NLRP3 maintains healthy pericytes in the brain

Wenqiang Quan
Universitat des Saarlandes
Qinghua Luo
Universitat des Saarlandes
Qiqiang Tang
University of Science and Technology College of Medicine and Health Sciences
Tomomi Furihata
Tokyo Toshi Daigaku
Dong Li
Tongji University School of Medicine
Klaus Fassbender
Universitat des Saarlandes
Yang Liu (a.liu@mx.uni-saarland.de)
Universitat des Saarlandes

Research

Keywords: Alzheimer's disease, Neuroinflammation, NLRP3, Pericyte

Posted Date: December 13th, 2019

DOI: https://doi.org/10.21203/rs.2.18793/v1

Abstract

Background Pericytes regulate structure and function of cerebral capillaries. Growing evidence shows that pericytes are damaged in the brain of Alzheimer's disease (AD), which potentially contributes to AD pathogenesis. NLRP3-contained inflammasome is activated in AD brain and considered as a promising target for therapy. However, how NLRP3 affects brain pericytes is unclear. In our study, we investigated physiological function of NLRP3 in pericytes.

Methods Immunohistological methods and Western blot were used to investigate pericytes and vasculature in the brains of 9-month-old NLRP3-deficient and wild-type littermate mice. Pericytes were also cultured and treated with NLRP3 inhibitor, recombinant IL-1β and AKT inhibitor. Then, proliferation, apoptosis and expression of PDGFRβ and CD13 in pericytes were analysed with biochemical methods. To investigate underlying molecular mechanisms, phosphorylation of protein kinases such as AKT, ERK and NF- k B were quantified.

Results We observed that NLRP3 deficiency reduced the coverage of PDGFRβ-positive pericytes and collagen type IV-immunereactive vasculature in the brain. NLRP3 deficiency was also shown to decrease PDGFRβ and CD13 proteins in isolated cerebral microvessels. In cultured pericytes, inhibition of NLRP3 with MCC950 attenuated cell proliferation but did not induce apoptosis. NLRP3 inhibition also decreased protein levels of PDGFRβ and CD13. On the contrary, treatments with IL-1β increased protein levels of PDGFRβ and CD13 in pericytes. The alteration of PDGFRβ and CD13 protein levels was correlated with phosphorylation of AKT. Inhibition of AKT reduced PDGFRβ and CD13 in cultured pericytes.

Conclusions NLRP3 might be essential to maintain healthy pericytes in the brain through activating AKT. Adverse effects on brain pericytes should be considered in the possible clinical therapies with NLRP3 inhibitors.

Background

Brain pericytes wrapping around endothelial cells regulate various functions in the brain, which include blood-brain barrier (BBB) permeability, angiogenesis, and capillary hemodynamic responses [1]. Pericytes express platelet-derived growth factor receptor β (PDGFRβ) and CD13. The binding of PDGFRβ with endothelial cells-released platelet-derived growth factor (PDGF)-B is essential for pericyte proliferation and integration into the blood vessel [2]. CD13 promotes angiogenesis in hypoxic tissues, and response to stimulation of angiogenic growth factors, such as vascular endothelial growth factor, basis fibroblast growth factor, and transforming growth factor [3]. Deficiency of PDGFRβ decreases pericyte number, accumulates blood-derived fibrin/fibrinogen, reduces vasculature and attenuates blood flow in the mouse brain, which finally leads to the white matter lesions characterized by loss of oligodendrocytes, demyelination, and axonal degeneration [4]. Growing evidence suggests that pericyte impairment mediates vascular dysfunction and contributes to pathogenesis of Alzheimer's disease (AD) [5]. In AD human brain, pericytes are lost in association with increased BBB permeability at very early disease stage

[6, 7]. In AD mouse models that overexpress Alzheimer's precursor protein (APP) in neurons, deletion of pericytes increases deposition of amyloid β peptide (Aβ) in both brain parenchyma and blood vessels, which potentially exaggerates cognitive deficits [8]. However, molecular mechanisms that regulate pericyte survival and activation in the brain are largely unknown.

Pericytes express pattern recognition receptors, such as Toll-like receptor 2 and 4 (TLR-2 and − 4) and NACHT, LRR and PYD domains-containing protein 1 and 3 (NLRP-1 and − 3) [9–11]. Cultured brain pericytes release cytokines and chemokines after being challenged with lipopolysaccharide (LPS), tumor necrosis factor (TNF)-α or E. coli infection [9, 10, 12]. Cultured pericytes secret active interleukin (IL)-1β when they are intracellularly stimulated with LPS, although how NLRP3-contained inflammasome is activated remains unclear [9]. It is interesting to ask whether innate immune signaling regulates cellular fate and functions of pericytes in the brain.

In AD research, NLRP3-contained inflammasome attracted great attention, as it is activated in AD brain and potentially mediates microglial inflammatory responses, exaggerates Aβ and Tau protein aggregation in APP or Tau-transgenic mice [13–16]. NLRP3 is considered as a promising therapeutic target for AD patients [17]. However, effects of NLRP3 activation on pericytes and vascular dysfunction were not addressed. The animal models used in published studies have also limited AD-associated vascular pathology. Thus, whether NLRP3 inhibition protects or damages microvascular circulation in AD brain remains unclear. Moreover, AD pathology is mainly localized in temporal and parietal lobes, instead of covering the whole brain. Between AD lesion sites as shown with Aβ deposits, neurofibrillary tangles and gliosis, the brain tissues are relatively or absolutely healthy [18]. It is, therefore, necessary to understand the physiological functions of NLRP3 in brain pericytes, which is helpful to predict potential off-target effects of NLRP3 inhibitors in the future anti-AD therapies.

In this study, we used NLRP3-knockout mice and treated cultured pericytes with NLRP3 inhibitor, MCC950, or IL-1β, a major product of NLRP3-contained inflammasome. We observed that NLRP3 might be essential for the maintenance of healthy pericytes in the brain. We further observed that AKT (also known as protein kinase B) might mediate the physiological function of NLRP3 in pericytes.

Materials And Methods

Mice

NLRP3-encoding gene knockout ($NLRP3^{-/-}$) mice were kindly provided by N. Fasel (University of Lausanne, Lausanne, Switzerland) [19]. Mice with knockout of gene encoding myeloid differentiation primary response 88 ($MyD88^{-/-}$) were originally provided by S. Akira and K. Takeda (Osaka University, Osaka, Japan) [20]. Breeding between heterozygous mutants (+/-) on a C57BL/6 background were used to maintain mouse colonies. Mice were compared only between littermates. Animal experiments were performed in accordance with all relevant national rules and were authorized by the local research ethical committee.

Tissue collection and isolation of blood vessels

Animals were euthanized by inhalation of isofluorane and perfused with ice-cold phosphate-buffered saline. The brain was removed and divided. The left hemisphere was immediately fixed in 4% paraformaldehyde (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) in phosphate-buffered saline and embedded in paraffin for immunohistochemistry. The cortex and hippocampus were carefully dissected from the right hemisphere, snap-frozen in liquid nitrogen and stored at -80°C for biochemical analysis. Cortex and hippocampus were also used for isolation of brain vessel fragments according to the published protocol [21]. 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) to delete myelin. The vessel pellet was re-suspended in HEPES-HBSS buffer supplemented with 1% bovine serum albumin (Sigma-Aldrich) and filtered with 20 μm -mesh. The blood vessel fragments were collected on the top of filter and frozen at -80°C for further biochemical analysis.

Histological image acquisition and analysis

Serial 40-μm-thick sagittal sections were cut from the paraffin-embedded hemisphere. Four serial sections per mouse with 400μm of interval between two neighboring sagittal sections were stained with rabbit anti-PDGFRβ monoclonal antibody (clone: 28E1; Cell Signaling Technology Europe, Frankfurt am Main, Germany) and Alexa488-conjugated goat anti-rabbit IgG (Thermo Fisher Scientific, Darmstadt, Germany). The coverage of PDGFRβ staining-positive cells in the whole hippocampus and cortex was estimated with the *Cavalieri* method on a Zeiss AxioImager.Z2 microscope (Carl Zeiss Microscopy GmbH, Göttingen, Germany) equipped with a Stereo Investigator system (MBF Bioscience, Williston, VT, USA). The grid size was set at 10 μm, which provided coefficient of error estimates of < 0.05.

To quantify vasculature in the brain, our established protocol was used [22]. Briefly, 4 serial paraffin-embedded sections per mouse were deparaffinized, heated at 80°C in citrate buffer (10mM, pH = 6) for 1 hour and digested with Digest-All 3 (Pepsin) (Thermo Fisher Scientific) for 20 minutes. Thereafter, brain sections were stained with rabbit anti-collagen IV polyclonal antibody (Catalog: # ab6586; Abcam, Cambridge, UK) and Alexa488-conjugated goat anti-rabbit IgG (Thermo Fisher Scientific). After being mounted, the whole brain including hippocampus and cortex was imaged with Microlucida (MBF Bioscience). The length and branching points of collagen type IV staining-positive blood vessels were analyzed with a free software, AngioTool (http://angiotool.nci.nih.gov) [23]. The parameters of analysis for all compared samples were kept constant. The length and branching points were adjusted with area of interest.

Western blot analysis of PDGFRβ and CD13 in cerebral blood vessels

Isolated blood vessels were lysed in RIPA buffer (50mM Tris [pH 8.0], 150mM NaCl, 0.1% SDS, 0.5% sodiumdeoxy-cholate, 1% NP-40, and 5mM EDTA) supplemented with protease inhibitor cocktail (Roche Applied Science, Mannheim, Germany) on ice. The tissue lysate was sonicated before being loaded onto

10% SDS-PAGE. For Western blot detection, rabbit monoclonal antibodies against PDGFRβ and CD13/APN (clone: 28E1 and D6V1W, respectively; Cell Signaling Technology Europe) were used. In the same sample, β-actin was detected as a loading control using rabbit monoclonal antibody (clone: 13E5; Cell Signaling Technology Europe). Western blots were visualized via the ECL method (PerkinElmer LAS GmbH, Rodgau, Germany). Densitometric analysis of band densities was performed with ImageJ software (https://imagej.nih.gov/ij/). For each sample, the protein level was calculated as a ratio of target protein/β-actin.

Culture of pericytes

Human primary brain vascular pericytes (HBPC) were immortalized by infecting cells with tsSV40T lentiviral particles [24]. 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, USA) 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.

Analysis of pericyte proliferation and apoptosis

Pericytes were seeded at 1.0×10^4 cells on 96-well plate /100μl (day 0), and cultured in pericyte medium containing NLRP3 inhibitor, MCC950 (Catalog: # PZ0280; Sigma-Aldrich), at 0, 25, 50 and 100 nM. The cell survival was detected with MTT-based Cell Proliferation Kit I (Catalog: # 11465007001; Sigma-Aldrich) on days 1, 2, 3, 4, 5, 6 and 7. In order to further detect cell death and proliferation of pericytes, cells were cultured in 12-well plate at 5.0×10^5 cells/well, and treated with MCC950 as described in MTT assay. After 24 hours, pericytes were collected and lysed in RIPA buffer. Quantitative Western blot was used with rabbit monoclonal antibody against cleaved caspase-3 (clone: 5A1E; Cell Signaling Technology Europe), mouse monoclonal antibody against proliferating cell nuclear antigen (PCNA) (clone: PC10; Cell Signaling Technology Europe) and rabbit monoclonal antibody against Ki-67 (clone: SP6; Abcam). α-tubulin and β-actin were detected as an internal control with mouse monoclonal antibody (clone: DM1A; Abcam) and rabbit monoclonal antibody (clone: 13E5; Cell Signaling Technology Europe), respectively.

Treatments of pericytes for detection of PDGFRβ and CD13 and phosphorylated kinases

Pericytes were cultured in 12-well plate at 5.0×10^5 cells/well. Before experiments, we replaced culture medium with serum-free pericyte medium and cultured cells at 37°C for 3 days to facilitate cell differentiation [24]. Thereafter, pericytes were treated for 24 hours with MCC950, at 0, 25, 50 and 100 nM, recombinant human IL-1β (Catalog: # 201-LB; R&D Systems, Wiesbaden, Germany) at 0, 5, 10 and 50 ng/ml, or AKT Inhibitor VIII (Catalog: # 124018; Sigma-Aldrich) at 0, 0.5, 1 and 5 μM. Cell lysate was prepared in RIPA buffer supplemented with protease inhibitor cocktail (Roche Applied Science) and phosphatase inhibitors (50 nM okadaic acid, 5 mM sodium pyrophosphate, and 50 mM NaF; Sigma-Aldrich). For Quantitative Western blot, the following antibodies were used: rabbit monoclonal antibodies

against PDGFRβ, CD13/APN, phosphorylated AKT (Ser473), phosphorylated ERK1/2 (Thr202/Tyr204), phosphorylated NFkB p65 (S536), NFkB p65, β-actin, GAPDH (clone: 28E1, D6V1W, D9E, D13.14.4E, 93H1, D14E12, 13E5, and 14C10, respectively; Cell Signaling Technology Europe), rabbit polyclonal antibodies against AKT and phosphorylated GSK-3β (Ser9) (Catalog: # 9272 and Catalog: # 9336, respectively; Cell Signaling Technology Europe) and mouse monoclonal antibodies against ERK1/2 and GSK-3β (clone: L34F12 and 3D10, respectively; Cell Signaling Technology Europe) and α-tubulin (clone: DM1A; Abcam).

Statistics

Data was presented as mean ± SEM for mice and mean ± SD for cells. For multiple comparisons, one-way or two-way ANOVA followed by Bonferroni or Tukey *post hoc* test. Two independent-samples Students *t* test was used to compare means for two groups of cases. All statistical analyses were performed with GraphPad Prism 5 version 5.01 for Windows (GraphPad Software, San Diego, CA, USA). Statistical significance was set at $p < 0.05$.

Results

NLRP3 deficiency reduces pericyte cell coverage and decreases protein levels of PDGFRβ and CD13 in the brain.

To explore effects of NLRP3 on the maintenance of pericytes in the brain, we estimated the coverage of PDGFRβ-positive cells in brains from 9-month-old NLRP3-knockout ($NLRP3^{-/-}$) and wild-type ($NLRP3^{+/+}$) littermate mice. As shown in Fig. 1, A and B, deficiency of NLRP3 significantly decreases the coverage of pericytes (t test, $p < 0.01$; $n = 10$ per group). We then isolated blood vessels from brains of 9-month-old $NLRP3^{-/-}$, $NLRP3^{+/-}$ and $NLRP3^{+/+}$ littermate mice for the detection of pericyte protein markers, PDGFRβ and CD13. We observed that deletion of NLRP3 significantly reduces PDGFRβ and CD13 proteins in the cerebral blood vessels in a gene dose-dependent manner (Fig. 1, C and D; one-way ANOVA, $p < 0.05$; $n \geq 7$ per group). Unfortunately, we failed to detect cleaved caspase-3 and PCNA in the tissue lysate of blood vessels (data not shown), which are markers for cell apoptosis and cell proliferation, respectively.

In further experiments, we asked whether innate immune signaling serves a common effect on pericyte survival in the brain. We detected PDGFRβ and CD13 proteins in cerebral blood vessels isolated from 6-month-old $MyD88^{-/-}$, $MyD88^{+/-}$ and $MyD88^{+/+}$ littermate mice. MyD88 is a common adaptor down-stream to most TLRs and also mediates the inflammatory activation of IL-1β [25]. We observed that protein levels of CD13 and PDGFRβ were significantly lower in MyD88-deficient mice than in MyD88-wildtype controls (Fig. 1, E and F; one-way ANOVA, $p < 0.05$; $n \geq 6$ per group).

NLRP3 deficiency reduces vasculature in the brain.

Pericytes are essential for the development of cerebral circulation. Dysfunction of pericytes reduces vasculature [1, 4]. We asked whether NLRP3 deficiency affects the structure of cerebral blood vessels. We observed that, in 9-month-old mouse brains, deficiency of NLRP3 significantly reduced the total length and branching pints of collagen type IV-positive blood vessels (Fig. 2, A - C; one-way ANOVA, $p < 0.05$; $n \geq 6$ per group). The reduction of brain vasculature was dependent on the copies of NLRP3-encoding gene.

NLRP3 inhibition attenuates cell proliferation in cultured pericytes.

After we observed that NLRP3 deficiency decreased the number of pericytes in the brain, we continued to investigate underlying mechanisms mediating the effects of NLRP3 on pericytes. After treating cultured pericytes with NLRP3 inhibitor, MCC950, at different concentrations, we observed that NLRP3 inhibition significantly reduced the conversion of MTT into its colorful product in a dose-dependent manner (Fig. 3, A; one-way ANOVA, $p < 0.05$; $n = 4$ per group). In further experiments, we detected no cleavage of caspase-3 in MCC950-treated cells (Fig. 3, B), while MCC950 treatments significantly decreased protein levels of both PCNA (Fig. 3, C and D; one-way ANOVA, $p < 0.05$; $n = 3$ per group) and Ki-67 (Fig. 3, E and F; one-way ANOVA, $p < 0.05$; $n = 4$ per group), which are two typical protein markers for cell proliferation. Thus, inhibition of NLRP3 potentially suppressed proliferation of pericytes.

NLRP3 inhibition attenuates expression of PDGFRβ and CD13 in cultured pericytes.

PDGFRβ and CD13 are two protein markers of pericytes in the brain, which mediate physiological and pathophysiological functions of pericytes [2, 3]. We observed that treatments with MCC950 inhibited expression of PDGFRβ and CD13 in pericytes in a dose-dependent manner (Fig. 4, A; One-way ANOVA, $p < 0.05$; $n = 4$ per group). In order to analyze underlying mechanisms, through which NLRP3 drives pericyte differentiation, we detected phosphorylation of AKT, ERK and NF-kB in MCC950-treated cells. Activation of AKT and ERK is involved in pericyte proliferation and migration [26, 27]. We observed that inhibition of NLRP3 reduced the protein levels of both phosphorylated AKT and ERK in a dose-dependent manner (Fig. 4, A; One-way ANOVA, $p < 0.05$; $n = 4$ per group). However, phosphorylation of NF-kB in pericytes was not significantly altered by treatments with MCC950 (Fig. 4, A; One-way ANOVA, $p = 0.094$; $n = 3$ per group).

IL-1β increases expression of PDGFRβ and CD13 in cultured pericytes.

NLRP3-contained inflammasome activates caspase 1 and subsequently cleaves pro-IL-1β into active IL-1β [28]. Due to the low level of IL-1β released from non-activated pericytes, we could not detect reduction of IL-1β secreted from NLRP3-deficient pericytes compared to NLRP3-wild-type pericytes (data not shown). However, we hypothesized that IL-1β affects differentiation of pericytes. We treated cultured pericytes with IL-1β at different concentrations. Very interestingly, IL-1β increases the protein expression

of PDGFRβ and CD13 also with a concentrations-dependent pattern (Fig. 5, A-C; One-way ANOVA, $p < 0.05$; n = 3 per group). As potential mechanisms mediating effects of IL-1β activation, we observed that IL-1β treatments significantly increased phosphorylation of AKT but of ERK (Fig. 5, D-F; One-way ANOVA, $p < 0.05$; n = 3 per group).

Inhibition of AKT suppresses expression of PDGFRβ and CD13 in cultured pericytes.

As activation of AKT in pericytes was suppressed by NLRP3 inhibition but enhanced upon IL-1β activation, we supposed that AKT signaling plays an important role in the differentiation of pericytes. We treated pericytes with AKT inhibitors at 0, 0.5, 1 and 5 μM. Phosphorylation of AKT and phosphorylation of GSK3β, a kinase down-stream to AKT, were both reduced (Fig. 6, A-C; One-way ANOVA, $p < 0.05$; n = 4 per group), which verified the successful inhibition of AKT signaling. With such an inhibition, expression of both PDGFRβ and CD13 was significantly down-regulated in a dose-dependent manner (Fig. 6, D-F; One-way ANOVA, $p < 0.05$; n = 3 per group).

Discussion

Pericytes play a central role in regulating microvascular circulation and BBB function in the brain [1]. Our study demonstrated that deletion of NLRP3 under physiological conditions decreases the coverage of pericytes and protein levels of PDGFRβ and CD13 in cerebral blood vessels. PDGFRβ and CD13, together with neural/glial antigen-2 and CD146 are expressed in capillary-associated pericytes, and often used as protein markers of pericytes [29]. PDGFRβ and CD13 also trigger proliferation and migration of pericytes after stimulation with angiogenesis-associated growth factors [2, 3]. Thus, the reduction of CD13 and PDGFRβ represents not only loss of pericytes but also dysfunction of pericytes in the brain. Indeed, we observed that deletion of NLRP3 reduces the vasculature in the brain, which corroborates a recent observation that dysfunction of pericytes decreases the length of cerebral blood vessels in PDGFRβ-mutated mouse brain [4]. Our study suggests that NLRP3 is essential in the maintenance of functional pericytes in healthy brains.

Activation of NLRP3-contained inflammasome produces active IL-1β [28]. MyD88 mediates inflammatory activation after challenges of TLRs ligands and IL-1β [25]. We observed that deficiency of either NLRP3 or MyD88 decreases protein levels of PDGFRβ and CD13 in the mouse brain. We supposed that NLRP3 drives a basal inflammatory activation in pericytes and promotes pericyte survival, although it was difficult to detect the secretion of IL-1β from pericytes in the brain. In cultured pericytes, we did find that inhibition of NLRP3 attenuated phosphorylation of multiple inflammation-related kinases, such as AKT and ERK, and perhaps also NFkB ($p = 0.094$), which is correlated with decreased cell proliferation and PDGFRβ and CD13 expression. Moreover, treatments with IL-1β increase PDGFRβ and CD13 expression in our cultured pericytes. It is consistent with a report that TNF-α at 10ng/ml promotes cultured pericytes to

proliferate and migrate [30]. Thus, it is not surprising that angiogenesis is activated with pericyte proliferation in inflammatory lesion sites of multiple sclerosis [31]. During early wound healing, NLRP3 facilitates angiogenesis; however, production of IL-1β appeared not to be always necessary for angiogenesis [32].

It should be noted that NLRP3 might not provide protective effects on pericyte when the inflammatory activation surrounding pericytes is severe and lasts for a long term. In AD brain, pericytes are damaged and lost [6, 7], while NLRP3-contained inflammasome is activated [13]. However, there are potentially other mechanisms than sustained NLRP3 activation, which damage pericytes. For example, brain-delivered neurotrophic factor (BDNF) drops down and the activation of BDNF receptor, TrkB, is impaired in AD brain [33]. TrkB signaling regulates pericyte migration. Deletion of TrkB in pericytes reduces pericyte density and causes abnormal vasculogenesis in the heart [34]. In our unpublished experiments, we observed that deletion of BDNF in astrocytes or neurons decreases protein levels of PDGFRβ and CD13 in cerebral blood vessels (Wenqiang Quan, Qinghua Luo, Zhengyu Tang, Michael Menger, Klaus Fassbender and Yang Liu). Deletion of NLRP3 in APP or Tau-transgenic mice was reported to rescue neuronal functions and shift microglial activation from pro-inflammatory to anti-inflammatory profile [13, 16]. Unfortunately, the microvascular circulation in NLRP3-deficient AD mice has not been investigated.

AKT is a known kinase to regulate cell survival, proliferation, and angiogenesis in response to extracellular signals [35]. AKT activation prevents pericyte loss in diabetic retinopathy [36]. In our experiments, AKT activation might mediate protective effects of NLRP3 on pericytes, as AKT phosphorylation is reduced by NLRP3 inhibition but enhanced by IL-1β activation. Moreover, inhibition of AKT down-regulates expression of PDGFRβ and CD13 in pericytes. Interestingly, PDGFRβ activation induces phosphorylation of AKT [37]. Thus, PDGFRβ and AKT activate each other and form a potential positive feed-back to maintain healthy pericytes in the brain.

Conclusions

Our study suggested that NLRP3 activation maintains healthy pericytes in the brain through activating AKT signaling pathway. In the potential anti-AD or other therapies, in which NLRP3 inhibitors are administered, the potential adverse effects of NLRP3 inhibition on pericyte function and microcirculation should be considered.

Availability Of Data And Materials

The data and materials published in this study are available from the corresponding author on reasonable request.

Abbreviations

Aβ = amyloid β peptide; AD = Alzheimer´s disease; APP = Alzheimer's precursor protein; BBB = blood-brain barrier; BDNF = brain-delivered neurotrophic factor; HBPC = human primary brain vascular pericytes; HBSS = Hanks' balanced salt solution; IL-1β = interleukin-1β; LPS = lipopolysaccharide; MyD88 = myeloid differentiation primary response 88; NLRP = NACHT, LRR and PYD domains-containing protein; PCNA = proliferating cell nuclear antigen; PDGFRβ = platelet-derived growth factor receptor β; TLR = Toll-like receptor; TNF-α = tumor necrosis factor α

References

1. Sweeney MD, Ayyadurai S, Zlokovic BV: **Pericytes of the neurovascular unit: key functions and signaling pathways.** *Nat Neurosci* 2016, **19:**771-783.
2. Lindahl P, Johansson BR, Leveen P, Betsholtz C: **Pericyte loss and microaneurysm formation in PDGF-B-deficient mice.** *Science* 1997, **277:**242-245.
3. Rangel R, Sun Y, Guzman-Rojas L, Ozawa MG, Sun J, Giordano RJ, Van Pelt CS, Tinkey PT, Behringer RR, Sidman RL, et al: **Impaired angiogenesis in aminopeptidase N-null mice.** *Proc Natl Acad Sci U S A* 2007, **104:**4588-4593.
4. Montagne A, Nikolakopoulou AM, Zhao Z, Sagare AP, Si G, Lazic D, Barnes SR, Daianu M, Ramanathan A, Go A, et al: **Pericyte degeneration causes white matter dysfunction in the mouse central nervous system.** *Nat Med* 2018, **24:**326-337.
5. Love S, Miners JS: **Cerebrovascular disease in ageing and Alzheimer's disease.** *Acta Neuropathol* 2016, **131:**645-658.
6. Nation DA, Sweeney MD, Montagne A, Sagare AP, D'Orazio LM, Pachicano M, Sepehrband F, Nelson AR, Buennagel DP, Harrington MG, et al: **Blood-brain barrier breakdown is an early biomarker of human cognitive dysfunction.** *Nat Med* 2019, **25:**270-276.
7. Sengillo JD, Winkler EA, Walker CT, Sullivan JS, Johnson M, Zlokovic BV: **Deficiency in mural vascular cells coincides with blood-brain barrier disruption in Alzheimer's disease.** *Brain Pathol* 2013, **23:**303-310.
8. Sagare AP, Bell RD, Zhao Z, Ma Q, Winkler EA, Ramanathan A, Zlokovic BV: **Pericyte loss influences Alzheimer-like neurodegeneration in mice.** *Nat Commun* 2013, **4:**2932.
9. Nyul-Toth A, Kozma M, Nagyoszi P, Nagy K, Fazakas C, Hasko J, Molnar K, Farkas AE, Vegh AG, Varo G, et al: **Expression of pattern recognition receptors and activation of the non-canonical inflammasome pathway in brain pericytes.** *Brain Behav Immun* 2017, **64:**220-231.
10. Guijarro-Munoz I, Compte M, Alvarez-Cienfuegos A, Alvarez-Vallina L, Sanz L: **Lipopolysaccharide activates Toll-like receptor 4 (TLR4)-mediated NF-kappaB signaling pathway and proinflammatory response in human pericytes.** *J Biol Chem* 2014, **289:**2457-2468.
11. Leaf IA, Nakagawa S, Johnson BG, Cha JJ, Mittelsteadt K, Guckian KM, Gomez IG, Altemeier WA, Duffield JS: **Pericyte MyD88 and IRAK4 control inflammatory and fibrotic responses to tissue injury.** *J*

Clin Invest 2017, **127:**321-334.

12. Kovac A, Erickson MA, Banks WA: **Brain microvascular pericytes are immunoactive in culture: cytokine, chemokine, nitric oxide, and LRP-1 expression in response to lipopolysaccharide.** *J Neuroinflammation* 2011, **8:**139.
13. Heneka MT, Kummer MP, Stutz A, Delekate A, Schwartz S, Vieira-Saecker A, Griep A, Axt D, Remus A, Tzeng TC, et al: **NLRP3 is activated in Alzheimer's disease and contributes to pathology in APP/PS1 mice.** *Nature* 2013, **493:**674-678.
14. Stancu IC, Cremers N, Vanrusselt H, Couturier J, Vanoosthuyse A, Kessels S, Lodder C, Brone B, Huaux F, Octave JN, et al: **Aggregated Tau activates NLRP3-ASC inflammasome exacerbating exogenously seeded and non-exogenously seeded Tau pathology in vivo.** *Acta Neuropathol* 2019, **137:**599-617.
15. Venegas C, Kumar S, Franklin BS, Dierkes T, Brinkschulte R, Tejera D, Vieira-Saecker A, Schwartz S, Santarelli F, Kummer MP, et al: **Microglia-derived ASC specks cross-seed amyloid-beta in Alzheimer's disease.** *Nature* 2017, **552:**355-361.
16. Ising C, Venegas C, Zhang S, Scheiblich H, Schmidt SV, Vieira-Saecker A, Schwartz S, Albasset S, McManus RM, Tejera D, et al: **NLRP3 inflammasome activation drives tau pathology.** *Nature* 2019.
17. Dempsey C, Rubio Araiz A, Bryson KJ, Finucane O, Larkin C, Mills EL, Robertson AAB, Cooper MA, O'Neill LAJ, Lynch MA: **Inhibiting the NLRP3 inflammasome with MCC950 promotes non-phlogistic clearance of amyloid-beta and cognitive function in APP/PS1 mice.** *Brain Behav Immun* 2017, **61:**306-316.
18. DeTure MA, Dickson DW: **The neuropathological diagnosis of Alzheimer's disease.** *Molecular Neurodegeneration* 2019, **14:**32.
19. Martinon F, Petrilli V, Mayor A, Tardivel A, Tschopp J: **Gout-associated uric acid crystals activate the NALP3 inflammasome.** *Nature* 2006, **440:**237-241.
20. Adachi O, Kawai T, Takeda K, Matsumoto M, Tsutsui H, Sakagami M, Nakanishi K, Akira S: **Targeted disruption of the MyD88 gene results in loss of IL-1- and IL-18-mediated function.** *Immunity* 1998, **9:**143-150.
21. Boulay AC, Saubamea B, Decleves X, Cohen-Salmon M: **Purification of Mouse Brain Vessels.** *J Vis Exp* 2015**:**e53208.
22. Decker Y, Muller A, Nemeth E, Schulz-Schaeffer WJ, Fatar M, Menger MD, Liu Y, Fassbender K: **Analysis of the vasculature by immunohistochemistry in paraffin-embedded brains.** *Brain Struct Funct* 2018, **223:**1001-1015.
23. Zudaire E, Gambardella L, Kurcz C, Vermeren S: **A computational tool for quantitative analysis of vascular networks.** *PLoS One* 2011, **6:**e27385.
24. Umehara K, Sun Y, Hiura S, Hamada K, Itoh M, Kitamura K, Oshima M, Iwama A, Saito K, Anzai N, et al: **A New Conditionally Immortalized Human Fetal Brain Pericyte Cell Line: Establishment and Functional Characterization as a Promising Tool for Human Brain Pericyte Studies.** *Molecular Neurobiology* 2018, **55:**5993-6006.

25. O'Neill LA, Bowie AG: **The family of five: TIR-domain-containing adaptors in Toll-like receptor signalling.** *Nat Rev Immunol* 2007, **7:**353-364.
26. Bonacchi A, Romagnani P, Romanelli RG, Efsen E, Annunziato F, Lasagni L, Francalanci M, Serio M, Laffi G, Pinzani M, et al: **Signal transduction by the chemokine receptor CXCR3: activation of Ras/ERK, Src, and phosphatidylinositol 3-kinase/Akt controls cell migration and proliferation in human vascular pericytes.** *J Biol Chem* 2001, **276:**9945-9954.
27. Yao Q, Renault MA, Chapouly C, Vandierdonck S, Belloc I, Jaspard-Vinassa B, Daniel-Lamaziere JM, Laffargue M, Merched A, Desgranges C, Gadeau AP: **Sonic hedgehog mediates a novel pathway of PDGF-BB-dependent vessel maturation.** *Blood* 2014, **123:**2429-2437.
28. Gross O, Thomas CJ, Guarda G, Tschopp J: **The inflammasome: an integrated view.** *Immunol Rev* 2011, **243:**136-151.
29. Smyth LCD, Rustenhoven J, Scotter EL, Schweder P, Faull RLM, Park TIH, Dragunow M: **Markers for human brain pericytes and smooth muscle cells.** *J Chem Neuroanat* 2018, **92:**48-60.
30. Tigges U, Boroujerdi A, Welser-Alves JV, Milner R: **TNF-alpha promotes cerebral pericyte remodeling in vitro, via a switch from alpha1 to alpha2 integrins.** *J Neuroinflammation* 2013, **10:**33.
31. Girolamo F, Coppola C, Ribatti D, Trojano M: **Angiogenesis in multiple sclerosis and experimental autoimmune encephalomyelitis.** *Acta Neuropathol Commun* 2014, **2:**84.
32. Weinheimer-Haus EM, Mirza RE, Koh TJ: **Nod-like receptor protein-3 inflammasome plays an important role during early stages of wound healing.** *PLoS One* 2015, **10:**e0119106.
33. Tanila H: **The role of BDNF in Alzheimer's disease.** *Neurobiol Dis* 2017, **97:**114-118.
34. Anastasia A, Deinhardt K, Wang S, Martin L, Nichol D, Irmady K, Trinh J, Parada L, Rafii S, Hempstead BL, Kermani P: **Trkb signaling in pericytes is required for cardiac microvessel stabilization.** *PLoS One* 2014, **9:**e87406.
35. Manning BD, Cantley LC: **AKT/PKB signaling: navigating downstream.** *Cell* 2007, **129:**1261-1274.
36. Yun JH, Jeong HS, Kim KJ, Han MH, Lee EH, Lee K, Cho CH: **beta-Adrenergic receptor agonists attenuate pericyte loss in diabetic retinas through Akt activation.** *FASEB J* 2018, **32:**2324-2338.
37. Lehti K, Allen E, Birkedal-Hansen H, Holmbeck K, Miyake Y, Chun TH, Weiss SJ: **An MT1-MMP-PDGF receptor-beta axis regulates mural cell investment of the microvasculature.** *Genes Dev* 2005, **19:**979-991.

Declarations

Acknowledgements

Not applicable

Funding

This work was supported by National Natural Science Foundation of China (Grant 81771371 to Y.L. and Grant 81902984 to W.Q.) and SNOWBALL, an EU Joint Programme for Neurodegenerative Disease (JPND) (01ED1617B; to Y.L. and K.F.).

Contributions

YL designed the study and wrote the manuscript. WQ, QL and QT conducted experiments, acquired data and analyzed data. TF provided pericyte cell line. DL and KF supervised the study. All authors read and approved the final manuscript.

Ethics declarations

Ethics approval and consent to participate

This study was carried out in accordance with all relevant national rules and was authorized by Landesamt für Verbraucherschutz, Saarland, Germany.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests

Figures

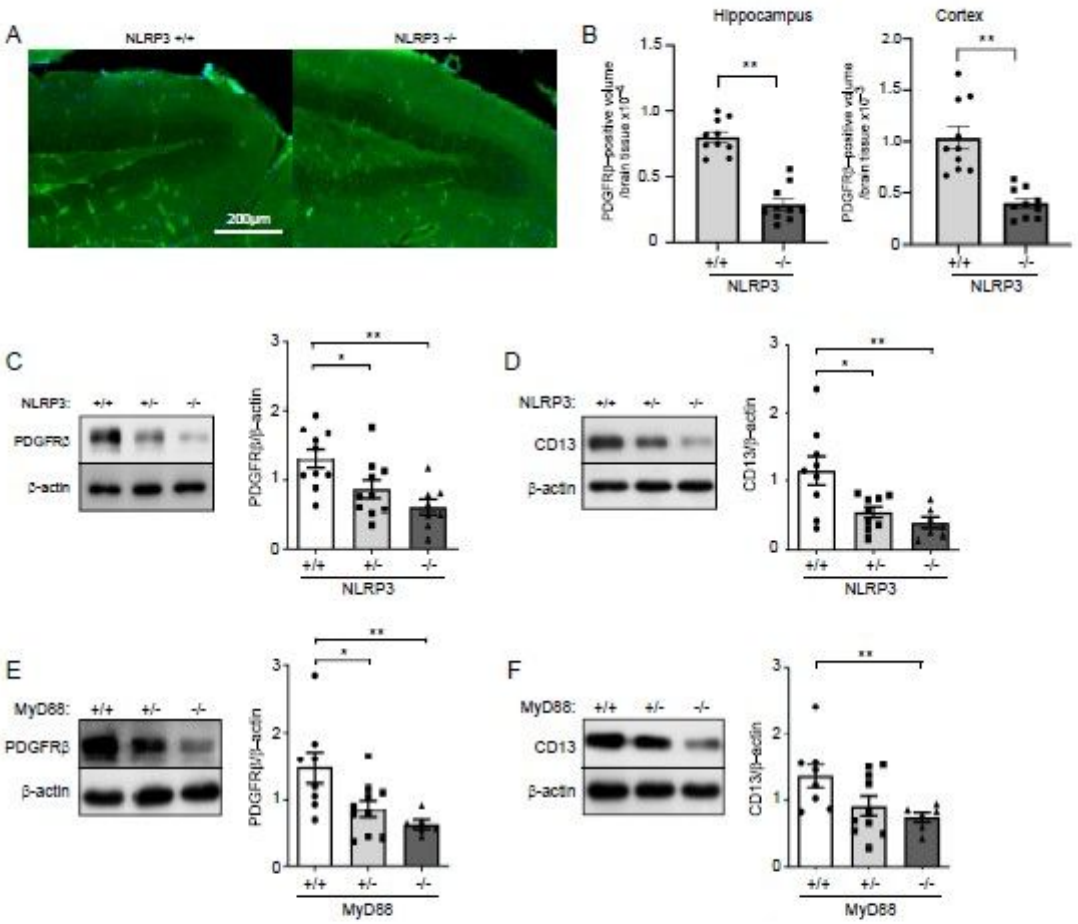

Figure 1

NLRP3 deficiency reduces pericyte cell coverage and decreases protein levels of PDGFRβ and CD13 in the brain. A, brain tissues from 9-month-old NLRP3-knockout (-/-) and wild-type (+/+) littermate mice were stained for PDGFRβ (in green fluorescence). B, the coverage of PDGFRβ-positive pericytes was estimated with Cavalieri method. t test, n = 10 per group. C-F, 9-month-old NLRP3 and 6-month-old MyD88 littermate mice with homozygous (-/-), heterozygous (+/-) and wild-type (+/+) of nlrp3 and myd88 genes, respectively, were analyzed for protein levels of PDGFRβ and CD13 in isolated cerebral blood vessels. One-way ANOVA followed by Bonferroni post hoc test, n ≥ 7 per group for NLRP3 mice and ≥ 6 per group for MyD88 mice. *: $p < 0.05$, and **: $p < 0.01$.

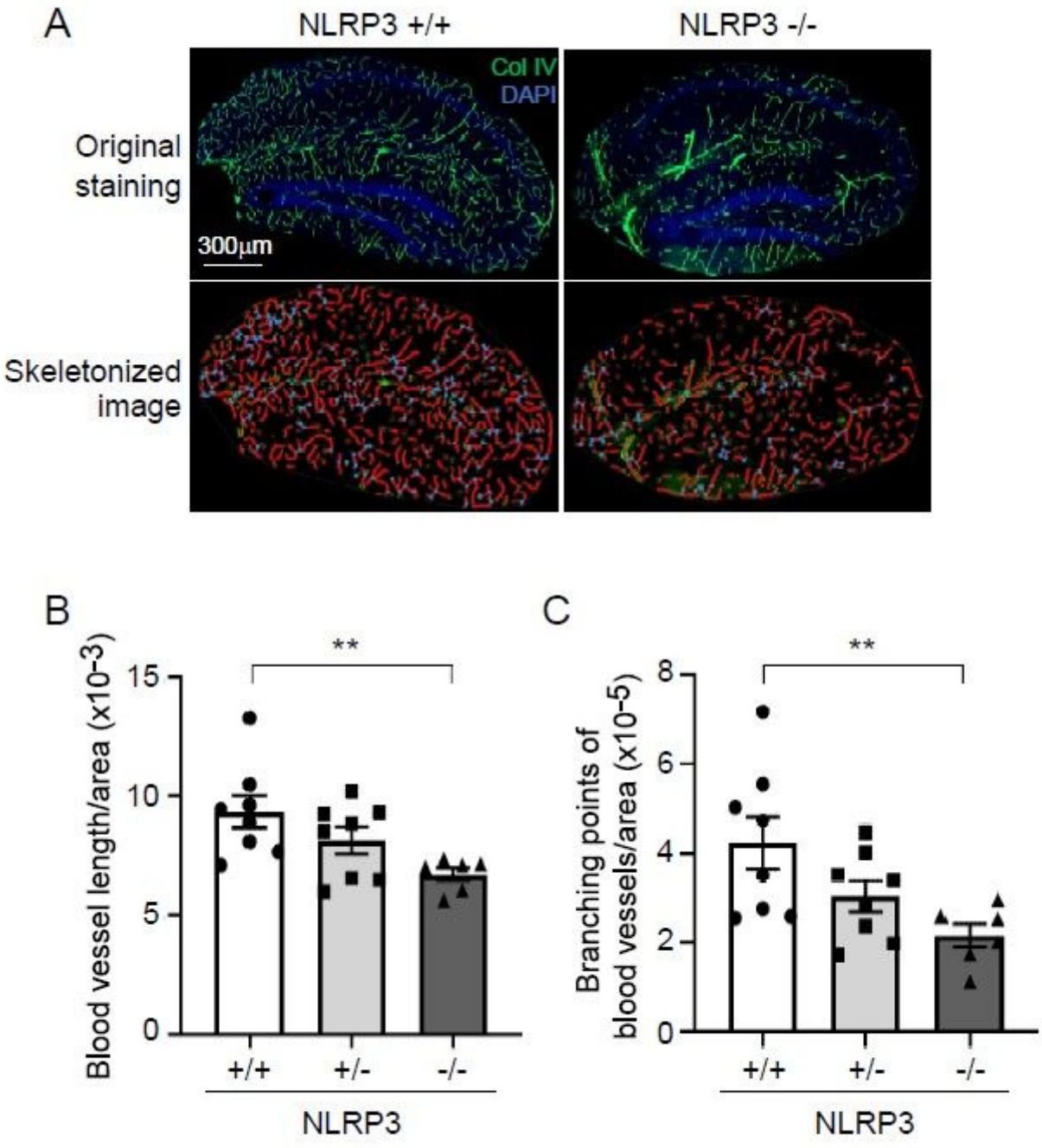

Figure 2

NLRP3 deficiency reduces vasculature in the brain. A, brains of 9-month-old littermate mice with homozygous (-/-), heterozygous (+/-) and wild-type (+/+) of nlrp3 gene were stained for collagen type IV (Col IV). The blood vessels in hippocampus were thresholded and skeletonized. The skeleton representation of vasculature is shown in red and branching points of blood vessels are in blue. B, the total length and branching points of blood vessels were calculated and adjusted by area of analysis. One-way ANOVA followed by Bonferroni post hoc test, n ≥ 6 per group. **: $p < 0.01$.

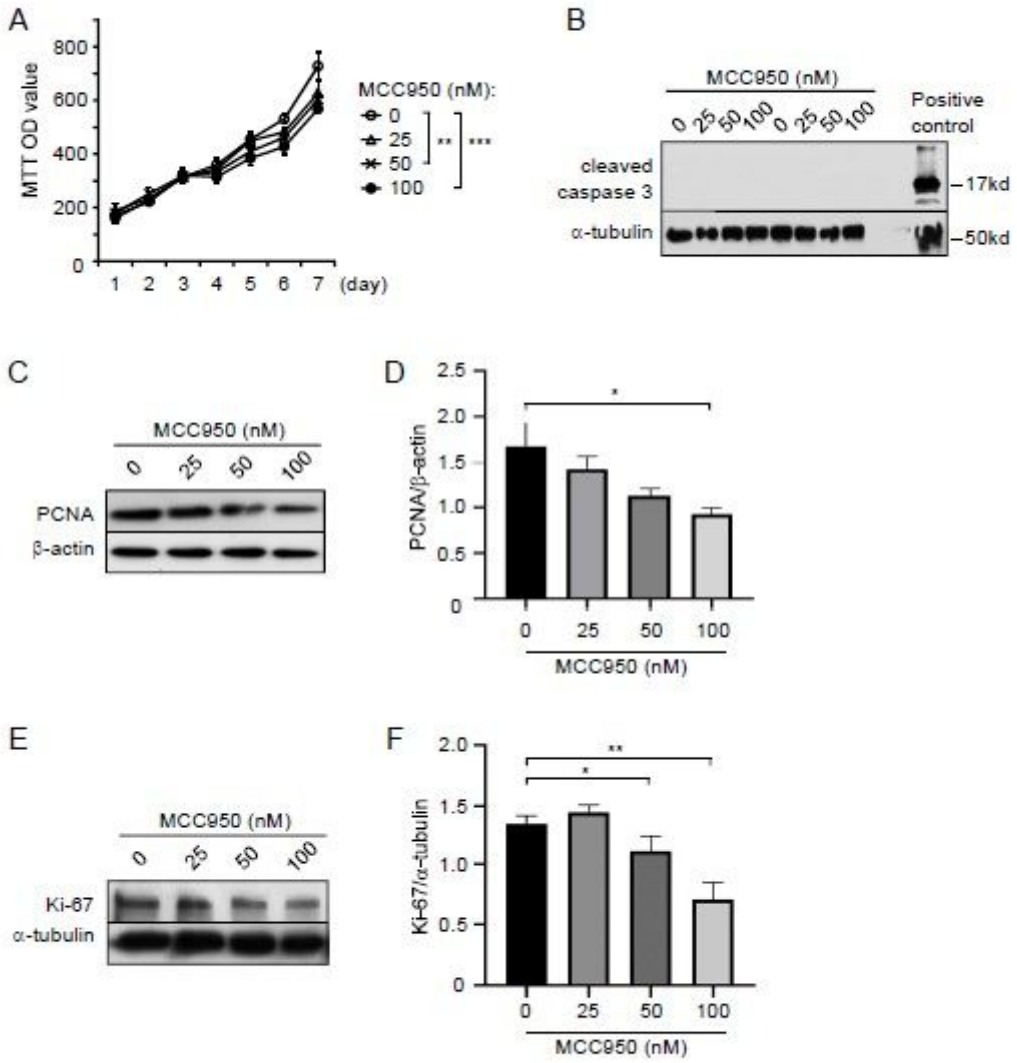

Figure 3

NLRP3 inhibition attenuates cell proliferation in cultured pericytes. A, cultured pericytes were treated with NLRP3 inhibitor, MCC950, at 0, 25, 50 and 100 nM and analyzed for proliferation with MTT assay every day for 7 days. Two-way ANOVA followed by Tukey post-hoc post hoc test, n = 4 per group. **: $p < 0.01$ and ***: $p < 0.001$. B, C and E, pericytes were cultured and treated with MCC950 at indicated concentrations for 24 hours. Cell lysate were detected for cleaved caspase-3, PCNA and Ki-67 with quantitative Western blot. As a positive control for cleavage of caspase-3, the brain lysate from neuronal ATG5-deficient mice was used. D and F, inhibition of NLRP3 reduces protein levels of PCNA and Ki-67 in a dose-dependent manner. One-way ANOVA followed by Tukey post-hoc post hoc test, n = 3 per group for PCNA and n = 4 per group for Ki-67. *: $p < 0.05$, and **: $p < 0.01$.

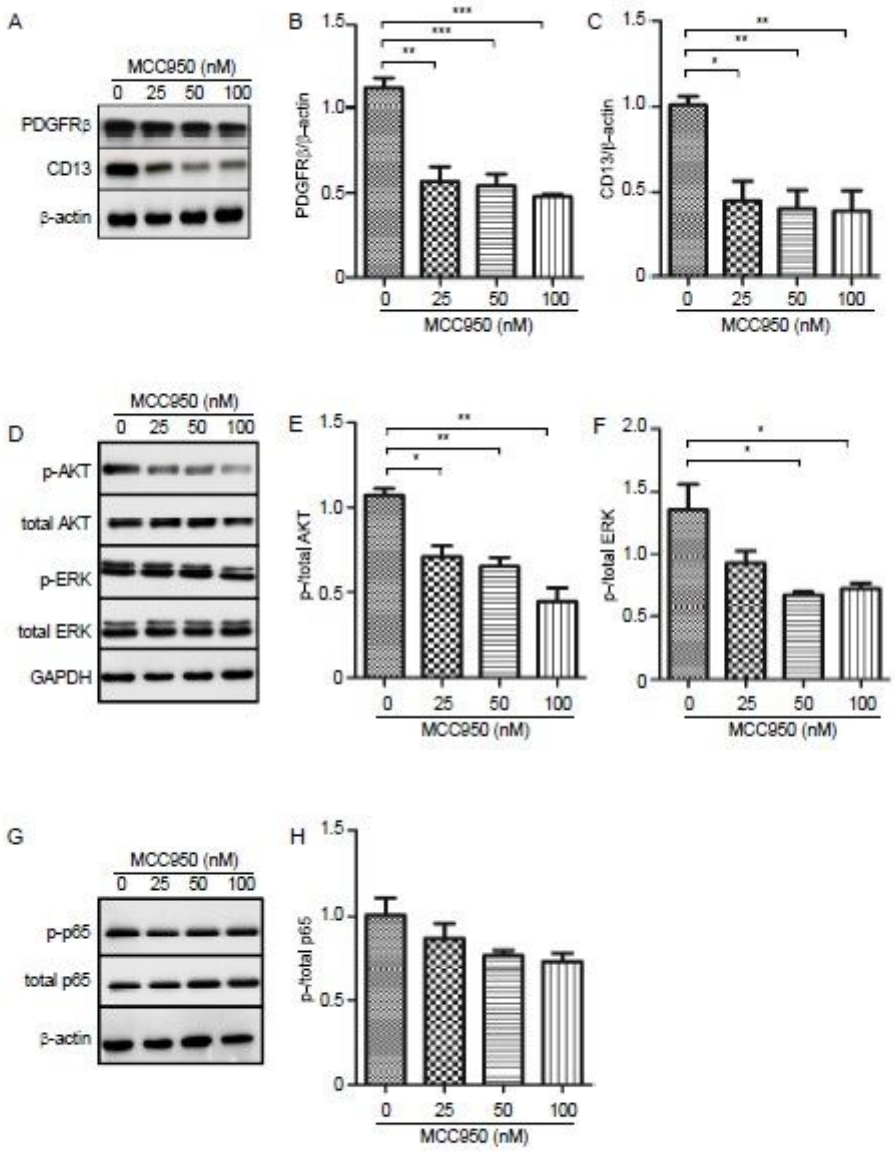

Figure 4

NLRP3 inhibition attenuates protein expression of PDGFRβ and CD13 and inhibits phosphorylation of AKT and ERK in cultured pericytes. Pericytes were cultured and treated with MCC950 at 0, 25, 50 and 100 nM for 24 hours. A, D and G, Western blot was used to detect PDGFRβ and CD13, as well as phosphorylated and total protein levels of AKT, ERK and NF□B p65. B, C, E and F, inhibition of NLRP3 reduces protein levels of PDGFRβ and CD13, and inhibits phosphorylation of AKT and ERK with a dose-dependent pattern. One-way ANOVA followed by Tukey post hoc test, n = 4 per group. *: $p < 0.05$, **: $p < 0.01$, and ***: $p < 0.001$. G, phosphorylation of NF□B p65 is not significantly changed by inhibition of NLRP3. One-way ANOVA, n = 3 per group.

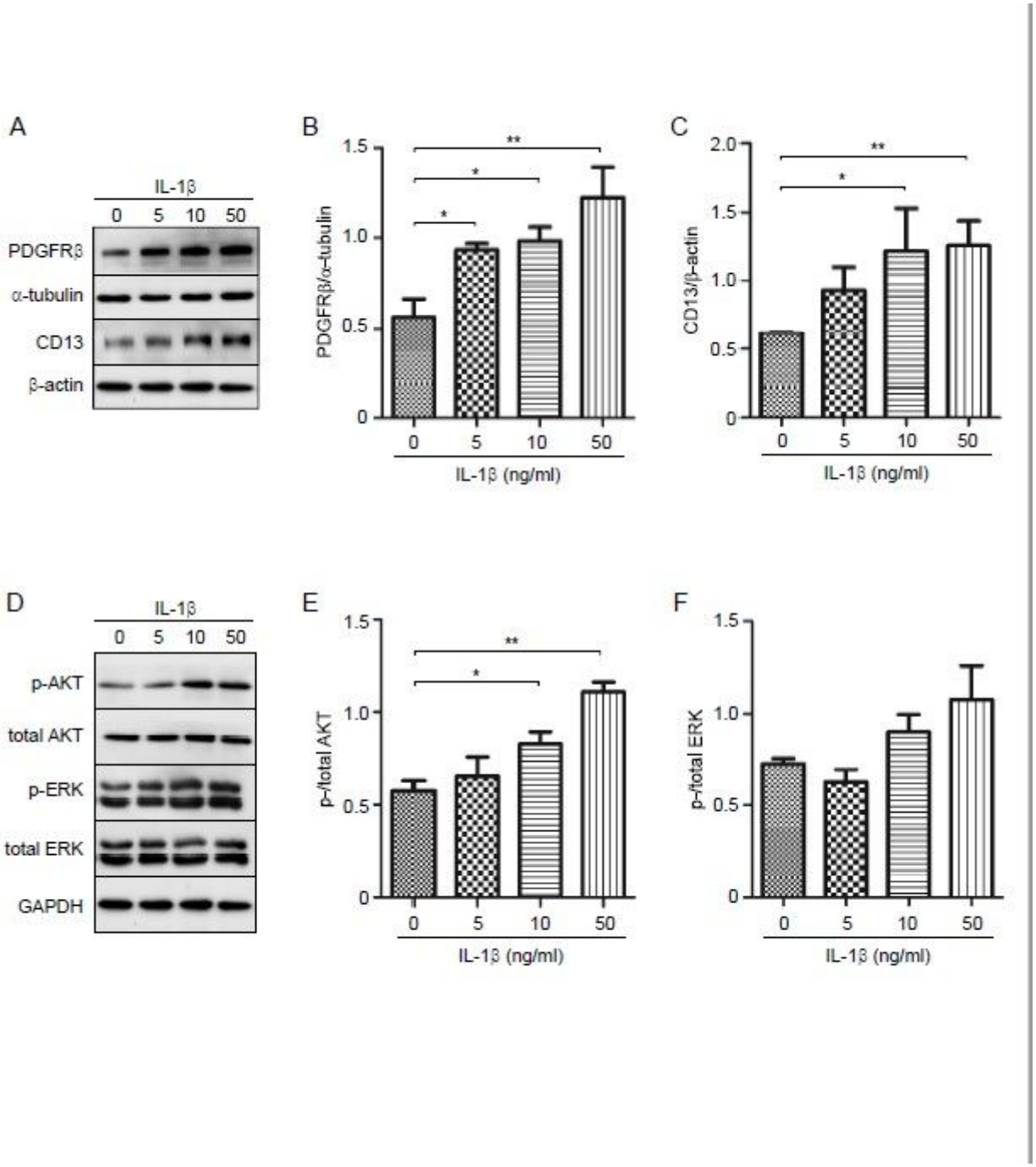

Figure 5

IL-1β increases protein expression of PDGFRβ and CD13 in cultured pericytes. Pericytes were cultured and treated with recombinant human IL-1β at 0, 5, 10 and 50 ng/ml for 24 hours. A and D, Western blot was used to detect PDGFRβ and CD13, as well as phosphorylated and total protein levels of AKT and ERK. B, C, E and F, stimulation of IL-1β increases protein levels of PDGFRβ and CD13, and activates phosphorylation of AKT, but not ERK, in a dose-dependent manner. One-way ANOVA followed by Tukey post hoc test, n = 3 per group. *: $p < 0.05$, and **: $p < 0.01$.

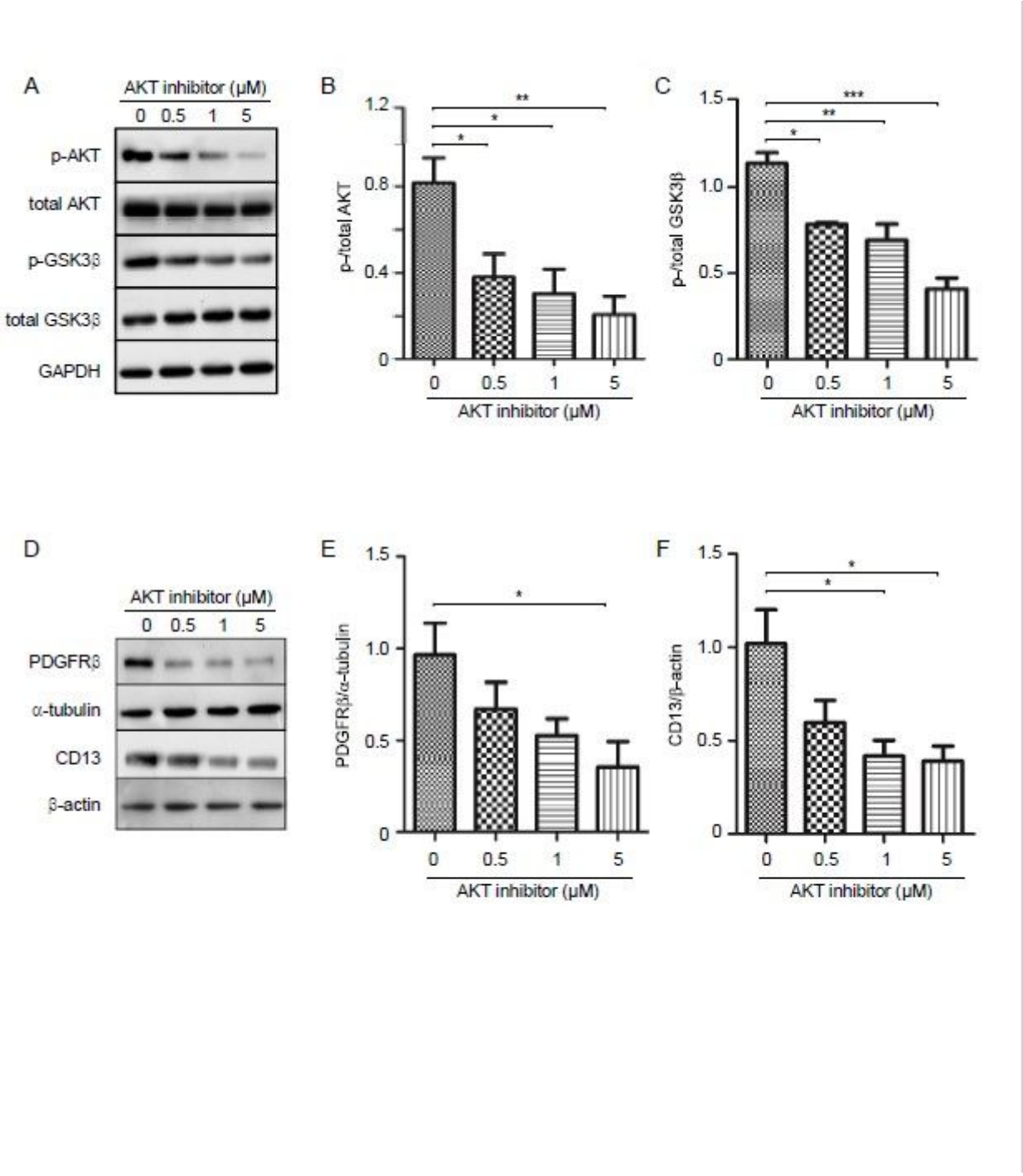

Figure 6

Inhibition of AKT signaling pathway suppresses protein expression of PDGFRβ and CD13 in cultured pericytes. Pericytes were cultured and treated with AKT inhibitor at 0, 0.5, 1 and 5 μM for 24 hours. A-C, Western blot was used to detect phosphorylated and total AKT, and GSK3β, which shows that phosphorylation of both AKT and GSK3β was significantly inhibited by treatments with AKT inhibitors. D-F, the cell lysate from AKT inhibitor-treated pericytes were detected for protein levels of PDGFRβ and CD13 with quantitative Western blot. Inhibition of AKT decreases protein levels of PDGFRβ and CD13 in a dose-dependent manner. One-way ANOVA followed by Tukey post hoc test, n = 4 per group for

phosphorylation of kinases, and n = 3 per group for PDGFRβ and CD13. *: $p < 0.05$, **: $p < 0.01$, and ***: $p < 0.001$.