Single-cell RNA sequencing of peripheral blood reveals that monocytes with high cathepsin S expression aggravate cerebral ischemia–reperfusion injury

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\textbf{ABSTRACT}

**Background:** Stroke is a major cause of morbidity and mortality worldwide. After cerebral ischemia, peripheral immune cells infiltrate the brain and elicit an inflammatory response. However, it is not clear when and how these peripheral immune cells affect the central inflammatory response, and whether interventions that target these processes can alleviate ischemia–reperfusion (I/R) injury.

**Methods:** Single-cell transcriptomic sequencing and bioinformatics analysis were performed on peripheral blood of mice at different times after I/R to analyze the key molecule of cell subsets. Then, the expression pattern of this molecule was determined through various biological experiments, including quantitative RT-PCR, western blot, ELISA, and in situ hybridization. Next, the function of this molecule was assessed using knockout mice and the corresponding inhibitor.

**Results:** Single-cell transcriptomic sequencing revealed that peripheral monocyte subpopulations increased significantly after I/R. Cathepsin S (Ctss) was identified as a key molecule regulating monocyte activation by pseudotime trajectory analysis and gene function analysis. Next, Cathepsin S was confirmed to be expressed in monocytes with the highest expression level 3 days after I/R. Infarct size (p < 0.05), neurological function scores (<0.05), and apoptosis and vascular leakage rates were significantly reduced after Ctss knockout. In addition, Ctss destroyed the blood–brain barrier (BBB) by binding to junctional adhesion molecule (JAM) family proteins to cause their degradation.

**Conclusions:** Cathepsin S inhibition attenuated cerebral I/R injury; therefore, cathepsin S can be used as a novel target for drug intervention after stroke.

1. Introduction

Stroke is a major cause of morbidity and mortality worldwide (Campbell et al., 2019). After a stroke, the innate immune system is rapidly activated to recognize a wide variety of molecular complexes that are perceived as foreign and potentially damaging (i.e., danger-associated molecular patterns DAMPs) (Matzinger, 2007). As resident cells in the brain, microglia are activated when they encounter DAMP signals and undergo morphological changes and secrete a variety of inflammatory factors (Bune et al., 2010; Burnstock, 2008). Moreover, peripheral immune cells can be detected in the brain within a few hours after the stroke onset. Neutrophils are the first cells to infiltrate into the central nervous system and promote the development of inflammation in the lesion by releasing a variety of cytokines. Subsequently, lymphocytes also infiltrate into the brain, which act with neutrophils to aggravate intracerebral inflammation in the acute phase after stroke.

\textbf{Abbreviations:} tMCAO, Intraluminal middle cerebral artery occlusion; BBB, Blood–brain barrier; S1PR, Sphingosine-1 phosphate receptor; CBF, Cerebral blood flow; CTSS/Ctss/CTSS, Cathepsin S; KO, Knockout; JAM, Junctional adhesion molecule; I/R, Ischemia–reperfusion; PBMCs, Peripheral blood mononuclear cells; IL-6, Interleukin-6; MCA, Middle cerebral artery; WT, Wildtype; TTC, 2,3,5-Triphenyltetrazoliumchloride; TNF-α, Tumor necrosis factor α; IL-1β, Interleukin-1β.

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lymphoid organs and reduces brain infiltration in an experimental model of acute ischemic stroke (Muhammad et al., 2008). In this study, we collected mouse peripheral blood samples at different time points after stroke for single-cell sequencing to reveal the dynamic changes in peripheral immune cells. The results showed that the proportion of monocyte subpopulations involved in the protease cleavage reaction significantly increased after stroke. In particular, cathepsin S, a representative molecule of this cell subgroup, was highly expressed after stroke. Next, we used a CTSS inhibitor and knockout (KO) mouse experiments to prove that inhibiting cathepsin S expression can significantly reduce infarct volume and blood–brain barrier (BBB) leakage, suggesting that cathepsin S can be used as a novel target for drug intervention after stroke.

2. Materials and methods

2.1. Animals

C57BL/6N mice (20–23 g, 6–8 weeks old) were provided by Army Medical University. C57BL/6N mice offspring (8 weeks old) of breeding pairs of C57BL/6N background were previously generated by Cyagen Biosciences Inc. (Suzhou, China). The mice were raised in a clean environment and light-controlled room (12-h light–dark cycle from 8 a.m. to 8 p.m.) with temperature of 25 ± 2 °C, 50 ± 10% humidity, and free access to food and water. The animal experiments were performed in accordance with the guidelines of the National Institutes of Health on the Care and Use of Animals and the Animal Management Committee of the Third Military Medical University (Chongqing, China).

2.2. Transient focal cerebral ischemia

Transient focal cerebral ischemia in mice was induced by intraluminal occlusion of the left middle cerebral artery (tMCAO), as described previously (Deng et al., 2019). Briefly, mice were numbered and operated on by different members of the research team. The animals were divided into an experimental group comprising littermate male mice and a control group, according to a completely randomized design (CRD) protocol. The mice were starved overnight and anesthetized with 2.5% isoflurane. Body temperature was maintained at 37 ± 0.5 °C with heating pads until the animals had recovered from surgery. A 2-cm-long rounded-tip nylon suture (Jialing [1800 model], Shanghai, China) was inserted into the internal carotid artery, approximately 8–9 mm distal to the suture until a mild resistance was encountered, and it was then advanced to block the left middle cerebral artery. After 90 min of tMCAO, the MCA-supplied region was reperfused by retracting the intraluminal suture. Blood flow occlusion was performed on all mice using a blood flow meter. We only included mice with residual cerebral blood flow (CBF) < 15% throughout ischemia. Except for arterial occlusion, the sham-operated mice underwent the identical surgical procedure, as described previously. The rectal temperature was generally maintained at 37.0 ± 0.5 °C during the operation until the animals woke after surgery. Experimental mice with similar clinical scores were randomly subdivided into two groups. One group (inhibitor group) received the CTSS inhibitor LY3000328 (diluted with corn oil, 30 mg/kg) (HY-15533, MedChemExpress, Monmouth Junction, NJ, USA) via intraperitoneal injection after ischemia–reperfusion (I/R), as described previously (Jadhav et al., 2014). The other group (control group) simultaneously received an intraperitoneal injection of corn oil. Then, the mice were allowed to recover for different periods of time until being subjected to further tests. After the operation, the mice were placed in an incubator at 37 °C, provided jelly and feed, and they were monitored regularly (Shichita et al., 2017).

2.3. Cerebral blood flow (CBF) measurements

Cortical CBF was monitored using RWD Laser Speckle Imaging System (PFLSI Pro+, RWD Instruments, Shenzhen, China). Briefly, a charge-coupled device camera was placed above the anesthetized mouse head and the intact skull surface was illuminated by a laser diode (785 nm) to allow the penetration to continue throughout the brain in a diffuse manner. Both cerebral hemispheres were measured and recorded 15 min before tMCAO and throughout the ischemic period until 15 min after reperfusion. Speckle contrast, defined as the ratio of the standard deviation of pixel intensity to mean pixel intensity, represents the speckle visibility relative to the velocity of the light-scattering particles (blood) and was used to measure cortical blood flow (Shi et al., 2016). Animals that did not show a reduction in CBF of at least 75% from the baseline level and those that died immediately after I/R induction (<10%) were excluded from further experiments. The areas of the ischemic core (0–20% of residual CBF) and penumbra (20–30% of residual CBF) were measured using laser speckle images.

3. Blood collection from the mouse Orbital Sinus

We collect blood from the orbital venous plexus of mice (Teilmann et al., 2014), the detailed steps are as follows: First, all pieces of surgical equipment, including animal, anesthetic agent, cotton, capillary tube, 1.5-ml centrifuge tubes, were sterilized and then coated with EDTA. Then, the mice were anesthetized via isoflurane inhalation. After that, the beard and hair around the eyes of each mouse were cut off with scissors by grabbing its tail and the skin on its neck with one hand. Next, a capillary is inserted into the medial canthus of the eye (30° angle to the nose). Slight thumb pressure to puncture the tissue and enter the plexus. 300µl volume of blood is collected from single Mouse’s plexus, the capillary tube is gently removed and wiped with sterile cotton. Bleeding can be stopped by applying gentle finger pressure. Thirty minutes after blood collection, animal is checked for postoperative and periorbital lesions. Blood was collected in an ordinary centrifuge tube and centrifuged at 3000 rpm for 10 min after having been kept at room temperature for 2 h to absorb the supernatant. Moreover, blood was collected in...
an EDTA centrifuge tube, which was then placed on ice and separated for PBMCs.

3.1. Single-cell RNA-sequencing

Blood was obtained from the mice 1, 3, and 7 days after I/R and the blood samples used for single-cell sequencing were from 3 different mice in each group. Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll-Paque PLUS (GE healthcare, Madison, WI, USA) density gradient centrifugation. Cells were resuspended in RPMI media (GIBCO, Boston, MA, USA) to obtain a single-cell suspension with high cell viability. Next, cells were stained with a live/death dye (DAPI) and dead cells were removed using fluorescence-activated cell sorting (FACS) (Cano-Gamez et al., 2020). Live cells were resuspended in PBS buffer and recounted using AO/PI double staining kit to ensure cell viability. Finally, cell suspensions were processed for single-cell RNA-sequencing using the 10 × Genomics 3′ v2 kit, as specified by the manufacturer’s instructions (Zheng et al., 2017). About 1 × 10^6 cells from each condition were loaded on to separate inlets of a 10 × Genomics Chromium controller to create GEM emulsions. The targeted recovery was 6,000 cells per condition. Emulsions were used to perform reverse transcription, cDNA amplification, and preparation of the RNA-sequencing library. Libraries were sequenced on an Illumina HiSeq 4000 platform, using 75 bp paired-end reads and loading one sample per sequencing lane.

3.2. Single-cell RNA-seq data analysis

Single-cell RNA-sequencing data were processed using the Cell Ranger Single-Cell Software Suite (version 3.0.2, 10 × -Genomics) (Zheng et al., 2017). Each sample was aligned to the mouse reference genome (mm10) using Cell ranger, and raw expression data were analyzed by Genome (version 3.5.1; R Foundation for Statistical Computing, Vienna, Austria). Then, cells of all the datasets were analyzed for their unique molecular identifier (UMI) and mitochondrial gene counts; cells with low (<300) or high (>2500) UMI counts or high percentage of mitochondrial genes (>4%) were excluded from further analysis. Data were integrated in a standardized workflow, as recommended by the developers of the “Seurat” package (Stuart et al., 2019), including data normalization, identification of variable genes, finding anchors for integration based on variable genes, integration of all of the datasets, scaling of data, principle component analysis (PCA), and unsupervised clustering with a resolution of 0.7 based on Uniform Manifold Approximation and Projection (UMAP). Cell types were identified based on a marker gene panel and differentially expressed genes (DEGs) in every cluster. DEGs were calculated by Wilcoxon rank sum test with Bonferroni correction for adjusted p-values. As recommended by the “Seurat” developers, data in feature plots, violin plots, heat maps, and trajectories demonstrating features that vary across conditions were displayed based on the “RNA”-count slot, and data reflecting the entire dataset were displayed based on the “integrated” dataset. Gene ontology (GO) networks based on DEGs were created using the Functional Annotation Bioinformatics Microarray Analysis (DAVID) (Huang da et al., 2009).

3.3. Pseudotime analysis

Cells were ordered into a branched pseudotime trajectory using Monocle 3 and the analysis was restricted to the highly variable genes identified by Seurat. Monocle was used to test for a significant correlation between gene expression and pseudotime in each trajectory (Qiu et al., 2017). A gene was defined as significantly associated with pseudotime if its estimated q value was <0.01.

3.4. Neurological deficit scores

Neurological deficits after 3 days of reperfusion were evaluated on a 5-point scoring system using the Longa score test, as described previously (Tao et al., 2019), and the evaluation was performed by investigator who were blinded to the assigned experimental group. The scoring standards were as follows: 0: no neurological deficit; 1: failure to fully extend the right forepaw; 2: leaning toward the right; 3: falling to the right; 4: unable to walk independently; and 5: dead. The examination was performed in a blinded manner.

3.5. Pole climbing test

Pole tests were performed to detect the motor function of the mice 1, 3, 7, and 14 days after stroke (Haesebaert et al., 2018). In brief, each mouse was placed head down atop a vertical pole (diameter: 2.5 cm; height: 60 cm) to record the time it took them to turn around and climb to the bottom.

3.6. Rotarod

The rotarod test was used to evaluate the motor function of the mice 1, 3, 7, and 14 days after stroke (Liberalet al., 2018). Briefly, the mice were placed on an accelerating rotarod (4–40 rpm, >300 s), and the latency to fall from the rotarod was recorded, with a 10-min interval for the next time. All animals were trained three times prior to stroke.

3.7. TTC staining for infarct volume ratio

After 3 days of tMCAO, mice were sacrificed under deep anesthesia with pentobarbital sodium. The mice brains were dissected, sliced into seven 1-mm-thick coronal sections with a matrix, and stained with 2 % TTC (Sangon Biotech, Shanghai, China) at 37 °C for 15 min. The stained slices were transferred to 4 % paraformaldehyde in PBS for post fixation. Images were captured using a digital camera (Canon). White or pale pink areas were labelled as infarctions and measured using image analysis software (Adobe Photoshop CC2015). To exclude the influence of cerebral edema induced by ischemia on the infarct size, the percentage of infarct volume was calculated using a corrected algorithm: (total contralateral hemispheric volume – total ipsilateral hemispheric normal stained volume) / total contralateral hemispheric volume × 100 %, as described previously (Zeng et al., 2019).

3.7.1. ELISA

The CTSS Level in peripheral blood was measured using a Mouse CTSS Quantikine ELISA Kit (CUSABIO, Wuhan, China) according to the manufacturer’s instructions. Briefly, the plasma was collected using EDTA or heparin as an anticoagulant. The plasma was centrifuged for 15 min at 1000 × g and 2–8 °C within 30 min of collection. The assay was performed immediately or stored as aliquots at −20 °C or −80 °C. Repeated freezing and thawing was avoided. The samples were centrifuged again after thawing before the assay. The mouse plasma samples were diluted to 1:100. The Assay Layout Sheet was used to determine the number of wells to be used and the remaining wells and desiccant were placed in a pouch, sealed with Ziploc, and stored at 4 °C. Then, 100 μl of Biotin antibody (1 ×) was added to each well. The wells were covered with a new adhesive strip and incubated for 1 h at 37 °C. Then, 100 μl of HRP-avidin (1 ×) was added to each well. The microtiter plate was covered with a new adhesive strip and incubated for 1 h at 37 °C. Then, 90 μl of TMB Substrate was added to each well and the wells were incubated for 15–30 min at 37 °C, while protecting from light. Subsequently, 50 μl of Stop Solution was added to each well and the plate was capped to ensure thorough mixing. Finally, the optical density of each well was determined within 5 min using a microplate reader set to 450 nm using Flash Varioskan software. Inflammatory factors of IL-1, IL-6 and TNF in brain tissues are detected also by Mouse CTSS Quantikine ELISA Kit (CUSABIO, Wuhan, China) according to the manufacturer’s instructions.
3.8. Quantitative real-time PCR

The total RNA was extracted from mouse brain tissues using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The total RNA quantity and purity were analyzed using ThermoScientific ND2000 (Thermo Fisher Scientific, Waltham, MA, USA), and RNA (1 μg) was reverse transcribed into DNA and quantified by SYBR RT-PCR according to the manufacturer’s instructions (Takara, Shiga, Japan). Briefly, the PCR was run at 95 °C for 10 s, 61 °C for 30 s, and 72 °C for 15 s, for a total of 44 times. Then, the PCR was continued at 4 °C for an unlimited time, using the following primers (the housekeeping gene Gapdh was used as the control gene):

Ctss-forward: 5’-GAAGAAATCTTGTTGCGATGG-3’; reverse:5’-CACAGAGACCTCCTGTATTTTCAC-3’;
Gapdh-forward: 5’-GATGTTGCGATGGTGATGCG-3’; reverse: 5’-GTGGTGCAAAGATGCTGTGA-3’;

3.9. Western blot (WB) analysis

For the in vivo analysis, WB analysis was performed on proteins obtained from the ipsilateral cortex after tMCAO or the perihematomal area. The detailed protocol was described in our previous article (Meng et al., 2017). In brief, fresh tissue on the infarcted side was extracted and the proteins were separated using protein lysate with EDTA-free protease inhibitor (Roche Diagnostics, Mannheim, Germany). Electrophoresis and membrane transfer were performed using 12 % SDS-PAGE and polyvinylidene fluoride membranes. We used 5 % BSA-blocked membranes at room temperature for 2 h and then incubated the membranes with primary antibodies against the following targets overnight at 4 °C: β-tubulin (1:1000, Abcam, Cambridge, MA, USA) and CTSS (1:500, Abcam). The membranes were incubated with HRP-conjugated goat anti-mouse secondary antibodies and goat anti-rabbit secondary antibodies for 1 h at room temperature. Finally, we used the enhanced chemiluminescence (ECL) substrate method to visualize the proteins at the membranes and ImageJ software to evaluate the grayscale values. The WB-related reagents were obtained from Beyotime Biotechnology (Shanghai, China).

3.10. Immunofluorescence staining

As described in detail in our previous article (Xiong et al., 2016), mouse brain samples were fixed with PBS and 4 % paraformaldehyde, and cut into 30-μm slices. After incubation in 4 % paraformaldehyde and 30 % sucrose, the brain slices were fixed and dehydrated. The slices were blocked with 5 % goat serum supplemented with 0.3 % Triton-100 at room temperature for 1 h and placed in solutions of the primary antibodies overnight at 4 °C: CD31 (1/100, Abcam, Cambridge, MA, USA) and Neu-N (1/100, Abcam). Then, the slices were incubated with the corresponding fluorescent secondary antibodies: Alexa Fluor 488 (1/500, donkey anti-mouse), Alexa Fluor 647 (1:500, donkey anti-rabbit), Alexa Fluor 555 (1:500, donkey anti-rat), and Alexa Fluor 555 (1:500, donkey anti-goat), which were purchased from Invitrogen (Carlsbad, CA, USA). Mouse brain slices from the right cortex (designated as penumbra) 2 mm away from the midline were observed with the naked eye (the infarcted area appeared white). Then, DAPI (infected nuclear fragmentation) was observed at low magnification and the infarcted area was identified and inspected at high magnification. The mouse infarct area was used for further experiments. Then, the sections were immersed in 6-diamidino-2-phenylindole (DAPI, 1:5000) for 15 min at room temperature. Finally, we used a confocal fluorescence microscope (Leica Sp5, Leica Microsystems, Mannheim, Germany) to capture the images, and positive cells were quantified using ImageJ software in three random fields. Images were analyzed by a person blinded to the grouping.

3.11. Fluorescence in situ hybridization (FISH)

The brains were harvested from post-stroke animals and control animals, fixed with PBS and 4 % paraformaldehyde, dehydrated in increasing gradients of ethanol, and baked at 60 °C for 1 h. The sections were treated with protease for 8 min and washed. Following the washes, the sections were hybridized with commercial oligo probes (mouse gene-specific probes for Cts and C1q), according to the manufacturer’s instructions for RNAscope® detection (Advanced Cell Diagnostics, Newark, CA, USA). Confocal microscopy (Leica Sp5) and ImageJ software were used for image acquisition and analysis.

3.12. TUNEL staining

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay was used to demonstrate the apoptotic cells. The apoptotic cells were demonstrated using the In Situ Cell Death Detection Kit. Fluorescein staining (Roche Diagnostics, 5421700) of brain tissues was performed following the manufacturer’s protocol. We randomly selected three visual fields of the infarct area for detection and calculated the average value for statistical analysis.

3.13. Coinmunoprecipitation

Fresh wild-type (WT) or tMCAO mice brain were harvested in ice-cold lysis buffer containing 50 mM Tris-HCl, 150 mM NaCl, 1 % NP-40, 2 mM EDTA, 1 mM Na-orthovanadate (pH 7.4), and a protease inhibitor mixture. After centrifugation at 13,000×g for 10 min at 4 °C, debris was discarded and the protein concentrations in the extracts were determined using the bicinchoninic acid (BCA) assay. The extracts (500 μg of protein) were incubated with nonspecific IgG (5 μg) or anti-JAM-A antibody (5 μg; Abcam) overnight at 4 °C, followed by the addition of 40 µl of protein G-Sepharose for 4 h at 4 °C. Then, the solution was washed and washed four times with a lysis buffer, denatured with SDS sample buffer, and separated by 4–12 % SDS-PAGE. Subsequently, SDS-PAGE and immunoblotting were performed as described previously. The lanes marked input were loaded with 1 % of the starting material used for immunoprecipitation, in accordance with the manufacturer’s recommendations (Thermo Fisher Scientific, 88288).

3.14. Vascular permeability quantitation

Cerebral blood vessels were visualized using dextran-conjugated fluorophores to estimate the BBB disruption caused by systemic inflammation. We injected fluorescence-tagged dextrans into the tail vein before imaging. Dextran beads have three different sizes: 10 kDa dextran-Texas Red, 40 kDa dextran-tetramethylrhodamine (TMR), and 70 kDa dextran-fluorescein (all at concentrations of 2 mg/ml; Sigma-Aldrich, St. Louis, MO, USA; 60842-46-8). None of these dextrans cross an intact BBB so the presence of fluorescence in the brain parenchyma in response to a dextran injection indicates BBB disruption, as described in detail in our previous study (Ozen et al., 2018). Briefly, after 3 days of reperfusion, Alexa 488-fluorescein thiocarbamoyl dextran (Sigma, Carlsbad, CA, USA) was injected into WT or CTSS KO mice through the tail vein. After 10 min, brain tissues were removed, sliced into 10-μm sections, and stained with fluorescent antibodies against CD31 (1:100, Santa Cruz Biotechnology, Dallas, TX, USA) to visualize the mouse vascular morphology. Slices were imaged using a Leica confocal fluorescence microscope (Leica Sp5), as described previously. Three random fields of infarction were captured to calculate the average value for statistics.

3.15. In vitro cleavage of recombinant protein

We obtained recombinant proteins from R&D Systems. Inactive CTSS was activated at 37 °C in 50 mM sodium acetate, 0.25 M NaCl (pH 4.5,
7.2, or 9.8), and 5 mM DTT for 1.5 h. Then, activated CTSS was incubated with recombinant JAM-A (50463-M02H, R&D), JAM-B (50464-M02H, R&D), and JAM-C (50465-M08H, R&D) in the presence or absence of the inhibitor (9 μM, pH: 7.2), LY3000328 (HY-15533, MCE), for 20 min. The reaction was stopped by adding SDS and boiling at 95–100 °C for 7 min. Then, the solution was mixed and washed four times with lysis buffer, denatured with SDS sample buffer, and separated using 4–12 % SDS-PAGE. We used Quick Silver Staining Kit from Beyotime (P0017S) to visualize the protein bands on SDS-PAGE. The assays were described in detail previously (Sevichet et al., 2014). Briefly, after the electrophoresis, the gel was placed in 100 ml of fixing solution and shaken on a shaker at room temperature for 2 h at a shaking speed of 60–70 rpm. Then, 100 ml of 30 % ethanol was added and shaken again at room temperature for 10 min at a shaking speed of 60–70 rpm; 100 ml of silver-stained color developing solution was added and shaken for 3–10 min at room temperature and shaking speed of 60–70 rpm, until the expected protein band appeared. Finally, 100 ml of silver staining stop solution (1X) was added to the shaker and the solution was shaken at room temperature for 10 min at a shaking speed of 60–70 rpm. Images were captured using a digital camera (Canon).

3.16. Isolation of cells and flow cytometry

The brain tissues of the control mice and I-R mice obtained after 3 days of I-R were separated and incubated with papain and DNase for 30 min at 37 °C. Horse serum was used to terminate the digestion, and the tissue was pipetted into a single-cell suspension. Then, the myelin sheath and tissue debris were removed using a solution (3 ml of DMEM, 1 ml of Percoll, and 4 ml of PBS), and the red blood cells were lysed. The obtained single cells were blocked with 10 % FBS at room temperature and the corresponding primary antibodies (anti-CD45) were added for 10 min at room temperature. After washing three times, DAPI was incubated for 5 min at room temperature and the samples were washed and analyzed using flow cytometry (BD, GOLI IOS).

3.17. Statistical analysis

All our animal experiments were carried out in a blinding procedure. In the stroke experiment, all of the mice were male. The experimental group consisted of age-matched animals that were randomly assigned to the experimental groups so that equal numbers from of mice from the original cages were randomly allocated to the different experimental groups. Blinding was performed by coding the animals and keeping the code and the corresponding experimental condition blinded from the rater who performed the testing and analyses (Shichita et al., 2017). The sample size required for the animal study was empirically determined based on the results of previous experiments and was similar to the size generally used in similar studies.

Data are presented as mean ± standard error of mean (SEM); *p < 0.05, **p < 0.01, and ***p < 0.001 were considered to indicate a significant difference. P values, the statistical analyses performed, and sample sizes are described in the figure legends. Statistical analyses were performed using GraphPad Prism 9 Software (GraphPad Software Inc., San Diego, CA, USA). Shapiro-Wilk tests were used to assess the normality of data distribution in each group and Levene’s tests were used to test for homogeneity of variance.

One- or two-way ANOVA and Bonferroni’s post hoc tests were used to compare three or more groups. Statistical significance of the data presented in Fig. 4A–E, S3, SBSC, SF5G, 6A-E was computed using one- or two-way ANOVA.

Significance was assessed using Student’s t-test (two-tailed) for comparisons of two groups. Statistical significance for data presented in Fig. 5A, 5D, 5E, 8A-C were calculated using Student’s t-test.

4. Results

4.1. Single-cell transcriptome profiling of peripheral blood immune cells in mice

In general, researchers refer to days 1–3 after the onset of stroke as the acute phase, days 3–7 as the subacute phase, and the period after 2 weeks as the chronic phase. Immune cell subsets change significantly during these different time periods. To explore the dynamic changes in peripheral blood immune cells in mice after stroke and its relationship with ischemia, we collected fresh peripheral blood samples from mice with tMCAO at day 1 (acute phase), day 7 (subacute phase), and day 14 (chronic phase), and the control mice were used for single-cell sequencing (Fig. 1A). After filtering out cells with low quality, we obtained transcriptome datasets from 36,905 cells with an average of 9,000 cells for each sample and time point. The cell clusters were annotated with expression of canonical marker genes. Major cell types comprising PBMCs were well captured by scRNA-seq, including CD4+ T cells (CD4), CD8+ T cells (CD8), Treg (FOXP3), B cells (CD19), monocytes (CD68), natural killer (NK) cells (KLRB1), and proliferative cells (MKI67) (Fig. 1B). We found that, compared to controls, tMCAO-treated mice showed an increased proportion of monocytes and decreased proportion of B cells. Other types of peripheral blood cells (proliferative cells, CD8+ T cells, and Treg cells) accounted for <10 % of the total cells; therefore, their dynamic changes could not be clarified in this experiment (Fig. 1C).

In addition, we found 28 clusters representing different cell types using t-distributed stochastic neighbor embedding (t-SNE) analysis (Fig. 1D). The top 10 featured genes of each cluster were displayed in the heat map (Fig. S1 and Table S1). We calculated the proportion of each cell subgroup at different I/R time points (Fig. 1E). Then, we divided the subgroups into four categories. Group 1 represents a subpopulation with a continuous increase in the proportion of cells from the acute phase (day 1) to the chronic phase (day 14), while group 2 represents a subpopulation with a decreasing proportion of cells during the above process. Group 3 represents a subset of cells that increased in the acute phase (day 1) and decreased in the subacute (day 7) and chronic (day 14) phases, suggesting that this group of cells mainly regulates the inflammatory process in the acute phase after I/R. In contrast, group 4 comprises a subpopulation of cells that decreased in the acute phase and increased in the subacute and chronic phases, suggesting that this group of cells is mainly involved in the inflammatory process in the chronic phase after I/R. Compared with the other groups, group 1 represents a subset of cells with increased expression throughout the acute, subacute, and chronic phases after I/R, indicating that these cell subsets (clusters 2, 9, 10, 18, 24, 26) play a key role in the entire inflammatory response after I/R. Moreover, as clusters 24 and 26 contained too few cells for further bioinformatic analysis, we excluded them from subsequent analyses (Fig. 1F).

4.2. Pseudotime analysis reveals the dynamic changes in cell subpopulations

To understand the hypothetical developmental relationships that might exist within the monocyte and macrophage clusters, we performed trajectory analysis on clusters 2, 9, 10, and 18 using the Monocle algorithm. Two branch points were determined based on changes in monocyte gene expression, which was plotted in pseudotime. Clusters were superimposed on the pseudotime plot, which revealed that clusters 18 and 2 were located toward the beginning, while cluster 9 was located on the tail of pseudotime (Fig. 2A–C). Interestingly, cluster 10 was located in the entire pseudotime trajectory, suggesting that cluster 10 may play a key role in the differentiation process of monocytes (Fig. 2C).

Therefore, we checked the expression levels of the characteristic genes (including Apoe, Csfir, and Ctsi) in cluster 10 in the pseudotime
We found that the expression levels of these genes were significantly increased (Fig. 2D), suggesting that these may be key molecules involved in the activation of monocytes after I/R. In contrast, the expression of characteristic genes in cluster 2 (including Ccl6, Ccr2, Fn1, and Chil3) gradually decreased with the pseudotime trajectory (Fig. 2D), indicating their limited effect in promoting monocyte activation.
4.3. Gene functional analysis of cell clusters

Based on the previous data (Fig. 2), we found that clusters 2 and 10 may play key roles in regulating monocyte activation. Thus, we performed GO analysis on these subpopulations. Through biological process and molecular function analyses, we found that the biological functions of cluster 2 mainly involve inflammation and toll-like receptor pathways (Fig. 3A). In order to identify the core genes that regulate the biological functions of cluster 2, we merged the data to identify the intersection and found that $S100a8$ and serum amyloid A-3 protein ($Saa3$) were the key regulatory genes in cluster 2 (Fig. 3B). However, we excluded them in the follow-up study because the functions of these two genes in stroke have been widely reported.

Then, we performed gene function analysis on cluster 10. The biological functions of this subgroup were mainly enriched in defense response, inflammatory response, proteoglycan binding, and collagen binding (Fig. 3C). We also identified the core gene that regulates cluster 10 by combining the results of biological process and molecular function analyses (Fig. 3D). We found that $Ctss$ was the only molecule that can regulate inflammation as well as bind with collagen. Since the activation of inflammatory cells and degradation of collagen in the BBB are important pathological features after stroke, we speculated that $Ctss$ may act on these two pathways to affect the stroke outcome.

Furthermore, we analyzed the molecular functions of other clusters and found that each group has different functions (Fig. S2). In group 2, the molecular functions of the main T cell subgroups (including clusters 3, 13, and 14) were concentrated on ribosomal composition and RNA binding, suggesting that these molecules may be involved in transcriptional regulation of T cells. The main gene function enrichment in groups 3 and 4 were related to the binding of the antigen and Major Histocompatibility Complex (MHC), and the activation of chemokines. The genes involved in these functions and their regulatory mechanisms remain to be identified.

4.4. Cathepsin S expression increases after stroke

To verify the expression of $Ctss$ in single-cell sequencing, we detected the expression levels of CTSS in peripheral blood at 0, 1, 3, 7, and 14 days after tMCAO by ELISA. We found that the expression of CTSS increased from day 1 ($p = 0.001$), peaked at day 3 ($p = 0.000$), and then declined to baseline (Fig. 4A). The peripheral immune cells (monocytes, neutrophils, and lymphocytes) infiltrate the brain and aggravate neuroinflammation when the BBB is destroyed. Then, we evaluated the expression of cathepsin S in the central nervous system. Quantitative real-time PCR revealed significantly increased $Ctss$ mRNA in the I/R brain from the days 3 ($p = 0.003$) to 7 ($p = 0.002$), which gradually returned to baseline at day 14. The WB analysis showed that the CTSS protein expression gradually increased at different time periods after I/R (Fig. 4B and C). These results indicated that cathepsin S expression was significantly increased mainly during the acute and subacute phases of I/R.

Our single-cell sequencing results showed that $Ctss$ was mainly derived from peripheral blood monocyte subpopulations, thus we assessed the location of $Ctss$ in the central nervous system using FISH analysis. We found that $Ctss$ was colocalized with C1q (marker of microglia/macrophages) in I/R brains ($p = 0.007$) (Fig. 4D and E), which was consistent with a previous study that reported that cathepsin S displayed tissue-specific distribution and was selectively expressed on antigen-presenting cells, including monocytes/macrophages, microglia, and other cells. (Chen et al., 2021; Clark and Malcangio, 2012).

In addition, we obtained human data from the Gene Expression Omnibus database (GSE16561) and found that CTSS expression was significantly increased in the peripheral blood of stroke patients at 24 h compared to healthy individuals (Fig. 4F). These results confirmed that cathepsin S expression was significantly increased after stroke and may be a potential target for intervention.
4.5. Ablation of cathepsin S ameliorates cerebral ischemic damage

First, we verified the KO efficiency of Ctss-KO mice by quantitative real-time PCR ($p = 0.006$) (Fig. 5A), and we found the Ctss mRNA level was significantly reduced (almost 80%). The expression of CTSS showed a peak around day 3 after I/R (Fig. 4B and 4C), suggesting that day 3 is an important time point to focus on the function of CTSS. Then, we determined the impact of cathepsin S on cerebral I/R injury by...
subjecting male Ctss-KO mice and their WT littermates to 90 min of ischemia followed by reperfusion for 3 days and sham operation. Next, we evaluated changes in cerebral blood flow in WT and Ctss-KO mice after ischemia and found no significant difference in cerebral blood flow between the two groups of mice (Fig. 5B and C). However, the brain infarct volume was smaller in Ctss-KO mice than in WT mice (relative to the contralateral hemisphere, p = 0.013) (Fig. 5D). To further evaluate the effect of cathepsin S on motor function after stroke in mice, we used the Longa score, pole test, and rotarod test, and the neurological function was found to be better in Ctss-KO mice than in WT mice (Fig. 5E–G). Moreover, neuronal apoptosis was reduced in the CTSS knockout group (Fig. S3), further confirming that Cts ablation ameliorates cerebral

Fig. 4. Peripheral and central cathepsin S are increased in mice after tMCAO. C57BL/6N mice were subjected to ischemia for 90 min or sham operation followed by 0, 1, 3, 7, and 14 days of reperfusion. (A) ELISA showed that peripheral CTSS levels were significantly increased after tMCAO (n = 4). (B) Representative immunoblots and quantification of CTSS (relative to β-Tubulin) in the ischemic ipsilateral brain at various time points after tMCAO (n = 4). (C) The mRNA abundance of Cts in the ischemic ipsilateral brain normalized to that of the Gapdh gene (n = 5). (D and E) Representative images and quantitation of in situ hybridization results for Cts and C1q in the sham group 3, 7 days after tMCAO (n = 4, magnification, 100 or 200; scale bar, 100 or 50 μm; yellow triangle indicates C1q + Cts + cells; the dotted line depicts the edge of the infarct). (F) Peripheral CTSS levels 24 h after stroke in humans from the GEO analysis database. Data are presented as mean ± SEM, *p < 0.05; **p < 0.01; ***p < 0.001; n.s. not significant.
ischemic damage.

The molecular function prediction showed that CtsS was involved in the degradation of collagen and glycoproteins (Fig. 3D, E), which serve as the main components of the tight junction and basement membrane of the BBB. Therefore, we speculated that CTSs would increase the leakage of the BBB by degrading the tight junction and the basement membrane. Future, we used FITC-dextran staining to detect the BBB leakage phenotype. The results showed that the vascular leakage was reduced in the CtsS-KO group compared to the WT group (Fig. 6A), thereby confirming that cathepsin S promotes vascular leakage.

Our results showed that CtsS may be related to the activation and function of monocytes (Fig. 2), and vascular leakage can promote infiltration of inflammatory cells (Niu et al., 2019), we found that the immune cell infiltration caused by I/R was significantly reduced, as
evidenced by the reduced inflammatory CD45+ cells in the Ctss-KO mice brain (p = 0.016) by flow cytometry (Fig. 6B). Moreover, we revealed that the inflammatory factors IL-1β (p = 0.001), IL-6 (p = 0.002), and TNF-α (p = 0.001) were significantly reduced in KO animal brains after I/R (Fig. 6C–E), which was consistent with a previous study that reported that CTSS promotes the release of the inflammatory factor IL-1β (Hughes et al., 2016) from macrophages and microglia (Seo et al., 2016). These results showed that Ctss-KO can attenuate neuroinflammation after I/R in mice.

### 4.6. Cathepsin S promotes BBB destruction through junctional protein cleavage

In our previous experiments, we found that cathepsin S inhibition can alleviate stroke damage, but the specific molecular mechanism of cathepsin S is still unclear. Therefore, we used the interacting protein database to predict proteins that might interact with CTSS, including HLA, CD72, and TLR9 (Fig. 7A). Among them, the junctional adhesion molecules (JAMs) are one of the components of tight junction proteins and are critical for maintaining the BBB integrity. We confirmed the interaction between JAM-A and CTSS through co-immunoprecipitation experiments on brain tissue, indicating that JAMs is very likely to be one of the substrates of CTSS. Moreover, this interaction is enhanced after cerebral ischemia, which may be due to the increase in CTSS in the brain after stroke (Fig. 7B). CTSS can also increase tumor brain metastasis by degrading JAMs of the BBB (Sevenich et al., 2014), thus we evaluated whether CTSS may aggravate I/R injury through increasing the mice BBB permeability by degrading JAMs.

CTSS is a lysosomal cysteine protease that exerts proteolysis activity across a wide range of pH in macrophages (Doherty et al., 2019). Therefore, we observed the cleavage efficiency of JAMs by CTSS under different pH conditions during in vitro enzyme cleavage experiments.
with acidic (4.5), neutral (7.2), and alkaline (9.8) pH (Fig. 7C). JAM-A and JAM-B can be cleaved into small fragments of approximately 25 kDa under neutral and acidic conditions, but slightly cleaved under alkaline condition by CTSS. In comparison, JAM-C could not be cleaved under neutral and acidic conditions, but it was slightly cleaved under alkaline condition. This is not consistent with a previous study that found that JAM-A and JAM-B can be degraded under acidic conditions, but only JAM-B can be degraded under neutral conditions (Sevenich et al., 2014). This discrepancy may be due to the different pathological conditions of tumor and stroke. It was also discovered for the first time that the JAM family can be slightly cleaved by CTSS under alkaline conditions. However, the cleavage of JAMs by CTSS can be absolutely inhibited by adding the CTSS inhibitor LY3000328 at pH of 7.2. These results indicated that CTSS can aggravate I/R injury through increasing the BBB permeability by degrading JAMs in mice.

4.7. Pharmacological inhibition of cathepsin S alleviates cerebral ischemic damage in mice

All of the mice, including those in the inhibitor group (intraperitoneal injection of CTSS inhibitor dissolved in corn oil) and control group (intraperitoneal injection of corn oil), were subjected to ischemia for 90 min and reperfusion for 3 days. The CTSS inhibitor significantly decreased the infarct volume \( p = 0.026 \), neurological deficits \( p = 0.040 \) (Fig. 8A and B), and BBB permeability \( p = 0.044 \) compared to the control group (Fig. 8C) after I/R injury. These results suggest that pharmacological inhibition and deficiency of CTSS alleviates I/R injury in mice.

5. Discussion

Single-cell RNA sequencing (scRNA-seq) is widely used to characterize the dynamics of various cells to identify disease-related cell subgroups (Gao et al., 2021; International Multiple Sclerosis Genetics, 2019). Recent studies have revealed through peripheral blood single-cell sequencing technology that T cell subsets are involved in the regulation of AD and aging (Huang et al., 2021; Xu and Jia, 2021), but there is still a lack of research on peripheral blood changes after stroke. According to the changes in the proportion of each cell subpopulation at different times after I/R, we further divided the cells into four categories and
analyzed the cell subpopulations with progressively rising proportion over time. The results showed that peripheral blood monocyte subpopulations significantly increased after I/R, which was consistent with previous reports. In particular, through pseudotime trajectory analysis, we identified the key cell subpopulations that regulate monocyte activation; GO analysis revealed that this subpopulation was significantly enriched in the protease cleavage pathway. These results enhanced our understanding of the biological functions of monocytes after I/R.

Furthermore, we found that cathepsin S, a key molecule involved in the regulation of protease cleavage, was significantly highly expressed in monocytes after I/R. Cathepsin S, a lysosomal enzyme, is expressed in a wide variety of immune cells and plays a significant role in various intracellular and extracellular processes, including proteolysis and MHC Class II-mediated immune responses (Kim et al., 2017; Shi et al., 1999; van Dalen et al., 2020). Dysregulated expression and activity of cathepsin S is linked to the pathogenesis of multiple diseases, including those affecting the lungs, liver, and heart (Baugh et al., 2011; Sena et al., 2018; Wilkinson et al., 2016). Unlike other members of the lysosomal cathepsin family that require an acidic pH, CTSS has potent endoproteolytic activity at a broad range of pHs (Vasiljeva et al., 2005), indicating that CTSS is proteolytically active at the neutral pH found in the extracellular microenvironment. Previous research found that CTSS efficiently cleaved the JAM family members, JAM-A, JAM-B, and JAM-C, at pH 4.5, which is the acidic pH found in lysosomes, and maintained robust cleavage of JAM-B specifically at pH 6.0, the acidified pericellular pH in solid tumors. Previous studies also found that CTSS specifically mediates BBB transmigration through proteolytic processing of the JAMs to accelerate breast-to-brain metastasis (Sandoval and Witt, 2008; Sevenich et al., 2014). This is consistent with our results that CTSS destroys the BBB by degrading JAM family proteins at a broad pH range.

JAM family proteins are essential for maintaining the structural integrity of the BBB (Jia et al., 2013; Liu et al., 2012). A large number of previous studies have found that after stroke, metalloproteinase (MMP) family proteins (specifically MMP-2, MMP-7, and MMP-9), which are released from the infiltrated immune cells, disrupt the BBB by degrading JAMs and other extracellular matrix proteins (Sandoval and Witt, 2008) (Ludewig et al., 2013). Inhibition of peripheral immune cell infiltration through blood replacement or fingolimod treatment can effectively reduce the expression of MMP proteins, thereby reducing I/R injury (Bobinger et al., 2019). However, the protease cleavage activity of MMP proteins is sensitive to the pH value (Abdul-Muneer et al., 2013; Chen et al., 2009; Hua et al., 2021; Yang et al., 2007). Whether the change in pH value during I/R has an effect on MMP activity needs to be further evaluated. In this study, we found that CTSS degrades JAM family proteins at a broad pH range. Additionally, the use of CtsS-KO mouse models or cathepsin S inhibitors prevent BBB destruction caused by JAM degradation, suggesting that cathepsin S is an important treatment target for I/R.

Although we found that cathepsin S may be an important molecule that aggravates the BBB destruction by peripheral immune cells, there is still a large quantity of single-cell sequencing data that needs to be deciphered. For example, the cluster 2 and 9 subgroups, which are highly expressed throughout the acute and chronic phases of stroke, probably have stroke-associated functions related to oxidative stress,
Toll receptors, and SH3/SH2 receptors. The cluster 1 and 4 subgroups are increased in the acute phase but begin to decrease 7 days after ischemia, indicating that they probably participate in stroke recovery. Therefore, it is necessary to further verify whether these groups of cells are involved in acute ischemia or chronic repair.

6. Conclusions

In summary, through single-cell sequencing at different time points in cerebral I/R mice, we found that cluster 10 was involved in the enzyme digestion process. In this group, CTSS was significantly higher expressed after stroke, and the CTSS inhibitor or KO alleviated BBB destruction by inhibiting JAM cleavage and neuroinflammation. We conclude that cathepsin S inhibitor may serve as new targets for clinical brain protection against ischemia.

7. Declarations

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Ethical approval: The animal experiments were performed in agreement with the guidelines of the National Institutes of Health on the Care and Use of Animals and the Animal Management Committee of the Third Military Medical University.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The data that has been used is confidential.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbl.2022.11.001.

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