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ORIGINAL RESEARCH

Identification and Evaluation of Lipocalin-2 in Sepsis-Associated Encephalopathy via Machine Learning Approaches

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Purpose: Sepsis-associated encephalopathy (SAE) critically contributes to poor prognosis in septic patients. Identifying and screening key genes responsible for SAE, as well as exploring potential targeted therapies, are vital for improving the management of sepsis and advancing precision medicine.

Patients and Methods: Single-cell RNA sequencing (scRNA-seq) was administrated to identify cell subpopulations related to poor prognosis in septic patients. Next, hierarchical dynamic weighted gene co-expression network analysis (hdWGCNA) was employed to identify genes associated with specific neutrophil subpopulations. Enrichment analysis revealed the biological functions of these genes. Subsequently, neuroinflammation-related genes were obtained to construct a neuroinflammation-related signature. The AddModuleScore algorithm was used to calculate neuroinflammation scores for each cell subpopulation, whereas the CellCall algorithm was used to assess the crosstalk between neutrophils and other cell subpopulations. To identify key genes accurately, four binary classification machine learning algorithms were utilized. Finally, Western blotting and behavioral tests were used to confirm the role of LCN2-related neuroinflammation in septic mice.

Results: This study utilized scRNA-seq to reveal the critical role of peripheral neutrophils during sepsis, identifying these neutrophils as contributors to poor prognosis and associated with neuroinflammation. On the basis of various machine learning algorithms, we discovered that Lipocalin-2 (LCN2) may be the key gene involved in neutrophil-induced SAE. To prove these findings, we conducted in vivo experiments and an animal model. Increased LCN2 expression and cognitive dysfunction occurred in septic mice. Additionally, the levels of markers of astrocytes and microglia and inflammatory factors such as TNF-α and IL-6 were significantly increased. All these phenomena were reversed by the downregulation of LCN2.

Conclusion: The upregulation of LCN2 expression on peripheral neutrophils is a critical step that triggers neuroinflammation in the central nervous system during SAE.

Keywords: sepsis-associated encephalopathy, cognitive dysfunction, lipocalin-2, single-cell RNA sequencing, machine learning, neuroinflammation

Introduction

According to statistical data, up to 70% of patients suffering from severe sepsis will develop SAE, which significantly contributes to the high mortality rates and poor prognoses observed in septic patients.[1,2] Given the increasing incidence

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and decreasing mortality rates of sepsis, SAE has emerged as a critical public health issue that poses a threat to individual health and societal development, necessitating urgent intervention.

SAE is characterized primarily by diffuse brain dysfunction, with clinical manifestations including alterations in consciousness, cognitive decline, neurological deficits, and behavioral abnormalities.[3] The pathological mechanisms underlying SAE are highly complex and influenced by numerous factors. Important mechanisms associated with SAE include excessive microglial activation,[4] impaired endothelial barrier function, cerebral ischemia and blood-brain barrier (BBB) dysfunction.[5] During sepsis, impaired cerebral circulation can lead to insufficient cerebral blood flow, which may be associated with electrophysiological and neurological changes.[6,7] At the same time, the dopaminergic, β-adrenergic, GBAB receptors, and cholinergic nervous systems are impaired to a certain extent.[8] Recent investigations into sepsis have elucidated the pivotal roles of neuroinflammation and blood–brain barrier (BBB) disruption in precipitating SAE.[9,10] Increased permeability of the BBB due to elevated levels of proinflammatory cytokines is considered an initial trigger for SAE.[11] Compromised BBB integrity permits peripheral proinflammatory cytokines to infiltrate the central nervous system (CNS), resulting in the activation of microglia and astrocytes.[12–14] Notably, infiltration of peripheral immune cells into the CNS followed by subsequent microglial activation within the CNS is believed to contribute significantly to both acute and chronic brain dysfunction.[15–17] Astrocytes are recognized as key regulators within the nervous system; they release various mediators, such as tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), and interleukin-1β (IL-1β), which play crucial roles in neuroinflammatory processes,[18–20] thereby contributing substantially to the neuronal damage associated with SAE.

In this study, we conducted a detailed analysis of hub genes that are overexpressed in deceased sepsis patients compared with recovered patients, employing both scRNA-seq and bulk data. Then, machine learning algorithms were utilized to identify several genes related to poor sepsis prognosis and neuroinflammation, ultimately revealing key feature genes associated with post-sepsis neuroinflammation. The reliability of our findings was confirmed through animal experiments. This research aims to enhance the understanding of neuroinflammation following sepsis, laying a theoretical groundwork for targeted treatments for systemic inflammatory response syndrome.

Materials and Methods

Transcriptome Data Acquisition

ScRNA-seq data (GSE167363) were obtained from the Gene Expression Omnibus (GEO) database (https://www.ncbi.nlm.nih.gov/geo/), which contains 12 human peripheral blood mononuclear cell (PBMC) samples (2 healthy controls, 4 non-survivors of sepsis patients, and 6 survivors of sepsis patients). The bulk RNA sequencing data were also acquired from the GEO database (GSE198861, GSE65682). To ensure the uniqueness of the variables, we retained 4 samples from normal diet-fed mice that underwent either (polymicrobial peritoneal sepsis) PCI treatment or not (sham), after excluding the samples from mice that received the PLX5622 diet in GSE198861. GSE65682 is an RNA sequencing cohort that includes blood samples from healthy controls and sepsis patients. On the basis of GeneCards data (https://www.genecards.org/), we identified 984 neuroinflammation-related genes for further analysis.

Analysis of the scRNA-Seq Data

Using the Seurat R package, we filtered cells on the basis of the following criteria: gene expression counts exceeding 200 and less than 10% mitochondrial gene expression. The expression matrix was normalized via the Normalized Data function. After computing principal components, the UMAP function was employed to visualize different cell types. Ultimately, we identified six distinct cell types on the basis of classical cell markers. To further investigate the relationship between neuroinflammation and PBMCs, we calculated neuroinflammation scores for each cell type via the AddModuleScore function, categorizing the cells into high and low groups on the basis of the median value.

hdWGCNA Analysis and Functional Enrichment Analysis

We employed hdWGCNA to identify pivotal genes related to special subpopulations of neutrophils. On the basis of the hdWGCNA, we identified modules that were highly related to specific subpopulations. On the basis of intra-module

correlation, the top 100 genes were considered hub genes closely associated with the specific neutrophil subpopulations in samples from non-survivors. We performed functional enrichment analysis base on Metascape database (https://Metascape.org/) after we obtained the hub genes.

Analysis of cell–cell Communication and Signaling Pathways

The CellCall R package was employed to analyze intercellular communication. The TransCommuProfile function calculated the communication weights among cells and inferred signaling pathways based on transcription factor activity. Finally, the plotBubble function visualized the signaling pathways.

Machine Learning Analysis

To identify key genes involved in the occurrence of SAE, we used multiple machine learning algorithms. Firstly, we converted the mouse gene names to human gene names on the basis of the human reference genome GRCh38. For the binary classification matrix, the LASSO-LR, random forest, Boruta, and svmREF algorithms were used to select feature variables. The intersection of the results from the four algorithms ultimately identified LCN2 as the key gene.

Receptor Ligand Docking

The structure of the protein LCN2 was acquired from the Protein Data Bank (https://www.rcsb.org/), while the molecular structures of the inhibitors were obtained from the PubChem database (https://pubchem.ncbi.nlm.nih.gov/). The molecular structures were converted into various file formats via Open Babel software. Molecular docking was conducted with AutoDock to predict the binding interactions between the ligands and the target protein. During protein structure preparation, water molecules were removed, and polar hydrogens were added, while the ligands undergo geometric optimization and appropriate charge distribution. The docking results were visualized and analyzed via PyMOL.

Animals

Male C57BL/6J wild-type mice (8 weeks old, 20–25 g) used in this research were purchased from the Animal Experiment Center of Anhui Medical University. The mice were fed at a room temperature of 23°C-25°C and a humidity of 50% using a controlled day–night cycle (12 hours/dark) and received food and water ad lib. The mice were randomly grouped, and the researchers remained blinded to the group information throughout the experiment. In this study, all animal experiments adhered to ethical and welfare standards and were approved by the Institutional Animal Care and Use Committee at Anhui Medical University.

Cecal Ligation and Puncture (CLP) Surgery

Intraperitoneal anesthesia was induced using pentobarbital at a dosage of 50 mg/kg. To access the abdominal cavity, an approximately 2–3 cm incision was made 1.5 cm below the xiphoid. The cecum was sutured with 3–0 silk thread and punctured twice with a 21G needle. The cecum was then compressed to release the fecal contents into the abdominal cavity. The same surgical procedure was administrated to the mice of sham group without suturing or puncturing the cecum. Postoperatively, the mice were treated with 0.9% saline (50 mL/kg) and clindamycin (30 mg/kg) for 3 days.

Intracisternal Injection for LCN2 Neutralization

After anesthetizing the mice with pentobarbital at a dosage of 50 mg/kg, the mice's head were fixed in a stereotactic frame. Following exposure of the skull, a small craniotomy was performed via a dental drill under a surgical microscope. One hour before CLP, neutralizing antibody targeting LCN2 (5 μg, R&D Systems, catalog number: MAB1857) or isotype IgG control antibody (5 μg, Absin, catalog number: abs1890066) was injected into the cerebral ventricle via a 30G Hamilton syringe. The syringe was held in position for 5 minutes before removal. The incision was then closed via 4–0 absorbable sutures.

Novel Object Recognition Test (NOR)

Seven to eleven days after CLP surgery and cerebral ventricle injection, a novel object recognition test was conducted on the basis of a predefined protocol. Before the baseline test, a ten-minute acclimation to the testing chamber was conducted for 3 days. During the training phase, two identical objects were placed in the testing chamber, allowing the mice to explore freely for 6 minutes. In the subsequent novel object phase, one object was replaced with a new object of similar size but different shapes, and the mice were allowed to explore for another 6 minutes. To maintain consistency in the experimental conditions, the testing chamber was cleaned with 75% ethanol and distilled water successively between trials. The time spent exploring the familiar object (Tf) and the novel object (Tn) was recorded. The preference index was calculated via the formula Tn/ (Tn + Tf) × 100%. The discrimination index was calculated via the following formula: (Tn - Tf)/ (Tn + Tf) × 100%.

Morris Water Maze (MWM)

The Morris water maze test was conducted to assess mice's spatial learning, memory, and cognitive flexibility, all of which depend on normal hippocampal function. The water maze composed of a circular pool with a diameter of 120 cm, filled with opaque water, and was maintained at a temperature of 23 ± 1°C. The diameter of the escape platform is 10 cm, and the water surface was 1 cm above the platform. The mice were placed in the four quadrants with a 20-minute interval between each trial, and the time taken to find the platform was recorded as the escape latency. The training lasts for five consecutive days. The mice will be placed on the platform for 10 seconds if they failed to find the platform within 60 seconds. The average escape latency across all trials was assessed. After the learning phase, a probe trial without the platform was conducted to record the first time the platform was found, the number of entries and the total time spent in the target quadrant within 60 seconds.

Immunofluorescence Staining

The animals were first anesthetized with pentobarbital sodium and then perfused with phosphate-buffered saline (PBS) and 4% paraformaldehyde successively. The brain tissue was fixed in 4% paraformaldehyde overnight and then dehydrated in 20%, 30%, and 40% sucrose solutions in turns. Coronal frozen sections 1.46~ 1.92 mm posterior from bregma were prepared at a thickness of 30 microns. After washing the slices with PBS, they were treated with 0.5% Triton X-100 (Sigma) and 0.5% bovine serum albumin (BioFroxx) for one hour. For double immunofluorescence analysis, the tissue slices were incubated with GFAP antibody (Cell Signaling, catalog number: 3670s, 1:400) and Iba-1 antibody (Wako, catalog number: 011–27,991, 1:400). Secondary antibodies conjugated with Alexa Fluor-488 or Alexa Fluor-568 (1:1000) were used, followed by DAPI staining provided by Invitrogen. Images were captured via a laser confocal microscope with a 20x objective lens (Carl Zeiss AG, LSM900).

Western Blotting

Cold RIPA lysis buffer (Beyotime, China) was used to lysed the hippocampal tissue for 15 minutes, followed by centrifugation at 12,000 × g for 15 minutes. The protein concentration in the lysate was measured by a BCA assay kit (Beyotime). After denaturation in sample buffer at 95°C for 10 minutes, 20–25 μg protein samples were separated by 10% SDS–PAGE gel at 70 V for 30 minutes and then at 120 V for 90 minutes. The proteins were subsequently transferred to a PVDF membrane (Millipore) at 200mA. 5% non-fat milk melted in Tris-buffered saline with Tween-20 (TBST) was used to blocked the membrane at room temperature for 2 hours, and then incubated overnight at 4°C with GFAP antibody (Cell Signaling, catalog number: 3670s, 1:2000), LCN2 antibody (ABclonal, catalog number: A2092, 1:1000), IL-6 antibody (Proteintech, catalog number: 21865-1-AP, 1:2000), TNF-α antibody (Proteintech, catalog number: 17590-1-AP, 1:2000), and GAPDH antibody (Proteintech, catalog number: 60004-1-Ig). After a 1-hour incubation at room temperature with HRP-conjugated secondary antibody, the membrane was exposured by enhanced chemiluminescence (Affinity, catalog number: KF8003). To quantify protein abundance, the band density was analyzed by ImageJ.

Statistical Analysis

All bioinformatics analyses and data processing in this study were conducted via R software, whereas experimental data analysis was performed via GraphPad Prism 8.0 software. The results are presented as the means ± standard error of the mean (SEM). Escape latency was analyzed by two-way repeated measure ANOVA during the learning phase. One-way ANOVA followed by Tukey's post hoc test was employed in the remaining data. Differences were considered significant when $P < 0.05$.

Results

Landscape of the scRNA-Seq Data of PBMCs from Sepsis Patients

The workflow diagram of this research is shown in Figure 1. A total of 46,459 cells from peripheral blood mononuclear cell (PBMC) samples of 12 patients were annotated as B cells (CD79A, MS4A1), monocytes (LYZ, S100A4), T cells (CD3E, CD3D), neutrophils (CD177), natural killer cells (NKG7, KLRD1), and platelets (PPBP) (Figure 2A). The heatmaps subsequently illustrated these classic cell markers on the basis of their expression levels (Figure 2B and C). The bar chart enabled visual observation of the differences in the cell composition of PBMCs among the patients. In non-surviving septic patients, the proportions of neutrophils and platelets were significantly higher (Figure 2D). Therefore, our subsequent research focused on neutrophils.

Identification of Hub Genes Within Special Subpopulations of Neutrophils

To further investigate the role of neutrophils in sepsis, we conducted additional dimensionality reduction and clustering analysis on neutrophils (Figure 3A). Neutrophils in Cluster 0 may play a crucial role in the progression of sepsis, as their proportion was significantly higher in PBMC samples from non-surviving patients than in those from other cell types (Figure 3B). hdWGCNA analysis was employed to identify gene modules closely associated with specific subpopulations (Figure S1A), resulting in the identification of five gene modules on the basis of the co-expression network (Figure 3C and D). On the basis of the intra-module correlation, the blue gene module was found to be strongly correlated with cluster 0 neutrophils (Figures 3E, S1B and C). We subsequently performed functional enrichment analysis and determined that the genes included in the blue module were enriched in neutrophil degranulation and the EPH-Ephrin signaling pathway (Figure 3F).

Cell–cell Communication Network of Neuroinflammation-Related Signaling Pathways

Neuroinflammation plays an imperative role in the onset and progression of SAE. To further investigate the potential relationship between SAE and neuroinflammation, we identified 19 neuroinflammation-related genes within the blue gene module (Figure 4A). In the GSE65682 cohort, these genes were predominantly overexpressed in sepsis patients (Figure 4B). Furthermore, a neuroinflammation-related gene signature was developed on the basis of these genes, and cells were scored accordingly. According to the single-cell atlas, the neuroinflammation scores of neutrophils were significantly greater than those of other cell types (Figure 4C and D). Additionally, the neuroinflammation scores of neutrophils in non-surviving sepsis patients were significantly greater than those in other patients (Figure 4E).

Neutrophils were categorized into high and low groups on the basis of the median neuroinflammation score. Cell–cell communication analysis revealed that neutrophils with high neuroinflammation scores in sepsis patients presented increased intercellular crosstalk with other cells (Figure 4F and G). Additionally, the communication co-expression network revealed significant differences in signaling pathways, particularly the MAPK and HIF-1 signaling pathways, between the high- and low- neuroinflammation groups (Figure 4H). During SAE development, neutrophils activated by sepsis likely serve as key mediators.

Identification of LCN2 as a Key Gene via Machine Learning Algorithms

To more accurately identify key targets in the development of SAEs, four machine learning algorithms were employed. The Lasso-LR algorithm identified four key genes: LTF, LCN2, CFL4, and ACTG1 (Figure 5A–C). The random forest algorithm also identified four genes: ALOX5AP, MMP8, LCN2, and MMP9 (Figure 5D). Both the Boruta and svmREF

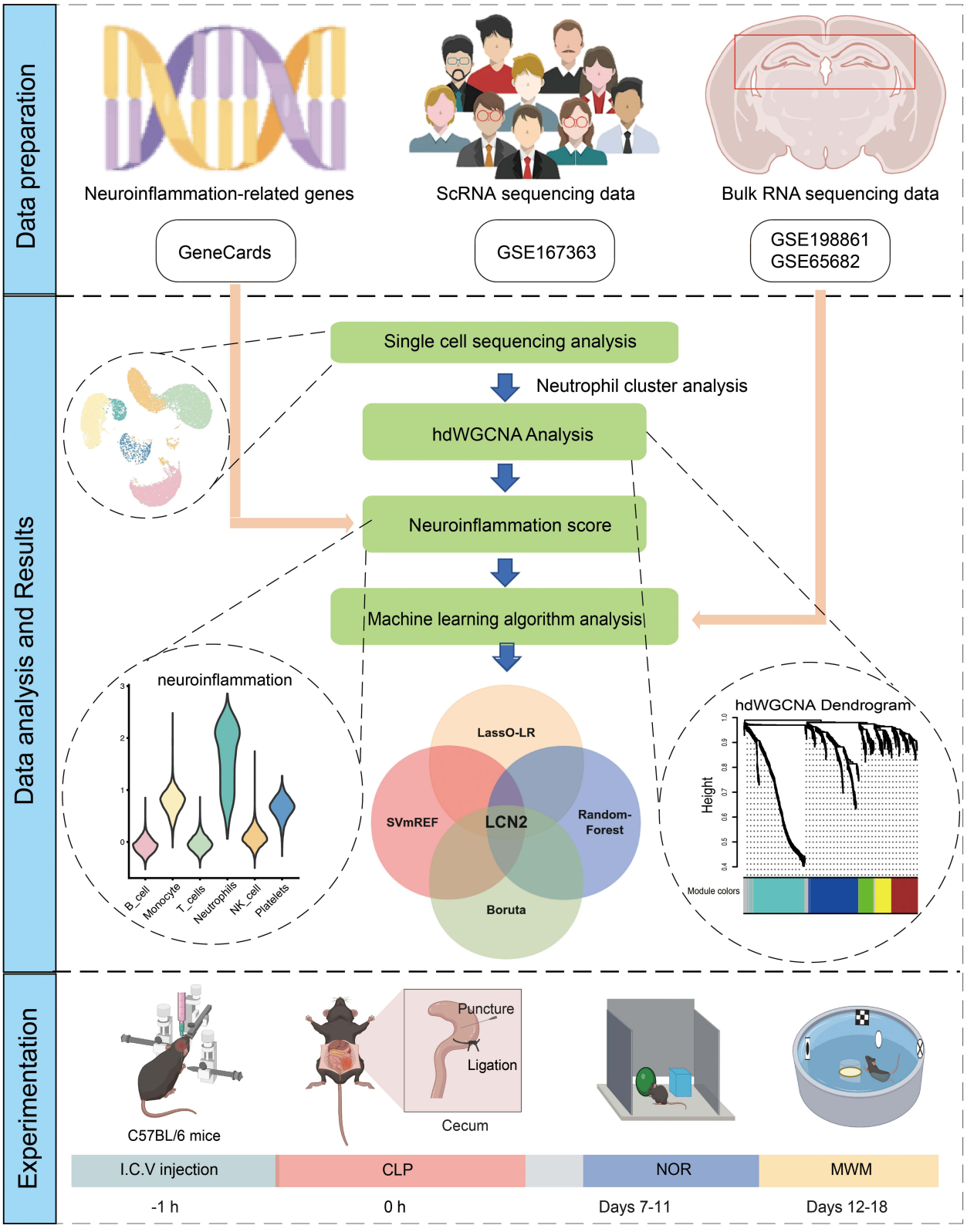

Figure 1 The general workflow of this study is illustrated. "Experimentation" section is created in BioRender. Xu, A. (2025)https://BioRender.com/f02h512..

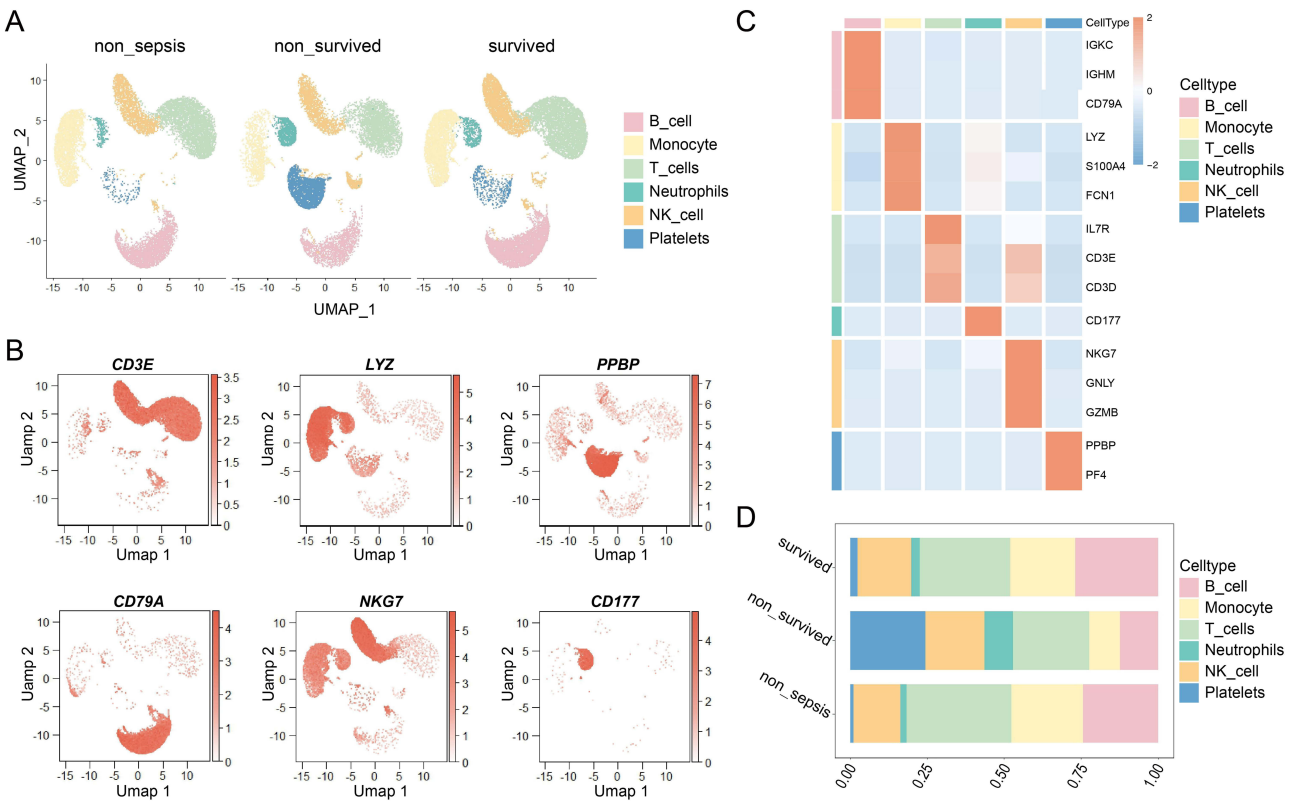

Figure 2 The landscape of sepsis scRNA data. (**A**) Single-cell atlas of PBMCs from different patients. (**B**) The expression of classic cell markers in single-cell atlas. (**C**) The heatmap showed the highly expressed genes in different kinds of cells. (**D**) The barplot showed the differences in cell composition among different patients.

algorithms identified additional key genes (Figure 5E and F). To ensure sufficient accuracy, we took the intersection of the results from the four algorithms, ultimately identifying LCN2 as the target gene (Figure S2).

Small Molecule-Targeted Drugs for LCN2

We selected three drugs—aspirin, carmustine, and acetaminophen—targeting LCN2 from the PubChem database. Molecular docking analysis revealed that all three drugs demonstrated favorable binding energies with LCN2, indicating potential binding interactions between the ligands and the receptor (Figure 6A–C).

Downregulation of LCN2 Rescues Sepsis-Related Cognitive Dysfunction and Neuroinflammation

The scRNA-seq results indicated that LCN2 plays a crucial role in SAE. First, an SAE model was constructed by cecal ligation and puncture. We investigated cognitive impairment and LCN2 upregulation via behavioral tests and Western blotting. Neuroinflammation was also observed through immunofluorescence staining and Western blotting. We subsequently injected a neutralizing antibody targeting LCN2 into the lateral ventricle. The results of the NOR test (Figure 7A–C) and MWM test (Figure 7D–H) revealed that downregulation of LCN2 alleviated cognitive dysfunction in CLP-induced septic mice. Compared with those in the CLP + IgG group, the expression levels of LCN2, GFAP, IL-6, and TNF-α were significantly lower in the CLP + anti-LCN2 group, confirming the effectiveness of the antibody (Figure 7I–M). CLP increased the mean fluorescence intensity of both IBA1 and GFAP, but these increases were less pronounced in the neutralizing antibody-treated group, providing strong evidence that SAE is related to LCN2-mediated neuroinflammation (Figure 8A–D). At the same time, the result of immunofluorescence staining shows that the elevated LCN2 originated from neurons, and the neutralizing antibodies of LCN2 mainly inhibit LCN2 on neurons (Figure S3A and B).

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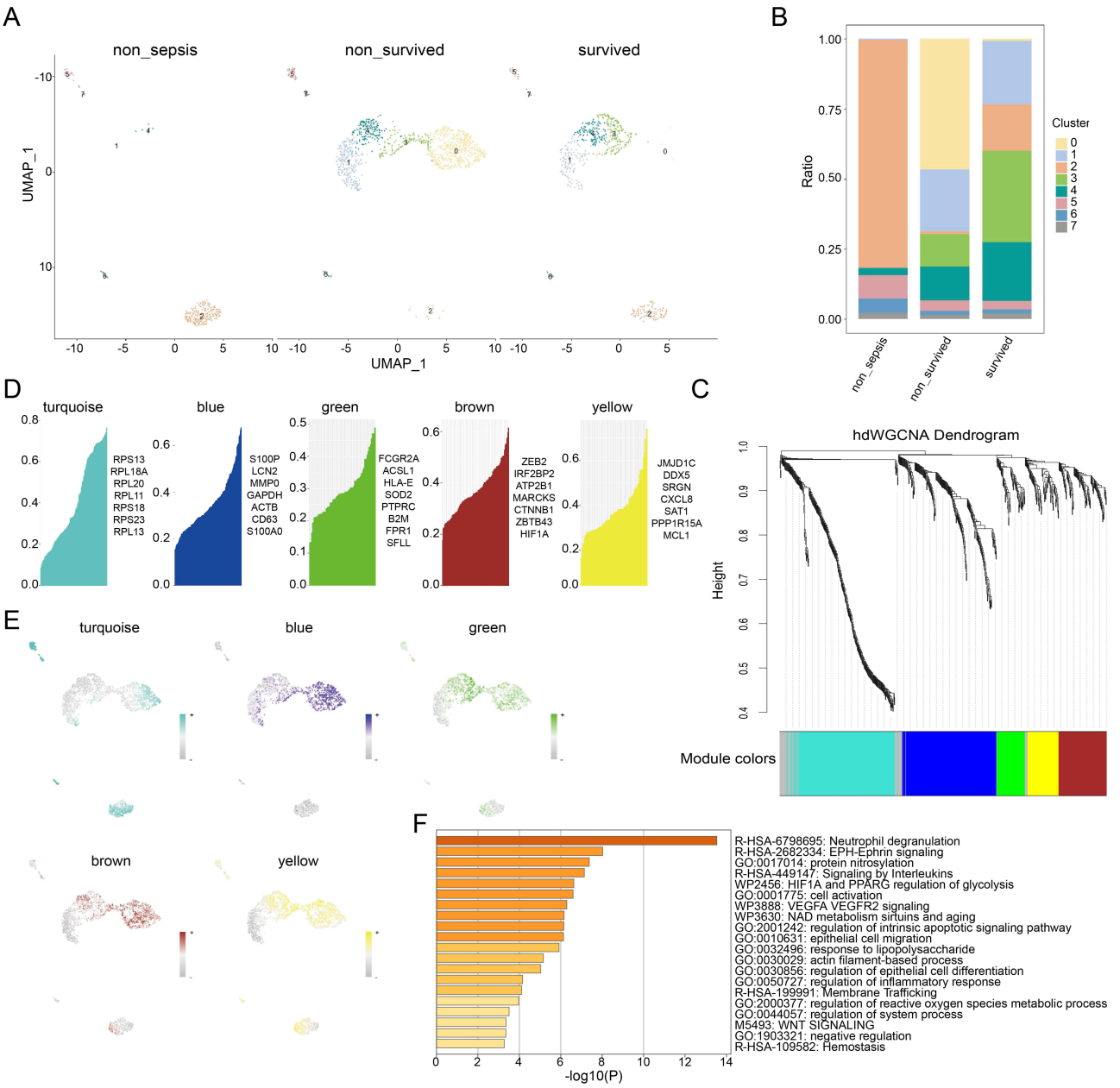

Figure 3 hdWGCNA and function enrichment analysis. (**A**) Atlas of neutrophils from different patients. (**B**) Composition of neutrophils from different patients. (**C**) Five distinct modules were identified. (**D**) Five different modules and their respective highly expressed genes0. (**E**) Relationship between different gene modules and cell subpopulations. (**F**) The bar chart displayed the results of the functional enrichment analysis.

Discussion

In numerous cases of human sepsis-associated multiorgan dysfunction, SAE is a severe and common complication associated with delirium, coma, and long-term cognitive dysfunction.[21] Patients with sepsis-related delirium still suffer significant cognitive impairments at 12–18 months after hospital discharge.[2] In particular, spatial memory and recognition memory, rather than other cognitive functions (new task learning), are consistently present in both septic patients and murine sepsis models. In the context of fecal peritonitis, lipopolysaccharide (LPS) injection, or CLP surgery, SAE is associated with impairments in hippocampus-dependent recognition memory, spatial memory and learning ability. Memory deficits differ between the Morris water maze test, fear conditioning test and novel objective recognition test

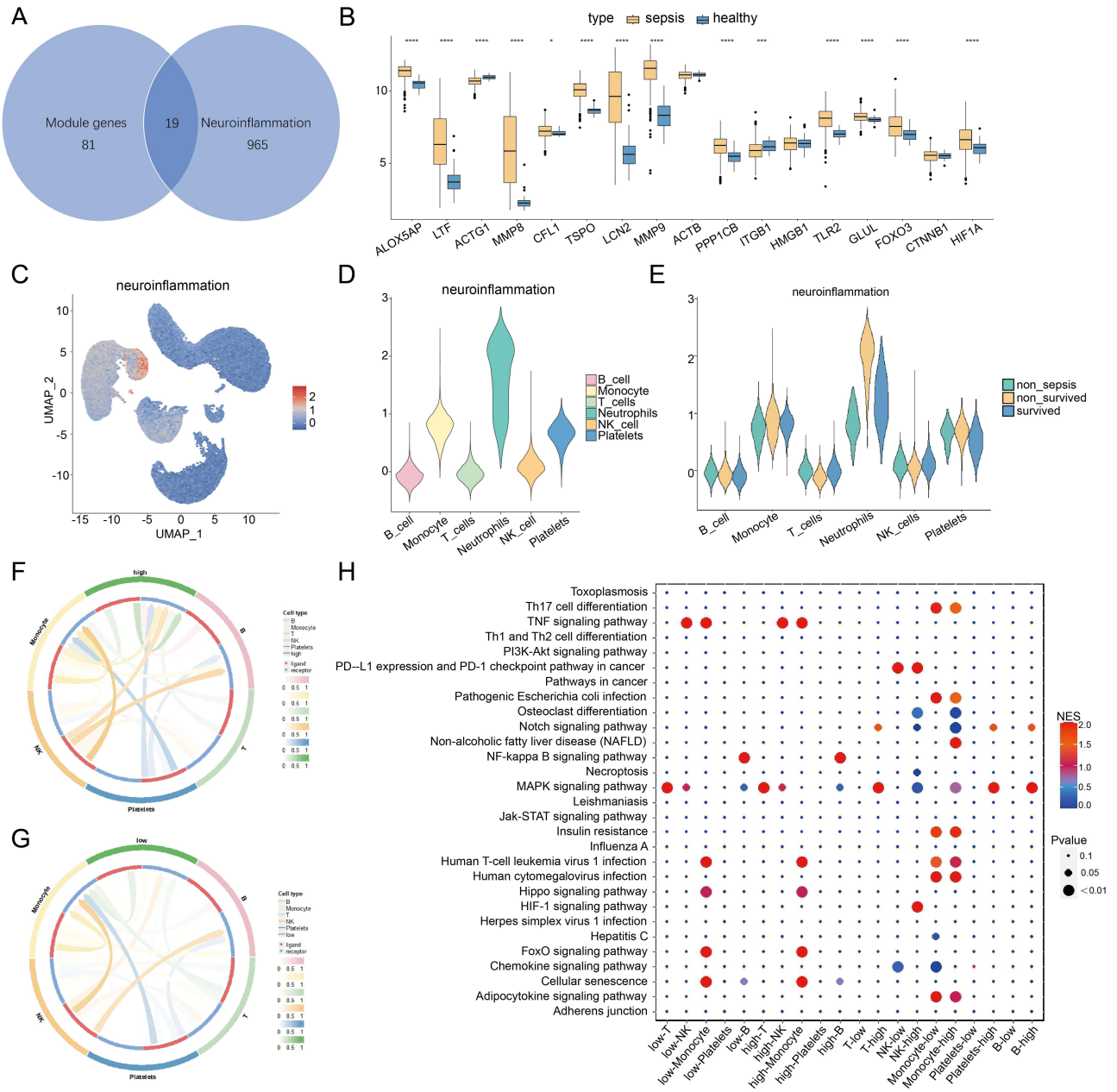

Figure 4 Neutrophils in sepsis had a potential link to SAE. (**A**) 19 sepsis-related genes were identified. (**B**) The box plot showed the differences in gene expression. (**C**) The scRNA atlas illustrated the neuroinflammation scores in sepsis PBMC. (**D** and **E**) The violin plot displayed the differences in neuroinflammation scores between different cells and samples. (**F** and **G**) The circular plot showed the differences in cell crosstalk intensity among cells with different scores. (**H**) Different neutrophils exhibited distinct signaling pathways. All data were presented as mean ± SD, *P<0.05, ***P<0.001, ****P<0.0001.

but not between the open-field, Y-maze, and elevated plus-maze behavioral tests. Cognitive deficits initiate as early as post-septic day 1 and persist for 14 days or even 18 weeks after infection.[2,22,23]

In septic patients and animals, the major manifestations include neuroinflammatory reactions, cerebral ischemic changes, and excitatory toxicity.[8] Non-surviving septic patients have a distinct immune profile with an imbalance between proinflammatory and anti-inflammatory effects according to longitudinal analysis and biochemical assays.[24] Cytokine storms affect septic patients 24 hours after hospital admission. At the same time, after sepsis, neutrophils heavily infiltrate the CNS and disrupt the BBB, and subsequently inducing neuronal apoptosis and glial activation in the hippocampus.[25,26] The accumulation of neutrophils in the hippocampus further promotes SAE progression and impairs hippocampus-dependent memory.[27] In this study, we identified a neutrophil subpopulation linked to poor prognosis in

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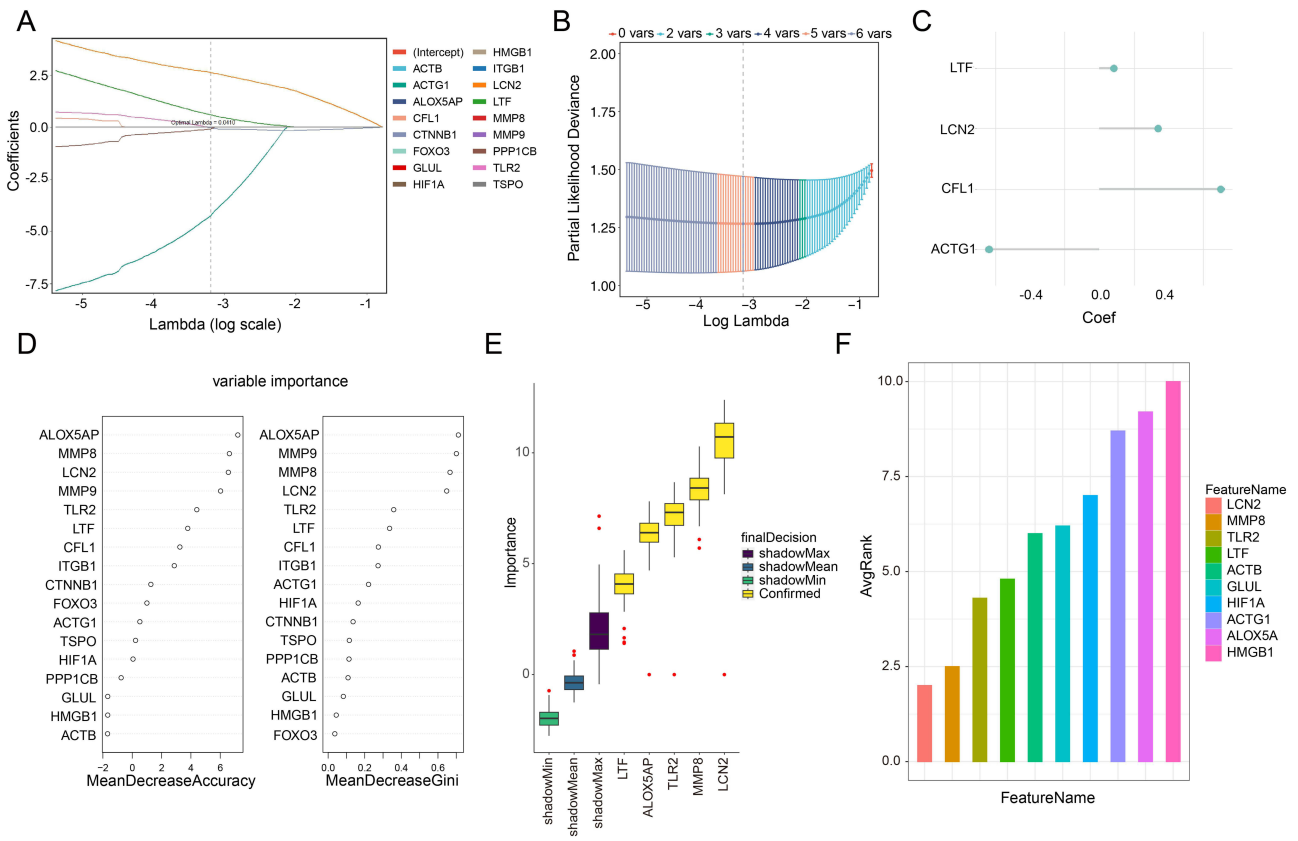

Figure 5 Machine learning identified feature characters. (**A**–**C**) Four genes were identified by the Lasso-LR algorithm. (**D**) The result of the RandomForest algorithm. (**E**) The result of the Boruta algorithm. (**F**) The result of the svmREF algorithm.

septic patients via hdWGCNA analysis. Further functional enrichment analysis revealed that this subpopulation was involved in neutrophil degranulation and the EPH-Ephrin signaling pathway, which plays a role in immune cell development, activation, and migration. Additional bioinformatic analysis revealed several genes highly expressed in neutrophils and are linked to both poor prognosis in sepsis and neuroinflammation. This neutrophil subpopulation in the peripheral blood plays a crucial role in the poor prognosis of septic patients and subsequent neuroinflammation.[28] CLP operation elevated the mRNA and protein levels of IL-6 and IL-1β in both the cortex and hippocampus at days 1 and 7.[22] The mRNA levels of TNF-α are concentrated only in the hippocampus.[29]

Lipocalin-2 (LCN2) is a 25 kDa member of the lipocalin superfamily and is an acute-phase protein with multiple functions.[30] Its structural features include a funnel-shaped binding pocket formed by α-helices and an 8-stranded antiparallel β-barrel, enabling the transport of small molecules such as lipids, steroids, and iron ions.[31,32] Under normal physiological conditions, LCN2 is expressed primarily in peripheral organs such as the kidneys, liver,[33] and adipose tissue.[34] Under conditions of inflammation, infection, or injury, LCN2 is synthesized and secreted from activated microglia, astrocytes, neurons, and endothelial cells to modulate cell death (apoptosis, pyroptosis, or ferroptosis), survival, morphology, migration, invasion, differentiation, and functional polarization.[35] LCN2 has been identified as a biomarker for cognitive dysfunction due to its elevated levels in conditions such as vascular dementia,[36] mild cognitive impairment,[37] and the preclinical stage of Alzheimer's disease[38] as well as aging.[39] Elevated LCN2 expression in cerebrospinal fluid and plasma is also a critical predictor of cognitive decline in patients after coronary artery bypass surgery.[40] A potential explanation for this phenomenon is that it take responsibility to neuronal death by promoting glial activation and inflammation in central nervous system,[41] as well as BBB disruption by decreasing the expression of tight junction protein.[42,43] Therefore, LCN2 may be a potential target for treating neuroinflammation-related diseases via transgenic deletion[44] or pharmacological inhibition or neutralization. We acquired bulk RNA sequencing data from the hippocampi of septic mice to perform four machine learning algorithms and accurately identified LCN2 as a potential key

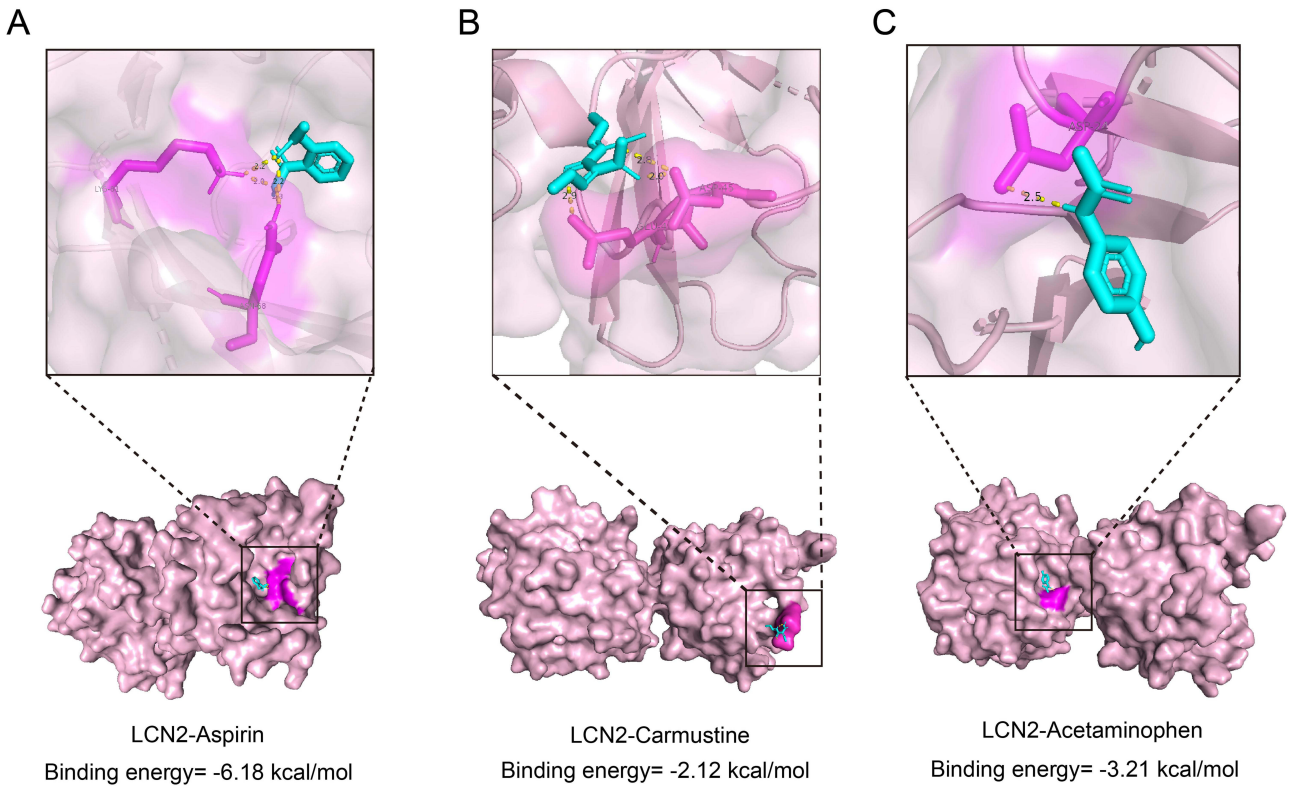

Figure 6 Results of molecule docking. (**A**–**C**) The molecular docking results for the three groups of compounds.

target contributing to SAE. When the increase in LCN2 in the hippocampi of CLP mice was neutralized, neuroinflammation and cognitive deficits were consistently attenuated.

As one of the main neuropathological features of SAE,[18,45,46] reactive astrocytes release chemokines and inflammatory cytokines that increase BBB permeability and facilitate immune cell infiltration.[29,47] Astrocyte activation is promoted by LCN-2 to aggravate neuroinflammation and cognitive impairment[48] by mediating the upregulation of CXCL10 with activating the JAK2/STAT3 pathway[49] or through the Rho kinase-GFAP pathway.[50] On the other hand, diffuse parenchymal and microglial C1q deposits are observed in septic mouse tissue and postmortem patient brain sections.[15,51] Microglial stimuli, as another physiopathological mechanism in SAE, offered various points for prospective intervention, such as NLRP3 inflammasome formation,[52] increased expression of cytokines,[53] and complement system activation.[54] Microglial Kdm6a epigenetically silences Lcn2 expression for the attenuation of photoreceptor dysfunction in diabetic retinopathy.[55] In intracerebral hemorrhage shock, LCN2 directly enhances microglial ferroptosis or indirectly through the specific interaction between LCN2-positive microglia and oligodendrocytes, which is mediated by the colony stimulating factor 1 (CSF1)/CSF1 receptor pathway, ultimately leading to subsequent neurological deficits.[56,57] In this study, glial activation was significantly suppressed after the neutralization of LCN2 in CLP mice.

In this study, the successful establishment of the model was confirmed through behavioral tests. We then administered neutralizing antibodies into the lateral ventricle to reduce the levels of hippocampal LCN2.[58] The administration of these antibodies effectively mitigated sepsis-related cognitive dysfunction and neuroinflammation, strongly suggesting that LCN2 plays a significant role in the development of SAE via its proinflammatory effects. This finding enhances our understanding of the SAE process and offers new insights for potential treatment strategies. Then, we screened small molecules from public databases that can inhibit LCN2 and performed molecular docking analysis. Future studies may build upon our findings to develop more effective small-molecule drugs or nanodrug carriers that specifically target LCN2, highlighting the potential clinical translational value of our results.

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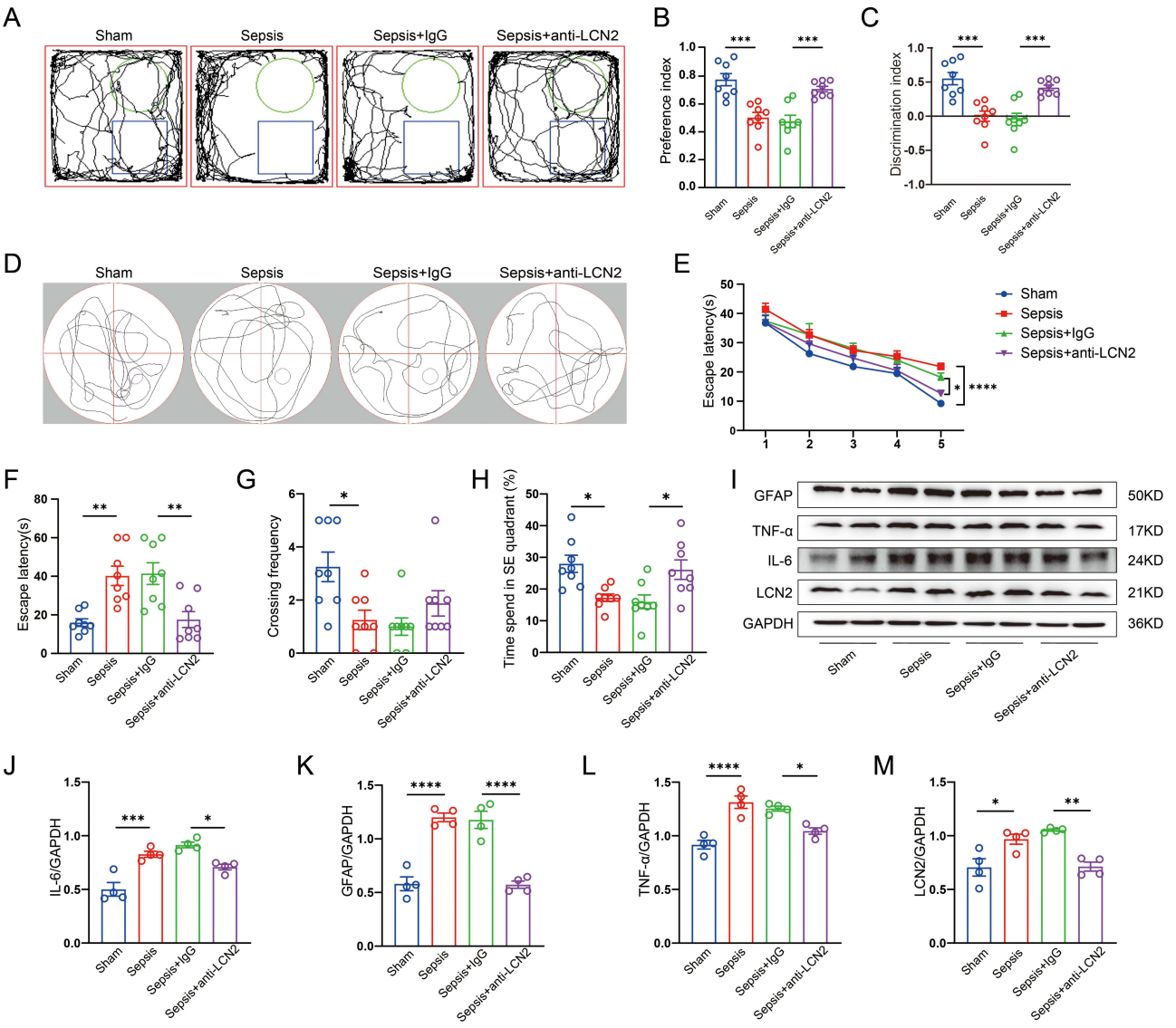

Figure 7 Down-regulation of LCN2 alleviates cognitive deficits in septic mice. (**A**–**D**) Representative video tracks, preference index, and discrimination index from the novel object recognition test (n=8 per group). (**E**–**H**) Representative video tracks and data from the Morris water maze, including the learning phase, time spent in the target quadrant, escape latency time, and number of crossings over the target (n=8 per group). (**I**–**M**) Representative Western blot images illustrating the expression of LCN2, glial fibrillary acidic protein (GFAP), IL-6, and TNF-α in the hippocampi from the sham, CLP, CLP + IgG, and CLP + anti-LCN2 groups. GAPDH was served as an internal control (n=4 per group). All data were presented as mean ± SEM, *P<0.05, **P<0.01,***P<0.001, ****P<0.0001.

However, limitations still need to be acknowledged. It is necessary to further explore the underlying mechanisms or signaling pathways by which LCN2 is involved in SAE. Notably, LCN2 triggers the phosphorylation of NF-κB by binding to cell membrane receptors such as 24p3R, thereby increasing the expression of inflammatory factors.[35] LCN2 can also activate the JAK-STAT pathway.[59] However, whether LCN2 regulates SAE via these two pathways remains unknown and needs further investigation. Illustrating the specific mechanisms underlying this phenomenon will be helpful to achieve a more comprehensive and detailed understanding in SAE. In addition, we did not elucidate the specificity of LCN2 as a biomarker across diverse patient populations. In future studies, we will collect a certain number of patient-derived clinical samples from different genders, regions, and ethnic backgrounds to address current limitations through clinically based research.

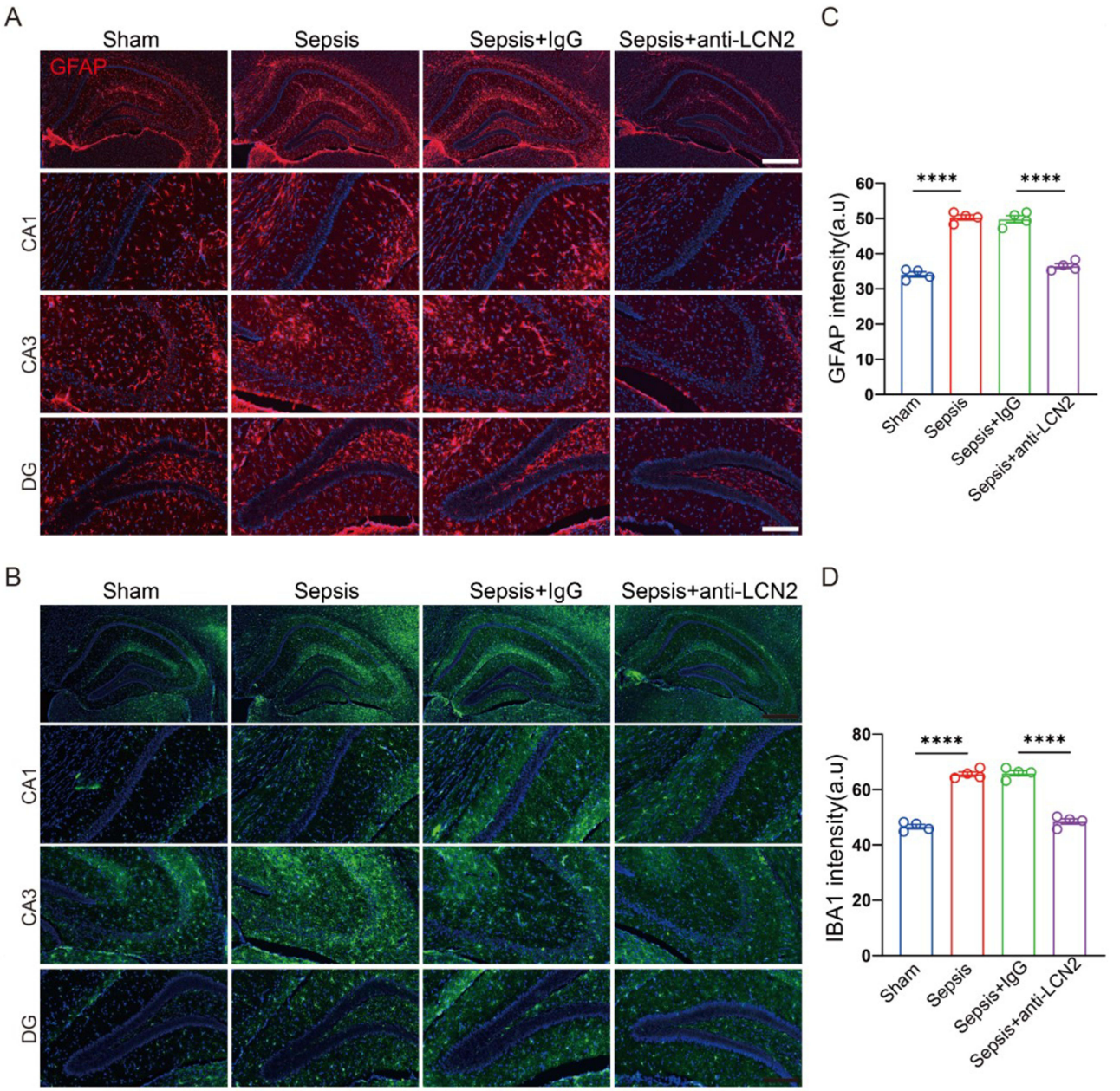

Figure 8 Sepsis-related neuroinflammation was rescued by LCN2 neutralization. (**A**)Representative immunofluorescence micrographs illustrating GFAP expression in the CA1, CA3 and DG region of hippocampi. Magnification: 20×. Scale bar: 200 μm. (**B**) Quantitative analysis of the expression of GFAP in overall hippocampi (n=4 per group). (**C**) Representative immunofluorescence micrographs depicting ionized calcium-binding adapter molecule (IBA1) expression in the CA1, CA3 and DG region of hippocampi. Magnification: 20×. Scale bar: 200 μm. (**D**) Quantitative analysis of the expression of IBA1 in overall hippocampi (n=4 per group). All data were presented as mean ± SEM, ****P<0.0001.

Conclusions

Based on single-cell data from PBMCs in septic patients and bulk RNA data from SAEs, we integrated hdWGCNA with binary classification machine learning models to identify the key gene LCN2. Next, we established a sepsis animal model via CLP surgery, and behavioral tests confirmed the occurrence of SAE in these mice. Subsequent molecular experiments demonstrated that LCN2 and several inflammatory markers were upregulated in the hippocampal tissue of the mice. Our results indicated that LCN2 plays a critical role in the cognitive impairment caused by neuroinflammation.

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Abbreviations

SAE, Sepsis-associated encephalopathy; CLP, Cecal ligation and puncture; scRNA-seq, Single-cell RNA sequencing; hdWGCNA, Hierarchical dynamic weighted gene co-expression network analysis; LCN2, Lipocalin-2; BBB, Blood–brain barrier; CNS, Central nervous system; TNF-α, Tumor necrosis factor-alpha; IL-6, Interleukin-6; IL-1β, Interleukin-1β; PBMC, Peripheral blood mononuclear cell; PCI, Polymicrobial peritoneal sepsis; PBS, Phosphate-buffered saline; TBST, Tris-buffered saline with Tween-20; NOR, Novel object recognition test; MWM, Morris water maze; LPS, Lipopolysaccharide; CSF1, Colony stimulating factor 1; IBA1, Ionized calcium-binding adapter molecule; GFAP, Glial fibrillary acidic protein.

Data Sharing Statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories can be found in the article. The datasets used and analyzed during the current study are available from the corresponding author (Min Yang) on reasonable request.

Ethics Approval and Informed Consent

All animal experiments followed the Guide for the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 8023, revised 2011) and were approved by the Institutional Animal Care and Use Committee of Anhui Medical University (LLSC20211456).

Since this study involves human databases, this study is exempt from ethical review under items 1 and 2 of Article 32 of the Measures for Ethical Review of Life Science and Medical Research Involving Human Subjects, effective February 18, 2023, in China.

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors declare that they have no competing interests in this work.

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