SUPPLEMENTAL MATERIALS AND METHODS

Mouse model of TBI

Mouse experiments were performed on male C56BL/6J mice (12-16 weeks old and 22-25g; Jackson Laboratory, Bar Harbor, ME), as we previously described.[1,2] Briefly, a 3-mm hole was drilled over the right parietal cortex (2.0-mm posterior from the bregma and 2.0-mm lateral to the sagittal suture) of an anesthetized mouse constrained to a stereotaxic frame (Harvard Apparatus, Holliston, MA) with the dura mater intact. A luer-lok connector filled with sterile saline was affixed to the site of craniotomy with dental cement and connected to a lateral fluid percussion injury device (Custom Design & Fabrication, Richmond, VA). The mouse was subjected to a single impact of 1.9±0.2 atmospheres (atm) and skin incision was then closed. Sham mice underwent the identical craniotomy without TBI. In a subset of experiments, ADAMTS-13 null mice and their wild-type littermates were subjected to identical TBI to evaluate the mortality and neurological dysfunctions, as we recently reported.[3]

Water contents in the brain and lungs

Tissue water content was measured using the dry–wet weight method at day 1 and day 3 post-injury to define the level of TBI-induced tissue edema in the brain and lungs, as we previously described.[4] Briefly, mice were euthanized under anesthesia to collect the brains and lungs. The ipsilateral and contralateral hemispheres of each brain and the right lower lobe of each lung were immediately dissected and weighed to obtain the wet weight (WW). These organs were then dried at 100°C for 24 hrs and weighted again to obtain the dry weight (DW). The water content was calculated as: (WW − DW)/WW×100%.

Quantification of cerebral hemorrhage

Cerebral hemorrhage was quantified using two complementary methods. First, images of cerebral tissues with H&E staining were captured under an Olympus IX81 inverted light microscope (Waltham, MA), as we previously described.[5,6] The area of hemorrhage (extravascular accumulation of erythrocytes) was marked and measured using NIH ImageJ software (Version 1.46r, Wayne Rasband, USA) to calculate the total cerebral hemorrhagic volume (hemorrhagic area × the thickness of the section).

This method is limited in measuring the volume of irregular hematoma areas. To address this concern, we also measured the total hemoglobin (Hb) in whole brain

homogenate, as previously described.[7] In brief, a mouse was perfused transcardially with PBS immediately after euthanasia. The brain was then dissected, weighed, and homogenized in 1 ml of PBS. The brain homogenate was centrifuged at 16,000×g for 30 min at RT to remove cellular debris. The supernatant (50μl) was incubated with Drabkin's reagent (200μl, Sigma-Aldrich) for 15 min at RT followed by centrifuging at 3,000×g for 15 min. Free Hb in the supernatant was measured at OD 540 nm using a SpectraMax M5 plate-reader (Molecular Devices, Sunnyvale, CA) and normalized to wet tissue weight (μg/g tissue).

Brain-derived extracellular vesicles (BDEVs)

BDEVs were generated from mouse brains subjected to snap-frozen and rapid-thawing injury, as previously described.[1,3,8] Briefly, the brain was removed from a non-injured mouse immediately after euthanasia, cut into 2-5 mm pieces, washed extensively with saline, snap-froze in liquid nitrogen followed by rapidly thawing in 37°C. The brain blocks were homogenized in 1 ml of PBS using a glass Dounce homogenizer (ThermoFisher Scientific). The brain homogenates were centrifuged at 1,500×g for 20 min at 4°C to collect cell-free supernatants, which were centrifuged at 13,000×g for 2 min at 4°C to remove large cellular debris. The supernatants thus obtained underwent two cycles of ultracentrifugation, each at 100,000×g for 60 min at 4°C. The BDEV pellets were re-suspended in PBS to desirable concentrations depending on experimental needs and, if necessary, the supernatant was tested as the EV-free control.

Neuron- and glial cell-derived EVs (collectively termed BDEVs) were identified by a modified flow cytometry protocol first by their size (< 1 μm) using standard microbeads that were 0.5, 0.9, and 3 μm in diameter (Biocytex, Marseille, France).[8] They were then examined for their expression of neuron-specific enolase (NSE) or glial fibrillary acidic protein (GFAP) by fluorescein isothiocyanate (FITC)-conjugated monoclonal rat-anti-mouse NSE antibody (Abcam, Cambridge, MA) or a polyclonal rabbit anti-mouse GFAP antibody (eBioscience Inc., San Diego, CA) on a LSR II flow cytometer (Beckon Dickinson, San Jose, CA). An isotype specific IgG and a rabbit non-immune IgG were used as negative controls. To reduce small particle contaminants, the buffers used for EV suspension and analyses were filtered with a 0.1 μm filter (EDM Millipore) before use.

Vascular permeability

The cerebral vascular permeability was evaluated in mice using two methods in order to cross-validate results. First, we used 70 kDa (FITC)-labeled dextran (EDM Millipore, Billerica, MA), as previously described.[9,10] Briefly, FITC-dextran (100 mg/kg in PBS) was infused into a mouse through the tail vein and allowed to circulate for 10 min. The mouse was euthanized and transcardially perfused with PBS to remove the intravascular dextran. The brain was immediately harvested and weighed. The brains collected from experimental mice were either sectioned to mark where FITC-dextran was located or homogenized to quantify FITC fluorescence intensity. For fluorescence images, the brains were fixed in 4% paraformaldehyde overnight at 4°C and dehydrated sequentially in 20% and 30% phosphate-buffered sucrose solutions. The brains were then embedded in O.C.T. medium (Tissue-Tek, Torrance, CA), sectioned, and counterstained with DAPI (Abcam). The sections were reviewed under an inverted fluorescence microscope (Olympus IX81). For quantifying the fluorescence intensity, the brains were homogenized in PBS and centrifuged at 16,000×g for 30 min at RT. FITC fluorescence intensity in the supernatant was measured in a SpectraMax M5 plate-reader (Molecular Devices) at 485 nm excitation and 535 nm emission wavelengths.

For the Evans blue (EB) dye extravasation test,[3,8] 100 μl of 2% EB (Sigma-Aldrich, St. Louis, MO) dissolved in PBS was injected over 30 seconds into the tail vein of a mouse and allowed to circulate for 2 hrs. The mouse was then sacrificed under anesthesia and immediately perfused transcardially with PBS to remove intravascular EB. The brain was removed, weighed, homogenized in formamide (1:20 w/v), and incubated at 60°C overnight. The homogenate was centrifuged at 16,000×g for 30 min and the absorbance determined at OD 620 nm using a SpectraMax M5 plate-reader (Molecular Devices).

Endothelial permeability was also assessed in vitro by the diffusion of FITC-dextran (EDM Millipore) and the leakage of PKH26-labeled EVs through the monolayer of confluent HUVECs (Lonza, Allendale, NJ), as we have previously described.[1,2] Briefly, HUVECs were seeded on collagen-coated transwell inserts (0.4 μm pore, Corning, Tewksbury, MA) and cultured in Endothelial Cell Growth Medium-2 (EGM-2, Lonza) until confluent. They were then incubated with plasma, or purified EVs from sham and TBI mice, or purified BDEVs from mouse brains for 3 hrs at 37°C in the presence of 300,000 platelets/μl and A2 (an equal volume of the vehicle TBST as control). The culture media in the bottom chambers were collected to measure FITC-dextran fluorescence (excitation at 485 nm and emission at 535 nm) using a SpectraMax M5

plate-reader (Molecular Devices) or to count the numbers of PKH26-labeled EVs on a LSR II flow cytometer (Beckon Dickinson) using standard counting microbeads to quantify EVs. Cells treated with either medium alone or VWF multimers purified from human cryoprecipitates[11] were equally examined as controls.

To purify plasma EVs, citrated blood samples were collected from mice under anesthesia and centrifuged at 120×g for 20 min at RT to collect platelet-rich plasma (PRP), which was further centrifuged at 1,500×g for 20 min at RT and followed by centrifugation at 13,000×g for 2 min at RT. The cell-free plasma thus obtained was centrifuged at 100,000×g for 60 min at 4°C (twice). The EV pellets were resuspended in PBS and the supernatant was tested as EV-free plasma (EVFP). In a subset of experiments testing the rate of EV transendothelial migration, purified EVs were labeled with the fluorescent dye PKH26 immediately before experiments, as we previously described.[1] After 3 hours of incubation at 37°C, medium in the bottom chambers was collected to quantify PKH26-labeled EVs on a LSR II flow cytometer (Beckon Dickinson), using standard counting microbeads to calculate EV numbers.

Cerebral blood flow

Mice were anesthetized with isoflurane and positioned in a temperature-controlled stereotaxic frame (37 ± 0.5°C; Harvard Apparatus). A midline incision was made to expose the skull, and the charge-coupled device (CCD) camera was installed 10 cm above the skull. Cerebral perfusion images were captured within a 2.0cm x 2.0cm scanning area, and cortical blood flow (perfusion unit, PU) was calculated within a standardized regions of interest (ROIs) using vendor supplied PIMSoft software (version 1.5; Perimed) and presented as mean perfusion values. After baseline collection, the mouse was subjected to TBI and monitored changes in cerebral cortical blood flow at 3, 24, and 72 hrs after injury. ROI was defined as areas in the contusional and peri-contusional cortex from mice with TBI, and the equivalent area from sham mice.

Immunoprecipitation and immunoblots

To assess the interaction between A2 and plasma VWF in the circulation of mice subjected to TBI or sham surgery, we performed co-immunoprecipitation experiments. Briefly, anti-His antibody (10 μg, Abcam) was mixed and incubated with 25 μl of sepharose-conjugated protein A/G microbeads for 1 hr at RT on a shaker to allow the antibody to crosslink with beads, according to the manufacturer's protocol

(ThermoFisher Scientific). Plasma samples from sham or TBI mice receiving A2 or TBST were incubated with the antibody-crosslinked beads overnight at 4°C on a parallel shaker set at 200 rpm/min. The immunoprecipitated plasma was then eluted, separated by 4-12% gradient SDS-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to nitrocellulose membranes (EDM Millipore), and blotted for VWF using a HRP-conjugated rabbit polyclonal anti-human VWF antibody (DAKO, Carpinteria, CA) that cross-reacts with mouse VWF. In reciprocal experiments, plasma samples immunoprecipitated by the polyclonal VWF antibody (DAKO) coupled to the protein A/G beads were probed with an anti-His antibody (Abcam) in an identical fashion. For in vitro validation, plasma (100 μl) from 1) TBI mice, 2) sham mice, 3) TTP patients, and 4) pooled from 30 normal subjects were incubated with A2 for 1 hr at 37°C and immunoprecipitated with the anti-His antibody, as described above. Immunoprecipitants were subjected SDS-PAGE and immunoblotted with a polyclonoal VWF antibody (DAKO). In addition, plasma from TBI mice incubated with A2 and an equal molar concentration of recombinant VWF A1 domain (A1, D^{1261}-T^{1468}, U-PROTEIN EXPRESS BV, Utrecht, Netherlands) was examined in the identical fashion.

Surface Plasmon Resonance Spectroscopy

The interaction between the A1 and A2 domains of VWF was quantitatively measured on a Biacore® T100 instrument (Biacore AB, Uppsala, Sweden) using a protocol modified from our previous study.[12] Briefly, 200 nM of a recombinant VWF-A1 protein (D^{1261}-T^{1468}, U-PROTEIN EXPRESS BV) was coupled to the surface of the CM5 sensor chips (GE Healthcare, Uppsala, Sweden). A2 suspended in HBS buffer (10 mM HEPES, 0.15 M NaCl, 3 mM EDTA, and 0.005% Tween 20, pH 7.4) was perfused over coupled A1 at increasing concentrations (12.5 nM to 400 nM) at 25°C and at a flow rate of 5 μl/min. The resonance signals from various concentrations of A2 over the A1 surface were recorded. After the signal from the control surface was subtracted, the specific A2 resonance signal was collected to calculate the dissociation constant (K_d) of the A1-A2 interaction.

Tail bleeding time

The mouse hemostasis was measured by tail bleeding, as we previously described.[3,8] In brief, the tail of an anesthetized mouse was transected at 1/10 distance to the tail tip using a sterile surgical scalpel and was immediately immersed in 50 ml of PBS that was pre-warmed to 37°C. The time to the full cessation of tail bleeding was recorded as the

tail bleeding time. The PBS suspension was then centrifuged at 4,000 rpm for 5 min at RT and the pelleted erythrocytes were lysed with 2 ml of the red blood cell lysis buffer (Sigma-Aldrich, 10 min at RT). After centrifugation at 10,000 rpm for 5 min to remove cellular debris, cell-free Hb was measured in the supernatant at OD 550 nm using a SpectraMax M5 plate reader (Molecular Devices). The Hb data were used for validating results and are not included in the manuscript.

SUPPLEMENTAL RESULTS

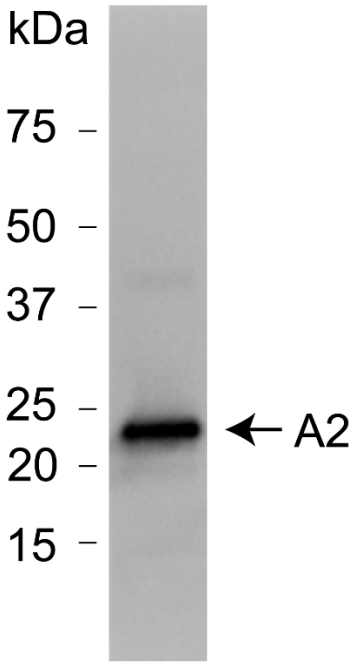

Fig. S1. A representative immunoblot of A2 purified using a HisPur Cobalt Spin Column and stained with a goat anti-A2 antibody (Bethyl Laboratories, Houston, TX).

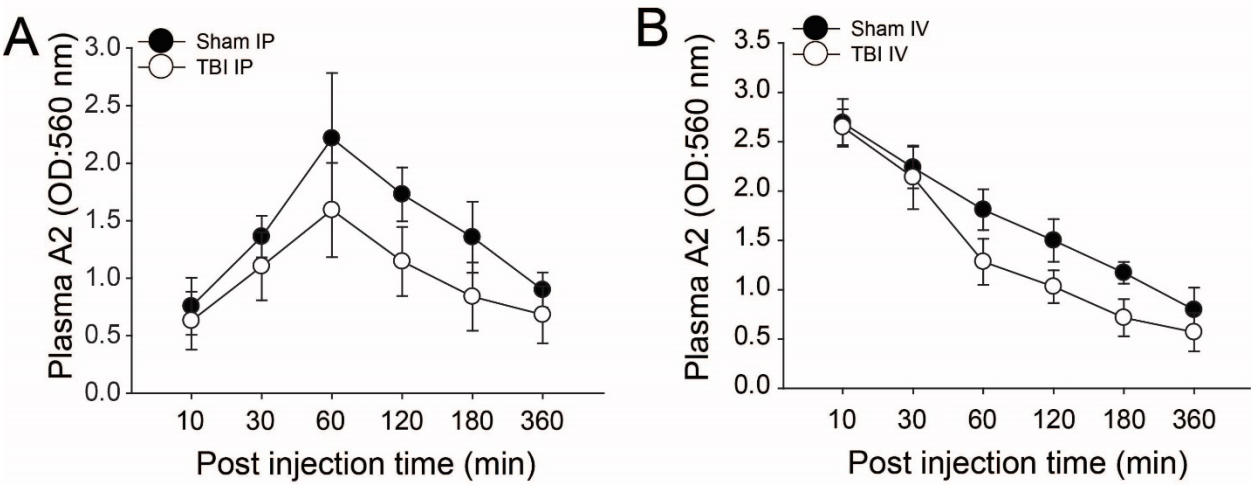

Fig. S2. Dynamic changes of circulating levels of A2 after infusion. A2 was given to sham or TBI C57BL/6J mice through intraperitoneal injection (IP) at 4 mg/kg (**A**) or intravenous injection (IV) at 3.2 mg/kg (**B**). Citrated blood samples were collected at the times indicated and plasma levels of A2 were detected by an anti-A2 antibody using ELISA.

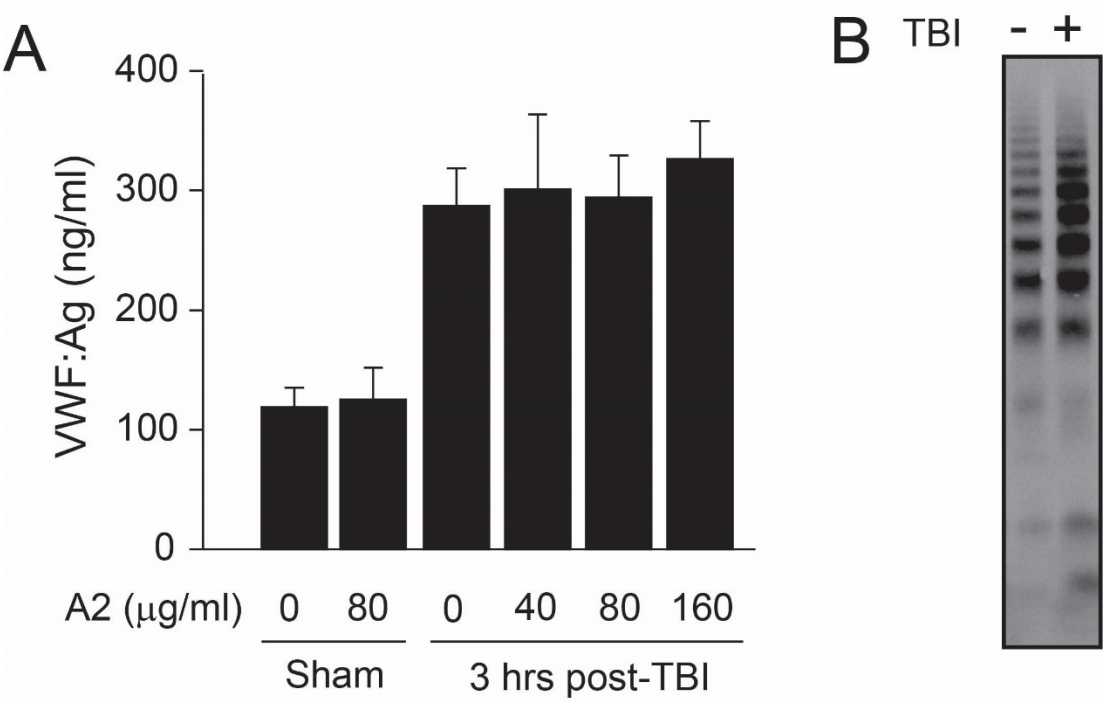

Fig. S3. (A) Plasma VWF:Ag was measured using ELISA in the presence of A2 at the concentration similar to those used in the study (e.g., 4 mg/kg is approximately 80 μg/ml, assuming that an adult mouse has 1 ml of circulating blood, n = 8, One-way ANOVA). **(B)** Distribution of VWF multimers before and after TBI (representative of 6 separate experiments).

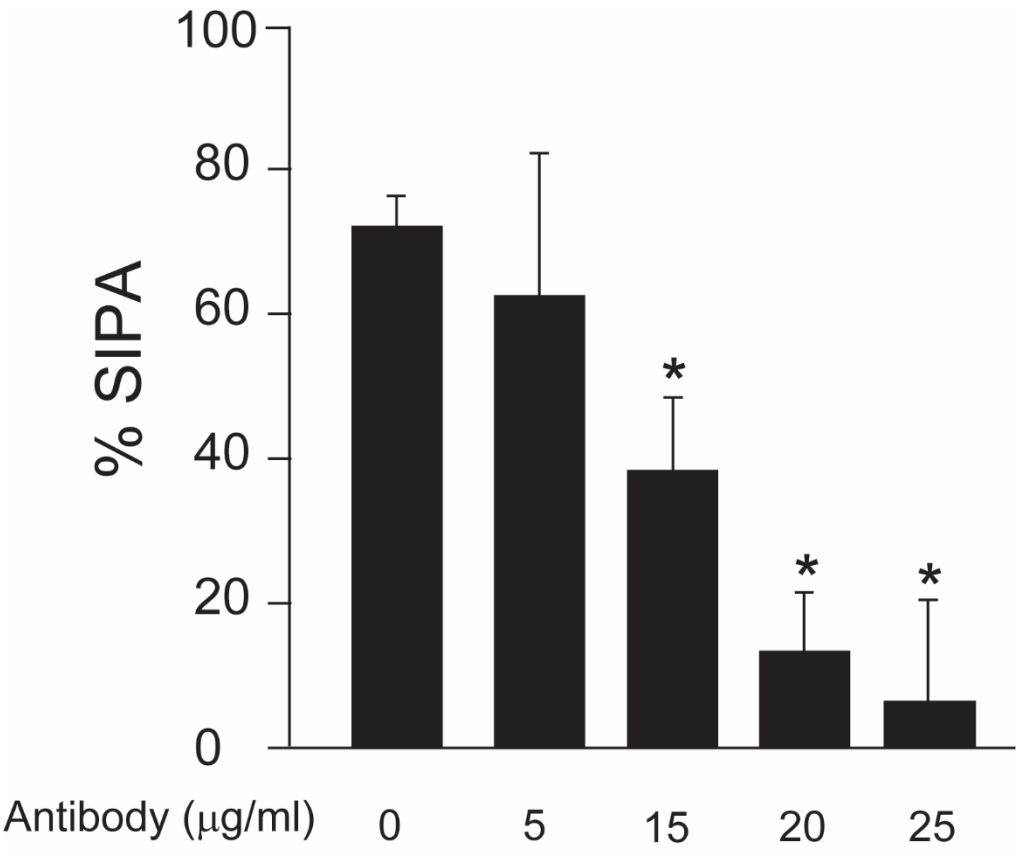

Fig. S4: The rabbit anti-VWF polyclonal antibody blocked shear-induced platelet aggregation (100 dynes/cm^2, 1 min at 37^{o}C) in a dose-dependent manner (n=32, one-way ANOVA, *$p<0.01$ vs. untreated). This antibody was used in the study for blocking VWF-mediated endothelial permeability induced by extracellular vesicles.

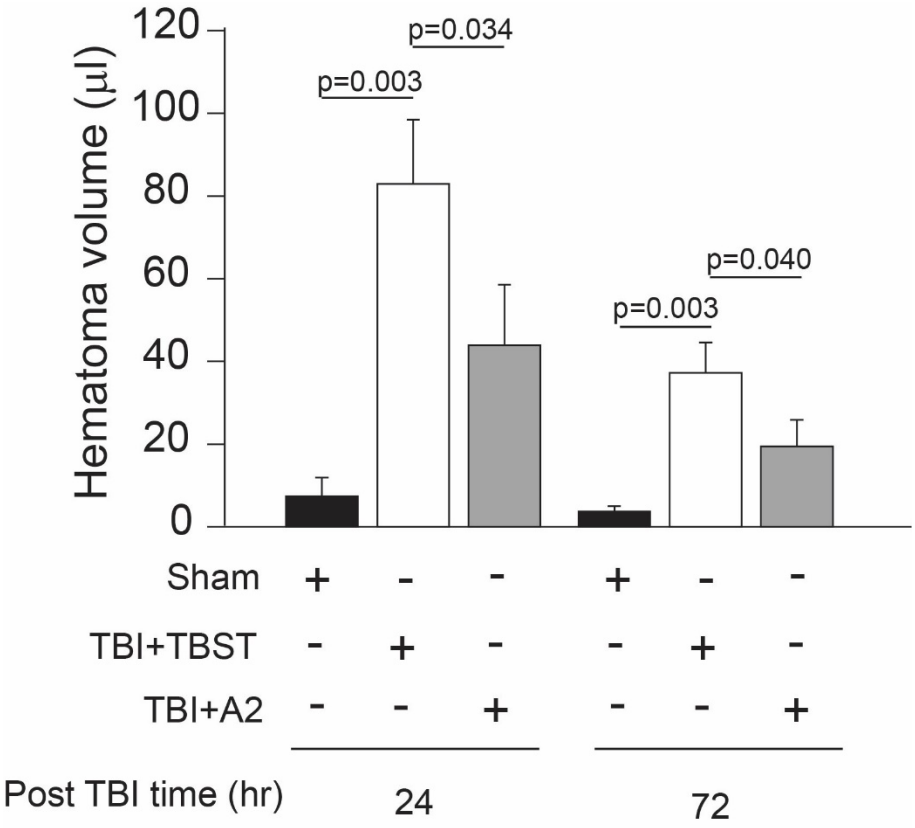

Fig. S5. A2 reduced intracranial bleeding after TBI. Mice were subjected to sham surgery or TBI. The TBI mice received either 4mg/kg A2 or TBST 30 min after injury. The brains were collected at 1 and 3 days after injury and processed to tissue sections. Hematoma and intracerebral bleeding were quantified in H&E stained tissue section (n=12, one-way ANOVA).

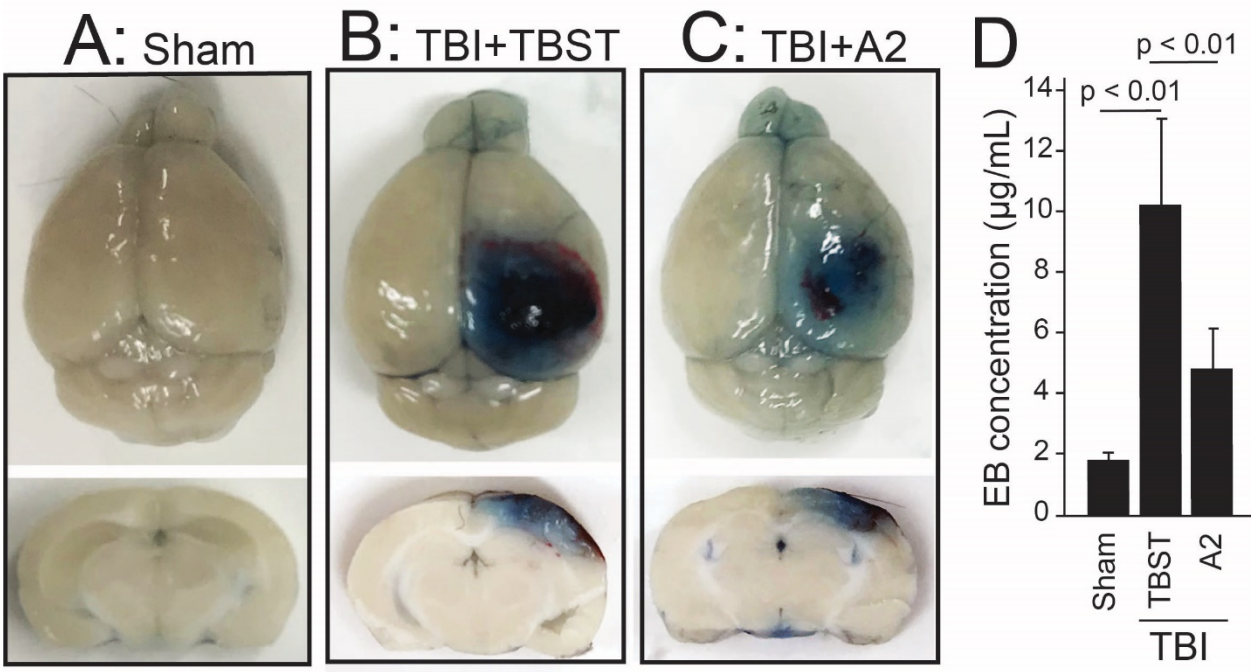

Fig. S6: A2 protected against vascular leakage in mice subjected to TBI. Evans blue (EB) dye extravasation test was used to measure cerebral vascular leakage. Top and coronal views of brains from sham mice (**A**), TBI mice receiving TBST (**B**), and TBI mice receiving A2 (**C**) subjected to EB dye extravasation test (**D:** summary from 15 mice, one-way ANOVA on ranks, EB: Evans blue).

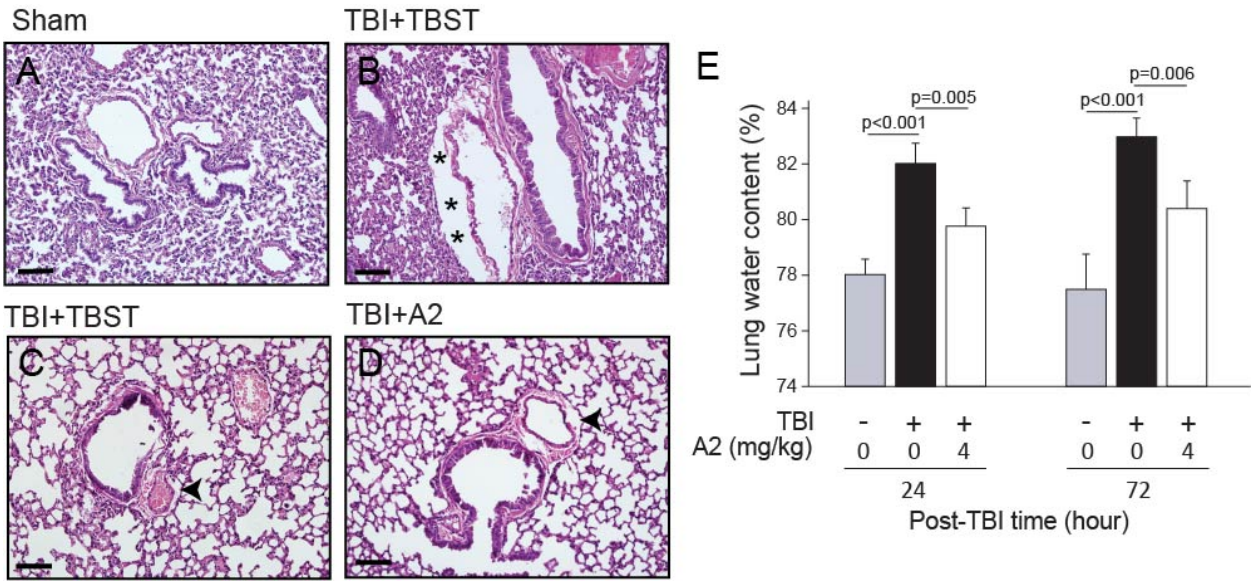

Fig. S7: A2 reduced TBI-induced pulmonary interstitial bleeding and tissue edema. The lung sections stained with H&E show the intact vessel wall in sham mice (**A**), but enlarged perivascular space (**B**, indicated by *) and tissue edema (**C**, indicated by arrow head) in TBI mice receiving the vehicle TBST. The vascular leakage is less severe in TBI mice receiving A2 (**D**, arrow: perivascular edema). The images are representative of samples from 36 mice. (**E**) The lung water content of mice in the three treatment groups was measured at 24 and 72 hrs after TBI (n=12, one-way ANOVA).

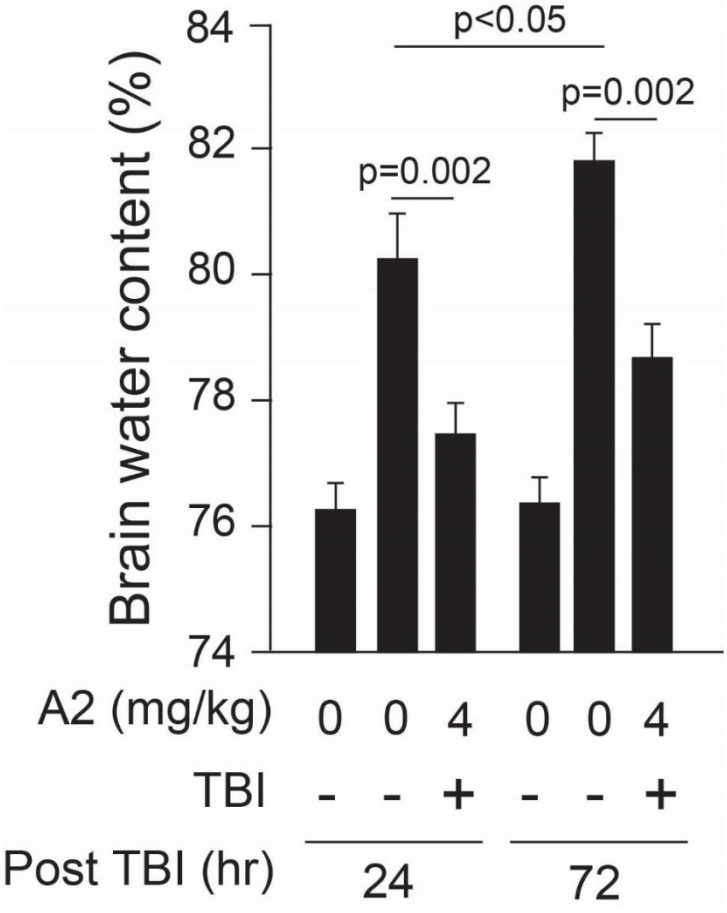

Fig. S8: A2 reduced TBI-induced cerebral edema. Mice were subjected to TBI. Their brains collected on day 1 and day 3 immediately after euthanasia and processed to measure the water content of the whole brain (n=12, one-way ANOVA).

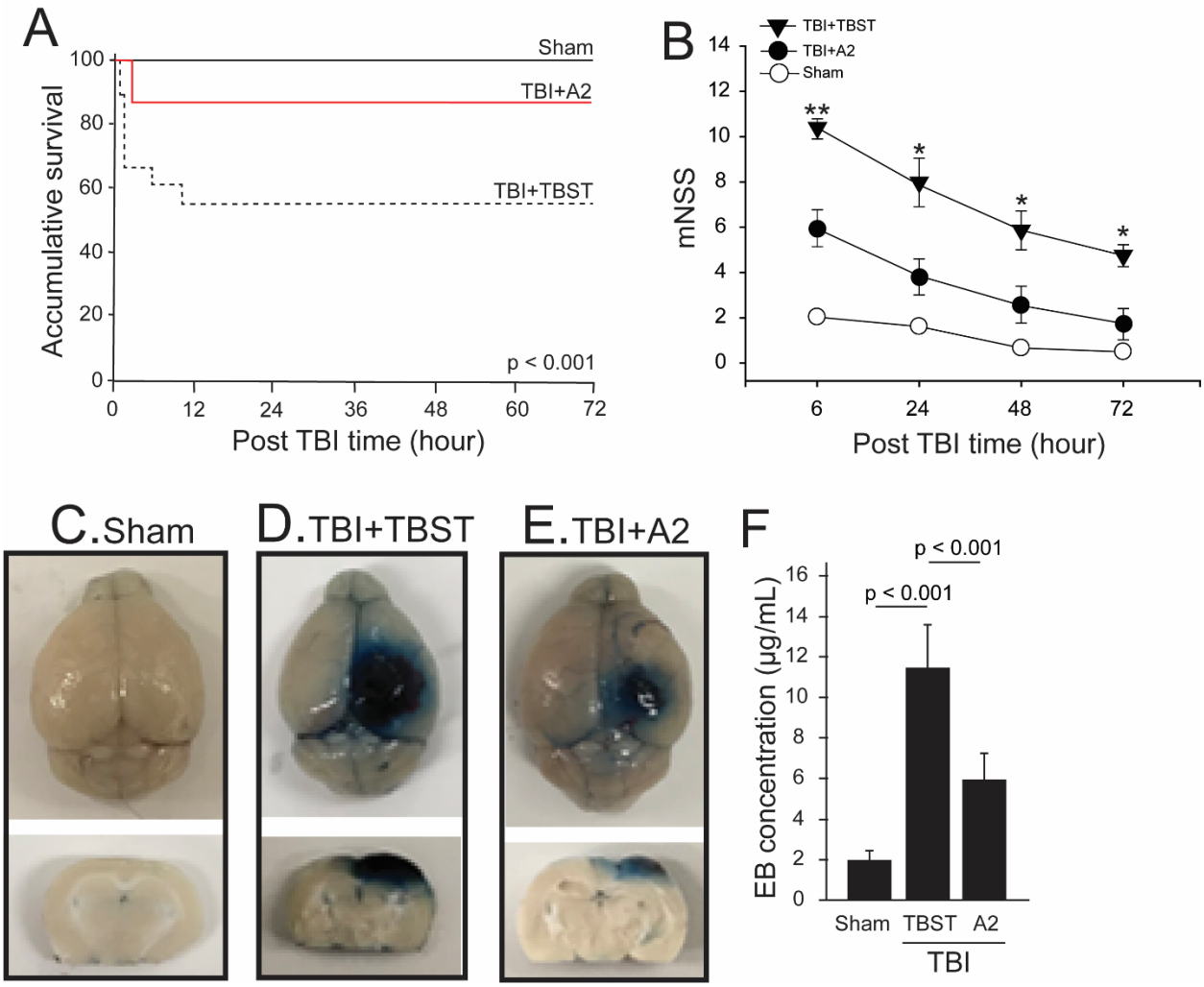

Fig. S9. A2 through IV infusion improved the survival and neurological function of TBI mice. C57BL/6J male mice were subjected to TBI and infused with 3.2 mg/kg of A2 through the tail vein 30 min after injury. Their survival (**A**, n = 32, Kaplan Meier survival analysis) and neurological functions (**B**, n = 20, one-way ANOVA on rank, *p < 0.001 and **p<0.005 between A2 and TBST) were measured. Evans blue extravasation tests on C57BL/6J mice subjected to sham surgery (**C**) and TBI receiving either the vehicle buffer TBST (**D**) or A2 (**E**, 3.2 mg/kg) were performed. (**F**) The summary from multiple experiments (n = 9, one-way ANOVA).

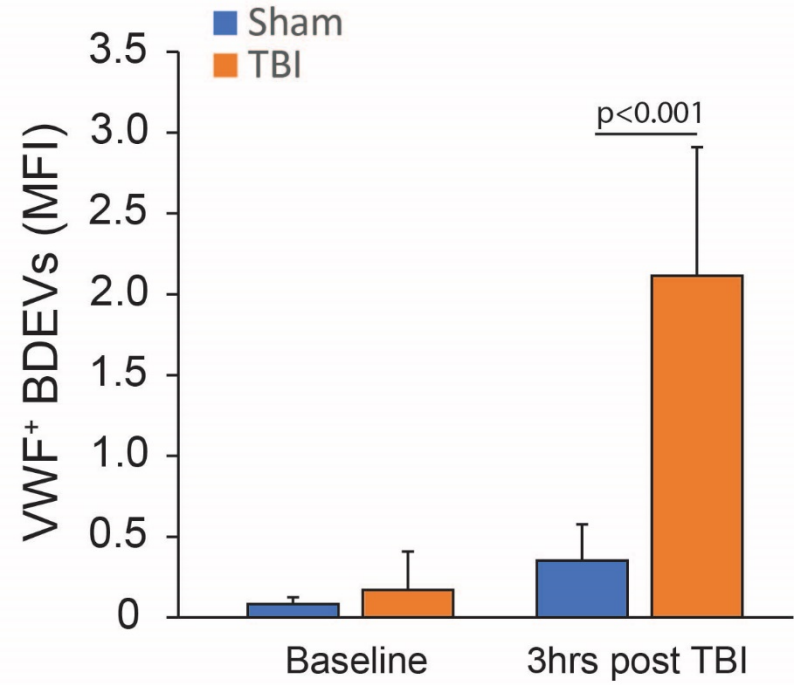

Fig. S10. VWF was detected on surface of BDEVs ($GFAP^+$) found in peripheral blood of TBI mice, but minimally in sham mice (n=6, paired t test).

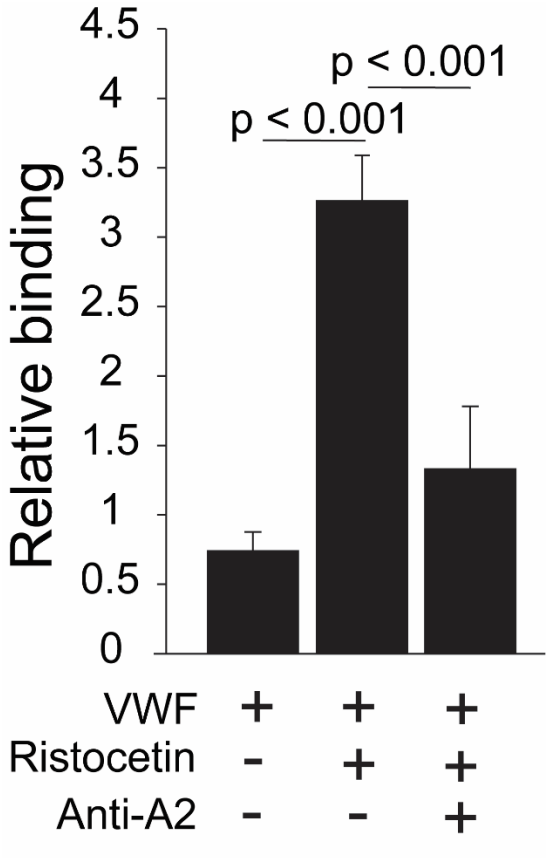

Fig. S11. An A2 antibody blocked the interaction between A2 and ristocetin-activated VWF. A2 (5 μg/ml) was captured to Nickel-coated microtiter plates through His-tag. A2-coated plates were then incubated with plasma VWF in the presence of either 1 mg/ml of ristocetin (which activates VWF) or ristocetin plus an A2 blocking antibody for 60 min at 37°C. After washing, VWF bound to A2 was detected by a polyclonal VWF antibody (n=12, one-way ANOVA).

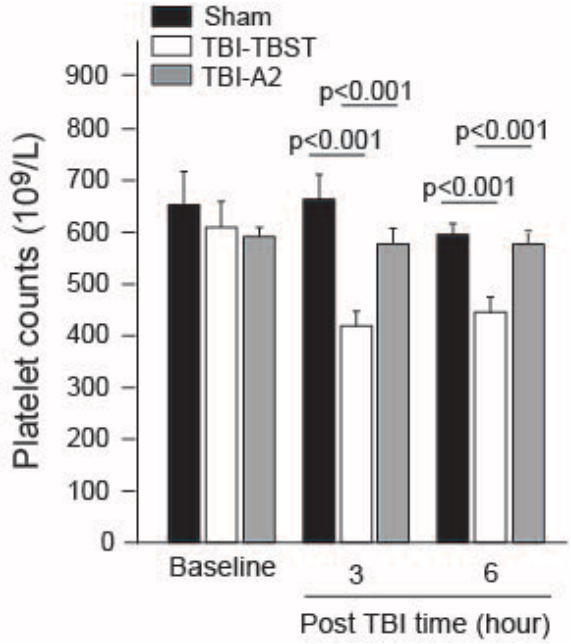

Fig. S12: A2 corrected TBI-induced low platelet counts. Mice were subjected sham surgery or TBI. The TBI mice received either 4 mg/kg of A2 or an equal volume of TBST 30 min after injury. Blood samples were collected at 3 and 6 hrs after TBI to measure platelet counts using VetScan HM5 Veterinary hematology analyzer (Abaxis, Union City, CA, n=21, one-way ANOVA).

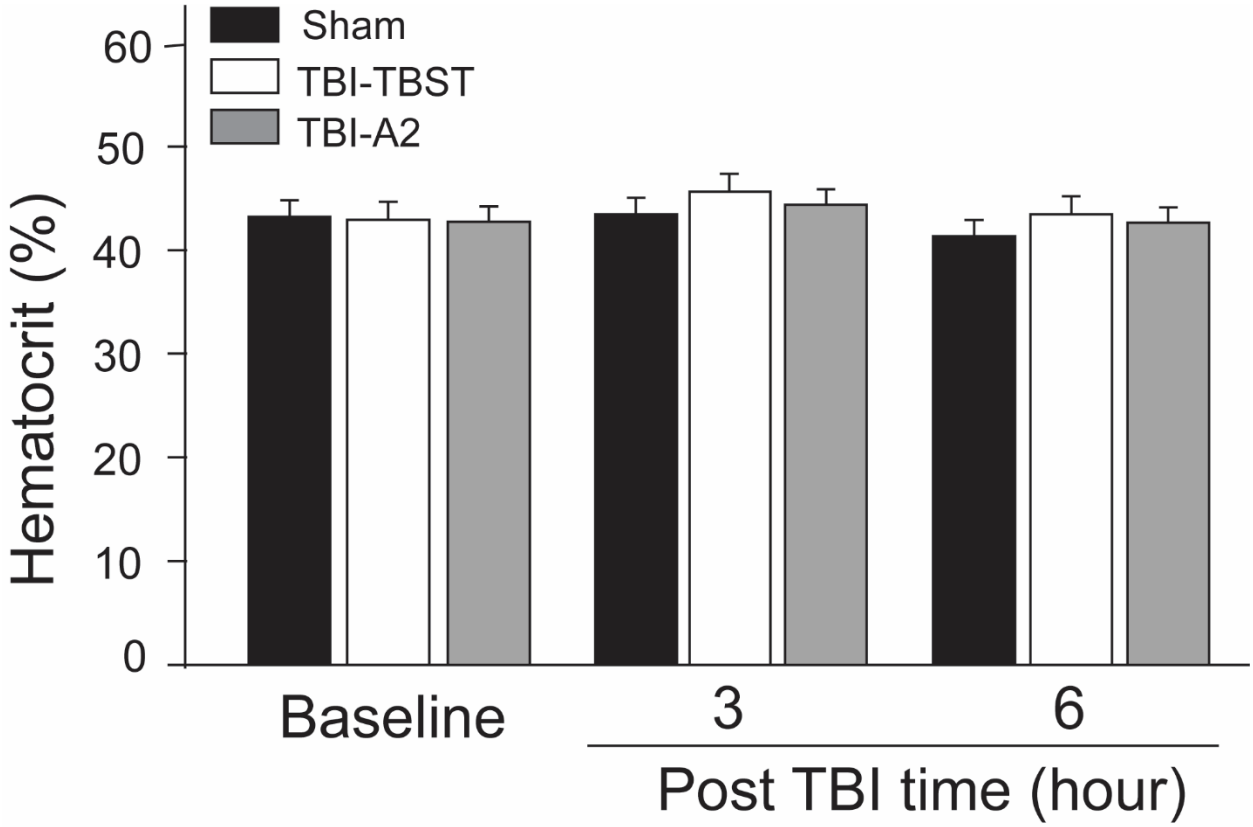

Fig. S13. A2 did not change the level of hematocrits in sham and TBI mice. C57BL/6J male mice were subjected to TBI and receiving A2 or an equal volume of TBST 30 min after injury (n = 9, one-way ANOVA). Their hematocrits were measured at the baseline and at 3 hrs and 6 hrs after injury using a VetScan HM5 Veterinary hematology analyzer (Abaxis).

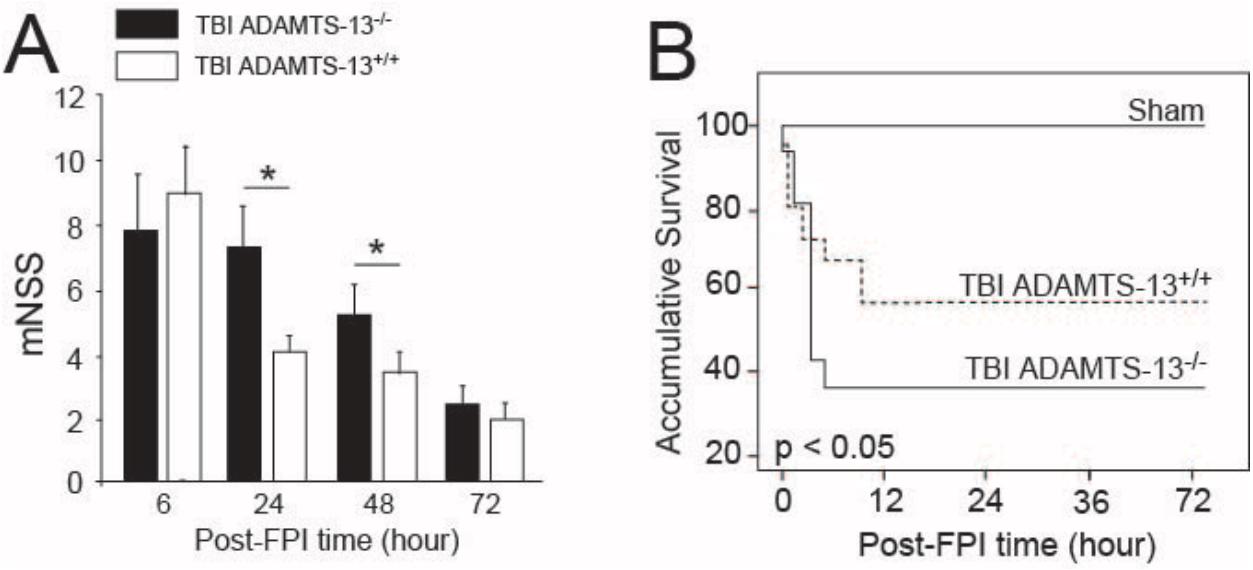

Fig. S14: ADAMTS-13$^{-/-}$ mice developed worse neurological deficits and had a lower survival rate. ADAMTS-13$^{-/-}$ mice and their wild type littermates were subjected to TBI of 1.9±0.2 atm. They were then evaluated for neurological deficits, measured by mNSS (n= 26, one-way ANOVA, *p<0.01) and monitored for survival (Kaplan Merrier accumulative survival analysis) for 3 days post injury.

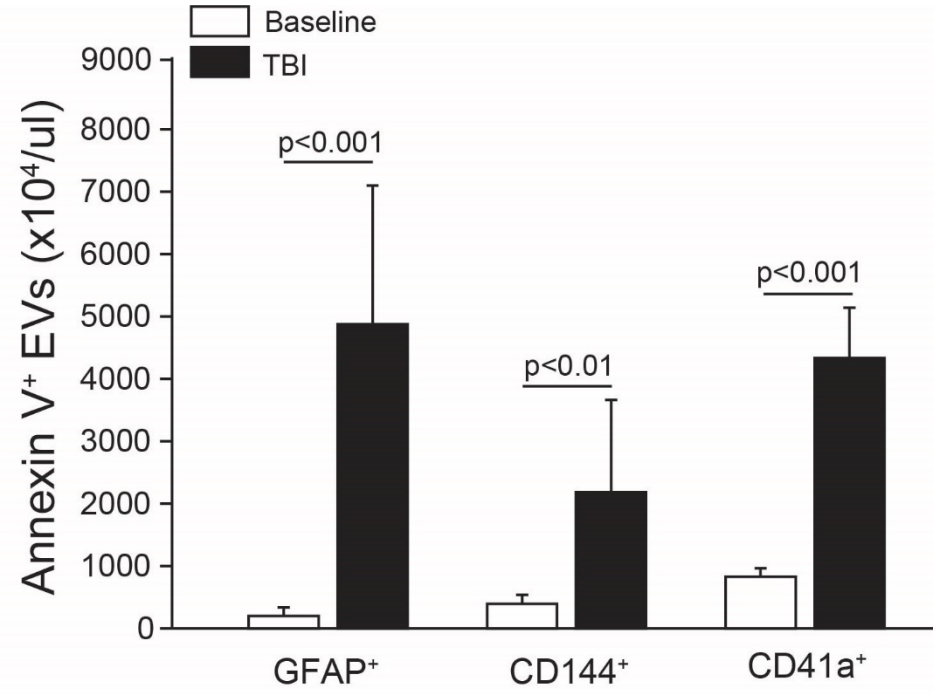

Fig. S15. TBI-induced the release of EVs from the brain ($GFAP^+$), endothelial cells ($CD144^+$) and platelets ($CD41a^+$). These EVs also coexpressed the procoagulant anionic phospholipids that bound annexin V (n=26, paired t test).

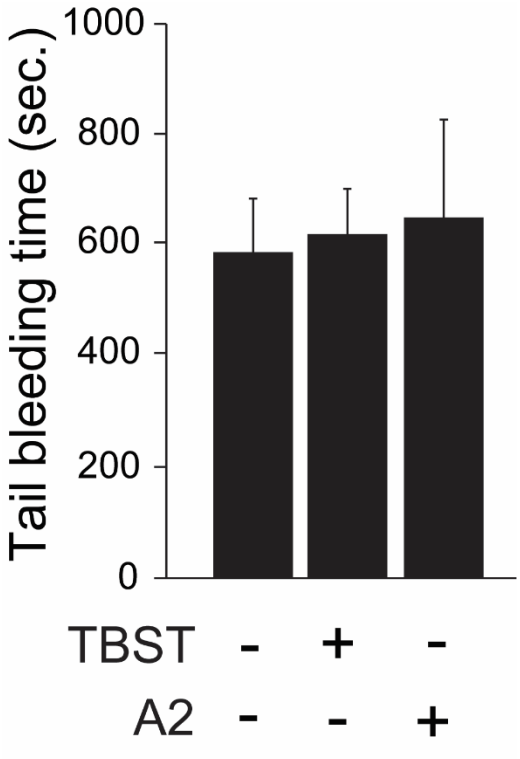

Fig. S16. A2 did not prolong tail bleeding of non-injured mice. Tail bleeding times measured in non-injured mice 30 min after they were injected through IP with either 4 mg/kg of A2 or an equal volume of TBST (n = 12, one-way ANOVA). A similar finding was also made in mice with endotoxemia.[13]

REFERENCES:

1. Tian Y, Salsbery B, Wang M, et al. Brain-derived microparticles induce systemic coagulation in a murine model of traumatic brain injury. *Blood*. 2015;125(13):2151-2159.
2. Zhao Z, Wang M, Tian Y, et al. Cardiolipin-mediated procoagulant activity of mitochondria contributes to traumatic brain injury-associated coagulopathy in mice. *Blood*. 2016;127(22):2763-2772.
3. Wu Y, Liu W, Zhou Y, et al. von Willebrand factor enhances microvesicle-induced vascular leakage and coagulopathy in mice with traumatic brain injury. *Blood*. 2018;132(10):1075-1084.
4. Xu X, Yin D, Ren H, et al. Selective NLRP3 inflammasome inhibitor reduces neuroinflammation and improves long-term neurological outcomes in a murine model of traumatic brain injury. *Neurobiology of Disease*. 2018;117:15-27.
5. Zhang Y, Chopp M, Emanuele M, et al. Treatment of Traumatic Brain Injury with Vepoloxamer (Purified Poloxamer 188). *J Neurotrauma*. 2018;35(4):661-670.
6. Yang G-L, Han Z, Xiong J, et al. Inhibition of intracranial hemangioma growth and hemorrhage by TNFSF15. *The FASEB Journal*. 2019;33(9):10505-10514.
7. Zhao BQ, Chauhan AK, Canault M, et al. von Willebrand factor-cleaving protease ADAMTS13 reduces ischemic brain injury in experimental stroke. *Blood*. 2009;114(15):3329-3334.
8. Zhou Y, Cai W, Zhao Z, et al. Lactadherin promotes microvesicle clearance to prevent coagulopathy and improves survival of severe TBI mice. *Blood*. 2018;131(5):563-572.
9. Hajek AS, Joist JH, Baker RK, Jarett L, Daughaday WH. Demonstration and partial characterization of insulin receptors in human platelets. *JClinInvest*. 1979;63(5):1060-1065.
10. Hue CD, Cho FS, Cao S, et al. Time Course and Size of Blood-Brain Barrier Opening in a Mouse Model of Blast-Induced Traumatic Brain Injury. *J Neurotrauma*. 2016;33(13):1202-1211.
11. Dong JF, Moake JL, Nolasco L, et al. ADAMTS-13 rapidly cleaves newly secreted ultralarge von Willebrand factor multimers on the endothelial surface under flowing conditions. *Blood*. 2002;100(12):4033-4039.
12. Dong JF, Moake JL, Bernardo A, et al. ADAMTS-13 metalloprotease interacts with the endothelial cell-derived ultra-large von Willebrand factor. *J Biol Chem*. 2003;278(32):29633-29639.
13. Nguyen TC, Gushiken F, Correa JI, et al. A recombinant fragment of von Willebrand factor reduces fibrin-rich microthrombi formation in mice with endotoxemia. *Thromb Res*. 2015;135(5):1025-1030.