1. SUPPLEMENTAL MATERIALS AND METHODS

Assessment of ICH lesion volume in patients

For each patient's computed tomography (CT) scan, uncompressed CT images were collected in a digital imaging format. These images were identifiable only with the patient's unique study number and were analyzed centrally by trained staff blinded to the clinical data. CT image analysis was based on computer-assisted multi-slice planimetric and voxel threshold techniques in MIStar (version 3.2).

Western blot analysis

We resuspended extracellular vesicles and cells using 0.1% Triton and lysated them through five rounds of ultrasound, each lasting 10 seconds followed by a 10-second cooldown. Subsequently, we added 5X loading buffer and denatured the mixture by heating at 95 °C for 5 minutes. We then electrophoresed equal amounts of protein on 4%–20% SDS-polyacrylamide gels at 80 V for 50 minutes. Protein transfer to a 0.45 μm nitrocellulose membrane was facilitated by a semi-dry transformation lasting 8 minutes. We blocked the membranes with 5% skim milk in TBST (20 mM Tris, pH 7.5, 137 mM NaCl, 0.1% Tween 20) and incubated them overnight at 4 °C with primary antibodies from Abcam (CD63 ab134045, TSG101 ab125011) at a 1:1000 dilution in TBST-5% BSA. After three 10-minute washes with TBST, we incubated the membranes in secondary antibodies conjugated to horseradish peroxidase at a 1:5,000 dilution in TBST-5% skim milk. Following three additional 10-minute washes in TBST, we detected bands using enhanced chemiluminescence (Syngene, UK).

Label-free quantitative proteomics

To further perform proteomic analysis, we randomly divided thirty extracellular vesicles into 3 groups. We utilized MALDI-TOF-MS/MS and database searching to identify extracellular vesicle proteins. For all LC-MS/MS experiments, we employed an online system consisting of an EasynanoLC system. It also includes a Q-Exactive HF mass spectrometer (Thermo Scientific, CA) with a nanoelectrospray ion source.

(i) We subjected the samples to proteolysis and digestion using the High pH Recovery kit. Subsequently, we diluted them with mobile phase A (a solution comprising 0.1% formic acid and 5% acetonitrile (pH=9.8)). 5% Mobile phase B (a solution comprising 0.1% formic acid and 95% acetonitrile (pH=9.8)) was used for gradient elution over 10 minutes, followed by 5% to 35% mobile phase B over 40 minutes, and 35% to 95% mobile phase B over 2 minutes. Finally, 5% mobile phase B was used for equilibration over 10 minutes. The

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flow rate was maintained at 1000 nL/min.

(ii) We set the voltage of the ion source at 1.6 kV. For full MS survey scans, we configured the scan range from 350 m/z to 1500 m/z to reach a final resolution of 120,000. Employing 50 milliseconds as a maximum ion implantation time, we chose the top 30 intense peaks (charge state: ≥2) and fragmented them in the ion trap via HCD (normalized collision energy: 28). We enabled the exclusion of isotope item and set the dynamic exclusion time to 30 seconds. 1st 3E6 and 2nd 1E5 were the automatic gain control (AGC) settings.

(iii) We utilized MaxQuant (V1.5.3.30) to search the Raw MS files against the UniProt database. The fixed modification was C (carbamidomethyl), and the variable modifications were M (oxidation) and protein N-term (acetyl). The false discovery level of PSM and protein was set at 1%. To verify the proteins whose expression change were ≥ 2-fold and a $p \leq 0.05$, we employed the MSstats software package (SP/ 21 FragPipe-Msstats v4.2.0).

Biological information analysis

We conducted protein-protein interaction (PPI) network analysis to gain a deeper understanding of differentially expressed proteins within a biological context. Protein interactions between identified or predicted proteins can be obtained from the STRING database, which is a vital resource within ELIXIR's core and it aggregates existing protein-protein correlation data for a large number of organisms. We inputted the list of differential protein IDs into the STRING database (https://string-db.org) to determine the functional correlation networks of the proteins.

Immunostaining and histology

We cut OCT-embedded brain tissue from mice with intracerebral hemorrhage into 9 μm coronal sections. These sections underwent permeabilization and were then incubated with a blocking solution containing 5% donkey serum, 3% BSA, and 0.3% Triton X-100. Subsequently, primary antibodies were applied and allowed to incubate overnight at 4 °C. The primary antibodies used were anti-mouse Iba1 (ab178847, Abcam, UK) and anti-mouse GFAP (3670, Cell Signaling Technology, USA). After three washes with phosphate-buffered saline (PBS), the slices were incubated with the corresponding AF488 fluorochrome-conjugated secondary antibody (A21206, Invitrogen, USA) at room temperature for 1.5 hours. Following another three washes with PBS, the slices were incubated with DAPI (ab104139, Abcam) for nuclear

staining. Subsequently, the sections were observed using the PerkinElmer 6 Launches Vectra® Polaris™ Automated Quantitative Pathology Imaging System (AKOYA, USA) and Laser scanning confocal microscope 880 (Zeiss, Germany). Further analysis was conducted using two software programs: Phenochart (Version 1.0.9) and Zen (Version 3.8), specifically designed for these microscopes. Cell counting was performed manually by two investigators who were blinded to the experimental groups.

Murine neuroimaging

We utilized the 7.0T small-animal magnetic resonance imaging (MRI) scanner (Clinscan, Bruker, Germany) to assess lesion volume. T2-weighted imaging (T2) was employed to evaluate the overall lesion volume. The setup parameters were as follows: repetition time (TR) = 3000 ms, echo time (TE) = 41 ms, field of view (FOV) = 21 × 30 mm, image matrix = 168 × 320, and slice thickness of 0.5 mm. Hematoma measurement was conducted using susceptibility-weighted imaging (SWI). The setup parameters were as follows: TR = 21 ms, TE = 8 ms, flip angle = 40°, FOV = 22 × 30 mm, and image matrix = 256 × 184. Volumes were delineated manually and subsequently calculated automatically using ITK-SNAP software (Version 4.0.1). Perihematomal edema (PHE) volumes were derived by subtracting hematoma volume from total lesion volume. The MRI data underwent analysis by two investigators who were blinded to the experimental groups.

Supplementary Table 1. National Institutes of Health Stroke Scale (NIHSS)[1]

Tested Item	Title	Responses and Scores
1A	Level of consciousness	0 = Alert 1 = Drowsy 2 = Obtunded 3 = Coma/unresponsive
1B	Orientation questions: 1) Current month 2) His/her age	0 = Answers both questions correctly 1 = Answers one question correctly 2 = Answers neither question correctly
1C	Response to commands	0 = Performs both tasks correctly. 1 = Performs one task correctly. 2 = Performs neither task correctly.
2	Best gaze	0 = Normal. 1 = Partial gaze palsy 2 = Complete gaze palsy
3	Visual fields	0 = No visual loss. 1 = Partial hemianopia. 2 = Complete hemianopia. 3 = Bilateral hemianopia
4	Facial movement	0 = Normal symmetrical movements 1 = Minor paralysis 2 = Partial paralysis 3 = Complete paralysis
5	Motor arm a. Left arm b. Right arm	0 = No drift 1 = Drift before 5 second 2 = Fall before 10 second 3 = No effort against gravity 4 = No movement
6	Motor leg a. Left leg b. Right leg	0 = No drift 1 = Drift before 5 second 2 = Fall before 5 second 3 = No effort against gravity 4 = No movement
7	Limb ataxia	0 = Absent 1 = Present in one limb 2 = Present in two limbs
8	Sensory	0 = Normal; no sensory loss 1 = Mild-to-moderate sensory loss 2 = Severe to total sensory loss
9	Language	0 = No aphasia; normal 1 = Mild-to-moderate aphasia 2 = Severe aphasia 3 = Mute, global aphasia
10	Dysarthria	0 = Normal

		1 = Mild-to-moderate dysarthria 2 = Severe dysarthria
11	Extinction and inattention	0 = No abnormality 1 = Visual, tactile, auditory, spatial, or personal inattention 2 = Profound hemi-inattention or extinction to more than one modality

Supplementary Table 2. Modified Neurological Severity Score (mNSS) tests and scoring values.[2]

Motor test score values and descriptions (Normal score = 0; maximum possible summary score = 6)	
0 or 1*	Flexion of forelimb after raising rat by the tail
0 or 1*	Flexion of hindlimb after raising rat by the tail
0 or 1*	Head moved >10° to vertical axis within 30 seconds after raising rat by the tail
0	Normal walk after placing rat on the floor
1	Inability to walk straight after placing rat on the floor
2	Circling toward paretic side after placing rat on the floor
3	Falls down to paretic side after placing rat on the floor
Sensory test score values and descriptions (Normal score = 0; maximum possible summary score = 2)	
0 or 1*	Placing test (visual and tactile test)
0 or 1*	Procioceptive test (deep sensation, pushing paw against table to stimulate limb muscles)
Beam and balance test score values and descriptions (Normal score = 0; maximum possible summary score = 6)	
0	Balances with steady posture
1	Grasps side of beam
2	Hugs beam and 1 limb falls down from beam
3	Hugs beam and 2 limbs fall down from beam, or spins on beam (60 seconds)
4	Attempts to balance on beam, but falls off (> 40 seconds)
5	Attempts to balance on beam, but falls off (> 20 seconds)
6	Falls off; no attempt to balance or hang on to beam (< 20 seconds)
Reflex absence and abnormal movements test score values and descriptions (Normal score = 0; maximum possible summary score = 4)	
0 or 1*	Pinna reflex (head shakes when auditory meatus is touched with cotton)
0 or 1*	Corneal reflex (eye blink when cornea is lightly touched with cotton)
0 or 1*	Startle reflex (motor response to a brief noise from snapping a clipboard paper)
0 or 1*	Seizure, myoclonus, myodystony

Supplementary Table 3. Identification of the upregulated proteins in the intracerebral hemorrhage extracellular vesicles group vs. the control group.

No.	NCBI No.	Gene name	Protein name	log2 (Fold change) (ICH/CTRL)	P value
1	AAB88187.1	PSMC5	proteasome 26S subunit	4.993	0.011
2	AAA60231.1	PYGM	Glycogen phosphorylase	4.582	0.034
3	AAA64799.1	SAA1	Serum amyloid A-1 protein	4.243	0.028
4	AAH03616.1	BLMH	Bleomycin hydrolase	4.155	0.031
5	CAA58788.1	NEB	Nebulin	3.561	0.007
6	ABC40673.1	TPM3	Tropomyosin alpha-3 chain	3.523	0.012
7	BAA36587.1	BAIAP2	Brain-specific angiogenesis inhibitor 1-associated protein 2	3.510	0.039
8	AAB51316.1	TPI1	Triosephosphate isomerase	3.372	0.012
9	AAA51690.1	ALDOA	Fructose-bisphosphate aldolase A	3.357	0.032
10	AAA52387.1	ENO1	Alpha-enolase	2.763	0.017
11	AAA36745.1	TMSB4X	Thymosin beta 4, x-linked	2.703	0.018
12	AAA36170.1	LGALS1	Galectin-1	2.593	0.017
13	AAB37397.1	——	protein disulphide isomerase isoform	2.477	0.0018
14	AAA35844.1	FKBP1A	Peptidyl-prolyl cis-trans isomerase	2.458	0.00013
15	AAA36486.1	PFN1	Profilin-1	2.446	0.000021
16	AAH07560.1	LASP1	LIM and SH3 domain protein 1	2.429	0.00062
17	AAB32876.1	——	Neuro polypeptide h3	2.396	0.030
18	AAD12230.1	GNAI2	Guanine nucleotide-binding protein G(i) subunit alpha-2	2.306	0.035
19	AAH00689.1	PPIA	Peptidyl-prolyl cis-trans isomerase A	2.242	0.0068
20	AAC50537.1	SNAP23	Synaptosomal-associated protein 23	2.228	0.0052
21	AAC39924.1	SKAP2	Src kinase-associated phosphoprotein 2	2.228	0.0478
22	AAA64501.1	CFL1	Cofilin-1	2.190	0.0012
23	AAA92644.1	FLNA	Filamin-α	2.159	0.0115
24	AAA59546.1	SFN	14-3-3 protein sigma	2.109	0.0010

25	AAA36186.1	PECAM1	Leukocyte surface protein	2.048	0.0085
26	AAA62175.1	HSPB1	Heat shock protein family b (small) member 1	2.045	0.0432
27	AAC67374.1	BASP1	Brain abundant membrane attached signal protein 1	1.949	0.0132
28	AAA59554.1	MARCKS	Myristoylated alanine-rich C-kinase substrate	1.831	0.0054
29	AAC16892.1	ILK	Integrin-linked protein kinase	1.802	0.0135
30	AAA02950.1	HLA-B	Major histocompatibility complex, class I, B	1.782	0.0443
31	AAA58453.1	EIF5A	Eukaryotic translation initiation factor 5A-1	1.744	0.0058
32	AAA51567.1	ACTB	Actin cytoplasmic 1	1.730	0.0383
33	AAA77058.1	CORO1A	Coronin-1A	1.694	0.0090
34	AAH11384.1	ATP5A1	ATP synthase subunit alpha	1.688	0.0124
35	AAB28688.1	RNase 4	Ribonuclease 4	1.628	0.0312
36	AAB36687.1	PTPRJ	Receptor-type tyrosine-protein phosphatase eta	1.597	0.0458
37	AAG41412.1	SH3BGRL3	SH3 domain-binding glutamic acid-rich-like protein 3	1.550	0.0134
38	AAB35096.1	RNASE1	Ribonuclease pancreatic	1.541	0.0344
39	AAA61272.1	VDAC1	Voltage-dependent anion-selective channel protein 1	1.522	0.0480
40	AAA51678.1	ANG	Angiogenin	1.502	0.0327
41	AAC50206.1	GNG11	Guanine nucleotide-binding protein G(I)/G(S)/G(O) subunit gamma-11	1.472	0.0399
42	AAA35635.1	CALM1	Calmodulin-1	1.448	0.0255
43	AAA58448.1	GRB2	Growth factor receptor-bound protein 2	1.422	0.0096
44	AAC31959.1	TUBA1B	Tubulin alpha-1B chain	1.401	0.0124
45	AAA61283.1	VCL	Vinculin	1.399	0.0199
46	AAH04186.1	GNB1	Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-1	1.392	0.0063
47	AAC04572.1	SLC9A3R1	Na(+)/H(+) exchange regulatory cofactor	1.336	0.0104

48	AAA59539.1	ARHGDIB	Rho GDP-dissociation inhibitor 2	1.096	0.0033
49	AAA51582.1	ACTN1	Alpha-actinin-1	1.036	0.0478

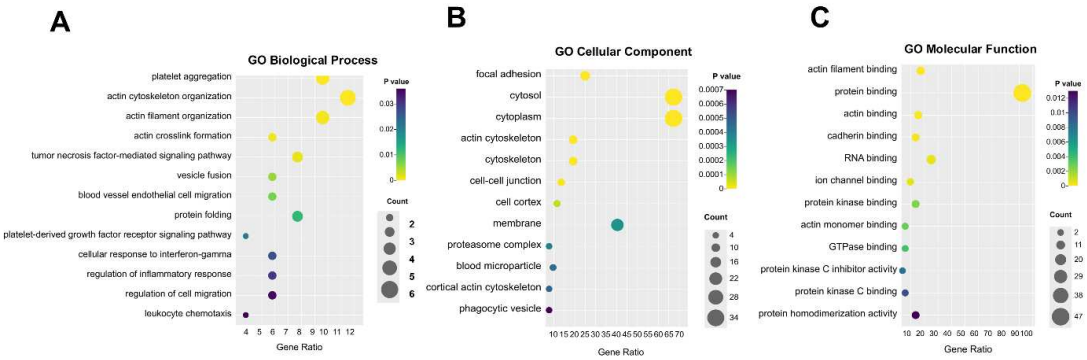
NCBI, National Center of Biotechnology Information; ICH, intracerebral hemorrhage; CTRL, control.

Supplementary Table 4. Identification of the downregulated proteins in the intracerebral hemorrhage extracellular vesicles group vs. the control group.

No.	NCBI No.	Gene name	Protein name	log2 (Fold change) (ICH/CTRL)	P value
1	AAF89993.1	ABCB9	ATP-binding cassette sub-family B member 9	-2.573	0.024
2	AAA50465.1	PRDX2	Peroxiredoxin-2	-2.409	0.028
3	AAH69670.1	FETUB	Fetuin-B	-2.341	0.020
4	AAH50456.1	THBS4	Thrombospondin 2/3/4/5	-2.212	0.018
5	AEE60974.1	——	Testicular tissue protein Li 61	-2.133	0.035
6	AAI00921.1	FGF14	Fibroblast growth factor 14	-2.065	0.032
7	AAC41775.1	ST8SIA4	Alpha-n-acetyl-neuraminate alpha-2,8-sialyltransferase	-1.712	0.028
8	AAB17099.1	FBLN1	Fibulin-1	-1.553	0.011
9	AAA51683.1	AHSG	Alpha-2-HS-glycoprotein	-1.438	0.015
10	AAH26033.1	GSN	Gelsolin	-1.143	0.013
11	AAF86334.1	KIF4A	Chromosome-associated kinesin	-1.120	0.047
12	AAA36506.1	C4BPA	C4b-binding protein alpha chain	-1.089	0.037

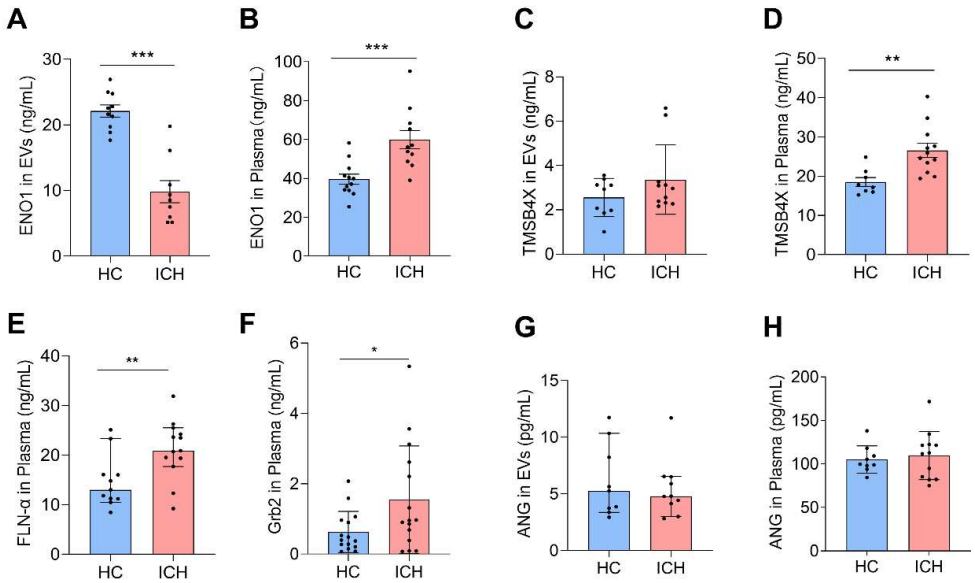
NCBI, National Center of Biotechnology Information; ICH, intracerebral hemorrhage; CTRL, control.

Supplementary Figure 1.



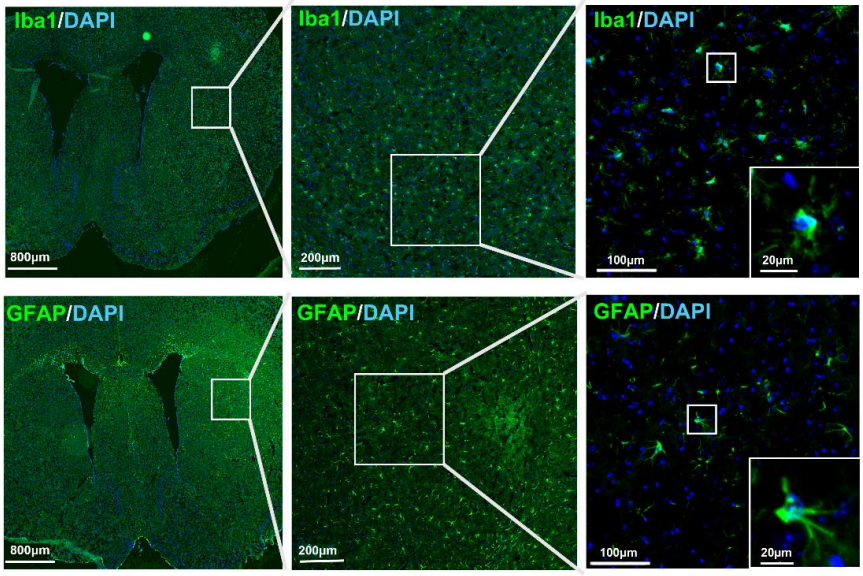
Supplementary Figure 1. Gene Ontology (GO) analysis results of upregulated proteins in intracerebral hemorrhage extracellular vesicles. (A-C) Top enriched proteins identified through a GO analysis between intracerebral hemorrhage patients and healthy controls. The x axis represents the gene ratio, while the size of each circle corresponds to the number for each function. The color indicates the P-value for each function. The analysis covers biological process (A), cellular components (B), and molecular functions (C) of the upregulated proteins.

Supplementary Figure 2



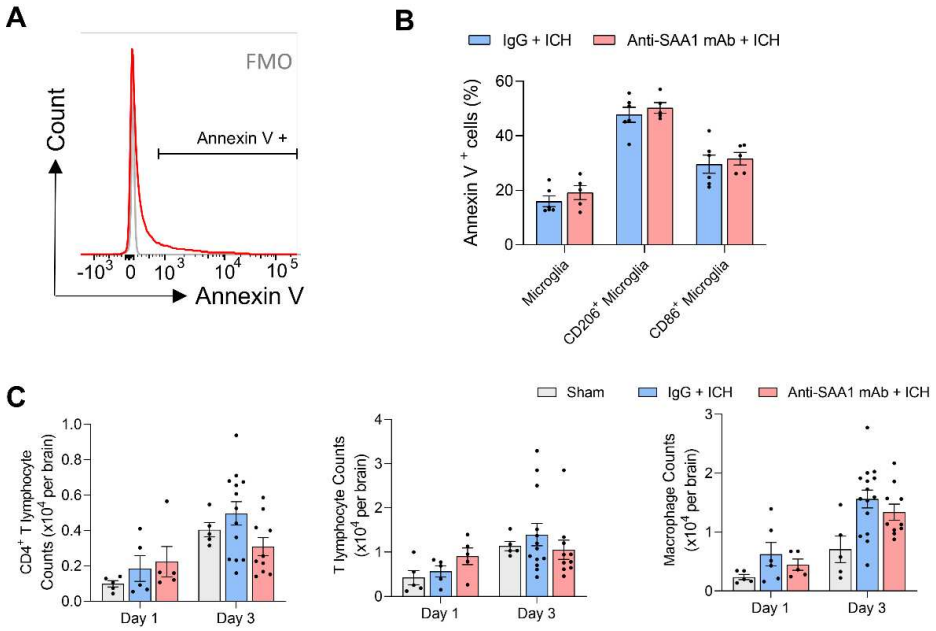
Supplementary Figure 2. Verification of other proteins in the plasma and extracellular vesicles of intracerebral hemorrhage patients as compared to the control group using ELISA. (A,B) Concentration of alpha-enolase (ENO1) in extracellular vesicles (n=10, 9 in the control group and the ICH group, respectively) and in plasma (n=12, 11 in the control group and the ICH group, respectively). Unit: ng/mL. (C,D) Concentration of thymosin beta 4 (TMSB4X) in both extracellular vesicles (n=9 in the control group and n=11 in the patients' group, respectively) and in plasma (n=8 in the control group and n=12 in the patients' group, respectively). Unit: ng/mL. (E) Concentration of filamin-α (FLN-α) in plasma (n=11 in the control group, n=13 in the ICH group, respectively). (F) Concentration of growth factor receptor-bound protein-2 (Grb2) in plasma (n=15 in the control group, n=15 in the patients' group, respectively). Unit: ng/mL. (G,H) Concentration of angiogenin (ANG) in both extracellular vesicles (n=9 in the control group and n=10 in the patients' group, respectively) and in plasma (n=9 in the control group and n=12 in patients' group, respectively). Units: pg/mL. *P < 0.05, **P < 0.01, ***P < 0.001. All data were analyzed by two-tailed unpaired Student's t-test and presented as means ± SEM.

Supplementary Figure 3.



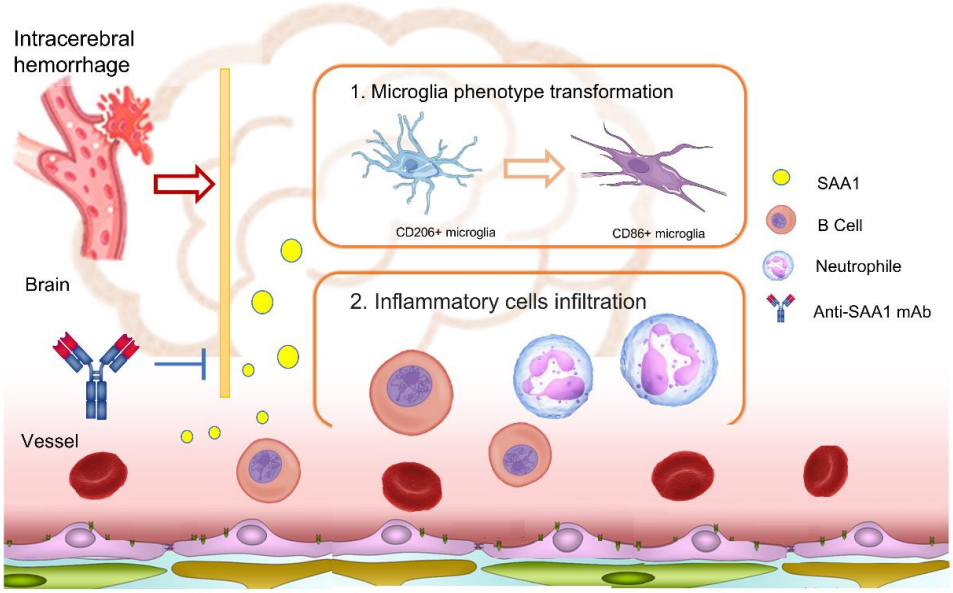
Supplementary Figure 3. Images of immunostaining of microglia (Iba1$^+$) and astrocytes (GFAP$^+$) in different sizes around the ipsilateral basal ganglia region of injection of 250 ng exogenous SAA1. Scale bars = 800 μm, 200 μm, 100 μm, and the inset scale bars = 20 μm separately.

Supplementary Figure 4.



Supplementary Figure 4. (A) Gating strategy of Annexin V in microglia by flow cytometry analysis. The FMO of annexin V was shown by the grew line. (B) Bar graph indicating the percentage of Annexin V-positive microglia and its subtypes with anti-SAA1 mAb or vehicle treatment. n= 6, 5 for IgG and mAb group. (C) Bar graph indicating additional cell quantification statistics, including the total number of leukocytes. This includes macrophages, as well as T cells, and their subtypes, including the $CD4^+$ phenotype. n= 5, 6, 5 on day 1 and n = 5, 13, 10 on day 3 for sham, IgG and mAb group. All data are expressed as means ± SEM, and analyzed by one-way ANOVA.

Supplementary Figure 5.



Supplementary Figure 5. Schematic diagram depicting the role of SAA1 in aggravating intracerebral hemorrhage injury by regulating the inflammatory response. During the acute phase of intracerebral hemorrhage, the SAA1 protein is synthesized and enters the bloodstream, subsequently crossing the compromised brain-blood barrier. It inhibits the transition of $CD86^+$ microglia to the $CD206^+$ phenotype and facilitates the migration of B lymphocytes and neutrophils into the brain. Treatment with anti-SAA1 mAb could attenuate this process, potentially reducing brain injury in intracerebral hemorrhage mice.

Reference:

1 NIH Stroke Scale | National Institute of Neurological Disorders and Stroke. https://www.ninds.nih.gov/health-information/stroke/assess-and-treat/nih-stroke-scale (accessed 14 August 2024)

2 Alam JJ, Krakovsky M, Germann U, *et al.* Modified Neurological Severity Score (mNSS) tests and scoring values. Published Online First: 4 December 2020. doi: 10.1371/journal.pone.0233073.s001