Pyruvate Kinase Isoenzyme M2 Impairs Cognition in Systemic Lupus Erythematosus by Promoting Microglial Synaptic Pruning via β-Catenin Signaling Pathway

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Research

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Abstract

Background Neuropsychiatric systemic lupus erythematosus (NPSLE) is the severest complication of SLE, which often involves pathological damage to the brain and cognitive function. Glucose metabolic changes are observed in SLE patients with cognitive impairments by medical imaging. Pyruvate kinase isoform M2 (PKM2) is a vital catalyzer of glucose catabolic pathways and in neurological diseases. However, PKM2 regarding the progress of NPSLE remains poorly studied. Thus, this study aimed to analyze and compare the central carbon metabolites in the validated neuropsychiatric lupus model and control mice.

Methods MRL/Mp-Faslpr (MRL/lpr) female mice were used as NPSLE mouse model, C57BL6 as control. Metabolomics to assess hippocampal glycolysis level. Glucose, lactic acid, IL-6 and IL-1β of hippocampal were detected by ELISA. The expression of PKM2 was detected by qRT-PCR and western blotting, and the localization of PKM2 in microglia and neurons was assessed with IBA-1, NeuN and PKM2 immunohistochemistry. Flow cytometry was used to detect the number and phenotype of microglia. In vitro, after transfected PKM2 overexpression plasmid on BV2, the effect on microglia and β-catenin signaling pathway were detected. Finally, PKM2 inhibitor Shikonin was injected into MRL/lpr mice, behavioral testing were performed to assess cognition, HE and FJB staining were used to evaluate brain damage.

Results Glycolysis was elevated in the hippocampal tissues from MRL/lpr lupus mice, accompanied by an increase in glucose consumption and lactate production. Based on these metabolic variations, PKM2 activation was revealed in hippocampal microglia from lupus mice. Furthermore, PKM2 facilitated microglial phagocytic activity and engulfment of neurons via β-catenin signaling. In vivo, an inhibitor of PKM2, Shikonin, was shown to reduce microglial activation, loss of neuronal synapses, and block β-catenin signaling. Accordingly, the cognitive impairment and brain damage of MRL/lpr mice were relieved.

Conclusion These results indicated that abnormal glycolytic metabolism in the brain tissue of NPSLE mice was induced by PKM2 overexpression, which increased the activation of microglia and the ability of phagocytizing neuronal synapses, leading to neuronal loss and cognitive dysfunction in lupus. These phenomena indicated that inhibition on PKM2 would be a novel therapeutic target for the treatment of lupus encephalopathy.

1. Introduction

Systemic lupus erythematosus (SLE) is a complex autoimmune disease, affecting various organs in the body [1, 2]. Clinically, 12–95% of the lupus patients exert significant psychiatric and neurologic manifestations for neuropsychiatric systemic lupus erythematosus (NPSLE), severely affecting the life quality of patients and increases the disease-related mortality [3]. Among the 19 NPSLE syndromes defined by the American College of Rheumatology (ACR) [4], cognitive dysfunction has been identified by
lupus patients as one of the most distressing symptoms of the disease [5]. It often develops insidiously, can present and progress independently of the systemic lupus erythematosus disease activity index (SLEDAI), and might not respond to standard immunosuppression [6, 7]. Accumulating evidence indicates that complex and interconnected mechanisms jointly promote the development of SLE-related cognitive impairment; however, the specific pathogenesis is yet unclear [8].

Brain function is correlated to an adequate metabolic cost, accounting for 20% of the whole-body energy consumption in humans [9, 10]. In 2000, positron emission tomography (PET) demonstrated that lupus cerebritis was positively associated with significant alterations in glucose metabolism with regional specificity in the brains of SLE patients [11, 12]. Using $^1$H and $^{13}$C nuclear magnetic resonance (NMR) spectroscopy, a well-established MRL/Mp-Faslpr (MRL/lpr) lupus mouse model was established, which verified that total brain glutamine, glutamate, and lactate concentrations increased significantly as compared to the MRL$^{+/+}$ control mice [13]. In SLE patients with cognition impairment, metabolic changes were observed in specific brain regions by multimodal magnetic resonance imaging (MRI) [14]. Interestingly, aging brains had high brain lactate levels and several neurodegenerative diseases that were accompanied by metabolic alterations, contributing to cognitive impairment [15]. In anti-DNA antibodies (DNRAbs)$^+$ mice, significant metabolic changes and spatial memory impairment were observed with neuronal loss in hippocampal regions [16]. Taken together, these studies suggested that the regional metabolic abnormalities are critical neuropathological findings on the cognitive performance in NPSLE. Pyruvate kinase isoform M2 (PKM2), is a crucial mediator of cellular energetics, catalyzes the conversion of phosphoenolpyruvate to pyruvate during glycolysis, and regulates neurological diseases. It was also identified as the hub network protein for identifying the causes of idiopathic autism [11]. Furthermore, a proteomics-based approach revealed that PKM2 was one of the main differentially expressed proteins in the pathogenesis of infantile spasms with severe cognitive dysfunction [12]. Propofol protective effects on ketamine-induced neonatal cognition damage was linked to PKM2 expression in the hippocampus [17]. Moreover, PKM2 was significantly oxidized in the hippocampi of mild cognitive impairment patients and found to be functionally involved in energy metabolism and synaptic plasticity [18]. Furthermore, methamphetamine-induced neurocognitive deficits exhibited PKM2 activity impairment [19]. Nonetheless, in the progress of NPSLE, PKM2 in cognitive dysfunction remains poorly studied.

Microglia are tissue-resident macrophages of the central nervous system (CNS) that play a major role in brain homeostasis [20, 21]. In addition to their intended role in host defense, microglia continuously stretch, retract, and restructure to monitor the functional status of their surroundings and remove the accumulated metabolites or cell debris continually to maintain brain homeostasis [22–24]. In the adult brain, microglia regulate high cognitive functions, such as learning and memory [25, 26]. When homeostasis is lost or brain tissue structure is, microglia undergo several dynamic changes, including changes in cell morphology, such as shortening and swelling of the cellular processes, changes in the surface phenotypes and secretory mediators, and increased proliferation response, known as “activated state” [27]. Microglia activation is a common pathological feature of a range of neurodegenerative diseases, including Alzheimer’s Disease (AD) [28–30]. Also, microglia play a major role in NPSLE, and
their abnormal activation has been detected in the hippocampus of several strains of lupus-prone mice (NZB/NZW, MRL/Ipr, and FcyRIIB−/−·Yaa) [31]. In the process of continuous inflammation, activated microglia-phagocytosed astrocytes promoted neuronal apoptosis and aggravated depression index and cognitive dysfunction in lupus mice [32–34]. Lupus antibodies, DNRAbs, directly activated microglia-mediated neuronal damage and impaired cognitive performance [35]. Hence, in this study, we elucidated the microglia-regulated cognitive dysfunction in NPSLE.

The present study analyzed and compared the metabolites in the hippocampal tissues of the validated neuropsychiatric lupus model and control mice. Based on these variations, the key molecules inducing NPSLE and the pathogenesis of SLE-mediated cognition and brain injury were explored.

2. Materials And Methods

2.1 Materials and reagents

Flow cytometry antibodies and reagents: FITC-conjugated anti-mouse CD11b, APC-conjugated anti-mouse CD45, PE-conjugated anti-mouse LAMP1 and PE-conjugated anti-mouse CD86 were purchased from Biolegend (USA), anti-CD16/CD32 Fc Block and Myelin Removal Beads was purchased from Miltenyi Biotec (Germany). Western blot and immunofluorescence (IF) antibodies: IBA-1 (R&D systems, USA); VGLUT1 (Santa Cruz Biotechnology, USA); LAMP1 and CD68 were purchased from Abcam (United Kingdom); albumin, PSD95 and PKM2 were purchased from Proteintech (China). β-catenin, Cyclin-D1, c-Myc, ENO1, PFKFB3, HK1, LDHA, PDK1, GLUT1 were purchased from Cell Signaling Technology (USA), Horseradish peroxidase (HRP)-linked goat anti-rabbit IgG (FcMACS, China), NeuN (Santa Cruz Biotechnology), donkey anti-rat IgG H&L (Alexa Fluor® 647) (Abcam), goat anti-mouse IgG-TRITC (Abcam), rabbit anti-goat IgG-FITC (FcMACS). ELISA kit: Maltose and Glucose assay kit (RayBiotech, USA), L-lactate assay kit (Cayman, USA), interleukin (IL)-6 (FcMACS), IL-1β (FcMACS). TRizol reagent and SYBR green dye were bought from Invitrogen (Carlsbad, USA). PKM2 overexpression plasmid was purchased from Nanjing Jereh Company (China), and RFectPM Eukaryotic DNA Transfection Kit was purchased from Changzhou EMI Company (China). PKM2 inhibitor Shikonin and β-catenin protein inhibitor (KYA1797K) were purchased from MCE Company (USA). Fluoro-Jade B (FJB) Detection Kit was bought from Merck & Millipore Company (Germany).

2.2 Animals

About 6–8-week-old female MRL/Mp-FasIpr (MRL/Ipr) and C57BL/6 (control) mice [36] were obtained from Shanghai Slaccas Laboratory Animal Breeding Company (Shanghai, China). All mice were maintained under specific pathogen-free (SPF) conditions at a 12 h light/dark cycle and 20–22 °C. The animals were allowed free access to drinking water. The mice were acclimatized to these housing conditions for at least one week. The mice were randomly divided into the following groups with 10 mice in each group: control, administration (Shikonin), MRL/Ipr, treatment (MRL/Ipr + Shikonin). When mice were grown to 10th week, Shikonin and MRL/Ipr + Shikonin groups were administered with Shikonin (5
mg/kg) via intraperitoneal injection, three times/week. When grown to week 20, the mice were sacrificed. The experiments on mice were approved by Institutional Animal Care and Use Committee, Nanjing University, and all experiments were performed in accordance with relevant guidelines and regulations.

2.3 Metabolites extraction

Hippocampus was dissected on a cold plate and frozen in liquid nitrogen. The tissue was homogenized in 80% methanol (made with methanol and water) on ice, vortexed for 30 s, sonicated for 10 min in an ice-water bath, and incubated for 1 h at −20 °C to precipitate the proteins. The supernatant from the remaining sample was obtained by centrifugation at 12000 × g for 15 min at 4 °C. The extracts were dried in a vacuum concentrator without heating. A volume of 100 μL extraction solvent (V acetonitrile: V water = 1:1) was added for reconstitution, which was vortexed 30 s and sonicated for 10 min in a 4 °C water bath, followed by centrifugation for 15 min at 12000 × g at 4 °C. The supernatant (60 μL) of each sample was analyzed for metabolites [37].

2.4 Metabolomic analysis by liquid chromatography-mass spectrometry (LC-MS)

LC-MS/MS analyses were performed using a UHPLC system (1290, Agilent Technologies Santa Clara, CA, USA) with a UPLC HSS T3 column (2.1 mm × 100 mm, 1.8 μm) coupled to Q Exactive (Orbitrap MS, Thermo, USA). The mobile phase A comprised of 0.1% formic acid in water for normal phase (NP-HPLC) and 5 mmol/L ammonium acetate in water for reverse phase (RP-HPLC). The mobile phase B was acetonitrile. The elution gradient was set as follows: 0 min, 1% B; 1 min, 1% B; 8 min, 99% B; 10 min, 99% B; 10.1 min, 1% B; 12 min, 1% B. The flow rate was 0.5 mL/min, and the injection volume was 2 μL. A QE mass spectrometer was used to record MS/MS spectra in an information-dependent manner during the LC-MS experiment. In this mode, acquisition software (Xcalibur 4.0.27, Thermo) continuously evaluates the full-scan survey MS data as it collects and triggers MS/MS spectra acquisition based on the preselected criteria. The electrospray ionization (ESI) source conditions were set as follows: sheath gas flow rate of 45 Arb, Aux gas flow rate of 15 Arb, capillary temperature of 400 °C, full MS resolution of 70000, MS/MS resolution of 17500, collision energy of 20/40/60 eV in normalized collisional energy (NCE) model, and spray voltage of 4.0 kV (positive, POS) or −3.6 kV (negative, NEG).

The raw data were converted into the mzXML format using ProteoWizard and processed by MAPS software (version 1.0). The preprocessing results generated a data matrix that consisted of retention time, mass-to-charge ratio (m/z), and peak intensity. An in-house tandem mass spectrometry (MS²) database was utilized for metabolite identification. The resulting three-dimensional (3D) data involving peak number, sample name, and normalized peak area were entered into SIMCA14.1 software package (V14.1, Sartorius Stedim Data Analytics AB, Umeå, Sweden) for principal component analysis (PCA) and orthogonal projections to latent structure-discriminate analysis (OPLS-DA). PCA showed the distribution of the original data. Supervised OPLS-DA was applied to obtain a high level of group separation and identify variables responsible for classification. Seven-fold cross-validation was used to estimate the robustness and predictive ability of our model. The permutation test further validated the model. A
loading plot was constructed on the basis of OPLS-DA and showed the contribution of variables to differences between the two groups. The first principal component of variable importance in projection (VIP) was obtained to refine the analysis. If \( P < 0.1 \) and VIP > 1, then the variable was defined as a significantly differential metabolite (SDM) between the groups. The SDMs, obtained from LC-MS, were imported into MetaboAnalyst 4.0 to explore different potential metabolic pathways in the hippocampus between MRL/lpr and control groups. As shown in Fig. 1C, the bubble plots demonstrated the main influential metabolic pathways [37, 38].

### 2.5 Measuring glucose consumption and lactate production

The hippocampal tissues of different groups of mice were lysed to obtain hippocampal tissue homogenates. According to the manufacturer's instructions, the Maltose and Glucose Assay Kit was used to determine the glucose concentration in the hippocampal tissue homogenates, while the L-Lactate Assay Kit was used to determine the concentration of lactic acid in the hippocampal tissue homogenate according to the manufacturer's protocol.

### 2.6 Flow cytometry analysis of mouse microglia

The flow cytometry labeling and experimental method of mouse microglia were designed by Bennett et al [39]. (Fig. 4A). The mice were sacrificed by cervical dislocation, hippocampus/cortex was excised on ice immediately, and the tissue dispersed to prepare a single-cell suspension. According to the manufacturer's instructions, the myelin removal beads were used to enrich microglia [40]. After sorting, single cells were pre-blocked with anti-CD16/CD32 Fc Block for 10 min, stained on ice for 30 min with combinations of CD11b-FITC, CD45-APC and CD86-PE, and CD86-PE or LAMP1-PE, rinsed twice with phosphate-buffered saline (PBS), and finally resuspended in 200 µL buffer for subsequent evaluation by flow cytometry (BD Accuri™ C6, BD Biosciences, USA), according to the manufacturer's instructions [41]. Data were analyzed using FlowJo software. Microglia were defined as CD11b\(^+\)CD45\(^{lo}\), proinflammatory microglia were defined as CD11b\(^+\)CD45\(^{lo}\)CD86\(^+\).

### 2.7 Brain pathological evaluation

The whole-brain of the mice was fixed with 4% polyoxymethylene (PFA) and sliced into 5-mm-thick sections for hematoxylin/eosin (HE) staining. The images were observed and photographed under a microscope. The pathological changes in the brain of the control and lupus groups were observed to evaluate brain damage. FJB kit was used to stain the degenerated neurons in the brain to evaluate the neuronal degeneration, according to the manufacturer's instructions [42].

### 2.8 PKM2 detection in microglia and neurons by IF staining

Double IF staining was carried out to detect PKM2 expression in microglia and neurons in hippocampus tissue. Briefly, 8-µm-thick frozen liver slices were fixed in cold methanol/acetone (1:1) for 10 min at −20 °C. After PBS washes, the samples were blocked with 3% bovine serum albumin (BSA) in PBS for 60 min
at room temperature, followed by incubation with anti-IBA-1, anti-PKM2, and anti-NeuN primary antibodies (1:200) overnight at −4 °C. Subsequently, the samples were incubated with goat anti-mouse IgG-TRITC and rabbit anti-goat IgG-FITC secondary antibodies (1:400) for 1 h at room temperature in the dark, followed by nuclei staining with 2-(4-aminophenyl)-1H-indole-6-carboxamidine (DAPI). The slides were visualized using a Nikon Eclipse Ti-U fluorescence microscope equipped with a digital camera (FV300, Olympus, Japan).

### 2.9 RNA extraction and quantitative real-time PCR (qPCR)

Total RNA was extracted from cells using TRIzol reagent, and 1 μg total RNA was reverse transcribed in a 20-μL reaction, according to the manufacturer’s instructions. The oligonucleotide primers used for PCR amplification are listed in Table 1. All reactions were carried out in triplicate. The expression levels of the target genes were normalized to that of GAPDH.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>GCATGGCCTTCCGTGTTCC</td>
<td>GGGTGGTCCAGGGTTTTCTTACTC</td>
</tr>
<tr>
<td>PKM2</td>
<td>TCAGAGCTCAACGCTTGTAGAACTCACTC</td>
<td>CCGCTCGAGAAATGGAAGGTGGAGGG</td>
</tr>
<tr>
<td>ENO1</td>
<td>GCCTCCTGCTCAAAGTCAAC</td>
<td>AACGATGAGACACCATGACG</td>
</tr>
<tr>
<td>HK1</td>
<td>TGCCATGCGCTCTCTTGATG</td>
<td>CTTGACGGAGGCCTGTTGGTT</td>
</tr>
<tr>
<td>PFKFB3</td>
<td>ATTCGGTTTTCGATGCCAC</td>
<td>GCCACAACTGTAGGGTCGT</td>
</tr>
<tr>
<td>LDHA</td>
<td>ATGGCACTCACTAAAGGATCA</td>
<td>GCAACTTGCAGTTCGGGC</td>
</tr>
<tr>
<td>PDK1</td>
<td>AGGCAAGGAAGTCCATCTCA</td>
<td>CCCATGCATTGTGCTACC</td>
</tr>
<tr>
<td>GLUT1</td>
<td>CAATGCTGATGATGCTGGGAGGGAGGATG</td>
<td></td>
</tr>
</tbody>
</table>

### 2.10 Western blot

Proteins were extracted by standard techniques [43]. Typically, total proteins were separated by Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore Co, Bedford, MA, USA). Then, the membranes were blocked in 5% BSA dissolved in TBST (50 mM Tris/HCL, pH 7.6, 150 mM NaCl and 0.1% Tween-20) for 2 h at room temperature and probed with indicated primary antibodies overnight at 4 °C, followed by incubation with appropriate HRP-linked secondary antibody 2 h at room temperature. The immunoreactive bands were visualized using enhanced Chemiluminescence (ECL) plus western blot detection reagents (SupersignalTM West Pico PLUS, Thermo, USA). The gray values were analyzed by Image J software (National Institutes of Health, Bethesda, MD, USA).

### 2.10 Cytokine detection by enzyme-linked immunosorbent assay (ELISA)
ELISA was used to detect the content of inflammatory factors (IL-1β and IL-6) in the hippocampus of mice using the commercial kits, according to the manufacturer's instructions. The mouse hippocampal tissue protein extract was at dilution of 1:5.

2.11 Cell culture and treatment

Microglia cell line BV2 cells and neuronal cell line HT22 cells were obtained from Nanjing Gulou Hospital and cultured in Dulbecco's modified eagle's medium (DMEM) medium with high glucose (Gibco) containing 10% fetal bovine serum (FBS; Gibco) at 37 °C with 5% CO₂. An equivalent of 2×10⁶ BV2 cells/mL was seeded in 6-well or 12-well plates. After cell confluence exceeded 30%, the cells were transfected with PKM2 plasmid. The cells were collected and treated with β-catenin inhibitor (KYA1797K), at a 200 ng/mL concentration. After transfection with PKM2 plasmid or treatment with KYA1797K, BV2 cells were co-cultivated with HT22 cells at a ratio of 1:3 for 24 h and collected cells for subsequent experiments.

2.12 Detection of microglial phagocytosis

The phagocytic capacity of microglia was determined using the method described previously [44]. Briefly, BV2 cells were seeded into 6-well plates at a density of 50,000 cells/well in DMEM medium. FBS and fluorescent yellow-green-labeled 1-mm amine-modified polystyrene latex beads were mixed at a ratio of 1:5 and incubated in a 37 °C water bath for 1 h. Subsequently, the cells were washed twice with PBS and incubated with DMEM-containing fluorescent beads for 1 h at 37 °C. Finally, the cells were collected for flow cytometry detection.

2.13 Behavioral assays

The majority of the 16-week-old MRL/lpr mice exhibited characteristic cognitive dysfunctions [45], as assessed by behavioral assays. Morris water maze (MWM) test was conducted to assess hippocampus-dependent spatial learning and memory functions in rodents [46, 47]. The MWM tests included two parts: the spatial acquisition and probe trials. The spatial acquisition trial was performed for three consecutive days. In each trial, the mouse was allowed to search the platform within 60 s, and could stay on the platform for 5 s after it was located. The mouse that failed to find the platform in 60 s was guided to it manually and ordered to remain on the platform for 15 s and 60 s, respectively, which was regarded as latency. The time spent on searching and mounting the platform (latency) was calculated. On day 4, a probe trial was performed for reference memory by removing the platform. The mice were randomly placed into two selected quadrants, which had different distances to the platform and were allowed to swim freely for 60 s. The percentage of time spent in the target quadrant and platform crossings was recorded, analyzed, and considered as an indicator of memory retention.

The fear-conditioning paradigm was assessed, as described previously [48, 49]. Briefly, mice were trained to associate cage context or an audiovisual cue with an aversive stimulus (foot-shock). The test was administered over two days. On day 1, mice were placed in a cage and exposed to two periods of 30 s
each of paired cue light and 3000-Hz tone, followed by a 2-s foot shock (0.8 mA) with a 180-s interval. On day 2, mice were subjected to two trials. In the first trial assessing contextual memory, mice were re-exposed to the same cage context, and freezing behavior was measured over 1–3 min, using a FreezeScan tracking system (CleverSys, Inc., Reston, VA). In the second trial measuring cued memory, mice were placed in a novel context and exposed to the same cue light and tone from day 1 after 2 min of exploration. The freezing behavior was measured for 1–3 min following the cue.

2.14 Statistical analysis

All data are presented as mean ± standard error of mean (SEM), and each experiment included triplicate sets. The significant differences among groups with one independent variable was determined by one-way analysis of variance (ANOVA) with a Tukey's multiple comparisons test for planned comparisons. P-value < 0.05 was considered significant. Graph Pad Prism 5 was used for data analysis (GraphPad Software Inc., CA, USA).

3. Results

3.1 Central carbon metabolome analysis reveals that elevated glycolysis is involved in hippocampal tissues in MRL/lpr lupus mice

In patients with neuropsychiatric SLE, structural lesions have been identified in the hippocampus [50]. Similarly, structural abnormalities in the hippocampus of MRL/lpr mice have also been reported by Sakic et al. [51]. Moreover, the hippocampus is responsible for memory processing and cognitive function. These findings suggested that the hippocampus is a vital region in the study of the pathogenesis of NPSLE. The metabolic activity in the mammalian brain is tightly regulated to ensure adequate energy substrates for neuronal activity. Surprisingly, the alterations on central carbon metabolism in the hippocampus of neuropsychiatric lupus mice are poorly understood. In the current study, the hippocampus from 20-week-old female MRL/lpr and control mice was harvested and dissected for targeted metabolite analysis. In order to characterize the alterations in the metabolic profiles, 11 types of metabolites, including 6-phosphogluconic acid, were identified from the two groups (Table 2).

Table 2 Metabolic overall analysis among the NPSLE model group and control group.
### Metabolite Name, LOG-Fold Change, P-Value, VIP, Q-Value

<table>
<thead>
<tr>
<th>Metabolite Name</th>
<th>LOG-Fold Change</th>
<th>P-Value</th>
<th>VIP</th>
<th>Q-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>6-Phosphogluconic acid</td>
<td>6.176E-03</td>
<td>0.986</td>
<td>0.346</td>
<td>0.329</td>
</tr>
<tr>
<td>D-Glucose</td>
<td>9.690E-02</td>
<td>0.176</td>
<td>0.528</td>
<td>0.080</td>
</tr>
<tr>
<td>D-ribose-5-phosphate</td>
<td>4.066E-01</td>
<td>0.03</td>
<td>1</td>
<td>0.017</td>
</tr>
<tr>
<td>Fructose 6-phosphate</td>
<td>-1.717E+00</td>
<td>4.312E-04</td>
<td>1.325</td>
<td>5.190E-04</td>
</tr>
<tr>
<td>Glucose 6-phosphate</td>
<td>-2.832E+00</td>
<td>0.001</td>
<td>1.378</td>
<td>6.017E-04</td>
</tr>
<tr>
<td>L-Lactic acid</td>
<td>3.554E-01</td>
<td>9.050E-06</td>
<td>1.474</td>
<td>3.301E-05</td>
</tr>
<tr>
<td>Malic acid</td>
<td>6.597E-03</td>
<td>0.964</td>
<td>0.148</td>
<td>0.324</td>
</tr>
<tr>
<td>Oxoglutaric acid</td>
<td>3.296E-01</td>
<td>0.026</td>
<td>0.829</td>
<td>0.016</td>
</tr>
<tr>
<td>Pyruvic acid</td>
<td>7.167E-01</td>
<td>0.038</td>
<td>0.855</td>
<td>0.020</td>
</tr>
<tr>
<td>Sedoheptulose 7-phosphate</td>
<td>-1.841E+00</td>
<td>1.881E-04</td>
<td>1.414</td>
<td>3.430E-04</td>
</tr>
<tr>
<td>Succinic acid</td>
<td>-2.718E-01</td>
<td>0.261</td>
<td>0.580</td>
<td>0.115</td>
</tr>
</tbody>
</table>

Based on PCA and OPLS-DA, a significant difference was noted in the metabolites of the hippocampus between MRL/lpr and control mice (Figs. 1A, 1SA, 1SB). All the samples were within the 95% confidence interval (CI) (Hotelling’s T-squared ellipse). As shown in Fig. 1B, the metabolite levels were altered in the hippocampus between the two groups. The levels of pyruvic acid, L-lactic acid, and oxoglutaric acid (P < 0.05) were significantly elevated in the hippocampus in MRL/lpr mice (Fig. 1A, 1B), while those of sedoheptulose 7-phosphate, fructose 6-phosphate, and glucose 6-phosphate (P < 0.05) were significantly decreased (Fig. 1A, 1B). In addition, signaling pathway analysis revealed elevated glycolysis and pyruvate in hippocampal tissues in these mice (Fig. 1C), indicating that the glycolysis metabolic pathway in the hippocampus of lupus mice was abnormal.

### 3.2 NPSLE induces increases glucose consumption and lactic acid production in the hippocampus

In the brain, glycolysis produces ATP from glucose, ending with pyruvate conversion to lactate to recycle NADH and continue rapid ATP production during neuronal stimulation. We further determined the glucose consumption and lactic acid production in the hippocampal tissues based on the metabolic profiles in the lupus hippocampus. Compared to the control group, the glucose consumption in the NPSLE group increased significantly, while the amount of lactate production increased markedly (Fig. 2A). These two metabolites were also detected in the cortical tissues, and the results were similar to another region with NPSLE pathology in this mouse strain (Fig. 2A). Furthermore, in the hippocampus and cerebral cortex in imiquimod (IMQ)-induced lupus mice, the glucose consumption and lactate production ex vivo 24 h were significantly increased, which was consistent with the current results from the conventional MRL/lpr NPSLE mice model (Fig. 2SA). Notably, glucose was transported across the plasma membrane by glucose transporters (GLUT), and the mRNA and protein expression of GLUT1 (5-fold and 3-fold increase,
respectively, P < 0.05) was significantly upregulated in the hippocampus of lupus mice (Figs. 2B, 2C, and 2SB) compared to control mice. Hippocampal pyruvate usage, i.e. lactic dehydrogenase kinase A (LDHA) and pyruvate dehydrogenase kinase 1 (PDK1) (4-fold and 1-fold increase, respectively, P < 0.05), also responded by strongly upregulating the mRNA and protein levels (Fig. 2B, 2C). The mRNA and protein expression of GLUT1, LDHA, and PDK1 levels were similar in the cortical tissue, except that no significant change was detected in the PDK1 gene (Figs. 2D, 2E, and 2SC). These results indicated that glycolysis was abnormally elevated in the brain tissue of NPSLE mice, with increasing glucose consumption and lactic acid production.

### 3.3 Glycolysis promotes PKM2 activation in hippocampal microglia

Based on the glycolysis pathway (Fig. 3A), we detected the expression of several critical glycolytic enzymes (HK1, PFKFB3, ENO1, and PKM2) in the hippocampus. Interestingly, only PKM2 expression was significantly increased, while the other key enzymes remained unchanged (Figs. 3B, 3C, and 3SA). These data suggested that the abnormal metabolism of glycolysis in the brain of NPSLE mice could be attributed to the upregulation of PKM2. The results of these key enzymes in cortical tissue are shown in Figs. 3D and 3E, and 3SB. Next, to explore the cell types related to the increased expression of PKM2, we stained PKM2, the microglia marker (IBA-1), and the neuronal marker (NeuN) by IF to analyze the colocalization of PKM2 with microglia and neuron and found that PKM2 is colocalized with IBA-1 or Neun in the hippocampus of these mice (Fig. 3F, 3G). Next, we observed a significant increase in PKM2 intensity colocalized with microglia in the hippocampus of MRL/lpr mice compared to the control mice (Fig. 3F, 3G). These data indicated that the abnormal aerobic glycolysis might be related to the upregulation of PKM2 on microglia.

### 3.4 Increased microglial engulfment of neuronal synapses in the hippocampus of NPSLE mice

A study by Lynch et al. determined that microglia prepared from a glycolytic amyloid precursor protein/presenilin-1 (APP/PS-1) mouse had compromised phagocytic function [52]. Thus, to understand how microglia responds to the pathological PKM2 in the MRL/lpr hippocampus, we counted the percentage of microglia in the tissue area in comparison to the control mice using flow cytometry. Following the experimental procedure shown in Fig. 4A and 4B, MRL/lpr mice had significantly more CD45\(^{low}\)CD11b\(^{+}\)microglia population than the control mice (Fig. 4C). The cells labeled with microglia surface antigen CD86 and analyzed by flow cytometry showed activated microglia in the hippocampi lobe; also, the expression of CD86 on microglia was upregulated in MRL/lpr lupus mice (Fig. 4D). Concurrently, the positive expression of lysosome-associated membrane protein 1 (LAMP1, a marker for activation for phagocytosis) was significantly increased in microglia cells [53], i.e., up to about 80% of microglial cells showed LAMP-1 expression (Fig. 4E). Furthermore, we examined the expression of microglial cell marker (IBA-1) and activated microglia glycoprotein (CD68) levels in the total hippocampus extract by immunoblotting and found abundant levels of hippocampal IBA-1 in both the MRL/lpr mice and control groups (Fig. 4F). The results showed that the expressions of IBA-1 and CD68 were obviously increased in the hippocampus of NPSLE mice compared to the control mice (Fig. 4F), indicating that both
the number and activation of microglia were upregulated. In addition, the IF images of hippocampus in NPSLE mice confirmed that the population of IBA-1$^+$ microglia cell was significantly increased (mean 28 cells/3mm$^2$ [CI 200–300 cells/mm$^2$] vs. 716 cells/mm$^2$ [CI 600–800 cells/mm$^2$] controls; P < 0.01), and also the abundance of activated CD68$^+$IBA-1$^+$ microglia was elevated (Fig. 4G). Additionally, the morphological changes in the microglia included thick cell bodies and abundant branches (Fig. 4G). Also, in IMQ-induced mouse lupus model, a high number and activation of microglia were observed in the hippocampus (Fig. 4SA-B, D-E).

Abnormal phagocytosis of microglia plays a major important role in CNS and impairs cognitive functions such as learning and memory [54-56]. LAMP1 expression on microglia was found to be upregulated by flow cytometry (Fig. 4E), which was also confirmed in the total hippocampal tissue protein extract of NPSLE mice by western blotting (Figs. 4F and 4SC), which indicated a significant anomaly in the phagocytic function of hippocampal microglia in NPSLE mice. Since abnormal neuronal synapses are mainly engulfed by microglia, we detected VGLUT1 (presynaptic membrane marker) and PSD95 (neuronal postsynaptic membrane marker) in the murine hippocampus. Compared to control mice, the expression of both PSD95 and VGLUT1 decreased in NPSLE mice (Fig. 4H). The confocal images confirmed an increase in the fraction of PSD-95 colocalized with IBA-1 in these mice (Fig. 4I), despite the decrease in the number of PSD-95 puncta that reflects synapse loss (Fig. 4I). Microglia-mediated synapse engulfment was further assessed by measuring the phagocytosis of BV2 microglial cells after stimulation with Toll-like receptor7 (TLR7) agonist R848. In vitro experiments indicated that the phagocytosis of neuronal synapses in BV2 cells increased after R848 stimulation (Fig. 5SA–D). Taken together, it could be deduced that NPSLE promotes the activation of microglia in the mice hippocampus, which enhances the removal of synapses by microglia.

3.5 PKM2 facilitates microglial phagocytic activity and engulfment of neurons via β-catenin signaling

In order to elucidate the correlation between PKM2 and microglia engulfment of neuronal structures, we analyzed the expression of PKM2 in BV2 cells after R848 stimulation and found an increasing trend of expression in a dose and time-dependent manner (Figs. 5SF-G). Next, PKM2-overexpressing plasmids were transfected in BV2 microglia (Fig. 5A, 5B). Flow cytometry and western blot experiments showed that the protein level of LAMP1 was significantly increased after overexpression of PKM2 in BV2 cells (Fig. 5C, 5D). Fluorescent microsphere experiments proved that the phagocytosis of BV2 cells was enhanced significantly after overexpression of PKM2 (Fig. 5E). The coculture of BV2 and HT22 cells indicated that the expression of PSD95 on HT22 was downregulated after cotransfection with PKM2 overexpression plasmid (Fig. 5F). Thus, the high expression of PKM2 promoted the phagocytosis of BV2 cells and the engulfment of neurons.

Furthermore, the PKM2-mediated upregulation of microglia phagocytosis through β-catenin signaling pathway is involved in the development of various CNS diseases [57-59]. Hence, we speculated that PKM2 upregulates microglia phagocytosis through β-catenin signaling pathway. This phenomenon was further substantiated by the downstream target genes and proteins of β-catenin signaling pathway on
BV2 cells. These results showed that β-catenin and the downstream target genes/proteins c-Myc and Cyclin-D1 were upregulated after the overexpression of PKM2 (Fig. 6A, 6B). In addition, the level of these genes/proteins also increased after R848 stimulation (Fig. 6SA–D), and the mRNA levels of these genes showed a significant positive correlation with PKM2 (Fig. 6SA, 6B). Similarly, in vivo, the level of β-catenin, c-Myc, and Cyclin-D1 was elevated in the hippocampus of NPSLE mice (Fig. 6C). These findings indicated that PKM2 upregulates the β-catenin signaling pathway.

To further demonstrated the involvement of β-catenin in neuron and synapse loss in NPSLE mice, we selected KYA1797K to inhibit β-catenin pharmacologically [60]. The safe dose of KYA1797K was determined by the CCK8 experiment, and the final concentration of KYA1797K was selected to be 200 ng/mL for subsequent experiments (Fig. 6D). Then, fluorescent bead experiments validated the effect of β-catenin inhibition by KYA1797K, which decreased phagocytosis and the level of LAMP1 in BV2 cells post-PKM2 overexpression (Fig. 6E–G). Moreover, the application of KYA1797K in BV2 cells stimulated by R848 also showed similar results (Fig. 6SE–G). Based on these findings, we concluded that treatment with KYA1797K inhibits the PKM2-induced abnormal phagocytic ability of microglial cells.

### 3.6 Inhibition of PKM2 reduces microglial activation and loss of neuronal synapses and blocks β-catenin signaling

Since PKM2 is required to promote synapse loss, we sought to determine whether chronic inhibition of PKM2 would be effective in preventing neuronal synapse loss in NPSLE mice. Shikonin, an inhibitor of PKM2, inhibits neuronal PTEN after systemic application and reduces apoptosis in the development and under pathological conditions, such as ischemia, trauma, and oxidative stress [61-63]. First, we validated the effect of Shikonin by intraperitoneal (i.p.) injection of 5 mg/kg Shikonin or saline as a control into 10-week-old MRL/lpr mice and determined the PKM2 expression. Next, we found that levels of PKM2 were decreased in murine brain tissues after Shikonin treated (Fig. 7S). Therefore, flow cytometry data showed that the number and activation of microglia in the hippocampus of the MRL/lpr + Shikonin group mice were significantly reduced compared to the MRL/lpr mice (Fig. 7A–C). The expression of PSD95 and VGLUT1 in the MRL/lpr + Shikonin group mice was upregulated compared to the MRL/lpr group (Fig. 7D-E), indicating that the synaptic pruning of neurons was suppressed by inhibiting PKM2. In order to confirm this phenomenon, we performed IF staining of the murine hippocampus in each group. The colocalized microglia IBA-1 and neuronal synaptic PSD95 in mouse hippocampus tissue showed that the synaptic phagocytosis of neurons on microglia in the MRL/lpr + Shikonin group was significantly reduced compared to MRL/lpr group (Fig. 7F). Also, β-catenin signaling pathway was inhibited after Shikonin treatment, and the expression of β-catenin, Cyclin-D1, and c-Myc proteins was decreased (Fig. 7G-H).

We also examined the effect of Shikonin on the peripheral tissues of all groups. The comparison between the spleen and groin lymph node tissues revealed that the recovery post-damage to these tissues in the MRL/lpr + Shikonin group was satisfactory (Fig. 9SA–C). Interestingly, the peripheral immune cells had also undergone tremendous changes. T, B, and Th17 cells in the peripheral blood, spleen, and inguinal
lymph node of mice in each group were examined. We found that T and B cells in the peripheral blood, spleen, and inguinal lymph node of NPSLE mice were activated, and Th17 cells were increased in the spleen. However, Shikonin relieved the activation of T and B cells and blocked the differentiation of Th17 cells, which requires further exploration (Fig. 8SD).

3.7 PKM2 inhibition alleviated cognitive disorders and brain damage in MRL/lpr mice

Hippocampus is responsible for memory processing and cognitive function. Microglia effectuates critical physiological functions in neurocognition and related synapse pruning [64, 65]. Morris water maze and conditional fear tests were applied to investigate whether the PKM2 inhibitor improves the cognitive ability of NPSLE mice. The Morris water maze is an experimental method for testing the learning ability of mice. We found that the swimming trajectories of these two groups of mice are distributed in the four quadrants, and the values averaged on day 1, after three days of training, and the last day of testing on mice. However, on day 3 of training, we observed that the mice in the MRL/lpr group could not log on the platform hidden in the water, and the swimming trajectory map was an exploration mode. The MRL/lpr + Shikonin group mice not only successfully landed on the platform but also swam through the track diagram, indicating that the mouse had more exploration time in the quadrant where the platform was located. On day 4, we counted the number of times the mice crossed the platform and stayed in the quadrant where the platform was located (Figs. 8A, 8B and 9SA, B). The results showed that the mice in the MRL/lpr + Shikonin group surpassed the mice in the MRL/lpr group, irrespective of the bouts they crossed the platform or the times they stayed in the quadrant where the platform was located (Figs. 8C, 8D and 9SC, D). The water maze suggested that PKM2 inhibitor Shikonin improved the learning ability of NPSLE mice. Next, we detected the memory of mice in each group and found that the percentage of rigidity in environmental fear and in fear of sound stimulation was higher in the MRL/lpr + Shikonin group than that in the MRL/lpr group. The MRL/lpr + Shikonin group mice had a better ability to remember fear than the MRL/lpr group (Figs. 8E and 9SE). In summary, both the Morris water maze and the conditional fear test results suggested that PKM2 inhibition improves the learning and memory of NPSLE mice.

Based on the effects of Shikonin on cognitive function, we hypothesized a functional outcome in NPSLE-induced brain damage. Hence, we analyzed the hippocampus Cornu Ammonis fields (CA1, CA2, CA3) and dentate gyrus (DG), and cerebral cortex of the hippocampus of the mouse brain. The neurons in the CA1, CA3, and DG brain areas of the control and Shikonin groups were neatly arranged, and the structure was intact without obvious ghost cells, while the neurons in the MRL/lpr group were scattered and the structure was not distinct. The neurons in the CA1, CA3 and DG brain regions of the MRL/lpr + Shikonin group were neatly arranged, and the number of ghost cells was significantly reduced compared to MRL/lpr mice (Fig. 9A). In the cerebral cortex, the MRL/lpr group showed a large number of neurophages and microglial nodules, which results in severe brain damage, while the MRL/lpr + Shikonin group showed a decline in neuropathy. These results indicated that the PKM2 inhibitor Shikonin relieved brain damage (Fig. 9A). FJB staining determined neuronal degeneration and showed that the neurons in the MRL/lpr group have severe neuronal degeneration, while that in the MRL/lpr + Shikonin group was
significantly reduced (Figs. 9B and 9SF). Next, we detected the integrity of the mouse brain blood-brain barrier (BBB), and the expression of albumin (Albumin) in the hippocampus and cerebral cortex of mice was detected by western blotting. A higher level of intermediate albumin (Albumin) was observed in the MRL/lpr group mice than the MRL/lpr + Shikonin group, indicating that the BBB permeability of the brain of the MRL/lpr group increased, and the BBB of the MRL/lpr + Shikonin group restored (Figs. 9C-D and 9SG). We detected the levels of IL-6 and IL-1β in the hippocampus tissue by ELISA, and the results showed that Shikonin significantly reduces the level of IL-6 and IL-1β (Fig. 9E-F). In summary, the application of the PKM2 inhibitor improved the brain damage of NPSLE mice.

4. Discussion

Herein, we showed that PKM2 is upregulated in lupus microglia and positively regulates the phagocytosis of microglia by activating aerobic glycolysis through the β-catenin signaling pathway, which aggravates the loss of neuronal synapses, promotes the occurrence of cognitive dysfunction in NPSLE, and decreases the expression of PKM2 that relieves the above symptoms. These results indicated that PKM2 might be a new target for the treatment of cognitive dysfunction in NPSLE.

As the pathogenesis of NPSLE is intensively investigated, potential pathogenic factors are being identified. Neurocognitive disorders, considered as a distinct subset of NPSLE, affect attention, memory, executive function, and the processing speed of patients [66]. Intriguingly, neurocognitive decline and depression have been reported in 60% of the patients with SLE [67]. Due to the lack of consensus on the inclusion or exclusion criteria for NPSLE, an overdiagnosis of NPSLE and the administration of unnecessary immunosuppressive treatments is a common occurrence. Currently, several factors, such as cerebrospinal fluid (CSF) IL-6 levels with seizures and interferon-alpha (IFNα) with lupus psychosis, are closely related to the development of NPSLE [68]. In addition, autoantibodies and RNA-protein antigens in CSF form immune complexes and initiate a proinflammatory cascade [69]. Functional neuroimaging, such as PET is being increasingly used in the field of neurology and psychiatry. Previous studies have reported that brain 18F-fluoro-d-glucose PET (FDG-PET) scans frequently reveal a decrease in cerebral metabolism in SLE patients with NP manifestations [70–72]. Although these reports indicate an association between cerebral hypometabolism and NPSLE, only a few studies have focused on the mechanism.

Cellular metabolism provides the means by which cells store and use macromolecules that are necessary for growth and for the generation of energy. Depending on nutrient availability and external or intracellular cues, cells use different substrates and distinct pathways to produce energy [73]. Accumulating evidence states that metabolic reprogramming plays a role in the regulation of innate inflammatory responses [74]. The flexibility of immune cells to adapt to different metabolic demands and diverse metabolic milieu via dynamic regulation of intracellular metabolism is essential for inflammation and tissue homeostasis [75, 76]. For example, macrophages and dendritic cells (DCs), the frontline cells of innate immunity, flip a metabolic switch towards glycolysis and away from oxidative phosphorylation (OXPHOS) as a response to proinflammatory stimuli [77]. In addition, Baik et al. demonstrated that a
switch from OXPHOS to glycolysis following microglia interaction with amyloid β acutely in AD [78]. Since dysfunctional metabolic reprogramming can directly influence and exacerbate defective immune responses, interrogation of the metabolic status of immune cells in SLE has become a topic of interest [79]. In this study, we extracted the metabolites in the hippocampal tissues and conducted metabolomics analysis (LC-MS analysis), which exhibited abnormal aerobic glycolysis metabolites in the hippocampus of MRL/lpr mice. Also, glucose consumption and lactic acid production in the hippocampus and cortex of MRL/lpr mice increased, indicating that the hippocampus tissue of MRL/lpr mice had elevated aerobic glycolysis. Typically, B cells chronically stimulated with B-cell activating factor (BAFF), a cytokine associated with SLE [80], has been shown to undergo enhanced glycolysis and subsequently synthesize more antibodies [81], indicating that the metabolism reconfiguration might influence the pathogenesis of SLE, and increased glycolysis could be linked to mitochondrial oxidative stress in lupus-prone mice [82]. In SLE patients, T cells exhibited persistent mitochondrial hyperpolarization (MHP) due to increased mitochondrial reactive oxygen species (ROS) production, depletion of reduced glutathione (GSH), and diminished mitochondrial ATP synthesis, which predisposes to proinflammatory death by necrosis [83]. These phenomena indicated that immune cells in SLE patients and lupus mice underwent metabolic reprogramming, which was closely related to the development of lupus, rendering metabolism might be a target for modulation in SLE [84].

Glycolysis is the metabolic pathway that converts glucose into pyruvate, controlled by various glycolytic enzymes [85]. PKM2 is an important rate-limiting enzyme in glycolysis that catalyzes the irreversible conversion of phosphoenolpyruvate to pyruvate; this phenomenon could fuel the tricarboxylic acid (TCA) cycle or convert pyruvate to lactate, which is then secreted [86]. PKM2 is present in a few types of proliferating normal cells, but highly expressed in cancer cells and activated immune cells [87, 88] and upregulated in most human cancers [89]. In recent years, the regulatory role of PKM2 in autoimmune diseases has gained increasing attention. Some studies have shown that pharmacological activation of PKM2 inhibits CD4+ T cell pathogenicity and suppresses experimental autoimmune encephalomyelitis (EAE) [90]. PKM2-mediated aerobic glycolysis contributes to macrophage activation and inflammatory response, while PKM2 inhibitor protects the mice from lethal endotoxemia and sepsis [91]. The current data indicated that PKM2 was abnormally highly expressed in the hippocampus of MRL/lpr mice, mainly expressed on microglia, affected the phagocytic function of microglia through β-catenin signaling pathway, and involved in neuronal apoptosis on hypoxic-ischemic encephalopathy in neonatal rats. Double IF labeling showed that PKM2 was mainly localized in the neurons of the ipsilateral cerebral cortex and not in astrocytes or microglia [92]. Some studies revealed that early treatment of recombinant PKM2 (rPKM2) exerts an acute neuroprotective effect against ischemic brain damage, whereas delayed rPKM2 treatment promotes regenerative activities in the poststroke brain, thereby improving functional recovery [93]. These phenomena deduced that PKM2 could be a target for the treatment of inflammatory brain diseases.

Microglia serve as first responders to neuronal damage and infections to restore/maintain homeostasis, which also plays critical roles in neural circuit connectivity [94]. Previous studies have shown histological
evidence of perivascular microglial activation [95, 96] in SLE patients and SLE mouse models based on positive microglial staining of CD68 and Iba-1 antigens [97, 98]. In a recent study, excessive synaptic pruning by microglia was associated with behavioral deficits in the 564Igi model of SLE, which displayed anxiety and cognitive defects [98]. Supposedly, microglia depletion ameliorates disease in multiple models of NPSLE [99]. Herein, we found that microglia proliferate and are activated in the hippocampus and cortex of MRL/lpr mice, which also enhances phagocytosis, which might be related to the abnormal expression of PKM2 on these microglia, thereby resulting in the loss of neurons and the occurrence of MRL/lpr cognitive dysfunction. The use of drugs to inhibit the expression of PKM2 alleviated the activation of microglia and the abnormal enhancement of their phagocytosis, ultimately alleviating the cognitive dysfunction of MRL/lpr mice. B-cell transgenic 564Igi SLE mice microglia exhibited enhanced synaptic pruning leading to synapse loss and development of NPSLE-like disease [100]. The spatial memory impairment in DNRAb + mice is facilitated by microglial-mediated C1q-dependent neuronal damage [101]. Similarly, in another SLE model, SLE-prone mice (CreCOM) microglia showed increased uptake of synaptic material, implying excessive synaptic pruning by this population [102]. These phenomena indicated that the proliferation and activation of microglia are closely related to the occurrence of lupus encephalopathy.

On the other hand, CNS lupus is a heterogeneous disease with many symptoms and causes. Thus, our data suggested that the upregulation of PKM2-mediated aerobic glycolysis enhanced the activation and phagocytosis of microglia, resulting in the loss of neuronal synapses and inducing cognitive dysfunction in lupus. Together, these findings suggested a novel mechanism for CNS lupus and provided a rationale for expanding future clinical trials to include CNS lupus patients, especially those with detectable aerobic glycolysis signatures.

**Conclusions**

In conclusion, we proved that PKM2 plays an important role in the development of cognitive impairment in NPSLE mice, that is, PKM2 up-regulated glycolysis in the brain tissue of NPSLE mice, and increases the activation of microglia and the ability of phagocytizing neuronal synapses, leading to neuronal loss and cognitive dysfunction in lupus. Inhibiting the expression of PKM2 alleviated cognitive impairment and brain damage in NPSLE mice. These phenomena indicated that inhibition on PKM2 would be a novel therapeutic target for the treatment of lupus encephalopathy.

**Abbreviations**

NPSLE
Neuropsychiatric systemic lupus erythematosus; PKM2:Pyruvate kinase isoform M2; SLE:Systemic lupus erythematosus; ACR:American College of Rheumatology; SLEDAI:systemic lupus erythematosus disease activity index; PET:Positron emission tomography; NMR:Nuclear magnetic resonance; MRI:magnetic resonance imaging; DNRAb:anti-DNA antibody; CNS:Central nervous system; AD:Alzheimer’s Disease; HPLC:High-performance liquid chromatography; LC/MS:Liquid chromatography-mass spectrometry;
Declarations

Ethics approval and consent to participate

All animal experiments were complied with the protocols approved by the Jiangsu Provincial Animal Care. The experiments on mice were approved by Institutional Animal Care and Use Committee, Nanjing University, and all experiments were performed in accordance with relevant guidelines and regulations.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Authors’ contributions
Huan Dou, Yayi Hou, Jun Liang and Yang Sun conceived and designed the project. Li Lu, Hailin Wang, Xuan Liu and Xiaoyue Qiao performed the experiments. Li Lu, Hailin Wang, Liping Tan and Jiali Ni analyzed the data. Li Lu wrote the manuscript, and Huan Dou revised the manuscript. All authors read and approved the final manuscript.

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Figures

Figure 1

Non-targeting metabolomics analysis reveals elevated metabolites in glycolysis in MRL/lpr mice. A. PCA model score scatter plot and OPLS-DA model show separation of glycolysis metabolites in the
NPSLE promotes cell glycolysis in the hippocampus, with increased expression of GLUT1, PDK1, and LDHA. A: Glucose levels and lactate production in the hippocampus and cerebral cortex of the MLR/lpr mice were normalized to that of the control group. B: The mRNA expression of GLUT1, LDHA, and PDK1 in the hippocampus of control and MLR/lpr groups. C: Western blot quantification of GLUT1, LDHA, and PDK1 in the hippocampus of control and MLR/lpr groups. D: The mRNA expression of GLUT1, LDHA, and PDK1 in the cerebral cortex of the control and MLR/lpr groups. E: Western blot quantification of GLUT1, LDHA, and PDK1 in the cerebral cortex of control and MLR/lpr groups. Data are presented as mean scores ± SEM. *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001. n = 6 mice per group.
Figure 3

In the NPSLE group, PKM2 was upregulated in aerobic glycolysis and expressed in hippocampal microglia. A: The simplified scheme indicating the main metabolic routes followed by glucose, the key intermediates and enzymes involved, and only the enzymes studied in the text are highlighted for simplicity. HK1: hexokinase 1; PFKFB3: 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 3; ENO1: alpha-enolase; PKM2: pyruvate kinase M2 isoform; LDHA: lactate dehydrogenase A. Dashed arrows indicated downstream intermediates or metabolic pathways. B: The mRNA expression of PKM2, HK1, PFKFB3, and ENO1 in the hippocampus of control and MLR/lpr groups. C: Western blot quantification of PKM2, HK1, PFKFB3, and ENO1 proteins in the hippocampus of control and MLR/lpr groups. D: The
mRNA expression of PKM2, HK1, PFKFB3, and ENO1 in the cerebral cortex of control and MLR/lpr groups. E: Western blot quantification of PKM2, HK1, PFKFB3, and ENO1 in the cerebral cortex of control and MLR/lpr groups. F: The expression levels of IBA-1 (red) and PKM2 (green) and their colocalization (yellow) in the hippocampus of control and MLR/lpr groups by IF staining; Bar = 20 μm. G: The expression levels of NEUN (red) and PKM2 (green) and their colocalization (yellow) in the hippocampus of control and MLR/lpr groups were detected by IF staining; Bar = 20 μm. Data represent the mean scores ± SEM. *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001. n = 6 mice per group.

Figure 4

NPSLE activated microglia and induced the engulfment of neurons. A: The schematic of sample preparation before flow cytometry detection of mouse hippocampal microglia. B: Gating strategy of microglia (CD45loCD11b+) in the hippocampus by flow cytometry. C: The percentage of microglia
(CD45loCD11b+) in the hippocampus of control and MLR/lpr groups. D: The expression level of CD86 in the microglia of control and MLR/lpr groups was detected by flow cytometry. E: The expression level of LAMP1 in the microglia of control and MLR/lpr groups was detected by flow cytometry. F: Western blot quantification of LAMP1, CD86, and IBA-1 in the hippocampus of control and MLR/lpr groups. G: The expression levels of CD68 (green), IBA-1 (red), and their colocalization (yellow) in the hippocampus of control and MLR/lpr groups were detected by IF staining; Bar = 20 μm. H: Western blot quantification of PSD95 and VGLUT1 in the hippocampus of control and MLR/lpr groups. I: The expression levels of PSD95 (green), IBA-1 (red), and their colocalization (yellow) in the hippocampus of control and MLR/lpr groups were detected by IF staining; Bar = 20 μm. Data are presented as mean scores ± SEM. *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001. n = 6 mice per group.

**Figure 5**

PKM2 overexpression stimulated phagocytic activity and engulfment of neurons. Transient transfection of BV2 cells with the negative control plasmid (NC) and PKM2 plasmid (PKM2) induced high expression of PKM2. At 24 h post-transfection, the cells were collected for RT-PCR, western blot, and flow cytometry. A: The expression of PKM2 mRNA was detected by RT-PCR at 24 h after PKM2 plasmid transfection. B: Western blot quantification of PKM2 at 24 h after PKM2 plasmid transfection. C: Western blot quantification of LAMP1 at 24 h after PKM2 plasmid transfection. D: The expression level of LAMP1 was detected by flow cytometry at 24 h after PKM2 plasmid transfection. E: Phagocytic function test. Firstly,
FBS and fluorescent beads were mixed in a ratio of 1:5 and incubated at 37 °C for 1 h. This mixture was diluted with DMEM complete medium to achieve the final concentration of fluorescent beads and FBS in DMEM as 0.01% (v/v) and 0.05% (v/v), respectively. Then, the original medium was replaced with DMEM containing fluorescent beads, and the mixture was cultured in an incubator for 2 h. Finally, BV2 cells were collected, and the fluorescent beads in BV2 cells were detected by flow cytometry. F: Western blot analysis of the PSD95 after HT22 cells cocultured with BV2 cells were transfected with NC and PKM2 high expression plasmids. Data represent the mean scores ± SEM. *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001. n = 3 mice per group.
PKM2 facilitated phagocytosis via β-catenin signaling pathway. Transient transfection of BV2 cells with negative control plasmid (NC) and PKM2 plasmid (PKM2) elevated the expression of PKM2. A: The expression of β-catenin, c-Myc, and Cyclin-D1 mRNA was detected by RT-PCR analysis at 24 h after PKM2 plasmid transfection. B: The protein expression of β-Catenin, c-Myc, and Cyclin-D1 was detected by western blot analysis at 24 h after PKM2 plasmid transfection. C: Western blot quantification of β-catenin, c-Myc, and Cyclin-D1 in the hippocampus of control and MLP/lpr mice. D: BV2 cells were treated with different concentrations of β-catenin inhibitor (KYA1797K) for 24 h, CCK8 detected the cytotoxicity of KYA1797K to BV2 cells. E: Phagocytic function test. Transient transfection of BV2 cells with negative control plasmid (NC) and PKM2 plasmid (PKM2) elevated PKM2 expression. After transfection, BV2 cells were treated with or without 200 ng/mL β-catenin inhibitor (KYA1797K) for 24 h. BV2 cells were cultured with DMEM- containing fluorescent beads in the incubator for 2 h. Finally, the cells were collected and detected by flow cytometry. F: Transient transfection of BV2 cells with negative control plasmid (NC) and PKM2 plasmid (PKM2)-induced PKM2 high expression. After transfection, BV2 cells were treated with or without 200 ng/mL β-catenin inhibitor (KYA1797K) for 24 h. The expression level of LAMP1 was detected by flow cytometry. G: Transient transfection of BV2 cells with negative control plasmid (NC) and PKM2 plasmid (PKM2) increased the expression of PKM2. After transfection, BV2 cells were treated with or without 200 ng/mL β-catenin inhibitor (KYA1797K) for 24 h. Western blot analysis of PSD95 protein. after HT22 cells were cocultured with BV2 cells transfected with NC plasmid and PKM2 high expression plasmid. Data are presented as mean scores ± SEM. *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001. n = 3 mice per group.
PKM2 inhibitor downregulated microglia excessive phagocytosis of neurons and blocked β-catenin signaling pathway. Female mice, aged 7-8-week-old, were divided into four groups: control, Shikonin, MRL/lpr, and MRL/lpr + Shikonin. Each group consisted of 10 mice, and the mice were maintained until week 10. The PKM2 inhibitor (KYA1797K) was injected intraperitoneally into mice at a dose of 5 mg/kg, three times a week until week 20. A: The percentage of microglia (CD45loCD11b+) in the hippocampus of

Figure 7
each group mice. B: The expression level of CD86 in microglia of each group mice was detected by flow cytometry. C: Western blot quantification of LAMP1 in the hippocampus of each group mice. D: Western blot quantification of PSD95 and VGLUT1 in the hippocampus of each group. E: Western blot quantification of PSD95 and VGLUT1 in the cerebral cortex of each group. F: The expression levels of PSD95 (green), IBA-1 (red), and their colocalization (yellow) in the hippocampus of the mice in each group were detected by IF; Bar = 10 μm. G: Western blot quantification of β-catenin, c-Myc, and cyclin-D1 in the hippocampus of each group. H: Western blot quantification of β-catenin, c-Myc, and cyclin-D1 in the cerebral cortex of each group. Data are represented as mean score ± SEM. *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001. n = 6 mice per group.

Figure 8

Inhibiting PKM2 alleviated cognitive disorders in MRL/lpr mice. A: Morris water maze test. Representative swimming traces of mice from different groups on training day 1 and 3. The hidden platform is located in quadrant III. B: The time latency to find the hidden platform in different groups of mice during consecutive 3 training days. C: The swimming trajectory of the test mice on day 4 of the water maze experiment. D: On day 4 of the water maze experiment, the time they stayed in the target quadrant (left) and the number of times the mice crossed the platform (right) was recorded. D: Fear conditioning tests. The percentage of the stiffness of mice was recorded during the test: different effects of 40-Hz light intervention, 2000-Hz sound intervention, and photoacoustic treatment on contextual and cue memory,
respectively. Data are represented as mean ± SEM. *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001. n = 10 mice per group.

**Figure 9**

Inhibiting PKM2 improves brain damage in lupus mice. A: HE staining of the coronal area of the mouse brain of each group mice; Bar = 200 μm. B: FJB staining of the hippocampus of each group mice; Bar = 50μm. C: Western blot quantification of albumin in the hippocampus of each group mice. D: Western blot
quantification of albumin in the cerebral cortex of each group mice. E: The expression levels of IL-6 of the hippocampus of each group was measured by ELISA. F: The expression levels of IL-1β in the hippocampus of each group was measured by ELISA. Data are represented as mean ± SEM. *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001. n = 6 mice per group.

**Supplementary Files**

This is a list of supplementary files associated with this preprint. Click to download.

- Supplementarymaterials.pdf