Blood-derived plasminogen drives brain inflammation and plaque deposition in a mouse model of Alzheimer's disease

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Two of the most predominant features of the Alzheimer's disease (AD) brain are deposition of β-amyloid (Aβ) plaques and inflammation. The mechanism behind these pathologies remains unknown, but there is evidence to suggest that inflammation may predate the deposition of Aβ. Furthermore, immune activation is increasingly being recognized as a major contributor to the pathogenesis of the disease, and disorders involving systemic inflammation, such as infection, aging, obesity, atherosclerosis, diabetes, and depression are risk factors for the development of AD. Plasminogen (PLG) is primarily a blood protein synthesized in the liver, which when cleaved into its active form, plasmin (PL), plays roles in fibrinolysis, wound healing, cell signaling, and inflammatory regulation. Here we show that PL in the blood is a regulator of brain inflammatory action and AD pathology. Depletion of PLG in the plasma of an AD mouse model through antisense oligonucleotide technology dramatically improved AD pathology and decreased glial cell activation in the brain, whereas an increase in PL activity through α-2-antiplasmin (A2AP) antisense oligonucleotide treatment exacerbated the brain's immune response and plaque deposition. These studies suggest a crucial role for peripheral PL in mediating neuroimmune cell activation and AD progression and could provide a link to systemic inflammatory risk factors that are known to be associated with AD development.

Alzheimer's disease | plasminogen | neuroinflammation

Alzheimer's disease (AD), classically identified by the presence of extracellular β-amyloid (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 (1). This neuroimmune response is activated to clear Aβ or pathogens from the brain and to tag impaired neurons for destruction (2). However, 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 (3). Thus, when chronically activated, the neuroimmune system, which is designed to be neuroprotective, becomes toxic by overloading the brain with inflammatory signals.

Plasmin (PL) is the primary enzyme that degrades fibrin clots. PL also plays a role in a variety of pathways, including cell signaling, cell adhesion, cancer metastasis, and wound healing (4). The fibrinolytic system is tightly regulated; PL is a serine protease formed through cleavage of its precursor, plasminogen (PLG), by tissue PLG activator (tPA) or urokinase PLG activator (uPA), and enzymatically degrades fibrin through hydrolysis (5). tPA and uPA activity is mainly controlled by PLG activator inhibitor 1 (PAI-1). PL can be inactivated through its main inhibitor, α-2-antiplasmin (A2AP), which regulates PL activity after PLG activation (6).

PL is also involved in the regulation of inflammatory events. PLG is a chemoattractant for macrophages, which play a key role in both increasing inflammation and inducing an immune response (7). Monocytes and monocytic cells have binding sites for PLG (8), and PL is a direct activator of these cells (9). When PL is depleted pharmacologically in a model of excessive immune activation, mice have higher survival rates and less inflammation, suggesting that PL plays a key role in production of inflammatory cytokines and chemokines (10). In cases where PL is generated in excess in inflamed tissues, PL is able to activate the classic complement cascade (11–13), which is involved in innate immunity, and this activation can lead to vasodilation and vascular permeability (14). In addition, studies using PLG-knockout mice have shown that PLG deficiency leads to the inability to mount a full immune response in the brain when injected with lipopolysaccharide (LPS) (15) and delays onset of multiple sclerosis (MS) and neuroinflammation (16). Previous studies looking at PLG function in vivo have used knockout mice, not allowing for the distinction between brain-produced PLG and PLG synthesized in other tissues.

Because of the recognized role of PLG and PL in regulating inflammation, we investigated the potential for PLG in modulating the neuroinflammatory activity associated with AD. We show that conditional depletion of PLG in peripheral blood, but not in the brain, is highly protective from Aβ deposition and a neuroinflammatory response in a 5XFAD (Tg6799) AD mouse model. Furthermore, activation of PL through depletion of A2AP in blood results in exacerbated neuroinflammatory activation and AD pathology, suggesting that PL, and not its precursor PLG, is a main peripheral modulator of neuroinflammation in this AD mouse model. We show that PLG in the periphery modulates the neuroinflammation and Aβ deposition characteristic of AD.

Significance

We demonstrate that depletion of blood plasminogen is sufficient to protect against both innate immune cell activation in the brain and Alzheimer's disease (AD) pathology in a mouse model of AD. This work provides a molecular mechanism for initiation of AD-related brain inflammation and for regulation of β-amyloid deposition, and could lead to therapeutic strategies in human AD patients, including the targeting of systemic molecules.

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Results

The PLG System Is Altered in the Peripheral Blood of PLG Antisense Oligonucleotide-Treated Mice, but Not in the Brain. We used an antisense oligonucleotide (ASO)-mediated gene knockdown strategy to deplete PLG in AD and WT mice. To analyze the extent of PLG depletion, we collected plasma after 2, 4, and 10 wk of PLG ASO injections. Plasma PLG was efficiently knocked-down by 90–95% from control (CTRL) ASO-treated plasma levels throughout the course of treatment in both AD and WT animals (plasma levels after 10 wk of treatment are shown in Fig. 1 *A* and *B*). Plasma PLG was not significantly different between WT CTRL ASO- and AD CTRL ASO-treated animals (Fig. 1 *A* and *B*). tPA and PAI-expression levels were examined in the plasma after PLG ASO treatment and were found to be unchanged compared with controls (Fig. 1 *A*, *C*, and *D*). We also analyzed levels of PLG, 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 PLG system activator proteins was not altered in response to PLG ASO treatment (Fig. 1 *E*–*H*). Therefore, although PLG ASO is effective at specifically reducing PLG levels in the blood of these mice, plasma tPA and PAI-1 are unaltered in response to PLG depletion, and brain levels of PLG, tPA, and PAI-1 are unaffected by ASO administration.

Depletion of Plasma PLG Reduces the Brain's Innate Immune Response in AD Mice. We analyzed whether plasma PLG, 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 (Fig. 2) and hippocampus (*SI Appendix*, Fig. S1). In WT mice, the expression levels of CD11b (Fig. 2 *A* and *B*) and GFAP (Fig. 2 *C* and *D*) were similar

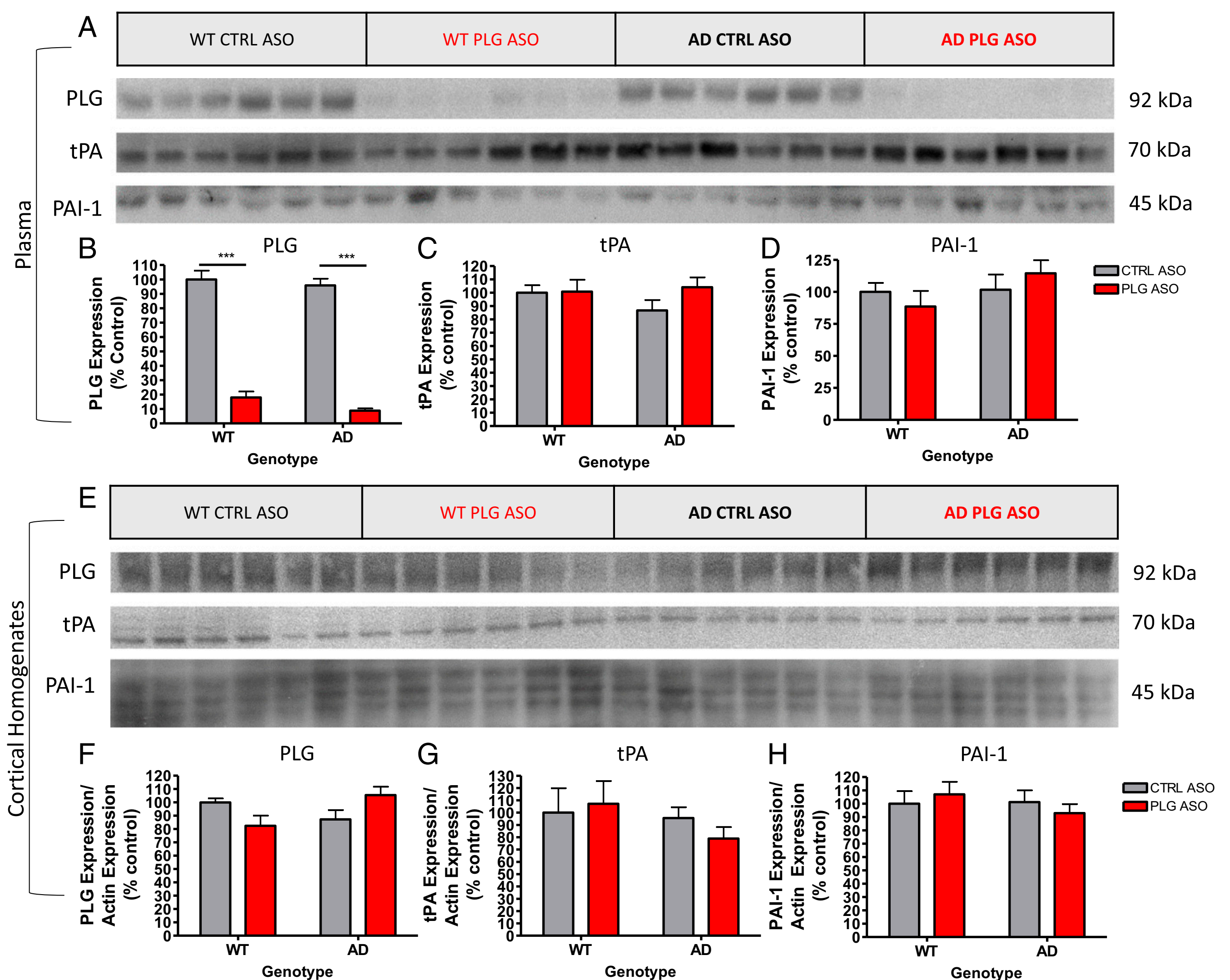

Fig. 1. PLG levels, but not all components of the PLG activator system, are depleted in the plasma of ASO-treated mice, but are unaffected in the brain. (*A*) Representative Western blots of PLG, tPA, and PAI-1 levels in the plasma of WT CTRL ASO, WT PLG ASO, AD CTRL ASO, and AD PLG ASO mouse groups. (*B*) PLG levels decrease in plasma of PLG ASO-treated AD and WT mice compared with 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 ($P = 0.95$). (*E*) Representative Western blots of PLG, 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 PLG ($P = 0.42$ 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 three independent experiments. ***$P < 0.0001$.

after CTRL ASO and PLG ASO treatment. In AD mice treated with CTRL ASO, both CD11b and GFAP levels were increased in the cortex (Fig. 2 *B* and *D*) and hippocampus (*SI Appendix*, Fig. S1 *B* and *D*) compared with WT mice, but levels of microglial/macrophage activation are decreased to WT levels in both the hippocampus and cortex in AD animals treated with PLG ASO (Fig. 2 *A* and *B* and *SI Appendix*, Fig. S1 *A* and *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 (Fig. 2 *C* and *D* and *SI Appendix*, Fig. S1 *C* and *D*). This decrease in inflammatory activity in PLG-depleted AD animals further corresponds with a two-thirds reduction in 6E10 staining, indicating reduced Aβ deposition (Fig. 2 *E* and *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 (Fig. 2 *B*, *D*, and *F*) than the hippocampus (*SI Appendix*, Fig. S1 *B*, *D*, and *F*).

Depletion of Plasma PLG Reduces AD Pathology in AD Mice. AD pathology includes neuronal degeneration, plaque deposition, and increased autophagy in the brain. To investigate whether decreased neuroinflammatory activation was associated with less AD pathology, we examined neuronal staining (NeuN), fibrillar Aβ plaque deposition (Congo red), and lysosome presence

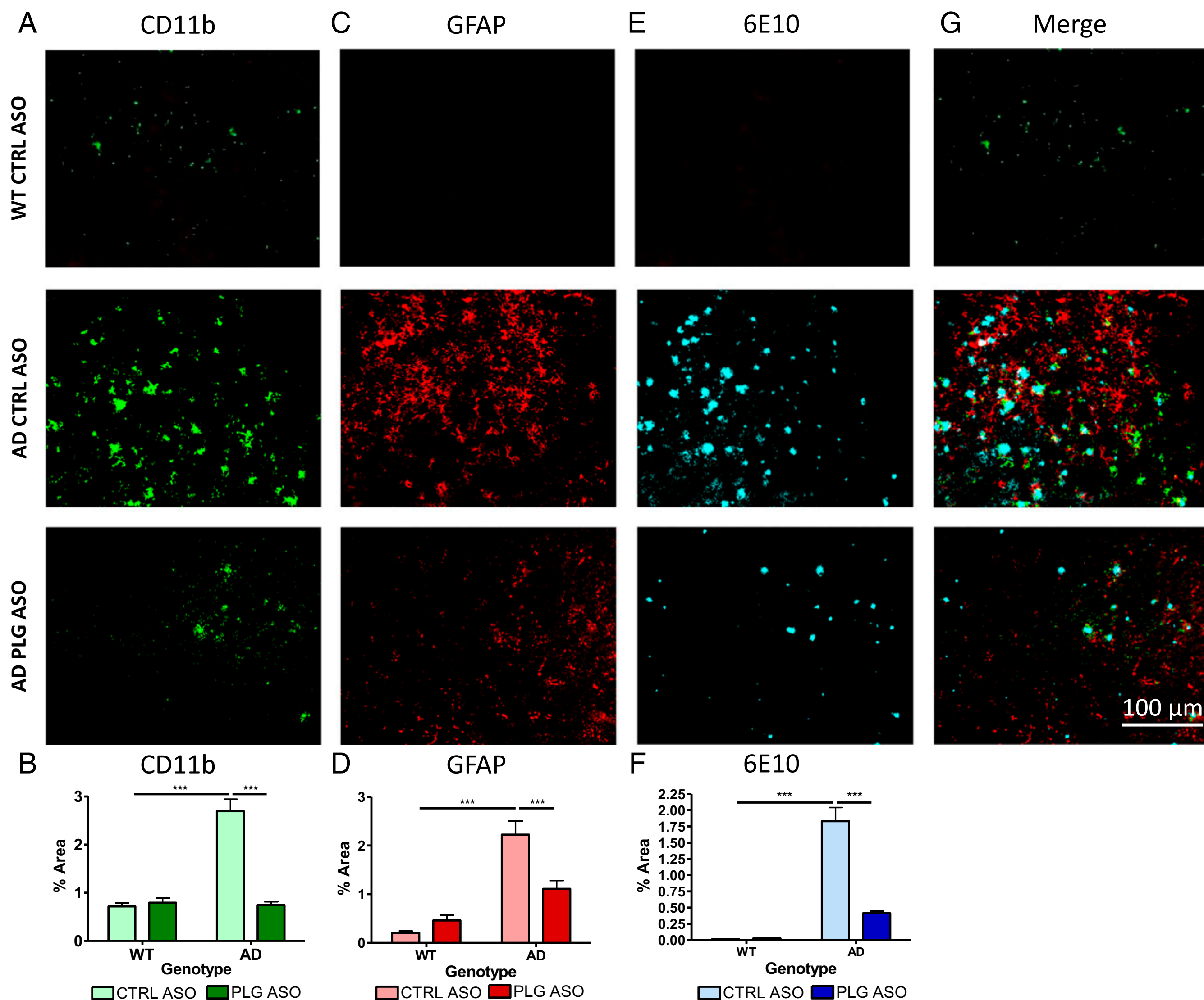

Fig. 2. Microglia/macrophage activation, astrocyte activation, and plaque load are reduced in the cortex of PLG ASO-treated AD mice compared with CTRL ASO-treated AD mice. (*A*) Representative images of CD11b staining in the cortex of WT CTRL ASO-treated mice and AD mice with CTRL or PLG ASO treatment. (*B*) Microglial/macrophage activation in the cortex is significantly increased in AD CTRL ASO-treated animals compared with WT groups ($P < 0.0001$). CD11b staining is significantly reduced in AD PLG ASO-treated animals compared with 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 with WT CTRL ASO mice ($P < 0.0001$). However, GFAP staining is significantly reduced in AD PLG ASO-treated animals compared with 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 with CTRL ASO-treated AD animals ($P < 0.0001$). (*G*) 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 three independent experiments. ***$P < 0.0001$.

(LAMP-1) in the cortex of AD animals treated with CTRL or PLG ASO. NeuN staining was increased in PLG ASO-treated AD animals compared with CTRL ASO-treated AD mice (Fig. 3 *A* and *B*), suggesting reduced neuronal damage with PLG depletion. In addition, PLG ASO reduced fibrillar plaque deposition (Fig. 3 *C* and *D*), 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 amyloid precursor protein (APP) (shown in Fig. 2*E*), Congo red specifically detects fibrillar amyloid protein. Furthermore, LAMP-1 staining was increased in AD animals with PLG ASO treatment, indicating these animals had less autophagy through lysosome recruitment in the brain (Fig. 3 *E* and *F*). Together, these results suggest that depletion of PLG in the plasma not only reduced neuroinflammation, but also improved AD pathology in these mice. Although insoluble Aβ plaque deposition is decreased in PLG ASO-treated AD mice, APP expression in the brain is unaffected (*SI Appendix*, Fig. S2), indicating that plaque deposition differences may be a result of differential APP processing or clearance of Aβ.

A2AP ASO Treatment Effectively Depletes the Protein in the Plasma, but Does Not Alter Other Plasma PLG System Proteins. Because we had evidence that plasma PLG contributed to the neuroinflammatory response in AD, we explored whether this inflammatory action was due to PLG's cleavage into the active

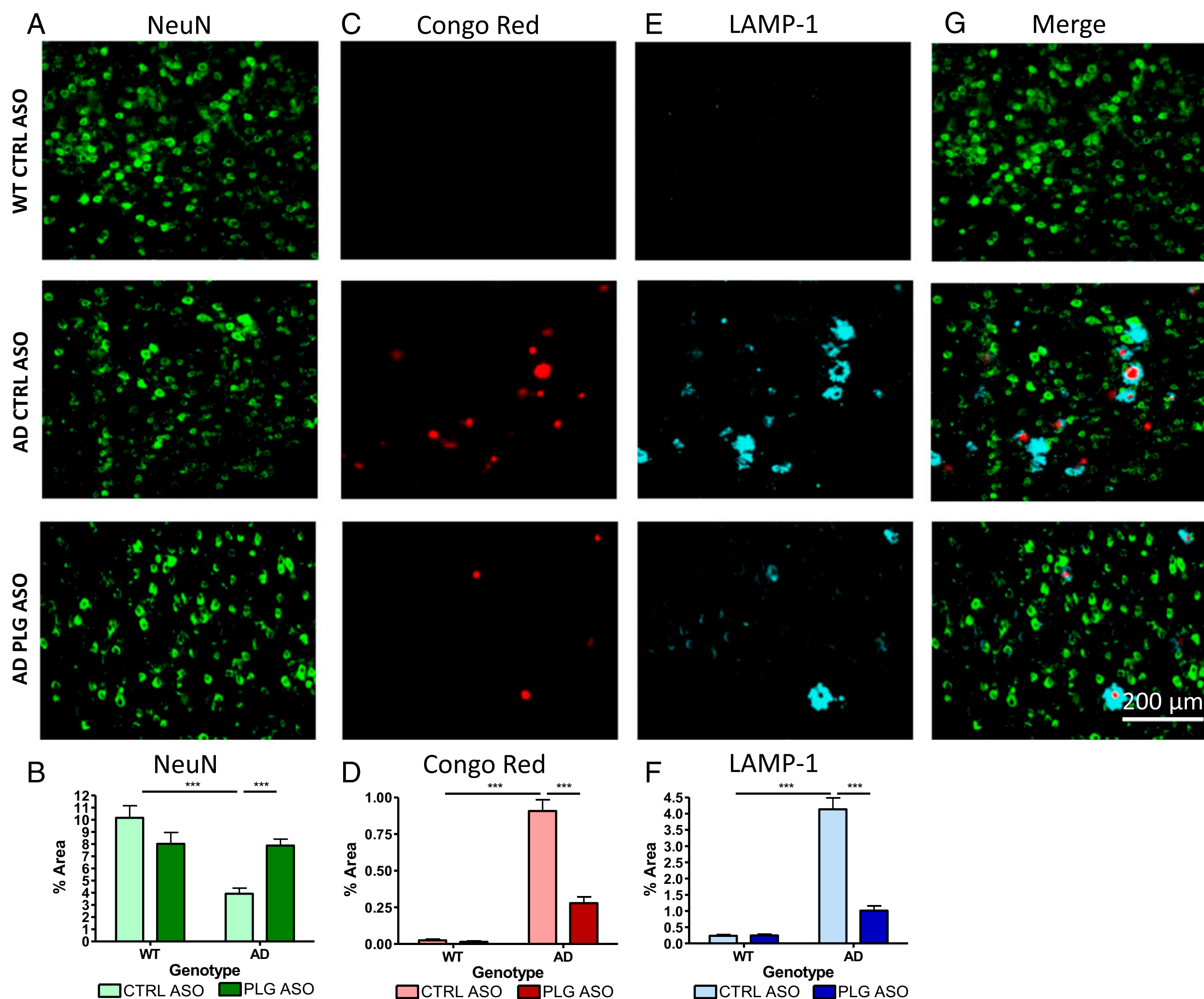

Fig. 3. PLG ASO treatment attenuates AD pathology by reducing neuronal death, fibrillar plaque deposition, and autophagy signaling in the cortex of AD mice. (*A*) Representative images of NeuN staining in the cortex of WT CTRL ASO-treated mice and AD mice treated with either CTRL or PLG ASO. (*B*) Neuronal 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 ($P < 0.0001$). (*C–G*) Images shown for Congo red and LAMP-1 staining are of AD animals only, because this staining is minimal in WT mice. Staining in WT animals is quantified in all statistical analyses. (*C*) Representative images of Congo red staining show fibrillar amyloid plaque deposition in WT CTRL ASO-treated mice and CTRL- or PLG ASO-treated AD mice. (*D*) Fibrillar plaque deposition is decreased in the cortex of PLG ASO-treated AD animals compared with CTRL ASO-treated AD animals ($P < 0.0001$). (*E*) 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. (*F*) LAMP-1 staining is increased ($P < 0.0001$) in AD animals compared with WT, but decreased in PLG ASO-treated animals compared with CTRL ASO-treated AD mice ($P < 0.0001$). (*G*) Merged images compare AD pathology marked by neuronal staining (NeuN), fibrillar plaque deposition (Congo red), and autophagy signaling (LAMP-1) in AD CTRL ASO- and AD PLG ASO-treated animals. Two-way ANOVA, n = 15–18 mice per group; all values presented as mean ± SEM. Results are from three independent experiments. ***$P < 0.0001$.

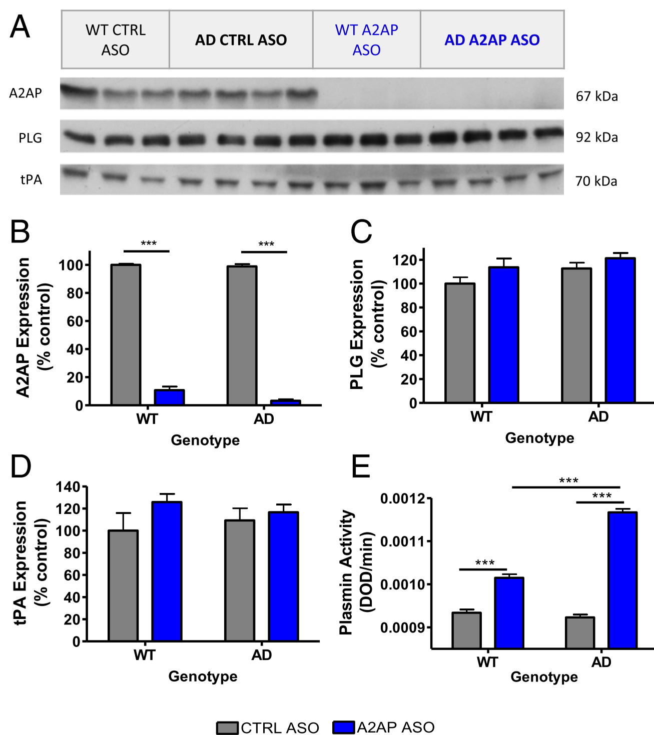

Fig. 4. A2AP levels are depleted in the plasma of A2AP ASO-treated mice, without affecting plasma levels of PA system proteins. (*A*) Representative Western blot of A2AP, PLG, and tPA levels in the plasma of WT CTRL ASO, WT A2AP ASO, AD CTRL ASO, and AD A2AP ASO-treated mice. (*B*) A2AP levels are significantly depleted in the plasma of A2AP ASO-treated animals compared with controls ($P < 0.0001$). PLG (*C*) and tPA (*D*) expression levels in the plasma are unaffected by A2AP ASO treatment ($P = 0.65$, $P = 0.40$, respectively). (*E*) PL 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. ***$P < 0.0001$.

serine protease PL. To do this, we investigated whether depleting A2AP in the plasma, and thus increasing PL through reduced inhibition, would increase the inflammatory response. We used an ASO-mediated gene knockdown strategy to deplete A2AP in AD and WT mice. To assess A2AP depletion in the circulation, plasma was collected after 2 and 10 wk of subcutaneous A2AP ASO injections. Plasma A2AP was knocked down efficiently in AD and WT animals to less than 5–10% of CTRL plasma levels (levels after 10 wk of treatment are shown in Fig. 4 *A* and *B*), while plasma PLG and tPA levels were not affected (Fig. 4 *A*, *C*, and *D*). We also analyzed PL activity in the plasma of ASO-treated animals (Fig. 4*E*) and observed that A2AP ASO injections increased PL activity in both WT and AD animals. We believe this result is a measure of free PL in the plasma, rather than PL bound to an inhibitor as a PAI-1 complex would be unlikely to efficiently cleave the assay's chromogenic substrate. The increase in PL activity is more drastic in AD A2AP ASO-treated animals compared with their WT counterparts, perhaps due to the fact that PL activity is increased in the presence of Aβ (17, 18). However, if this is the case, it is surprising that there is no difference in PL activity between AD and WT animals treated with CTRL ASO.

Depletion of Plasma A2AP Increases Microglial/Macrophage Activation and Fibrillar Plaque Deposition 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 (Fig. 5). In AD mice treated with CTRL ASO, CD11b levels were increased in the cortex compared with WT mice, and increased further in AD animals with A2AP ASO treatment (Fig. 5 *A* and *B*), suggesting that monocytic cells have enhanced activation due to increased PL activity. The expression levels of CD11b (Fig. 5*B*) 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 (Fig. 5 *C* and *D*), indicating an exacerbated fibrillar plaque load. We also examined NeuN and GFAP levels and did not see any difference in levels of these proteins between AD CTRL ASO and AD A2AP ASO-treated mice. This may be due to the fact that astrocyte activation

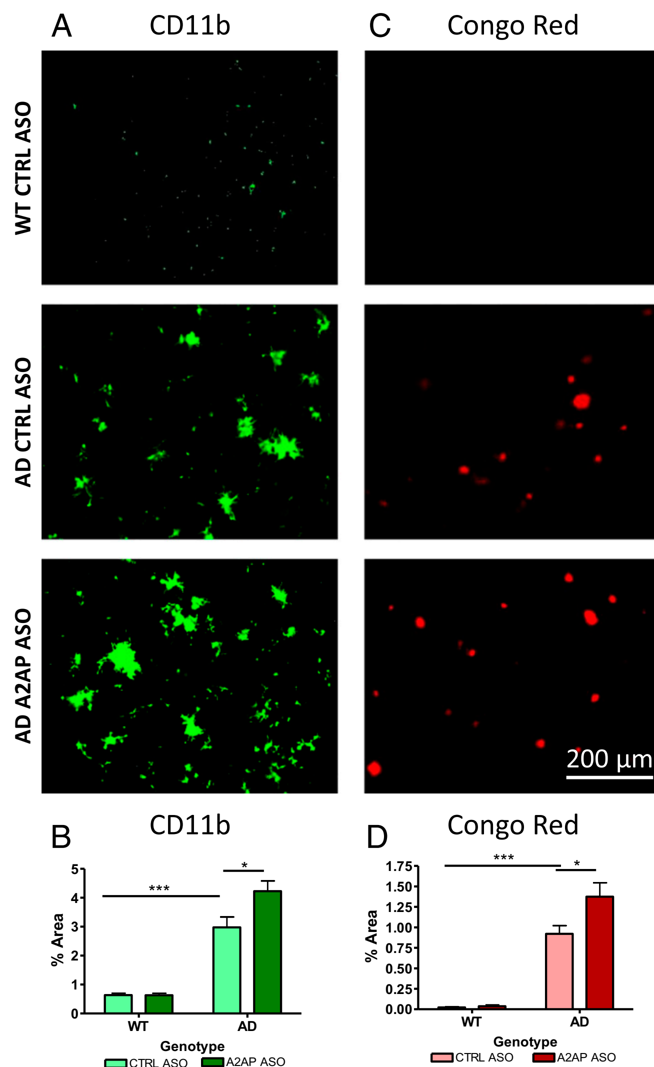

Fig. 5. Microglia/macrophage activation and fibrillar plaque load, but not overall plaque load, are increased in the brains of A2AP ASO-treated AD mice. (*A*) Representative images of CD11b staining in the cortex of WT CTRL ASO-treated mice and AD mice with CTRL or A2AP ASO treatment. (*B*) Microglial/macrophage activation in the cortex is significantly increased in AD animals ($P < 0.0001$), and this increase is exacerbated in mice with depleted plasma A2AP ($P = 0.02$). (*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 with CTRL ASO ($P = 0.03$). Two-way ANOVA, $n = 7$ mice per group; all values presented as mean ± SEM. *$P < 0.05$; ***$P < 0.0001$.

is saturated in these animals and that the disease has not yet progressed in mice of this age to see exacerbated neuronal death compared with AD controls.

Peripheral PL 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 because they share many of the same cell-surface markers. 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 (Fig. 6*A*); TMEM119, the only known microglia-specific marker (Fig. 6*B*) (19, 20); and CD206,

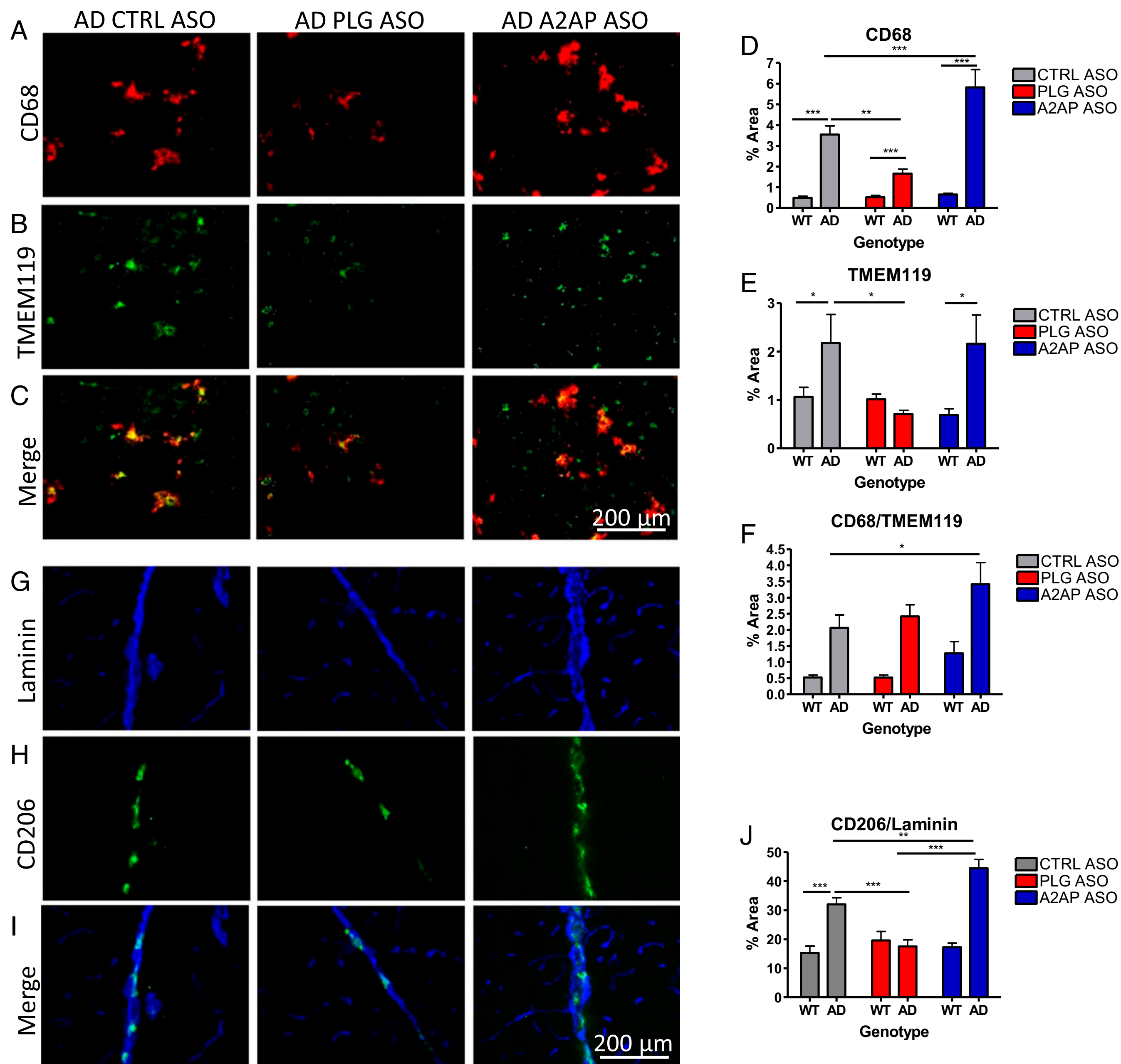

Fig. 6. Monocytic cell activation in AD mice is primarily driven by activated microglia. Representative images of CD68 (*A*) and TMEM119 (*B*) staining in CTRL ASO-, PLG ASO-, or A2AP ASO-treated AD animals. (*C*) Merged images show that $CD68^+$ cells are also $TMEM119^+$, suggesting they are activated microglia, not macrophages. (*D*) CD68 level is increased in all AD animals compared with 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 with WT controls ($P = 0.02$). (*F*) The amount of CD68 expression per TMEM119 expression is increased in AD animals treated with A2AP ASO compared with 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 $CD206^+$ cells are perivascular macrophages, contained in vessels of the mouse brain. (*J*) Percentage of vessels containing $CD206^+$ macrophages is increased in CTRL ASO-AD animals compared with WT groups ($P < 0.0001$). PLG ASO treatment of AD animals significantly decreases the level of peripheral macrophages around vessels compared with 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. *$P < 0.05$; **$P < 0.01$; ***$P < 0.0001$.

a marker for perivascular macrophages (Fig. 6*H*) (21). Representative images of these stainings in AD animals are shown (Fig. 6 *A*–*C* and *G*–*I*), and quantification from WT and AD mice is presented in the accompanying bar graphs (Fig. 6 *D*–*F* and *J*). In the cortex, around Aβ plaques, most CD68 expression is colocalized with TMEM119 staining (Fig. 6*C*), suggesting that activated microglia are primarily responsible for the innate immune response around plaques in the AD mouse brain. Peripheral PLG depletion decreased both CD68 (Fig. 6 *A* and *D*) and TMEM119 (Fig. 6 *B* and *E*) expression levels compared with CTRL ASO-treated AD animals. However, in AD A2AP ASO-treated animals, TMEM119 levels were unchanged relative to CTRL ASO-treated AD animals (Fig. 6 *B* and *E*), while CD68 levels were increased (Fig. 6 *A* and *D*). We observed an increase in the expression of CD68/TMEM119 in plasma A2AP-deficient AD animals. These data suggest that in the presence of greater PL 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 (Fig. 6*G*), we examined the expression levels of CD206 (Fig. 6*H*) on $CD68^+$ cells (all $CD206^+$ cells examined were also $CD68^+$). $CD206^+$ 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 with WT controls (Fig. 6*J*). 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 PL 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 ROS that may be contributing to microglial activation in AD.

Discussion

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 (22, 23). 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 (2, 24). 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 IFN-γ, TNF-α, IL-1β, and IL-6 (23). Inflammatory cytokines and activated microglia are key pathological features of the AD patient brain (25), and chronic use of antiinflammatory drugs can delay or limit the severity of AD (26). 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 (27, 28). In addition, many of the risk factors for AD have an inflammatory component, including obesity, traumatic brain injury, and systemic inflammation or infection (29).

The Aβ peptide can lead to an immune response through activation of pattern recognition receptors on glial cells and neurons (30), but the relationship between Aβ plaque formation and inflammation is bidirectional because 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 up-regulation of Aβ production and plaque deposition (23, 31, 32). 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.

PLG 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 PLG can contribute to inflammation through modulation of cell-signaling, as has been shown with monocytes, macrophages, dendritic cells, and other inflammatory cells (33). PLG is a chemoattractant for monocytic cells (9) and also plays a role in gene-expression changes crucial to phagocytosis by macrophages (7). Inflammation is not observed in PLG-deficient mice when injected with collagen type II to induce autoimmune arthritis, unless mice are also supplied with intravenously injected PLG (34). PLG-knockout mice also show a compromised immune response in the brain following hippocampal injection of LPS (15), an inflammatory agent used to activate microglia and proinflammatory cytokine expression. In addition, PLG deficiency in a mouse model of MS delays both the onset of MS symptoms and decreases neuroinflammation in these mice (16). 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 (35).

Because most previous in vivo studies are knockout studies that eliminate PL as a result of eliminating its precursor PLG, it is challenging to distinguish between the role of PL and PLG modulation of inflammation. In the present study, we show that PL is the major mediator of inflammation in an AD mouse model. Knocking down PLG using ASO technology reduces a neuroimmune response, whereas increasing the level of PL without affecting the PLG level through use of an A2AP ASO leads to an increased neuroimmune response. In this case, PL appears to be acting by way of a peripheral mechanism, as the ASO treatments target liver production, and therefore plasma levels, of PLG and A2AP 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 (36, 37). The contact system is also activated in the plasma and cerebral spinal fluid of AD patients (37–39). Our laboratory recently 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 (40). Furthermore, FXIIa, FXIa, kallikrein, and bradykinin—key molecules in the contact pathway—are known activators of PLG to PL (41–44). Thus, if contact activation is increased, PL generation is also increased, which could propagate the inflammatory response. In addition, PL can activate FXII, leading to contact system activation and bradykinin release, or to thrombin generation through the coagulation pathway (45, 46). Bradykinin is a proinflammatory mediator of vascular permeability (47), and thrombin also plays a proinflammatory role in the CNS through activation of protease-activated receptors (48).

Fibrin(ogen) deposition in the CNS is known to cause inflammation (49). Fibrin(ogen) can enter the brain when there is blood–brain barrier (BBB) damage (50–52), and depleting PLG or PL in instances where there is increased fibrin deposition could be detrimental, as it could lead to increased inflammation in the brain, because fibrin would not be readily cleared by its main fibrinolytic agent (49). A previous study demonstrated this

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effect (49), showing that inhibition of PLG by tranexamic acid (TXA) leads to an increased inflammatory response. However, inhibition of PL activity using TXA does not influence inflammatory pathways, even though both PL generation and fibrin degradation are inhibited with this reagent (53). This result may be because TXA can only inhibit free PL or fibrin-bound PL, but cannot successfully inhibit cell-bound PL, which is the major contributor to PL's modulation of inflammatory signaling (8, 33). Thus, while use of TXA may be an effective method to explore PL's effects on fibrinolysis and thus fibrin's contribution to an inflammatory response, this body of literature may be confounded by the fact that PL has many cell-mediated functions, including regulation of inflammation.

Because 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 PLG depletion. We ended treatment and killed animals before cognitive deficits are easily detectable by behavioral tests because chronic PLG depletion can lead to multiple pathologies (54), and therefore were unable to evaluate memory improvement. Nonetheless, this treatment protocol and timing allowed us to distinguish between PL's inflammatory functions and fibrinolytic functions early in disease progression. In addition, PLG is still expressed in the brain with this ASO treatment compared with a global PLG knockout model, so it is likely that PLG continues to participate in fibrinolysis in the brain.

Temporal profiling of gene expression in the 5XFAD mouse model of AD (55) 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 mo of age, it is clear that the immune and inflammatory processes are predominant in this mouse model. These mice develop Aβ plaques and gliosis by 2 mo of age, and between the first and fourth month of age there is a huge up-regulation of inflammatory and immune markers in both the cortex and the hippocampus. Later, between 4 and 9 mo of age, continuous microglial activation becomes the principal feature and increasing neuroinflammation is present, modulated by sustained complement system activation (55). It is likely that peripheral PLG depletion in these mice beginning early in disease (at 3 mo 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 PLG depletion in later disease stages would have the same effect.

Aβ plaque deposition was significantly decreased in PLG-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 PLG depletion. There is some evidence that PL can enhance both α- and β-cleavage of APP (56), although this result has not been extensively studied. The PLG activation (PA) system is induced by Aβ (57), and PL can degrade oligomeric and fibrillar Aβ (18, 56, 57). In addition, tPA-activated PL can digest Aβ oligomers, inhibiting both its aggregation and neurotoxicity (58). While tPA and PLG 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 PL (59). Genetic deletion of PAI-1, the major inhibitor of tPA and uPA, in an AD mouse model led to increased tPA and PLG activity and decreased amyloid plaque deposition in these animals, further implicating PL in Aβ clearance (60). These studies suggest that PL may play a beneficial role in degrading Aβ and that depletion of PL would therefore lead to more persistent plaques. However, it is also important to consider that these studies have focused on the fibrinolytic function of PLG, without considering the role of PLG in inflammation. We suggest here that it is important to understand that PLG 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. PLG depletion limits the ability to mount a full inflammatory response, and therefore less Aβ may be generated because the activation cycle is dampened in the absence of this protein. In our model of peripheral PLG depletion, PLG and tPA levels are unaltered in the brain, and thus the PA system in the brain may still have the ability to degrade Aβ.

While we have shown that depletion of PLG in the plasma affects neuroinflammation and brain pathology, the mechanism by which this plasma protein modulates inflammation in the brain is not yet understood. The BBB—which is composed of endothelial cells, the basement membrane, and astrocytic endfeet—forms a barrier that prevents large molecules from entering the brain from the blood (61). PLG levels are unaffected in the brain, and tight junction proteins are intact in these animals, suggesting that there is no BBB damage. However, there are many other routes of communication between the blood and the brain, and the brain and periphery are in constant communication with one another (61). Systemic immune activation can affect the CNS through signaling across the BBB via cytokines, ROS, and other enzymes (62), diffusion by way of the circumventricular organs of the brain that are not surrounded by the BBB, and signaling through peripheral nerves, such as the vagus nerve (63, 64). In addition, peripheral immune cells like macrophages and T cells can migrate through the BBB or through the choroid plexus (61, 65). We have evidence that the predominant monocytic cells that surround plaques in the AD mouse brain in this study are the brain's innate immune cells, the microglia. However, we also see that the migration of perivascular macrophages into the major vessels of the brain is affected by the presence of PLG in our AD mouse model. Studies have shown that perivascular macrophages are frequently replaced in the CNS with fresh cells from the blood (66), and PLG likely plays a role in their migration (67, 68). Through local interactions, CNS macrophages and microglia play a major role in relaying information between the systemic immune system and the brain (69). Inflammatory mediators in the blood signal to the cerebral endothelial cells, which signal to perivascular macrophages, which then signal to microglia (70). It is possible in our study that because PLG-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 PLG depletion and increased glial activation with plasma A2AP depletion (increased PL activity) suggest that PLG is modulating the neuroinflammatory response via one of these peripheral mechanisms.

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. PLG and the PA 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, PLG may play a detrimental role due to its function in mediation of inflammatory action. Excessive activation of PL is seen in chronic inflammatory and autoimmune diseases, and PLG 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 PL 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.

Materials and Methods

Animals. All animal experiments were conducted in accordance with the guidelines of the NIH *Guide for the Care and Use of Laboratory Animals* (71) and with approval from the Animal Care and Use Committee of the Rockefeller University. Tg6799 transgenic mice (referred to as AD mice) are double-transgenic for human APP/Presenilin 1 that express five early-onset familial AD mutations on a B6SJLF1/J background. WT littermates were used as controls in all experiments. ASO were prepared against PLG and A2AP along with a relevant scrambled CTRL (Ionis Pharmaceuticals). The PLG and A2AP ASOs are 20 nucleotides long and chemically modified with a phosphorothioate backbone and 2-O-methoxyethyl wings 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 nongenomic 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, and AD-A2AP ASO (n = 7–18 mice per group). Mice were treated for 2 wk at a dose of 150 mg/kg/wk starting at 3 mo of age, followed by 8 wk of treatment at a dose of 100 mg/kg/wk. The weekly ASO dose was divided into two injections per week. Plasma was collected by tail bleed after 2 and 4 wk of treatment and again by retro-orbital bleed on the day of killing to determine the extent of PLG or A2AP depletion.

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 μL 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 (SC-134220; Santa Cruz). Plasma from both bleeding methods was prepared by centrifugation of whole blood and was frozen before Western blot analysis.

Immunohistochemistry. Mice were deeply anesthetized and perfused with saline before 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/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), and laminin (blood vessels; Sigma). Brain sections were incubated with primary antibodies for 3 h 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 min, followed by 70% isopropanol for 15 min. All brain sections were washed and a coverslip was applied along with fluorescence mounting media (Vectashield).

Western Blotting. Mice were deeply anesthetized and perfused with saline before 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 mixture (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), incubated overnight at 4 °C in primary antibody [rabbit anti-PLG (Abcam); rabbit anti-tPA (Molecular Innovations); rabbit anti–PAI-1 (Abcam); mouse anti-actin (Sigma-Aldrich); rabbit anti-A2AP (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 ImageJ (NIH). Western blot results for brain protein extracts were normalized to actin.

Imaging Analysis. Following immunostaining, brain sections were imaged with a microscope (Axiovert 200; Carl Zeiss) equipped with Plan-Neofluar (10× NA 0.3, 20× NA 0.5, and 40× NA 0.75) objective lenses at room temperature using air as the imaging medium. AxioVision software was used to collect images from an AxioCam color camera (Carl Zeiss). Images of areas with positive staining were thresholded using ImageJ (NIH). 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 7 to 18 mice per group).

PL 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 PL. The change in OD per minute at 405 nM was measured for each plasma sample as a determination of activity.

Statistical Analysis. Statistical analyses were conducted using GraphPad Prism software for two-way ANOVA, as indicated in each figure legend. All values presented in graphs are mean ± SEM.

Supporting Information. Representative images and statistical analyses of inflammatory markers in the hippocampus of PLG ASO-treated animals, as well as a representative Western blot and quantification of brain APP expression levels can be found in *SI Appendix*.

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