA, n = 7 mice per group; all values presented as mean ± SEM.

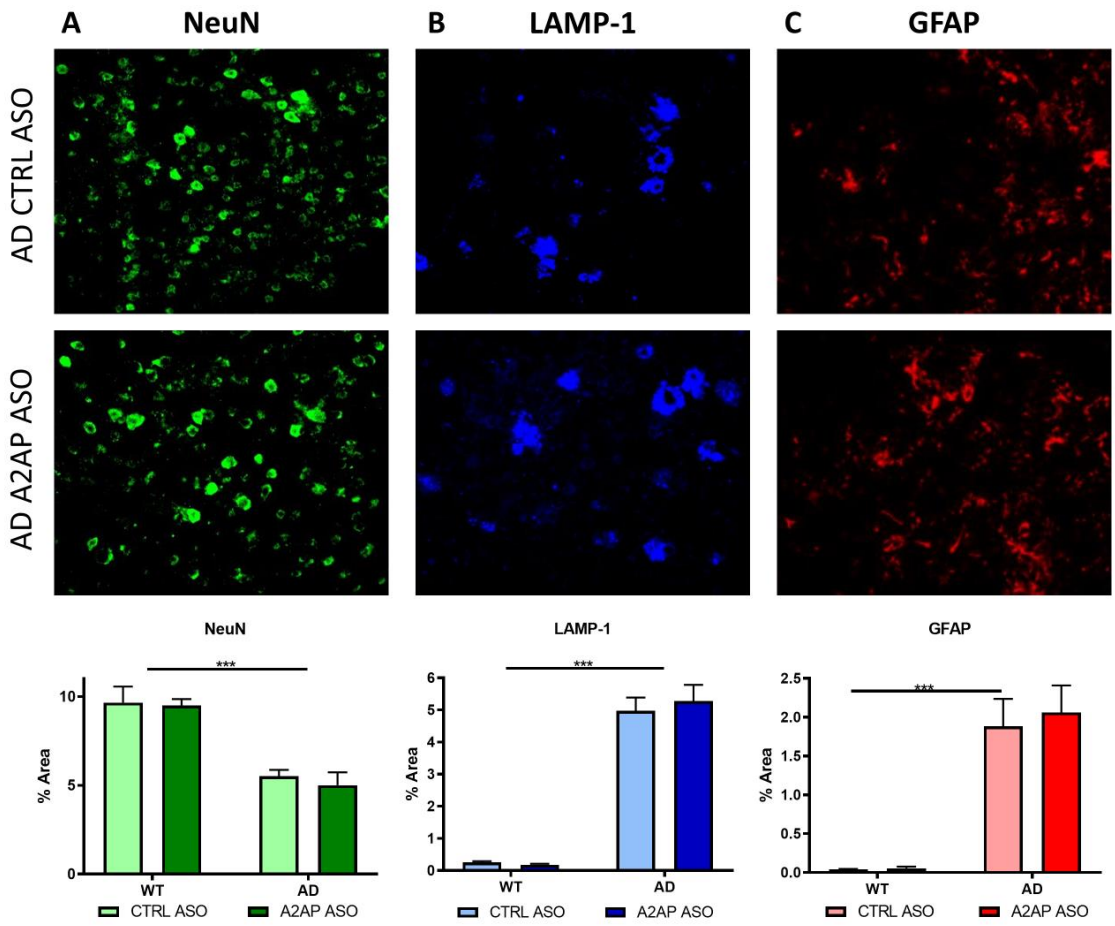


Figure 3.13. A2AP ASO does not further affect neuronal death, lysosome presence, or astrocyte activation in AD animals compared to control. Representative images of (A) NeuN, (B) LAMP-1, and (C) GFAP staining in the cortex of AD CTRL ASO and AD A2AP ASO-treated mice. Below the images, these markers are quantified in WT and AD animals treated with CTRL or A2AP ASO.

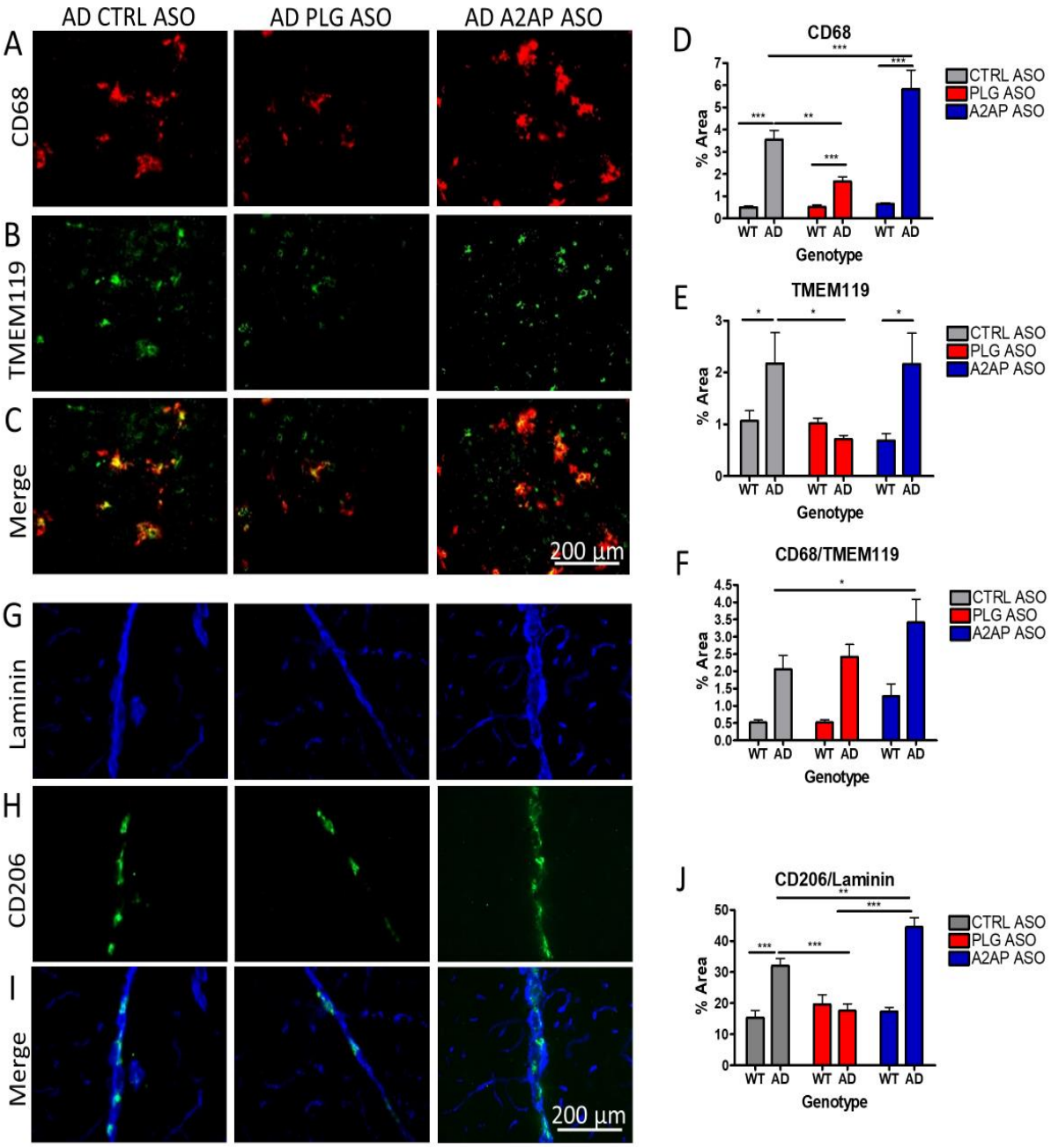
3.8 Peripheral plasmin level regulates degree of microglial activation and recruitment of perivascular macrophages in the AD mouse brain.

It is difficult to differentiate between activated microglia and infiltrating macrophages since they share many of the same cell-surface markers. In order to distinguish between activated resident microglia and infiltrating peripheral macrophages in the AD mouse brain, we examined the expression of a variety of markers: CD68, which is preferentially expressed on both activated microglia and on macrophages (Figure 3.14A); TMEM119, the only known microglia-specific marker (Figure 3.14B) (Bennett et al., 2016, Satoh et al., 2016); and CD206, a marker for perivascular macrophages (Figure 3.14H) (Galea et al., 2005). Representative images of these stainings in AD animals are shown (Figure 3.14A-C, 3.14G-I), and quantification from WT and AD mice is presented in the accompanying bar graphs (Figure 3.14D-F, 3.14J). In the cortex, around Aβ plaques, most CD68 expression is co-localized with TMEM119 staining (Figure 3.13C), suggesting that activated microglia are primarily responsible for the innate immune response around plaques in the AD mouse brain. Peripheral plasminogen depletion decreased both CD68 (Figure 3.14A, 3.14D) and TMEM119 (Figure 3.14B, 3.14E) expression levels

compared to CTRL ASO-treated AD animals. However, in AD A2AP ASO-treated animals, TMEM119 levels were unchanged relative to CTRL ASO-treated AD animals (Figure 3.14B, 3.14E), while CD68 levels were increased (Figure 3.14A, 3.14D). We observed an increase in the expression of CD68/TMEM119 in plasma α_2-antiplasmin-deficient AD animals. These data suggest that in the presence of greater plasmin activity, microglia are more highly activated, as indicated by increased signal overlap, even though the number of activated cells may not change.

We also investigated the presence of perivascular macrophages in major arteries of the brain. Using laminin as a vascular marker (Figure 3.14G), we examined the expression levels of CD206 (Figure 3.14H) on CD68 positive cells (all CD206 positive cells examined were also CD68 positive). CD206 positive cells in these animals were always found associated with vessels, and we quantified the amount of CD206/laminin to determine the percentage of the vessel that contained perivascular macrophages. We found that perivascular macrophages are increased in AD CTRL ASO-treated animals compared to WT controls (Figure 3.14J). However, PLG ASO treatment in AD animals reduced the presence of perivascular macrophages to WT control levels, while A2AP ASO treatment led to an increase in perivascular macrophage staining. These results suggest that plasmin is required for perivascular macrophage recruitment in the major blood vessels of the mouse brain during the progression of AD. Furthermore, although perivascular macrophages are not the main species surrounding plaques in the AD brain, they can communicate with microglia through signaling pathways such as cytokine and reactive ROS that may be contributing to microglial activation in AD.

Figure 3.14. Monocytic cell activation in AD mice is primarily driven by activated microglia, but perivascular macrophage migration is also upregulated in AD and this is mediated by plasminogen. Representative images of CD68 (A) and TMEM119 (B) staining in CTRL ASO-, PLG ASO-, or A2AP ASO-treated AD animals. (C) Merged images show that CD68-positive cells are also TMEM119-positive, suggesting they are activated microglia, not macrophages. (D) CD68 level is increased in all AD animals compared to WT controls ($p < 0.0001$), but this increase is reduced by PLG ASO treatment ($p < 0.01$) and exacerbated by A2AP ASO treatment ($p < 0.001$). (E) TMEM119 level is increased in AD CTRL ASO- and A2AP ASO-treated animals compared to WT controls ($p = 0.02$). (F) The amount of CD68 expression per TMEM119 expression is increased in AD animals treated with A2AP ASO compared to CTRL ASO ($p < 0.05$). Representative images of laminin (G) and CD206 (H) staining in CTRL ASO-, PLG ASO-, or A2AP ASO-treated AD animals. (I) Merged images show that CD206-positive cells are perivascular macrophages, contained in vessels of the mouse brain. (J) Percentage of vessels containing CD206-positive macrophages is increased in CTRL ASO-AD animals compared to WT groups ($p < 0.0001$). PLG ASO treatment of AD animals significantly decreases the level of peripheral macrophages around vessels compared to AD PLG ASO mice ($p < 0.01$), whereas A2AP ASO treatment of AD animals leads to an increase in perivascular macrophage staining ($p < 0.0001$). Two-way ANOVA, $n = 6$ mice per group; all values presented as mean ± SEM.


AD CTRL ASO
AD PLG ASO
AD A2AP ASO
A
CD68
B
TMEM119
C
Merge
200 µm
G
Laminin
H
CD206
I
Merge
200 µm
D
CD68
% Area
Genotype
WT AD WT AD WT AD
CTRL ASO
PLG ASO
A2AP ASO
E
TMEM119
F
CD68/TMEM119
J
CD206/Laminin

3.9 Plasminogen depletion in AD mice may be associated with modest cognitive improvement but behavioral data is inconclusive.

Cognitive decline was assessed in AD mice using two behavioral tests: contextual fear conditioning and Barnes maze. Contextual fear conditioning is used to test for hippocampal impairment, a brain region involved in remembering location and highly affected in AD. Cognitive function was assessed in a small cohort of mice (n = 3-4 per group) using contextual fear conditioning. On the training day, mice were allowed to habituate to a chamber and then administered a pair of shocks 2 minutes apart. On the testing day, mice were placed back in the chamber and levels of freezing were measured as an assessment of how well they remembered the negative stimulus of the shocks in the chamber. AD-CTRL ASO treated mice froze less (~20% of the time) than WT mice (~50% of the time) during the testing period (p = 0.09, n.s.). PLG ASO treatment ameliorated this effect (Figure. 3.15), returning freezing levels to >50% (p = 0.06). Due to the small number of animals used for this experiment, statistical significance was not met and cognitive benefits of plasminogen-depletion remained inconclusive.

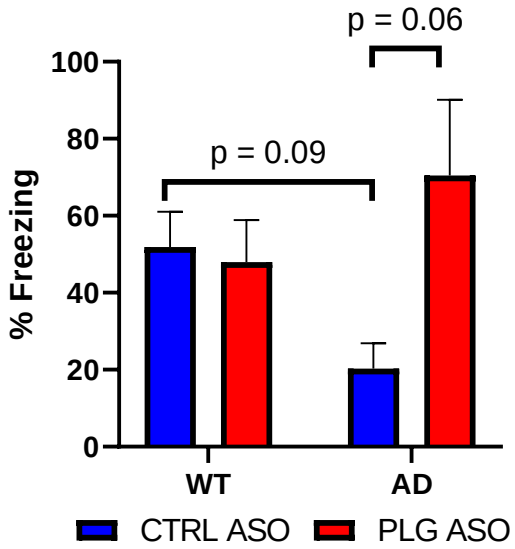


Figure 3.15. Depletion of plasma plasminogen may improve cognitive function in AD mice as measured by contextual fear conditioning. Contextual memory of WT and AD mice was assessed by measuring freezing behavior upon exposure to the shock chamber 24 hours after fear conditioning training. WT mice froze more than AD CTRL ASO-treated animals (p = 0.09), but plasminogen depletion returned AD freezing levels to that of WT mice (p = 0.06). Two-way ANOVA, n = 3-4 mice per group; all values presented as mean ± SEM.

Due to technical problems with the fear conditioning method, we were unable to replicate this experiment with a larger cohort of mice. As an alternative measure of cognition, we conducted Barnes maze testing. The Barnes maze assesses spatial learning and memory in rodents. Mice are trained to find an escape hole on a platform over several days of repeated training. During the memory retention test, the latency to visit the target hole, the number of errors in going to other holes, and the number of target hole visits were recorded. There were no differences between WT and AD CTRL ASO-treated animals by any of the measures (Figure

3.16). and thus this test could not be used to assess whether plasminogen deficiency affected cognition. It is possible that these animals do not show memory retention impairment at this age (~5 months of age) and that testing at later ages would be better to assess whether cognitive improvement changes in AD mice are seen along with lessened AD pathology and neuroimmune activation seen with PLG ASO treatment.

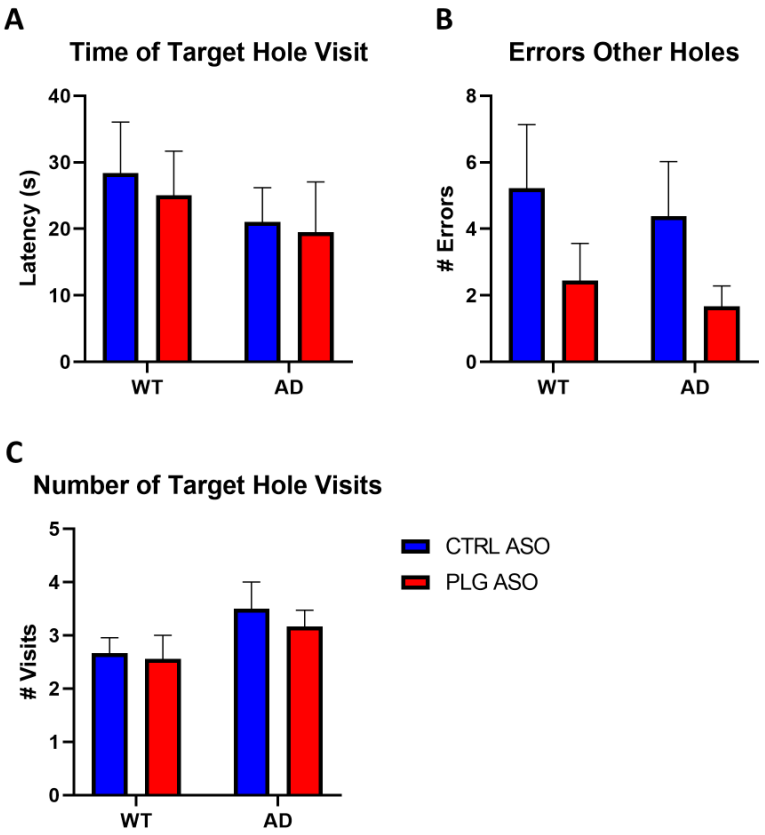


Figure 3.16. Barnes maze testing of spatial learning and memory indicated no changes between animals by ASO treatment or genotype. Cognitive performance was assessed by the Barnes maze behavioral test. Following training, on the testing day (A) time to visit the target hole, (B) the number of errors visiting other holes, and (C) the number of target hole visits were assessed. No significant changes by either of the 3 measures were noted based on genotype or ASO treatment. Two-way ANOVA, n = 8-10 mice per group; all values presented as mean ± SEM.

CHAPTER 4: DIRECT INJURY TO THE BRAIN ELUCIDATES THE PLEIOTROPIC FUNCTIONS OF PLASMINOGEN IN THE BODY

To explore whether peripheral plasminogen depletion modulates the neuroinflammatory response in the brain in response to a direct brain injury, we depleted plasminogen in the blood and then injected Aβ or LPS directly into the brain and looked at the brain's inflammatory response.

4.1 Treatment with plasminogen ASO for 2 weeks is sufficient to knock-down plasminogen levels in the plasma and Aβ injected into the brain consisted of aggregated species.

We treated C57Bl/6 (WT) mice with PLG or CTRL ASO for 2 weeks. PLG level was depleted in the plasma compared to CTRL ASO-treated mice (Figure 4.1A). In addition, we ran a Western blot of the Aβ (6E10 staining) that was injected into the brains of ASO-treated mice to determine the size of aggregates. Aβ was largely oligomeric (monomeric Aβ is ~4.5 kD) with the majority of injected species ranging from 25 to 150 kD (Figure 4.1B).

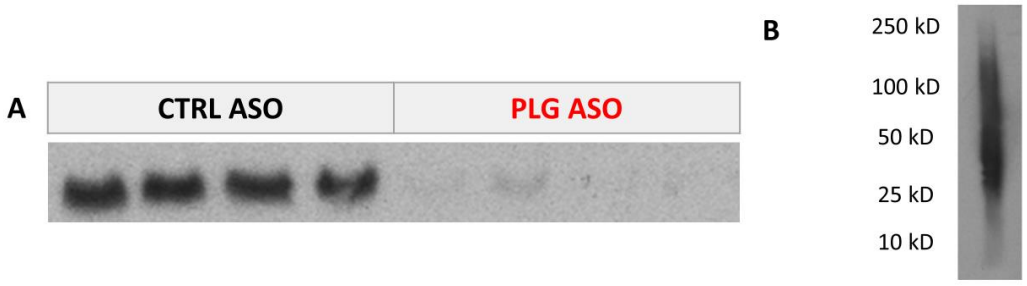


Figure 4.1. Plasminogen is depleted in the plasma of PLG ASO-treated animals and the Aβ injected into the brain consisted of a mix of aggregated Aβ with a range of sizes. (A) Representative Western blot of plasminogen levels in the plasma of CTRL ASO- and PLG ASO-treated mice. (B) Western blot showing 6E10 staining of the Aβ that was injected into the brains of mice. Aβ is aggregated and clumped into a wide range of oligomeric species.

4.2 Injections were accurately conducted using stereotactic coordinates.

To confirm that injections were precise, we stained for CD11b to look at gliosis around injection sites (Figure 5.2). Microglia were activated around the tracts where the needle was injected into the brain, indicating positioning of injections.

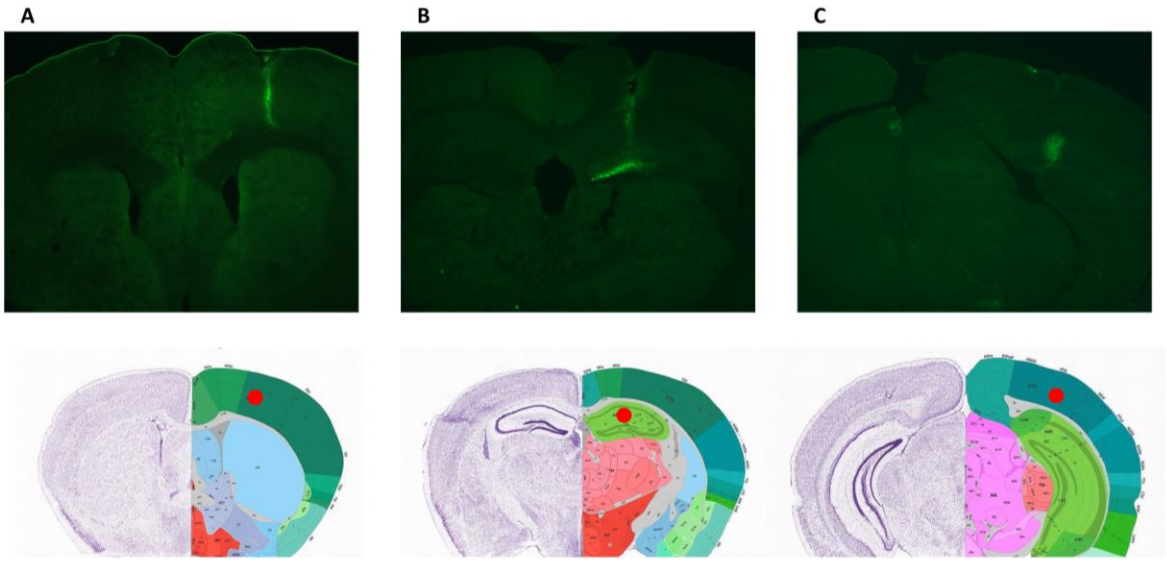


Figure 4.2. Reagents are accurately injected into the cortex and hippocampus following intracerebral injection using stereotactic coordinates. Representative CD11b staining of coronal sections of brain taken from animals injected in 3 different locations in the brain: (A) anterior cortex, (B) hippocampus, and (C) posterior cortex, as shown with corresponding Allen Brain Atlas positions. The red dot indicates the intended injection site.

4.3 Peripheral plasminogen depletion does not affect amount of Aβ deposited in the brain one week after intracerebral injection.

To see if peripheral plasminogen depletion affected Aβ clearance in the brain one week after intracerebral injection, we stained for 6E10 in CTRL or PLG ASO-treated mice (Figure 4.3). The amount of Aβ was unchanged between plasminogen-deficient and control mice indicating that plasminogen did not play a major role in clearing the Aβ injected into the brains of these animals, at least within a week of Aβ injection into the brain.

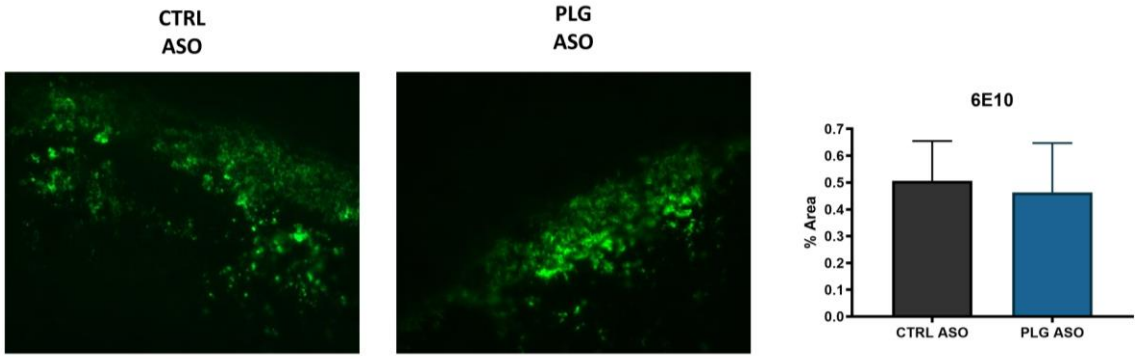


Figure 4.3. Aβ clearance is not altered by peripheral plasminogen depletion within one week of Aβ injection into the brain. Representative 6E10 staining around the injection site of Aβ-injected brains. Aβ deposition/clearance is not affected by peripheral plasminogen depletion, at least within a week of injection of Aβ into the brain. T-test, all values presented as mean ± SEM.

4.4 Injection with Aβ or LPS does not affect astrogliosis around the injection site.

Following sectioning of brains, we looked at signs of inflammatory cell activation in the brains of treated animals. To check if astrocyte activation was influenced by intracerebral injection type or peripheral plasminogen depletion, we stained for GFAP (Figure 4.4). GFAP expression around the injection sites was unchanged in response to Aβ or LPS injected into the brain or peripheral plasminogen depletion.

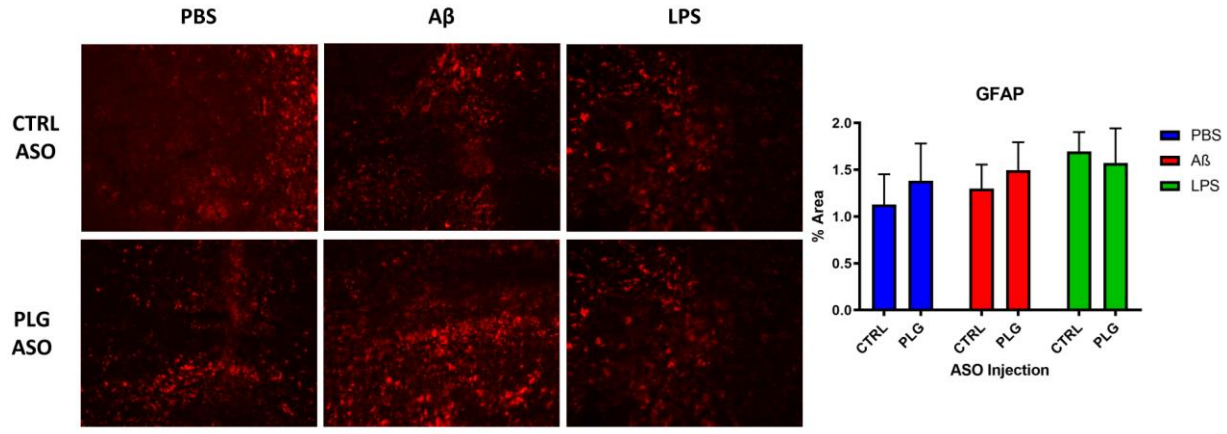


Figure 4.4. Astrocyte activation is not increased in response to injection of PBS, Aβ, or LPS into the brain, regardless of ASO injection. Representative staining of GFAP (astrocytes) in the brains of CTRL and PLG ASO-treated animals injected with PBS, Aβ, or LPS. GFAP levels are unchanged by injection type or ASO treatment. Two-way ANOVA; all values presented as mean ± SEM.

4.5 Microglia activation around injection site is dependent on peripheral levels of plasminogen and reagent injected into the brain.

Although astrogliosis was unaffected by intracerebral injections into the brain, we also looked to see if microglial activation was affected. We stained for CD11b expression around the injection sites (Figure 4.5). Both injection of Aβ and LPS increased CD11b expression compared to PBS-injected animals. CD11b was upregulated in PLG ASO-treated mice injected with either PBS or LPS. In Aβ-injected animals, Aβ seemed to be the driving factor of CD11b upregulation, independent of peripheral plasminogen levels.

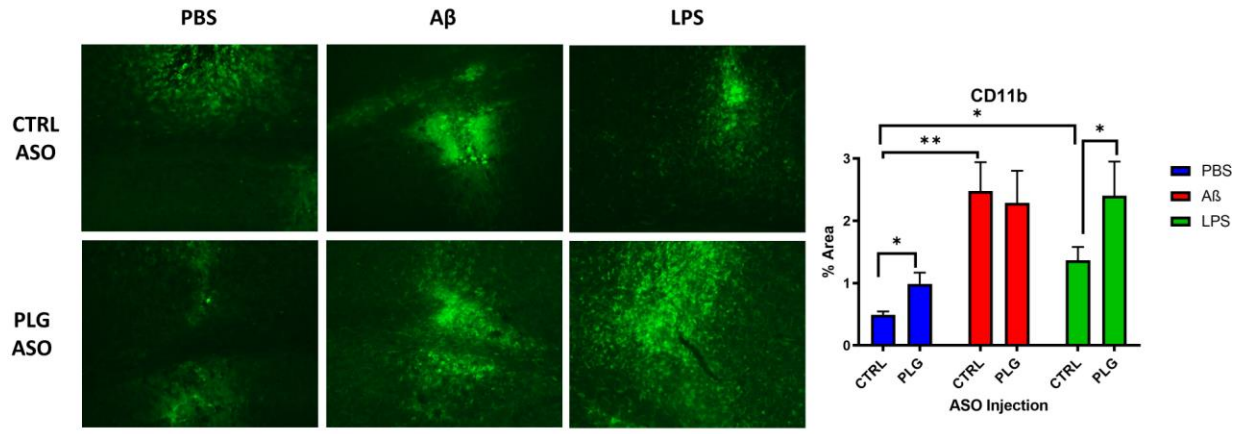


Figure 4.5. Microglia activation is dependent on both peripheral plasminogen depletion and type of inflammatory stimulus injected into the brain. Representative staining of CD11b (microglia) in the brains of CTRL and PLG ASO-treated animals injected with PBS, Aβ, or LPS. CD11b levels are increase around the injection site in response to Aβ ($p < 0.01$) or LPS injection ($p < 0.05$). CD11b levels are also increased in plasminogen-deficient animals injected with PBS ($p < 0.05$) or LPS ($p < 0.05$) compared to CTRL ASO-treated animals injected with PBS or LPS. Two-way ANOVA; all values presented as mean ± SEM.

4.6 Fibrinogen deposition may be a driving factor for differences in LPS-induced CD11b expression in CTRL ASO vs. PLG ASO-treated mice.

LPS is known to induce BBB breakdown. To see if BBB damage effects in response to LPS were plasminogen-dependent, we stained for fibrinogen (Figure 4.6). Fibrinogen levels in the brain were similar across all treatment groups, except for plasminogen-deficient LPS-injected animals which had a large degree of fibrinogen deposition around the injection site. Since LPS induced BBB damage and thus fibrinogen leakage into the brain, this fibrinogen likely came from the blood when LPS was injected. In CTRL ASO-treated animals with normal levels of plasminogen, plasmin was able to clear this fibrinogen away. However, in plasminogen-deficient LPS-injected animals, the fibrinogen was not as readily cleared away, which could be one mechanism for an increased inflammatory response by microglia in PLG ASO-LPS treated mice compared to CTRL ASO-LPS treated mice. In Aβ-injected mice, the Aβ did not exacerbate BBB damage and fibrinogen leakage into the parenchyma, but Aβ itself served as an inflammatory stimulus to activate microglia, independent of peripheral plasminogen levels.

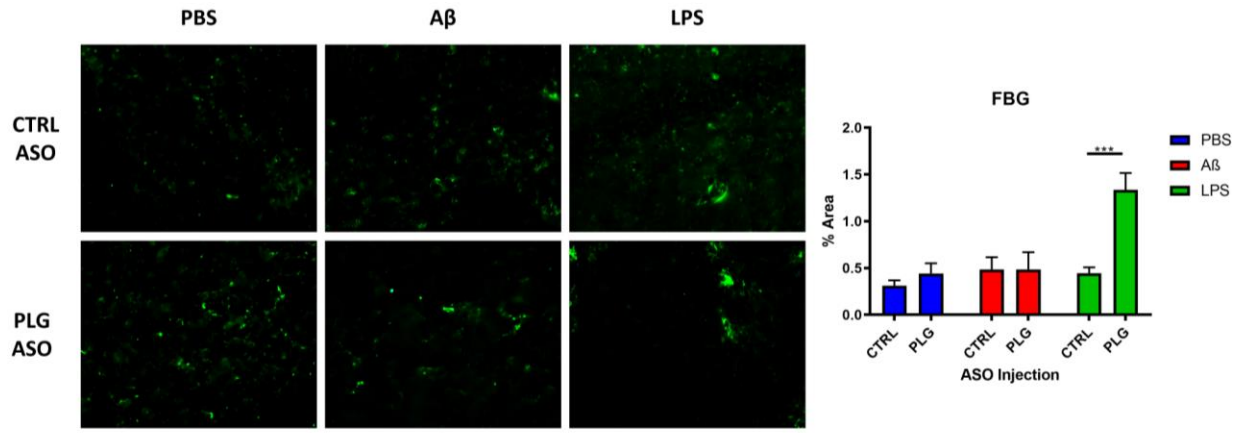


Figure 4.6. Fibrinogen levels in the brains of CTRL and PLG ASO-treated mice injected with PBS, Aβ, or LPS. Representative staining of fibrinogen in the brains of CTRL and PLG ASO-treated animals injected with PBS, Aβ, or LPS. FBG levels are unchanged by injection type or ASO treatment, except in plasminogen-deficient animals treated with PLG ASO ($p < 0.0001$). Two-way ANOVA; all values presented as mean ± SEM.

CHAPTER 5. PLASMINOGEN MEDIATES COMMUNICATION BETWEEN THE PERIPHERAL AND CENTRAL IMMUNE SYSTEMS DURING SYSTEMIC IMMUNE CHALLENGE WITH LIPOPOLYSACCHARIDE

Because of the link between systemic inflammation and cognitive decline, I was interested to see whether plasminogen plays a key role in the regulation of CNS inflammation in cases of a systemic inflammatory challenge. In this study, we depleted plasminogen peripherally in mice and then challenged the animals with LPS injected into the intraperitoneal cavity. LPS injections have long been used as a mouse model of systemic inflammation (Juskewitch et al., 2012). LPS is a gram-negative bacterial toxin that causes activation of Toll-like receptor 4 (TLR-4), which is expressed on myeloid cells including monocytes, macrophages, granulocytes, and dendritic cells (Vaure and Liu, 2014). LPS is a good model to study the connection between peripheral and central immune responses; when injected systemically, LPS leads to activation of microglia, the major immune cells of the brain, which then release proinflammatory signals into the parenchyma (Hoogland et al., 2015).

To investigate the role of plasminogen in the interaction between the periphery and the brain during the innate immune response, we treated mice with CTRL or PLG ASO. Mice were treated at a dose of 150 mg/kg/week for 2 weeks starting at 10 weeks of age. This dose and timing was sufficient to deplete plasminogen level in the plasma PLG ASO-treated mice compared to CTRL ASO-treated mice.

Figure 5.1. Plasminogen is depleted in the plasma of PLG ASO-treated animals after 2 weeks of treatment. Representative Western blot of plasminogen and transferrin (TF) levels in the plasma of CTRL ASO- and PLG ASO-treated mice.

5.1 Plasma plasminogen depletion dramatically decreases microglial and astrocytic cell responses to systemic LPS challenge.

When exposed to systemic LPS on 3 consecutive days, mice showed a global increase in CD11b (microglia/macrophages) and GFAP (astrocytes) expression throughout whole coronal brain sections (Figure 5.2A), indicating activation of the brain's resident microglia and astrocytes, and possible migration of peripheral macrophages into the brain. More specifically in the cortex, LPS injection robustly induced expression of astrocyte maker GFAP (~3-fold increase), as well as two microglial/macrophage markers, CD11b and CD68 (~4-fold increase for both) (Figure 5.2B).

Remarkably, peripheral plasminogen depletion led to a markedly decreased immune response by astrocytes and microglia/macrophages in the brain after LPS challenge (Figure 5.2A, B).

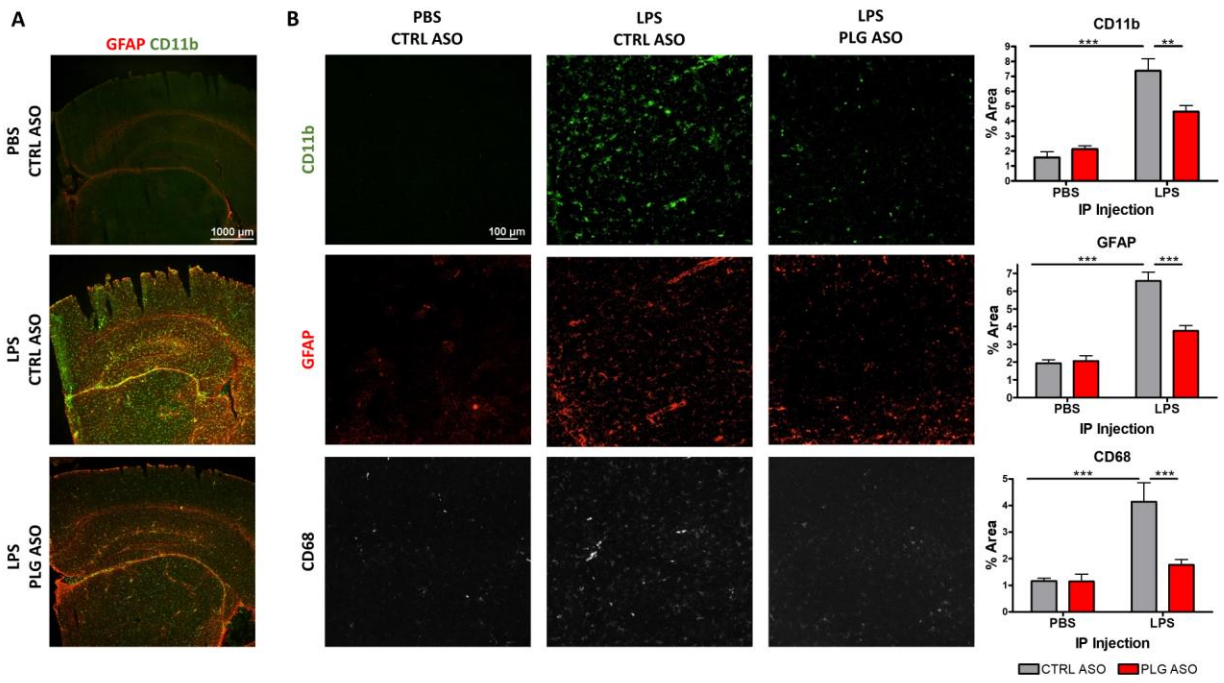


Figure 5.2. Glial cell activation is reduced in the brains of plasminogen-depleted mice following injection with LPS. (A) GFAP (red) and CD11b (green) staining in PBS-CTRL ASO, LPS-CTRL ASO, and LPS-PLG ASO mouse brains. Scale bar = 1000 µm. n = 4 sections/brain, 5-7 mice/group. (B) GFAP (red), CD11b (green), and CD68 (white) staining and quantification in the cortex of CTRL and PLG ASO-treated mice injected with PBS or LPS (20X magnification). PBS-injected PLG ASO images are not shown because they are indistinguishable from those of PBS-injected CTRL ASO. CD11b, GFAP, and CD68 expression levels are increased significantly in LPS-treated animals compared to PBS-treated animals following CTRL ASO injection ($p < 0.001$ for all comparisons). CD11b ($p < 0.01$), GFAP ($p < 0.001$), and CD68 ($p < 0.001$) are decreased significantly in LPS injected plasminogen-deficient animals compared to LPS-injected CTRL ASO mice. Scale bar = 100 µm. n = 4 sections/brain, 5-7 mice/group.

5.2 Plasma plasminogen depletion decreases perivascular macrophage migration to the brain during systemic LPS infection.

We analyzed whether a difference in immune response between CTRL ASO and PLG ASO animals treated with LPS could be attributed to a decrease in PVM migration in plasminogen-deficient animals. Therefore, we examined the expression of CD206, a marker of PVMs, around brain blood vessels of CTRL or PLG ASO animals injected with either LPS or PBS (Figure 5.3). PVM staining was observed in major vessels of the brain that contain a perivascular space, and PVM accumulation in these spaces increased dramatically with LPS injection. Brains of LPS-injected PLG ASO mice had significantly reduced CD206 expression levels compared to that of LPS-injected CTRL ASO animals, similar to the expression level found in PBS-injected CTRL ASO mice (Figure 5.3A). The lack of systemic plasminogen in PLG ASO mice may function by inhibiting the migration of PVMs into the brain during a systemic challenge with LPS.

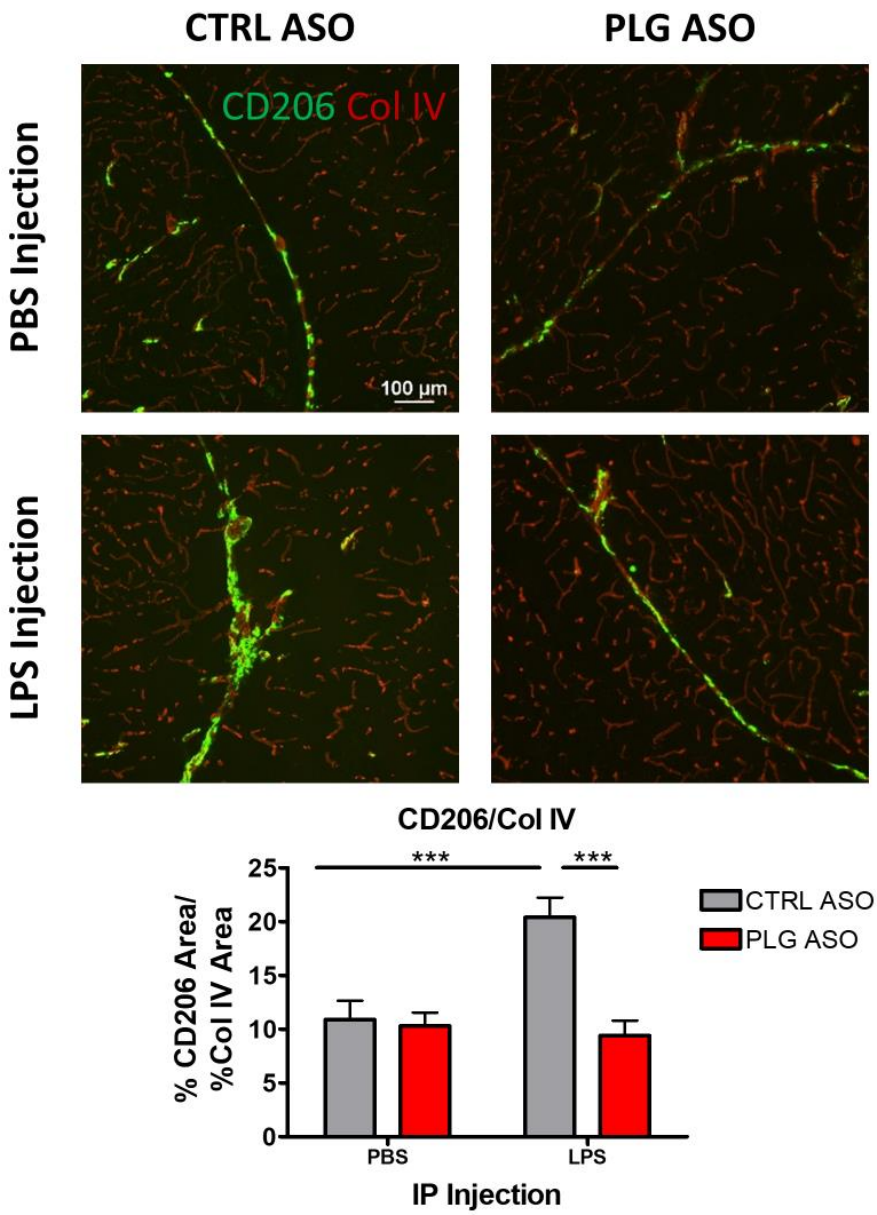


Figure 5.3. PVM accumulation in the brain increases with LPS challenge and is mediated by plasma plasminogen. Representative images and quantification of PVMs (CD206, green) in blood vessels of the brain (Col IV, red) in CTRL or PLG ASO-treated mice challenged with LPS or PBS. CD206 staining increases with LPS injection ($p < 0.001$) in CTRL ASO mouse brains, but returns to control levels in plasminogen-deficient mice challenged with LPS ($p < 0.001$). Scale bar = 100 µm. n = 3 sections/brain, 5-7 mice/group.

5.3 Perivascular macrophage migration is affected in response to LPS prior to microglial activation.

Following chronic LPS injection across 3 days, both PVM and microglial activation levels are elevated compared to controls (Figures 5.2, 5.3), but which response occurs first is unclear. In order to investigate the timing of effects on both PVMs and microglia and response to

LPS, we injected mice with LPS and perfused the animals at various time points following LPS injection (4 hours, 12 hours, or 12 hours). We stained brains from animals treated with LPS or PBS for CD206 (PVMs) and CD68 (microglia) and saw that within 4 hours of LPS injection, PVM levels are increased compared to controls (Figure 5.4A). By 12 hours and 24 hours-post-injection, PVM levels drop so that they are no longer significantly different from saline injected controls at those time points (Figure 5.4A). Conversely, CD68 levels in LPS-treated animals remain similar to controls at 4 hours and 12 hours-post-injection, but greatly elevate relative to controls 24 hours after injection (Figure 5.4B). Taken together, this data suggests that systemic LPS affects PVM-migration into the major vessels of the brain prior to microglial activation.

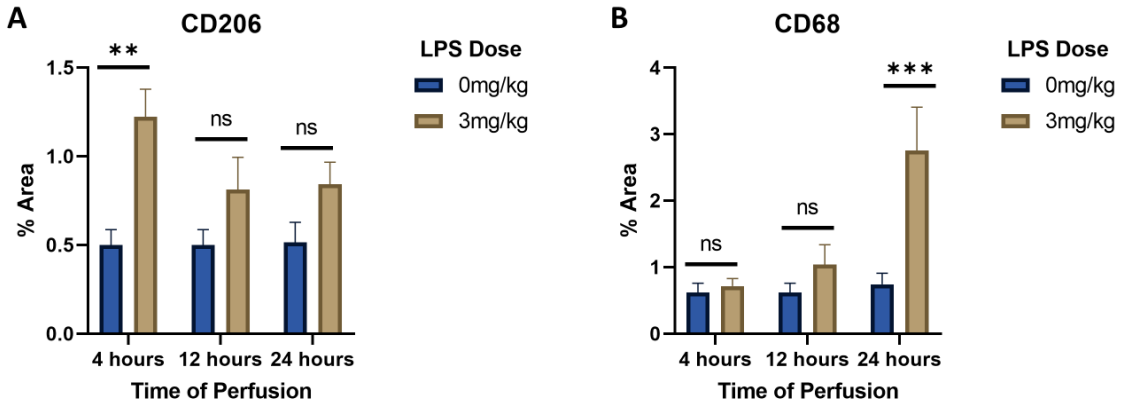


Figure 5.4. Perivascular macrophage migration is affected prior to microglial activation in response to systemic LPS. (A) CD206 staining increases compared to controls 4 hours after LPS injection ($p = 0.0014$) but then drops again within 12 hours of LPS injection. (B) CD68 levels remain similar to controls within the first 12 hours of LPS injection, but are elevated compared to controls 24 hours after injection with LPS ($p = 0.0003$).

These studies indicate that PLG plays a crucial role in regulating the neuroimmune response to a peripheral immune challenge with LPS by aiding in migration of PVMs into the brain during LPS challenge.

CHAPTER 6. P11 (S100A10) IS A PLASMINOGEN RECEPTOR THAT IS PARTIALLY RESPONSIBLE FOR A NEUROIMMUNE RESPONSE IN THE BRAIN DURING SYSTEMIC IMMUNE CHALLENGE WITH LIPOPOLYSACCHARIDE

Although plasminogen depletion leads to insights into the role of plasminogen in modulating communication between peripheral and central inflammatory signals, plasminogen itself is not a viable therapeutic target due to its pleotropic roles in physiological functions within the body. For this reason, it would be useful to identify a receptor specific to plasminogen-mediated perivascular macrophage migration into the brain in response to inflammatory stimuli. Several plasminogen receptors have been reported as chemotactic receptors for plasmin-mediated monocytic cell migration, including H2B, α-enolase, annexin 2, p11 (S100A10), and PLG-R_{KT} (Das et al., 2007, Das et al., 2009, Andronicos et al., 2010, Miles et al., 2014). In models of thioglycollate induced peritonitis, genetic p11 and PLG-R_{KT} depletion led to the greatest reduction in macrophage migration into the peritoneal cavity, with 50% and 80%

reduction in macrophages migrating into the peritoneum, respectively (O'Connell et al., 2010, Andronicos et al., 2010).

P11 binds with annexin A2 to form a heterotetramic complex made up of a p11 dimer connected to two annexin A2 molecules on the outside of the complex (Godier and Hunt, 2013). The p11-annexin A2 complex has been shown to bind both tPA and plasminogen (Miller et al., 2017). Annexin A2 binds to the cell membrane, whereas p11 has a carboxyl-terminal lysine reside that binds plasminogen (Godier and Hunt, 2013) as shown in Figure 6.1. The carboxyl-terminal lysine is not the only lysine that can bind plasminogen and as tPA-mediated activation of plasminogen occurs on the cell surface, plasminogen binding to other lysines can accelerate degradation via this pathway (Miller et al., 2017).

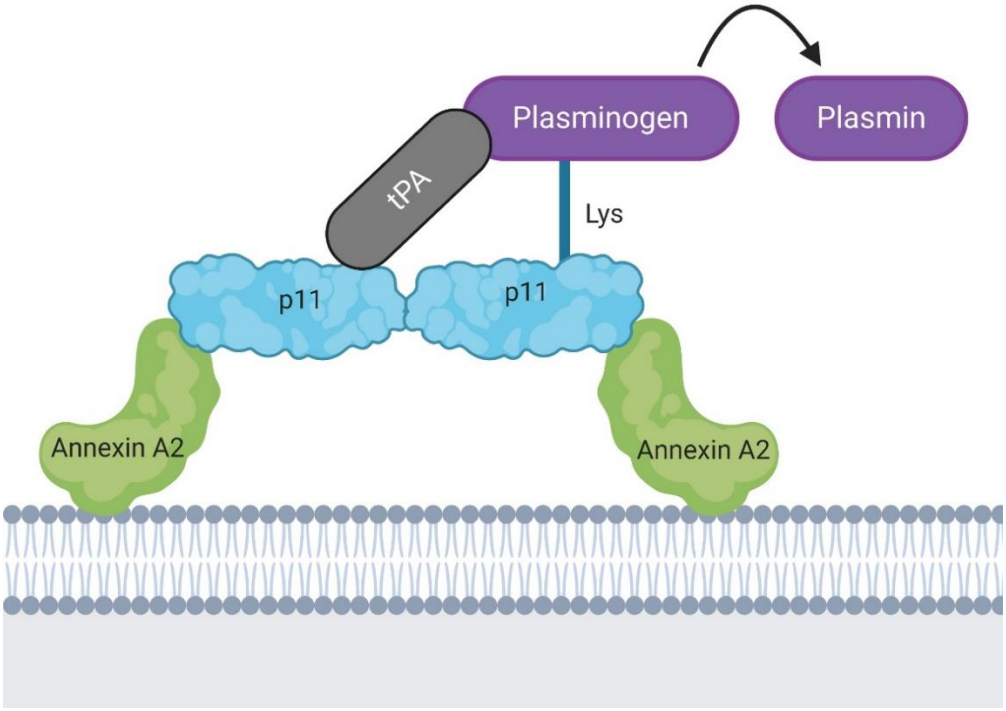


Figure 6.1. Binding of plasminogen to the p11-annexin A2 complex. Annexin A2 binds to the cell membrane surface and forms a heterotetramer with a dimer of p11 on the inside of the complex. P11 binds to plasminogen via its lysine terminal and also to tPA which leads to plasmin generation.

We sought to explore whether genetic depletion of p11 would limiting perivascular macrophage migration and glial cell activation in mice injected systemically with LPS, similar to the effects seen with depletion of peripheral plasminogen.

6.1 P11 deficiency is associated with a decreased microglial and astrocytic cell responses to systemic LPS challenge.

We injected *p11*$^{-/-}$, *p11*$^{+/-}$, and *p11*$^{+/+}$ mice with LPS on 3 consecutive days prior to sacrifice. When exposed to systemic LPS on 3 consecutive days, mice showed a global increase in CD68 (Figure 6.2) and GFAP (Figure 6.3) compared to PBS injected mice and these responses were attenuated in the absence of p11.

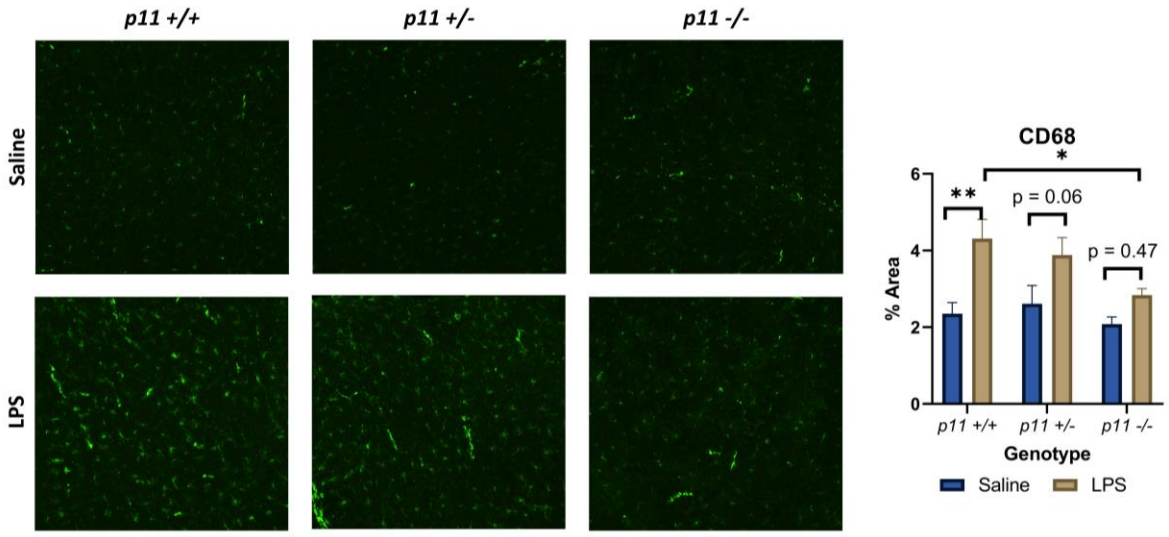


Figure 6.2. Microglia/macrophage activation is reduced in the brains of *p11*$^{-/-}$ mice following injection with LPS. Representative images and quantification of CD68 staining in saline or LPS-injected p11 knockout, partial knockout, or wild type mice. In wild type (*p11*$^{+/+}$) mice, LPS injection induces an increase in CD68 staining in the cortex of animals compared to PBS controls of the same genotype ($p = 0.0014$). Partial knockouts (*p11*$^{+/-}$) have a slight increase in CD68 levels compared to PBS-injected controls of the same genotype ($p = 0.06$). LPS-injected p11 knockout mice (*p11*$^{-/-}$) have no significant increase in CD68 levels compared to PBS injected p11 knockout mice ($p = 0.47$). Wild type mice injected with LPS have significantly more CD68 expression than full knockouts injected with LPS ($p = 0.03$). n = 4 sections/brain, 5-7 mice/group.

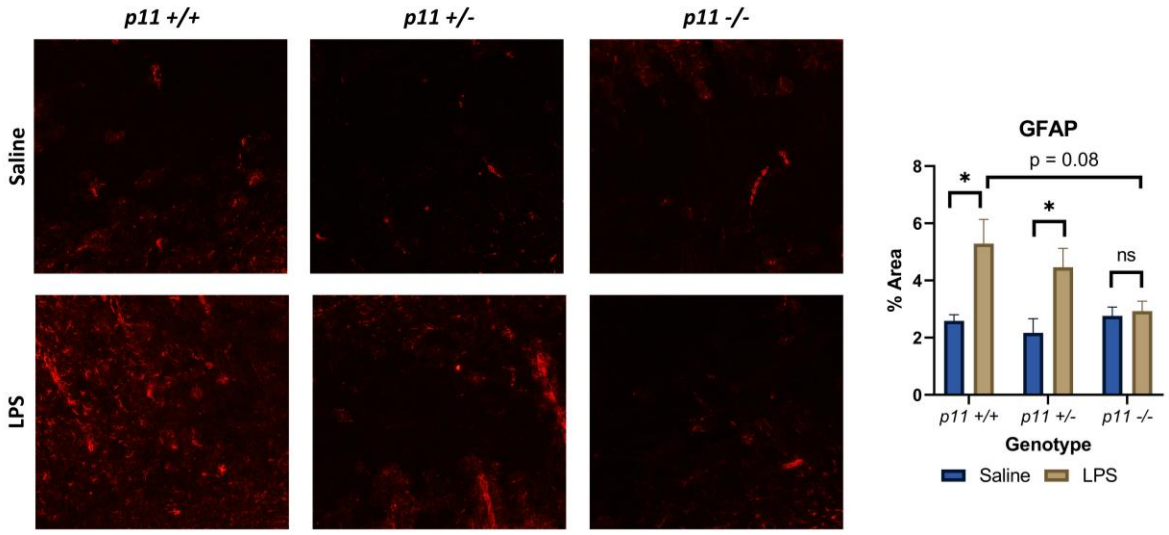


Figure 6.3. Astrocyte activation is reduced in the brains of *p11*$^{-/-}$ mice following injection with LPS. Representative images and quantification of CD68 staining in saline or LPS-injected p11 knockout, partial knockout, or wild type mice. In wild type (*p11*$^{+/+}$) mice, LPS injection induces an increase in GFAP staining in the cortex of animals compared to PBS controls of the same genotype ($p = 0.018$). Partial knockouts (*p11*$^{+/-}$) have a slight increase in CD68 levels compared to PBS-injected controls of the same genotype ($p = 0.0384$). LPS-injected p11 knockout mice (*p11*$^{-/-}$) have no significant increase in CD68 levels compared to PBS injected p11 knockout mice ($p > 0.999$). n = 4 sections/brain, 5-7 mice/group.

6.2 P11 deficiency leads to decreased perivascular macrophage migration to the brain during systemic LPS infection.

We analyzed whether a difference in immune response in animals with varying levels of p11 could be attributed to a decrease in PVM into their brains with p11 deficiency. We examined the expression of CD206, a marker of PVMs, around brain blood vessels of *p11*$^{+/+}$, *p11*$^{+/-}$, or *p11*$^{-/-}$ animals injected with either LPS or PBS (Figure 6.4). Mice with full levels of p11 had an increase in CD206 expression in the brain in response to LPS injection and this response was mediated by p11, as LPS-injected mice with partial p11 levels or no p11 expression had reduced differences in CD206 expression compared to PBS controls.

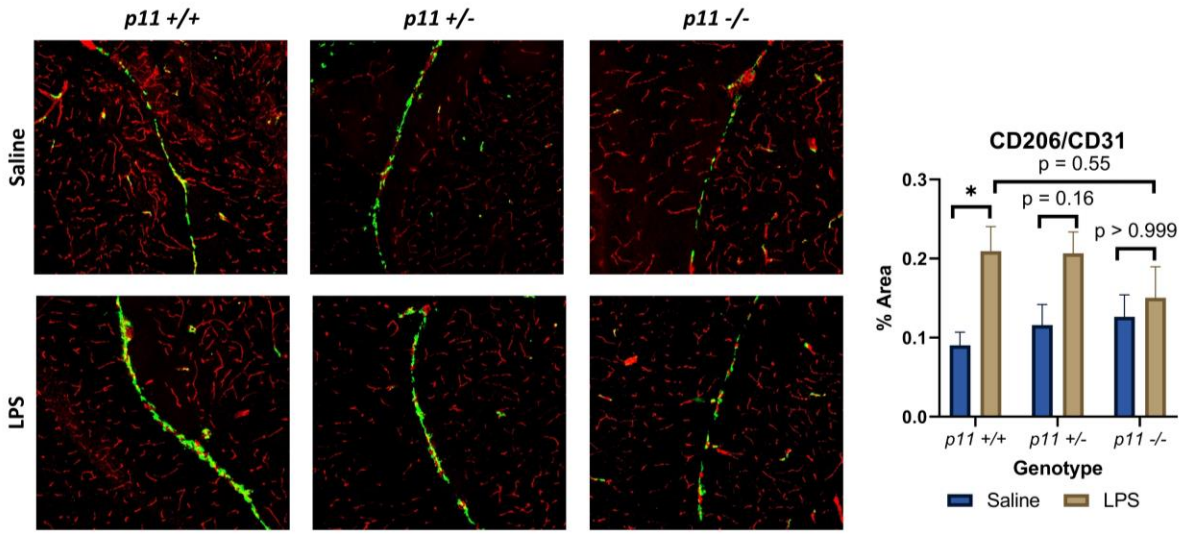


Figure 6.4. PVM accumulation is mediated by p11. Representative images and quantification of PVMs (CD206, green) in blood vessels of the brain (CD31, red) in $p11^{+/+}$, $p11^{+/-}$, or $p11^{-/-}$ mice challenged with LPS or PBS. CD206/CD31 staining increases with LPS injection (p = 0.049) in WT ($p11^{+/+}$) mouse brains, but is not statistically increased (p = 0.16) from PBS-injected mouse levels in partial knockouts ($p11^{+/-}$). PVM level completely returns to PBS-injected animal level with full p11 deficiency ($p11^{-/-}$). n = 4 sections/brain, 5-7 mice/group.

6.3 P11 expression in the brain is widespread and is found on neurons, endothelial cells, and perivascular macrophages.

p11 is expressed widely throughout the body and has been detected in a variety of cells including circulating monocytes/macrophages, endothelial cells in blood vessel walls, reactive astrocytes, splenocytes, fibroblasts, and tumor cells. Profiling of p11 in the brain indicated that this protein is expressed in some subsets of neurons in the cortex and hippocampus, as well as in several nonneuronal cells, including glia, choroid plexus cells, ependymal cells, endothelial cells, and meningeal cells (Milosevic et al., 2017). Although the authors who profiled p11 in the brain did not specifically look at p11 expression in PVMs, images published in Milosevic et al. (2017) indicate that p11 is likely expressed in PVMs. To confirm, we conducted staining of p11 with CD68 in $p11^{+/+}$and $p11^{-/-}$ animals (Figure 6.5).

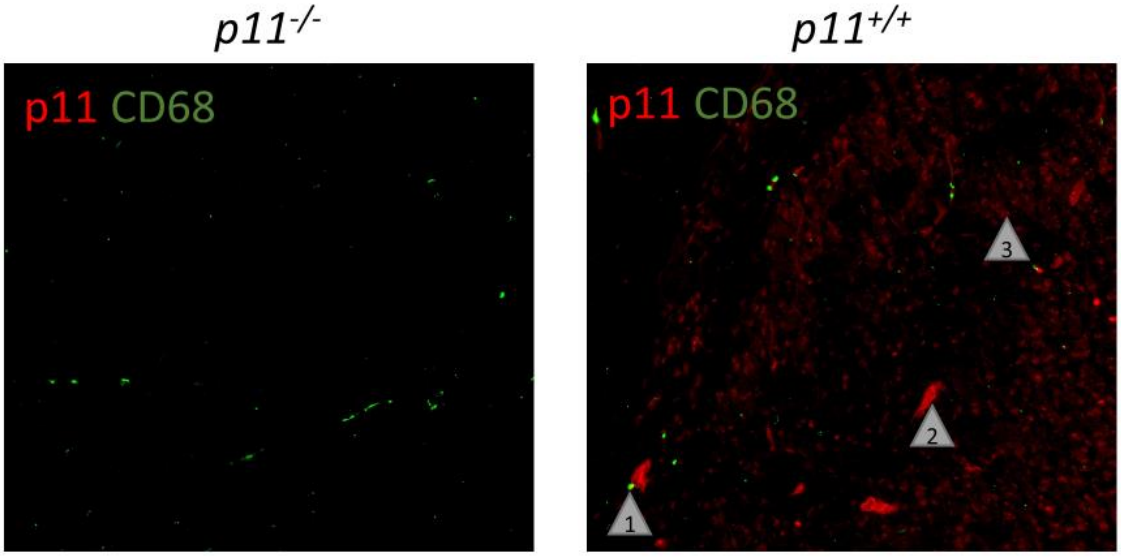


Figure 6.5. p11 is expressed on a variety of cell types in the brain including perivascular macrophages, endothelial cells, and neurons. Representative images of p11 and CD68 expression in the brains of *p11*$^{+/+}$and *p11*$^{-/-}$ mice. As expected, there is no staining of p11 in the brains of *p11*$^{-/-}$ mice. In the brains of *p11*$^{+/+}$ mice and labelled with the gray arrows, p11 is expressed on 1) CD68+ cells of the vasculature, likely PVMs, 2) along blood vessels, likely endothelial cells, and 3) in neurons.

We conclude that p11 is expressed widely and the primary expression in the brain is not primarily on PVMs. Furthermore, brain-produced p11 has functions in membrane trafficking, vesicle secretion, and endocytosis, and has been implicated in major depressive disorder (Milosevic et al., 2017). Although this receptor does play a role in the plasminogen-mediated neuroimmune response to peripheral LPS injection, it is not specific. For these reasons, this receptor is not optimal to specifically target PVM migration into the brain in response to systemic inflammatory stimuli.

6.4 Monocytic cells collected from the blood and peritoneal cavity of mice, as well as perivascular macrophages, express PLG-R$_{KT}$, a plasminogen receptor important for chemotaxis.

PLG-R$_{KT}$ has gained much recent attention due to the fact that it may be more specialized as a plasminogen receptor specific to chemotactic cell migration and it is highly expressed on proinflammatory macrophages (Miles et al., 2014, Flick and Bugge, 2017). In addition, *Plg-R$_{KT}$*-KO mice do not show any of the phenotypes observed in *Plg*-KO mice, such as rectal prolapse or ligneous conjunctivitis (Flick and Bugge, 2017). We examined the expression of PLG-R$_{KT}$, the purported receptor responsible for plasminogen-mediated cell migration, and found that it is highly expressed in leukocytes circulating in the blood (Figure 6.6A), IP macrophages (Figure 6.6B), and perivascular macrophages (Figure 6.6C). PLG-R$_{KT}$ is expressed on all of these cell types, and because it is highly expressed on PVMs, it may be a receptor worth studying and targeting for its role in plasminogen-mediated cell migration of PVMs.

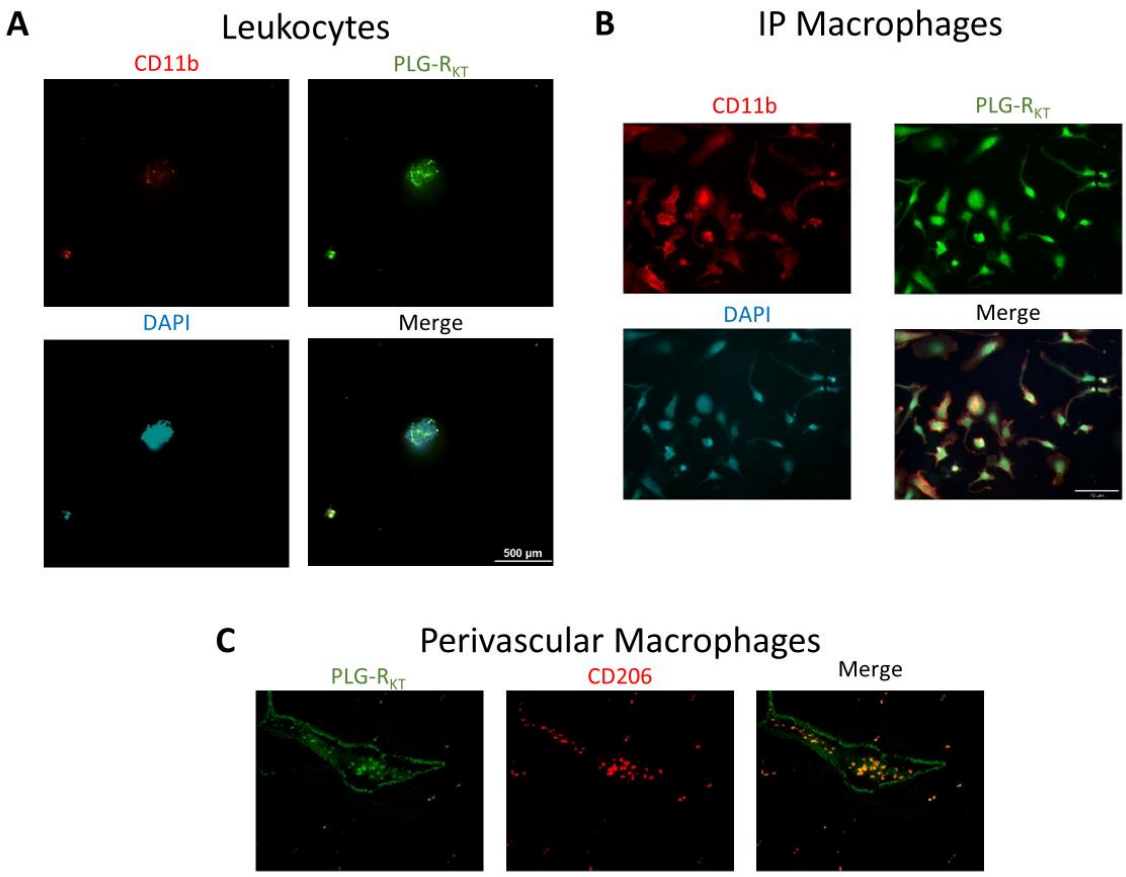


Figure 6.6. PLG-R$_{KT}$, a purported receptor responsible for plasminogen-mediated chemotactic migration is expressed on leukocytes, intraperitoneal macrophages, and perivascular macrophages in mice. (A) PLG-R$_{KT}$ (green) is expressed on leukocytes (CD11b+ cells, red) in the blood, (B) on IP macrophages (CD11b+ cells, red) isolated from the peritoneal cavity, and (C) on perivascular macrophages (CD206+ cells, red) in the brains of mice.

CHAPTER 7: DISCUSSION

7.1 The Role of Plasminogen in Alzheimer's Disease Progression

In the first part of my thesis work, I focused on the role that peripheral plasminogen may play in regulating neuroinflammation and AD pathology in a genetic model of AD (Baker et al., 2018). Although the mechanisms behind the progression of AD pathology remain poorly understood, developing evidence supports contributions of the neuroinflammatory response as one driver of the disease (Hong et al., 2016, Heneka et al., 2015a). Glial cells, including microglia and astrocytes, are part of the brain's innate immune system. Microglia are able to clear soluble and some aggregated forms of Aβ through phagocytosis (Mandrekar-Colucci and Landreth, 2010, Paresce et al., 1996). However, these glial cells can also become chronically activated by misfolded and aggregated proteins, such as Aβ. Furthermore, Aβ can bind to receptors on glial cells, inducing an inflammatory response, including the release of proinflammatory cytokines such as interferon γ, tumor necrosis factor α, IL-1β, and IL-6 (Heneka et al., 2015a). Inflammatory cytokines and activated microglia are key pathological features of the AD patient brain (Lee et al., 2010), and chronic use of anti-inflammatory drugs can delay or limit the severity of AD (Combs et al., 2000). Specific mutations linked to sporadic occurrence of AD in genes such as TREM2 and APOE are related to the ability of microglial cells to clear Aβ, implicating the innate immune system in development of AD (Pimenova et al., 2017, Krasemann et al., 2017). In addition, many of the risk factors for AD have an inflammatory component, including obesity, traumatic brain injury, and systemic inflammation or infection (Glass et al., 2010).

The Aβ peptide can lead to an immune response through activation of pattern recognition receptors on glial cells and neurons (Salminen et al., 2009), but the relationship between Aβ plaque formation and inflammation is bidirectional since molecules involved in the inflammatory response can also increase Aβ generation. Inflammatory mediators such as cytokines, chemokines, free radicals, nitric oxide, and complement system proteins are all known to lead to an upregulation of Aβ production and plaque deposition (Heneka et al., 2015a, Cai et al., 2014, Lee et al., 2008). Thus, there is a complex relationship between neuroinflammation and Aβ accumulation such that Aβ production, microglial activation, and subsequent cytokine release will lead to increased APP processing, Aβ deposition, and inflammation, all contributing to a vicious cycle.

Plasminogen and the fibrinolytic system proteins have diverse physiological functions, including a role in mediating the inflammatory response. Several *in vitro* and *in vivo* studies have demonstrated that plasminogen can contribute to inflammation through modulation of cell-signaling, as has been shown with monocytes, macrophages, dendritic cells, and other inflammatory cells (Syrovets et al., 2012). Plasminogen is a chemoattractant for monocytic cells (Syrovets et al., 1997) and also plays a role in gene expression changes crucial to phagocytosis by macrophages (Das et al., 2014). Inflammation is not observed in plasminogen-deficient mice when injected with collagen type II to induce autoimmune arthritis, unless mice are also supplied with intravenous injected plasminogen (Li et al., 2005). Plasminogen-knockout mice also show a compromised immune response in the brain following hippocampal injection of LPS (Hultman et al., 2014), an inflammatory agent used to activate microglia and pro-inflammatory cytokine expression. In addition, plasminogen deficiency in a mouse model of MS delays both the onset of MS symptoms and decreases neuroinflammation in these mice (Shaw et al., 2017). In the brain, it is possible that the uPA receptor plays a role in microglial activation and the

inflammatory response, and the uPA receptor is used as a microglial activation marker (Mehra et al., 2016).

Since most previous *in vivo* studies are knockout studies that eliminate plasmin as a result of eliminating its precursor plasminogen, it is challenging to distinguish between the role of plasmin and plasminogen in modulation of inflammation. In the present study, we show that plasmin is the major mediator of inflammation in an AD mouse model. Knocking down plasminogen using ASO technology reduces a neuroimmune response, whereas increasing the level of plasmin without affecting the plasminogen level through use of an A2AP ASO leads to an increased neuroimmune response. In this case, plasmin appears to be acting by way of a peripheral mechanism, as the ASO treatment targets liver production, and therefore plasma levels, of plasminogen and α_2-antiplasmin, without appearing to affect brain expression levels of these proteins.

AD pathology is a great example of a link between vascular inflammation and neuroinflammation, as both AD mouse models and human patients show not only neuroinflammation, but also systemic inflammation. The Aβ peptide can activate the proinflammatory contact system in plasma *in vitro* and *in vivo* (Maas et al., 2008, Zamolodchikov et al., 2015). The contact system is also activated in plasma and cerebral spinal fluid of AD patients (Zamolodchikov et al., 2015, Bergamaschini et al., 2001, Bergamaschini et al., 1998). Our lab showed that depletion of coagulation factor XII (FXII), the initiator of the intrinsic pathway of coagulation and contact activation, decreases brain pathology, inflammation, and cognitive impairment in an AD mouse model (Chen et al., 2017). Furthermore, FXIIa, FXIa, kallikrein, and bradykinin, key molecules in the contact pathway, are known activators of plasminogen to plasmin (Kluft et al., 1987, Colman, 1969, Mandle and Kaplan, 1979, Schousboe et al., 1999). Thus, if contact activation is increased, plasmin generation is also increased, which could propagate the inflammatory response. In addition, plasmin can activate FXII, leading to contact system activation and bradykinin release, or to thrombin generation through the coagulation pathway (de Maat et al., 2016, Ewald and Eisenberg, 1995). Bradykinin is a proinflammatory mediator of vascular permeability (Kaplan and Joseph, 2014), and thrombin also plays a proinflammatory role in the CNS through activation of protease activated receptors (Ebrahimi et al., 2017).

Fibrinogen deposition in the CNS is known to cause inflammation (Paul et al., 2007). Fibrinogen can enter the brain when there is BBB damage (Yamazaki and Kanekiyo, 2017, Ryu and McLarnon, 2009, Bardehle et al., 2015), and depleting plasminogen or plasmin in instances where there is increased fibrin deposition could be detrimental, as it could lead to increased inflammation in the brain, since fibrin would not be readily cleared by its main fibrinolytic agent (Paul et al., 2007). A previous study demonstrated this effect (Paul et al., 2007), showing that inhibition of plasminogen by tranexamic acid (TXA) leads to an increased inflammatory response. However, inhibition of plasmin activity using TXA does not influence inflammatory pathways, even though both plasmin generation and fibrin degradation are inhibited with this reagent (Renckens et al., 2004). This result may be because TXA can only inhibit free plasmin or fibrin-bound plasmin, but cannot successfully inhibit cell-bound plasmin, which is the major contributor to plasmin's modulation of inflammatory signaling (Syrovets et al., 2012, Syrovets and Simmet, 2004). Thus, while use of TXA may be an effective method to explore plasmin's effects on fibrinolysis and fibrin's contribution to an inflammatory response, this body of

literature may be confounded by the fact that plasmin has many cell-mediated functions, including regulation of inflammation.

Since our 5XFAD mice underwent PLG ASO treatment and sacrifice in the early stages of AD, there is no significant BBB damage or fibrin deposition in the brains of these animals. In addition, it would be interesting to study whether memory deficits in this AD mouse line are rescued by peripheral plasminogen depletion. We ended treatment and sacrificed animals before cognitive deficits were easily detectable by behavioral tests since chronic plasminogen depletion can lead to multiple pathologies (Bugge et al., 1995), and therefore were unable to evaluate whether memory improvement corresponded to pathological changes due to peripheral plasminogen depletion. Nonetheless, this treatment protocol and timing allowed us to distinguish between plasmin's inflammatory functions and fibrinolytic functions early in disease progression. In addition, plasminogen is still expressed in the brain with this ASO treatment as compared to a global plasminogen KO model, so it is likely that plasminogen continues to participate in fibrinolysis in the brain as needed.

Temporal profiling of gene expression in the 5XFAD mouse model of AD (Landel et al., 2014) gives further hints as to why this may be a useful model to study the effect of inflammatory modulation on AD pathology and progression. By studying gene expression in the cortex and hippocampus of 5XFAD mice at 1, 4, 6, and 9 months-of-age, it is clear that the immune and inflammatory processes are predominant in this mouse model. These mice begin to develop Aβ plaques and gliosis by 2 months-of-age, and between the first and fourth month of age there is a huge upregulation of inflammatory and immune markers in both the cortex and the hippocampus. Later, between 4 and 9 months-of-age, continuous microglial activation becomes the principal feature and increasing neuroinflammation is present, modulated by sustained complement system activation (Landel et al., 2014). It is likely that peripheral plasminogen depletion in these mice beginning early in disease (at 3 months-of-age) was successful in ameliorating the disease because inflammation was attenuated before this later phenotype had fully developed. To test this hypothesis, it would be interesting to explore whether plasminogen depletion in later disease stages would have the same effect.

Aβ plaque deposition was significantly decreased in plasminogen-depleted AD mice using ASO treatment, even though APP expression level in the brain was unchanged. Considering previous literature, it is unclear why Aβ plaque deposition was decreased with plasminogen depletion. There is some evidence that plasmin can enhance both α- and β-cleavage of APP (Ledesma et al., 2000), although this result has not been extensively studied. The plasminogen activator system is induced by Aβ (Tucker et al., 2000b), and plasmin can degrade oligomeric and fibrillar Aβ (Ledesma et al., 2000, Exley and Korchazhkina, 2001, Tucker et al., 2000b). In addition, tPA-activated plasmin can digest Aβ oligomers, inhibiting both its aggregation and neurotoxicity (Tucker et al., 2000a). While tPA and plasminogen decrease with age in an AD mouse model, the expression of both proteins is increased around Aβ plaques, indicating an attempt at tPA-mediated proteolysis of Aβ by plasmin (Bi Oh et al., 2015). Genetic deletion of PAI-1, the major inhibitor of tPA and uPA, in an AD mouse model led to increased tPA and plasminogen activity and decreased amyloid plaque deposition in these animals, further implicating plasmin in Aβ clearance (Liu et al., 2011). These studies suggest that plasmin may play a beneficial role in degrading Aβ and that depletion of plasmin would therefore lead to more persistent plaques. However, it is also important to consider that these studies have focused on the fibrinolytic function of plasminogen, without considering the role of plasminogen in

inflammation. We suggest here that it is important to understand that plasminogen in the brain may have a different function than in the periphery and also that there is a cyclic link between inflammation and Aβ generation. Plasminogen depletion limits the ability to mount a full inflammatory response, and therefore less Aβ may be generated since the activation cycle is dampened in the absence of this protein. In our model of peripheral plasminogen depletion, plasminogen and tPA levels are unaltered in the brain, and thus the plasminogen activator system in the brain may still have the ability to degrade Aβ.

While we have shown that depletion of plasminogen in the plasma affects neuroinflammation and brain pathology, the mechanism by which this plasma protein modulates inflammation in the brain is not yet understood. It is possible in our study that since plasminogen-depletion reduces perivascular macrophage presence in the cerebral vasculature, these mice have less cytokine or ROS signaling to, and thus less activation of, microglia. Decreased glial cell activation in the brain with plasma plasminogen depletion and increased glial cell activation with plasma α_2-antiplasmin depletion (increased plasmin activity) suggests that plasminogen is modulating the neuroinflammatory response via one of these peripheral mechanisms.

In addition, blood-derived plasminogen may only be a crucial modulator of CNS inflammation when peripheral inflammation plays a role. Although depletion of plasminogen early in life when genetic upregulation of Aβ production is induced in an AD mouse model has a strong effect on neuroinflammatory and AD pathology, this is not the case when there is a direct insult to the brain, such as local injection of Aβ or LPS into the cortex or hippocampus. In our intracerebral injection experiments, the same amount of Aβ remained deposited in the brain in both control and plasminogen-deficient animals, upregulating microglial similarly. In animals where LPS was injected directly into the brain, plasminogen-deficiency led to more microglial activation than in control animals. This seemed to be driven by fibrinogen deposition in the brains of these animals; LPS is a potent activator of BBB breakdown, allowing fibrinogen to leak into the parenchyma. Decreased plasminogen levels reduced ability to clear fibrinogen, a potent proinflammatory microglial activator. Therefore, plasminogen has different effects on inflammation depending on the type of insult induced.

Finding a key regulator in the periphery that links vascular inflammation to the inflammation characteristic of the AD brain has potential for use as a therapeutic treatment for the disease. Plasminogen and the plasminogen activator system play a crucial role in response to vascular injury. However, in cases where chronic inflammation is contributing to disease pathogenesis, such as in AD, plasminogen may play a detrimental role due to its function in mediation of inflammatory action. Excessive activation of plasmin is seen in chronic inflammatory and autoimmune diseases, and plasminogen and tPA are found localized around Aβ plaques in AD, which would lead to an increased local inflammatory response. We have demonstrated a principle role for peripheral plasmin in modulation of the immune response to the Aβ peptide. The results of this study are further evidence that inflammation is a double-edged sword – this ancient system of battling pathogens is well-intentioned but can sometimes be a destructive driver of pathology.

7.2 The Role of Plasminogen in Regulating Communication between the Periphery and Central Nervous System in Response to a Systemic Inflammatory Stimulus

We were interested to explore whether plasminogen plays a role in mediating inflammatory signaling between the CNS and periphery in contexts outside of AD and showed that plasminogen plays a crucial role in regulating the neuroimmune response to a peripheral immune challenge with LPS (Baker et al., 2019). With the PLG ASO experiments in AD mice, we had previously showed that plasmin, the active serine protease form of plasminogen, is likely responsible for regulation of this activity, and showed that treatment with PLG ASO specifically knocks down plasma levels of plasminogen without affecting brain expression of various proteins in the plasminogen activator pathway (Baker et al., 2018). The LPS experiment adds evidence that plasmin is a key modulator of communication between the nervous system and the immune system.

Activation of the innate immune system is a crucial step for the body when battling pathogens. However, when this activation becomes chronic, it can have detrimental effects. Microglia are the brain's primary immune cells that are continually surveying their surroundings to release other inflammatory mediators or to phagocytose cells and protein aggregates (Janda et al., 2018). Chronic microglial activation has been linked to neurodegeneration and clinical manifestations of dementia (Lull and Block, 2010). Furthermore, many of the risk factors for cognitive decline are associated with systemic inflammation, including obesity, hypertension, infection, cerebral infarction, smoking, and diabetes (Cunningham, 2013, Holmes, 2013, Heneka et al., 2015a, Glass et al., 2010).

As a broad-spectrum protease, plasminogen has many physiological functions, and it is understood that plasminogen interacts with cell surfaces for many of these roles. Plasminogen, considered a proinflammatory cell activator, is a potent chemoattractant for monocytes, macrophages, and dendritic cells (Syrovets et al., 1997, Silva et al., 2019). There is also evidence that plasminogen plays a role in innate immunity by regulating macrophage phagocytosis (Das et al., 2014) and can affect inflammatory cell function through production of cytokines, ROS, and other inflammatory mediators (Miles et al., 2014). Previous work in our lab has shown that *Plg*$^{-/-}$ mice have a decreased neuroimmune response following hippocampal injection of LPS (Hultman et al., 2014). In this study, however, there was no way to distinguish between the role of brain- and liver-derived plasminogen in regulation of this response. We extended this study by using an ASO that specifically targets liver-produced plasminogen, and found that it is plasma plasminogen that is crucial to regulating this response. Furthermore, we found that systemic challenge with LPS is sufficient to drive a neuroinflammatory response, and direct injection into the brain is not required. This situation better recapitulates what happens physiologically in the body in response to pathogens.

Plasminogen receptors are distributed broadly on many types of cells, including monocytes, macrophages, endothelial cells, and platelets (Miles et al., 2014). The proteolytic functions of plasminogen allow it to degrade extracellular matrices and activate growth factors that aid in cell migration. Studies with *Plg*$^{-/-}$ mice have shown that monocytes and macrophages have an impaired ability to migrate to the peritoneum when thioglycollate, an inducer of neutrophils, granulocytes, monocytes, and lymphocytes, is injected (Ploplis et al., 1998). Furthermore, mice with increased plasmin activity due to knockout of α_2-antiplasmin, a plasmin inhibitor, have increased macrophage recruitment in the thioglycollate injection model (Gong et al., 2008).

Several receptors for plasminogen have been shown to play a role in the migration of monocytic cells in response to an inflammatory stimulus. H2B, α-enolase, annexin A2, and p11 have all been implicated in macrophage migration into the peritoneum following thioglycollate-induced peritonitis. These receptors have variable effects on macrophage migration, with 48%, 24%, and 53% less recruitment of macrophages into the peritoneal cavity when histone H2B, α-enolase, and p11, respectively, are reduced using a monoclonal antibody or genetic knockout (Das et al., 2007, Das et al., 2009, O'Connell et al., 2010, Andronicos et al., 2010).

In response to inflammatory stimuli, the p11-annexin A2 complex binds to plasminogen on macrophages and monocytes to induce plasmin generation and lead to matrix protein and ECM degradation. In addition, plasmin cleaves annexin A2 to induce intracellular signaling pathways involved in inflammation, such as MAP kinase phosphorylation, and activation of cytokines and chemokines. Together, plasminogen with p11-annexin A2 receptor mediates chemotactic migration of macrophages and pro-inflammatory cell recruitment (Godier and Hunt, 2013). The use of a thioglycollate-induced peritonitis model in p11 knockout mice showed that the p11-annexin A2 tetramer is an important mediator of a plasminogen-dependent inflammatory response in the peritoneum (O'Connell et al., 2010). Other studies have indicated that p11-annexin A2 mediates tumor-promoting macrophage migration, fibrinolysis, and ECM invasion in cancer (Phipps et al., 2011, Huang et al., 2017, Bydoun et al., 2018). Our results indicate that this complex is also involved in migration of PVMs in the neuroinflammatory response in an LPS-induced inflammation model.

We have shown that p11 plays a role in PVM migration in the brain in response to peripheral LPS injection and that a genetic knockout of p11 leads to decreases in PVM levels and microglia and astrocyte activation levels similar to peripheral depletion of plasminogen using an ASO. This effect is most striking in a homozygous p11 knockout, but wanes with a heterozygous knockout of p11, suggesting that partial levels of this receptor are enough to allow plasminogen to function to mediate inflammatory signals between the blood and the parenchyma. In addition, the effects are not as striking as with PLG ASO treatment, indicating that other plasminogen receptors are likely involved in plasminogen-mediated chemotaxis of PVMs. Different plasminogen receptors may be involved in plasminogen-mediated migration of immune cells during different steps of the inflammatory response or may be tissue or stimulus specific and it will be interesting to further explore other known plasminogen receptors to determine if they have a role in the LPS-induced neuroinflammation model.

Various studies linking plasminogen to immune cell migration led to a proteomics-based search for a migration-specific receptor for plasminogen, leading to the discovery and characterization of PLG-R_{KT} (Andronicos et al., 2010). A PLG-R_{KT} mAb inhibits invasion of monocytes *in vitro* into a matrigel in response to a chemotactic stimulus, MCP-1, and *in vivo* in a thioglycollate model (Lighvani et al., 2011), and this regulation of chemotactic migration appears specific to PLG-R_{KT} (Andronicos et al., 2010). We show that PLG-R_{KT} is expressed broadly on many types of immune cells in our mouse line, including IP macrophages, blood monocytes, and PVMs and thus may be an important receptor to target CNS inflammation induced by systemic inflammatory cues.

PVMs are replenished by bone marrow-derived cells (Kierdorf et al., 2013) and are phagocytic cells that have a close association to the cerebral vasculature (Faraco et al., 2017). Because of their location in the perivascular space of blood vessels in the brain, PVMs are in an

optimal location for interacting with circulating immune cells and cerebral endothelial cells and for communicating signals to the brain. There is also evidence that these cells may play a crucial role in maintaining homeostasis of the brain (Faraco et al., 2017).

Previous studies with LPS have shown that sickness behavior occurs due to proinflammatory cytokines acting on the brain (Hoogland et al., 2015). A recent study showed that bone marrow-derived PVMs play a crucial role in producing this proinflammatory effect in the brain; these cells release IL-1β as a response to LPS circulating in the blood in the subfornical area, one of the circumventricular organs that lacks a blood brain barrier (Morita-Takemura et al., 2019). Our work supports the idea that PVMs are crucial for this neuroimmune response to systemic LPS challenge, and also suggests that plasma plasminogen is crucial for migration of PVMs into the brain.

Although LPS is too large (50-100 kDa) to enter the brain parenchyma and directly activate microglia, PVMs exposed to LPS can penetrate the parenchyma and induce release of IL-1β and other cytokines, thereby leading to microglial activation and an astrocytic response. There is a full recovery of PVMs to PBS-treated CTRL ASO levels in mice treated with PLG ASO prior to LPS injection, but activation of microglia/macrophages and astrocytes is still increased compared to controls, indicating that PVMs are not fully responsible for the neuroimmune response to LPS. For example, LPS can act directly on endothelial cells to stimulate release of cytokines and other inflammatory mediators such as cyclooxygenase-2 into the parenchyma of the brain (Holmes, 2013).

In the context of p11-deficient mice, LPS's action on endothelial cells via the TLR-4 receptor may explain why p11-KO mice showed a significant decrease in microglia and astrocyte activation in response to LPS, despite only a modest effect on PVM recruitment differences. The annexin A2-p11 is expressed on endothelial cells where it assembles plasminogen and tPA to promote plasmin generation. Furthermore, plasmin phosphorylates annexin A2 and this requires TLR-4 activation on the cell surface (He et al., 2011). In our LPS model, TLR-4 is activated by circulating LPS which may lead to plasmin activation and downstream signaling to the glial cells of the parenchyma.

While it has been known that LPS causes activation of microglial cells, the mechanism remains elusive. Our current study increases evidence that PVMs likely play a crucial role in this response, and our temporal profiling of PVMs and activated microglia showed that PVMs are affected prior to microglia in response to LPS, adding support to this theory. However, endothelial signaling may also play an important role in neuroinflammatory cell activation in response to a peripheral inflammatory stimulus. A summary of our hypothesized mechanism of this effect is indicated in Figure 7.1.

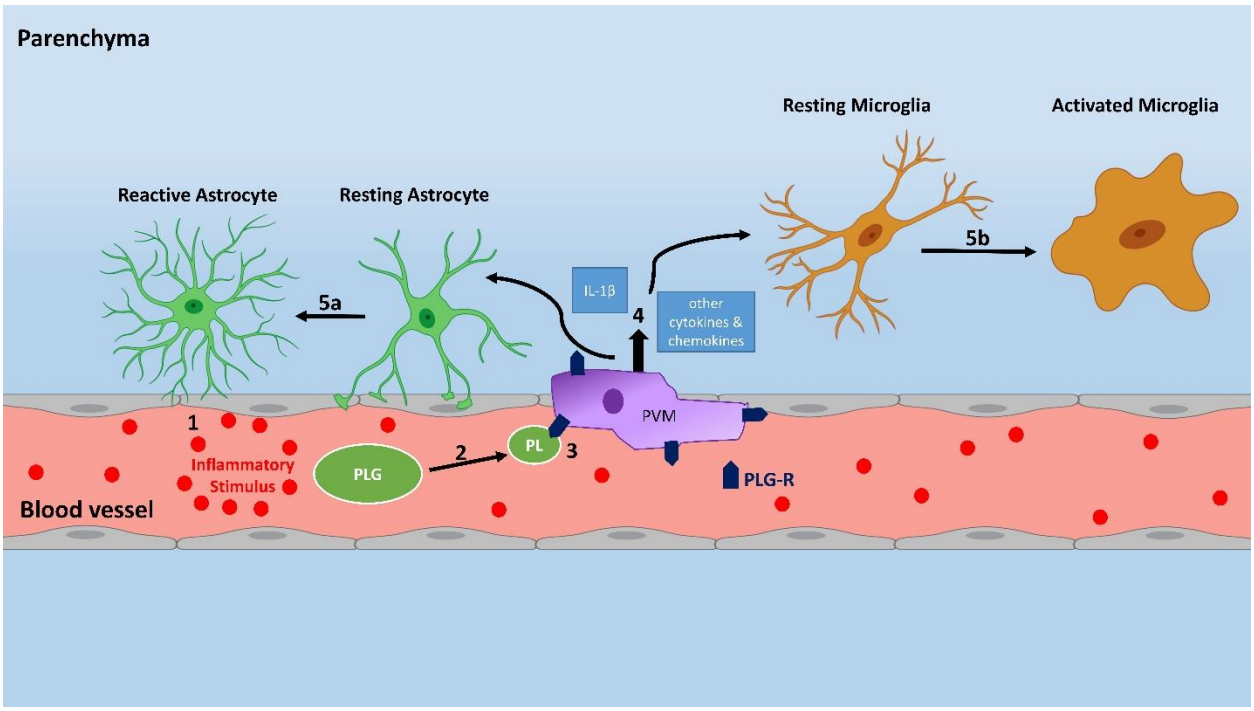


Figure 7.1. Proposed mechanism of how plasminogen mediates communication between systemic inflammatory cues and the central nervous system. (1) An inflammatory stimulus, such as LPS, gets into the blood stream of mice. (2) Inflammatory signals in the blood lead to plasminogen cleavage into plasmin. (3) Plasmin, which mediates macrophage migration, acts on plasminogen receptors on PVMs. (4) PVMs release IL-1β and other cytokines and inflammatory mediators, either directly or through endothelial cell signaling, into the parenchyma of the brain which (5a) causes astrocyte reactivity and (5b) activation of microglial cells.

Notably, our data also suggest that plasma plasminogen depletion may be an effective treatment for neuroinflammatory conditions. However, plasminogen deficiency is known to cause other disorders, such as conjunctivitis of the eye, woody deposits on the gums, and gingivitis, all of which are linked to the inability to clear fibrin (Miles et al., 2014). Because plasminogen has many physiological functions in the body, it may be challenging to just target those linked to chemotaxis and immunity, and thus it may not be the best therapeutic target for neuroinflammatory disease. It may be most effective to target PLG-R_{KT} or another plasminogen receptor to interfere with chemotaxis of immune cells like PVMs without affecting overall plasmin activity. The role plasminogen receptors such as PLG-R_{KT} may play in modulating neuroinflammation merits further studies to validate it as an important clinical target since it may be specific to the chemotactic functions of plasminogen.

7.3 Conclusions

Plasminogen, its activators, and its receptors comprise pathways that play roles in various inflammation regulatory processes. These roles span functions in fibrinolysis, interaction with complement proteins, extracellular matrix degradation, inflammatory cell migration, and resolution of inflammation and wound healing. As a consequence, plasminogen has been associated with many pathologies spanning different organ systems including the blood (infection, systemic inflammation, complement activation, cardiovascular disease), the brain (neuroinflammation, neurodegenerative disease), the joints (arthritis), the lungs (asthma), and the

skin (psoriasis). In addition, the plasminogen activator system is found to be differentially regulated in plasma or inflammatory lesions in a variety of these diseases (Baker and Strickland, 2020).

We reviewed the role of plasminogen in these diseases extensively in Baker and Strickland (2020). One common link between diseases across organ systems is that plasminogen seems to play a role in development of pathology in several autoimmune models. For example, in arthritis models where collagen immunization is used to induce joint inflammation, plasminogen is necessary for cell migration into the joints. In models of neurodegenerative disease, such as EAE where myelin immunization is used to model MS or AD mouse models where the Aβ protein is overexpressed in the brain, plasminogen is also necessary for full inflammatory cell migration and responses in the nervous system, contributing to the chronic inflammatory associated with neurodegenerative disease.

In models of peripheral disease, such as infection, arthritis, and acute myocardial infarction, fibrinogen-dependence was found to be a contributor to plasminogen's inflammatory functions (Berri et al., 2013, Silva et al., 2019, Raghu et al., 2014, De Nardo et al., 2010, DeFilippis et al., 2016). Studies using different models of arthritis give further insight into fibrin-dependence of plasminogen-mediated processes. In two different models of arthritis, systemic and acute, fibrin-dependence was only seen when arthritis was acutely-induced (Raghu et al., 2014). Thus, in acute processes, when fibrinolysis is necessary for wound healing, plasminogen may be protective against chronic inflammation. However, during chronic inflammatory insults and diseases, plasminogen may be detrimental because it allows for persistent migration of inflammatory cells and activation of inflammatory modulators.

In the central nervous system, fibrin-independence and fibrin-dependence are both reported for plasminogen's roles in neuroinflammatory disease (Tsirka et al., 1997b, Paul et al., 2007, Hultman et al., 2014, Shaw et al., 2017). These results may be confounded by the fact that plasminogen and its activators are produced endogenously in the brain, separate from liver-produced plasminogen. Conditional or tissue-specific knockouts of plasminogen and its activators may be necessary to further understand the specific roles of brain-plasminogen in neurological disease.

Plasminogen and related proteins may serve as targets for a wide variety of pathologies, all connected to their role in inflammatory regulation. My thesis works further implicates plasminogen as a key regulator of cross-talk between CNS inflammatory signals and peripheral inflammatory cues. However, the pleotropic functions of plasminogen also serve as a limitation to its use as a therapeutic target unless highly specific therapeutics are found that could alter one function of plasminogen without affecting another. Humans with plasminogen deficiency have well-characterized pathologies related to deposition of fibrous deposits on mucosal membranes throughout the body, limiting plasminogen's potential as a therapeutic target itself. Mice treated with PLG ASO long-term also showed significant failure to gain weight, indicating that they were not physically healthy.

Future work in the field of plasminogen and inflammation should focus on better defining the pathways that lead to inflammatory effects. Several receptors for plasminogen have been discovered that contribute to its inflammatory roles, such as PLG-R_{KT}, histone H2B, α-enolase, and the p1-annexin A2 complex. These receptors have all been shown to be involved in macrophage migration in a peritoneal thioglycollate model, with 49%, 48%, 24%, and 53% less

recruitment of macrophages into the peritoneal cavity when PLG-R_{KT}, histone H2B, α-enolase, and p11, respectively, are reduced using a monoclonal antibody or genetic knockout (Das et al., 2007, Das et al., 2009, O'Connell et al., 2010, Andronicos et al., 2010). I have preliminary results suggesting that p11 plays a role in plasminogen-mediated PVM migration into the brain in response to a systemic inflammatory stimulus. Attention to this receptor and others to determine their unique or combined roles in inflammatory processes may yield important insights into the inflammatory diseases in which plasminogen plays a role. In addition, it will be interesting to study each of these receptor's expression and ability to regulate perivascular macrophage migration in an LPS or AD mouse model. Specific modulation of these receptors has the potential to bypass the detrimental effects that targeting plasminogen itself would have on other functions within the body and warrants future study.

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