Supplemental Methods

Thrombin generation assay

We used a commercial kit to measure BDMP-induced thrombin generation. Briefly, MP-Reagent (20 µl, Diagnostica Stago, Inc., Parsippany-Troy Hills, NJ), 70 µl of phospholipid-deficient porcine plasma (IMVS, Adelaide, Australia), 20 µl of FluCa solution (Diagnostica Stago) and 25,000 BDMPs/1 µl (close to the maximal number of BDMPs found in TBI mice) were mixed and incubated at 37°C. The MP-Reagent contained PS, PC, and phosphatidylethanolamine at a ratio of 20:60:20, providing 4 µM of total phospholipids to the reaction mix. Thrombin generation was measured on a Thrombinoscope Fluoroskan Ascent® reader (Thermo Labsystems, Helsinki, Finland; 390 nm excitation and 460 nm emission). The peak concentration of thrombin that was generated, reaction lag time and area-under-the curve (AUC) were calculated with Thrombinoscope™ software (Thrombinoscope, BV, Maastricht, The Netherlands). Thrombin generation induced by 1 pM of TF was used as a reference.

Production of BDMPs by freeze-thawing and mechanical injury: We generated BDMPs from mouse brain using a method adopted from a report for generating platelet microparticles.¹ Briefly, the mouse brains were removed, washed with saline, and quickly frozen in liquid nitrogen. Before the experiments, they were rapidly thawed at 37°C and homogenized in 1 ml of PBS using a glass Dounce homogenizer (Fisher Scientific Co., Federal Way, WA). The brain tissue was centrifuged at 1,500 x g for 20 min at 4°C to remove the intact cells. The supernatant was centrifuged at 13,000 x g for 2 min at 4°C to remove large cellular debris, and then twice at 100,000 x g for 1 hr at
4°C, using a TLA-100.4 rotor (Beckman Coulter, Miami, FL). The BDMP pellet was re-suspended in 500 μl of PBS. Microparticles were quantified by flow cytometry in a time-fixed mode in the presence of counting beads (Spherotech, Lake Forest, IL). Megamix microbeads (0.5, 0.9 and 3 μm, (Biocytex, Marseille, France) were used to gate microparticles based on the particle size. A total of 20 mouse brains were processed and tested.

**Production of platelet microparticles:** Blood was collected from adult C57BL/6J mice using 0.38% sodium citrate (final concentration) as the anti-coagulant. The blood sample was then centrifuged at 150 x g for 15 min at 25°C. Platelet-rich plasma (PRP) was stimulated with 10 μg/ml type I fibrillar collagen (Helena Laboratories, Beaumont, TX) for 10 min at room temperature, followed by centrifugation at 1,500 x g for 20 min at 22°C, to yield platelet-poor plasma (PPP), which was then centrifuged at 13,000 x g for 2 min at 4°C to remove large cellular debris. The supernatant underwent two cycles of ultra-centrifugations, each at 100,000 x g for 1 hr at 4°C. The platelet microparticle pellet was re-suspended in 500 μl of PBS and counted by flow cytometry.

**Measurement of calcium flux by flow cytometry:** PRP (20 μl) was mixed with 180 μl of Tyrode’s buffer (137 mM NaCl, 2.8 mM KCl, 1 mM MgCl₂, 1.8 mM CaCl₂, 12 mM NaHCO₃, 0.4 mM Na₂HPO₄, 5.5 mM glucose, pH 7.5) containing 5 μM eFluor 514 Calcium Sensor Dye (final concentration, eBioscience, San Diego, CA). A baseline level of calcium was established using flow cytometry to measure the mean fluorescence intensity (MFI) in the platelets gated on forward scatter. These platelets were then
treated with BDMPs and monitored for an increase in MFI over time. Dye-labeled platelets incubated with PBS were tested as negative control.

**Evans blue extravasation:** Evans blue (EB, Sigma Aldrich, St. Louis, MO, 2% in PBS) was slowly injected into the jugular vein of a mouse 1 hr after it was subjected to FPI or sham surgery. The mouse was sacrificed 1.5 hrs after injection and transcardially perfused with 5 ml of 1 × PBS and 5 ml 0.9% saline. The brain was then quickly removed to allow visual detection of EB leakage.

**Tissue histology:** Mice were sacrificed after being injected with PBS or BDMPs to excise the lungs, kidneys and heart. These organs were fixed immediately with 4% neutral formaldehyde (overnight at room temperature), dehydrated, embedded in paraffin and sectioned. Tissue sections were de-paraffinized, rehydrated and washed in distilled water for 1 min. They were then fixed in a potassium permanganate solution for 5 min, washed in distill water and bleached by oxalic acid for 5 min. After being washed twice in distill water, the sections were incubated with phosphotungastic acide hematoxylin (PTAH), which specifically recognizes fibrin deposited onto the vessel wall (also stain for muscles), for 18 hrs at room temperature. The sections were dehydrated, mounted, and examined under an optical upright microscope (Nikon eclipse 80i, Nikon, Japan). Images were captured using a Nikon DS-Ri1 camera and processed with the NIS-Elements F3.0 Imaging program (Nikon).
For scan microscopy, acid-cleaned glass coverslips were coated with human fibrinogen (2 mg/ml, 4°C, overnight). These fibrinogen-coated coverslips were incubated with PRP for 30 min at 37°C and, after washing to remove unbound platelets, with 40 μl BDMPs (1 x 10⁴/μl final density) for an additional 30 min at 37°C. After washing, the coverslips were fixed with 2.5% glutaraldehyde at 4°C for 24 hrs, followed by additional washing and fixation in a 1% osmium tetroxide at 4°C for 1.5 hrs. Platelet-bound coverslips were dehydrated in 50%, 70%, 80%, 90% and 100% ethanol and air-dried. The samples were then sputter-coated with a thin metal coating for view under scanning electron microscopy (JSM-6380Lv, JEOL, Japan). A modified method was also used to detect BDMPs that have transmigrated through endothelial cells in a transwell assay. Briefly, confluent HUVECs were stimulated with histamine and then incubated with BDMPs either alone or with platelets for 30 min at 37°C. The transwell membranes were removed and fixed in 3% glutaraldehyde overnight at 4°C, washed with PBS and treated with 1% osmium tetroxide for 1 hr at 4°C. After washing with PBC, they were dehydrated with 50%, 70%, 80%, 90%, and 100% of alcohol (10 min at each treatment) and air-dried for SEM.
Fig. S1: Protocol for detecting BDMPs in plasma samples: (A) Microbeads measuring 0.5, 0.9, and 3 μm were ran on forward (FSC) and side (SSC) scatters to set up an acquisition gate that included particles measuring up to 0.9 μm in diameter. (B) Plasma samples were then analyzed on the same settings to identify particles within the preset gate (P1 included all particles of ≤ 1 μm in size). (C) The gated particles were subsequently analyzed for the expression of GFAP and PS detected by a polyclonal antibody and annexin V, respectively.
Fig. S2: Calcium-dependent annexin V binding to BDMPs: BDMPs were incubated with APC-conjugated annexin V and a FITC-conjugated GFAP antibody in the presence and absence of 5 mM EDTA for 20 min at room temperature. They were then selected first on particle size and then on GFAP positivity to specifically identify BDMPs, which were then examined for PS expression. Graphs show that the chelation of calcium eliminates annexin V binding to PS on the surface of BDMPs.
Fig. S3: The calcium-dependence of the PS-dependent clot formation assay: PS-dependent clot formation was measured in the presence and absence of 4 mM EDTA, which quenches free calcium to block coagulation. This is a control experiment to show that clot formation (measured as a shortened clotting time) requires calcium.
Fig. S4: Detection of BDMPs transmigrated through the endothelial barrier:
BDMPs pre-labeled with PKH26 were incubated in the upper chamber of a transwell system with HUVECs for 3 hrs at 37°C. The medium collected from the bottom chamber was analyzed for the presence of PKH26-labeled BDMPs. For the assay, BDMPs were initially gated based on size (A) and then on PKH26 positivity (B), using flow cytometry (fixed time-mode with counting beads, acquisition time of 3 min). The graph in (B) is from a control experiment using non-activated HUVECs, showing no BDMPs (in P2 region) detected in the bottom chamber.
Fig. S5: Detection of neuronal and glial cell markers on BDMPs: Lysates from purified BDMPs and platelets were separated on 4-20% gradient SDS-PAGE and probed for Na\(^+\)/K\(^+\) ATPase \(\alpha3\) (neuronal marker) and glutamate transporter 1 (astroglial marker). Platelet lysate served as a negative control and the cytoskeleton protein, tubulin, as a loading control.
Fig. S6: Calculated levels of BDMPs: The numbers of BDMPs/μl were calculated by extrapolating the quantitative data from 3 hr post TBI (*p < 0.01 and ** p < 0.005 compared to sham surgery controls).
Fig. S7: Detection of GFAP and PS on BDMP from FPI mice and mouse brain subjected to freeze-thawing injury: (A & B) Plasma samples from TBI mice and (D & E) BDMPs isolated from uninjured brains (normalized to an equal number) were incubated with APC-conjugated annexin V, and either a FITC-conjugated isotype control IgG (A & D) or a FITC-conjugated GFAP antibody (B & E), for 20 min at room temperature. They were then quantitatively analyzed by flow cytometry for PS and GFAP levels. (C) BDMPs that were not incubated with annexin V and the GFAP antibody were tested to set baseline values. Panel F summarizes data from 6 separate preparations of BDMPs.
Fig. S8: Expression of non-neural markers on BDMPs: BDMPs made from freeze-thawing injured mouse brains were incubated with each of the three FITC-conjugated antibodies against markers for (A) leukocytes (CD45), (B) endothelial cells (CD144), and (C) erythrocytes (CD235a), for 30 min at room temperature, and then analyzed by flow cytometry. Levels of antibody binding are marked with solid red lines and those from isotype specific IgGs (negative controls) with solid blue lines. The graphs represent 3-5 separate experiments.
**Fig. S9: A flow cytometric detection of bovine lactadherin binding to BDMPs:**

FITC-conjugated bovine lactadherin were incubated with BDMPs for 30 min in 37°C in the presence or absence of a 100-fold excess of unlabeled bovine lactadherin. BDMPs were then stained with a monoclonal antibody to glutamate transporter 1. FITC-lactadherin were detected on Glutamate transportor 1 positive BDMPs (n = 3, paired t-test).
Fig. S10: Partial blockage of lactadherin binding to BDMPs by PS: BDMPs were first incubated with a PE-conjugated monoclonal antibody against glutamate transporter 1 for 20 min at room temperature, followed by incubation with FITC-labeled lactadherin for 30 min at room temperature, in the presence of increasing doses of either purified brain PS or purified brain PC. The reaction mix was analyzed by flow cytometry. BDMPs were initially identified by size and then by glutamate transporter 1 positivity. FITC fluorescence conjugated to lactadherin was detected on gated BDMPs. The panels are representatives of three separate sets of experiments.
Fig. S11: Comparison of procoagulant activities of BDMPs and platelet microparticles: BDMPs and platelet microparticles were normalized to an equal count in PBS and tested for their ability to induce clot formation in a PS-dependent assay.
Fig. S12: The minimal expression of TF on platelet microparticles: Microparticles generated from collagen-stimulated platelets were incubated with a FITC-conjugated antibody against the platelet specific marker CD42. CD42+ platelets were stained with Annexin V or a TF antibody, in the presence or absence of 5 mM EDTA, for 20 min at room temperature. Platelet microparticles were first gated on size and then on CD42 positivity. CD42b+ microparticles were analyzed for PS and TF expression. Non-immune IgGs were used as control. The data shows 1) a minimal TF expression on the surface of platelet microparticles and 2) the calcium-dependence of annexin V binding to platelet microparticles.
Reference