

Therapeutic Potential of Microglial SMEK1 in Regulating H3K9 Lactylation in Cerebral Ischemia-Reperfusion

Corresponding Author: Dr Ruonan Duan

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Attachments originally included by the reviewers as part of their assessment can be found at the end of this file.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

In this study, the author mainly found that histone lactylation in microglia promotes glycolysis in ischemia-reperfusion injury, and elucidated the potential role of SMEK1 as an upstream regulatory molecule for histone lactylation after cerebral ischemia.

The research logic is clear, the workload is sufficient, and the results are reliable. And the relevant conclusions may guide the initiation of clinical research.

Comments:

1. From Figure 1D and E, it can be seen that the level of SMEK1 significantly increased on the first day after reoxygenation. Figure 1I also shows that in the OGD3h/R1h group, the level of SMEK1 significantly increased but then decreased over time. Please revise the description on page 16, lines 340-342 in the article.
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3. On page 26, line 542. The reference cited here is prone to ambiguity, it is recommended to place it at the end of the sentence.

Reviewer #2

(Remarks to the Author)

Using transient middle cerebral artery occlusion (tMCAO) and oxygen-glucose deprivation and reoxygenation (OGD/R) models, the authors reveal that reduced SMEK1 expression in microglia after ischemic stroke may promote lactate production by inhibiting the pyruvate dehydrogenase kinase 3-pyruvate dehydrogenase (PDK3-PDH) pathway, which results in H3 lysine 9 lactylation (H3K9la), which activates *Ldha* and *Hif-1 α* transcription in microglia and is associated with subsequent neuroinflammation. These results suggest that microglial SMEK1 may be a potential therapeutic target for acute ischemic stroke.

This study is a fairly comprehensive study that is well designed, the methods used are feasible, and the results are reliable. The manuscript is well written, the results are clearly presented and explained, and it will be useful to the research community. Please find my comments below:

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8. In Fig. 7F, "Relative SMEK1 protein expression" should be corrected.

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(Remarks to the Author)

The author has addresses all the questions I raised and made revisions.

Reviewer #2

(Remarks to the Author)

The authors have addressed all my concerns, and the manuscript has been much improved. I recommend the revised manuscript for publication in Communications Biology.

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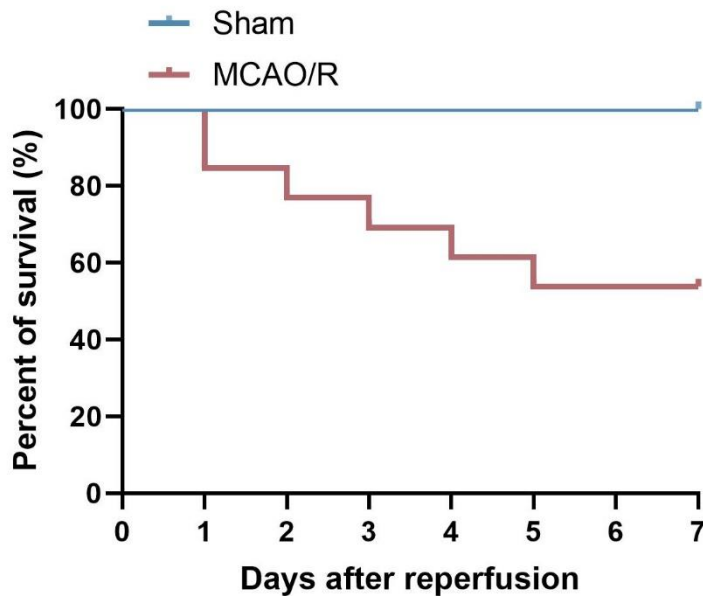
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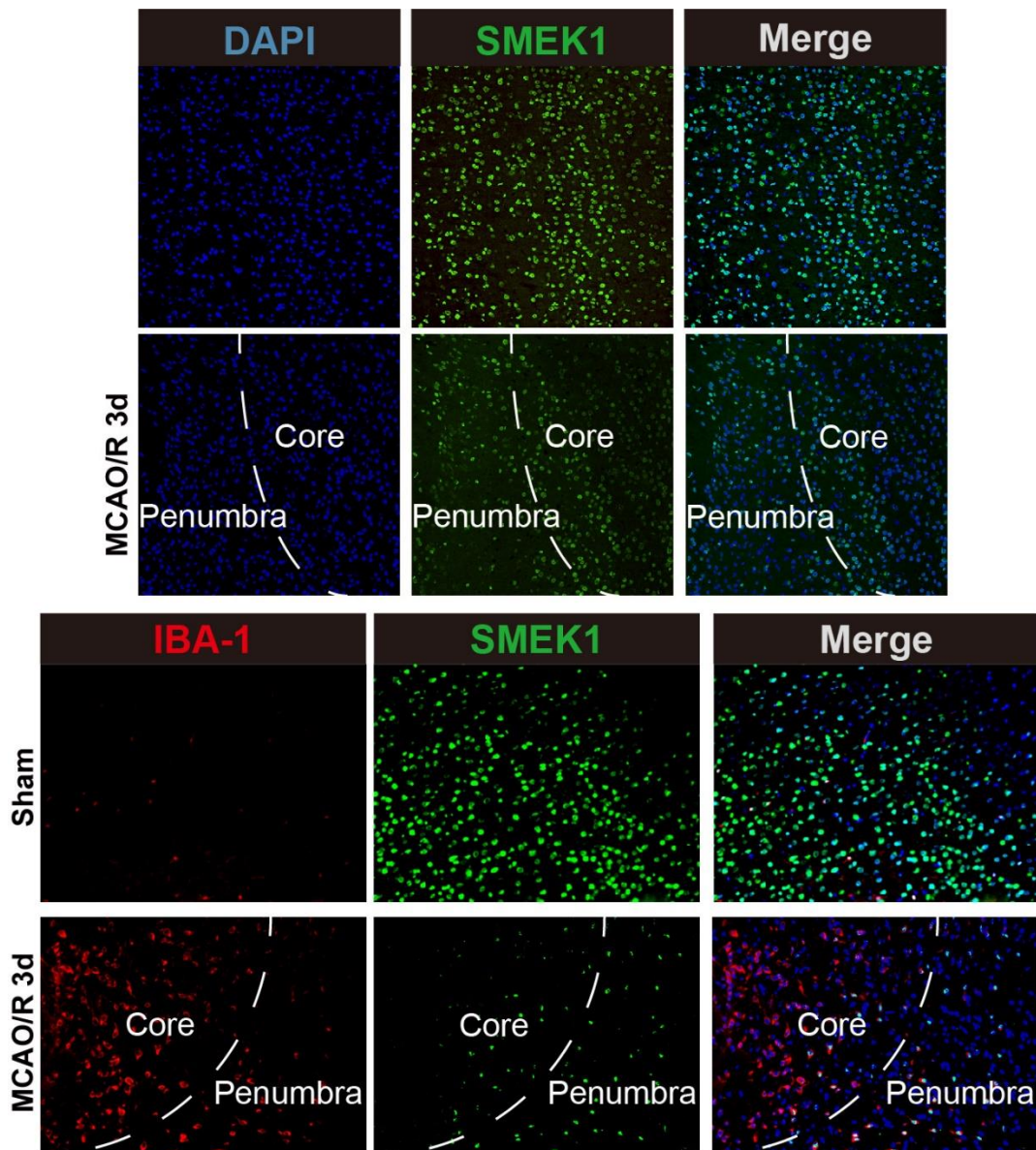
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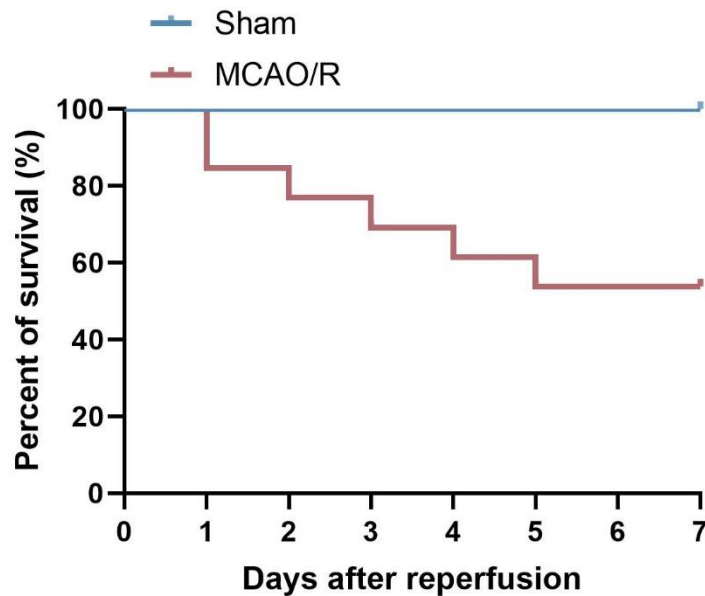
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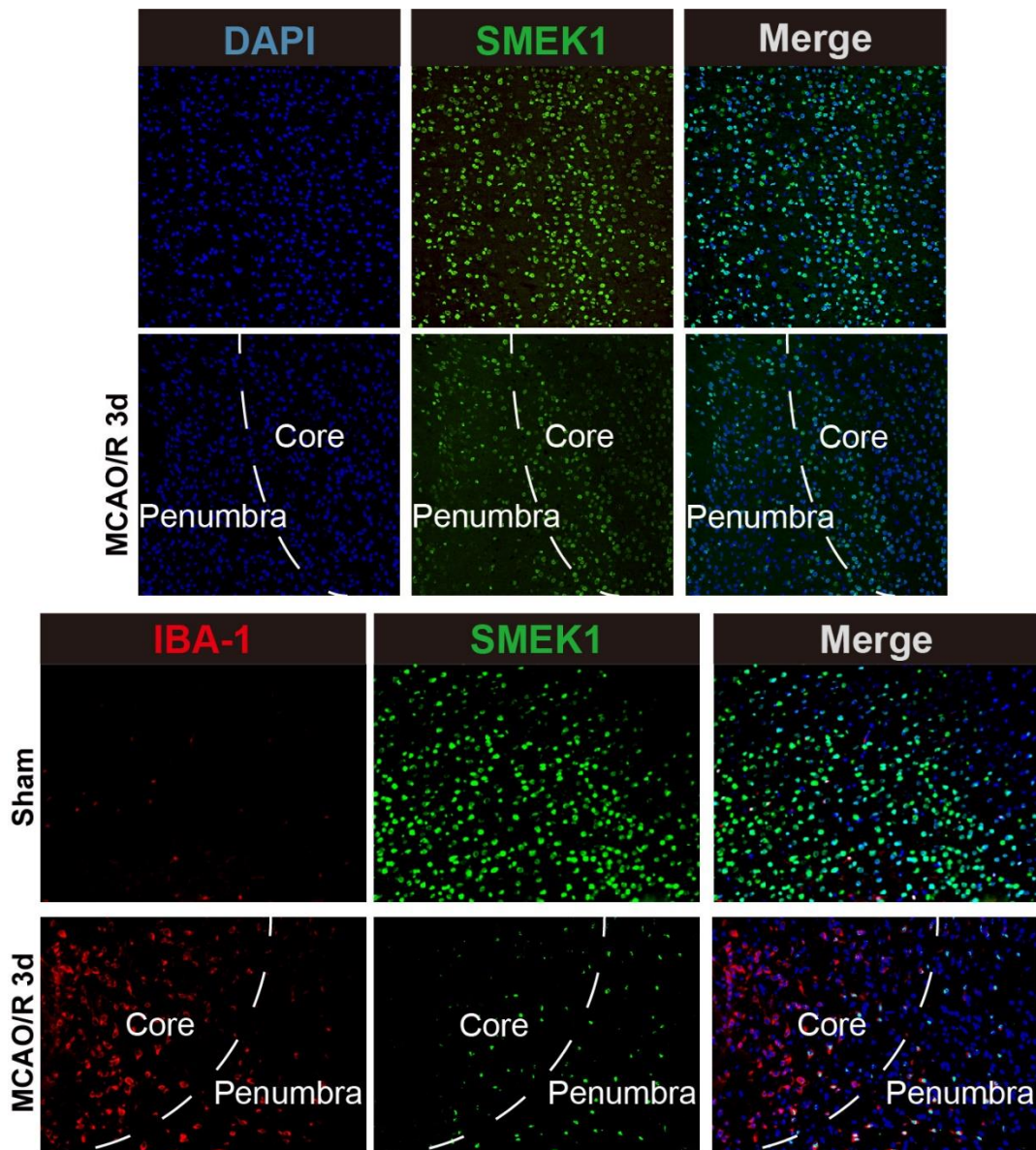
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1 **Therapeutic potential of microglial SMEK1 via regulation of H3 lysine 9**

2 **lactylation in cerebral ischemia-reperfusion injury**

3 **Data sharing statement**

4 All data will be made available by the corresponding author upon reasonable request.

5 **Funding declaration**

6 **Conflict of interest disclosure**

7 The authors declare that they have no known competing financial interests or personal

8 relationships that could have appeared to influence the work reported in this paper.

9 **Ethics approval and consent to participate**

10 All experiments involving animals were approved by the Institutional Animal Care and

11 Use Committee.

12

Abstract

Immune responses and neuroinflammation occurring after acute ischemic stroke (AIS) are closely related to brain injury. Histone lactylation is a metabolic stress-related histone modification that participates in the pathogenesis of various diseases. However, the role of histone regulation in cerebral ischemic stroke remains unknown. In this study, a transient middle cerebral artery occlusion (tMCAO) model and an oxygen–glucose deprivation and reoxygenation (OGD/R) model were used to simulate in vivo/in vitro ischemia–reperfusion injury. The underlying mechanism of microglial histone lactylation was investigated using microglia-specific SMEK1-overexpressing mice and BV2 cells. The results showed that lactate overload resulted in elevated histone lactylation after AIS. Decreased SMEK1 expression in microglia after ischemic stroke was associated with increased lactate levels and subsequent neuroinflammation. Microglia-specific SMEK1 deficiency in microglia after ischemia can promote lactate production by inhibiting the pyruvate dehydrogenase kinase 3-pyruvate dehydrogenase (PDK3-PDH) pathway. Specifically, H3 lysine 9 lactylation (H3K9la) activated *Ldha* and *Hif-1 α* transcription in microglia and promoted glycolysis. SMEK1-overexpressing mice exhibited better neurologic recovery after ischemic stroke than control mice. Mechanistically, we provided new evidence that microglial histone lactylation promoted glycolysis in ischemia–reperfusion injury and elucidated the potential role of SMEK1 as an upstream regulatory molecule in histone lactylation after cerebral ischemia. According to our results, microglial SMEK1 may be potential therapeutic targets for AIS.

Keywords: SMEK1; microglia; histone lactylation; ischemic stroke; neuroinflammation

1. Introduction

Stroke has become the second leading cause of death worldwide ¹ and the leading cause of death in China ², and ischemic stroke accounts for 80% of stroke-related deaths ³. Ischemic stroke, characterized by high incidence, disability and mortality, is a general term for necrosis of brain tissue caused by stenosis or insufficient blood supply to the brain. However, the treatment of acute ischemic stroke (AIS) is currently limited because of the narrow window of time available for thrombolysis and adverse reactions such as reperfusion injury after AIS ⁴. Immune responses and neuroinflammation occurring after AIS are strongly related to brain injury ⁵. Numerous clinical studies have demonstrated that drugs targeting immune responses after stroke can be promising alternative treatment strategies for stroke⁶.

Histone lactylation is a newly identified epigenetic modification that facilitates the transcription of specific genes⁷. Research has shown that histone lactylation regulates gene expression and plays a pivotal role in mediating immune responses in macrophages⁷⁻⁹. Microglia are resident immune cells in the brain that are derived from primitive macrophages¹⁰. However, the significance of microglial histone lactylation in various brain disorders remains unknown. Microglia are activated and migrate to the infarct area and penumbra immediately after ischemic stroke. There are two major phenotypic subtypes of microglia: classically activated M1 microglia and alternatively activated M2 microglia ¹¹. M1 microglia highly express cluster of differentiation 86

(CD86) and release proinflammatory factors such as interleukin-1 β (IL-1 β), tumor necrosis factor- α (TNF- α) and inducible nitric oxide synthase (iNOS), which exacerbate brain damage. M2 cells express CD206 and secrete anti-inflammatory cytokines such as IL-10 and transforming growth factor- β (TGF- β), which promote tissue repair ^{11,12}. After ischemic stroke, alterations in the brain microenvironment cause microglia to polarize to the M1/M2 phenotype. Dysregulation of M1/M2 microglia contributes to subsequent neuroinflammation ¹³. Metabolic reprogramming is now recognized as a key driver of microglial immunity. Microglia in proinflammatory states are mainly powered by glycolysis, whereas microglia in anti-inflammatory states preferentially depend on oxidative phosphorylation and fatty acid oxidation for energy production ¹⁴. However, the mechanism of microglial metabolic reprogramming in AIS remains unclear.

Suppressor of MEK1 (SMEK1) is a regulatory subunit of the PP4 enzyme. Previous studies have shown that SMEK1 participates in chemotaxis ¹⁵, neurogenesis ^{16,17}, neuronal differentiation ¹⁸ and aging ¹⁹. Previously, we found that SMEK1 deficiency promoted inflammation in microglia and exacerbated autoimmune diseases ²⁰. Herein, we revealed a novel mechanism by which miR-125a-5p targets the 3'UTR of the SMEK1 transcript and downregulates SMEK1 after AIS. SMEK1 deficiency in microglia caused elevated lactate production through the pyruvate dehydrogenase kinase 3-pyruvate dehydrogenase (PDK3-PDH) pathway and further induced histone lactylation. H3K9la, the site with the greatest increase in lactylation after cerebral ischemia, promoted the transcription of glycolytic genes. Our results revealed that

targeting microglial histone lactylation and modulating SMEK1 expression may constitute a new strategy for the treatment of AIS.

2. Materials and methods

2.1. Animals and transient middle cerebral artery occlusion (tMCAO) procedure

This study used C57BL/6J male mice purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. The mice were bred under specific pathogen-free conditions with a 12-hour light/dark cycle, controlled temperature and humidity, and free access to food and water. The Institutional Animal Care and Use Committee approved all the experimental procedures.

C57BL/6J male mice (21-25 g) aged 8-10 weeks were anesthetized and placed in a supine position under a SZX10 research stereomicroscope system (Olympus, Japan). The left common carotid artery, external carotid artery, and internal carotid artery were separated. The occlusion was accomplished by slowly inserting monofilaments (0.20-0.21 mm, RWD Life Science, Shenzhen, China) to block the middle cerebral artery. After 1 h of occlusion, the monofilament was withdrawn to complete reperfusion. Sham mice underwent the same procedure apart from monofilament insertion. After surgery, the animals were sacrificed at 1, 3, and 7 days (Fig. 1A). The ischemic core was selected as the sampling site for our subsequent studies (Fig. 1B).

2.2. SMEK1-overexpressing mice

CAG Pr-loxP-Stop-loxP-SMEK1 CDS-HA Tag-WPRE-pA was inserted at the ROSA26 site to produce C57BL/6J model mice based on the EGE system developed by CRISPR/Cas9. ROSA26-SMEK1^{fl/fl} mice were mated with Ubc-Cre-ERT2 mice and

Tmem119-Cre-ERT2 mice (Tmem119-Cre-ERT2 knock-in mice have the endogenous transmembrane protein 119 promoter/enhancer sequences directing expression of tamoxifen-inducible Cre recombinase primarily in microglia, and are also designed to retain endogenous Tmem119 expression) to obtain ROSA26-SMEK1^{fl/fl} Ubc-Cre-ERT2 mice and ROSA26-SMEK1^{fl/fl} Tmem119-Cre-ERT2 mice. We administered tamoxifen (100 mg/kg, T5648, Sigma, USA) to transgenic mice (male, 21-25 g, 8-10 weeks old)²¹ through intraperitoneal injection to induce SMEK1 overexpression. Littermate SMEK1^{fl/fl} mice (male, 21-25 g, 8-10 weeks old) were used as controls. Mice were injected for 7 days and then bred for 7 days. Subsequently, the mice underwent the tMCAO procedure.

2.3. Neurobehavioral test

2.3.1. mNSS test

Based on comprehensive motor, sensory, reflex, and balance tests, we used the modified neurological severity score (mNSS) test to evaluate overall neurological deficits in mice. Functional deficits were assessed 1, 2, and 3 days after surgery, with 0 representing normal performance and 18 representing the most severe deficit.

2.3.2. Corner test

We used the corner test to assess sensory motor and postural asymmetries. Between two plastic sheets, the mice were placed at a 30° angle facing the corner. Intact mice turned to both sides at the same frequency, while tMCAO mice preferentially turned to the ipsilateral side¹². The number of turns in each of the 10 trials was recorded, and the average percentage of turns on the ipsilateral side was calculated.

2.3.3. Foot-fault test

Sensorimotor coordination of the forelimbs was evaluated by the foot-fault test. Foot failure was recorded when mouse forelimbs were inaccurately placed, dropped, or slipped between wires on a horizontal steel grid with openings of different sizes¹². Foot errors in 3 trials were recorded, and the average percentage was calculated.

2.4. Cerebral cortical blood flow (CBF) and 2,3,5-triphenyltetrazolium chloride (TTC) staining

Cerebral CBF was measured at baseline, at 0 h (onset of tMCAO), and at 1 d, 2 d and 3 d after MCAO using a zoom laser speckle flow imaging system (MoorFLPI-2, Moor Instruments Ltd., UK). Briefly, the anesthetized mice were incised along the midline of their heads to expose their skulls and then placed under the lens of the imager to observe CBF (Fig. S1A).

The infarct volume was determined by TTC staining (G3005, Solarbio, Beijing). The brain tissue was fixed on a flat surface, frozen at -20 °C for 20 min, and then cut into 4-6 pieces with a blade. TTC was added, and the cells were placed in the incubator chamber at 37 °C for 40 min. The infarct volume was observed after staining (Fig. S1B).

2.5. Immunofluorescence staining

At room temperature, frozen brain sections were permeabilized with 0.3% Triton X-100 (T8200, Solarbio, Beijing) for 30 minutes. Nonspecific staining was blocked with 10% normal donkey serum (SL050, Solarbio, Beijing) at room temperature for 1 hour. The sections were incubated overnight at 4 °C with primary antibodies. Primary antibodies included mouse anti-IBA-1 (1:500, GB12105, Servicebio, Wuhan), rat anti-

IBA-1 (1:4000, ab283346, Abcam, UK), rabbit anti-SMEK1 (1:1000, HPA002568, Sigma, USA) and rabbit anti-H3K9la (PTM-1419RM, PTO BIO, Hangzhou). After washing with PBS, the sections were incubated with the appropriate secondary antibodies at room temperature for two hours. Finally, the nuclei were stained with DAPI (P0131, Beyotime, Shanghai). Paraffin sections were dewaxed and subjected to antigen retrieval before staining. The climbing cells were fixed with 4% paraformaldehyde (G1101, Servicebio, Wuhan) at room temperature for 20 min and then permeabilized with 0.3% Triton X-100 (T8200, Solarbio, Beijing) at room temperature for 20 min. Nonspecific staining was blocked with 10% normal donkey serum (SL050, Solarbio, Beijing) at room temperature for 20 min. The other steps were the same as those described above.

2.6. Flow cytometry

Single-cell suspensions were prepared from brain tissues. The mononuclear cells in the brain were separated with 30% Percoll (P8370, Solarbio, Beijing) and stained according to standard protocols with fluorochrome-conjugated antibodies against the following molecules: CD45 (103116, Biolegend, USA), CD11b (101236, Biolegend, USA), iNOS (25-5920-82, eBioscience, USA), and IL-10 (505010, Biolegend, USA). Anti-mouse SMEK1 (HPA002568, Sigma, USA)-labeled cells were detected with the corresponding fluorochrome-conjugated secondary antibody. Flow cytometry was performed using a CytoFLEX analyzer platform (A00-1-1102, Beckman Coulter, USA). All the data were analyzed using FlowJo 10.4 software. The gating of microglia from mononuclear cells is shown in Fig. S1I.

2.7. Single-cell RNA-seq analysis

Single-cell RNA-seq was carried out as previously described²⁰. In brief, cerebral cortices (both halves) and hippocampi from 2-month-old mice (wild type and SMEK1^{fl/fl} Sox2-Cre, N = 2 for each group) were collected. The library was prepared according to the instructions of 10X Chromium. The alignment of the clean data (GSE171986) to the mouse reference genome (hg19) was processed with Cell Ranger (version 7.1.0, 10X Genomics) to generate the feature-barcode gene expression matrix. The dataset was analyzed using the Seurat R package (version 5.0.0) for downstream principal component analysis (PCA) and uniform manifold approximation and projection (UMAP) analysis. Clusters were then annotated based on the DEGs in each cluster. The metabolic profiles were compared between different cells through scMetabolism by calculating pathway scores²².

2.8. RNA sequence and data analysis

Bulk RNA sequencing was performed by Novogene (Novogene.com). Briefly, the samples were sent for library preparation and sequencing. Raw data (raw reads) in fastq format were first processed into low-quality reads by in-house Perl scripts. Based on the number of reads and length of each gene, the FPKM was calculated after mapping to the reference genome with HISAT2 (v2.0.5). Additionally, the DESeq2 R software package (version 1.20.0) was used for differential expression analysis between the two groups. To control the error discovery rate, Benjamini and Hochberg's methods were used to adjust the p value, with $\text{padj} \leq 0.05$ and $|\log_2(\text{fold change})| \geq 1$ considered to indicate statistical significance. Gene Ontology (GO) enrichment analysis of

differentially expressed genes was conducted using clusterProfiler R (3.8.1).

2.9. Cell culture and lentiviral transfection

Mouse BV2 microglia (CX0103, Boster, Wuhan) were cultured in Dulbecco's modified Eagle's medium (DMEM, C11995500BT, Gibco, USA) supplemented with 10% fetal bovine serum (A3161001C, Gibco, USA) and 1% penicillin–streptomycin (15070063, Gibco, USA). The cells were incubated in 5% CO₂ at 37 °C, and the medium was changed every 2 days.

For knockdown/overexpressing SMEK1 BV2 cells (shSMEK/oe-SMEK), the target gene fragment was amplified with the following primers: 49830FW-65237: GCGAATTCGAAGTATACCTCGAGGC, 49830RW-65238: CGATCGCAGATCCTTGGATCC. The fragment was subsequently cloned and inserted into the lentiviral vectors PGMLV-hU6-MCS-CMV-Puro-WPRE and lenti-CMV-MCS-PGK-Puro (Genomeditech, Shanghai). Then, lentiviruses containing the corresponding target fragments or negative controls were transfected into BV2 cells. After transfection for 48 h, the medium was discarded, and the cells were cultured for another 48 hours. The transfected cells were selected with puromycin (2.5 µg/ml, HY-B1743A/CS-6857, MCE, Shanghai). Validation was performed using RT–qPCR and Western blotting.

2.10. Dual-luciferase reporter assays

The dual-luciferase reporter plasmid (pmirGLO-SMEK1 WT/MUT) was designed and synthesized by Scientific Research Cloud Biotechnology Co., Ltd. (Jinan, China). BV2 cells were seeded in 6-well plates overnight (5 x 10⁵/well). The dual-luciferase

reporter plasmid (2500 ng/well) and miR-NC or miR-125a-5p mimics (20 μ M, 5 μ l/well) were added to the medium for cotransfection. Luciferase activity was analyzed using the Dual-Luciferase Reporter Assay System (Berthold Technologies, Germany).

2.11. Oxygen and glucose deprivation (OGD) treatment

To mimic ischemic stroke in vitro, cells were subjected to OGD ²³. In brief, the medium was replaced with glucose-free DMEM (11966025, Gibco, USA), and the cells were cultured in 94% N₂ and 5% CO₂ at 37 °C for 3 h ²⁴. Then, the medium was replaced with normal DMEM, and the cells were reoxygenated for 1 h, 6 h, 12 h, 21 h, or 24 h (Fig. 1C).

2.12. Seahorse assays

SMEK1-knockdown/overexpressing BV2 cells (shSMEK1/oe-SMEK1) were cultured at 2×10^5 cells/ml for 2 days and then reseeded at 4×10^4 cells/well in a Seahorse 24-well microplate. The metabolic status of the cells was evaluated with a Seahorse XF Glycolytic Rate Assay Kit (103344-100, Agilent Technologies, USA), a Seahorse XF Cell Mito Stress Test Kit (103015-100, Agilent Technologies, USA), and a Seahorse XF24 Extracellular Flux Analyzer ²⁵. To determine the extracellular acidification rate (ECAR), rotenone/antimycin A (0.5 μ M) and 2-deoxy-D-glucose (50 mM) were added to the microplate. To measure the oxygen consumption rate (OCR), oligomycin (1 μ M), FCCP (1 μ M), and rotenone/antimycin A (0.5 μ M) were added sequentially to the microplate ²⁶. The data were analyzed using Wave software (Agilent Technologies, USA).

2.13. Lactate (LA) content assay

A lactate (LA) content assay kit (BC2235, Solarbio, Beijing) was used to measure the LA content. The treatment of cells or supernatants and the experiment were carried out according to the standard protocol. The absorbance at 570 nm was measured using a microplate reader (BioTek Epoch, USA).

2.14. Pyruvate dehydrogenase (PDH) and lactate dehydrogenase (LDH) activity assays

PDH activity and LDH activity were measured using a PDH activity assay kit (BC0385, Solarbio, Beijing) and an LDH activity assay kit (BC0685, Solarbio, Beijing), respectively. The treatment of cells and the experiment were carried out according to standard protocols. The absorbances were measured at 605 nm and 450 nm using a microplate reader (BioTek Epoch, USA).

2.15. Fatty acid oxidation (FAO) detection

Fatty acid beta-oxidation in SMEK1-knockdown/overexpressing BV2 cells was detected by fatty acid oxidation detection reagent (FDV-0033, Funakoshi, Japan) ²⁷. Briefly, the reagent was added to the medium, and the cells were cultured at 37 °C for 2 h. The images were observed via confocal microscopy (LU-N4, Nikon, Japan) at a wavelength of 405 nm, or the mean fluorescence intensity was recorded using a CytoFLEX analyzer platform (A00-1-1102, Beckman Coulter, USA) with a PB450 channel.

2.16. qRT-PCR

For the extraction of total RNA from BV2 cells or brain tissues, we used an RNA Easy Fast Tissue/Cell Total RNA Extraction Kit (DP451, Tiangen, Beijing) or TRIzol

reagent (15596026, Thermo Fisher Scientific, USA). For the extraction of microRNA from brain tissues and BV2 cells, we used a miRcute miRNA extraction and separation kit (DP501, Tiangen, Beijing). Then, cDNA was synthesized using a PrimeScript™ RT reagent kit (RR047A, Takara, Japan). qPCR was carried out on a Bio-Rad CFX96™ instrument using ChamQ SYBR qPCR Master Mix (Q321-02, Vazyme, Nanjing). Protocols were followed during the experimental procedures. The primers used for amplification are shown in Supplementary Table 1.

2.17. Western blots

Brain tissues and cells were lysed in RIPA buffer (P0013B, Beyotime, Shanghai) supplemented with PMSF (P0100, Solarbio, Beijing) and phosphatase inhibitor cocktail (P1081, Beyotime, Shanghai). We used a histone extraction kit (B015, KROMATIN, Zhengzhou) to extract the histone. Protocols were followed during the experiment. Proteins were electrophoretically separated on 10% or 12.5% SDS–PAGE gels (PG112, Epizyme, Shanghai) and then transferred onto PVDF membranes (ISEQ00010, Merck, Germany). After blocking with western rapid blocking buffer (GF1815, Genefist, Shanghai), the membranes were incubated with primary antibodies overnight at 4 °C. The following antibodies were used: anti-SMEK1 (1:1000, HPA002568, Sigma, USA), anti-β-actin (1:20000, 66009-1-Ig, Proteintech, Wuhan), anti-β-tubulin (1:1000, 10068-1-AP, Proteintech, Wuhan), anti-PDH (1:6000, 18068-1-AP, Proteintech, Wuhan), anti-P-PDH (1:2000, ab177461, Abcam, UK)²⁸, anti-HIF-1α (1:1000, ab179483, Abcam, UK), anti-PDK3 (1:500, 12215-1-AP, Proteintech, Wuhan), anti-IL-1β (1:1000, 31202, CST, USA), anti-CD206 (1:1000, 24595, CST, USA), anti-Pan-Kla (1:1000, PTM-

1401RM, PTO BIO, Hangzhou), anti-H2BK16la (1:1000, PTM-1424RM, PTO BIO, Hangzhou), anti-H3K9la (1:1000, PTM-1419RM, PTO BIO, Hangzhou), anti-H3K14la (1:1000, PTM-1414RM, PTO BIO, Hangzhou), anti-H3K18la (1:1000, PTM-1406RM, PTO BIO, Hangzhou), anti-H4K5la (1:1000, PTM-1407RM, PTO BIO, Hangzhou), anti-H4K8la (1:1000, PTM-1415RM, PTO BIO, Hangzhou), anti-H4K12la (1:1000, PTM-1411RM, PTO BIO, Hangzhou), anti-H4K16la (1:1000, PTM-1417RM, PTO BIO, Hangzhou), anti-H3 (1:1000, PTM-1001RM, PTO BIO, Hangzhou), anti-H4 (1:1000, PTM-1009RM, PTO BIO, Hangzhou). The blots were incubated with appropriate secondary antibodies at room temperature for 2 h.

2.18. Nasal delivery of miR-125a-5p

MiR-NC, miR-125a-5p mimics, inhibitor-NC and miR-125a-5p inhibitors (GenePharma, Shanghai) were administered intranasally at a dose of 25 μ l (1 nmol, 40 nmol/ml) per mouse 24 h before tMCAO²⁹. Alternate drops were applied between the left and right nose. Mice were sacrificed 3 days after tMCAO.

2.19. Fluorescence in situ hybridization (FISH)

The miR-125a-5p probe and SMEK1 probe were purchased from Shanghai GenePharma Co., Ltd. For hybridization, we used an RNA FISH kit (paraffin section/frozen section/cell climbing tablet) (GenePharma, Shanghai). The experiments were conducted according to the manufacturer's protocols.

2.20. MicroRNA transfection

MiR-NC, miR-125a-5p mimics, inhibitor-NC and miR-125a-5p inhibitors were purchased from Shanghai GenePharma Co., Ltd. The sequences are included in

Supplementary Table 2. For microRNA transfection, BV2 cells were seeded in a 6-well plate overnight and transfected using LipofectamineTM 3000 (L3000008, Thermo Fisher Scientific, USA) according to standard protocols. The cells were collected 48 h after transfection for qRT-PCR and Western blot analysis.

2.21. Chromatin immunoprecipitation (ChIP) and qPCR

ChIP was performed using a Pierce Agarose ChIP kit (26156, Thermo Fisher Scientific, USA) according to the manufacturer's protocol. BV2 cells were seeded in 21 cm² plates. To obtain nuclear pellets, BV2 cells were fixed with 1% (w/v) formaldehyde (28906, Thermo Fisher Scientific, USA), quenched with glycine, incubated with lysis buffer, and centrifuged. We then digested the nuclei with micrococcal nucleases to generate chromatin fragments. After 10% of the digested chromatin was used as the input, the remaining samples were incubated with rabbit anti-H3K9la antibody (PTM-1419RM, PTO BIO, Hangzhou) or rabbit IgG and recovered with protein A/G agarose beads. The samples were incubated at 65 °C for 40 minutes and digested with proteinase K. A column-based isolation method was used to recover the DNA, and qPCR analysis was performed using the primers listed in Supplementary Table 3.

2.22. Statistical analysis

The mean $\pm$ SD was used to present the results. Preliminary analysis of data normality was carried out with Shapiro-Wilk's test. Statistical comparisons were performed by unpaired Student's t test or one-way analysis of variance (ANOVA). $p < 0.05$ was considered to indicate statistical significance. Statistical analyses were

performed, and charts were drawn using GraphPad Prism 8 (GraphPad Software, San Diego, CA, USA).

3. Results

3.1. SMEK1 expression in microglia fluctuates with time after tMCAO/OGD

At present, the role of microglial SMEK1 in the inflammatory response after ischemic stroke is unclear. To investigate this issue, we first examined total SMEK1 mRNA and protein expression in the brain tissue of mice after MCAO (Fig. 1D, E). We found that SMEK1 expression peaked on day 1 after MCAO and decreased at days 3-7, with SMEK1 expression decreasing to its lowest level on day 3 after MCAO (Fig. 1D, E). Additionally, immunofluorescence staining verified that SMEK1 expression was decreased in the brain tissue of mice 3 days after MCAO (Fig. 1F). Furthermore, immunofluorescence staining (Fig. 1G) and flow cytometry (Fig. 1H) revealed that SMEK1 expression in microglia was reduced at 3 days after MCAO. The gating strategy of microglia in flow cytometry is shown in Fig. S1I. Correspondingly, SMEK1 expression in total mRNA and protein from mouse BV2 microglia after OGD (Fig. 1C) was detected in vitro. SMEK1 expression in BV2 microglia increased first and then decreased with increasing reoxygenation time after OGD, and SMEK1 expression decreased the least in the OGD3h/R21h group (Fig. 1I, J and Fig. S1F-H). In addition, immunofluorescence staining demonstrated that SMEK1 expression in BV2 microglia was downregulated after OGD3h/R21h (Fig. 1K). Taken together, our results demonstrated that SMEK1 expression in microglia after tMCAO/OGD decreased over time and that microglial SMEK1 may participate in the pathophysiology of AIS.

3.2. Microglial SMEK1 protects against AIS injury by suppressing inflammation

We first confirmed that the levels of proinflammatory cytokines were increased in the MCAO 3d mouse brain (Fig. S2A, B and K). To demonstrate the role of microglial SMEK1 in the inflammatory response after ischemic stroke, we examined the cytokine expression of SMEK1⁺ microglia and SMEK1⁻ microglia in the brain tissue of MCAO 3d mice. Compared with SMEK1⁻ microglia, SMEK1⁺ microglia exhibited increased secretion of IL-10 and decreased secretion of IL-1 β (Fig. S3A, B). Subsequently, we constitutively overexpressed SMEK1 in ROSA26-SMEK1^{fl/fl}Ubc-Cre-ERT2 mice, and tMCAO experiments were carried out 7 days after tamoxifen (TAM) or corn oil induction (Fig. S3C). The upregulated SMEK1 mRNA and protein expression in brain tissues was confirmed by qRT-PCR and Western blotting (Fig. S2C, D). The mNSS and cerebral CBF were measured at 1, 2 and 3 days after MCAO. On the third day after MCAO, the mice were sacrificed, and the mononuclear cells in the infarction area were extracted for flow cytometry. SMEK1-overexpressing mice (SMEK1-KI) had significantly lower mNSSs than control mice, and the difference was most significant on day 3 after MCAO (Fig. S3D). A zoom laser speckle flow imaging system showed better cerebral blood flow (CBF) recovery in SMEK1-KI mice than in control mice (Fig. S3E). Flow cytometry showed that IL-10 was increased in SMEK1-overexpressing microglia, while iNOS were decreased (Fig. S3F, G). Furthermore, immunofluorescence staining revealed that SMEK1 was overexpressed in microglia from transgenic mice with microglia-specific overexpression of SMEK1 (SMEK1-mCKI) (Fig. S2E). SMEK1-mCKI mice had better CBF recovery (Fig. 2B) and

neurobehavioral scores (Fig. 2C-E) than control mice. Flow cytometry revealed that SMEK1-overexpressing microglia exhibited increased expression of the anti-inflammatory factor IL-10, decreased expression of the proinflammatory factors iNOS (Fig. 2F, G), upregulated expression of the surface marker CD206 and downregulated expression of the CD86 (Fig. 2H, I) after MCAO. Taken together, these results suggested that microglial SMEK1 participates in protecting against ischemia-induced brain damage.

As shown in previous results and the present study, BV2 microglia were activated after OGD (Fig. S1C-E). To determine whether microglial SMEK1 is involved in immunoregulation after ischemic stroke, we constructed SMEK1-knockdown/overexpressing BV2 cells via lentiviral transfection (Fig. 3A, B). Interestingly, the expression of the proinflammatory factor IL-1 β increased in SMEK1-knockdown BV2 cells (shSMEK1) without hypoxia stimulation (Fig. 3C). Overexpressing SMEK1 in BV2 cells (oe-SMEK1) increased the expression of the anti-inflammatory mediator IL-10 in the absence of hypoxia stimulation (Fig. 3C). After OGD3h/R21h, shSMEK1 BV2 cells exhibited increased expression of CD86, IL-1 β , iNOS and TNF- α and decreased expression of CD206, IL-10 and TGF- β compared to those in the negative controls (shNCs) (Fig. 3D). Fortunately, compared with the negative controls (oeNCs), oe-SMEK1 BV2 cells showed increased expression of CD206, IL-10 and TGF- β and decreased expression of CD86, IL-1 β , iNOS and TNF- α after OGD3h/R21 h (Fig. 3E). These findings indicated that SMEK1 deficiency in microglia promoted inflammation, while overexpression of microglial SMEK1 had a

neuroprotective effect after ischemic stroke.

3.3. SMEK1 deficiency in microglia causes metabolic reprogramming

To elucidate the relationship between the inflammatory response and metabolic processes in SMEK1-deficient microglia, we reanalyzed our previous single-cell RNA sequencing (scRNA-seq) dataset (Fig. S5A). A total of 21 clusters were identified (Fig. S4A and Fig. S5B). Subsequently, the cells were categorized into ten major cell types according to cell type-specific markers (Fig. S4B and C). Notably, clusters 0, 1, 2, 3, 6, 8 and 13 were classified as microglia but were distinctly different between SMEK1^{-/-} and wild-type microglia (Fig. S4D). Specifically, clusters 0 and 1 were more abundant in the wild type, while clusters 2, 3 and 6 were more abundant in the SMEK1^{-/-} plants (Fig. S4E). There were no significant differences in the number of microglia (Fig. S5C). All microglial subclusters were enriched for the marker gene *Tmem119* (Fig. S4F) but showed distinct inflammatory characteristics. Proinflammatory cytokines, including *Il-1α*, *Il-1β* and *Tnf*, were highly expressed in cluster 3 and cluster 13 (Fig. 4A and Fig. S5D-F). In addition, the marker genes of these clusters also showed that cluster 3 and cluster 13 displayed M1-like features (Fig. 4B). Aberrant metabolism plays a key role in microglial immune responses. We next used scMetabolism to quantify the metabolic pathway scores of the SMEK1^{-/-} and wild-type mice. We found that microglia in SMEK1^{-/-} mice exhibited decreased pyruvate metabolism, TCA cycle progression and oxidative phosphorylation (Fig. 4C). Furthermore, the activities of pyruvate metabolism, the TCA cycle and oxidative phosphorylation in cluster 13 were lower in the SMEK1^{-/-} group than in the WT group (Fig. 4D). This suggested that mitochondrial

oxidative phosphorylation might be suppressed in SMEK1-deficient microglia.

To uncover the functional mechanism of abnormal metabolism in SMEK1-deficient microglia, we performed transcriptome sequencing analysis using SMEK1-knockdown BV2 microglia (shSMEK1) and negative controls (shNC) (Fig. 5A). Among the DEGs, PDK3, a dehydrogenase kinase that inhibits the PDH complex by phosphorylating the E1 alpha subunit, was dramatically upregulated in shSMEK1 BV2 cells (Fig. 5B). Moreover, we found that DEGs were enriched in inflammation-related biological processes (Fig. 5C), suggesting that SMEK1 deficiency is involved in microglial immune/inflammatory functions. Consistent with the scRNA-seq results, GSEA showed that microglial SMEK1 deficiency was closely associated with fatty acid metabolism and pyruvate metabolism (Fig. 5D, E).

To reveal the metabolic changes induced by SMEK1 knockdown/overexpressing BV2 cells, the ECAR and OCR, which reflect glycolysis and oxidative phosphorylation, respectively, were next assessed using a Seahorse XF24 Extracellular Flux Analyzer. The ECAR of SMEK1-knockdown BV2 cells was significantly greater than that of control cells (Fig. 6A), as evidenced by basal glycolysis and post-2-deoxy-D-glucose (2-DG) acidification (Fig. 6B). The oxygen consumption rates of SMEK1-knockdown BV2 cells were significantly lower than those of control cells (Fig. 6C), as evidenced by basal respiration, maximal respiration, proton leakage and spare respiratory capacity (Fig. 6D). These data suggested that SMEK1 deficiency enhanced glycolysis and impaired oxidative phosphorylation and mitochondrial function. The oxygen consumption rates of SMEK1-overexpressing BV2 cells were significantly greater than

those of control cells (Fig. 6E), as indicated by enhanced basal respiration and proton leakage (Fig. 6F). The extracellular acidification rates of SMEK1-overexpressing BV2 cells were significantly lower than those of control cells (Fig. 6G), with reduced basal glycolysis and 2-DG post acidification (Fig. 6H). These data suggested that SMEK1 promoted oxidative phosphorylation and mitochondrial function and inhibited glycolysis. Subsequently, we measured the levels of fatty acid oxidation (FAO). The results showed that FAO levels decreased after SMEK1 deficiency (Fig. 6I-K). However, after SMEK1 overexpression, FAO levels increased (Fig. 6J, K). This finding was further confirmed in SMEK1-overexpressing microglia extracted from SMEK1-mCKI MCAO mice (Fig. 6L, M). Taken together, these results suggested that SMEK1 deficiency in microglia can lead to metabolic reprogramming.

3.4. Microglial SMEK1 deficiency after ischemic stroke induces lactate production and histone lactylation by inhibiting PDH activity

Since PDK3 was significantly increased in shSMEK1 BV2 cells, we first proved that compared with those in the control group, PDK3 mRNA and protein levels were obviously increased in the shSMEK1 group but decreased in the oe-SMEK1 group (Fig. 7A, C). The activity of the PDH decreased in shSMEK1 cells but increased in the oe-SMEK1 group (Fig. 7B). The changes in P-PDH, an inactive form of pyruvate dehydrogenase, were in accordance with the changes in PDH activity (Fig. 7C). In addition, we measured intracellular and extracellular lactate (LA) levels. The results showed that lactate levels were increased significantly both inside and outside the cells after SMEK1 deficiency but decreased in SMEK1-overexpressing cells (Fig. 7D, E).

To investigate whether histone lactylation occurs in microglia after ischemic stroke, we examined microglial histone lactylation levels in OGD-induced BV2 cells and brain tissues from MCAO 3d mice. The results showed that histone lactylation levels were widely increased in the OGD and MCAO 3d groups, especially at the H3K9la site (Fig. 7F). Next, we confirmed the increase in H3K9la levels in the microglia of MCAO 3d mice by immunofluorescence staining (Fig 7G). Subsequently, chromatin immunoprecipitation (ChIP)-qPCR revealed that H3K9la can directly stimulate *Ldha* and *Hif-1 α* gene transcription (Fig. 8A). The mRNA levels of *Ldha* and *Hif-1 α* were increased in BV2 cells after OGD (Fig. 8B). Interestingly, knocking down SMEK1 in BV2 cells promoted histone lactylation, while overexpressing SMEK1 reversed this epigenetic modification after OGD3h/R21h or MCAO 3d (Fig. 8C). Immunofluorescence staining also showed that H3K9la expression in microglia decreased after SMEK1 overexpression (Fig. 8D). We next measured the mRNA level of *Ldha* and detected markedly increased *Ldha* expression in shSMEK1 cells after OGD3h/R21h (Fig. 8E). In contrast, the mRNA level of *Ldha* was downregulated in SMEK1-overexpressing cells. Consistent with the *Ldha* expression pattern, the activities of lactate dehydrogenase (LDH) in the shSMEK1 and oe-SMEK1 groups were elevated and reduced, respectively (Fig. 8F). Similarly, the mRNA and protein levels of HIF-1 α also significantly changed in shSMEK1 and oe-SMEK1 BV2 cells after hypoxia (Fig. 8G, H). Collectively, the above results demonstrate that reduced SMEK1 after cerebral ischemia results in histone lactylation and that H3K9la directly promotes the transcription of glycolytic genes.

3.5. MiR-125a-5p regulates the expression of microglial SMEK1

To demonstrate the role of miR-125a-5p in neuroinflammation after ischemic stroke, we first examined the alteration of miR-125a-5p in the brain tissues of mice after MCAO 3d. The results suggested that the expression of miR-125a-5p was greater in the MCAO group than in the sham and contralateral groups (Fig. 9A). Correspondingly, we found that the expression of miR-125a-5p in BV2 cells was also elevated after OGD3h/R21h (Fig. 9B). These results suggested that miR-125a-5p may participate in neuroinflammation after ischemic stroke.

To determine the colocalization of miR-125a-5p and SMEK1 in BV2 cells and brain tissue, we first used miR-125a-5p and SMEK1 RNA fluorescent probes to perform a FISH assay on cell climbing tablets and frozen brain sections. The results suggested that miR-125a-5p overlapped with SMEK1 in both BV2 cells and brain tissue (Fig. 9C, D). Interestingly, the FISH results also showed that miR-125a-5p in BV2 cells and brain tissue was indeed upregulated after OGD, while SMEK1 was downregulated (Fig. 9C, D). Next, we predicted from TargetScan (<https://www.targetscan.org/>)³⁰ that miR-125a-5p may bind to the 3'UTR of SMEK1 (Fig. 9E). The prediction needed to be verified by dual-luciferase reporter assays. Subsequently, luciferase reporter plasmids containing the wild-type (WT) and mutant (MUT) 3'UTRs of SMEK1 were constructed (Fig. 9E, F). As shown in Fig. 9G, miR-125a-5p significantly inhibited luciferase activity in the presence of the WT plasmid but not in the presence of the MUT plasmid. These results suggested that miR-125a-5p binds to the 3'UTR of SMEK1 and may inhibit its expression.

To clarify the regulatory effect of miR-125a-5p on the expression of microglial SMEK1, miR-125a-5p mimics and inhibitors were transfected into BV2 cells. The transfection efficiency was verified by qPCR (Fig. S2I, J). Next, we performed qRT-PCR and Western blotting to detect the expression of SMEK1. The results showed that miR-125a-5p mimics suppressed the expression of SMEK1 in BV2 cells compared with the negative control (Fig. 9H, J). However, miR-125a-5p inhibitors upregulated the expression of SMEK1 (Fig. 9I, J). The results showed that miR-125a-5p can inhibit the expression of microglial SMEK1.

3.6. MiR-125a-5p inhibition can improve the symptoms of tMCAO mice

To explore the effect of miR-125a-5p on neuroinflammation after ischemic stroke, miR-125a-5p mimics or inhibitors were delivered into the brains of C57BL/6J mice through nasal delivery one day before tMCAO surgery (Fig. 10A). The qRT-PCR results suggested that miR-125a-5p mimics increased the expression of miR-125a-5p in the brains of mice at 24 h after delivery compared to that in the negative control group, while the level of miR-125a-5p was reduced after nasal delivery of miR-125a-5p inhibitors (Fig. 10B, C). Western blots confirmed that miR-125a-5p mimics downregulated the expression of SMEK1 in brain tissue, while miR-125a-5p inhibitors upregulated SMEK1 in brain tissue (Fig. 10D). Subsequently, a mNSS test was performed on the mice after 3 days of MCAO, and the results indicated that the score of the miR-125a-5p mimic group was greater than that of the control group (Fig. 10E). And the score was lower in the miR-125a-5p inhibitor group than in the negative control group (Fig. 10E). Next, the infarct volume was also measured by TTC staining.

Compared with that in the respective control group, the infarction volume in the miR-125a-5p mimic group was greater, while the infarct volume decreased in the miR-125a-5p inhibitor group (Fig. 10F). Further qPCR confirmed that the proinflammatory factors IL-1 β and TNF- α were upregulated in the miR-125a-5p mimic group (Fig. 10G). The anti-inflammatory factor IL-10 was increased in the miR-125a-5p inhibitor group, and the proinflammatory mediators IL-1 β and TNF- α were decreased (Fig. 10H). The results indicated that the miR-125a-5p inhibitor suppressed inflammation and improved the symptoms of tMCAO mice by upregulating SMEK1 expression.

4. Discussion

Lactate-derived histone lactylation was initially identified in macrophages as a new epigenetic modification that directly stimulates gene transcription from chromatin⁷. Recent studies have shown that histone lactylation is increased in microglia under various conditions³¹⁻³³. In this study, we revealed that SMEK1 deficiency after brain ischemia, mediated by increased miR-125a-5p, promoted lactate production by inhibiting oxidative phosphorylation through the PDK3/PDH signaling pathway. Overloaded lactate resulted in elevated pan-Kla and histone KLa levels in both MCAO brain tissues and OGD-treated BV2 microglia. H3K9la regulates *Ldha* and *Hif-1 α* transcription in microglia and induces glycolysis. Overexpression of microglial SMEK1 in MCAO mice ameliorated clinical manifestations, inhibited histone lactylation and suppressed neuroinflammation. A recent study described the profile of lysine-lactylated proteins in the cerebral endothelium of cerebral ischemia–reperfusion rats and identified several lactylated proteins in the Ca²⁺ signaling pathway³⁴. Moreover,

a more recent study showed that H3K18la and LDHA were elevated in the OGD/R-treated neuronal cell line N2a³⁵ and demonstrated that the LDHA/H3K18la/HMGB1 signaling pathway mediates neuronal pyroptosis in cerebral ischemia.

Interestingly, HIF-1 α and LDHA seem to be a link between protein lactylation and glycolysis. The production of lactate is accelerated in glycolysis, while histone lactylation levels are correlated with increased intracellular lactate levels⁷, forming a positive feedback loop. Specifically, amyloid- β acutely induces metabolic reprogramming from OXPHOS to glycolysis in microglia through the mTOR/HIF-1 α pathway, resulting in increased lactate secretion in Alzheimer's disease (AD)³⁶. Lactate-derived histone lactylation H4K12la in turn induces mRNA transcription of *Hif-1 α* and *Ldha* and promotes glycolysis³³. Here, we screened several histone K1a sites in MCAO brain tissue and OGD-treated BV2 cells. Our results showed that H3K9la levels increased significantly in the MCAO/OGD group; however, H4K12la levels were not different between the control group and the MCAO and OGD groups. This suggested that the H3K9la site may be the fundamental site of microglial histone lactylation in cerebral ischemia, which differs from that in AD or senescence^{31,33}.

Notably, HIF-1 α binds to the promoter region of the *Ldha* gene and enhances its transcription³⁷. HIF-1 α is an oxygen-regulated transcription factor that can regulate various pathways, including energy metabolism pathways³⁸. In the hypoxic region of the ischemic brain, HIF-1 α governs metabolic reprogramming by activating a variety of glycolytic enzymes and glucose transporters³⁹. It is well known that the transcriptional level of HIF-1 α is independent of oxygen levels, but the degradation of

HIF-1 α by the ubiquitin–proteasome pathway under normoxia results in undetectable HIF-1 α protein levels. Initially, an in vivo study showed an increase in HIF-1 α mRNA in cerebral ischemia as well as in animal models exposed to hypoxia⁴⁰, which was further confirmed by subsequent studies⁴¹. While studies have revealed bidirectional roles of microglial HIF-1 α in ischemic stroke⁴², few have explained the underlying mechanism of elevated HIF-1 α mRNA levels. Our results showed that histone lactylation in cerebral ischemia, particularly H3K9la, could promote HIF-1 α transcription and thus further increase the protein level of HIF-1 α .

Metabolic process switching in immune cells is a crucial mechanism that enables them to change their metabolic status as they respond to and interact with their environment⁴³. As microglia are highly dependent on the energy supply for monitoring and activation, metabolic reprogramming plays an important role in the microglial immune response^{14 44}. Cellular metabolism shifts from OXPHOS to glycolysis in activated proinflammatory microglia. Here, we showed that SMEK1-deficient microglia displayed aberrant metabolic processes. According to the scRNA-seq data, pyruvate metabolism, the TCA cycle and oxidative phosphorylation were suppressed in SMEK1-deficient microglia. According to bulk RNA-seq, PDK3 was significantly upregulated in microglia after SMEK1 was knocked down. PDKs, particularly PDK3, are involved in a variety of diseases. Inactivation of PDHC by PDK is thought to promote cancer cell growth⁴⁵. However, whether PDK3 plays a role in the microglial inflammatory response remains to be investigated. PDK3 modulates the switch between glycolytic metabolism and mitochondrial oxidation⁴⁶. In the tricarboxylic acid cycle

(TCA cycle), PDHC catalyzes the oxidative decarboxylation of pyruvate to acetyl-CoA⁴⁷. However, PDK3 can convert active PDH to inactive phosphorylated pyruvate dehydrogenase (P-PDH) at the Ser293 site (Ser293-PDHE1 α)²⁸. This blockage prevents pyruvate entry into the TCA cycle, thus attenuating mitochondrial oxidative respiration and causing pyruvate to produce more lactate⁴⁷⁻⁴⁹, which further explains the decreased activity of PDH and the increased level of LA after SMEK1 deficiency. Because different energy metabolism pathways are strongly associated with different microglial polarization states, modulating metabolic processes to modulate the transition of microglia from the M1 to M2 phenotype could be a promising therapeutic approach⁵⁰.

Previous studies reported that miR-125a-5p and miR-125b-5p in the peripheral blood of ischemic stroke patients are elevated^{51,52}. However, controversial results exist regarding the changes in miR-125a-5p in brain ischemic stroke. The expression levels of miR-125a-5p and miR-125b-5p were decreased in HT22 cells under OGD/R⁵³, and the expression of miR-125a-5p was also reduced in the circulating endothelial microvesicles of patients with ischemic stroke⁵⁴. Hence, the diagnostic and therapeutic role of miR-125a-5p remain unclear. Here, we found that microglial miR-125a-5p was elevated after tMCAO/OGD in vivo and in vitro, while miR-125b-5p was not different between the control and tMCAO/OGD groups (Fig. S2F-H). MiR-125a-5p plays a role in the development and function of immune cells⁵⁵ and participates in the pathogenesis of autoimmune diseases, including experimental autoimmune encephalomyelitis (EAE)⁵⁶. Although the inhibition of miR-125a-5p ameliorated the clinical

manifestations of EAE in mice, most likely through binding to target genes in spinal neurons, astrocytic miR-125a-5p attenuated inflammation through the inhibition of macrophage recruitment in pathological retinas⁵⁷. To date, the biological function of miR-125a-5p in microglia has not been evaluated. Our study demonstrated that miR-125a-5p targets SMEK1 in microglia and that intranasal delivery of miR-125a-5p mimics exacerbates neuroinflammation in MCAO.

There are several limitations in our study. Since the proportion of microglia decreases after cerebral ischemia⁵⁸ and it is difficult to distinguish microglia from macrophages and neutrophils that migrate into the brain from the periphery¹⁰, the extraction of primary microglia for in vitro experiments has become relatively complicated, and the results are unclear. Therefore, we used BV2 cells for in vitro analysis. Another limitation is that nasal delivery of miR-125a-5p cannot target microglia. Because miR-125a-5p has been identified to target a variety of cell types⁵⁵, the therapeutic role of miR-125a-5p in MCAO demonstrated here might not be entirely due to suppressed SMEK1 expression in microglia. In the future, more accurate drug delivery systems that target microglia could be developed, such as DNA origami⁵⁹.

In summary, our findings demonstrate that decreased microglial SMEK1 after ischemic stroke induces neuroinflammation through H3K9la/HIF-1 α /glycolysis signaling. The crucial role of SMEK1 and histone lactylation-related molecules identified in this study may provide novel evidence and a mechanistic basis for therapeutic strategies for cerebral ischemia.

Authors' contributions

Ruo-Nan Duan designed, analyzed, and provided overall guidance for the experiments. Wei-Yue Si designed, performed, and analyzed the experiments and wrote the manuscript. Chun-Lin Yang, Shu-Li Wei, Tong Du, Liang-Kang Li, Jing Dong, Yang Zhou, Heng Li and Peng Zhang performed the experiments. Qi-Ji Liu and Rui-Sheng Duan performed statistical analyses. All authors have read and approved the final manuscript.

Consent for publication

All authors listed gave consent for publication.

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Figure legend

Figure 1. Microglial SMEK1 expression fluctuated with time after tMCAO/OGD.

(A) Schematic diagram of the mouse experimental design. (B) Schematic diagram of the sampling sites. (C) Schematic diagram of the experimental design for BV2 cells. Determination of SMEK1 mRNA (D) and protein (E) expression in the SHAM and MCAO 1 d, 3 d and 7 d groups. n = 3 per group. (F) Representative photos of SMEK1⁺ cells in the SHAM, MCAO 3d and contralateral groups. (G) Representative photos of SMEK1⁺ microglia in the SHAM and MCAO 3D groups. (H) Gating strategy for SMEK1⁺ microglia isolated from the brain after tMCAO and the proportion of SMEK1⁺ microglia in the MCAO 3D and contralateral groups. n = 8 per group. SMEK1 mRNA (I) and protein (J) expression in BV2 cells subjected to OGD for 3 h followed by exposure to a normal oxygen concentration for 0, 1, 6, 12, 21 or 24 h. n = 3 per group. (K) Representative photos of SMEK1⁺ BV2 cells in the control and OGD3h/R21h groups. *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001. ns, not significant.

Figure 2. Neuroinflammation was reduced after tMCAO in SMEK1-mCKI mice.

(A) Schematic diagram of the experimental design for the SMEK1-mCKI mice. (B) Representative photographs of laser speckle contrast images and quantitative analyses of cortical CBF. n = 5 per group. Neurobehavioral function was evaluated by the mNSS (C), corner test (D) and foot-fault test (E). (F) Gating strategy for IL-10⁺, iNOS⁺ microglia in the brains of control and SMEK1-mCKI mice after tMCAO 3d. (G) Flow cytometry analysis of IL-10, iNOS expression in microglia from the brains of control and SMEK1-mCKI mice after tMCAO 3d. C-G, n = 11 for each group. (H) Gating

strategy for CD86⁺ and CD206⁺ microglia in the brains of control and SMEK1-mCKI mice after tMCAO 3d. (I) Flow cytometry analysis of CD86 and CD206 expression in microglia from the brains of control and SMEK1-mCKI mice after tMCAO 3d. H and I, n = 5 per group. *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001. ns, not significant.

Figure 3. Microglial SMEK1 deficiency promotes neuroinflammation.

The expression of SMEK1 mRNA (A) and protein (B) in shNC, shSMEK1 (n = 4 per group), oeNC and oe-SMEK1 (n = 3 per group) BV2 cells was assessed using qPCR and WB. (C) The expression of IL-1 β mRNA in shNC and shSMEK1 BV2 cells and the expression of IL-10 mRNA in oeNC and oe-SMEK1 BV2 cells were assessed using qPCR. (D) The expression of CD86, IL-1 β , iNOS, TNF- α , CD206, IL-10 and TGF- β mRNA in shNC- and shSMEK1-treated BV2 cells subjected to OGD for 3 h/R21 h was assessed using qPCR. (E) CD86, IL-1 β , iNOS, TNF- α , CD206, IL-10 and TGF- β mRNA levels in oeNC and oe-SMEK1 BV2 cells subjected to OGD for 3 h/R for 21 h were assessed using qPCR. C-E, n = 3 for each group. *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001. ns, not significant.

Figure 4. Integrated scRNA-seq and bulk RNA-seq analysis revealed that microglial SMEK1 deficiency suppresses mitochondrial oxidative phosphorylation.

(A) Dot plots of microglia-related genes expressed among cell clusters. (B) Volcano map of marker genes in different microglial clusters. (C) Dot plots showing differences in metabolic characteristics between SMEK1^{-/-} and wild-type mice. (D) Dot plots

showing metabolic characteristics of seven microglial clusters between SMEK1^{-/-} and wild-type mice.

Figure 5. Integrated scRNA-seq and bulk RNA-seq analysis revealed that microglial SMEK1 deficiency suppresses mitochondrial oxidative phosphorylation.

(A) Heatmap from RNA sequencing analysis depicting genes differentially expressed between shNC-treated and shSMEK1-treated BV2 cells. (B) A total of 594 upregulated and 515 downregulated genes were identified using RNA sequencing analysis of shNC and shSMEK1 BV2 cells, and the expression of PDK3 was upregulated in shSMEK1 BV2 cells. (C) A number of cytokine-related biological processes and several biological processes related to lipid synthesis were enriched according to the GOBP analysis. GSEA of enriched fatty acid metabolism (D) and pyruvate metabolism (E).

Figure 6. Loss of SMEK1 causes metabolic reprogramming in microglia.

(A, G) The extracellular acidification rate (ECAR) of BV2 cells was measured using a Seahorse XF24 Extracellular Flux Analyzer. (B, H) Bar graphs demonstrating basal glycolysis and post-2-DG acidification. A and B, n = 4 per group; G and H, n = 3 per group. (C, E) Representative oxidative phosphorylation (OCR) Seahorse bioenergetics profiles before and after injections of oligomycin (1 μ M), FCCP (1 μ M), and rotenone/antimycin A (0.5 μ M). (D, F) Bar graphs demonstrating basal respiration, maximal respiration, proton leakage and spare respiratory capacity. n = 3 per group. (I) Representative photos of FAO levels in shNC and shSMEK1 BV2 cells. (J, K) Flow cytometry analysis of FAO levels in shNC, shSMEK1, oeNC and oe-SMEK1 BV2 cells.

n = 3 per group. (L) Gating strategy for FAOBlue⁺ microglia in the brains of control and SMEK1-mCKI mice after tMCAO 3d. (M) Flow cytometry analysis of FAO levels in microglia from the brains of control and SMEK1-mCKI mice after tMCAO 3d. n = 5 per group. *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001. ns, not significant.

Figure 7. Microglial SMEK1 deficiency promotes histone lactylation via the PDK3-PDH signaling pathway.

(A) Determination of PDK3 mRNA expression in shNC, shSMEK1, oeNC and oe-SMEK1 BV2 cells. n = 3 for each group. (B) PDH activity of shNC, shSMEK1, oeNC and oe-SMEK1 BV2 cells. n = 4 per group. (C) The protein expression of PDK3, PDH and P-PDH in shNC, shSMEK1, oeNC and oe-SMEK1 BV2 cells was assessed using WB. Extracellular (D) (n = 3 per group) and intracellular (E) (n = 4 per group) LA levels in shNC, shSMEK1, oeNC and oe-SMEK1 BV2 cells. (F) Determination of Pan-Kla, H2BK16la, H3K9la, H3K14la, H3K18la, H4K5la, H4K8la, H4K12la and H4K16la protein expression in control, OGD3h/R21h BV2 cells and sham, MCAO 3d mice. n = 3 for each group. (G) Representative photos of H3K9la⁺ microglia in the sham and MCAO 3d groups. *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001. ns, not significant.

Figure 8. H3K9la stimulates *Ldha* and *Hif-1α* gene transcription.

(A) BV2 cells were subjected to OGD3h/R21h, and ChIP assays were performed with antibodies against H3K9la. (B) Determination of *Ldha* and *Hif-1α* mRNA expression in control and OGD3h/R21h BV2 cells. n = 3 for each group. (C) Determination of Pan-Kla, H2BK16la, H3K9la, H3K14la, H3K18la, H4K5la, H4K8la, H4K12la and

H4K16la protein expression in shNC, shSMEK1, oeNC, and oe-SMEK1 BV2 cells after OGD3h/R21h and in SMEK1^{fl/fl} and SMEK1-mCKI mice after MCAO 3d. n = 3 for each group. (D) Representative photos of H3K9la⁺ microglia in SMEK1^{fl/fl} and SMEK1-mCKI mice. (E) Determination of *Ldha* mRNA expression in shNC, shSMEK1, oeNC and oe-SMEK1 BV2 cells after OGD3h/R21h. n = 3 for each group. (F) LDH activity of shNC, shSMEK1 (n = 3 per group), oeNC and oe-SMEK1 (n = 4 per group) BV2 cells. Determination of *Hif-1α* mRNA (G) and protein (H) expression in shNC, shSMEK1 (n = 3 per group), oeNC and oe-SMEK1 (n = 4 per group) BV2 cells subjected to OGD3h/R21h. *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001. ns, not significant.

Figure 9. MiR-125a-5p regulates the expression of microglial SMEK1.

(A) RT-qPCR analysis of the expression of miR-125a-5p in brain tissue from the sham, contralateral and MCAO 3d groups. n = 3 per group. (B) The expression of miR-125a-5p in BV2 cells subjected to OGD3h/R21h was assessed using qPCR. n = 4 per group. Fluorescence in situ hybridization (FISH) was used to detect the location of miR-125a-5p and SMEK1 in BV2 cells (C) subjected to OGD3h/R21h and in the brains (D) of MCAO 3d mice. (E) The binding position and complementary sequence of miR-125a-5p in the 3'UTR of SMEK1, as predicted by TargetScan. (F) Construction of wild-type (WT) and mutant (MUT) luciferase reporter vectors based on the predicted binding site of miR-125a-5p in the 3'UTR of SMEK1. (G) BV2 cells were cotransfected with the reporter vectors and miR-NC or miR-125a-5p. Luciferase activity was assessed 48 h after transfection. n = 6 per group. Determination of SMEK1 mRNA (H, I) and protein

(J) expression in BV2 cells transfected with miR-NC, miR-125a-5p mimics, inhibitor-NC and miR-125a-5p inhibitors. n = 6 per group. *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001. ns, not significant.

Figure 10. miR-125a-5p inhibitors improved the symptoms of tMCAO mice.

(A) The timeline of the experimental protocol. (B, C) The expression of miR-125a-5p in the brains of MCAO 3d mice after nasal delivery was assessed using qPCR. (D) Determination of SMEK1 protein expression in the brains of MCAO 3d mice after nasal delivery. (E) Neurological function was evaluated by the mNSS test. (F) Representative photographs of TTC staining. (G, H) The expression of IL-10, IL-1 β and TNF- α in the brains of MCAO 3d mice after nasal delivery was assessed using qPCR. (I) Schematic diagram showing the mechanism of SMEK1 in ischemic stroke. n = 3 for each group. *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001. ns, not significant.

Supplementary figure 1.

(A) Representative photographs of laser speckle contrast images of cortical CBF. (B) Infarct size was determined by TTC staining. Determination of IL-1 β mRNA (C) and protein (D) expression in BV2 cells subjected to OGD3h. n = 3 per group. (E) Representative images showing the morphology of BV2 cells in the control, LPS 3 h and OGD 3 h groups. Determination of SMEK1 mRNA (F) and protein (G) expression in BV2 cells subjected to OGD3h/R21h. n = 5 per group. (H) The protein expression of SMEK1 and IL-1 β in shNC and shSMEK1 BV2 cells subjected to OGD3h/R21h was assessed using WB. (I) Gating strategy for microglia isolated from the brains after MCAO 3d mice. *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001. ns, not

significant.

Supplementary figure 2.

(A, B) The expression of CD86, IL-1 β , iNOS, TNF- α , CD206, IL-10 and TGF- β in the brains of mice in the Sham, MCAO 3d and Contralateral groups was assessed using qPCR. n = 3 per group. Determination of SMEK1 mRNA (C) and protein (D) expression in SMEK1^{fl/fl} and SMEK1-KI mice. n = 4 per group. (E) Representative photos of SMEK1⁺ microglia in SMEK1^{fl/fl} and SMEK1-mCKI mice. (F-H) The expression of miR-125b-5p in the brains of mice in the Sham, MCAO 3d, and contralateral groups and in BV2 cells subjected to OGD3h/R21h. n = 3 per group. (I, J) The expression of miR-125a-5p in BV2 cells transfected with miR-125a-5p mimics or inhibitors. n = 3 per group. (K) Western blot analysis of CD206 and IL-1 β in the sham, MCAO 3d and contralateral groups. *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001. ns, not significant.

Supplementary figure 3.

(A) Gating strategy for IL-10⁺ and iNOS⁺ cells in SMEK1⁺ or SMEK1⁻ microglia of tMCAO mice. (B) Flow cytometry analysis of IL-10 and iNOS expression in SMEK1⁺ or SMEK1⁻ microglia from the brains of tMCAO 3d mice. n = 5 per group. (C) Schematic diagram of the experimental design for SMEK1-KI mice. (D) Neurological function was evaluated by the mNSS test. (E) Representative photographs of laser speckle contrast images and quantitative analyses of cortical CBF. (F) Gating strategy for IL-10⁺, iNOS⁺ microglia in the brains of control and SMEK1-KI mice after tMCAO. (G) Flow cytometry analysis of IL-10, iNOS expression in microglia from the brains of

control and SMEK1-KI mice after tMCAO. D-G, n = 8 for each group. *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001. ns, not significant.

Supplementary figure 4.

(A) scRNA-seq profiling of SMEK1^{-/-} and wild-type mouse cells obtained from the hippocampus and cortex (GSE171986). (B) Dot plots of the expression values of enriched cell type-specific markers for the cell types. (C) Uniform manifold approximation and projection (UMAP) plot showing the cell distribution of different cell types. (D) UMAP plot showing the cell distribution among different SMEK1^{-/-} and wild-type mice. (E) Sequenced cell distribution represented in each cluster. (F) UMAP plot showing Tmem119 as a microglia-specific marker gene.

Supplementary figure 5.

(A) The genes (features), counts, and mitochondrial gene percentages of each sample. (B) Correlation heatmaps showing hierarchical clustering of Pearson correlation scores calculated between averaged cell types. (C) Sequenced cell distribution represented in each cell type. (D) UMAP plot showing the distribution of cluster 3 among SMEK1^{-/-} and wild-type mice. (E) UMAP plot showing the distribution of cluster 13 among SMEK1^{-/-} and wild-type mice. (F) UMAP plot showing the distribution of proinflammatory cytokines in microglia.

Supplementary Table 1.

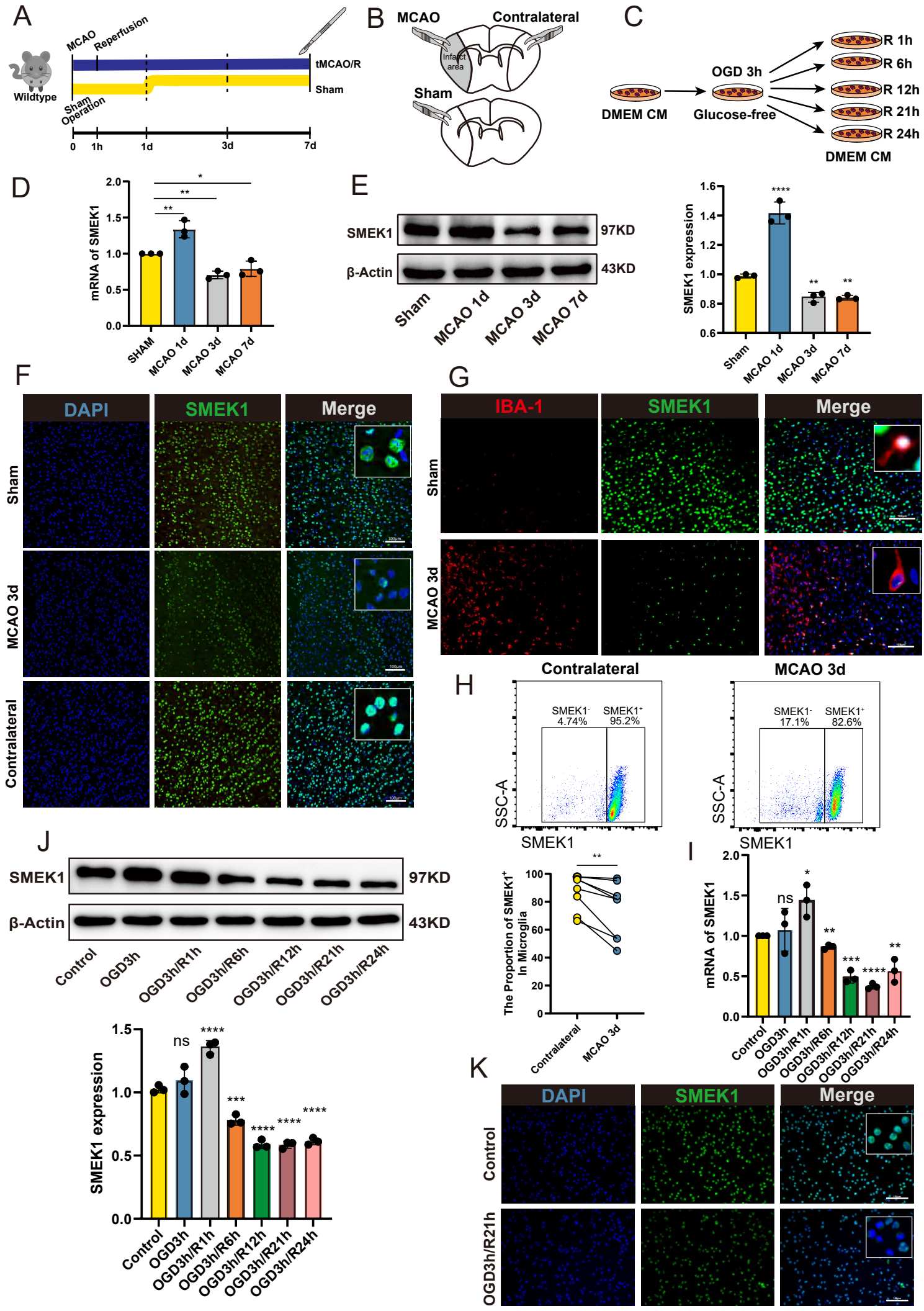
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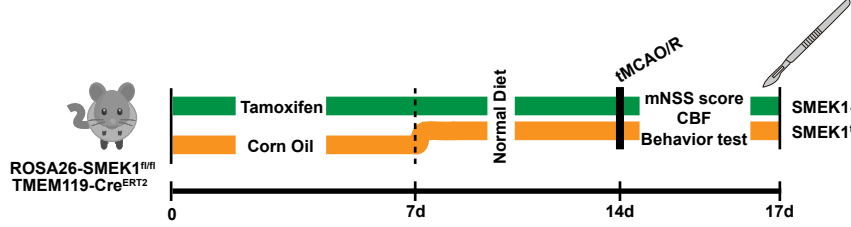
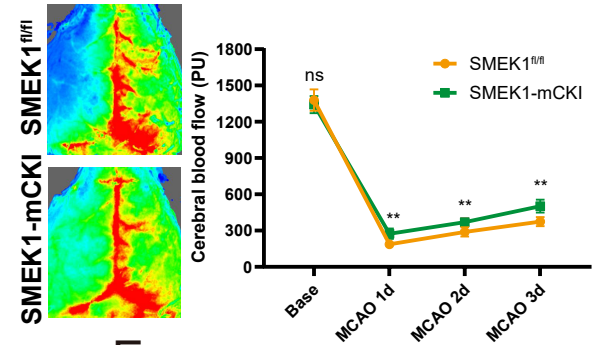
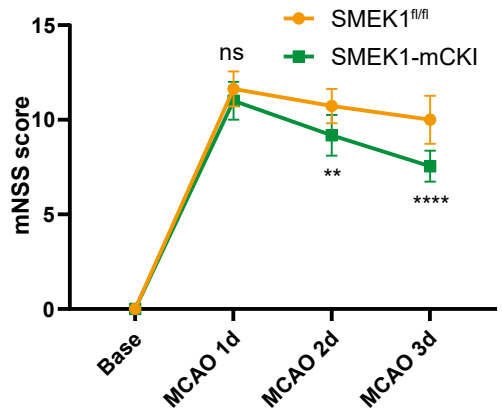
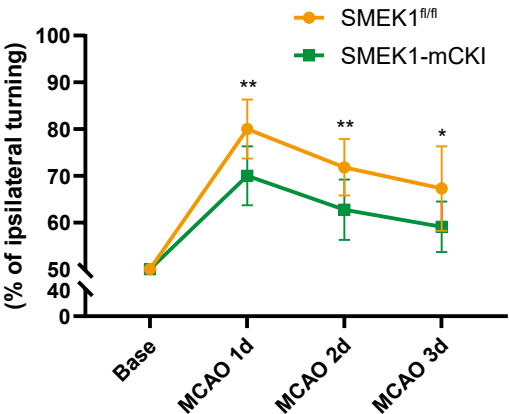
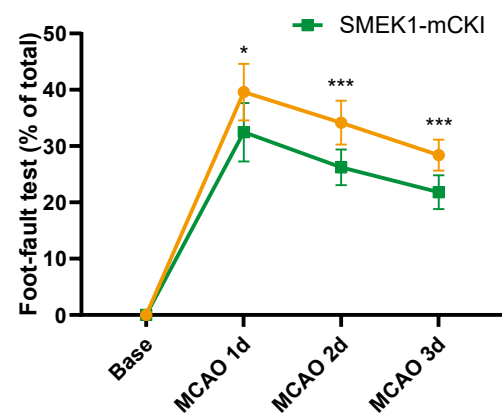
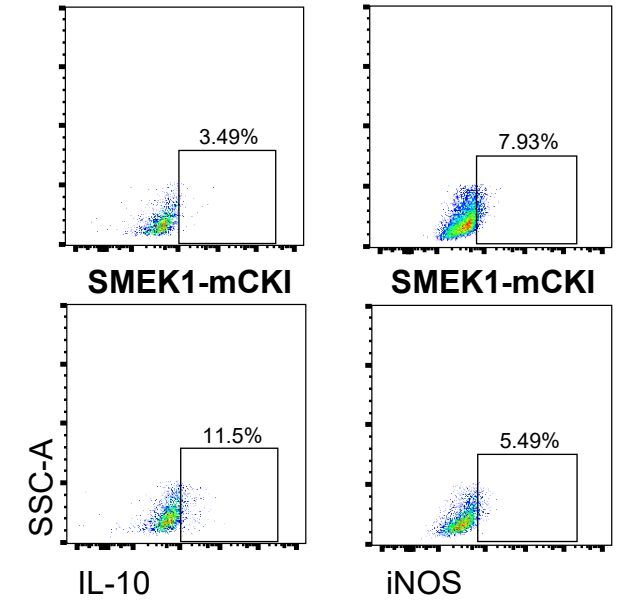
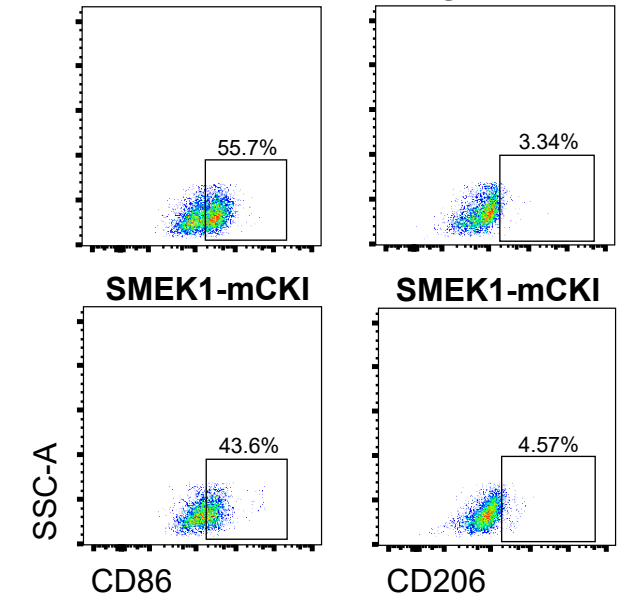
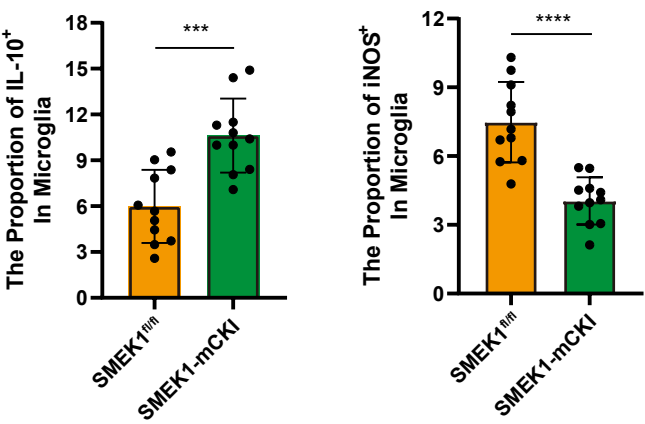
Supplementary Table 2.

The sequences used for miRNA synthesis.

995 **Supplementary Table 3.**

996 The primers used for amplification via ChIP-qPCR.



A**B****C****D****E****F****H****G****I**