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Traumatic brain injury results in rapid pericyte loss followed by reactive pericytosis in the cerebral cortex

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Accumulating evidence suggests a pivotal role of PDGFR β positive cells, a specific marker for central nervous system (CNS) pericytes, in tissue scarring. Identification of cells that contribute to tissue reorganization in the CNS upon injury is a crucial step to develop novel treatment strategies in regenerative medicine. It has been shown that pericytes contribute to scar formation in the spinal cord. It is further known that ischemia initially triggers pericyte loss *in vivo*, whilst brain trauma is capable of inducing pericyte detachment from cerebral vessels. These data point towards a significant role of pericytes in CNS injury. The temporal and spatial dynamics of PDGFR β cells and their responses in traumatic brain injury are poorly understood. Here we show that PDGFR β positive cells initially decline in the acute phase following experimental traumatic brain injury. However, PDGFR β positive cells increase significantly in the trauma zone days after brain injury. Using various pericyte markers we identify these cells to be pericytes that are demarcated by reactive gliosis. Our data indicate that brain trauma causes a biphasic response of pericytes in the early phase of brain trauma that may be of relevance for the understanding of pathological cellular responses in traumatic brain injury.

Pericytes play a pivotal role in a number of physiological and pathophysiological processes. They are of tremendous importance for vessel stability in various organ systems, e.g. the blood brain barrier (BBB) and in coronary vessels^{1,2}, but also in tumor growth and vascularization³. Importantly, pericytes contribute to the formation of the neurovascular unit, which is comprised of brain endothelial cells, pericytes, astrocytes, neurons, and glial cells⁴. Here, they are important in the regulation and development of the BBB⁵ and contribute to its proper function⁶. A loss of CNS pericytes induces BBB breakdown, neuroinflammation⁶, and CNS aneurysm formation⁷. In addition, pericytes are capable of regulating capillary diameter and microvascular perfusion within the CNS^{6,8,9}.

In disease states such as cerebral ischemia, it has been shown that hypoxia causes pericyte death and persistent CNS microvascular obstruction^{8,10}. We have previously shown a loss of pericytes in an *in vitro* preparation of the developing cerebral cortex upon ischemic and inflammatory stimuli¹¹.

On the other hand, abnormal pericyte proliferation has been reported upon pathophysiological stimuli such as spine trauma and it is known that pericytes contribute to organ scar formation and tissue fibrosis^{12–14}. Pericytes are further a promising cellular source for novel cellular therapy concepts. For example human pericytes have been reprogrammed recently into induced neuronal cells^{15,16}. More than

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a decade ago, Dore-Duffy *et al.* have demonstrated that pericytes are capable of migrating away from the vessel wall after traumatic brain injury (TBI)³². Experimental data from a murine cerebral ischemia model^{17,18} have shown that platelet-derived growth factor receptor beta (PDGFR β), a highly specific marker for pericytes within the CNS¹⁹, has an important role in ischemic tissue responses upon injury. Fernández-Klett *et al.* have observed a significant increase in PDGFR β cellular mass formation within the ischemic core in a murine middle cerebral artery occlusion (MCAO) starting at days 3–5 after ischemic insult¹⁷. A better characterization of PDGFR β positive cell responses in TBI may therefore be of clinical importance in order to better understand and modulate TBI pathophysiology.

We were therefore interested in the temporal and spatial dynamics of PDGFR β positive cell formation following cortical controlled impact (CCI).

Material and Methods

Induction of CCI and ethical statement. C57/Bl6 mice were subjected towards CCI as described in detail elsewhere²⁰. In order to detect CCI-induced cellular alterations, animals were compared with naïve mice in which the physiological arrangement of the neurovascular unit is preserved. In brief mice were anesthetized with isoflurane. Body temperature was monitored and maintained at 37 °C with the help of a heating pad throughout the experiment. For CCI a small skin incision above the skull was made and a burr hole was drilled. Then a precisely controlled pneumatic insult was applied above the right cortex with a custom made impactor (Supplementary Figure S1, diameter 3 mm, penetration depth 1.5 mm, velocity 8 m/s). After this procedure animals were allowed to recover and were clinically monitored regularly. Mice were sacrificed at several varying time points following injury and brains were collected for immunohistochemical processing or mRNA analyses (Fig. 1A). All experimental procedures were approved by the local ethical committee of the University Medical Center of the Johannes Gutenberg-University Mainz. The study protocol was approved by the Landesuntersuchungsamt RLP (protocol number: G12-1-010). All efforts were made to minimize the number of animals used and their suffering. European guidelines on the handling of animals were followed.

Immunohistochemistry. Immunoreactive stainings were performed as documented before^{11,20}. In brief brains were snap frozen in dry ice and cryosliced at 20 μ m thickness (at Bregma –1 mm according to mouse brain atlas, mbl.org) with a cryostat (CryoStarTM NX70 Kryostat, Fischer Scientific). Care was taken to acquire sections from the same coordinates. Following cryoslicing probes were fixed in ice cold acetone for 5 minutes. Then tissue was further processed for immunostaining. Brain slices were blocked and permeabilized in 0.01 mol/l PBS containing 7% normal goat serum (Jackson Immuno Research Dianova, Hamburg, Germany) and 0.3% triton (Sigma-Aldrich Steinheim, Germany) for 2 hours. Then probes were incubated with primary antibodies in 2% bovine serum albumin (Dianova) with 0.05% azide and 0.1% Triton in 0.01 mol/l PBS overnight at room temperature. Subsequently probes were carefully washed with 0.01 mol/l PBS and then incubated with secondary antibodies in 2% bovine serum albumin for 2 hours at room temperature. After washing in 0.01 mol/l PBS sections were embedded in Fluoromount (Southern Biotech, Birmingham, AL, USA).

Table 1 and Table 2 give an overview on primary and secondary antibodies and dilutions used in this study.

Confocal imaging. Imaging was performed with a Leica SP5 confocal microscope using Leica Application Suite Advanced Fluorescence (Leica Microsystems). Excitations wavelengths were 405, 488, 561 and 633 nm. Care was taken to perform imaging with same parameters regarding exposure time and laser intensity for all samples.

Image analysis. Quantitative analyses were performed with NIH “Fiji is just Image J” (<http://fiji.sc/Fiji>). In order to quantify PDGFR β positive stained cells, Z-stacks were collected from each animal in the pericontusional zone (yellow region in Supplementary Figure S1). Z-stacks were Z projected at maximum intensity and binary images were created. Finally average gray scale intensity was acquired by executing the Fiji command “measure”. Details on the calculation of gray scale values by Fiji are documented at the NIH webpage: <http://rsb.info.nih.gov/ij/docs/menus/analyze.html>. Animated 3 dimensional reconstructions from acquired z-stacks were created using IMARIS (Bitplane, Switzerland).

Gene expression (mRNA) analysis. For time series analysis tissue preparation was performed as follows: Brains were quickly removed and placed in a cooled brain matrix (Zivic Instruments, Pittsburgh, USA). Perilesional injured brain tissue was immediately dissected, rapidly frozen in liquid nitrogen and stored at –80 °C until processing. Quantification of mRNA was performed by real-time polymerase chain reaction as described previously²¹. Absolute copy numbers of target genes were normalized against cyclophilin A (PPIA) as housekeeping gene²². Applied primers and probes are shown in Table 3. Same amounts of cDNA were amplified in duplicates using AbsoluteTM Fast qPCR SYBR Green Mix (Thermo Fisher Scientific, Dreieich, Germany) for PPIA, PDGFR β , NG2, and Desmin according to the manufacturer’s instructions.

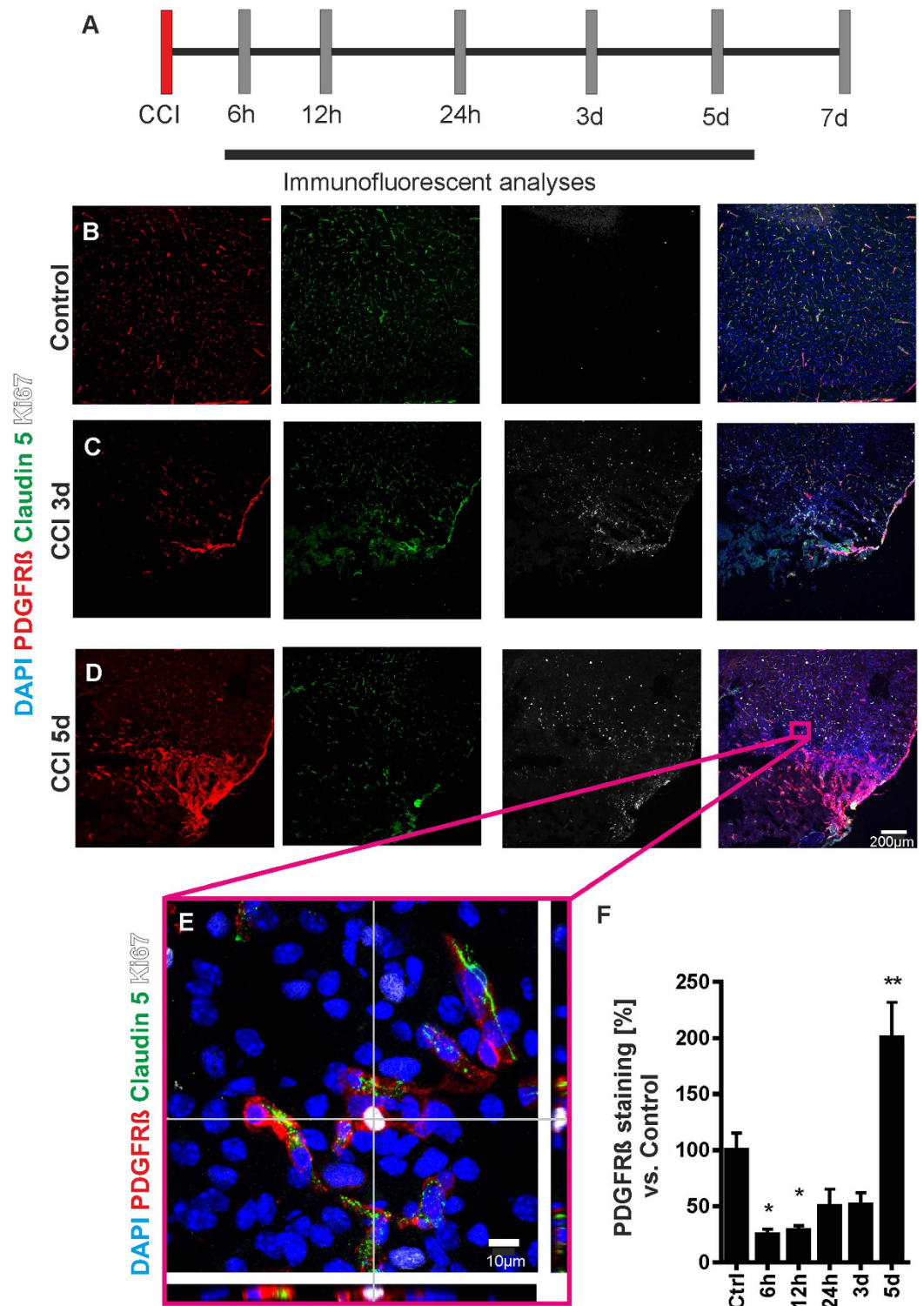


Figure 1. CCI results in pericontusional pericyte proliferation. Tissue was collected for immunohistochemical and mRNA analyses at varying time points after induction of CCI (A). Under physiological conditions pericytes were located in a typical perivascular manner within the cerebral cortex (Claudin 5: green; PDGFR β : red). Only few cells were positive for Ki67, a marker for cell proliferation (B). Three days after CCI PDGFR β positive cells at the CCI border were associated with a strong increase of Ki67 immunoreactivity (C). Five days after CCI a prominent increase of PDGFR β expressing cells was detected (D). Panel E shows an orthogonal section view of a pericontusional pericyte positive for Ki67 that is not in contact with a microvessel. Quantification of PDGFR β staining demonstrates an initial loss of PDGFR β that is followed by a significant increase of PDGFR β 5 days after CCI (F). N = 5–6 animals per group; *P < 0.05; **P < 0.01.

Primary antibodies	Application
anti-claudin 5 (Life technologies #35-2500), 1:50	Brain endothelial cell marker and tight junction protein
anti-GFAP (Dakocytomation, Z 0334), 1:200	Marker of reactive astrocytes
anti-PDGFR β (Neuromics, GT 15065), 1:200	Marker of pericytes
anti-Ki67 (ab 15580 Abcam), 1:200	Cell proliferation marker
DAPI (Sigma, 32670), 0.5 μ g/ml	Cell nuclei staining
anti-Desmin (4024 Cell Signaling), 1:100	Pericyte marker
anti-pan-Laminin (ab7463, Abcam), 1:1000	Staining for basement membrane and laminins
anti-NG2 (AB5320: Merck Millipore), 1:200	Staining for pericyte marker NG2

Table 1. Primary antibodies and their respective application.

Secondary antibodies	related primary antibodies
Cy3 (705-165-147, Jackson, Dianova), 1:200	PDGFR β
DyLight 488 (A90-337D2, Biomol), 1:200	Claudin 5, GFAP
Alexa Fluor 647 (711-605-152, Jackson, Dianova), 1:200	Ki67, Laminin, NG2

Table 2. Secondary Antibodies.

PCR Assay (amplicon size, annealing temp)	Oligonucleotide Sequence (5'-3')	Gene Bank No.
Cyclophilin A (PPIA, 146 bp, 58 °C)	Forw: 5'-GCGTCTCTTCGAGCTGTT-3' Rev: 5'-RAAGTCACCACCCTGGCA-3'	NM_008907
PDGFR β (152 bp, 58 °C)	Forw: 5'-AGGAGTGATACCAGCTTAGTCC-3' Rev: 5'-CCGAGCAGGTCAGAACAAAGG-3'	NM_001146268
NG2 (Cspg4, 132 bp, 58 °C)	Forw: 5'-GGGCTGTGCTGTCTGTTGA-3' Rev: 5'-TGATTCCTTCAGGTAAGGCA-3'	NM_139001
Desmin (243 bp, 58 °C)	Forw: 5'-CCTGGAGCGCAGAATCGAAT-3' Rev: 5'-TGAGTCAAGTCTGAAACCTTGGA-3'	NM_010043

Table 3. Primer sequences.

Statistics. For statistical analyses GraphPad Prism version 6.0 for Windows was used. All datasets were checked for normalization using the D'Agostino and Pearson method²³. All datasets following a Gaussian distribution were analyzed using ONE WAY ANOVA followed by Dunnett's test for multiple comparisons. Non-parametric data were analyzed with a Kruskal Wallis Test followed by a Dunns multiple comparison test. Differences were considered to be significant at alpha < 0.05. Data are displayed as mean \pm standard error of the mean.

Results

Impact of CCI on PDGFR β staining in the pericontusional zone. In the cerebral cortex of naïve control animals we found a physiological perivascular arrangement of PDGFR β positive cells. Under physiological conditions, only few cells were positive for the proliferation marker Ki67 (Fig. 1B). Three days after CCI the expression of the proliferation marker Ki67 increased within the pericontusional zone (Fig. 1C). Five days after CCI a significant cellular mass formation positive for the pericyte marker PDGFR β and Ki67 became apparent at the site of the lesion (Fig. 1D, inset E). High power imaging revealed PDGFR β positive cells that were immunoreactive for Ki67 (Fig. 1D). Many of these PDGFR β positive cells were not in contact with microvessels as documented in co-stainings with the microvascular marker Cl-5, which has been shown to be a specific marker for brain endothelial cells in brain trauma (Fig. 1E, Supplementary Movie 1)^{20,24}.

Quantitative analyses revealed a significant decrease in PDGFR β staining 6–12 hours following CCI in the pericontusional zone (Fig. 1F). At 24 hours and 3 days post CCI no significant difference in PDGFR β positive cells could be detected when compared with naïve controls (Fig. 1F). However, on day 5 there was a significant increase of PDGFR β positive cells compared with controls (Fig. 1F).

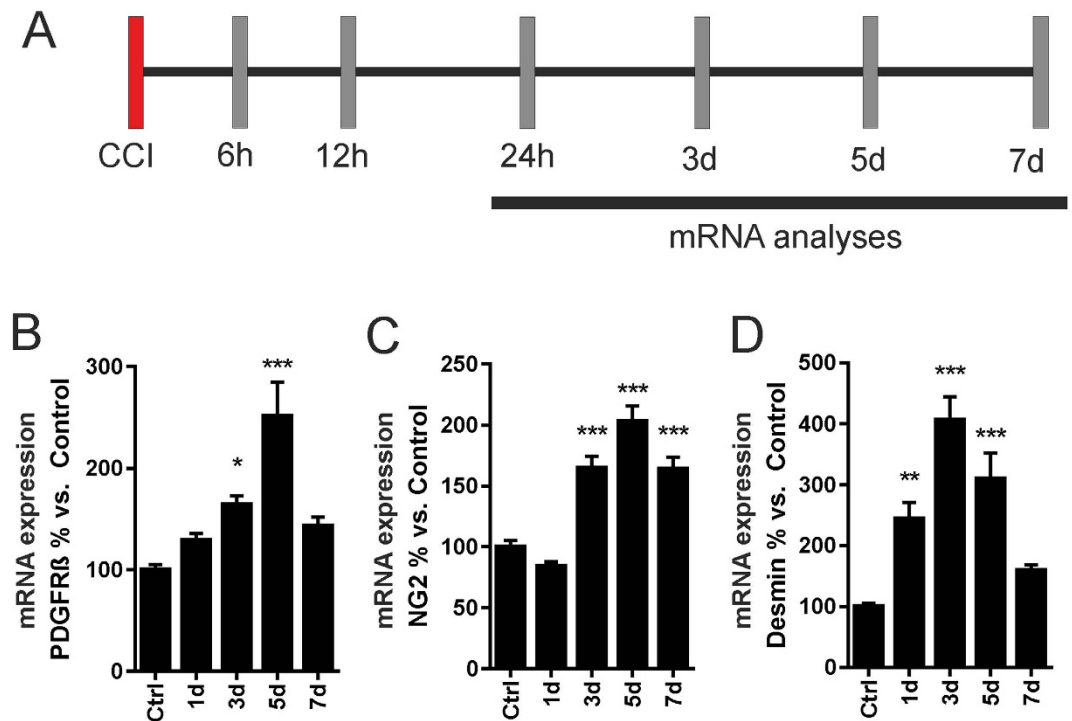


Figure 2. Pericyte differentiation markers are upregulated in a time dependent manner following CCI. The pericyte expression markers PDGFR β , NG2, and Desmin were significantly upregulated following CCI. mRNA analyses were carried out on days 1, 3, 5 and 7 following TBI (A). PDGFR β mRNA levels were found to increase significantly, starting on day 3 after CCI with a peak at day 5 (B). The pericyte marker NG2 was also upregulated upon CCI from days 3–7 (C). Here, maximum NG2 mRNA levels were detected 5 days after brain trauma induction. The pericyte marker Desmin was already significantly upregulated on day 1, reaching its maximum expression levels on day 3 after CCI (D). N = 8–10 animals per group; *P < 0.05; **P < 0.01; ***P < 0.001.

Since PDGFR β is a specific marker for pericytes within the CNS, we further analyzed mRNA levels of pericyte markers on day 1, 3, 5 and 7 after CCI induction (Fig. 2A). Here we found that mRNA levels of the pericyte markers PDGFR β and NG2 were significantly upregulated after CCI with a peak on day 5 (Fig. 2B,C). The pericyte marker Desmin was already significantly upregulated on day 1 following CCI reaching its maximum mRNA levels on day 3 (Fig. 2D).

PDGFR β positive cells in CCI are surrounded by a basement membrane. We and others have previously documented that PDGFR β is a specific marker for pericytes in the CNS^{11,19,25}. Another property of pericytes is the presence of a basement membrane (BM) that maintains several laminin isoforms^{11,26}. Association with a BM is an important criterion to identify pericytes²⁷. By using a pan-laminin antibody, that recognizes several laminin isoforms, we observed that pericytes in intact cortices (Fig. 3A, Supplementary movie 2), as well as PDGFR beta positive cells in the pericontusional tissue after CCI are surrounded by a BM (Fig. 3B, Supplementary movie 3). Interestingly, pericytes in the pericontusional zone display an amoeboid like morphology that is significantly different from the perivascular morphology of pericytes in physiological conditions (Fig. 3A,B, Supplementary movies 2–5).

PDGFR β positive cells express various pericyte differentiation markers. In order to further characterize PDGFR β positive cells in the pericontusional CCI zone, we performed co-stainings with a set of established pericyte differentiation markers such as NG2, Desmin, and PDGFR β (Fig. 4). Here we found that PDGFR β co-localized with NG2 and partly with Desmin, indicating that PDGFR β cells were indeed pericytes (Fig. 4A,B). However there is not a 100 percent overlap between all markers. This is due to the different cellular localization of the respective proteins. PDGFR β is a transmembrane tyrosine kinase, whilst Desmin is an intracellular intermediate filament protein²⁸. NG2 is mostly expressed in a perinuclear manner in mature CNS pericytes that is also reflected by the immunofluorescent image in Fig. 4. However, we would like to point out that this discrepancy of marker overlap might also be partly due to a lack of specificity of these cellular markers.

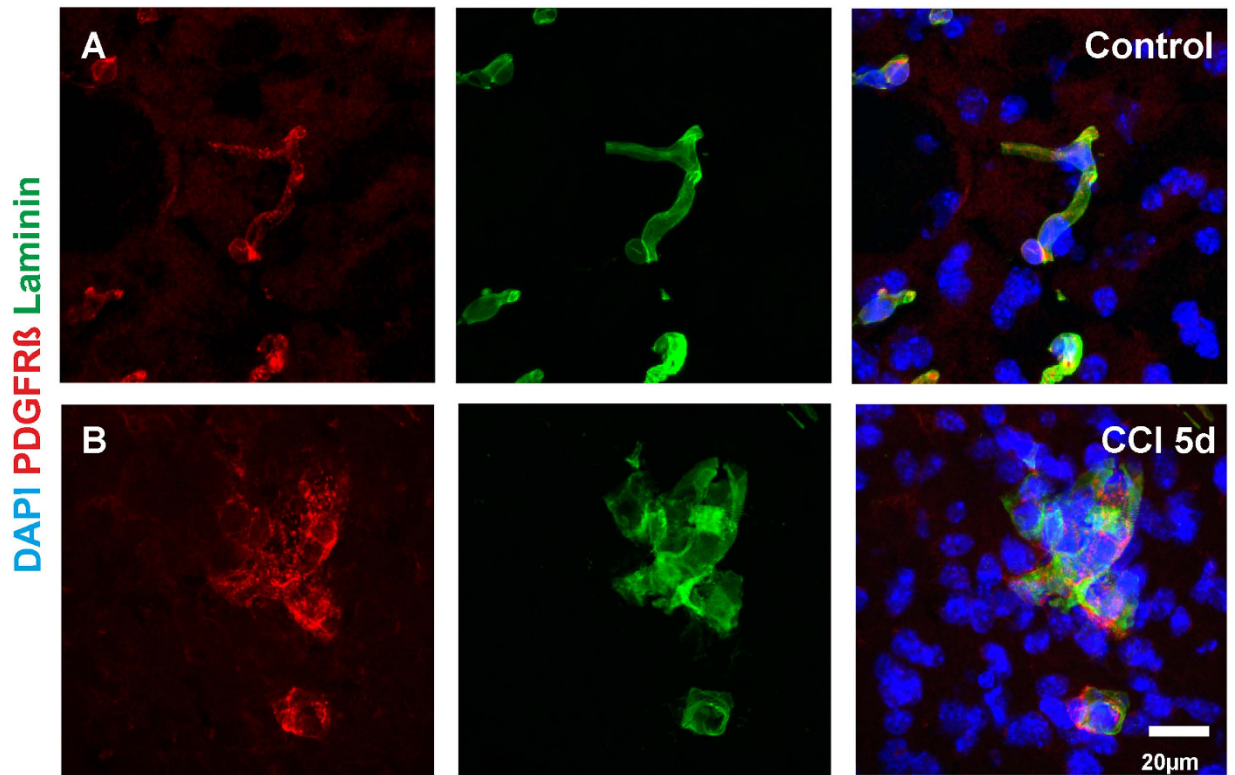


Figure 3. Pericytes in control and CCI conditions are sheathed by a basement membrane. In order to verify the pericyte identity of PDGFR β positive cells, we performed pan-laminin co-stainings. In both, control (A) and CCI animals (B), PDGFR β positive cells were found to be sheathed by a basement membrane, a hallmark to identify pericytes.

Reactive glia demarcates PDGFR β cell formation. It is well established that GFAP expressing reactive astrocytes are a major contributor to scar formation^{13,29} in the CNS. It has been recently documented in a model of murine ischemic stroke that proliferation of PDGFR β cells occurs in the ischemic core, which is demarcated by reactive astrogliosis²⁵. In order to evaluate whether the observed PDGFR β cell proliferation is demarcated in a similar manner by reactive astrogliosis in CCI, we performed co-stainings with PDGFR β and the reactive astrocyte marker GFAP following CCI. In line with the literature we found that a high number of reactive astrocytes were present in the pericontusional zone. Similar to the results of Fernandez-Klett *et al.* in cerebral ischemia²⁵, we found that Ki67 positive pericytes were demarcated by reactive astroglia 5 days after CCI (Fig. 5). In addition, PDGFR β and GFAP co-staining gave no evidence for an overlap of both immunohistochemical markers, indicating that PDGFR β and GFAP positive cells are distinct cell types.

Discussion

Here we show in a murine model of CCI that pericytes undergo a biphasic cell response within the first 5 days after TBI.

A rapid loss of PDGFR β cells within the first 12 hours was followed by a significant increase of PDGFR β positive cells on day 5 after TBI. In our study PDGFR β immunoreactive cells expressed several pericyte markers such as Desmin and NG2 on protein level, pointing towards a pericyte identity. In addition, our qRT-PCR results on the increase of the pericyte markers NG2, Desmin and PDGFR β following TBI further support the immunofluorescence data.

However, the morphology of PDGFR β cells in CCI was different compared with physiological conditions. PDGFR β cells in the pericontusional TBI zone were amoeboid shaped and not necessarily in vasular contact. Co-stainings with GFAP revealed that PDGFR β cell mass formation was demarcated by reactive glia. Further these immunohistochemical results indicate that PDGFR β cells do not express GFAP, indicating they are not a subpopulation of reactive astrocytes.

Three to five days following brain trauma, Ki67 was found to be expressed by PDGFR β positive cells, which was not the case in physiological conditions. In line with our data on PDGFR β staining on day 5 after TBI, Fernández-Klett *et al.* have shown that PDGFR β positive cells significantly increase in a model of middle cerebral artery occlusion followed by reperfusion 5 days after stroke in mice²⁵. In addition, the

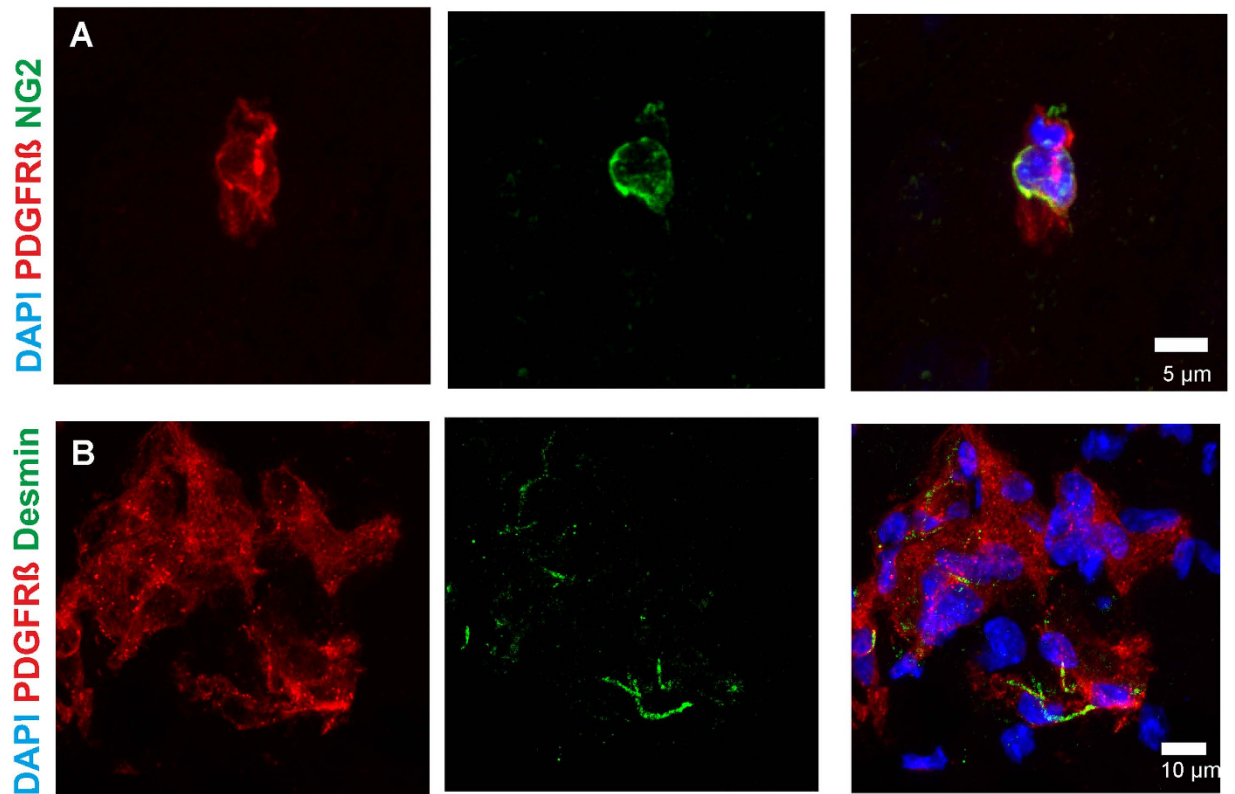


Figure 4. PDGFR β cells in the pericontusional zone express the pericyte markers NG2 and Desmin. Co-staining with the pericyte marker NG2 demonstrates that PDGFR β positive cells are immunoreactive for NG2 at day 5 after brain trauma (A). Note the typical perinuclear expression of NG2. In addition we found that some PDGFR β positive cells expressed the pericyte marker Desmin (B). Note that NG2 is a perinuclear protein whilst Desmin is an intracellular filament protein. Therefore both markers do not totally overlap with PDGFR β due to their different subcellular localization.

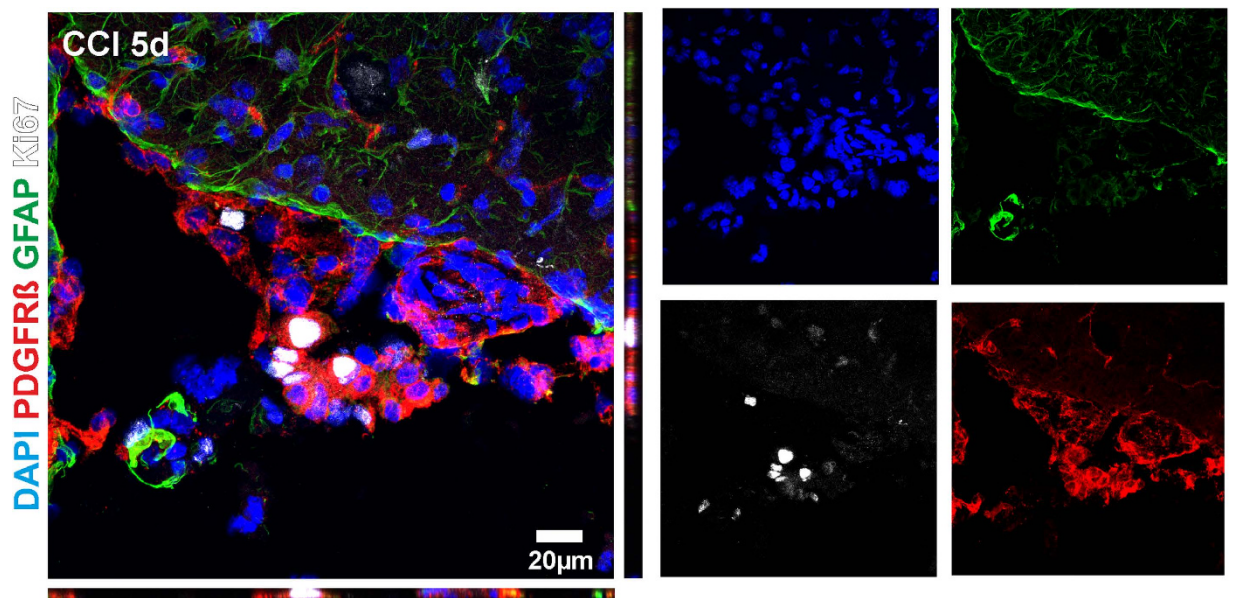


Figure 5. Reactive astrocytosis demarcates proliferative PDGFR β cellular mass formation. Immunofluorescent confocal analyses revealed that PDGFR β cells 5 days after CCI were a distinct cell population and different from reactive astrocytes. Co-stains for GFAP, a marker for reactive astroglia, and PDGFR β demonstrate that reactive astrocytes demarcate a large number of proliferating PDGFR β positive cells. Note the high expression level of Ki67 in PDGFR β positive cells.

authors from that study also observed a demarcation of PDGFR β cell mass formation by reactive glia, which was also the case in our experimental study.

Interestingly, an enhanced cell proliferation was absent 2 weeks after brain trauma insult in a report by Chen *et al.*³⁰. In our study we investigated proliferation for up to 5 days following trauma. Therefore our results may implicate that Ki67 is only transiently increased. However the trauma model used by Chen *et al.*³⁰ is different to that used in our present study and the investigated species was rat whilst we examined C57/Bl6 mice. Our finding that pericytes play an important role in cellular brain injury responses is in line with data from Birbair *et al.* who have recently shown that 14 days after brain trauma, pericytes contribute to scar formation in the cortex¹⁴. However, in the study by Birbair *et al.* there was a mismatch in NG2 and PDGFR β expression 14 days after injury. Since we observed a colocalization of NG2 and PDGFR β immunoreactivity up to 5 days after injury, we speculate that the expression of both pericyte markers may undergo regulatory changes over longer time periods following TBI.

PDGFR β ablation experiments have shown that loss of PDGFR β induces pericyte loss, leading to an increase of BBB permeability, which results in extravasation of acute phase proteins into the CNS parenchyma that enhance secondary brain injury due to inflammation^{6,31}. Therefore, therapeutic strategies to modulate PDGFR β related cell responses upon CNS injury may help to improve clinical outcome. We speculate that the observed biphasic response of PDGFR β positive cells within the first 5 days of TBI may present a promising target to modulate clinical complications such as evolution of brain edema or improve nerve fibre regeneration^{13,31}.

In summary, our *in vivo* study identifies CNS pericytes as a cell population that declines in the acute phase of TBI, which is followed by reactive pericytosis on day 5 following cortical brain trauma. More research needs to be done in order to unravel the pathophysiological relevance of the observed biphasic pattern of PDGFR β cell dynamics.

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Author Contributions

C.M.Z. and S.C.T. designed the study, performed experiments, and analyzed the data, C.M.Z. and H.L. wrote the main manuscript, A.S., F.B. and A.H. performed experiments, C.M.Z. designed all figures, C.M.Z. designed 3 dimensional reconstructions, S.C.T. designed supplementary figure 1.

Additional Information

Supplementary information accompanies this paper at <http://www.nature.com/srep>

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