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Contribution of brain pericytes to neuroinflammation following repetitive head trauma

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Abstract

Background Neuroinflammation is a prominent pathological hallmark of traumatic brain injury (TBI) and glia cells have been widely characterized in the onset or progression of brain inflammation. While an effect of inflammation on cerebrovascular breakdown has been observed, little is known about the specific contribution of brain pericytes to the inflammatory response in TBI. Here, we focused on studying the pericyte response to inflammatory stimuli commonly found in the brain following TBI.

Methods Mouse brain vascular pericytes were exposed to IL-1β, TNF-α and IFN-γ for 2 h and 24 h and probed for markers of pericyte health and a panel of inflammatory mediators. As the platelet-derived growth factor (PDGF) pathway is critical to pericyte function, we also assessed the effect of PDGF-BB stimulation on the inflammatory response in pericytes. Cultured pericytes were treated with PDGF-BB (10 ng/mL) prior to, simultaneously, and following inflammatory insult. To further investigate their role in brain immunosurveillance, we analyzed the cytokine secretome in mouse pericyte cultures treated with PDGF-BB, as well as in brain vascular pericytes isolated from repetitive mild TBI (r-mTBI) mice that were fed phenytoin-enriched chow, an inducer of PDGF-BB secretion.

Results Cytokine stimulation with TNF-α, IL-1β, and IFN-γ for 2 and 24 h led to significant upregulation of PDGFRβ in cultured pericytes, with an 8-fold increase after 24 h. MTT assays showed no significant change in cell viability, indicating that cytokine treatment did not induce cytotoxicity. Further, elevated levels of pro-inflammatory markers STAT1 and p-NFkB were observed in response to cytokine exposure, with a concurrent increase in VCAM1 and MMP9 expression. PDGF-BB treatment significantly attenuated the inflammatory response in pericytes, reducing PDGFRβ levels and the activation of inflammatory pathways, including STAT1 and NFkB. Cytokine secretion profiles also revealed that PDGF-BB, when administered post-inflammatory insult, selectively reduced pro-inflammatory cytokines such as IL-1β and IFN-γ. Additionally, phenytoin treatment in r-mTBI mice decreased IL-1β, TNF-α, IL-5, and KC/GRO levels in isolated brain pericytes, while IL-2, IL-4, and IL-6 levels were unchanged compared to untreated r-mTBI animals.

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Conclusions Our results indicate an immunoreactive role for brain pericytes in the propagation of neuroinflammation. Moreover, following brain insults, we found PDGF-BB stimulation can normalize pericyte function and reduce cerebrovascular inflammation, a key factor in secondary brain injury. Targeting brain pericytes may provide novel therapeutic opportunities to improve cerebrovascular health and reduce brain inflammation in the aftermath of TBI.

Keywords TBI, Neuroinflammation, Blood-brain barrier, Brain pericytes, Cytokines, PDGF-BB, Phenytoin

Background

Neuroinflammation and disruption of the blood-brain barrier (BBB) are prominent early events in neurodegenerative diseases such as Alzheimer's disease [12], epilepsy [14], multiple sclerosis [29], stroke [13] and brain injury [18]. Inflammation is a crucial process that is mainly beneficial in the initial stages post-stimulation; however prolonged and/or excessive inflammation is particularly damaging for the brain [3]. Notably, the brain possesses limited regenerative capacity and loss of neurons as a result of inflammation cannot be fully resolved [27]. Microglia and astrocytes are the most commonly studied neuroinflammatory cell types, though the BBB is a crucial site of cerebral inflammation. Indeed, the BBB plays a vital role in maintaining central nervous system (CNS) functioning and homeostasis [36], and BBB impairment has been widely observed in neurodegeneration [16]. The BBB is primarily composed of tightly joined endothelial cells from brain capillaries, which restrict many substances in the bloodstream from entering the brain. This barrier is part of a larger structure known as the 'neurovascular unit' (NVU), which includes not only endothelial cells but also other brain cells such as mural cells (pericytes and vascular smooth muscle cells), astrocytes, microglia and neurons [28]. Among these cells, perivascular pericytes occupy a unique position making them important regulators of vascular development, stabilization, maturation and remodeling. Furthermore, perivascular pericytes mediate both physiological and pathological processes, therefore becoming a potential target for therapeutics [22, 30]. Most recently, the emerging link between neurodegenerative and cerebrovascular disease has been studied with a particular focus on brain pericytes. Specifically, pericytes have gained more and more attention as a mediator of inflammation in the brain [7, 7]. With increasing knowledge of their role in normal and pathological conditions, new strategies to modulate pericyte function must be conceived. Understanding how pericytes contribute to brain inflammation and BBB dysfunction could provide new avenues to translate laboratory research into clinical outcomes. Furthermore, it will be especially useful to determine whether unique neuroinflammatory pericyte signatures are associated with specific neurodegenerative diseases. In the following studies, we investigated how pericytes contribute to the progression of neuroinflammation and their response to inflammatory mediators after traumatic brain injury. Lastly, we interrogate a possible therapeutic strategy that may be used to mediate the pericyte response and/or contribution to brain inflammation.

Materials and methods

Animals

Wild-type (WT) mice (C57BL/6J) were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). A mix of male and female mice were used for all studies. The mice were 11 weeks old at the start of the study and housed under controlled standard housing conditions (dark/light cycle of 12 h at a range of 20–23 °C). All procedures involving mice were performed in accordance with Office of Laboratory Animal Welfare and National Institutes of Health guidelines and the approval of The Roskamp Institute Institutional Animal Care and Use committee (AAALAC certified).

Traumatic brain injury protocol

A mouse model of closed head injury was used to investigate the effects repetitive mild traumatic brain injury (r-mTBI) as previously described [1, 32]. Mice were shaved at the injury site and then anesthetized with 1.5 L/min if oxygen and 3% isoflurane for 3 min. Animals were positioned on a heating pad to maintain their body temperature at 37 °C and the head was placed in a stereotaxic frame (Stereotaxic Instrument, Stoelting, Wood Dale, Illinois) connected to an impact device (Impact One Stereotaxic Motorized Impactor, Richmond, Illinois). The impact was performed using a 5 mm blunt metal impactor tip which was then retracted to a strike depth of 1 mm. The impact was delivered at 5 m/s with a force of 72 N. To prevent possible hypothermia, mice were allowed to recover on a heating pad set at 37 °C until they became ambulant again. Mice in the r-mTBI group received 2 impacts per week (approximately every 72 h) for 3 months. The r-sham group was exposed to isoflurane for the same frequency and time as the r-mTBI mice, but they did not receive an injury, to control for the effects of repeated anesthesia. For the pericyte isolation studies, mice were euthanized, and brains collected after 6 months post-injury. For the PDGF-BB disposition studies, mice were euthanized at post-injury time points of 24 h, 3 months, 6 months and 12 months. Additional

information on the injury timeline and tissue collections can be found in Fig. 1.

Animal treatment

Beginning on the fifth month post-injury, mouse chow incorporating phenytoin (PHT) (40 mg/kg, Inotiv) was administered to r-sham and r-mTBI mice *ad libitum* for a total of 4 weeks. The PHT dosage administered here was based on prior reporting [9]. The total cohort was split in 4 groups which included r-sham and r-mTBI fed with normal chow (control mice) and r-sham and r-mTBI fed with PHT chow (PHT-administered mice). On a weekly basis, we monitored the body weight and food intake of the PHT-administered mice for comparison to the control mice.

Human and primary mouse cerebrovessel isolation

Parenchyma and cerebrovasculature from brains were isolated using a protocol as previous described [32]. Freshly extracted mouse brains were collected (minus the cerebellum), the meninges and other outer vessels were removed with a dry cotton swab, and then minced with a blade in a Dounce homogenizer on ice. HBSS was added in fivefold excess of the brain volume, then the minced brain material was stirred with the spatula to break up larger material. After 6–8 passes with a Teflon pestle, a sample of the brain homogenate was collected as a representation of the whole brain. This homogenate fraction was stored in an equal volume of lysis buffer consisting of M-PER (Pierce Biotechnology, Rockford, IL, ISA) supplemented with phenylmethanesulfonylfluoride fluoride (PMSF) (1 mM) and Halt protease and phosphatase inhibitor cocktail (Thermo Scientific, Waltham, MA, USA). An equal volume of 40% dextran solution was added to the brain homogenate for a final concentration of 20% dextran, and subsequently centrifuged at 6000 x g for 15 min at 4 °C. This results in a compact mass at the top of the solution (parenchyma) and a pellet at the bottom of the container (cerebrovasculature) separated by a clear dextran interface (non-cell associated fraction). The parenchyma and dextran were collected and added to an equal volume of HBSS, then centrifuged at 6000 x g for 10 min at 4 °C. The parenchyma was resuspended in HBSS and centrifuged for 5 min at 4 °C to remove any residual dextran, and parenchymal pellet was collected in lysis buffer. After the parenchyma and dextran were removed, the remaining cerebrovascular pellet was washed with ice cold HBSS and collected in lysis buffer. All samples were stored at −80 °C until further analysis.

Human brain specimens were obtained from Dr. Thomas Beach, Director of the Brain and Body Donation Program at the Sun Health Research Institute (Sun City, AZ). Frozen cortical samples (500 mg) from the inferior frontal gyrus were collected from autopsied brains, representing non-demented control subjects (no history of TBI), and TBI. For the TBI group, donors reported one or two brain injuries accompanied by loss of consciousness, each lasting less than 30 min. Given our primary focus on the chronic phase post-injury, samples with a longer duration since the last injury were prioritized, with an average of approximately 40 years post-injury. A summary of the human brain specimens is provided in Table 1.

Primary brain vascular mouse pericyte isolation by magnetic cell sorting

Primary mouse pericytes were prepared from C57BL/6 mice at 6 months post-injury (r-mTBI) and r-sham.

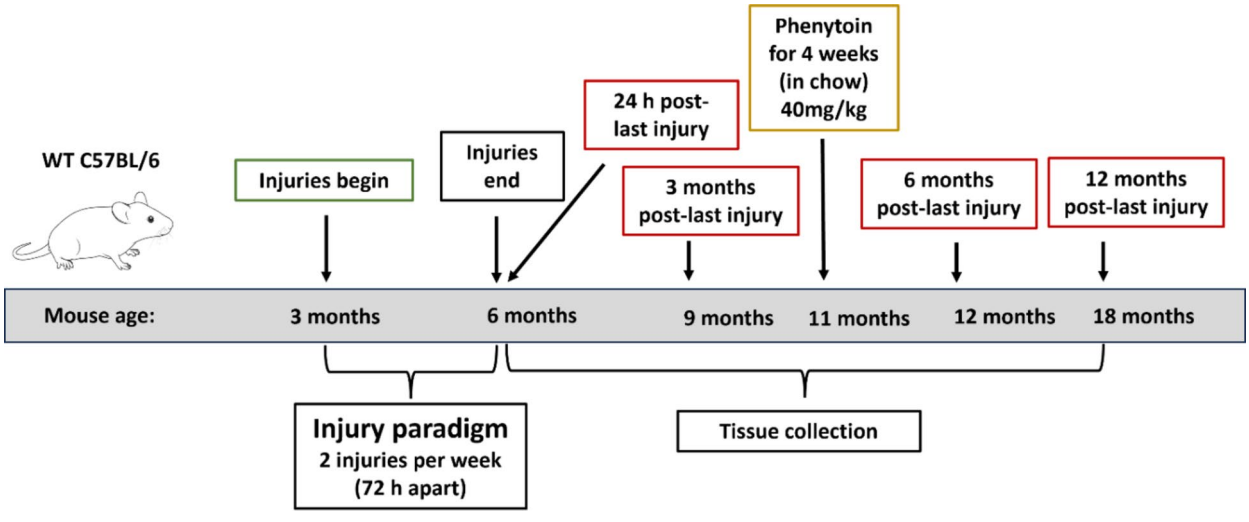

Fig. 1 Timeline of the brain injury paradigm and tissue collection for the mouse studies. C57BL/6 mice at 3 months of age received 2 injuries per week for 3 months. Mice were euthanized and tissue was collected at 24 h, 3 months, 6 months and 12 months after the final brain injury or anesthesia exposure for r-sham control mice. Moreover, for pericyte isolation studies, mice were fed with phenytoin for 4 weeks before euthanasia

Table 1 Specifications of human brain specimens

Group	Sample size	Age ± SEM (years)	Sex (M/F)	Years post-last injury ± SEM
Control	10	77.7 ± 2.8	7/3	
TBI	10	85.7 ± 2.2	6/4	32.8 ± 10.2

Entire brains were collected using stringent aseptic conditions, minced, dissociated using an enzyme-based brain dissociation kit (Cat No.130-107-667, MACS, Miltenyi Biotec), and passed through a sterile 70 µm nylon cell strainer (Cat No. 130-098-462, MACS, Miltenyi Biotec). After resuspending the cell pellets with 5 mL of cold PBS, cold debris removal solution was applied to the cell suspensions. Cells were centrifuged and resuspended with a primary FITC-conjugated CD13 + antibody (Cat No.) for 10 min in the dark at 4˚C. After centrifugating the cells to remove the unbound primary antibody, pellets were resuspended with anti-FITC Microbeads (Cat No. 130-048-701) for 15 min at 4˚C. Cells were applied onto the LS columns (Cat No. 130-042-401) and placed in the magnetic field of a suitable MACS separator and unlabeled cells were washed out 3 times with 3 mL of Dulbecco's Phosphate Buffered Saline (DPBS, Cat No. 20012-027, Gibco™). After removing the columns from the magnetic separator and placing them in new collection tubes, magnetically labeled cells were firmly flushed out by applying the plunger (Supplementary files: Figure S1). Cells were centrifuged and resuspended in culture media (complete pericyte medium). Cells were allowed to grow at 37 °C in a humidified chamber with 5% CO2 and cultured for 3–5 days before collecting conditioned media.

Mouse brain vascular pericyte cell cultures

Mouse brain vascular pericytes (MBVP) (Cat No. M1200-57) were purchased from ScienCell Research Laboratories. Cells were resuspended in poly-L-lysine-coated T-75 flasks, (PLL, Cat No. A005C, MilliporeSigma™), with 15 mL PM-medium (ScienCell) composed of DMEM (490 ml), 10 mL of fetal bovine serum (FBS, Cat No. 0010), 5 ml of growth supplement-mouse (PGS, Cat No. 1282), and 5 ml of penicillin/streptomycin solution (P/S, Cat No. 0503) and incubated at 37 °C with 5% CO2. Fresh medium was replaced every 2–3 days. When forming a confluent layer, cells were rinsed with DPBS followed by 2 ml of Trypsin/EDTA (0.25%), phenol red (Cat. No 25–200-072, Gibco™) and incubated at 37 °C until the cells were fully detached. Next, cells were counted and centrifuged for 5 min at 1000 rpm, resuspended in fresh culture medium, and finally plated at 50,000 cells/cm2. MBVP were used withing a narrow passage range P2-P6 for the experiments.

Table 2 Specifications for cytokines and growth factors used

Inflammatory stimulus	Conc. (ng/mL)	Incubation (h)	Supplier	Cat No.
TNFα	100	2, 24	R&D systems	410-MT-025/CF
IL1β	100	2, 24	R&D systems	201-LB-010/CF
IFN-γ	100	2, 24	R&D systems	485-MI-100/CF
Growth factor	**Conc. (ng/mL)**	**Incubation (h)**	**Supplier**	**Cat No.**
PDGF-BB	10	24	Abcam	Ab78593

Cytokines and PDGF-BB exposure to mouse brain vascular pericytes

MBVP were treated with either TNFα, IL1β, or IFN-γ alone (100 ng/mL) (resuspended in sterile PBS) or the combination of all three for either 2–24 h for the cell viability studies. PDGF-BB or vehicle was used at 1 ng/mL and 10 ng/mL for 1, 2–3 h to test PDGFRβ internalization. PDGF-BB was used either 24 h prior, simultaneously, or 24 h following cytokine stimulation at a concentration of 10 ng/mL (Table 2).

Human brain microvascular endothelial cell cultures

Human brain microvascular endothelial cells (hBMEC) (Cat No. 1000) were purchased from ScienCell. Cells were then resuspended in fibronectin-coated T-75 flasks, with 20 mL complete endothelial cell medium (ECM) (ScienCell, Cat No. 1001) and incubated at 37 °C with 5% CO2. Fresh medium was replaced every 2–3 days. When forming a confluent layer, cells were rinsed with Dulbecco's Phosphate Buffered Saline (DPBS, Cat No. 20012-027, Gibco™) followed by 2 ml of Trypsin/EDTA (0.25%), phenol red (Cat. No 25–200-072, Gibco™) and incubated at 37 °C until the cells were fully detached. Next, cells were counted and centrifuged for 5 min at 1000 rpm resuspended in fresh culture medium, and finally plated at 50,000 cells/cm2.

In vitro PDGF-BB stimulation via phenytoin treatment in human brain microvascular endothelial cells

HMBEC were treated with either 0, 0.2, 2, 20 µg/mL of PHT for 48 h. HBMEC lysates were collected and probed for PDGF-BB using an ELISA.

Pericyte viability assay

MTT (3-(4,5-dimethylthiazol-2-yl)–2,5-diphenyltetrazolium bromide) (cat No. M5655, Sigma-Aldrich) was dissolved in water to 5 mg/mL. Mouse brain vascular pericytes were seeded in 96-well plates containing a final volume of 100 µL/well and incubated for 2 and 24 h. MTT solution (10 µL) was added per well to achieve a final concentration of 0.45 mg/mL and plates were incubated for 2 h at 37 °C. Next, MTT solution was aspirated off and

100 µL solubilization solution (DMSO, cat No. D45, Sigma-Aldrich) was added to each well to dissolve the formazan crystals for 10 min on an orbital shaker. Plates were read at 570 nm with a plate reader.

Pericyte edu proliferation assay

MBVP proliferation was measured by an Edu proliferation Kit (iFluor 488) (cat No. ab219801, Abcam). Edu solution was added to wells for 2–4 h at 37 °C with 5% CO2 prior to completion of experiment. Next, cells were fixed with 4% paraformaldehyde for 15–20 min followed by three washes in a 3% BSA -PBS solution. Reaction buffer was added to the plate for 30 min at RT protected from light. After three PBS washes, mounting medium with DAPI (Cat No. ab104139, Abcam) was used to counterstain cell nuclei and to mount the rounded coverslips on slides. Images were captured with a ZEISS LSM 800 confocal microscope.

Conventional western blotting

Cell lysates were diluted in 4X Laemmli buffer (with 20x β-mercaptoethanol; Cat No. 1610747, BioRad) separated by 4–15% precast gels SDS-PAGE (BioRad) and transferred onto PVDF membranes (Cat No. 1620239, Bio-Rad). Membranes were blocked with 5% non-fat milk in 0.05% Tween 20 Tris-buffered Saline (TBST) for 1 h at RT, followed by an incubation with primary antibodies (Table 3) at 4 °C overnight. Membranes were exposed to horseradish peroxidase-conjugated secondary antibodies (secondary antibodies were obtained from Cell Signaling and used in 1:500 dilution) for 1 h at RT Then, membranes were washed in TBST and deionized water and developed using the enhanced chemiluminescence (ECL) system (Cat No. A38554,Thermofisher). β-actin was used as the housekeeping protein to normalize the protein levels. Band quantification was performed with ImageLab software.

Table 3 Details of antibodies used for these studies

Primary antibody	Species	Company	Catalogue	Dilution
VCAM1	Rabbit	Abcam	Ab134047	1:1000
STAT1	Rabbit	Cell Signaling	14,994 S	1:1000
p-NFkB	Rabbit	Cell Signaling	3033 S	1:1000
β-Actin	Mouse	Millipore Sigma	MAB1501	1:500
Secondary antibody	**Species**	**Company**	**Catalogue**	**Dilution**
Anti-rabbit HRP-linked	Goat	Cell Signaling	7074	1:1000
Anti-mouse HRP-linked	Horse	Cell Signaling	7076	1:1000

Characterization of PDGFRβ and total MMP-9 expression by ELISA

MBVP lysates were collected from adherent cells by first removing the media and washing the cells with ice-cold PBS. Lysis buffer consisting of M-PER (cat No. 78501, ThermoFisher Scientific) supplemented with phenylmethanesulfonylfluoride fluoride (1 mM) and Halt™ protease and phosphatase inhibitor cocktail (Cat No. 78440, ThermoFisher Scientific) was added to the plate, the cells were scraped off, and the solution was transferred to new tubes and stored at −80 °C. After thawing, cell lysates were centrifuged for 5 min at 5000 x g, 2–8 °C. Quantitative determination of mouse beta-type platelet-derived growth factor receptor (PDGFRβ) and total MMP-9 concentrations were evaluated using ELISA kits (respectively Cat No. MBS919047, MyBioSource Cat No. MMPT90, R&D Systems). Intra-assay precision: CV% <8%. Normalization to total protein was measured using the bicinchoninic acid (BCA) protein assay (cat No. 23225, ThermoFisher Scientific).

Characterization of PDGF-BB expression levels by ELISA

Human and mouse isolated cerebrovessels lysates, in addition to HBMEC lysates were probed to determine PDGF-BB levels. Quantitative determination of PDGF-BB was evaluated using an ELISA kit (Cat No. ab224879, Abcam). Normalization to total protein was measured using the bicinchoninic acid (BCA) protein assay (cat No. 23225, ThermoFisher Scientific).

Immunofluorescence assay

Cells were fixed with 4% paraformaldehyde (PFA, Cat No. 158127, Sigma-Aldrich) for 15–20 min followed by three PBS washes. Firstly, cells were permeabilized with a solution containing 0.1% Triton X-100 (Cat No. 93443, Sigma-Aldrich) in PBS for 15 min at RT. Then, cells were blocked with a solution of 5% donkey serum (Cat No. D9663, Sigma-Aldrich) and 0.1% Triton X-100 in PBS for 1 h at RT. After that, cells were incubated with a primary antibody (Table 3) solution containing 5% donkey serum and 0.1% Triton X- 100 overnight at 4 °C, followed by PBS washing to remove the excess primary antibody solution. Next, cells were incubated with a secondary antibody solution containing 5% of donkey serum 0.1% Triton X-100 and Alexa Fluor-647 conjugated donkey anti-rabbit (Cat No. A31573, Invitrogen) used at a dilution of 1:500 for 1.5 h at RT, protected from light. After three washes with PBS, mounting medium with DAPI (Cat No. ab104139, Abcam) was used to counterstain cell nuclei and to mount the rounded coverslips on slides. Images were captured with a ZEISS LSM 800 confocal microscope.

Confocal imaging analysis

Quantitative analysis of intensity and positively stained cells was performed using ImageJ software. Quantification of intercellular (both cytoplasmic and nuclear) pAkt, pPDGFRβ and VCAM1 was performed by setting a threshold level equal to all samples and measuring the corrected total cell fluorescence (CTCF) using the formula: CTCF = integrated density– (Area of selected cell X Mean fluorescence of background readings). Quantification of nuclear translocation of NF-kB and STAT1 was performed by setting a level equal to all samples and measuring the corrected total nuclear fluorescence (CTNF) using the same formula mentioned above.

Collection of conditioned media and cytokines measurement (secretome analysis) by MSD assay

Cytokine levels in the cell media of treated MBVP with cytokines and/or PDGF-BB and primary isolated pericytes were analyzed through electrochemiluminescence detection using the Meso Scale Discovery (MSD) 96-well MULTI-SPOT® Ultra-sensitive V-PLEX Proinflammatory Panel 1 Mouse Kit on an MSD Sector Imager™ 6000 with Discovery Workbench software (version 3.0.18) (MSD®, Gaithersburg). This assay measured ten cytokines as follows: IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, KC/GRO, IL-10, IL-12p70, TNF-α. All assays were performed according to manufacturer's instructions in triplicate. Cell supernatants (50 µL) were added to the plate wells which contained the capture antibody immobilized on a working electrode.

Statistical analysis

Data were analyzed by ordinary one-way analysis of variance (ANOVA) with Tukey's multiple comparisons statistical tests and t-test using GraphPad Prism 9.4.0. Significant statistical difference between groups was considered * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$, **** $p < 0.0001$. Data were reported as mean ± standard error of the mean (SEM).

Results

Effects of inflammatory insult on mouse brain vascular pericytes

PDGFRβ is a widely used marker for pericytes and it can be upregulated in response to stresses such as an injury or inflammation [11]. To identify the physiological response of pericytes to inflammatory insult, PDGFRβ expression levels were examined with an ELISA assay. Cultured pericytes were treated with TNF-α, IL-1β and IFN-γ at 100 ng/mL for 2–24 h and a 3-fold and 8-fold increase in PDGFRβ expression was observed at 2 h and 24 h following cytokine stimulation, respectively (Fig. 2A).

Inflammatory conditions generally constitute upregulation of many cytokines, chemokines, and other inflammatory molecules [6, 21]. To determine if the cytokine concentration used in these studies affected pericyte viability, MTT assays were used to measure cellular metabolic activity as an indicator of cell cytotoxicity. MBVP were exposed to TNF-α, IL-1β or IFN-γ alone or grouped (at a concentration of 100 ng/mL) for 2–24 h. As seen in Fig. 2B, overall, there was no significant difference in viability ratios between cells treated with these cytokine doses and the control group, suggesting this specific cytokine stimulation paradigm does not demonstrably alter cellular viability.

Pericyte inflammatory response through activation of STAT1-NFkB and upregulation of VCAM1 and MMP9

Inflammatory response protein expression is largely controlled by transcription factor-mediate gene transcription. To further understand how upregulation of such cytokines alters the pericyte immune response, the activation of the transcription factors signal transducer and activator of transcription 1 (STAT1) and phosphorylated nuclear factor kappa-light-chain-enhancer of activated B cells (p-NFkB) were examined. Following cytokine stimulation, elevated levels of both STAT1 and p-NFkB were detected after 2 h, which were maintained and significantly increased after 24 h (Fig. 2C). Furthermore, a significant upregulation of an immune mediator enhancing leukocyte extravasation (VCAM1) (Fig. 2C) was observed after 24 h of cytokine stimulation and a vascular permeability enzyme (MMP9) (Fig. 2D) after 2 and 24 h of stimulation. These results indicate that even acute cytokine stimulation can provoke an inflammatory phenotype in vascular brain pericytes (pericytosis).

Cytokines and PDGF-BB stimulates pericyte viability

To further characterize responsiveness of pericytes to cytokine stimulation, pericyte health was evaluated through an Edu-positive cell assay. Pericytes were stimulated with 10 ng/mL PDGF-BB since the PDGF-BB: PDGFRβ signaling is critical to pericytes survival during both basal and inflammatory conditions. This concentration was used for further experiments since pericytes treated with 10 ng/mL showed an increased number of PDGFRβ puncta in cells (Supplementary files: Figure S2). A significant proliferative response was observed in treated pericytes (the 24 h time point was used based on more significant changes represented in the experiment shown above) versus the untreated control. Both PDGF-BB and cytokines induced a strong proliferative response in cultured pericytes in vitro (Fig. 3).

PDGF-BB levels are diminished in the cerebrovasculature following mild TBI

As mentioned above, PDGF-BB: PDGFRβ signaling has a pivotal role in pericyte function under both basal and

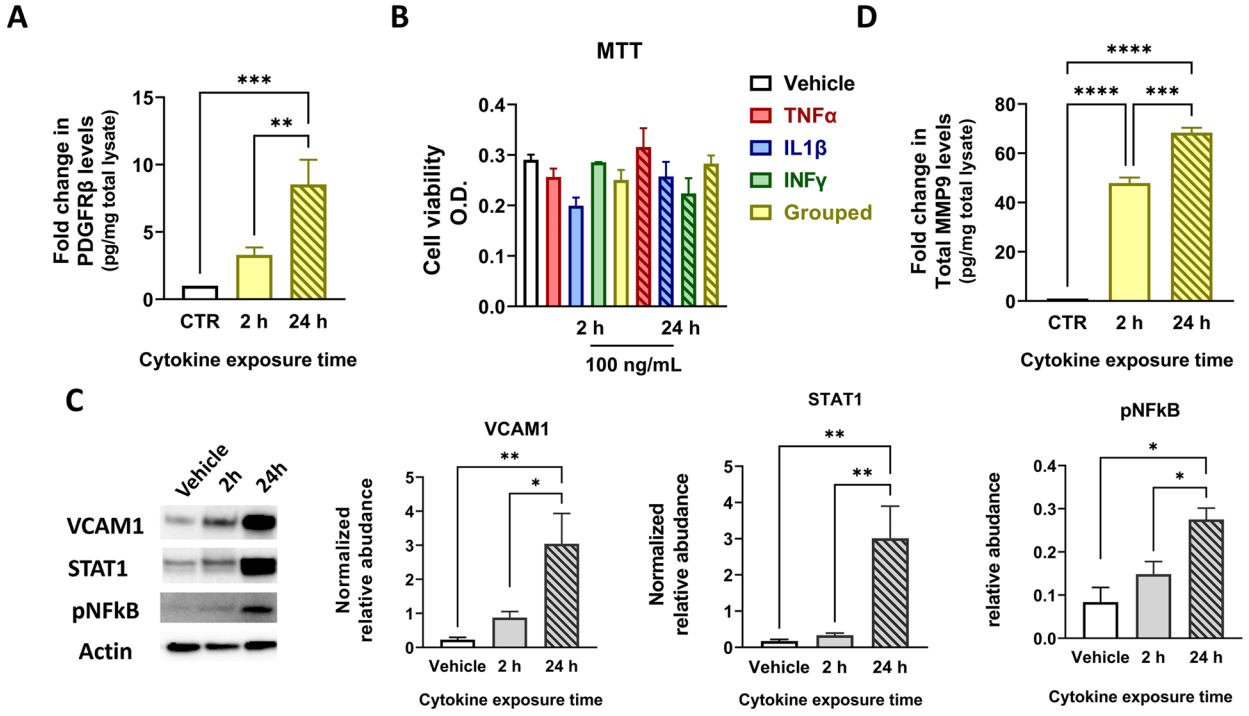

Fig. 2 Inflammatory insult does not have a cytotoxic effect on mouse brain vascular pericytes but drives pericytes towards an inflammatory phenotype in vitro. **A** PDGFRβ expression levels after treatment with a combination of the three cytokines for 2 and 24 h. Pericyte lysates were analyzed by a PDGFRβ ELISA assay. **B** Pericytes were treated with each individual cytokine (100 ng/ml) or with a combination of the three cytokines for 2 and 24 h. The MTT assay was used to measure cytotoxicity. **C** Representative western blot images of pericytes treated with a combination of the three cytokines for 2 and 24 h. Bands were quantified with ImageLab and normalized to actin. **D** Total MMP9 levels in pericytes treated with a combination of the three cytokines for 2 and 24 h. Pericyte lysates were analyzed by a total MMP9 ELISA assay. All results are presented as mean ± SEM. *$p < 0.05$; **$p < 0.01$; *** $p < 0.001$; ****$p < 0.0001$ Multiple comparisons (ANOVA)

disease conditions. Our work showed decreased levels of PDGF-BB in human and mouse r-mTBI brain homogenates (Fig. 4A and B). Moreover, we found freshly isolated r-mTBI mouse cerebrovessels (6 months post-injury) secreted less PDGF-BB than r-sham vessels, indicating reduced availability of PDGF-BB in chronic r-mTBI (Fig. 4C). Also, in our previous studies, we showed that at 6 months post-injury, there was no difference in the number of endothelial cells in the vascular fraction between r-mTBI and r-sham groups [32]. As such, the lack of PDGF-BB was not attributed to a reduction in endothelial cell quantity but rather to compromised endothelial cell health and disrupted cerebrovascular cell cross-talk, which may be contributing to pericyte-mediated inflammation.

Effect of PDGF-BB treatment on inflamed pericytes

Our work showed that after TBI, there is an insufficient supply of PDGF-BB available to pericytes, which may be altering their functionality. This led us to explore whether we could stimulate pericytes through PDGF-BB treatment to modulate the pericyte response to inflammation. Following cytokine exposure, pericytes were treated with 10 ng/mL of PDGF-BB at two different time intervals: (a) prior to inflammatory insult and (b) following inflammatory insult. After the treatment period, cellular lysates were probed for PDGFRβ via an ELISA assay. While PDGF-BB stimulation prior to cytokine stimulation did lead to a subtle reduction in PDGFRβ levels, administration of PDGF-BB following cytokine stimulation significantly mitigated the effect of the cytokine stimulation, lowering PDGFRβ levels by nearly 2-fold compared to cytokine stimulation alone (Fig. 5A). As a complement to these studies, pericytes were also fixed and immunostained for phosphorylated PDGFRβ at tyr571 as a marker of receptor activation, as it is known to be the major residue of the receptor that is phosphorylated in response to PDGF-BB stimulation. Here again, a significant increase in pPDGFRβ activation was observed upon cytokine stimulation compared to control. Moreover, PDGF-BB treatment both prior to and following cytokine stimulation significantly attenuated the cytokine effect, as pPDGFRβ levels were reduced to those observed under control conditions (Fig. 5B).

To further investigate whether PDGF-BB stimulation could modulate the response of pericytes to inflammatory stimuli, cells were fixed and immunostained for pericyte-specific pro-inflammatory transcription factor

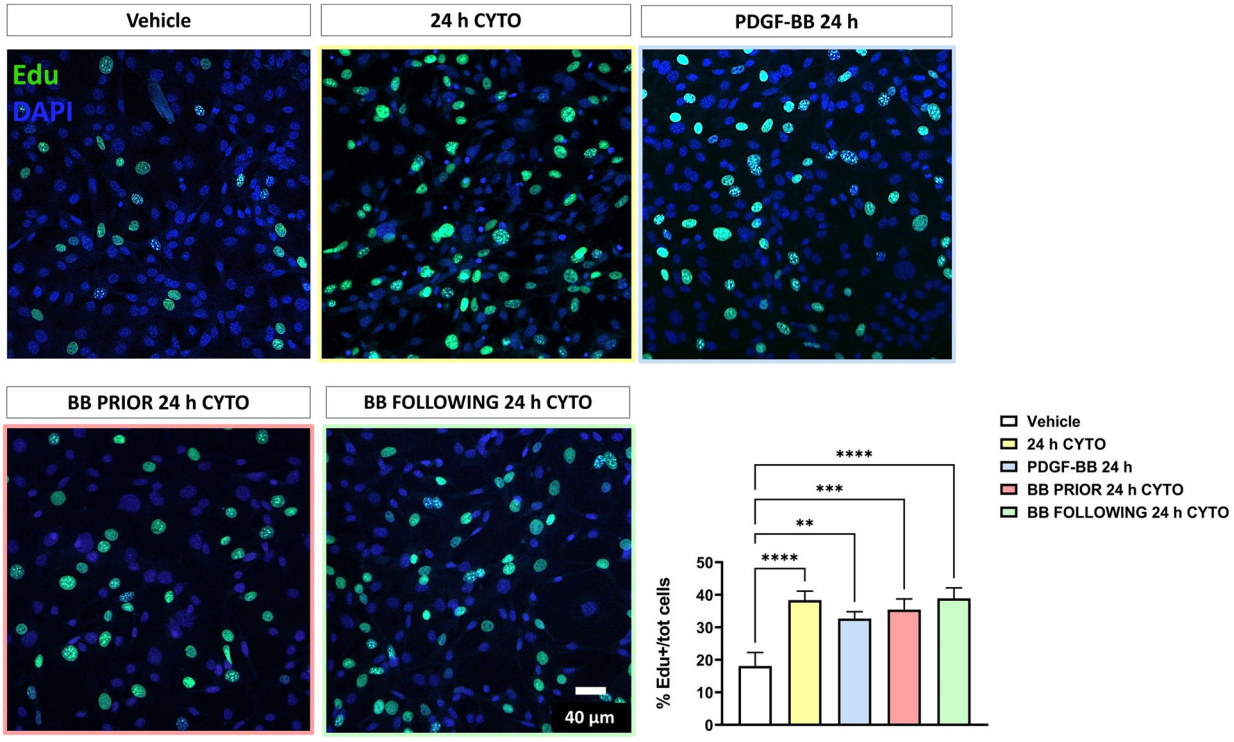

Fig. 3 PDGF-BB and/or cytokine treatment increases pericyte proliferation in vitro. The percentage of Edu+ cells was determined by image analysis using ImageJ Software. All results are presented as mean ± SEM.*$p < 0.05$; **$p < 0.01$; *** $p < 0.001$; ****$p < 0.0001$ Multiple comparisons (ANOVA). Scale bar 40 µm

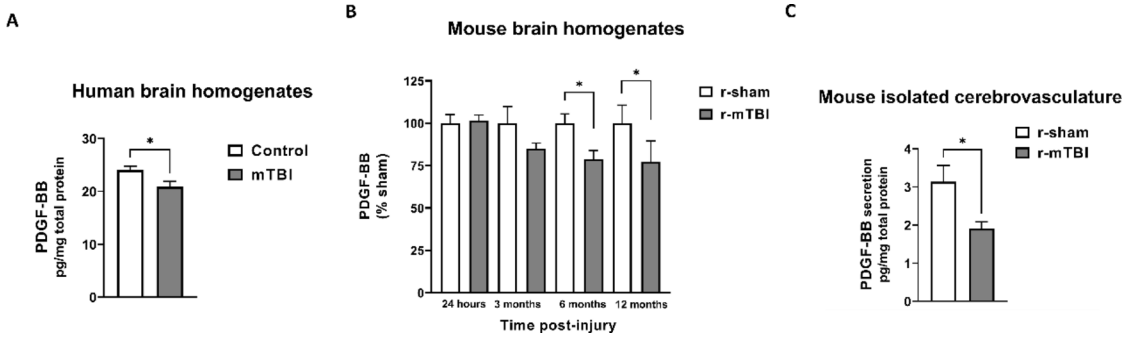

Fig. 4 Decreased PDGF-BB levels in the brain after mild TBI. **A** Human brain homogenates were analyzed by PDGF-BB ELISA assay. **B** r-sham and r-mTBI mouse brain homogenates were analyzed by PDGF-BB ELISA assay at 24 h, 3, 6, and 12 months post-injury **C** Conditioned media was collected from isolated cerebrovessels from r-sham and r-mTBI mice (6 months post-injury) after incubation for 72 h and analyzed by PDGF-BB ELISA. All results are presented as mean ± SEM. *$p < 0.05$; **$p < 0.01$ t-test and Multiple comparisons (ANOVA)

markers (STAT1, NFkB and pAkt) in addition to VCAM1 and MMP9. Pericytes were treated with cytokines for 24 h and PDGF-BB was given prior to and following the inflammatory insult. Translocation of NFkB and STAT1 to the nucleus were used as markers of a pro-inflammatory response. Treatment with cytokines significantly increased both NFkB and STAT1 levels. Here, PDGF-BB stimulation prior to and following cytokine stimulation significantly reduced cytokine-induced STAT1 levels. NFkB levels were largely decreased when PDGF-BB was administered after cytokine exposure while higher levels were detected when PDGF-BB was given before the cytokine stimulation (Fig. 6A and B). A significant increase in VCAM1 staining and total MMP9 was observed in the pericyte lysate after the 24 h cytokine stimulation. Once again, PDGF-BB stimulation significantly reduced the effect of cytokine stimulation on both VCAM1 (Fig. 6D) and total MMP9 expression (3-fold each) compared to cytokine stimulation alone (Fig. 6E). Furthermore, since it is well documented that PDGF-BB: PDGFRβ signaling can act through the Akt pathway, the effect of PDGF-BB treatment on this downstream pathway under

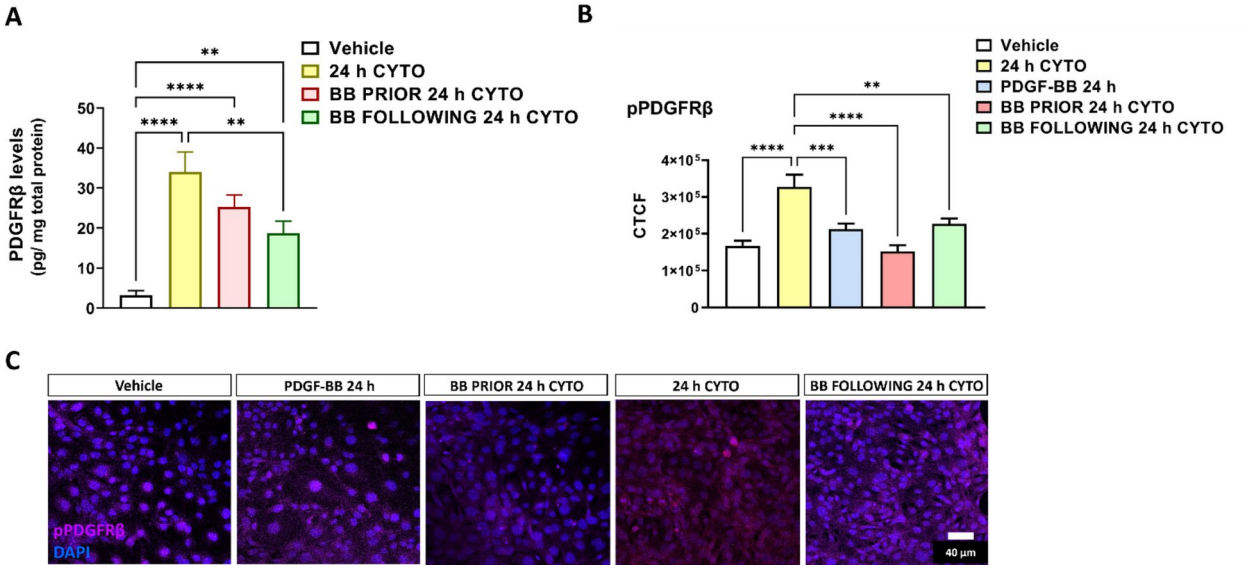

Fig. 5 PDGF-BB and cytokines alters PDGFRβ expression and signaling in inflamed pericytes. Pericytes were treated with cytokines for 24 h with and without PDGF-BB. **A** Pericyte lysates were analyzed using an ELISA assay. **B** Pericytes were fixed and immunostained for pPDGFRβ and quantification of intracellular pPDGFRβ was performed measuring the CTCF (Corrected Total Cell Fluorescence), which includes both nuclear and cytoplasmic levels. Representative images of pPDGFRβ (magenta) and DAPI (blue) for each treated group are shown. All results are presented as mean ± SEM.*$p < 0.05$; **$p < 0.01$; *** $p < 0.001$; ****$p < 0.0001$ Multiple comparisons (ANOVA). Scale bar 40 µm

inflammatory conditions was interrogated. Like the other molecules investigated, an increase in pAkt following cytokine stimulation and a subsequent reduction upon PDGF-BB treatment was observed, though none of these effects reached statistical significance. (Fig. 6C). These results indicate that cytokine stimulation provokes pericytes into an immune-responsive state that is mitigated by PDGF-BB stimulation.

Effect of inflammatory insult and PDGF-BB on immune-modulatory pericyte secretome

In addition to contributing to vascular function and maintenance of the BBB, pericytes also play an extremely important role in brain immunosurveillance and can modulate neuroinflammation via paracrine secretion of distinct inflammatory molecules and growth factors. Using an MSD assay, secretion of ten cytokines was measured from pericytes in response to inflammatory insult and PDGF-BB stimulation. For this experiment, PDGF-BB was given at three different time intervals (a) prior to inflammatory insult (b) simultaneous to inflammatory insult and (c) following inflammatory insult. After the treatment period, the conditioned media was collected and probed using the MSD assay.

All 10 cytokines were detectable, including IL-10, IL-4 and IL-5 (Fig. 7E, H and I). High levels of TNF-α, IFN-γ and KC/GRO (Fig. 7A, C and J) were identified alongside IL-6 and IL-12p70 (Fig. 7D and F). When PDGF-BB was given prior to the inflammatory insult, no effect of PDGF-BB on any of the selected cytokine secretions was observed; instead, when co-stimulation of PDGF-BB and cytokines was given to pericytes for 24 h, there was a decrease in the secretion of some of the inflammatory molecules, but overall, these effects were not statistically significant. Interestingly, when PDGF-BB was given following the inflammatory insult, IL-1β, IL-2 and IL-12p70 revealed a significant decrease close to control levels (Fig. 7B, G and F), while IFN-γ showed a significant decrease compared to cytokine stimulation alone after 24 h (Fig. 7C). However, TNF-α, IL-6, IL-10, and KC/GRO were not altered by PDGF-BB exposure when administered after the inflammatory insult (Fig. 7A, D, E and J). These results suggest that PDG-BB has limited or no effect on cytokine secretion when given before or alongside the inflammatory insult. However, when administered after the inflammatory event, PDGF-BB appears to have a selective impact on reducing certain pro-inflammatory cytokines.

Inflammatory secretome profiles in isolated mouse brain pericytes ex vivo following traumatic brain injury

Further research is still needed to understand the roles of pericytes in neuroinflammation and neurodegeneration. Pericytes can secrete a plethora of inflammatory mediators; but their inflammatory profile under disease conditions is largely unknown.

Primary pericytes were isolated from the mouse brain and the purity of the culture was determined (Supplementary files: Figure S1). In this study, we characterized the immunological properties by studying the cytokine secretome of brain pericytes isolated from r-sham and r-mTBI mice following administration of PHT in

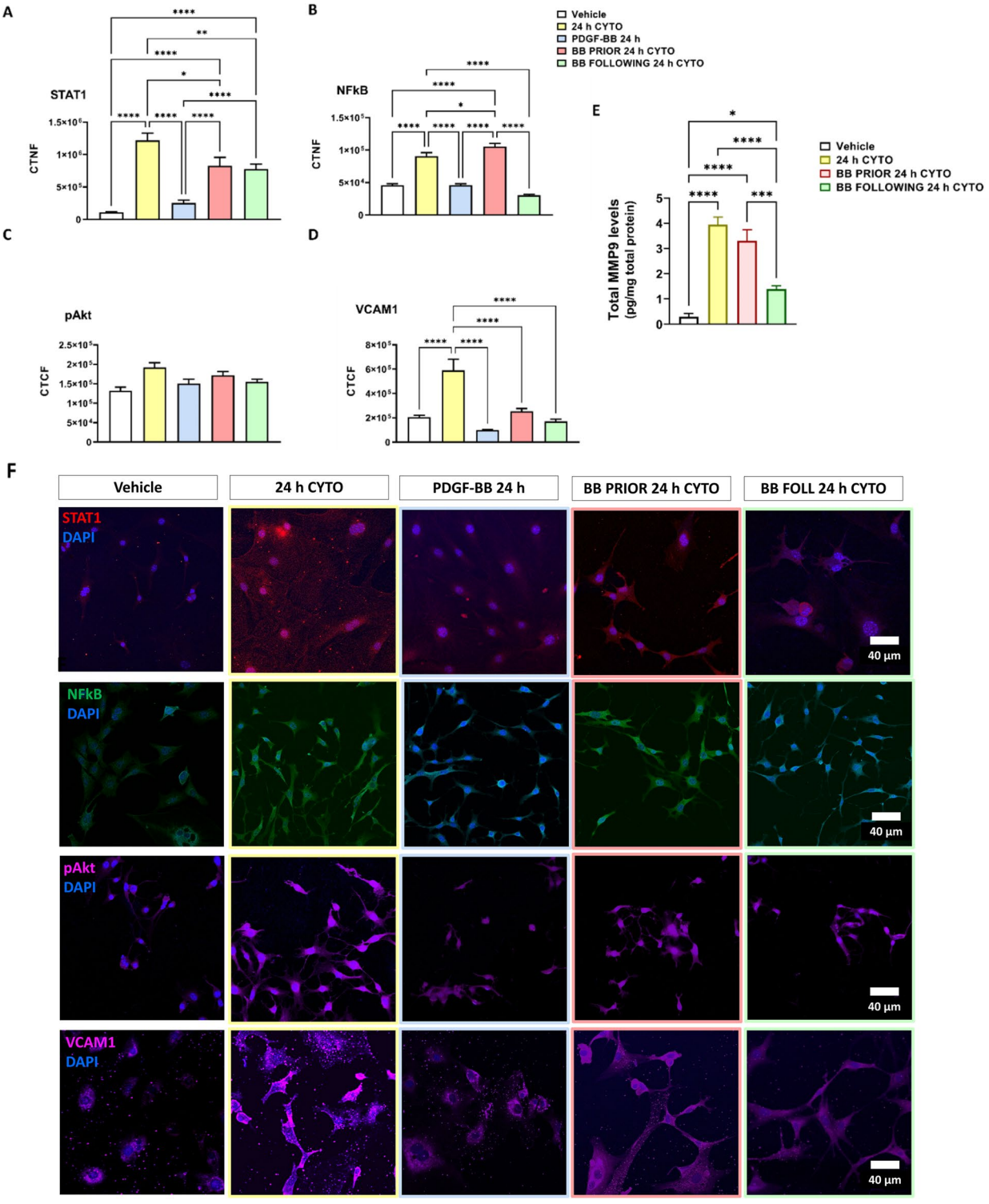

Fig. 6 PDGF-BB stimulation can prevent or mitigate the activated immune-response of pericytes upon exposure to pro-inflammatory molecules. Pericytes were treated with cytokines and/or vehicle for 24 h and PDGF-BB stimulation was given prior or following the inflammatory insult. Cells were fixed and immunostained for **A** VCAM1, **B** STAT1, **C** pAkt, and **D** NFkB. **E** Cell lysates were analyzed by a total MMP9 ELISA assay. **F** Representative images for each treatment group with DAPI (blue) are shown. All results are presented as mean ± SEM. *$p < 0.05$; **$p < 0.01$; *** $p < 0.001$; ****$p < 0.0001$ Multiple comparisons (ANOVA). Scale bar 40 µm

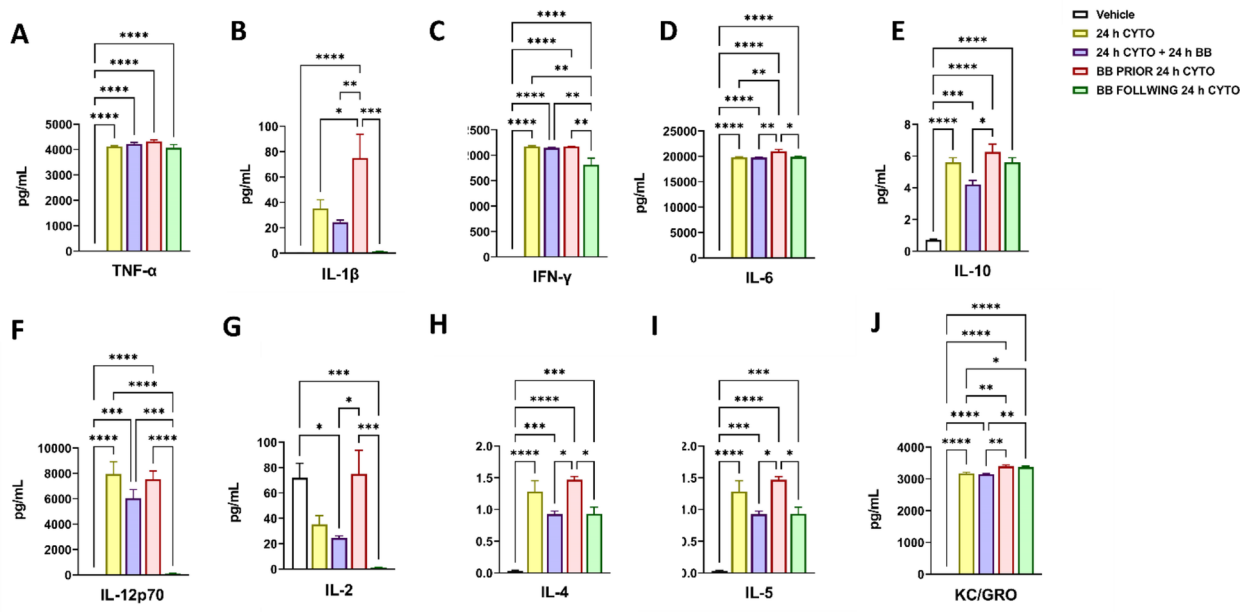

Fig. 7 Pericyte inflammatory protein secretion following cytokine insult and PDGF-BB stimulation. Conditioned media was collected from cultured mouse brain vascular pericytes (MBVP) treated with both cytokines and/or PDGF-BB for 24 h and probed for immunomodulatory factors using the pro-inflammatory panel 1 MSD assay. All results are presented as mean ± SEM. *$p < 0.05$; **$p < 0.01$; *** $p < 0.001$; ****$p < 0.0001$ Multiple comparisons (ANOVA)

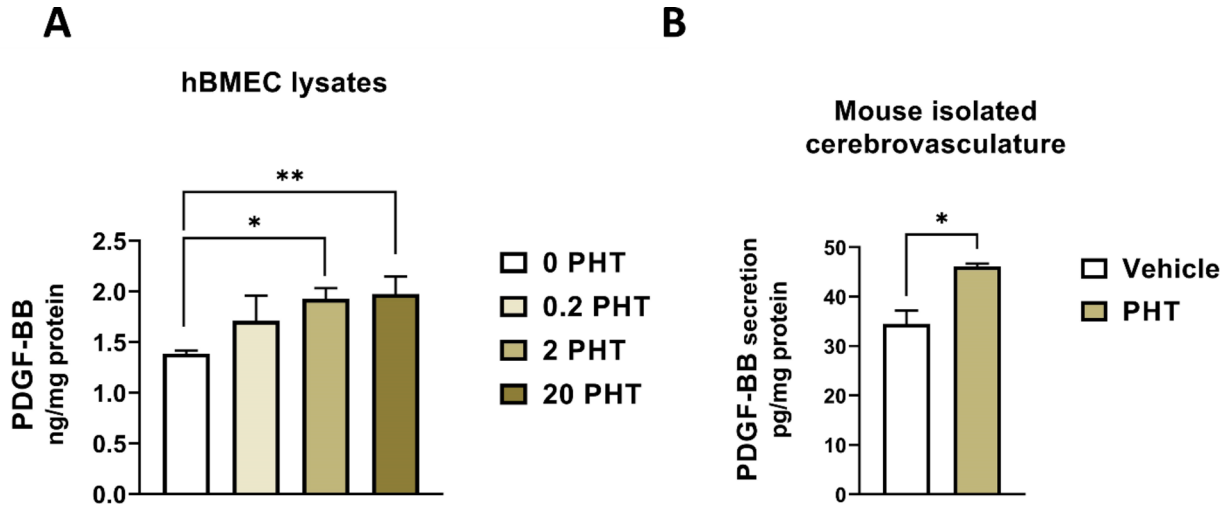

Fig. 8 PDGF-BB secretion levels increase after PHT stimulation both in vitro and ex vivo. **A** hBMEC were treated with different concentrations of phenytoin and cell lysates were analyzed by PDGF-BB ELISA assay. **B** Wild-type mice (3 months old) were administered normal mouse chow or phenytoin in chow (40 mg/kg) for 1 month. After the phenytoin treatment period, fresh cerebrovessels were isolated and lysates were analyzed by PDGF-BB ELISA assay. All results are presented as mean ± SEM. *$p < 0.05$; **$p < 0.01$ t-test and Multiple comparisons (ANOVA)

mouse chow. We showed that PHT can induce PDGF-BB secretion in human endothelia cells in vitro (Fig. 8A) and increase PDGF-BB secretion levels in isolated cerebrovessels from mice (Fig. 8B).

Primary mouse brain pericytes were isolated using a magnetic microbeads cell isolation method. Supernatants were collected from primary brain pericyte cultures and cytokine secretions were analyzed by MSD assay. We showed that all 10 cytokines were detectable, both IL-1β and TNF-α levels were significantly reduced in the r-mTBI- group treated with PHT compared to the untreated r-mTBI group (control). A similar decrease, but not statistically significant, was found in the r-sham group– treated with PHT compared to the untreated r-sham group (control vs. PHT) (Fig. 9A and B). However, no significant differences were observed in IFN-γ or IL-6 across groups (Fig. 9C and D). Interestingly, IL-10 was significantly elevated in both r-sham and r-mTBI -PHT treated groups compared to their untreated counterparts (Fig. 9E). The levels of IL-2 and IL-4 did not

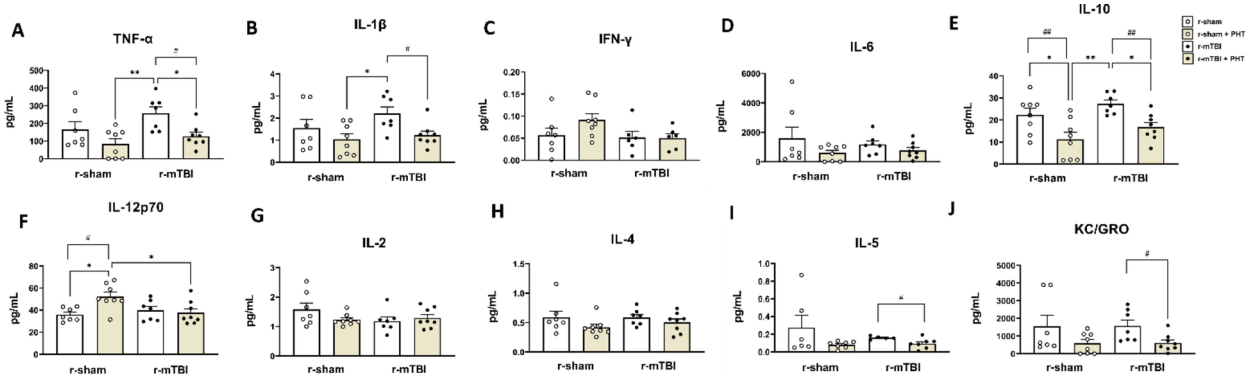

Figure 9. Inflammatory secretome profiles of brain pericytes following TBI. Primary mouse pericytes were isolated by magnetic cell sorting from r-sham and r-mTBI mice (6 months post-injury) following 4 weeks of treatment with phenytoinadministered in mouse chow. After 3-5 days, conditioned media from the isolated pericytes were collected and probed for immunomodulatory factors using the pro-inflammatory panel 1 MSD assay. All results are presented as mean ± SEM *$p < 0.05$; **$p < 0.01$; Multiple comparisons (ANOVA) and #$p < 0.05$; ##$p < 0.01$; t-test

differ significantly across all groups. A notable reduction in IL-5 was observed in the r-mTBI-PHT treated group compared to the r-mTBI control group (Fig. 9I). Finally, KC/GRO was significantly lower in the r-mTBI-PHT treated group compared to the r-mTBI control group (Fig. 9J). These findings highlight the modulatory effects of PHT (perhaps via PDGF-BB induction) on cytokine expression in pericytes following TBI.

Discussion

In this study, we aimed to explore the effect of inflammation on pericyte function and the contribution of pericytes to the inflammatory response in the brain. Additionally, we examined the effect of PDGF-BB stimulation on the immunomodulatory response of pericytes, focusing on their role in maintaining cerebrovascular integrity and regulating neuroinflammation following brain injury.

Our findings provide new insights into the dynamic role of pericytes in the brain's inflammatory response, particularly in the context of TBI, highlighting their potential as targets for modulating secondary brain damage. We observed a significant upregulation of PDGFRβ expression in pericytes after exposure to inflammatory cytokines commonly observed following TBI, such as TNF-α, IL-1β, and IFN-γ [5], indicating that pericytes are responsive to early inflammatory signals. This suggests that pericytes not only act as structural components of the BBB, but they also actively participate in the inflammatory response, contributing to tissue recovery through the modulation of immune cell migration and vascular remodeling, independent of the injury mechanism [25]. The increase in PDGFRβ expression observed after 24 h of cytokine exposure may represent an adaptive response in conditions that would require survival, proliferation, and migration, all critical processes in the recovery from injury [22]. These results complement previous in vivo studies of TBI, where a rapid loss or migration from vessels occurs 3–5 days after injury, followed by "reactive pericytosis" which is characterized by increased receptor expression [19].

The tightly regulated inflammatory response in the brain is partly due to the BBB, which restricts the exchange of inflammatory molecules and cells [35]. The perivascular localization of pericytes within the BBB makes them ideally situated to control several aspects of the brain immune response, including leukocyte extravasation [10]. Leukocyte migration through the BBB involves multiple steps regulated by cell adhesion molecules (CAMs) and chemokine signaling. VCAM-1 is an adhesion molecule expressed by endothelial cells and pericytes which facilitates leukocyte adhesion and transmigration across the BBB. During both basal and inflammatory states, pericytes enhance the expression of these mediators and secrete chemokines to attract circulating leukocytes to the brain via a concentration gradient. Additionally, matrix metalloproteinases (MMPs) derived from pericytes contribute to BBB breakdown, increasing permeability [33]. Importantly, inflammatory signals activate several transcription factors, including NF-κB and STAT1, both implicated in pericyte-mediated inflammation. We found significant upregulation of VCAM1, STAT1 and p-NFkB protein expression in pericyte lysate following cytokine exposure after 24 h, with an increasing trend already detected at 2 h. Furthermore, we detected an increase in MMP9 levels after 2 h, which significantly increased after 24 h. These results further indicate that pericytes serve as active regulators of vascular integrity, influencing both permeability and immune cell trafficking.

The PDGF-BB: PDGFRβ signaling axis has been identified as crucial regulator of pericyte function, particularly under inflammatory conditions [23, 26]. In line with this body of work, our findings highlight the potential

therapeutic significance of modulating the PDGF-BB: PDGFRβ axis to improve pericyte health and cerebrovascular function in the context of brain injury. Specifically, we observed a marked reduction in PDGF-BB levels in both human and mouse brain homogenates following mild brain injury, which indicates a disruption of the PDGF signaling cascade. Moreover, we found that freshly isolated r-mTBI mouse cerebrovessels secreted less PDGF-BB than control animals (r-sham); suggesting head injury diminishes the vascular production and availability of PDGF-BB in the brain, which not only impairs the integrity of the cerebrovascular network but also pericyte activity. This is especially concerning as pericytes are essential for maintaining vascular stability, regulating BBB function, and ensuring proper nutrient and oxygen delivery to the brain. Without adequate PDGF-BB signaling, pericytes may lose their protective function, resulting in vascular leakage, cerebral edema, and increased vulnerability to further damage. These findings combined with our prior observations of a progressive reduction in PDGFRβ expression in the brain vasculature following r-mTBI [32], highlight the extent of the disruption to the PDGF pathway in the chronic stages after brain trauma. The loss of PDGFRβ in pericytes would not only impair endothelial-pericyte cross-talk, but also pericyte-mediated immune modulation. The decrease in PDGF-BB secretion and the reduced expression of PDGFRβ observed in our studies highlight a concerning breakdown of the mechanisms that are crucial for maintaining vascular homeostasis after brain injury. This imbalance in the PDGF signaling pathway may lead to more than just structural damage to the vasculature; it may contribute to a less effective response to injury, leading to prolonged inflammation, secondary damage, and delayed recovery. Given the pivotal role of pericytes in regulating both vascular tone and the immune response, restoring PDGF-BB: PDGFRβ signaling could represent a promising approach to enhance cerebrovascular function and improve outcomes following TBI.

To further our investigation, we examined the effects of cytokines and PDGF-BB stimulation on pericyte disposition. We initially confirmed PDGFRβ activation and nuclear translocation following PDGF-BB stimulation with the 10 ng/mL concentration yielding the highest puncta count, which was used for the subsequent experiments (Supplementary files: Figure S2). Both PDGF-BB stimulation and cytokine exposure increased Edu + cells, demonstrating the role of PDGF-BB in activating proliferation signaling pathways, while the strong response to pro-inflammatory cytokines suggested specific regulation of pericyte growth. Once again, our results demonstrate the ability of pericytes to actively respond to stressful conditions by remaining proliferative and prepared to coordinate subsequent neuronal immune response events.

Next, we investigated the influence of PDGF-BB stimulation on the status of the PDGF pathway upon inflammatory insult. We exposed culture pericytes to PDGF-BB at two time-points: (a) prior to inflammatory insult, and (b) following inflammatory insult, and evaluated PDGFRβ protein expression. We found that while PDGF-BB stimulation of pericytes prior to cytokine exposure showed a subtle change in receptor expression compared to cytokines alone, pericytes treated with PDGF-BB stimulation after the inflammatory insult significantly normalized the cytokine effects on receptor expression levels. These data suggest PDGF-BB stimulation can stabilize the PDGF pathway following inflammatory insult, which may be a useful approach in regulating the immunoreactivity of vascular pericytes upon insults to the brain.

To further asses the response of the PDGF pathway to inflammatory exposure and PDGF-BB stimulation, we also evaluated PDGFRβ activation, specifically focusing on tyrosine 751 (Tyr751), a major residue of PDGFRβ that is phosphorylated upon PDGF-BB stimulation and associates with phosphatidylinositol-3-kinase (PI3K). While 24 h of PDGF-BB stimulation alone did not alter the pPDGFRβ levels compared to vehicle, PDGF-BB treatment both prior to and following cytokine exposure significantly normalized pPDGFRβ levels, again indicating PDGF-BB stimulation in pericytes can offset alterations in the PDGF pathway induced by inflammatory insults.

Furthermore, PDGF-BB stimulation after cytokine exposure alleviated the inflammatory-induced upregulation of key transcription factors such as STAT1 and NF-κB. These transcription factors are central to the inflammatory cascade, with NF-κB driving the expression of pro-inflammatory cytokines [4]. The observed time-dependent effects of PDGF-BB on NF-κB and STAT1 activation highlight the nuanced role of this pathway in modulating pericyte inflammatory responses. Early PDGF-BB stimulation may serve to prime pericytes for a protective immune response, while post-injury PDGF-BB treatment appears to exert a more pronounced anti-inflammatory effect. This underscores the potential therapeutic value of PDGF-BB in mitigating neuroinflammation and promoting vascular repair following brain injury.

We also observed the effect of PDGF-BB on the Akt pathway, which transmits extracellular stimuli to the nucleus for proliferation and survival. PDGF-BB stimulation after cytokine insult resulted in a slight decrease in pAkt levels, though these effects were not statistically significant. Conversely, we noted a significant reduction in VCAM1 and MMP9 levels when PDGF-BB stimulation was administered after the cytokine insult, while

PDGF-BB stimulation prior to cytokine exposure failed to reduce MMP9 levels.

Recent evidence suggests that brain pericytes regulate immune responses through the release of a heterogeneous secretome of pro-/anti-inflammatory molecules, cytokines, chemokines, and growth factors, indicating their potential as a target for modulating brain inflammation [8, 15]. Based on that, we aimed to investigate how pericytes respond to the common inflammatory cues in TBI and whether PDGF-BB could affect their immunomodulatory secretome. The observed effects of PDGF-BB on the pericyte secretome further reinforce the idea that PDGF-BB signaling shapes the pericyte inflammatory response. Specifically, we found that PDGF-BB stimulation prior to cytokine exposure increased the secretion of several pro-inflammatory interleukins, such as IL-6 and IL-12p70, which are key players in the innate immune response. Conversely, PDGF-BB treatment after cytokine exposure reduced the levels of certain cytokines, including IL-1β, IL-2, IFN-γ and IL-12p70, to near control levels, suggesting that PDGF-BB stimulation can redirect pericytes to a less inflammatory state. Interestingly, while PDGF-BB did not affect the levels of IL-10, a potent anti-inflammatory cytokine, when applied prior to the inflammatory insult, PDGF-BB did reduce IL-10 levels when administered post-insult. This indicates a complex interplay between PDGF-BB signaling and pericyte-mediated immune responses, which may vary depending on the timing and context of treatment. The cytokine profiling also revealed an interesting opposition in the response of pericytes to inflammation and PDGF-BB treatment. While pro-inflammatory cytokines such as TNF-α and IFN-γ were elevated across all experimental groups, we observed that PDGF-BB treatment post-cytokine exposure significantly reduced the secretion of IL-1β, IL-6, and IL-12p70 and increased KC/GRO levels, suggesting a shift toward a neuroprotective phenotype. These findings align with a growing body of literature suggesting that PDGF-BB may help pericytes transition from an inflammatory to a reparative state [20, 31]. Also, these results highlight the complexity of PDGF-BB's immunomodulatory potential and the need to consider the timing of any intervention and the specific immune-factor cascades involved.

While PDGF-BB holds promise as a therapeutic strategy to regulate pericyte activity and neuroinflammation, its translational application remains challenging. We explored an alternative approach to stimulate the PDGF pathway using PHT, which was administered in mouse chow over a four-week period. Previous work demonstrated PHT can induce PDGF-BB expression in brain endothelia [9]. Moreover, it was found that PDGF-BB mRNA was significantly elevated in PHT-treated human subjects compared to untreated individuals [9]. Our studies support these reports demonstrating that PHT administration increases PDGF-BB secretion in both human brain microvascular endothelial cells (hBMECs) and isolated cerebrovessels from PHT-treated mice. As such, by inducing PDGF-BB secretion from brain endothelia, PHT could provide a translatable strategy for modulating the response of brain pericytes to neuroinflammatory insults.

To complement the above in vitro studies and further evaluate the effects of PHT on neuroinflammation and pericyte immune modulation under disease conditions, we performed ex vivo secretome analyses using brain pericytes isolated from a mouse model of closed head injury (r-mTBI). The observed reduction in pro-inflammatory cytokines TNF-α and IL-1β, suggest that PHT can reduce cerebrovascular inflammation, which could promote tissue repair mechanisms and stabilize the neurovascular unit following brain insults, like TBI. Specifically, the decreased levels of IL-10 in both r-sham and r-mTBI-PHT treated groups indicate an attenuated systemic inflammatory effect, potentially mitigating excessive inflammatory responses that damage the tissue, which in this scenario, may protect against the secondary brain injury. Additionally, the significant decrease in KC/GRO, a chemokine involved in neutrophil recruitment, implies a dampening of acute inflammatory cell infiltration into the injury site. Interestingly, the lack of significant changes in IFN-γ and IL-6, alongside with the increase in IL-12p70 in r-sham-PHT treated group, may suggest that PHT treatment could be modulating the cytokine environment to promote a more controlled, localized inflammatory response that may be more conducive to tissue repair and neuroprotection. The absence of significant changes in some cytokines may indicate that the effects of PHT are not broad-based but rather focused on regulating certain inflammatory pathways (potentially through the modulation of PDGF-BB signaling in pericytes), emphasizing its therapeutic potential. In this way, PHT could be influencing the balance between pro-inflammatory and anti-inflammatory responses, rather than globally suppressing all immunomodulatory molecules.

Taken together, these data point to a nuanced effect of PHT on neuroinflammation, wherein PDGF-BB secretion, induced by PHT, potentially shifts the pericyte response toward a more neuroprotective role. By dampening pro-inflammatory molecules like TNF-α, IL-1β, IL-5, and KC/GRO, PHT may help reduce the harmful inflammatory cascade that often follows brain injury. In contrast, the shift in cytokine profiles also hints at the therapeutic potential of PHT to fine-tune the immune response, promoting a more balanced neuroinflammatory environment that could facilitate recovery. However, caution should be used when interpreting these

data as many conditions (media, supplements, treatments, etc.) may affect cell physiology and biology. The differences observed between the ex vivo and in vitro pericyte responses could be due to the more complex inflammatory environment present in the injured brain, where multiple overlapping inflammatory mediators and pathways might counterbalance or obscure the effects of PHT treatment. The r-mTBI condition may create a more severe or sustained inflammatory milieu that is less responsive to the effects of PDGF-BB stimulation compared to the in vitro condition. Additionally, PHT may be exerting its effects through pathways beyond PDGF signaling. Thus, the use of phenytoin in the context of brain injury requires further optimization, potentially in combination with other therapies that target the broader inflammatory cascade.

Conclusions

In conclusion, our results demonstrate that pericytes play a crucial role in the immune response within the brain, particularly in the context of neuroinflammatory disorders such as TBI. Notably, we observed a chronic reduction in PDGF-BB levels in both human and animal models of TBI, which appears to contribute to pericyte dysfunction and subsequent cerebrovascular inflammation following brain injury. Importantly, PDGF-BB stimulation in inflamed pericytes showed promise in reducing inflammatory molecules and markers of vascular injury, revealing the potential therapeutic value of the PDGF pathway. Furthermore, chronic treatment with phenytoin, a clinically relevant PDGF-BB inducer, demonstrated a significant decrease in key inflammatory markers in pericytes isolated from r-mTBI mouse brains. These findings not only emphasize the critical involvement of PDGF signaling in pericyte-mediated neuroinflammation, but also suggest that PDGF-BB based therapeutic strategies, such as phenytoin, hold promise for improving cerebrovascular outcomes after TBI. Overall, these studies highlight the importance in studying pericyte reactivity following head trauma to better understand the role of these cells in TBI pathogenesis.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12950-025-00439-9.

Supplementary Material 1.

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Authors' contributions

A.C. wrote the main manuscript text and performed all the experiments and procedures. M.E. and S.F. assisted with experimental design and planning. D.P. contributed to data collection for Figs. 7 and 9 C.B. was the main director of the study and contributed to writing and reviewing the manuscript. All authors reviewed the manuscript.

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Data availability

No datasets were generated or analysed during the current study.

Declarations

Competing interests

The authors declare no competing interests.

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