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RESEARCH ARTICLE

Haploinsufficiency of microglial MyD88 ameliorates Alzheimer's pathology and vascular disorders in APP/PS1-transgenic mice

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Abstract

Growing evidence indicates that innate immune molecules regulate microglial activation in Alzheimer's disease (AD); however, their effects on amyloid pathology and neurodegeneration remain inconclusive. Here, we conditionally deleted one allele of *myd88* gene specifically in microglia in APP/PS1-transgenic mice by 6 months and analyzed AD-associated pathologies by 9 months. We observed that heterozygous deletion of *myd88* gene in microglia decreased cerebral amyloid β (Aβ) load and improved cognitive function of AD mice, which was correlated with reduced number of microglia in the brain and inhibited transcription of inflammatory genes, for example, *tnf-α* and *il-1β*, in both brain tissues and individual microglia. To investigate mechanisms underlying the pathological improvement, we observed that haploinsufficiency of MyD88 increased microglial recruitment toward Aβ deposits, which might facilitate Aβ clearance. Microglia with haploinsufficient expression of MyD88 also increased vasculature in the brain of APP/PS1-transgenic mice, which was associated with up-regulated transcription of *osteopontin* and *insulin-like growth factor* genes in microglia. Moreover, MyD88-haploinsufficient microglia elevated protein levels of LRP1 in cerebral capillaries of APP/PS1-transgenic mice. Cell culture experiments further showed that treatments with interleukin-1β decreased LRP1 expression in pericytes. In summary, haploinsufficiency of MyD88 in microglia at a late disease stage attenuates pro-inflammatory activation and amyloid pathology, prevents the impairment of microvasculature and perhaps also protects LRP1-mediated Aβ clearance in the brain of APP/PS1-transgenic mice, all of which improves neuronal function of AD mice.

KEYWORDS
Alzheimer's disease, LRP1, microglia, MyD88, vasculature

Wenqiang Quan and Qinghua Luo authors contributed equally to this study.

1 | INTRODUCTION

Alzheimer's disease (AD) is a progressive neurodegenerative disease pathologically characterized by extracellular amyloid β (Aβ) deposits, intracellular neurofibrillary tangles, and microglial activation (Heneka et al., 2015). Genome-wide association studies correlated microglial genes (i.e., *TREM2*, *APOE*, *CD33*, *ABCA7*, and *PLCG2*) with the occurrence of late-onset AD, which highlights an essential role of microglia in AD pathogenesis (Lewcock et al., 2020). In AD mouse models, which overexpress Alzheimer's amyloid precursor protein (APP) in neurons, microglia are activated and recruited to Aβ deposits (Bolmont et al., 2008; Meyer-Luehmann et al., 2008), and are more closely correlated with the impairment of cognitive performance than Aβ deposition (Focke et al., 2018). Elimination of microglia at a late disease stage with noticeable Aβ already in the brain prevents the synaptic and neuronal loss in APP-transgenic mice (Spangenberg et al., 2016). However, activated microglia also exert a protective effect on neurons in AD mice by up-taking Aβ peptides (Michaud et al., 2013) and promoting degradation of phosphorylated tau proteins in neurons (Qin et al., 2016).

Many studies through cross-breeding or bone marrow reconstruction have shown that the innate immune signaling regulates microglial activation in AD mice. Deficiency of CD14 (Reed-Geaghan, Reed, Cramer, & Landreth, 2010), Toll-like receptor (TLR) 2 (S. Liu et al., 2012), TLR4 (Song et al., 2011), myeloid differentiation factor 88 (MyD88) (Hao et al., 2011), interleukin receptor-associated kinase 4 (IRAK4) (Cameron et al., 2012), inhibitor of nuclear factor κ-B kinase subunit β (IKKβ) (Y. Liu et al., 2014), or NLR family pyrin domain containing 3 (NLRP3) (Heneka et al., 2013) attenuates the degree of inflammation, shifts inflammatory activation from pro-inflammatory to anti-inflammatory profiles, or both in the brain of APP-transgenic mice. However, results on the effects of innate immunity on Aβ pathology and neuronal degeneration in AD mice are often contradictory. For example, deletion of MyD88 or its downstream signaling molecule, IRAK4, and IKKβ, or disruption of the interaction between TLR2 and MyD88, attenuates Aβ pathology and neuronal death in APP-transgenic mice (Cameron et al., 2012; Hao et al., 2011; Lim et al., 2011; Y. Liu et al., 2014; Rangasamy et al., 2018), whereas, wild-type MyD88 was also reported to promote Aβ clearance and protect neurons (Michaud, Richard, & Rivest, 2011, 2012). There was one study even showing that overall deletion of MyD88 in AD mice have no effects on neuroinflammation and Aβ deposition (Weitz, Gate, Rezai-Zadeh, & Town, 2014). It is difficult to explain the apparently conflicting results delivered from different animal models and different experimental methods. However, in the investigation of MyD88 and AD, it should be noted that: (a) MyD88 functions not only in microglia, but also in other brain cells (e.g., neurons, astrocytes and endothelial cells; Gosselin & Rivest, 2008; Hung et al., 2018; Shen et al., 2016); and (b) overall deletion of MyD88 in AD mice alters development of the brain and is potentially fatal (Michaud et al., 2011; Schroeder et al., 2021). Thus, to clarify the pathogenic role of MyD88 in AD, MyD88 expression should be manipulated specifically in microglia or other brain cells within a designed time window in AD animals.

Growing evidence suggests that vascular disorders contribute to AD pathogenesis. AD patients often have vascular pathologies, which are from large artery atherosclerosis, cerebral amyloid angiopathy (CAA) to microvascular disease, and blood–brain–barrier (BBB) impairment (Cortes-Canteli & Iadecola, 2020). We observed a reduction of vasculature and blood flow in the hippocampus of APP- or tau-transgenic AD mice (Decker et al., 2018). Pericytes wrapping around endothelial cells are essential for the maintenance of normal structure and function of cerebral blood circulation, which include BBB homeostasis and angiogenesis (Sweeney, Ayyadurai, & Zlokovic, 2016). Pericytes are injured in AD at an early disease stage (Montagne et al., 2020; Nation et al., 2019). Deletion of pericytes increases permeability of BBB, decreases vasculature and blood flow, and exaggerates Aβ accumulation in the brain of APP-transgenic mice (Montagne et al., 2018; Sagare et al., 2013). BBB is able to efficiently clean cerebral Aβ by transporting Aβ outside of brain (Roberts et al., 2014), which is at least partially mediated by low-density lipoprotein receptor-related protein 1 (LRP1) and ATP binding cassette subfamily B member 1 (ABCB1) (Kuhnke et al., 2007; Shinohara, Tachibana, Kanekiyo, & Bu, 2017). Interestingly, LRP1 also mediates Aβ internalization by pericytes, thereby cleaning Aβ locally at BBB (Ma et al., 2018). However, how microglia regulate microcirculation, pericyte function, and BBB-mediated Aβ clearance in AD brain remains unclear.

In this study, we knocked out one allele of *myd88* gene specifically in microglia in APP/PS1-transgenic mice by 6 months and investigated amyloid pathology, neuroinflammation, and cerebral vasculature by 9 months. We observed that haploinsufficiency of MyD88 in microglia attenuated AD-associated pathologies and protected neurons.

2 | MATERIALS AND METHODS

2.1 | Animal models and Cross-breeding

APP/PS1-double transgenic mice over-expressing human mutated APP (KM670/671NL) and PS1 (L166P) under Thy-1 promoters (Radde et al., 2006) were kindly provided by M. Jucker, Hertie Institute for Clinical Brain Research, Tübingen, Germany; *myd88*-floxed mice (B6.129P2[SJL]-Myd88tm1Defr/J; $MyD88^{fl/fl}$; Stock number: 008888; Hou, Reizis, & DeFranco, 2008) were imported from the Jackson Laboratory, Bar Harbor, ME; and Cx3Cr1-CreERT2 mice that express a fusion protein of Cre recombinase and an estrogen receptor ligand binding domain under the control of endogenous *cx3cr1* promoter/enhancer elements (Goldmann et al., 2013) were kindly provided by M. Prinz, University of Freiburg, Germany. APP/PS1-transgenic mice were cross-bred with $MyD88^{fl/fl}$ and Cx3Cr1-Cre mice to obtain mice with the following genotypes: $APP^{tg\ or\ wt}MyD88^{fl/wt}Cre^{+/-}$ and $APP^{tg\ or\ wt}MyD88^{fl/wt}Cre^{-/-}$. In order to minimize potential toxic effects of MyD88 deficiency on the physiological function, we used heterozygous *myd88*-floxed mice ($MyD88^{fl/wt}$) in the whole study. To induce the recombination of

myd88 gene, 6-month-old mice were injected (*i.p.*) with tamoxifen (Sigma-Aldrich Chemie GmbH, Munich, Germany; 100 mg/kg) in corn oil once a day over 5 days. The phenotype of APP/PS1-transgenic mice with or without haploinsufficient expression of MyD88 in microglia was compared between siblings. As a control experiment, APP/PS1-transgenic and Cx3Cr1-Cre mice were mated with *gpr43*-floxed mice (Tang et al., 2015) to obtain mice with $APP^{tg}Gpr43^{fl/fl}Cre^{+/-}$ and $APP^{tg}Gpr43^{fl/fl}Cre^{-/-}$ of genotypes. Control mice were treated completely the same as for MyD88-deficient mice. To identify cells, which express Gpr43, Gpr43-RFP reporter mice overexpressing monomeric red fluorescence protein (mRFP) under the control of *gpr43* promoter (Tang et al., 2015) were used. Both *gpr43*-floxed mice and Gpr43-RFP reporter mice were kindly provided by S. Offermanns, Max Planck Institute for Heart and Lung Research, Germany. All animal experiments were performed in accordance with relevant national rules and authorized by Landesamt für Verbraucherschutz, Saarland, Germany (permission numbers: 29/2016 and 14/2018).

2.2 | Morris water maze

The Morris water maze test, consisting of a 6-day training phase and a 1-day probe trial, was used to assess the cognitive function of APP/PS1-transgenic mice and their wild-type littermates, as previously described (Qin et al., 2016; Schnöder et al., 2020). During training phase, latency time, distance, and velocity were recorded with Ethovision video tracking equipment and software (Noldus Information Technology, Wageningen, the Netherlands). During the probe trial, the platform was removed and we measured the latency of first visit to the location of original platform, the frequency of crossing in that location, and the time spent in the platform area.

2.3 | Tissue collection and isolation of blood vessels

Animals were euthanized at 9 months of age by inhalation of isofluorane. Mice were then perfused with ice-cold PBS, and the brain was removed and divided. The left hemisphere was immediately fixed in 4% paraformaldehyde (Sigma-Aldrich Chemie GmbH) in PBS and embedded in paraffin for immunohistochemistry. For one part of mice, a 0.5-μm-thick sagittal piece of tissue was cut from the right hemisphere. The cortex and hippocampus were carefully separated and homogenized in TRIzol (Thermo Fisher Scientific, Darmstadt, Germany) for RNA isolation. The remainder of the right hemisphere was snap-frozen in liquid nitrogen and stored at −80°C until biochemical analysis.

For the other part of mice, the cortex and hippocampus from right hemisphere were carefully dissected and brain vessel fragments were isolated according to the published protocol (Boulay, Saubamea, Decleves, & Cohen-Salmon, 2015). Briefly, brain tissues were homogenized in HEPES-contained Hanks' balanced salt solution (HBSS) and centrifuged at 4,400*g* in HEPES-HBSS buffer supplemented with dextran from *Leuconostoc spp*. (molecular weight ~70,000; Sigma-Aldrich Chemie GmbH) to delete myelin. The vessel pellet was re-suspended in HEPES-HBSS buffer supplemented with 1% bovine serum albumin (Sigma-Aldrich Chemie GmbH) and filtered with 20 μm -mesh. The blood vessel fragments were collected on the top of filter and stored at −80°C for biochemical analysis.

2.4 | Histological analysis

Serial 30-μm-thick sagittal sections were cut from the paraffin-embedded hemisphere. For each animal, four sections with an interval of 10 layers between neighboring sections were examined. Human Aβ was stained with rabbit anti-human Aβ antibody (clone D12B2; Cell Signaling Technology Europe, Frankfurt am Main, Germany) and microglia labeled with rabbit anti-ionized calcium-binding adapter molecule (Iba)-1 antibody (Wako Chemicals, Neuss, Germany), and visualized with the VectaStain Elite ABC kit (Vector Laboratories, Burlingame, USA) or fluorescence-conjugated second antibodies. In the whole hippocampus, volumes of Aβ were estimated with the *Cavalieri* method, and Iba-1-positive cells were counted with Optical Fractionator as described previously (Y. Liu et al., 2014) on a Zeiss AxioImager.Z2 microscope (Carl Zeiss Microscopy GmbH, Göttingen, Germany) equipped with a Stereo Investigator system (MBF Bioscience, Williston, ND).

To evaluate the cerebral Aβ deposition, four serial brain sections from each animal were labeled with methoxy-XO4 (Tocris Bioscience, Wiesbaden-Nordenstadt, Germany) after deparaffinization. The whole cortex and hippocampus were imaged with Microlucida (MBF Bioscience) and merged. Fluorescence-labeled areas were measured using the Image J software (https://imagej.nih.gov/ij/) with fixed thresholds for all compared animals. The percentage of Aβ coverage in the brain was calculated.

To detect the deposition of Aβ at blood vessels, brain sections were co-stained with human Aβ antibody (clone D12B2) and biotin-labeled *Griffonia Simplicifolia* Lectin I isolectin B4 (Catalog number: B-1205; Vector Laboratories, Burlingame, CA), and Alexa488-conjugated anti-rabbit IgG and Cy3-conjugated streptavidin, respectively (Thermo Fisher Scientific). To identify Gpr43-expressing cells, brain sections from Gpr43-reporter mice were co-stained with rabbit anti-RFP antibody (Catalog number: 600-401-379; Rockland Immunochemicals, Limerick, PA) and mouse anti-Tmem119 antibody (clone: 195H4; Synaptic Systems GmbH, Göttingen, Germany), which were followed by incubation with Alexa488 or Cy3-conjugated second antibodies (Thermo Fisher Scientific).

The relationship between microglia and Aβ deposits was investigated as we did in a previous study (Hao et al., 2011). Serial brain sections were stained with Iba-1 antibody (Wako Chemicals) and Alexa546-conjugated anti-rabbit IgG (Thermo Fisher Scientific), and then co-stained with methoxy-XO4 (Bio-Techne GmbH). Under Zeiss microscopy with 40× objective, Aβ deposits were imaged with green fluorescence filter. Thereafter, Z-stack serial scanning from −10 to +10 μm was performed under both green and orange fluorescence

filters. From each section, more than 10 randomly chosen areas were analyzed. The total number (>200) of Iba-1-positive cells co-localizing with Aβ deposits were counted. The area of Aβ was measured with Image J and used for the adjudgment of microglial cell number.

To quantify vasculature in the brain, our established protocol was used (Decker et al., 2018; Quan et al., 2020). Four serial paraffin-embedded sections per mouse with 300 μm of distance in between were deparaffinized, heated at 80°C in citrate buffer (10 mM, pH = 6) for 1 hr and digested with Digest-All 3 (Pepsin) (Thermo Fisher Scientific) for 20 min. Thereafter, brain sections were stained with rabbit anti-collagen IV polyclonal antibody (Catalog number: ab6586; Abcam, Cambridge, UK) and Alexa488-conjugated goat anti-rabbit IgG (Thermo Fisher Scientific). After being mounted, the whole hippocampus was imaged with Microlucida (MBF Bioscience). The length, branching points, and density of collagen type IV staining-positive blood vessels were analyzed with a free software, AngioTool (http://angiotool.nci.nih.gov; Zudaire, Gambardella, Kurcz, & Vermeren, 2011). The mean diameter of blood vessels was calculated by dividing total area of blood vessels with the total length of vessels. The parameters of analysis for all compared samples were kept constant. The length and branching points were adjusted with brain area of interest.

2.5 | Western blot analysis

Frozen mouse brains were homogenized on ice in 5× radioimmunoprecipitation assay buffer (RIPA buffer; 50 mM Tris [pH 8.0], 150 mM NaCl, 0.1% SDS, 0.5% sodiumdeoxy-cholate, 1% NP-40, and 5 mM EDTA) supplemented with protease inhibitor cocktail (Sigma-Aldrich Chemie GmbH), followed by centrifugation at 16,000*g* for 30 min at 4°C to collect the supernatants. Isolated blood vessels were directly lysed in 2 × SDS-PAGE sample loading buffer containing 4% SDS and sonicated before loading. The protein levels of synaptic proteins: Munc18-1 protein mammalian homolog (Munc18-1), synaptosome-associated protein 25 (SNAP-25), postsynaptic density protein 95 (PSD-95) and synaptophysin were detected with rabbit polyclonal antibodies (Catalog numbers: 13414, 3926, and 2507, respectively; Cell Signaling Technology) and mouse monoclonal antibody (clone SY38; Abcam). In the same sample, β-actin was detected as a loading control using rabbit monoclonal antibody (clone: 13E5; Cell Signaling Technology). For the detection of proteins in cerebral capillaries, rabbit monoclonal antibodies against platelet-derived growth factor receptor β (PDGFRβ), CD13/APN, ABCB1, and vinculin (clone: 28E1, D6V1W, E1Y7S, and E1E9V respectively; Cell Signaling Technology) and rabbit polyclonal antibodies against LRP1 and α-tubulin (Catalog numbers: 64099 and 2144, respectively; Cell Signaling Technology), tight junction protein 1 (TJP1; Catalog numbers: NBP1-85047; Novus Biologicals, Wiesbaden-Nordenstadt, Germany), Claudin-5 (Thermo Fisher Scientific) and aquaporin 4 (AQP4; Proteintech Europe, Manchester, United Kingdom) were used. Western blots were visualized via the ECL method (PerkinElmer LAS GmbH, Rodgau, Germany). Densitometric analysis of bands was performed with the Image J software. For each sample, the protein level was calculated as a ratio of target protein/β-actin, α-tubulin or vinculin.

For detection of Aβ oligomers, the proteins in the brain homogenate or in the isolated blood vessels were separated by 10–20% precasted Tris-Tricine gels (Anamed Elektrophorese GmbH, Groß-Bieberau/Rodau, Germany). For Western blot, anti-human Aβ mouse monoclonal antibody (clone W0-2; Merck Chemicals GmbH, Darmstadt, Germany), anti-β-actin, or anti-α-tubulin antibodies (Cell Signaling Technology) were used.

2.6 | β- and γ-secretase activity assays

Membrane components were purified from the brain homogenate of 9-month-old APP/PS1-transgenic mice with and without heterozygous deletion of *myd88* gene and β- and γ-secretase activity were measured by incubating the crude membrane fraction with secretase-specific FRET substrates according to our established methods (Hao et al., 2011; Xie et al., 2013).

2.7 | Positive selection of CD11b-positive microglia in the adult mouse brain

To determine the gene expression in microglia, CD11b-positive cells were isolated from the entire cerebrum of 9-month-old APP/PS1-transgenic mice with our established protocol (Y. Liu et al., 2014). A single-cell suspension was prepared with Neural Tissue Dissociation Kit (papain-based) (Miltenyi Biotec B.V. & Co. KG, Bergisch Gladbach, Germany) and selected with MicroBeads-conjugated CD11b antibody (Miltenyi Biotec). Lysis buffer was immediately added to CD11b-positive cells for isolation of total RNA with RNeasy Plus Mini Kit (Qiagen, Hilden, Germany).

2.8 | Quantitative PCR for analysis of gene transcripts

Total RNA was isolated from mouse brains or selected CD11b-positive cells and reverse-transcribed. Gene transcripts were quantified with our established protocol (Y. Liu et al., 2014) using Taqman gene expression assays of mouse *tumor necrosis factor* (*tnf-α*), *interleukin-1β* (*il-1β*), *inducible nitric oxide synthase* (*inos*), *chemokine (C–C motif) ligand 2* (*ccl-2*), *il-10*, *chitinase-like 3* (*chi3l3*), *mannose receptor C type 1* (*mrc1*), *insulin-like growth factor* (*igf*)*-1*, *triggering receptor expressed on myeloid cells 2* (*trem2*), *apoe*, *CX3C chemokine receptor 1* (*cx3cr1*), *purinergic receptor P2Y12* (*p2ry12*), *C-type lectin domain family 7 member A* (*clec7a*), *lipoproteinlipase* (*lpl*), *transforming growth factor β receptor 1* (*tgfbr1*), *integrin α X* (*itgax*), *neprilysin*, *insulin-degrading enzyme* (*ide*), and *glyceraldehyde 3-phosphate dehydrogenase* (*gapdh*) (Thermo Fisher Scientific). The transcription of *osteopontin* (*opn*), *vascular endothelial growth factor* (*vegf*), and *peptidyl-prolyl cis-trans isomerase A* (*ppia*) genes in CD11b-positive cells was evaluated using the SYBR green binding technique with the following pairs of primers: *opn*, 5′-CAGCCATGAGTCAAGTCAGC-3′ and 5′-TGTGGCTGT GAAACTTGTGG-3′; *vegf*, 5′-CCCTTCGTCCTCTCCTTACC-3′ and

5′-AGGAAGGGTAAGCCACTCAC-3′; and *ppia*, 5′-AGCATACAGGT CCTGGCATCTTGT-3′ and 5′-CAAAGACCACATGCTTGCCATCCA-3′. The primer sequences for detecting the transcripts of various receptors of free fatty acids were listed in the legend of Figure S1.

2.9 | Pericyte culture and treatments

Human primary brain vascular pericytes (HBPC) were immortalized by infecting cells with tsSV40T lentiviral particles (Umehara et al., 2018). The selected immortalized HBPC clone 37 (hereafter referred to as HBPC/ci37) was used for our study. HBPC/ci37 cells were cultured at 33°C with 5% CO2/95% air in pericyte medium (Catalog: # 1201; Sciencell Research Laboratories, Carlsbad, CA) containing 2% (v/v) fetal bovine serum, 1% (w/v) pericyte growth factors, and penicillin–streptomycin. Culture flasks and plates were treated with Collagen Coating Solution (Catalog: # 125-50; Sigma-Aldrich). HBPC/ci37 cells were used at 40–60 passages in this study.

To investigate the effects of inflammatory activation on expression of LRP1, PDGFRβ, and CD13, pericytes were cultured in 12-well plate at 5.0×10^5 cells/well. Before experiments, the culture medium was replaced with serum-free pericyte medium and cells were cultured at 37°C for 3 days to facilitate the cell differentiation (Umehara et al., 2018). Thereafter, pericytes were treated with recombinant human IL-1β (Catalog: # 201-LB; R&D Systems, Wiesbaden-Nordenstadt, Germany) at 0, 5, 10 and 50 ng/ml for 24 hr, or for 8 days with and without withdrawal of IL-1β for the last 3 days. At the end of experiments, cultured cells were lysed in RIPA buffer supplemented with protease inhibitor cocktail. Protein levels of LRP1, PDGFRβ, and CD13 were detected with quantitative Western blot as described in Section 2.5.

2.10 | Statistical analysis

Data were presented as mean ± *SEM*. For multiple comparisons, we used one-way or two-way ANOVA followed by Bonferroni, Tukey, or Dunnett T3 post hoc test (dependent on the result of Levene's test to determine the equality of variances). Two independent-samples Students *t*-test was used to compare means for two groups of cases. All statistical analyses were performed with GraphPad Prism 8 version 8.0.2. for Windows (GraphPad Software, San Diego, CA). Statistical significance was set at $p < .05$.

3 | RESULTS

3.1 | Haploinsufficient expression of MyD88 in microglia protects neurons and improves cognitive function of APP/PS1-transgenic mice

To investigate effects of microglial MyD88 on AD pathogenesis, we mated APP/PS1-transgenic mice with $MyD88^{fl/fl}$ mice and Cx3Cr1-CreERT2 mice. Littermate mice with $APP^{tg}Myd88^{fl/wt}Cre^{+/-}$ and $APP^{tg}Myd88^{fl/wt}Cre^{-/-}$ of genotypes were injected with tamoxifen at 6 months of age. Tamoxifen-induced gene recombination should delete one allele of *myd88* gene in >93% microglia but not in other brain cells (Goldmann et al., 2013). Tamoxifen injection also knocks out loxP site-flanked *myd88* gene in peripheral Cx3Cr1-positive myeloid cells; however, normal MyD88-expressing myeloid cells produced from the bone marrow should replace these MyD88-deficient cells within 1 month (Goldmann et al., 2013). By detecting *myd88* gene transcripts in CD11b-positive brain cells from 9-month-old $APP^{tg}Myd88^{fl/wt}Cre^{+/-}$ ($MyD88^{het}$) and $APP^{tg}Myd88^{fl/wt}Cre^{-/-}$ ($MyD88^{wt}$) mice, we observed that the transcriptional level of *myd88* in microglia of $MyD88^{het}$ mice was only 11.4% of that in $MyD88^{wt}$ mice (*myd88/gapdh*: 0.178 ± 0.054 [wt] and 0.020 ± 0.004 [het]; *t* test, $p = .044$; $n = 3$ per group), which was in accordance with a previous observation that heterozygous knockout of *myd88* gene reduced *myd88* transcripts by 66% in the whole brain of APP-transgenic mice (Michaud et al., 2011). As a control, transcriptional levels of *myd88* (*myd88/gapdh*) in CD11b-positive blood cells were 0.100 ± 0.023 and 0.123 ± 0.025, in $MyD88^{het}$ and $MyD88^{wt}$ mice, respectively (*t* test, $p = .518$; $n = 4$ per group), which indicated that peripheral myeloid cells had been replenished by bone marrow-produced cells expressing MyD88 at a normal level.

We used the Morris water maze test to examine cognitive function of mice. During the acquisition phase, 9-month-old non-APP-transgenic (APP^{wt}) littermate mice with or without haploinsufficiency of microglial MyD88 ($APP^{wt}Myd88^{fl/wt}Cre^{+/-}$ and $APP^{wt}Myd88^{fl/wt}Cre^{-/-}$) showed no significant differences in either swimming time or swimming distance before climbing onto the escape platform (Figure 1a,b; two-way ANOVA, $p > .05$). Compared to $APP^{wt}Myd88^{fl/wt}Cre^{-/-}$ littermates, 9-month-old $APP^{tg}Myd88^{fl/wt}Cre^{-/-}$ mice with normal MyD88 expression spent significantly more time (Figure 1a; two-way ANOVA, $p < .05$) and traveled longer distances (Figure 1b; two-way ANOVA, $p < .05$) to reach the escape platform. Interestingly, $APP^{tg}Myd88^{fl/wt}Cre^{+/-}$ mice with the heterozygous deletion of microglial MyD88 performed significantly better than their $APP^{tg}Myd88^{fl/wt}Cre^{-/-}$ littermates in searching and finding the platform after 3 days of training (Figure 1a,b; two-way ANOVA, $p < .05$). The swimming velocity did not differ between MyD88-deficient and wild-type APP-transgenic mice or for the same mice on different training dates (data not shown).

Twenty-four hours after the end of training phase, the escape platform was removed and a 5-min probe trial was performed to test the memory of mice. Compared to $APP^{wt}Myd88^{fl/wt}Cre^{-/-}$ littermates, $APP^{tg}Myd88^{fl/wt}Cre^{-/-}$ mice crossed the region where the platform had been located with significantly less frequency, and remained for a significantly longer time in their first visit to the original platform region during the total 5-min probe trial (Figure 1c,d; one-way ANOVA followed by *post-hoc* test, $p < .05$). Interestingly, when compared to $APP^{tg}Myd88^{fl/wt}Cre^{-/-}$ mice, $APP^{tg}Myd88^{fl/wt}Cre^{+/-}$ mice were able to cross the region more frequently and reach the original platform region in less time (Figure 1c,d; one-way ANOVA followed by *post-hoc* test, $p < .05$). We observed differences in neither parameter analyzed in the probe trial between $APP^{wt}Myd88^{fl/wt}Cre^{+/-}$ and $APP^{wt}Myd88^{fl/wt}Cre^{-/-}$ littermate mice (Figure 1c,d; one-way ANOVA, $p > .05$).

In our previous study, we have observed that overexpression of APP/PS1 decreases protein levels of synaptic proteins

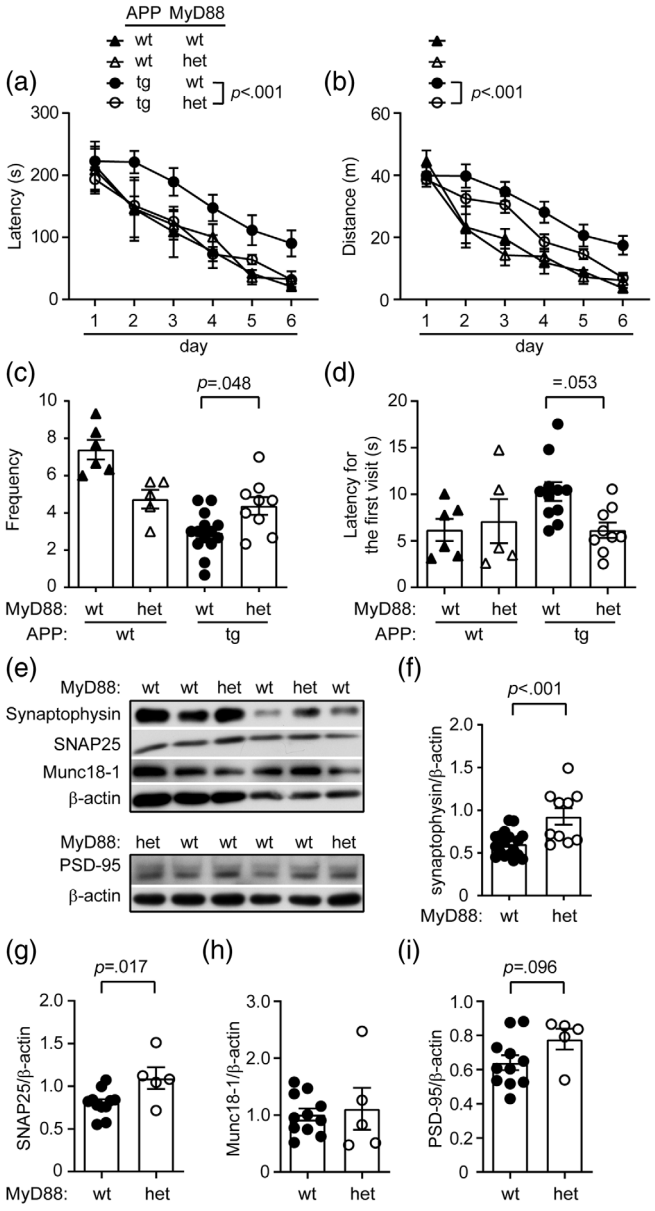

FIGURE 1 Haploinsufficiency of microglial MyD88 improves cognitive function and attenuates AD-associated loss of synaptic proteins in APP/PS1-transgenic mice. During the training phase of the water maze test, 9-month-old APP-transgenic mice (APPtg) spent more time and traveled longer distances to reach the escape platform than did their non-APP-transgenic littermates (APPwt). Compared to mice with normal expression of MyD88 (wt), heterozygous deletion of MyD88 in microglia (het) significantly reduced the traveling time and distance of APPtg mice but not of APPwt mice (a,b; two-way ANOVA followed by Bonferroni post hoc test; $n \geq 5$ per group). In the probe trial, APPtg mice crossed the region where the platform was previously located with significantly less frequency during the total 5-min experiment, and spent significantly longer time in the first visit to the platform region; heterozygous deletion of MyD88 in microglia recovered these APP expression-induced cognitive impairments (c,d; one-way ANOVA followed by Bonferroni post hoc test). Western blotting was used to detect the amount of synaptic structure proteins, Munc18-1, SNAP25, synaptophysin, and PSD-95 in the brain homogenate of 9-month-old APPtg mice (e–i). Haploinsufficiency of microglial MyD88 was associated with higher protein levels of synaptophysin and SNAP25 (*t* test; $n \geq 5$ per group)

(Schnöder et al., 2020). We further used Western blot analysis to quantify the levels of four synaptic structure proteins: Munc18-1, synaptophysin, SNAP-25, and PSD-95 in the brain homogenate of 9-month-old APP^{tg} littermate mice with different expression of MyD88. As shown in Figure 1e–g, protein levels of synaptophysin, and SNAP-25 in $APP^{tg}Myd88^{fl/wt}Cre^{+/-}$ mice were significantly higher than levels of these proteins derived from $APP^{tg}Myd88^{fl/wt}Cre^{-/-}$ littermate mice (*t* test, $p < .05$). Haploinsufficient expression of MyD88 in microglia tended to increase the protein level of PSD-95 in the brain as compared with MyD88-wildtype AD mice (Figure 1i; *t* test, $p = .096$).

3.2 | Haploinsufficient expression of MyD88 in microglia reduces Aβ load in the brain parenchyma and blood vessels of APP/PS1-transgenic mice

After observing that heterozygous deletion of MyD88 in microglia attenuated cognitive deficits of APP^{tg} mice but not of APP^{wt} littermates, we analyzed the effects of microglial MyD88 on Aβ load in the APP^{tg} mice, as Aβ is the key molecule leading to neurodegeneration in AD (Mucke & Selkoe, 2012). We stained brain sections from $APP^{tg}Myd88^{fl/wt}Cre^{+/-}$ and $APP^{tg}Myd88^{fl/wt}Cre^{-/-}$ mice with methoxy-XO4, which specifically recognizes β-sheet secondary structure of Aβ. We observed that haploinsufficiency of microglial MyD88 significantly reduced the area of methoxy-XO4 staining-positive Aβ plaques in both hippocampus and cortex of APP/PS1-transgenic mice (Figure 2a,b; *t* test, $p < .05$). We then used standard immunohistological and stereological *Cavalieri* methods to evaluate Aβ volume, adjusted relative to the volume of analyzed tissues, in the hippocampus of 9-month-old $APP^{tg}Myd88^{fl/wt}Cre^{+/-}$ and $APP^{tg}Myd88^{fl/wt}Cre^{-/-}$ mice. The volume of 7.67% ± 0.71% of $APP^{tg}MyD88^{fl/wt}Cre^{+/-}$ mice was significantly lower than that of $APP^{tg}MyD88^{fl/wt}Cre^{-/-}$ mice (10.83% ± 0.56%; Figure 2c,d; *t* test, $p < .05$).

To measure the amount of oligomeric Aβ in brain tissues, quantitative Western blot was performed as we did in previous studies (Schnöder et al., 2016, 2020). We observed that the protein level of dimeric but not monomeric Aβ in 9-month-old $APP^{tg}MyD88^{fl/wt}Cre^{+/-}$ mice was significantly lower than that in $APP^{tg}MyD88^{fl/wt}Cre^{-/-}$ littermates (Figure 2e,f; *t* test, $p < .05$). Dimer has been considered as a toxic species of Aβ aggregates (Shankar et al., 2008).

Aβ is deposited not only in the brain parenchyma, but also at blood vessels (Stakos et al., 2020). Blood circulation contributes to Aβ clearance (Roberts et al., 2014). APP/PS1-transgenic mice used in our study were not a typical animal model for amyloid angiopathy; however, we observed an impairment of vasculature in their brain (Decker et al., 2018). Thus, we examined the potential localization of Aβ at blood vessels. We did observe that Aβ deposited in and around cerebral blood vessels of APP/PS1-transgenic mice (Figure 2h). To quantify the blood vessels-associated Aβ, we isolated capillaries from the brain homogenate and detected Aβ in the tissue lysate. Interestingly, the protein level of dimeric Aβ in $APP^{tg}MyD88^{fl/wt}Cre^{+/-}$ mice was also significantly lower than that in $APP^{tg}MyD88^{fl/wt}Cre^{-/-}$ littermate mice (Figure 2i,j; *t* test, $p < .05$). As a negative experimental control,

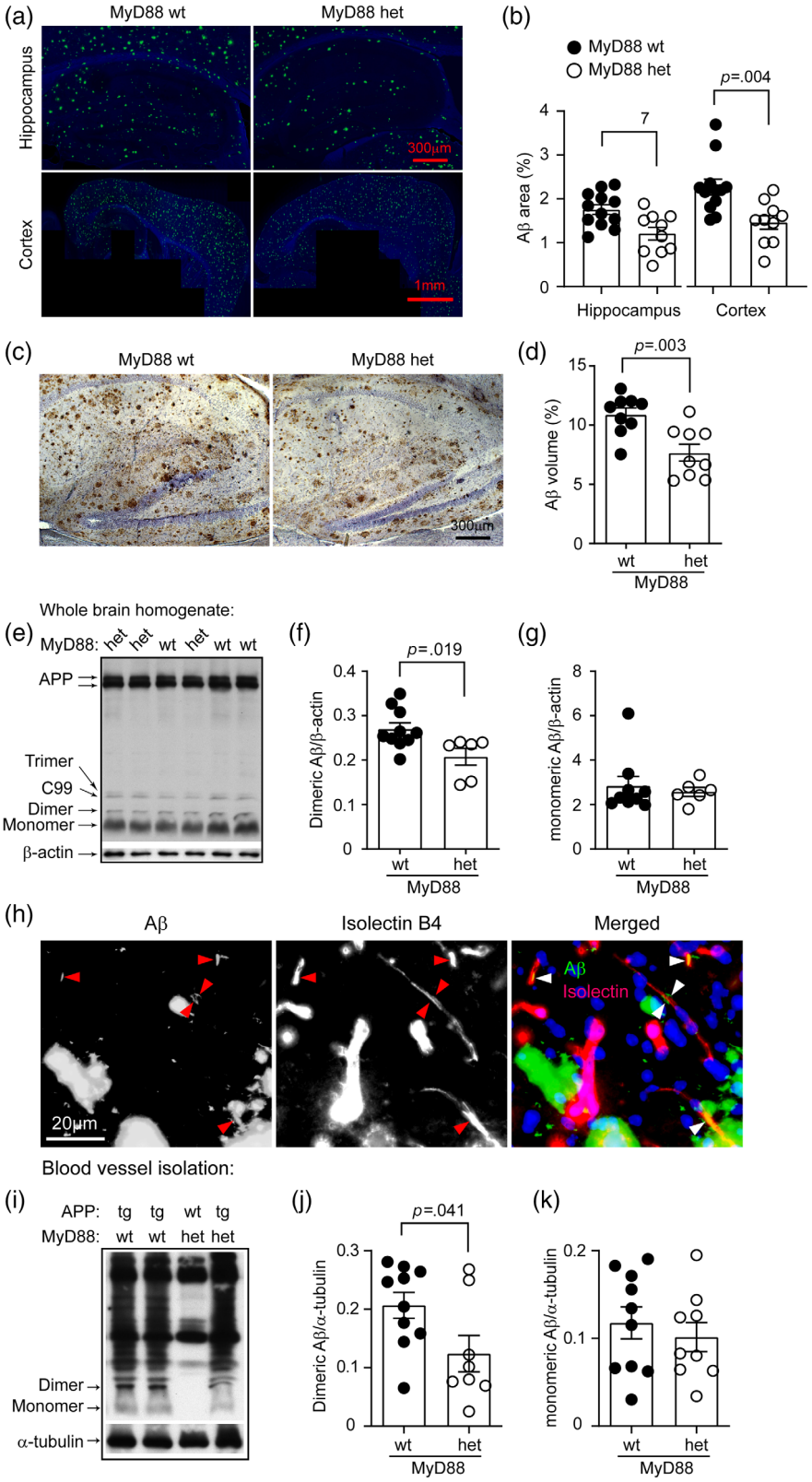

FIGURE 2 Haploinsufficiency of microglial MyD88 reduces cerebral Aβ load in APP/PS1-transgenic mice. Six-month-old $APP^{tg}MyD88^{fl/wt}Cre^{+/-}$ (MyD88 het) and $APP^{tg}MyD88^{fl/wt}Cre^{-/-}$ (MyD88 wt) were injected with tamoxifen and analyzed by 9 months of age for Aβ load in the brain. Brain sections were first stained with methoxy-XO4 and imaged with Microlucida (a). The area of Aβ plaques were quantified and adjusted by the area of analyzed brain tissue (b). Brain sections were further stained with human Aβ-specific antibodies (c) and the volume of immune reactive Aβ deposits in hippocampus were estimated with stereological *Cavalieri* method and adjusted by the volume of analyzed tissues (d). Heterozygous deletion of microglial MyD88 significantly decreases Aβ deposits in the brain (b,d; *t* test; $n \geq 9$ per each group). The brains derived from 9-month-old microglial MyD88-het and wt APP-transgenic mice were also homogenized in RIPA buffer for Western blot analysis of soluble Aβ (monomeric and dimeric) (e). To demonstrate the relationship between blood vessels and Aβ, brain sections were co-stained Aβ antibodies and isolectin B4. Some Aβ deposits are located in and around the vessels (h; marked with arrow heads). Thereafter, micro blood vessels were isolated from 9-month-old MyD88-het and wt APP-transgenic mice and homogenized in RIPA buffer for Western blot analysis of monomeric and dimeric Aβ. Haploinsufficiency of MyD88 significantly reduces dimeric Aβ in the whole brain homogenate and isolated cerebral blood vessels of APP-transgenic mice (f,g,j,k; *t* test; $n \geq 6$ per each group) [Color figure can be viewed at wileyonlinelibrary.com]

oligomeric Aβ species could not be detected in capillaries isolated from non-APP transgenic mice (Figure 2i).

In $APP^{tg}Myd88^{fl/wt}Cre^{+/-}$ mice, one allele of *cx3cr1* gene is replaced by the insertion of Cre-encoding sequence (Yona et al., 2013). To investigate the potential effects of heterozygous knockout of *cx3cr1* gene in $APP^{tg}Myd88^{fl/wt}Cre^{+/-}$ mice, we created APP/PS1-transgenic control mice with $APP^{tg}Gpr43^{fl/fl}Cre^{+/-}$ and $APP^{tg}Gpr43^{fl/fl}Cre^{-/-}$ of genotypes, in which *gpr43*, instead of *myd88* gene was loxP site-flanked. By detecting *gpr43* gene transcripts in CD11b-positive brain cells and staining RFP in the brain tissue of Gpr43-reporter mice, we observed that

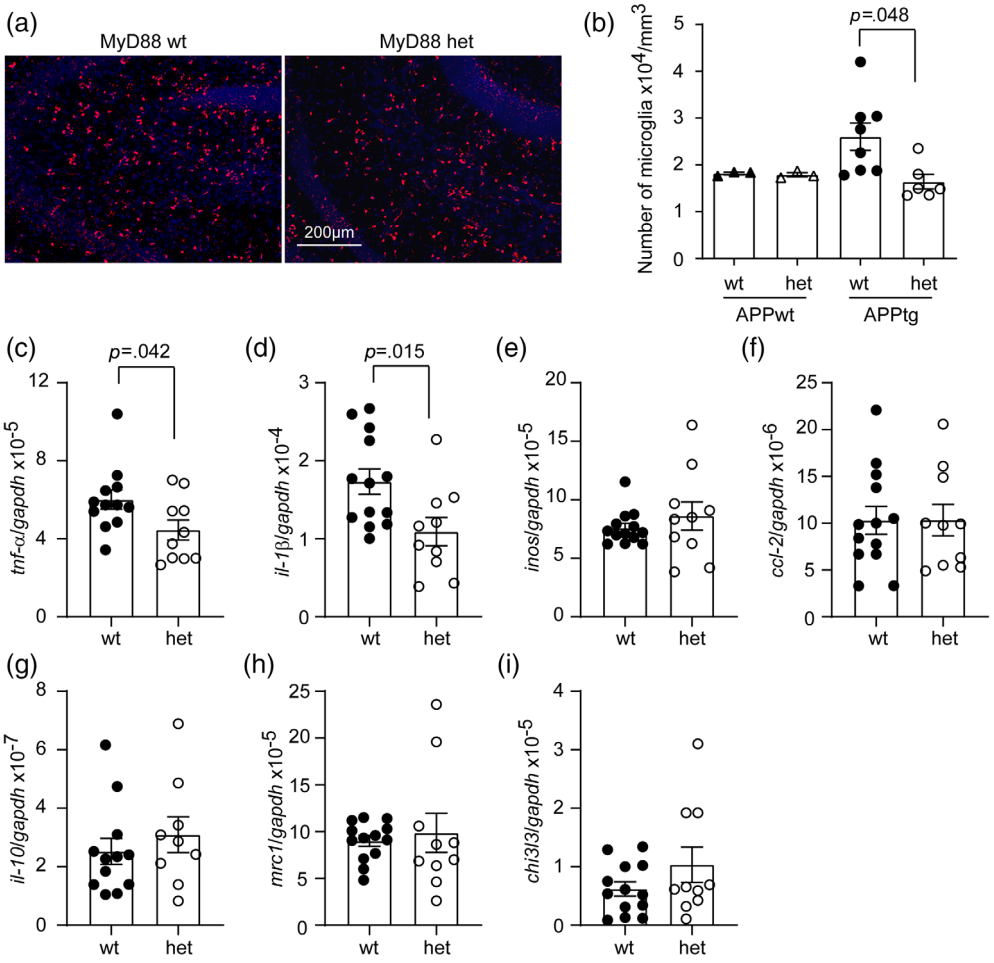

FIGURE 3 Haploinsufficiency of microglial MyD88 inhibits inflammatory activation in the brain of APP-transgenic mice. Six-month-old $APP^{tg}MyD88^{fl/wt}Cre^{+/-}$ (MyD88 het) and $APP^{tg}MyD88^{fl/wt}Cre^{-/-}$ (MyD88 wt) mice were injected with tamoxifen and analyzed at 9 months of age for neuroinflammation. Microglia were stained with fluorescence-conjugated Iba-1 antibodies (a) and counted with the stereological probe, Optical Fractionator. Haploinsufficiency of MyD88 reduced Iba-1-positive cells in 9-month-old APP-transgenic (APPtg) mice, but not in 9-month-old APP-wild-type (APPwt) mice (b; one-way ANOVA followed by Bonferroni post hoc test; $n \geq 6$ per group for APPtg mice and = 3 per group for APPwt mice). The inflammatory activation in brain was further analyzed with real-time PCR to detect transcripts of both pro- and anti-inflammatory genes (c–i). Transcription of pro-inflammatory genes, *tnf-α* and *il-1β*, was reduced by heterozygous deletion of MyD88 in 9-old APPtg mice (c and d; *t* test; $n \geq 9$ per group) [Color figure can be viewed at wileyonlinelibrary.com]

there was no expression (or expression at a very low level) of Gpr43 in microglia (see Figure S1a,b), which corroborated a previous observation (Erny et al., 2015). As Gpr43 was absent in microglia of both $APP^{tg}Gpr43^{fl/fl}Cre^{+/-}$ and $APP^{tg}Gpr43^{fl/fl}Cre^{-/-}$ mice, the phenotypical difference between these two groups of mice was caused by haploinsufficiency of Cx3Cr1. Surprisingly, with immunohistochemical methods, we did not detect changes of cerebral Aβ deposits in $APP^{tg}Gpr43^{fl/fl}Cre^{+/-}$ mice compared with $APP^{tg}Gpr43^{fl/fl}Cre^{-/-}$ littermate mice (see Figure S1c,d; *t* test, $p > .05$).

3.3 | Haploinsufficient expression of MyD88 in microglia inhibits pro-inflammatory activation in APP/PS1-transgenic mouse brain

Microglial inflammatory activation contributes to neuronal degeneration (Heneka et al., 2015). We observed that the number of Iba-1-immunoreactive cells (representing microglia) in the hippocampus was significantly fewer in 9-month-old microglial MyD88-haploinsufficient APP/PS1-transgenic mice than in MyD88-wild-type APP/PS1-transgenic littermates ($APP^{tg}Myd88^{fl/wt}Cre^{+/-}$ mice, $1.64 \pm 0.16 \times 10^4$ cells/mm^3 vs. $APP^{tg}Myd88^{fl/wt}Cre^{-/-}$ mice, $2.60 \pm 0.29 \times 10^4$ cells/mm^3; *t* test, $p < .05$; Figure 3a,b). Haploinsufficiency of MyD88 in microglia did not change the number of Iba-1-positive cells in 9-month-old non-APP transgenic mice (Figure 3b; *t* test, $p > .05$).

We further measured transcripts of M1-inflammatory genes (*tnf-α*, *il-1β*, *inos*, and *ccl-2*) and M2-inflammatory genes (*il-10*, *chi3l3*, and *mrc1*) in brains of 9-month-old $APP^{tg}Myd88^{fl/wt}Cre^{+/-}$ and $APP^{tg}Myd88^{fl/wt}Cre^{-/-}$ mice. As shown in Figure 3c,d, haploinsufficient expression of MyD88 in microglia significantly reduced cerebral *tnf-a* and *il-1β* transcripts in APP-transgenic mice compared to MyD88-wildtype AD littermate mice (*t* test, $p < .05$). However, the transcription of other genes tested was not changed by MyD88 deficiency in microglia (Figure 3e–i).

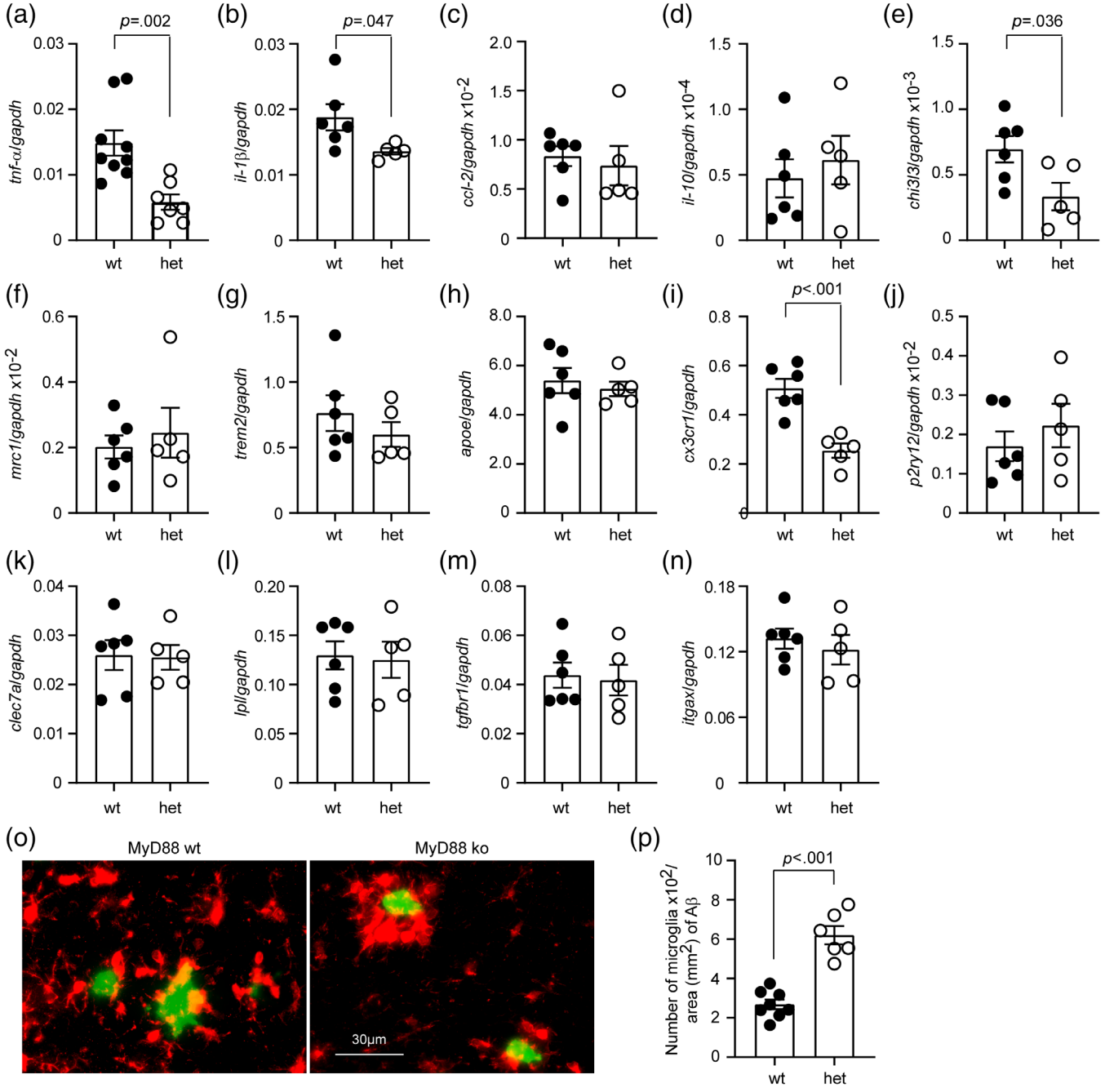

FIGURE 4 Haploinsufficiency of MyD88 inhibits pro-inflammatory activation in microglia and enhances microglial responses to Aβ deposits in the brain of APP-transgenic mice. Microglia were selected with magnetic beads-conjugated CD11b antibodies from 9-month-old tamoxifen-injected $APP^{tg}MyD88^{fl/wt}Cre^{+/-}$ (MyD88 het) and $APP^{tg}MyD88^{fl/wt}Cre^{-/-}$ (MyD88 wt) mice. Transcription of signature genes associated with neurodegenerative diseases were detected with real-time PCR. MyD88 deficiency significantly decreases transcripts of *tnf-α*, *il-1β*, *chi3l3*, and *cx3cr1* genes (a,b,e,i; *t* test; *n* ≥5 per group). Iba-1 was also co-stained with methoxy-XO4, which recognizes aggregated Aβ (o). Haploinsufficiency of MyD88 significantly increases recruitment of microglia toward Aβ deposits (p; *t* test; *n* ≥ 6 per each group) [Color figure can be viewed at wileyonlinelibrary.com]

In additional experiments, we also detected transcripts of inflammatory genes (*tnf-α*, *il-1β*, *ccl-2*, and *il-10*) in the brains of 9-month-old $APP^{tg}Gpr43^{fl/fl}Cre^{+/-}$ and $APP^{tg}Gpr43^{fl/fl}Cre^{-/-}$ mice, which were injected with tamoxifen by 6 months of age. As shown in Figure S1e–h, heterozygous deletion of *cx3cr1* gene did not change the inflammatory gene transcription in $APP^{tg}Gpr43^{fl/fl}Cre^{+/-}$ mice as compared with Cx3Cr1-wild-type $APP^{tg}Gpr43^{fl/fl}Cre^{-/-}$ mice (*t* test, $p > .05$).

3.4 | Haploinsufficient expression of MyD88 suppresses pro-inflammatory activation in microglia but enhances microglial responses to Aβ in APP/PS1-transgenic mice

Recently, disease-associated microglia (DAM) phenotype was defined after comparing microglial transcriptome between APP/PS1-transgenic

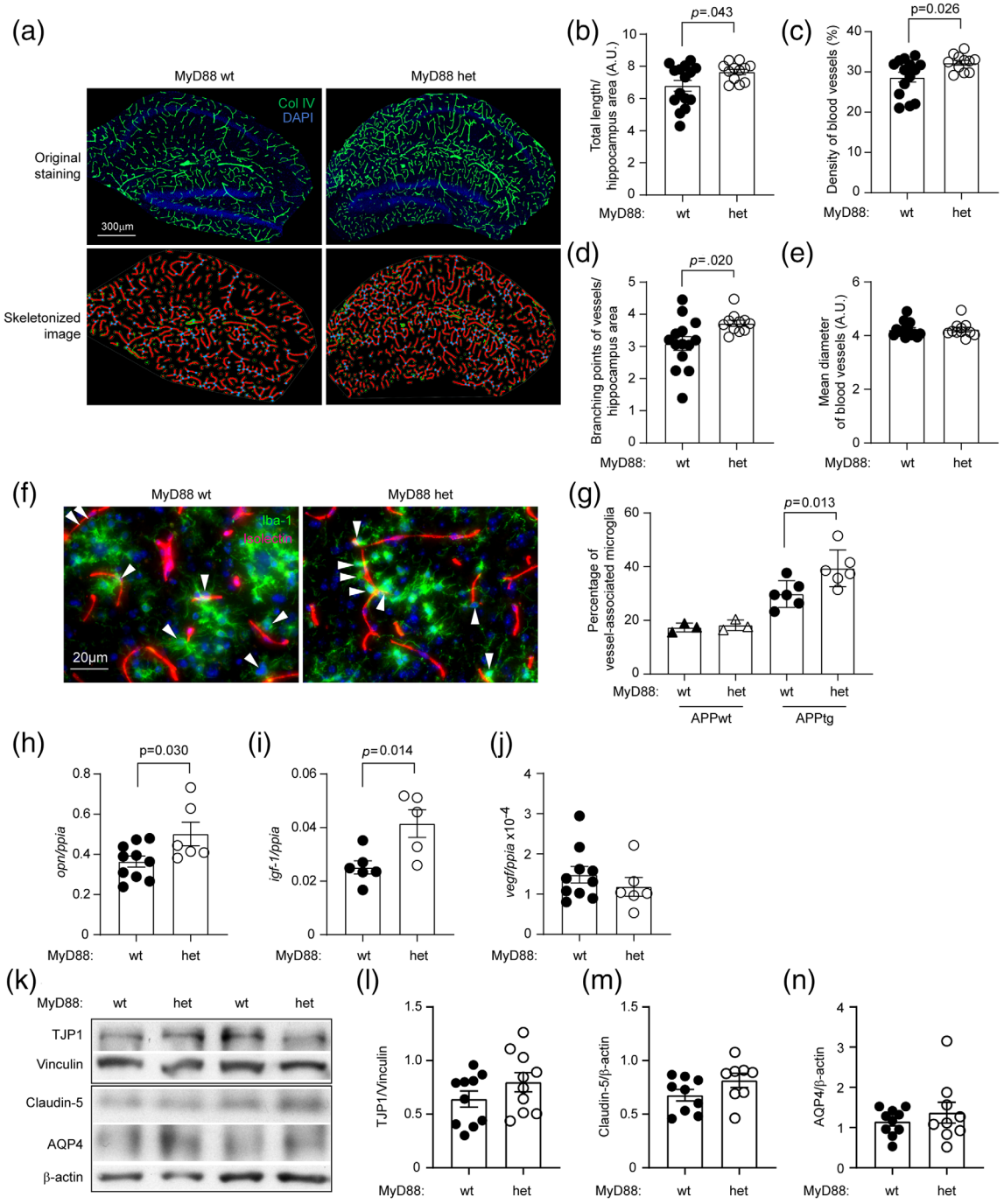

FIGURE 5 Haploinsufficiency of microglial MyD88 increases cerebral vasculature of APP/PS1-transgenic mice. The brains of 9-month-old tamoxifen-injected $APP^{tg}MyD88^{fl/wt}Cre^{+/-}$ (MyD88 het) and $APP^{tg}MyD88^{fl/wt}Cre^{-/-}$ (MyD88 wt) mice were stained for collagen type IV. The blood vessels in the hippocampus were thresholded and skeletonized. The skeleton representation of vasculature is shown in red and branching points of blood vessels are in blue (a) The total length, density and branching points of blood vessels were calculated and adjusted by area of analysis (b–d; *t* test, $n \geq 11$ per group). The mean diameter of blood vessels was calculated by dividing area of total blood vessels with total length of vessels (e). In order to analyze the relationship between vasculature and microglia, brain sections were co-stained with isolectin B4 (in red) and Iba-1 antibodies (in green) (f). Haploinsufficiency of MyD88 in microglia significantly increased the distribution of microglia around blood vessels (g; one-way ANOVA followed by Bonferroni post hoc test; $n = 6$ per group for APP-transgenic [tg] mice and = 3 per group for APP-wildtype [wt] mice). Moreover, CD11b-positive brain cells were quantified for the transcription of pro-angiogenic genes. The transcription of *opn* and *igf-1* genes, but not of *vegf* gene was significantly up-regulated by MyD88 deficiency (h,i; *t* test, $n \geq 5$ per group). In order to evaluate the integrity of BBB, isolated brain capillaries were detected for TJP1, Claudin-5, and AQP4 with quantitative Western blot (k). Haploinsufficiency of MyD88 in microglia did not significantly change the expression levels of all proteins tested (l–n; *t* test, $p > .05$, $n \geq 8$ per group) [Color figure can be viewed at wileyonlinelibrary.com]

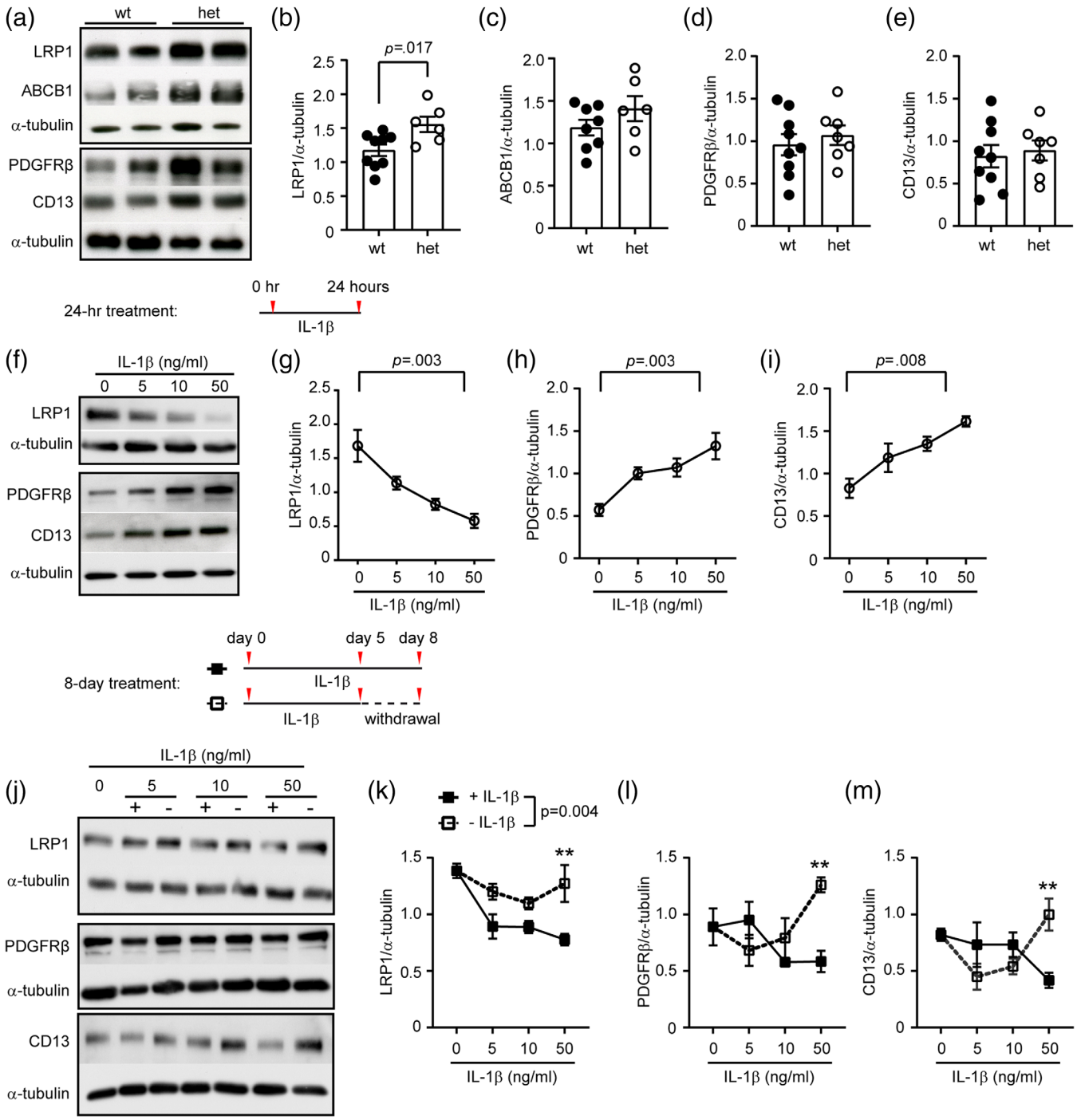

FIGURE 6 Haploinsufficiency of microglial MyD88 increases LRP1 in cerebral micro-vessels of APP/PS1-transgenic mice and IL-1β treatment decreases LRP1 in cultured pericytes. Micro blood vessels were isolated from the brains of 9-month-old $APP^{tg}MyD88^{fl/wt}Cre^{+/-}$ (MyD88 het) and $APP^{tg}MyD88^{fl/wt}Cre^{-/-}$ (MyD88 wt) mice, which were injected with tamoxifen 3 months ago. Protein levels of LRP1, ABCB1, PDGFRβ, and CD13 were determined with quantitative Western blot (a–e). Haploinsufficiency of MyD88 in microglia significantly elevates LRP1 protein level but not for other proteins tested, compared with MyD88-wildtype APP/PS1-transgenic mice (b; *t* test, $n \geq 6$ per group). Immortalized pericytes from human cerebral capillaries were cultured and treated with IL-1β at various concentrations for 24 hr (f–i) or for 8 days with and without withdrawal of IL-1β during the last 3 days (j–m). At the end of experiments, cell lysates were prepared from IL-1β-treated pericytes and detected for LRP1, PDGFRβ, and CD13 with quantitative Western blot. One-way ANOVA comparing levels of each tested protein at different concentrations of IL-1β shows that: (1) IL-1β treatments significantly decreases LRP1, but increases PDGFRβ and CD13 in a concentration-dependent manner after a 24-hr treatment (g–i; *p* values are shown in the figure); (2) IL-1β treatments significantly decreases LRP1 in a concentration-dependent manner after a 8-day treatment (k; $p < .001$); and (3) IL-1β treatments does not significantly changes protein levels of PDGFRβ and CD13 after a 8-day treatment (l,m). Two-way ANOVA comparing protein levels of LRP1, PDGFRβ, or CD13 with and without withdrawal of IL-1β in the last 3 days of experiments shows that withdraw of IL-1β recovers expression of LRP1 (k; $p = .004$), but not for PDGFRβ and CD13 (l,m). *t* test was used to analyze the difference of protein levels in cells treated with IL-1β at 50 ng/ml shows that withdrawal of IL-1β significantly recovers expression of LRP1, PDGFRβ, and CD13 (k–m; **: $p < .01$). $n = 3$ or 4 per group [Color figure can be viewed at wileyonlinelibrary.com]

and wild-type mice. This signature is characterized by an induction of pro-inflammatory genes (including *il-1β*, *ccl2*, and *itgax*), and a suppression of homeostatic genes (e.g., *cx3cr1*, *p2ry12*, *tmem119*, *tgfbr1*, *sall1*, and *csf1r*). APOE and TREM2 are two signaling proteins essential for DAM development (Keren-Shaul et al., 2017; Krasemann et al., 2017). We quantified the transcription of several DAM signature genes to characterize the effects of MyD88 on microglial activation. We observed that haploinsufficiency of MyD88 significantly decreased transcripts of pro-inflammatory genes, *tnf-α* and *il-1β* (Figure 4a,b; *t* test, $p < .05$), and also reduced the transcription of anti-inflammatory gene *chi3l3* (Figure 4e; *t* test, $p < .05$) and homeostatic gene *cx3cr1* (Figure 4i; *t* test, $p < .05$), as compared with MyD88-wildtype microglia.

In order to investigate the relationship between microglia and Aβ deposits, we co-stained brain sections of 9-month-old AD mice with Iba-1-specific antibodies and methoxy-XO4. There were significantly more microglia surrounding Aβ deposits in $APP^{tg}Myd88^{fl/wt}Cre^{+/-}$ mice ($6.21 \pm 0.47 \times 10^2$ cells/mm^2) than in $APP^{tg}Myd88^{fl/wt}Cre^{-/-}$ mice ($2.68 \pm 0.24 \times 10^2$ cells/mm^2; Figure 4o,p; *t* test, $p < .001$), which agrees with our previous observation in MyD88-deficient bone marrow chimeric APP-transgenic mice (Hao et al., 2011).

3.5 | Haploinsufficient expression of MyD88 in microglia increases cerebral vasculature in APP/PS1-transgenic mice

We observed that cerebral microvasculature is reduced in APP/PS1-transgenic mice (Decker et al., 2018). In this study, we asked whether haploinsufficiency of microglial MyD88 changed the vasculature in AD mouse brain. We stained brains of 9-month-old $APP^{tg}Myd88^{fl/wt}Cre^{+/-}$ and $APP^{tg}Myd88^{fl/wt}Cre^{-/-}$ mice with collagen type IV-specific antibodies, skeletonized and quantified the immunoreactive blood vessels (Figure 5a). As shown in Figure 5b–d, MyD88 deficiency in microglia significantly increased the total length, vessel density and branching points of micro-blood vessels (*t* test, $p < .05$), but did not change the mean diameter of blood vessels (Figure 5e; *t* test, $p > .05$).

As resident microglia serve pro-angiogenic effects in the brain (Brandenburg et al., 2016; Jiang et al., 2020; Mastorakos et al., 2021), we co-stained Iba-1 and isolectin B4 on brain sections of 9-month-old $APP^{tg}Myd88^{fl/wt}Cre^{+/-}$ and $APP^{tg}Myd88^{fl/wt}Cre^{-/-}$ mice and counted microglia with and without contact with blood vessels in CA1 area of the hippocampus (Figure 5f). We observed that haploinsufficiency of MyD88 significantly increased the distribution of microglia to blood vessels (Figure 5g; one-way ANOVA followed by *post-hoc* test, $p < .05$). Furthermore, we quantified gene transcription of pro-angiogenic genes in CD11b-positive brain cells. As shown in Figure 5h–j, MyD88 deficiency significantly up-regulated the transcription of *opn* and *igf-1* genes (*t* test, $p < .05$).

To evaluate the integrity of BBB in AD mice, we quantified TJP1, Claudin-5 and AQP4 in blood vessels isolated from 9-month-old $APP^{tg}Myd88^{fl/wt}Cre^{+/-}$ and $APP^{tg}Myd88^{fl/wt}Cre^{-/-}$ mice. As shown in Figure 5k–n, haploinsufficiency of MyD88 in microglia did not significantly alter the protein levels of all proteins tested (*t* test, $p > .05$).

3.6 | Haploinsufficient expression of MyD88 in microglia increases LRP1 in cerebral capillaries of APP/PS1-transgenic mice

LRP1 mediates Aβ efflux and local clearance by pericytes at BBB (Ma et al., 2018; Shinohara et al., 2017). We isolated micro-vessels from brains of 9-month-old $APP^{tg}Myd88^{fl/wt}Cre^{+/-}$ and $APP^{tg}Myd88^{fl/wt}Cre^{-/-}$ mice and observed that haploinsufficiency of MyD88 in microglia significantly increased protein levels of LRP1, but not ABCB1 in APP/PS1-transgenic mice, as compared with MyD88-wildtype AD mice (Figure 6a–c; *t* test, $p < .05$). The protein levels of pericyte markers, PDGFRβ, and CD13, were not changed by microglial deficiency of MyD88 (Figure 6d,e; *t* test, $p > .05$). In isolated blood vessels from non-APP-transgenic control mice ($APP^{wt}Myd88^{fl/wt}Cre^{+/-}$ and $APP^{wt}Myd88^{fl/wt}Cre^{-/-}$), haploinsufficiency of MyD88 in microglia did not alter the protein levels of all molecules examined (data not shown).

To examine the effects of inflammatory activation on LRP1 expression, we treated cultured pericytes with recombinant IL-1β either for 24 hr or for 8 days. As shown in Figure 6f,g,j,k, both short- and long-term treatments of IL-1β significantly decreased the protein levels of LRP1 in a dose-dependent manner (one-way ANOVA, $p < .05$). In the 8-day treatment experiment, withdrawal of IL-1β for the last 3 days restored expression of LRP1 in cultured pericytes (Figure 6j,k; two-way ANOVA, $p < .05$). The short-term treatment of IL-1β markedly increased expression of PDGFRβ and CD13, which corroborates our recent finding (Quan et al., 2020) (Figure 6f,h,i; one-way ANOVA, $p < .05$). The long-term treatment of IL-1β only at a high concentration (e.g., 50 ng/ml) tended to decrease the expression of PDGFRβ and CD13; however, it was not statistically significant (shown in Figure 6l,m with solid lines; one-way ANOVA, $p > .05$). The withdrawal of IL-1β recovered the expression of PDGFRβ and CD13 in cultured pericytes after treatment of IL-1β at 50 ng/ml (Figure 6l,m; *t* test comparing cells with and without treatment of IL-1β at 50 ng/ml, $p < .05$).

3.7 | Haploinsufficient expression of MyD88 in microglia decreases β- and γ-secretase activity but does not affect *neprilysin* and *ide* gene transcription in the brain of APP/PS1-transgenic mice

Cerebral Aβ level is determined by Aβ generation and clearance. We continued to ask whether MyD88-deficient microglia regulated Aβ production. Using our established protocols (Hao et al., 2011; Xie et al., 2013), we detected the activity of β- and γ-secretases in brains of 9-month-old $APP^{tg}Myd88^{fl/wt}Cre^{+/-}$ and $APP^{tg}Myd88^{fl/wt}Cre^{-/-}$ mice. Interestingly, the activity of both enzymes was significantly lower in the brain of $APP^{tg}Myd88^{fl/wt}Cre^{+/-}$ mice than in MyD88-wildtype $APP^{tg}Myd88^{fl/wt}Cre^{-/-}$ littermates (Figure 7a,b; two-way ANOVA, $p < .05$).

To further investigate the clearance of Aβ, we quantified gene transcripts of Aβ-degrading enzymes, *neprilysin*, and *ide* (Leissring et al., 2003). There were no changes in the transcription of *neprilysin*

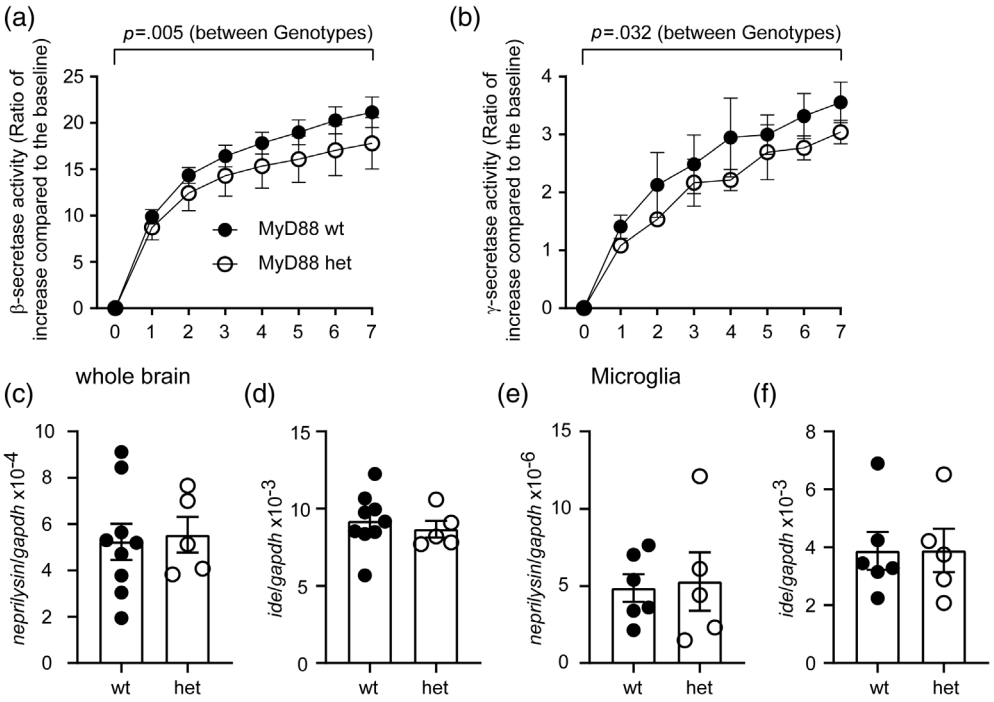

FIGURE 7 Haploinsufficiency of MyD88 in microglia decreases β- and γ-secretase activity, but does not affect the transcription of *neprilysin* and *ide* genes in the brain of APP/PS1-transgenic mice. The brains of 9-month-old tamoxifen-injected $APP^{tg}MyD88^{fl/wt}Cre^{+/-}$ (MyD88 het) and $APP^{tg}MyD88^{fl/wt}Cre^{-/-}$ (MyD88 wt) mice were used to prepare membrane components and RNA isolation. β- and γ-secretase activity was measured by incubating membrane components with fluorogenic β- and γ-secretase substrates, respectively (a,b, two-way ANOVA comparing MyD88 wt and het mice; $n = 4$ per group). The transcripts of *neprilysin* and *ide* genes in the brain tissue (c,e) and isolated microglia (d,f) were measured with real-time PCR, which showed that transcription of *neprilysin* and *ide* genes was not changed by the haploinsufficiency of MyD88 in microglia (*t* test; $n \geq 5$ per group)

and *ide* genes in both brain tissues and microglia from 9-month-old $APP^{tg}Myd88^{fl/wt}Cre^{+/-}$ mice compared with $APP^{tg}Myd88^{fl/wt}Cre^{-/-}$ mice (Figure 7c–f; *t* test, $p > .05$), suggesting that haploinsufficiency of MyD88 in microglia does not affect Aβ catabolism.

4 | DISCUSSION

Microglial activation has been extensively investigated in AD brain (Heneka et al., 2015), but microglial effects on Aβ pathology and neuronal degeneration remain inconclusive. In this study, we deleted one allele of *myd88* gene specifically in microglia in APP/PS1-transgenic mice by 6 months. Notably, by 9 months of age these animals showed attenuation both in the total number of microglia and in the transcription levels of pro-inflammatory genes (e.g., *tnf-α* and *il-1β*) within the whole brain and individual microglia, correlating with decreased Aβ load and improved cognitive function. Interestingly, MyD88-haploinsufficient microglia might prevent APP/PS1 overexpression-induced changes of cerebral vasculature and LRP1 expression at BBB.

The regulating effects of innate immune signaling on the role of microglia in AD pathogenesis are highly heterogenous. For example, TREM2 is essential for microglial response to Aβ in the brain (Ulland et al., 2017). One group reported that TREM2 deficiency in APP-transgenic mice increases hippocampal Aβ burden and accelerates neuron loss (Y. Wang et al., 2015), while another group showed that TREM2 deletion reduces cerebral Aβ accumulation (Jay et al., 2015). Subsequent work suggested that TREM2 may have a protective effect at the early disease stage through phagocytic clearance of Aβ, but display a pathogenic effect at the later disease stage by triggering neurotoxic inflammatory responses (Jay et al., 2017; Parhizkar et al., 2019). In our studies, we reduced MyD88 expression in microglia or in bone marrow cells of APP-transgenic mice after noticeable Aβ had already developed in the brain, which decreases cerebral Aβ load and protects neurons (Hao et al., 2011). In the experiments by other groups, MyD88 expression was manipulated in APP-transgenic mice before the birth (by cross-breeding) or at 2 months of age (by bone marrow reconstruction) before Aβ deposits appeared in the brain. With such an experimental setting, MyD88-deficient microglia promote Aβ accumulation in the brain and accelerate spatial memory deficits (Michaud et al., 2011, 2012). Thus, the pathogenic role of innate immune molecules in microglia is shaped by the evolving cellular environment and should be analyzed dynamically during AD progression.

MyD88, as a common signaling adaptor for most TLRs and IL-1 receptor, plays an essential role in the innate immune response (O'Neill, Golenbock, & Bowie, 2013). It is not surprising that the heterozygous deletion of *myd88* gene inhibits the inflammatory activation of microglia in APP/PS1-transgenic mice. MyD88 deficiency

might reduce the generation of new microglia, as we previously observed that deletion of IKKβ, a signaling molecule downstream to MyD88, decreases proliferating microglia in APP-transgenic mice (Y. Liu et al., 2014). It is unlikely that MyD88 deficiency induces microglial cell death in APP/PS1-transgenic mice, as blocking MyD88 signaling inhibits TLR4 activation-induced microglial apoptosis (Jung et al., 2005), while TLR4 is a receptor mediating microglial response to Aβ challenge (Walter et al., 2007). Interestingly, we observed that MyD88 deficiency promotes clustering of microglia around Aβ deposits as we observed in MyD88-deficient bone marrow chimeric AD mice (Hao et al., 2011). As deficiency of MyD88, IKK2, or TLR2 increases Aβ internalization by cultured microglia or macrophages (Hao et al., 2011; S. Liu et al., 2012; Y. Liu et al., 2014), the haploinsufficiency of MyD88 perhaps enhances microglial clearance of Aβ in AD brain. The relationship between microglial clustering and Aβ reduction in APP-transgenic mice has been described in many studies. For example, deficiency of TREM2 blocks microglial recruitment to Aβ, which is correlated with cerebral Aβ accumulation (Y. Wang et al., 2015). Administration of TREM2 agonist antibodies increases Aβ-associated microglia, which decreases Aβ in the brain (Fassler, Rappaport, Cuno, & George, 2021; Price et al., 2020; S. Wang et al., 2020). However, the molecular mechanisms, which mediate the migration of microglia toward Aβ and the following Aβ internalization, remain unclear.

The haploinsufficiency of MyD88 strongly decreases the transcription of *cx3cr1*, *chi3l3*, *tnf-α*, and *il-1β* genes in microglia of our APP/PS1-transgenic mice. Cx3Cr1 expression is up-regulated in the brain of AD patients or animal models (Gonzalez-Prieto et al., 2021). Deficiency of Cx3Cr1 decreases cerebral Aβ in various APP-transgenic mice (Hickman, Allison, Coleman, Kingery-Gallagher, & El Khoury, 2019; Lee et al., 2010; Z. Liu, Condello, Schain, Harb, & Grutzendler, 2010). Homozygous knockout of *cx3cr1* gene was also observed to increase Aβ deposits-associated microglia and microglial phagocytosis of Aβ (Z. Liu et al., 2010). However, it should be noted that the reduction of *cx3cr1* transcription in our $APP^{tg}Myd88^{fl/wt}Cre^{+/-}$ mice might be caused by the replacement of endogenous *cx3cr1* gene by Cre-encoding sequence (Yona et al., 2013). To address the question whether Cx3Cr1 haploinsufficiency in $APP^{tg}Myd88^{fl/wt}Cre^{+/-}$ mice affects amyloidosis, we created $APP^{tg}Gpr43^{fl/fl}Cre^{+/-}$ and $APP^{tg}Gpr43^{fl/fl}Cre^{-/-}$ mice, in which *gpr43* instead of *myd88* gene was loxP site-flanked. As Gpr43 is not expressed in microglia in both $APP^{tg}Gpr43^{fl/fl}Cre^{+/-}$ and $APP^{tg}Gpr43^{fl/fl}Cre^{-/-}$ mice (Erny et al., 2015), any changes in $APP^{tg}Gpr43^{fl/fl}Cre^{+/-}$ mice relative to $APP^{tg}Gpr43^{fl/fl}Cre^{-/-}$ littermates should be due to the haploinsufficiency of Cx3Cr1. Our experiments revealed that the haploinsufficiency of Cx3Cr1 alters neither Aβ deposition nor transcription of inflammatory genes (e.g., *tnf-α*, *il-1β*, *ccl-2*, and *il-10*) in the brain of our APP/PS1-transgenic mice. Thus, the pathological improvement in $APP^{tg}Myd88^{fl/wt}Cre^{+/-}$ mice is a result from the haploinsufficiency of MyD88 instead of Cx3Cr1 deficiency in microglia.

Chi3l3 is a known marker for alternative activation of microglia and macrophages. Its transcription is also elevated in APP-transgenic mouse brain (Colton et al., 2006). However, the pathogenic function of microglial Chi3l3 in AD mice is indeed unknown. Our serial studies revealed that neuroinflammation and Aβ level are often simultaneously attenuated in the brain of APP-transgenic mice (Hao et al., 2011; S. Liu et al., 2012; Y. Liu et al., 2014). As pro-inflammatory activation inhibits phagocytosis of cultured microglia (Koenigsknecht-Talboo & Landreth, 2005), inhibition of proinflammatory activation in microglia might help Aβ clearance in AD brain. However, systemic injection of TLR4 or TLR9 agonist induces both pro- and anti-inflammatory activation and decreases Aβ in the brain of AD mice (Michaud et al., 2013; Scholtzova et al., 2014). Thus, how inflammatory activation regulates Aβ clearance in the brain is still an unanswered question.

There is growing evidence showing that microvascular circulation is damaged in AD brain; for example, capillary density and cerebral blood flow decrease, while BBB permeability increases (Watanabe et al., 2020). Our previous study showed that the blood flow goes down in correlation with a reduced vasculature in the hippocampus of APP-transgenic mice (Decker et al., 2018). Aβ-activated perivascular macrophages injure the neurovascular coupling through producing reactive oxygen species (Park et al., 2017). However, microglia were observed to serve pro-angiogenic effects in brains with glioma, ischemia, or direct vascular injury (Brandenburg et al., 2016; Jiang et al., 2020; Mastorakos et al., 2021). Transcription of *opn*, *vegf* and *igf1* genes in microglia is associated with angiogenesis (Jiang et al., 2020). Our study showed that haploinsufficiency of MyD88 in microglia increases cerebral vasculature, and distribution of microglia around blood vessels. MyD88 deficiency also up-regulates the transcription of *opn* and *igf-1* genes in microglia. As OPN enhances VEGF expression in endothelial cells (Dai et al., 2009) and IGF-1 drives the tissue repairment, including angiogenesis, in the brain (Vannella & Wynn, 2017), MyD88-haploinsufficient microglia might prevent vascular impairment in AD brain. However, a postmortem tissue study showed a higher density of capillaries in the brain of AD patients (Fernandez-Klett et al., 2020). Tg4510 tau-transgenic mice display increased capillaries, but with atypical and spiraling morphologies, and reduced luminal diameter of blood vessels (Bennett et al., 2018). Thus, more studies, especially functional analysis of the effects of microglia on the microvascular circulation in AD, are required.

BBB breakdown is an early biomarker of AD (Nation et al., 2019). *APOE4* variant was recently linked to the loss of BBB integrity before the cognitive deficit (Montagne et al., 2020). The cerebrovascular leakage of ~100 nm nanoparticles was observed in APP-transgenic mice (Tanifum, Starosolski, Fowler, Jankowsky, & Annapragada, 2014). However, the effects of microglia on BBB integrity in AD brain remain unclear. In a mouse model of systemic lupus erythematosus, microglial activation around blood vessels protects BBB at the initial phase by expressing tight-junction protein Claudin-5, and impairs BBB by phagocytosing astrocytic end-feet after the inflammation is sustained (Haruwaka et al., 2019). In our study, we did not observe altered protein levels of TJP1, Claudin-5, and AQP-4 in microglial MyD88-haploinsufficient AD mice, although we were not able to exclude small damages in BBB integrity.

LRP1 contributes to Aβ clearance at BBB through mediating Aβ efflux and pericyte internalization of Aβ (Ma et al., 2018; Shinohara

et al., 2017). Deletion of LRP1 in endothelial cells accumulate Aβ in APP-transgenic mouse brain (Storck et al., 2016). LRP1 expression decreases in brain capillaries with aging and in AD (Shibata et al., 2000). Pro-inflammatory cytokines, such as IL-1β, IL-6, and TNF-α, down-regulate LRP1 in cultured microvascular endothelial cells (Hsu, Rodriguez-Ortiz, Zumkehr, & Kitazawa, 2021). Our experiments showed that LRP1 expression is decreased by IL-1β treatments in cultured pericytes. In APP/PS1-transgenic mice, MyD88-haploinsufficient microglia elevates LRP1 protein level in cerebral capillaries, which might be due to the inflammatory inhibition. It was supported by another observation that inflammatory activation in the brain by systemic administration of lipopolysaccharide decreases Aβ efflux at BBB (Erickson, Hansen, & Banks, 2012). PDGFRβ and CD13 are two receptors on pericytes and essential for the survival and integration of pericytes in blood vessels (Lindahl, Johansson, Leveen, & Betsholtz, 1997; Rangel et al., 2007). The expression of PDGFRβ and CD13 appeared to be resistant to inflammatory regulation, as haploinsufficiency of MyD88 did not change their protein levels in AD mice. Their expression in cultured pericytes was not altered, either, by IL-1β at the concentrations sufficient for the down-regulation of LRP1 expression. There was a recent study showing that the density of pericytes is reserved during AD pathogenesis (Fernandez-Klett et al., 2020).

Aβ is produced after serial digestions of APP by β-(BACE1) and γ-secretases (Haass, Kaether, Thinakaran, & Sisodia, 2012). The expression of BACE1 in neurons is up-regulated by inflammatory activation (He et al., 2007; Sastre et al., 2006). Our studies showed that p38α-MAPK deficiency promotes BACE1 degradation in neurons (Schnöder et al., 2016). Recently, inflammatory cytokines, such as interferon-γ and α, were shown to induce the expression of interferon-induced transmembrane protein 3 in neurons and astrocytes, which binds to γ-secretase and increases its activity (Hur et al., 2020). Thus, haploinsufficient expression of MyD88 in microglia in our APP/PS1-transgenic mice decreases neuroinflammation, and inhibits β- and γ-secretase activity in the brain, which might serve as another mechanism decreasing Aβ level in AD mice.

In summary, haploinsufficient expression of MyD88 in microglia at a late disease stage slows down the cognitive decline of APP/PS1-transgenic mice. MyD88 deficiency inhibits pro-inflammatory activation of microglia, but enhances microglial response to Aβ, which subsequently attenuates Aβ load in the brain. Haploinsufficiency of MyD88 might enhance pro-angiogenic effects of microglia, and prevent the loss of LRP1-mediated Aβ clearance at BBB in AD. However, the effects of microglia on the structure and function of micro-vessels in AD are far from being understood. To answer these questions will be a focus of our following studies.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest with the contents of this article.

AUTHOR CONTRIBUTIONS

Yang Liu: conceptualized and designed the study, acquired funding, conducted experiments, acquired and analyzed data, and wrote the manuscript. **Wenqiang Quan, Qinghua Luo, Wenlin Hao, and Inge Tomic:** conducted experiments, acquired data, and analyzed data. **Tomomi Furihata:** provided pericyte cell line. **Walter Schulz-Schäffer:** provided technical support. **Michael D. Menger:** offered animal facility and supervised the study. **Klaus Fassbender:** acquired funding and supervised the study. All authors contributed to the article and approved the submitted version.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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