SVN Stroke & Vascular Neurology

TIMP1 protects against blood-brain barrier disruption after subarachnoid haemorrhage by inhibiting ubiquitination of astrocytic β1-integrin

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To cite: Tang T, Chen H, Hu L, *et al*. TIMP1 protects against blood-brain barrier disruption after subarachnoid haemorrhage by inhibiting ubiquitination of astrocytic β1-integrin. *Stroke & Vascular Neurology* 2024;**0**. doi:10.1136/svn-2023-002956

► Additional supplemental material is published online only. To view, please visit the journal online (https://doi.org/10.1136/svn-2023-002956).

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Received 28 October 2023
Accepted 7 February 2024

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ABSTRACT

Background Astrocytes regulate blood-brain barrier (BBB) integrity, whereas subarachnoid haemorrhage (SAH) results in astrocyte dysregulation and BBB disruption. Here, we explored the involvement of tissue inhibitor of matrix metalloprotease-1 (TIMP1) in astrocyte-mediated BBB protection during SAH, along with its underlying mechanisms.

Methods C57BL/6J mice were used to establish a model of SAH. The effects of TIMP1 on SAH outcomes were analysed by intraperitoneal injection of recombinant mouse TIMP1 protein (rm-TIMP1; 250 µg/kg). The roles of TIMP1 and its effector β1-integrin on astrocytes were observed by in vivo transduction with astrocyte-targeted adeno-associated virus carrying TIMP1 overexpression plasmid or β1-integrin RNAi. The molecular mechanisms underlying TIMP1 and β1-integrin interactions were explored in primary cultured astrocytes stimulated with red blood cells (RBCs).

Results TIMP1 was upregulated after SAH. Administration of rm-TIMP1 mitigated SAH-induced early brain injury (EBI) in male and female mice. TIMP1 was primarily expressed in astrocytes; its overexpression in astrocytes led to increased β1-integrin expression in astrocytes, along with the preservation of astrocytic endfoot attachment to the endothelium and subsequent recovery of endothelial tight junctions. All of these effects were reversed by the knockdown of β1-integrin in astrocytes. Molecular analysis showed that TIMP1 overexpression decreased the RBC-induced ubiquitination of β1-integrin; this effect was partially achieved by inhibiting the interaction between β1-integrin and the E3 ubiquitin ligase Trim21.

Conclusion TIMP1 inhibits the interaction between β1-integrin and Trim21 in astrocytes, thereby rescuing the ubiquitination of astrocytic β1-integrin. It subsequently restores interactions between astrocytic endfeet and the endothelium, as well as BBB integrity, eventually mitigating SAH-induced EBI.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ The occurrence of subarachnoid haemorrhage (SAH) leads to the disruption of the blood-brain barrier (BBB), and safeguarding BBB integrity emerges as a promising therapeutic target for SAH. Tissue inhibitor of matrix metalloprotease-1 (TIMP1) has been identified as a potential candidate for BBB protection, yet the underlying cellular and molecular mechanisms remain inadequately elucidated.

WHAT THIS STUDY ADDS

⇒ Recombinant TIMP1 protein alleviates the early brain injury induced by SAH. In terms of the cellular mechanism, astrocytes secrete TIMP1 which acts on astrocytic endfeet, thereby restoring their coverage on endothelium and preserving the integrity of endothelial tight junctions and BBB after SAH. For the molecular mechanism, TIMP1 restores endfeet coverage in astrocytes by inhibiting ubiquitin-mediated degradation of β1-integrin, a molecule that mediates endfeet coverage on endothelium. This effect is partially achieved by inhibiting the interaction of β1-integrin with E3 ubiquitin ligase Trim21.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ This study elucidates a novel mechanism through which TIMP1 reinstates the integrity of the BBB, thereby highlighting its potential as a promising therapeutic target for SAH. Meanwhile, ubiquitination of β1-integrin in astrocytic endfeet results in detachment of the endfeet from the endothelium, thereby elucidating a novel mechanism through which SAH disrupts BBB integrity. This finding holds significant implications for future investigations into pathophysiological progresses associated with SAH.

INTRODUCTION

Subarachnoid haemorrhage (SAH) is a type of catastrophic haemorrhagic stroke mainly caused by intracranial aneurysm rupture.[1] Early brain injury (EBI) is the primary cause of SAH-related death,[2] which occurs within 72 hours after SAH.[3 4] During the EBI phase, haemorrhage causes dysregulation of intracranial pressure and cerebral blood flow, leading to blood-brain barrier (BBB) disruption, neuroinflammation and neuronal death.[3 4]

The BBB maintains central nervous system homeostasis by regulating molecular transport and preserving immune privilege,[5 6] while its disruption invariably exacerbates EBI by allowing neurotoxin influx and immune cell infiltration.[4] Thus far, there are no therapies that maintain the BBB and alleviate EBI; such therapies are urgently needed for the effective management of SAH.

The BBB is composed of endothelial cells and astrocytes. Its structure comprises tight junctions (TJs) among neighbouring endothelial cells to form the functional core of the BBB, along with astrocytic endfeet attached to the endothelium as support for the TJs.[4 7 8] Within astrocytic endfeet, β1-integrin plays a key role in regulating adhesive contacts between astrocytes and endothelial cells by interacting with endothelial Dab1.[9–12] Endfoot coverage of the endothelium is essential for BBB integrity,[13 14] while SAH results in the loss of endfoot coverage, which is strongly correlated with hippocampal atrophy after SAH, suggesting that interactions between endfeet and endothelium can be a therapeutic target for the management of SAH.[15]

Tissue inhibitor of matrix metalloproteases-1 (TIMP1), an endogenous inhibitor of matrix metalloproteases (MMPs) that destroy the BBB,[16] demonstrates its potential therapeutic effects on SAH by preserving BBB integrity. The protective effects of TIMP1 on the BBB have been verified in models of ischaemic stroke and traumatic brain injury[17–19]; however, these protective effects might be independent of MMP9 inhibition. Pharmacological inhibition of MMP9 cannot enhance ischaemia-induced vascular remodelling in TIMP1 knockout mice.[20] Regardless of its inhibitory effects on MMPs, TIMP1 can reverse BBB impairment[19] in a model of brain trauma. Overall, the mechanisms of TIMP1-mediated BBB protection remain unclear.

β1-integrin, a downstream effector of TIMP1, modulates cellular adhesion and communiation[16]; it has been unclear whether TIMP1 regulates the functions of astrocytic endfeet via β1-intergrin. The mechanisms by which TIMP1 protects against BBB disruption, particularly with respect to astrocytes, require further investigation. In this study, we explored whether TIMP1 preserves BBB integrity during SAH, then characterised the cellular and molecular mechanisms by which TIMP1 protects against BBB disruption.

METHODS

Patient blood samples

Blood sample collection was approved by the ethics committee of the university (approval no. IR2020001461). Patients in the SAH group were diagnosed with aneurysmal SAH by CT and DSA; their blood samples were collected within 24 hours after SAH onset. Patients in the control group were diagnosed with unruptured intracranial aneurysms; their blood samples were collected before embolisation or clipping. The baseline characteristics of the enrolled patients are shown in online supplemental table 1.

In vivo experiments

All animal procedures were approved by the ethics committee (approval no. 2019-108). Adult C57BL/6J mice (SLAC, Shanghai) aged 8–10 weeks (weighing 20–25 g (male mice) or 15–20 g (female mice)) were subjected to SAH model. All mice were labelled with ear tags and grouped by randomisation sequence generated by R-4.3.1. Mice exhibiting eating and conscious disturbances after surgery were euthanised and excluded from analysis. The surviving mice with evident SAH (Grade 8–15)[21] and the absence of hematoma in the skull base or brain parenchyma were included for further analysis. To evaluate the therapeutic effects of TIMP1 on SAH outcomes, mice were intraperitoneally administered with recombinant mouse TIMP1 (rm-TIMP1; 250 μg/kg).[19] To assess the roles of TIMP1 and β1-integrin in astrocytes, adeno-associated virus (AAV) vectors (serotype 2/9; glial fibrillar acidic protein promoter) with specific expression in astrocytes were constructed (Hanbio, Shanghai). Mice neurological deficiencies were recorded and brain samples were collected for histological (transmission electron microscopy (TEM) and immunofluorescence), cellular (flow cytometry and cell sorting) and molecular (RNA and protein) analysis. The study design and procedural details are provided in the online supplemental material.

In vitro experiments

Primary cultured astrocytes were stimulated by red blood cells (RBCs) to mimic SAH. The roles of TIMP1 were investigated through transfection with overexpressing plasmids (Genepharma, Shanghai). The regulatory progresses of β1-integrin were explored by administration with drugs including protein synthesis inhibitor cycloheximide (CHX), proteasome inhibitor MG132 and autophagy inhibitor chloroquine (CQ). RNA and protein samples were collected for further analysis. The procedural details are provided in the online supplemental material.

Statistics

The investigators and data collectors were not aware of the group assignments. The normality of the dataset in each group was analysed using the Shapiro-Wilk test. For comparisons between two groups, normally distributed data were compared using Student's t-test, otherwise the Mann-Whitney U test. For comparisons among three or more groups, normally distributed data were compared by one-way analysis of variance (ANOVA), otherwise the Kruskal-Wallis test. Two-way ANOVA was used for comparisons among different time points. All acquired images were analysed using ImageJ software. GraphPad Prism 8.0.2 and R-4.3.1 were used for statistical analysis, and p values <0.05 were considered statistically significant.

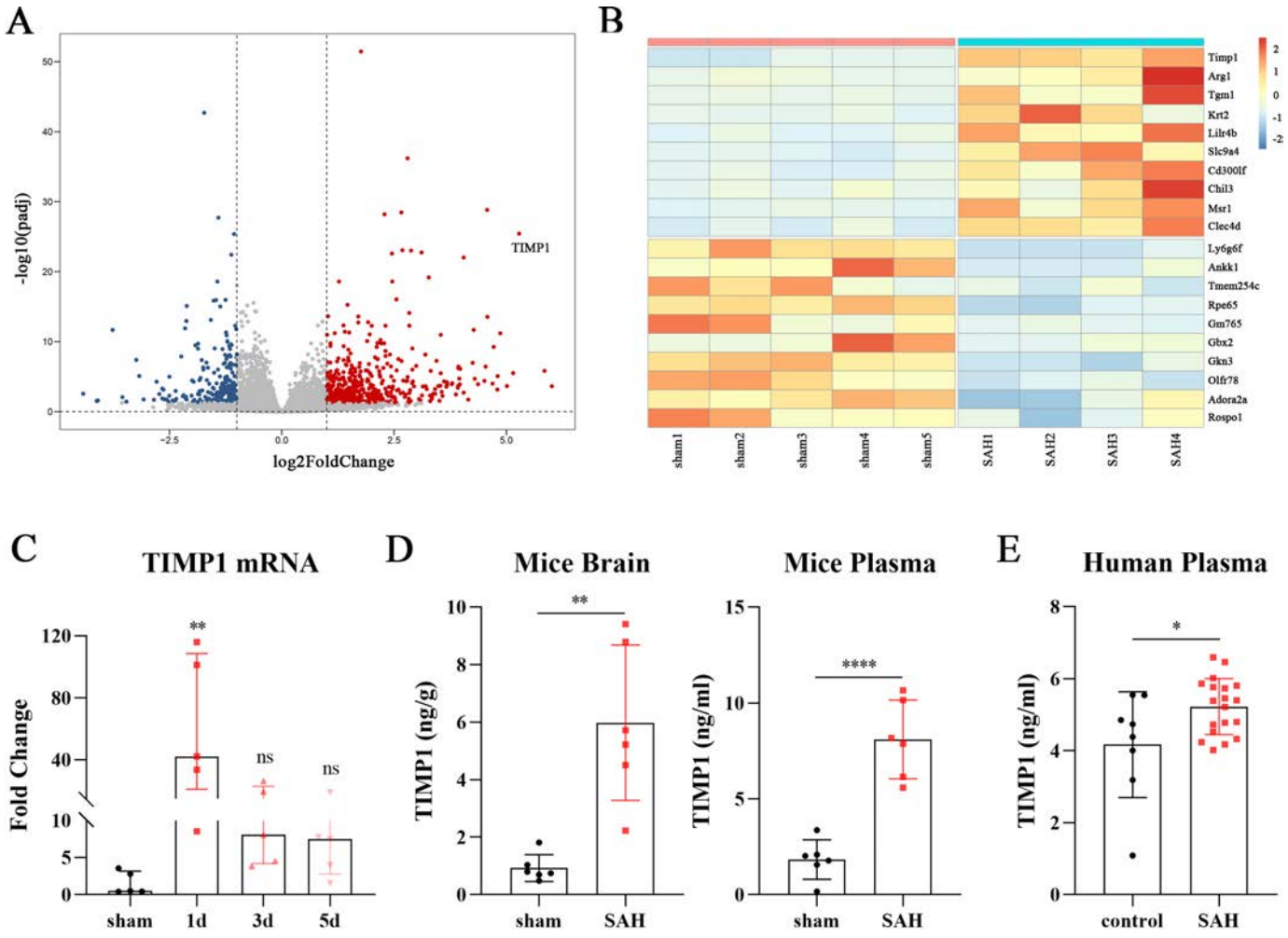

Figure 1 TIMP1 is upregulated after SAH. (A) Volcano plot of upregulated and downregulated genes at 1 day post-SAH. (B) Heatmap of the top 20 differentially expressed genes. (C) Tissue mRNA levels of TIMP1 within 5 days post-SAH. n=5 mice on each day. (D) Protein levels of TIMP1 in mouse brain tissues and plasma. n=6 mice in each group. (E) TIMP1 levels in human plasma. n=8 patients in control group and n=19 patients in SAH group. *p<0.05, **p<0.01, ****p<0.0001. SAH, subarachnoid haemorrhage; TIMP1, tissue inhibitor of matrix metalloprotease-1.

RESULTS

TIMP1 was upregulated after SAH

To investigate changes in transcription profiles after SAH, brain samples from mice that underwent sham and SAH surgery were subjected to RNA sequencing analysis. Among the differentially expressed genes, TIMP1 was significantly elevated 1 day post-SAH (figure 1A,B). Analysis of the mRNA levels of TIMP1 in mouse brains at various time points after SAH revealed that the peak occurred 1 day post-SAH (figure 1C). Concurrently, the protein levels of TIMP1 were significantly increased in mouse brain tissues and plasma at 24 hours post-SAH (figure 1D). Furthermore, analysis of patient plasma samples revealed significantly elevated levels of TIMP1 in the SAH group compared with the control group (figure 1E). These findings indicated that TIMP1 is upregulated after SAH in both mice and humans.

Administration of rm-TIMP1 alleviated EBI after SAH

To investigate the effects of TIMP1 on SAH outcomes, mice that underwent SAH surgery were intraperitoneally administered rm-TIMP1 (250 µg/kg), daily for consecutive 3 days. In male mice, after 3 days of rm-TIMP1 treatment, all behavioural tests (Garcia Score, grip test, pole test and rotarod test) revealed obvious improvement (figure 2A). Treatment with rm-TIMP1 also suppressed neuroinflammation, manifested by decreased mRNA levels of IL (Interleukin)-1α, IL-1β, TNF (Tumor Necrosis Factor)-α, IL-6, CD16, CD68 and iNOS (figure 2B). Moreover, rm-TIMP1 alleviated cellular apoptosis in samples of brain tissue, manifested by decreased levels of PARP, cleaved-PARP and cleaved-Caspase 9 (figure 2C,D). Furthermore, rm-TIMP1 reduced the percentage of apoptotic neurons both in cortical and hippocampal regions at 3 days post-SAH (figure 2E,F).

Considering the prominent sexual dimorphism in SAH,[22] the aforementioned experiments were repeated in female mice. Three days of treatment led to improvements in Garcia Score, grip test and rotarod test results; however, there was no statistically significant difference in pole test results (figure 3A). In terms of inflammatory

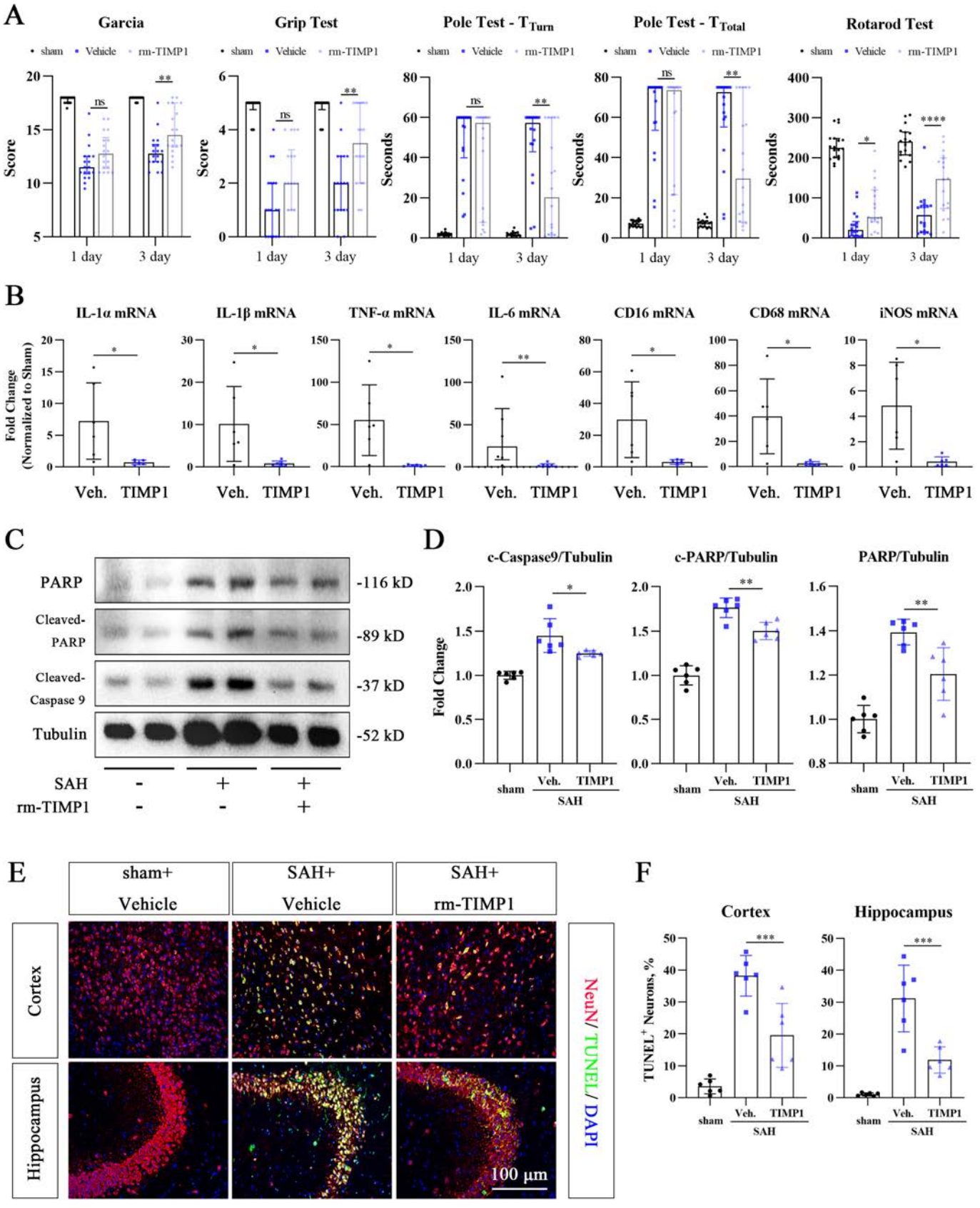

Figure 2 Administration of rm-TIMP1 mitigates EBI at 3 days post-SAH in male mice. (A) Behavioural function at 1 and 3 days post-SAH. n=18 mice in each group. (B) mRNA levels of inflammatory cytokines. n=6 mice in each group. (C) Apoptotic proteins in brain tissue. (D) Statistics for panel C. n=6 mice in each group. (E) Detection of TUNEL (Green) positive neurons (Neu, Red) after SAH and TIMP1 treatment. (F) Statistics for panel E. n=6 mice in each group. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. EBI, early brain injury; SAH, subarachnoid haemorrhage; TIMP1, tissue inhibitor of matrix metalloprotease-1.

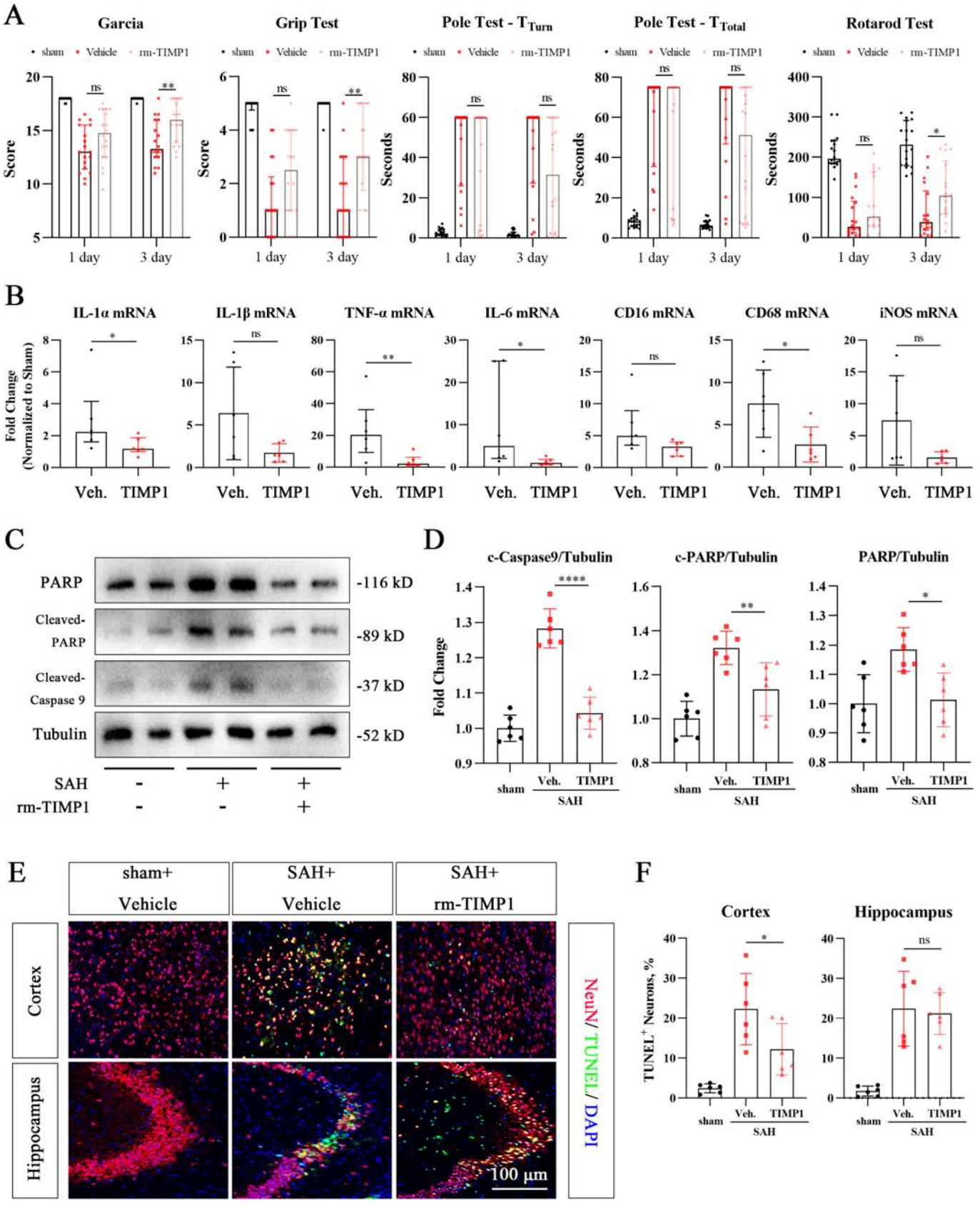

Figure 3 Administration of rm-TIMP1 mitigates EBI at 3 days post-SAH in female mice. (A) Behavioural function at 1 and 3 days post-SAH. n=18 mice in each group. (B) mRNA levels of inflammatory cytokines. n=6 mice in each group. (C) Apoptotic proteins in brain tissue. (D) Statistics for panel C. n=6 mice in each group. (E) Detection of TUNEL (Green) positive neurons (Neu, Red) after SAH and TIMP1 treatment. (F) Statistics for panel E. n=6 mice in each group. *p<0.05, **p<0.01, ****p<0.0001. EBI, early brain injury; SAH, subarachnoid haemorrhage; TIMP1, tissue inhibitor of matrix metalloprotease-1.

cytokines, rm-TIMP1 treatment significantly reduced the mRNA levels of IL-1α, TNF-α, IL-6 and CD68; the decreases in levels of IL-1β, CD16 and iNOS were not statistically significant (figure 3B). Treatment with rm-TIMP1 also suppressed apoptosis in samples of brain tissue from female mice (figure 3C,D). Additionally, rm-TIMP1 rescued neuronal apoptosis only in cortical regions (figure 3E,F). These findings suggested that treatment with exogenous TIMP1 can effectively mitigate EBI after SAH. Notably, male mice showed a more favourable therapeutic response to rm-TIMP1.

Overexpression of astrocytic TIMP1 mitigated BBB disruption

The subsequent analyses were performed in male mice. To explore the cellular mechanisms by which TIMP1 mitigated EBI, we analysed the cellular origin of TIMP1. RBCs were co-cultured in vitro with primary cells (eg, neurons, astrocytes, microglia and endothelial cells). Compared with control conditions, RBC stimulation upregulated TIMP1 in astrocytes alone (online supplemental figure 1). Subsequently, astrocytes, microglia and $CD45^{high}$ cells in mouse brains were sorted by flow cytometry at 1 day post-SAH, revealing that TIMP1 was mainly derived from astrocytes (online supplemental figure 2A). Examination of magnetically isolated astrocytes from sham and SAH brains further confirmed that SAH induced upregulation of astrocytic TIMP1 (online supplemental figure 2B).

Astrocytes are important components of the BBB. Therefore, we hypothesised that astrocytic TIMP1 plays a protective role in maintaining BBB integrity after SAH. To test this hypothesis, we constructed an AAV vector specifically expressing TIMP1 in astrocytes (online supplemental figure 3A). We then conducted an analysis of BBB integrity at 3 days post-SAH. Intriguingly, compared with SAH mice transduced with a control AAV vector, TIMP1 overexpression in astrocytes decreased IgG leakage (figure 4A,B) and brain water content (figure 4C). Additionally, TIMP1 increased levels of the TJ proteins ZO-1 and Occludin but decreased the levels of MMP9 (figure 4D,E). TIMP1 overexpression also increased both ZO-1 and Occludin fluorescent intensity (figure 4F,G). Microstructural analysis using TEM revealed that SAH led to the detachment of endothelial TJs, whereas overexpression of astrocytic TIMP1 restored TJ structural integrity (figure 4H,I). These findings indicated that overexpression of astrocytic TIMP1 improved endothelial TJ integrity and alleviated BBB disruption after SAH.

Overexpression of TIMP1 restored astrocytic endfoot attachment to the endothelium

Next, we investigated how astrocytic TIMP1 restored BBB integrity. Previous research showed that TIMP1 is involved in cellular adhesion through interactions with β1-integrin.[16] The expression of β1-integrin was predominantly observed in astrocytes and endothelial cells (online supplemental figure 4). At 3 days post-SAH, proportions of β1-integrin-positive cells exhibited a decline in both astrocytes and endothelial cells. However, the overexpression of TIMP1 rescued β1-integrin expression in astrocytes but not endothelial cells (figure 5A), suggesting that astrocytic β1-integrin plays a key role in the TIMP1-induced protection of BBB.

The attachment of endfeet to the endothelium plays a central role in barriergenesis to form the BBB through a mechanism mediated by β1-integrin.[11 13 14] The restoration of astrocytic β1-integrin expression by TIMP1 suggested that TIMP1 could promote endfoot attachment. Thus, adhesion between astrocytic endfeet and the endothelium was analysed at 3 days post-SAH.

Brain samples from mice in the sham group showed robust endfoot coverage (indicated by aquaporin-4 (AQP4)) on the endothelial surface (indicated by CD31). However, this coverage was disrupted by SAH; it was subsequently restored by overexpression of astrocytic TIMP1 (figure 5B,C). Furthermore, TEM-based microstructural analysis of normal brain tissue revealed close adhesion between astrocytic endfeet and the endothelium. SAH led to endfoot swelling and subsequent detachment from the endothelial surface. However, TIMP1 overexpression mitigated these structural changes (figure 5D,E). These findings suggested that TIMP1-induced restoration of BBB integrity was associated with the recovery of astrocytic endfoot attachment. This process could involve β1-integrin; thus, further experiments were conducted to determine its role.

β1-integrin was required for TIMP1-induced endfoot attachment and BBB recovery

An AAV vector was used for targeted transduction of β1-integrin siRNA into astrocytes (online supplemental figure 3B). Analyses of endfoot attachment and endothelial TJs were performed. Notably, knockdown of β1-integrin prevented the restoration of TIMP1-induced endfoot overlap with the endothelium (figure 6A,B). Microstructural analysis showed that knockdown of β1-integrin led to reappearance of swollen endfeet and detachment of endfeet from the endothelium (figure 6C,D). Considering the strong association between endfoot impairment and endothelial TJ disruption,[11 23 24] we conducted further analysis of TJ integrity. Knockdown of β1-integrin led to reversal of TIMP1-induced increases in expression levels of the TJ-associated proteins ZO-1 and Occludin, as well as the decrease in MMP9 expression (figure 7A,B). Knockdown of β1-integrin also abrogated the TIMP1-induced enhancements of fluorescence intensity for both ZO-1 and Occludin (figure 7C,D). β1-integrin silencing also resulted in endothelial TJ detachment, thereby neutralising the protective effects of TIMP1 (figure 7E,F). Additionally, at 3 days post-SAH, behavioural assessments such as the Garcia Score, grip test and rotarod test demonstrated the reversal of TIMP1-induced neurological recovery in mice subjected to β1-integrin silencing (figure 7G). These findings suggested that the restoration of β1-integrin plays a key role in TIMP1-induced endfoot attachment, BBB repair and subsequent neurological

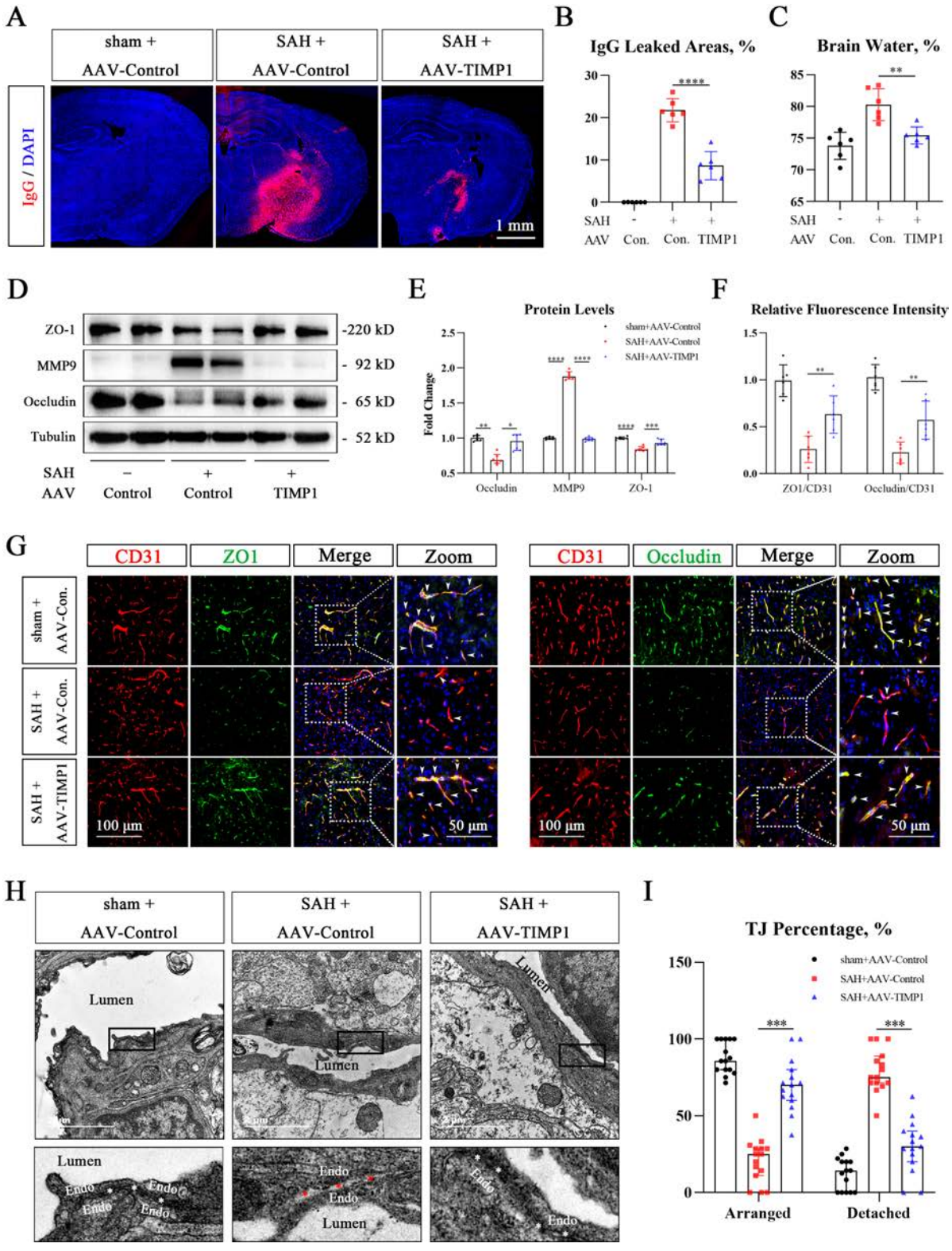

Figure 4 Astrocytic expression of TIMP1 reduced BBB disruption. (A) IgG leakage (Red) were detected at 3 days post-SAH. (B) Statistics for panel A. n=6 mice in each group. (C) Brain water content was measured at 3 days post-SAH. n=6 mice in each group. (D) Protein levels of ZO-1, Occludin and MMP9 were detected at 3 days post-SAH. (E) Statistics for panel D. n=6 mice in each group. (F) Statistics for panel G. n=6 mice in each group. (G) Brain sections (3 days post-SAH) were labelled with the endothelial marker CD31 and a TJ marker (ZO-1 or Occludin). (H) Microstructure of endothelial TJ (3 days post-SAH). The attached TJ is indicated with a white asterisk (*), and the detached TJ is indicated with a red asterisk (*). (I) Statistics for panel H. n=15 fields of view in each group (n=3 mice in each group, and five fields in each sample). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. AAV, adeno-associated virus; BBB, blood-brain barrier; SAH, subarachnoid haemorrhage; MMPs, matrix metalloproteases; TIMP1, tissue inhibitor of matrix metalloprotease-1; TJ, tight junction.

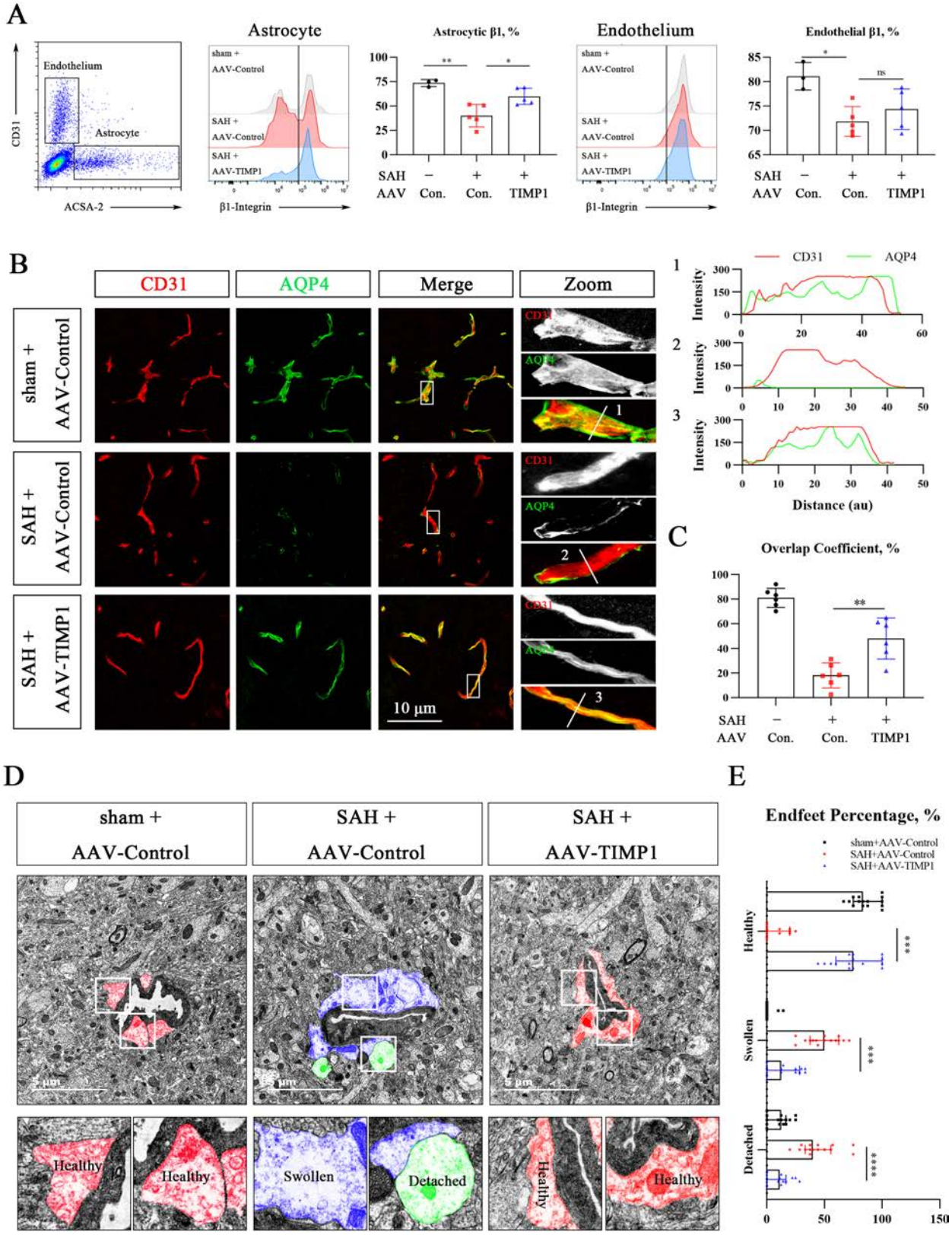

Figure 5 Astrocytic expression of TIMP1 promotes endfoot attachment to the endothelium. (A) Flow cytometry analysis of astrocytic and endothelial β1-integrin levels was performed at 1 day post-SAH. n=3 mice for sham group, n=5 mice for groups with SAH (with or without TIMP1 overexpression). (B) Overlap (3 days post-SAH) of endfeet (AQP4) and endothelium (CD31). (C) Statistics for panel B. n=6 mice in each group. (D) Microstructure of astrocytic endfeet (3 days post-SAH). Red, blue and green regions represent healthy, swollen and detached endfeet, respectively. (E) Statistics for panel D. n=15 fields of view in each group (n=3 mice in each group, and five fields in each sample). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. AAV, adeno-associated virus; AQP4, aquaporin-4; SAH, subarachnoid haemorrhage; TIMP1, tissue inhibitor of matrix metalloprotease-1.

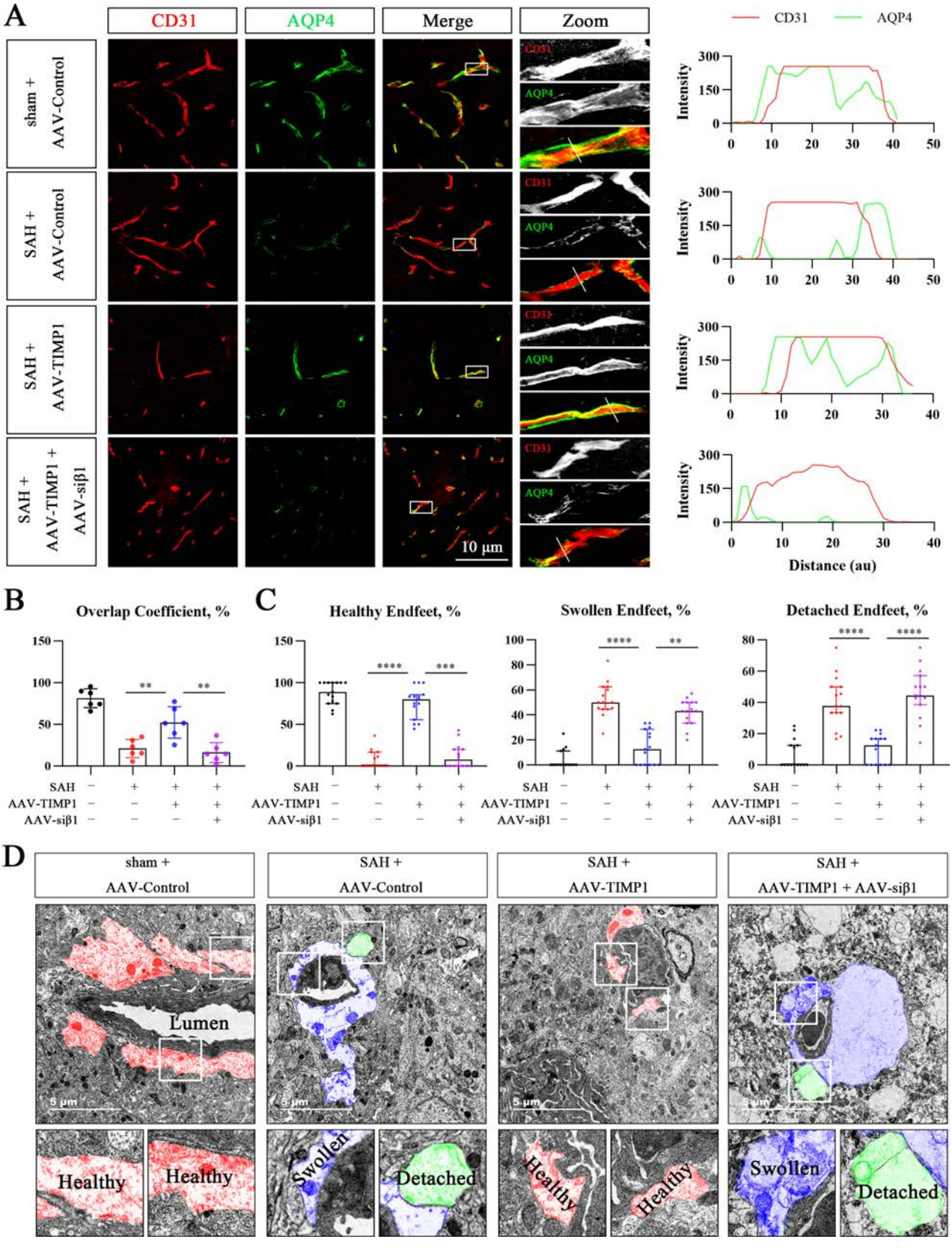

Figure 6 Silencing of β1-integrin in astrocytes reverses TIMP1-induced endfoot coverage. (A) Overlap (3 days post-SAH) of endfeet (AQP4) and endothelium (CD31). (B) Statistics for panel A. n=6 mice in each group. (C) Statistics for panel D. n=15 fields of view in each group (n=3 mice in each group, and five fields in each sample). (D) Microstructure of astrocytic endfeet (3 days post-SAH). Red, blue and green regions represent healthy, swollen and detached endfeet, respectively. **p<0.01, ****p<0.0001. AAV, adeno-associated virus; AQP4, aquaporin-4; SAH, subarachnoid haemorrhage; TIMP1, tissue inhibitor of matrix metalloprotease-1.

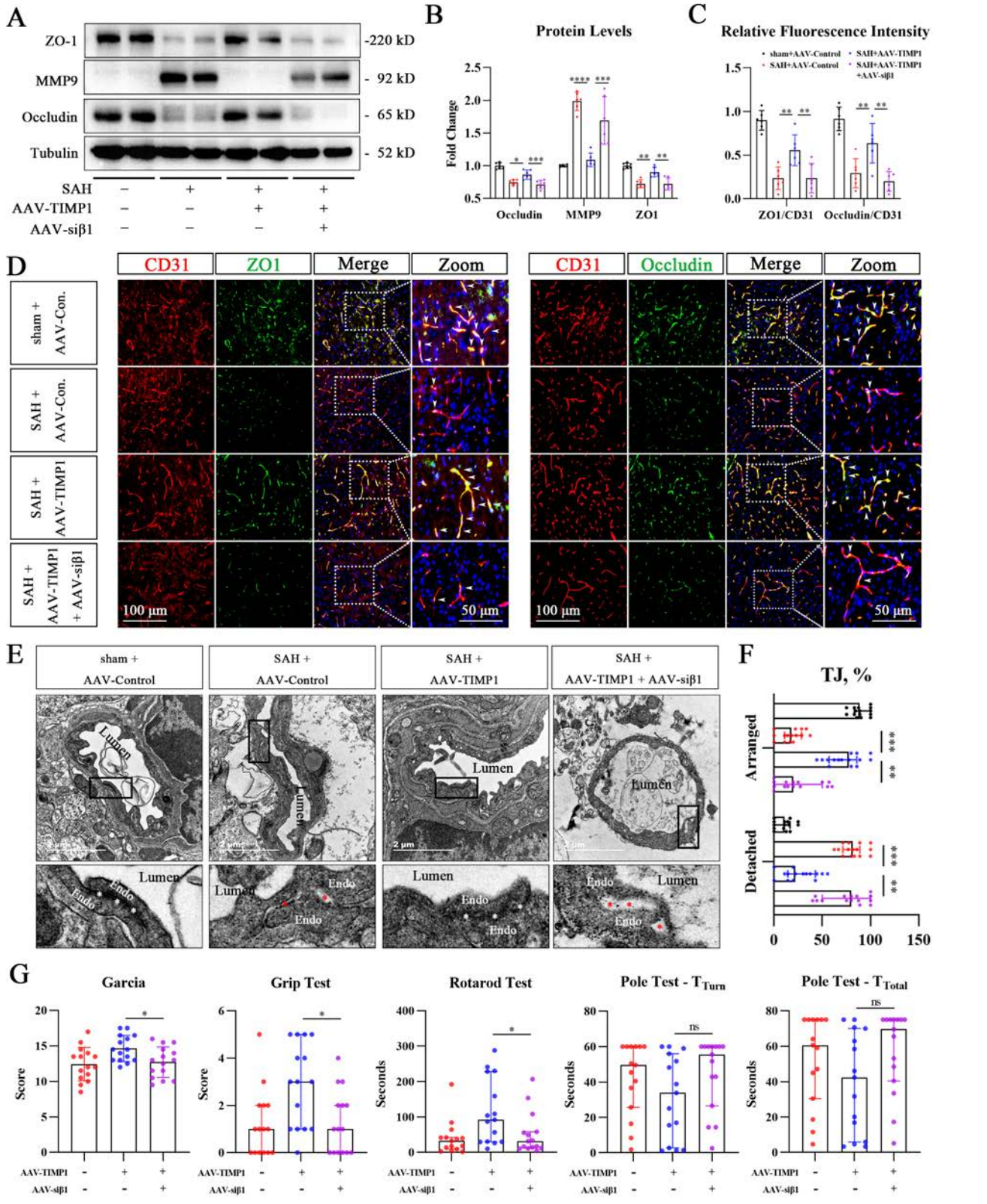

Figure 7 Silencing of β1-integrin in astrocytes reverses TIMP1-induced TJ protection. (A) Protein levels of ZO-1, Occludin and MMP9 were detected at 3 days post-SAH. (B) Statistics for panel A. n=6 mice in each group. (C) Statistics for panel D. n=6 mice in each group. (D) Brain sections (3 days post-SAH) were labelled with the endothelial marker CD31 and a TJ marker (ZO-1 or Occludin). (E) Microstructure of endothelial TJ (3 days post-SAH). The attached TJ is indicated with a white asterisk (*), and the detached TJ is indicated with a red asterisk (*). (F) Statistics for panel E. n=15 fields of view in each group (n=3 mice in each group, and five fields in each sample). (G) Behavioural tests at 3 days post-SAH. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. AAV, adeno-associated virus; MMPs, matrix metalloproteases; SAH, subarachnoid haemorrhage; TIMP1, tissue inhibitor of matrix metalloprotease-1; TJ, tight junction.

recovery. Thus, we explored the molecular mechanisms by which TIMP1 rescues astrocytic β1-integrin.

TIMP1 inhibited the ubiquitination of β1-integrin in RBC-stimulated astrocytes

We first evaluated the mRNA levels of β1-integrin in magnetically sorted astrocytes and observed that neither SAH nor TIMP1 overexpression exerted a significant impact on β1-integrin mRNA transcripts (online supplemental figure 5A). Similar outcomes were observed in the in vitro analysis (online supplemental figure 5B), indicating that the downregulation of β1-integrin during SAH and the TIMP1-induced rescue of β1-integrin occurred at the protein level.

Protein degradation and clearance primarily occur through the ubiquitin-proteasome and autophagy-lysosome pathways.[25] Therefore, we investigated the roles of these pathways in the downregulation of astrocytic β1-integrin in response to SAH; we also explored the effects of TIMP1 on these pathways.

The protein synthesis inhibitor CHX was added to primary cultured astrocytes that had been subjected to control treatment, RBC treatment and RBC treatment combined with transfection of the TIMP1 overexpression plasmid. We found that TIMP1 overexpression delayed the reduction of β1-integrin levels in RBC-stimulated astrocytes after CHX treatment (figure 8A,B), supporting the notion that TIMP1 prevented β1-integrin degradation. Cells were subsequently treated with the proteasome inhibitor MG132 (figure 8C,D) or the autophagy inhibitor CQ (figure 8E,F). Intriguingly, MG132 treatment enhanced the levels of β1-integrin in RBC-stimulated astrocytes (figure 8C,D), but no such effects were observed after CQ treatment (figure 8E,F), indicating that β1-integrin was degraded by the ubiquitin-proteasome pathway after RBC stimulation. Thus, the interaction between β1-integrin and ubiquitin was analysed by immunoprecipitation, revealing that RBC stimulation significantly enhanced the ubiquitination of β1-integrin. However, this process was reversed by TIMP1 overexpression (figure 8G). These findings suggested that TIMP1 inhibited the ubiquitination of β1-integrin in RBC-stimulated astrocytes.

TIMP1 inhibited the interaction between β1-integrin and Trim21

Next, we examined how TIMP1 inhibited the ubiquitination of β1-integrin. Because TIMP1 lacks ubiquitin ligase or deubiquitinase domains, we used β1-integrin antibody-precipitated protein samples from control or RBC-treated astrocytes for shotgun proteomics to determine the interactome of β1-integrin. In total, 990 proteins interacting with β1-integrin were identified in the control group, whereas 485 proteins were identified in the RBC-treated group; 397 proteins were shared between the two groups (figure 8H). Among these 397 proteins, GO analysis revealed proteins involved in the regulation of ubiquitination (figure 8I). A notable interacting protein was tripartite motif containing-21 (Trim21), an E3 ubiquitin ligase.[26] The docking score of Trim21 and β1-integrin was −279.40, and the confidence score was 0.9301, suggesting a high probability of interaction (figure 8Ja,b). Microstructure analysis revealed that the two proteins mainly interacted via hydrogen bonding, salt bridges and hydrophobic interactions (figure 8Jc,d). We thus validated the interaction between β1-integrin and Trim21, demonstrating that astrocytes exhibited an augmented interaction on stimulation of RBCs, while this effect was reversed by overexpression of TIMP1. Notably, neither RBC stimulation nor TIMP1 overexpression could affect the total protein level of Trim21 (figure 8K). Moreover, silence of Trim21 further augmented the levels of β1-integrin and inhibited its ubiquitination in comparison to solely overexpressing TIMP1 (online supplemental figure 6). Overall, these findings suggested that TIMP1 inhibited the interaction between β1-integrin and Trim21, thereby preserving astrocytic endfoot attachment to the endothelium, protecting BBB integrity and alleviating the EBI caused by SAH.

DISCUSSION

In this study, we discovered a novel mechanism by which TIMP1 reverses SAH-induced disruption of the BBB. TIMP1 rescued the level of β1-integrin in astrocytes, thereby restoring astrocytic endfoot coverage of the endothelium and repairing BBB integrity. Molecularly, TIMP1 rescued astrocytic β1-integrin by inhibiting the interaction between β1-integrin and the E3 ubiquitin ligase Trim21, which reduced the ubiquitin-mediated degradation of β1-integrin.

Based on a recent cohort study, Zhong *et al* suggested that a higher serum level of TIMP-1 at admission is associated with worse ischaemic stroke outcomes.[27] However, their results also suggested that a higher level of TIMP1 was associated with a higher serum level of MMP9,[27] and TIMP1 upregulation is a spontaneous response to increased MMP9 expression.[28] Furthermore, an increased MMP9/TIMP1 ratio is associated with worse stroke outcomes.[17 18] The evidence thus far suggests that endogenous TIMP1 expression is insufficient to counteract the acute brain injury, and a supply of exogenous TIMP1 is required for neuroprotection.

Disruption of the BBB is a leading cause of SAH-induced EBI, and therapies targeting BBB disruption may be useful in the treatment of SAH.[4 7] Elevated levels of MMPs after acute brain injury disrupt the BBB matrix and destroy cellular connections.[12 29] Therefore, MMPs have been regarded as therapeutic targets for vascular neurological diseases, with the aim of restoring BBB integrity.[30] Considering its ability to inhibit MMPs, it was reasonable to suspect that TIMP1-mediated protection of the BBB was achieved by inhibiting MMPs. However, a previous work showed that TIMP1 protects against BBB disruption through MMP-independent pathways.[19] Therefore, we assumed that the mechanism by which TIMP1 reduced

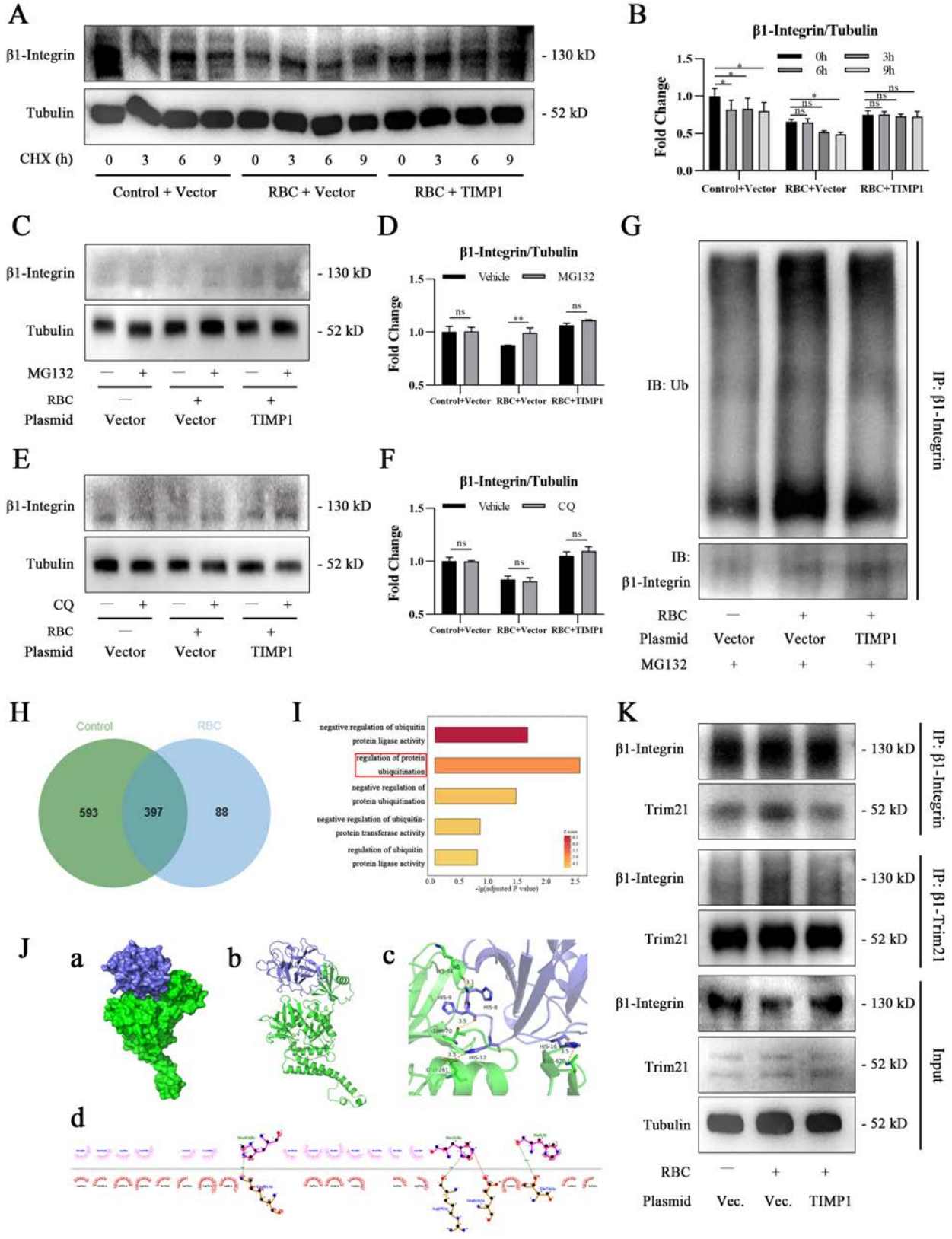

Figure 8 TIMP1 reduced ubiquitination of β1-integrin by inhibiting its interaction with Trim21. (A) β1-integrin levels in astrocytes treated with CHX. (B) Statistics for panel A. n=3 dependent experiments. (C) β1-integrin levels in astrocytes treated with MG132. (D) Statistics for panel C. n=3 dependent experiments. (E) β1-integrin levels in astrocytes treated with CQ. (F) Statistics for panel E. n=3 dependent experiments. (G) Ubiquitination of β1-integrin. (H) Venn diagram for interactome of β1-integrin in control and RBC-treated astrocytes. (I) Gene Ontology (GO) analysis. (J) Molecular docking analysis of β1-integrin and Trim21. (K) Verification of the interaction between β1-integrin and Trim21. *p<0.05, **p<0.01. CHX, cycloheximide; CQ, chloroquine; RBC, red blood cell; TIMP1, tissue inhibitor of matrix metalloprotease-1.

SAH-induced BBB disruption did not solely involve antagonising MMP activity.

Although astrocytic endfeet plays a central role in the construction of the BBB, their roles in acute cerebrovascular diseases (eg, SAH) remain poorly understood. β1-integrin is a downstream effector of TIMP1, and our data suggested that only astrocytic β1-integrin was responsive to TIMP1 overexpression (figure 5A). In astrocytes, β1-integrin mediates endfoot attachment to the endothelium by interacting the Dab1 on the endothelial cell surface.[11] Therefore, it was reasonable for us to hypothesise that TIMP1 rescued BBB integrity by restoring β1-integrin-mediated endfoot coverage. Our data suggested that SAH led to decreased endfoot coverage of the endothelium; TIMP1 overexpression restored endfoot coverage, and knockdown of β1-integrin abrogated this therapeutic effect. These findings reveal a novel effect of TIMP1 on interactions between astrocytic endfeet and the endothelium.

β1-integrin acts as a molecular bridge connecting astrocytic endfeet to the endothelium,[11] and the restoration of β1-integrin by inhibiting its ubiquitination is the molecular mechanism underlying TIMP1-mediated endfoot recovery. The process of ubiquitination has been widely documented in cerebrovascular diseases,[31–33] but its regulation in astrocytes under stressful conditions remains poorly understood. The present study showed that astrocytic β1-integrin can be degraded by ubiquitination in response to RBC stimulation, revealing an important mechanism by which cerebral vessels lose endfoot coverage. β1-integrin may be ubiquitinated through its interaction with Trim21, which is a novel regulatory mechanism for β1-integrin activities; this process can be reversed by TIMP1 overexpression (figure 8K). However, neither RBC stimulation nor TIMP1 overexpression influenced the total level of β1-integrin, suggesting that β1-integrin underwent allosteric regulation during SAH and TIMP1 treatment, and further studies are needed to unveil these regulations in the future.

This work used both male and female mice; the results showed that TIMP1 could alleviate SAH-induced EBI in both sexes, but females exhibited less sensitivity to TIMP1 treatment. Oestrogen exerts neuroprotection against acute brain injury.[34] The data shown in figures 2 and 3 suggested that overall neurological deficits were less severe in female mice than in male mice, indicating that oestrogen may partially obscure the therapeutic effects of TIMP1. Notably, female mice were more vulnerable to SAH in the hippocampal region, and TIMP1 treatment had no restorative effects on hippocampal neurons in those mice (figure 3E,F), which might be related to the sexual dimorphism in cerebrovascular anatomy.[35] Although female mice were less sensitive to exogenous TIMP1 treatment, female mice lacking TIMP1 or exposed to functional inhibition of TIMP1 might be more vulnerable to SAH. This hypothesis is based on the fact that TIMP1 is encoded by the X chromosome and can escape the X chromosome inactivation process, leading to redundant TIMP1 expression in female mice.[36] Future studies should more comprehensively explore sex differences regarding TIMP1 treatment of SAH, using TIMP1 knockout mice and castrated mice, to further characterise the clinical potential of TIMP1 as a treatment for SAH.

In summary, TIMP1 treatment inhibits the interaction between β1-integrin and Trim21 in astrocytes, promoting astrocytic endfoot attachment to the endothelium, maintaining BBB integrity and alleviating SAH-induced EBI.

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Acknowledgements We thank Professor Xiangnan Zhang in the College of Pharmaceutical Sciences, Zhejiang University for his instruction for the study design. We thank Dr Yinping Lv and Menghan Zhang in the Center of Cryo-Electron Microscopy (CCEM), Zhejiang University for their technical assistance on TEM. We thank Dr Li Liu and Xiaoli Hong in the Core Facilities, Zhejiang University School of Medicine for their technical assistance on confocal imaging.

Contributors TT contributed to experimental design, experimental operations (blinded to group allocation), data collection and manuscript draft. HC contributed to randomisation, blinding (aware of the group allocation), experimental operations, data analysis. LH contributed to experimental operations (blinded to group allocation) and data collection. JY and CJ contributed to experimental operations, validation and data. CX, XW and YC contributed to experimental operations. ZC and HZ contributed to data curation. LF and XF contributed to manuscript refinement. CQ, JC and ZT contributed to funding acquisition. JL contributed to patient blood sample collection. HZ contributed to conceptualisation, experimental design, data verification and review. GC and FL contributed to review, supervision, project administration and funding acquisition. TT, HC and LH contributed equally. HZ, GC and FL: collaborative correspondence.

Funding This work was supported by the National Natural Science Foundation of China (Nos. 82371299, 82171273, 82271314 and 82171275), Natural Science Foundation of Zhejiang Province (LGD22H090004 and LQ21H090008), Innovative Talent Program of Zhejiang Health Department (2021434317), Key R&D Program of Zhejiang (2022C03133 and WKJ-ZJ-2004) and Zheng Shu Medical Elite Scholarship Fund.

Competing interests None declared.

Patient consent for publication Not applicable.

Ethics approval This study involves human participants and was approved by Institutional Ethics Committee of the Second Affiliated Hospital, Zhejiang University of Medicine (approval no. IR2020001461). Participants gave informed consent to participate in the study before taking part.

Provenance and peer review Not commissioned; externally peer reviewed.

Data availability statement Data are available upon reasonable request.

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