SVN Stroke & Vascular Neurology

Protective effects of melatonin on stroke in diabetic mice: central and peripheral inflammation modulation

Cuiying Liu [1], Jiayi Guo,[2] Longfei Guan,[3] Junfa Li [2], Baohui Xu [4], Heng Zhao [5]

To cite: Liu C, Guo J, Guan L, *et al*. Protective effects of melatonin on stroke in diabetic mice: central and peripheral inflammation modulation. *Stroke & Vascular Neurology* 2025;**0**. doi:10.1136/svn-2024-003442

CL and JG contributed equally.

Received 1 June 2024
Accepted 17 January 2025

[1]School of Nursing, Capital Medical University, Beijing, China
[2]Department of Neurobiology, School of Basic Medical Science, Capital Medical University, Beijing, China
[3]China-America Institute of Neuroscience, Beijing Luhe Hospital, Capital Medical University, Beijing, China
[4]Division of Vascular Surgery, Department of Surgery, Stanford University School of Medicine, Stanford, California, USA
[5]Beijing Institute of Brain Disorders, Laboratory of Brain Disorders, Ministry of Science and Technology, Collaborative Innovation Center for Brain Disorders, Capital Medical University, Beijing, China

Correspondence to
Professor Heng Zhao;
hengzhao@ccmu.edu.cn

ABSTRACT

Background Melatonin protects against ischaemic stroke in diabetic animal models, though the mechanisms involving brain and peripheral immune responses remain underexplored. We aimed to clarify how melatonin interacts with these immune responses to protect against stroke in diabetic mice.

Methods Type 1 diabetes mellitus (T1DM) was induced in mice using streptozotocin. RNA sequencing of brain tissue and peripheral blood mononuclear cells (PBMCs) was performed 24 hours poststroke. Inflammatory responses were evaluated 72 hours after ischaemia/reperfusion.

Results Melatonin reduced infarction and improved neurological function in T1DM mice. In the ischaemic brain, melatonin downregulated inflammatory factor expression, with bioinformatics identifying 62 differentially expressed genes (DEGs) related to inflammation and 11 associated with inflammasomes. Western blotting confirmed reductions in NLRP3, HMGB1 and Cleaved Caspase-1 expression. Flow cytometry showed reduced infiltration of CD8+T cells and neutrophils. Melatonin decreased IL-6, IL-1β and IL-4 levels. In PBMCs, RNA sequencing revealed 939 DEGs following melatonin treatment. Kyoto Encyclopaedia of Genes and Genomes analysis indicated that downregulated DEGs were involved in metabolic pathways, and upregulated DEGs were enriched in the Jak-STAT signalling pathway. GO analysis showed that downregulated DEGs were enriched in the cytosol, and upregulated DEGs related to macromolecule modification. Protein–protein interaction analysis revealed that melatonin affected 38 inflammation-associated genes linked to key cytokines (Il6, Il1b, Ifng, Il4). Flow cytometry indicated melatonin increased CD8+T cells, monocytes and neutrophils in the blood, suggesting a reversal of immunosuppression. Multiplex cytokine assays showed melatonin decreased IL-6 and IFN-γ levels.

Conclusion Poststroke melatonin therapy reduces ischaemic brain damage in T1DM mice by modulating central and peripheral inflammatory responses.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Melatonin protects against stroke in diabetic mice, yet the underlying cellular and molecular mechanisms remain elusive.

WHAT THIS STUDY ADDS

⇒ Melatonin modulates inflammatory responses in the brain and peripheral blood, by reducing immune cell infiltration and inhibiting NLRP3 inflammasome activity.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ This study suggests melatonin's potential as a therapeutic agent for managing stroke in diabetic conditions, encouraging further research, clinical application and policy development to integrate melatonin into treatment protocols.

INTRODUCTION

Diabetes is the primary risk factor for ischaemic stroke and is related to a higher incidence of ischaemic stroke and a worse prognosis for recovery.[1 2] The effectiveness of various prospective therapies for stroke is diminished in diabetic stroke patients.[3] Therefore, it necessitates studying the protective effects of neuroprotectants against stroke with diabetes. Among various neuroprotectants, melatonin (N-acetyl-5-methoxytryptamine) is promising to protect against stroke.[4 5] It is released by the pineal gland in the brain to control the circadian rhythm in animals.[6] It is also released by organs such as the liver, retina, testes and ovary to stimulate the production of antioxidants.[4 6] Melatonin levels are altered in neurological disorders such as stroke, Parkinson's disease and Alzheimer's disease, indicating its crucial involvement in the pathogenesis of these ailments.[4]

Previous studies have demonstrated that melatonin offers significant neuroprotection in stroke by reducing oxidative stress, inflammation and neuronal apoptosis.[7 8] As an antioxidant, melatonin detoxifies reactive species and stimulates antioxidant enzymes.[9] It chelates harmful metals, preventing the formation of toxic radicals and its presence in mitochondria helps resist oxidative damage.[10] Additionally, melatonin modulates inflammation by decreasing proinflammatory markers and increasing anti-inflammatory ones. It inhibits NF-κB[11] and enhances Nrf2 and HO-1[12] pathways. Melatonin also regulates

apoptosis[13] and autophagy[14] through the Akt and mTOR pathways, promoting cell survival and reducing stress.

Melatonin is a hormone, which is synthesised in the pineal gland, and then released into the bloodstream and cerebrospinal fluid to control circadian rhythms, and participate in a variety of physiological processes, including emotional behaviour, blood pressure regulation, ovarian physiology and osteoblast differentiation.[15 16] Recently, several studies have shown the protective effects of melatonin against stroke in diabetic conditions.[2 17 18] Melatonin reduces inflammation and cell death to protect neuronal cells in diabetic stroke models.[18] It promotes autophagy via the SIRT1-BMAL1 pathway, aiding in cell repair and reducing oxidative stress.[2] Furthermore, melatonin improves mitochondrial function and reduces oxidative stress through the Akt-SIRT3-SOD2 pathway.[19] These effects make melatonin a promising candidate for stroke therapy in patients with diabetes.

The peripheral immune system plays a crucial role in regulating brain injury, with extensive interactions between peripheral immune cells and brain cells.[20 21] However, few studies have addressed the effects of melatonin on the interaction between peripheral immune responses and brain pathological mechanisms. Moreover, previous research primarily used low-throughput methods to study its protective effects.

In this research, we aim to investigate the effects of melatonin on ischaemic brain damage and related inflammatory mechanisms in mice with type 1 diabetes mellitus (T1DM) following a stroke. We will clarify the interaction between the peripheral immune system and brain injury using both traditional methods and high-throughput bulk RNA sequencing. Bulk RNA sequencing will assess transcriptomic changes in brain tissue and peripheral blood mononuclear cells (PBMCs) 24 hours after administration. The alterations in immune cells and inflammatory factors will be evaluated to validate the bioinformatics analysis results. Additionally, western blotting will be used to determine if melatonin influences NLRP3 inflammasome activities in the ischaemic brain of mice with T1DM. These findings may suggest a possible therapeutic target for diabetic individuals with ischaemic stroke.

MATERIALS AND METHODS

T1DM MODEL

Eight-week-old male C57BL/6J mice were obtained from Beijing Vital River Laboratory Animal Technology (Beijing, China). These animals were housed in cages with ad libitum access to food and water. To induce T1DM, the mice received a single intraperitoneal injection of 1% streptozotocin (STZ, Sigma, Buchs, Switzerland). The STZ dosage (dissolved in citrate buffer, pH 4.2) was adjusted to 110 mg/kg based on our previous study.[22] Mice with blood glucose levels >300 mg/dL (16.7 mmol/L) were confirmed using the ACCU-CHEK Performa 3 days after STZ administration. Melatonin (Sigma-Aldrich) was administered intraperitoneally at a dosage of 10 mg/kg[19] (dissolved in saline) immediately after reperfusion and daily for 3 days. The animals were then randomly assigned to one of three groups: (1) Sham; (2) middle cerebral artery occlusion (MCAO) plus vehicle (Vehicle) and (3) MCAO plus melatonin therapy (Melatonin).

Focal cerebral ischaemia

As previously documented, focal cerebral ischaemia was induced by transient MCAO.[22–24] Mice were anaesthetised with isoflurane inhalation, using 3%–5% for induction and 1%–3% for maintenance. A 7–0 silicon-coated nylon suture with a 0.19 mm tip diameter (Doccol, Redlands, California, USA) was gently introduced into the external carotid artery. The suture was then advanced into the lumen of the internal carotid artery and guided towards the middle cerebral artery to occlude it. The occlusion was maintained for 45 min, after which the suture was removed to allow reperfusion for 1 or 3 days. In the sham group, mice underwent the surgical procedure without inserting the filament. During the surgery, body temperature was continually monitored, and a heating pad was used to maintain the rectal temperature at 37°C±0.5°C.

Neurological score test

According to previous research, neurological scores were assessed 3 days after reperfusion by an investigator blinded to the animal group assignments.[25] The scoring criteria were as follows: 0—no deficit; 1—inability to extend the left forepaw; 2—circling to the left; 3—falling to the left; 4—inability to walk spontaneously and loss of awareness and 5—inability to walk spontaneously and loss of consciousness (death).

Infarct size and oedema measurements

The brains of mice were promptly removed under anaesthesia and sliced into 1 mm thick coronal sections 72 hours following cerebral ischaemia. The sections were then submerged in 2% 2,3,5-triphenyltetrazolium chloride (TTC, Sigma-Aldrich) at 37°C for 20 min, followed by a 2-hour fixation in 4% paraformaldehyde. Infarct sizes were calculated using ImageJ software and the following formula: (size of the contralateral hemisphere–the size of the non-ischaemic zone in the ipsilateral hemisphere)/size of the contralateral hemisphere×100%).[26] The oedema ratio was computed based on the method described in our previous report.[27]

Bulk RNA sequencing

The penumbra region of the cerebral cortex and PBMCs were obtained from diabetic mice 24 hours after ischaemic stroke. Following total RNA extraction, cDNA was synthesised, purified, screened and amplified to construct the gene library. The gene library was diluted and quantified to 1.5 ng/μL, with quality and integrity assessed using the Agilent 2100 bioanalyzer. After passing quality control, clustering and sequencing were performed on the Illumina NovaSeq 6000 system, generating 150 bp paired-end

reads. Sequencing used the Sequencing by Synthesis method, incorporating fluorescence-labelled dNTPs and DNA polymerase to capture sequence information. Gene library construction and sequencing were completed by Novogene Bioinformatics Technology.

Analysis of RNA sequencing data

Differential expression analysis between the melatonin and the control group was conducted using the DESeq2 R package (V.1.20.0). DESeq2 provides statistical methods to identify differential expression in digital gene expression data, employing a model based on the negative binomial distribution. We set the threshold for significant differential expression at a p value of <0.05 and |fold change (FC)|>1.2.

Gene Ontology (GO) is a standardised system that describes the functions of genes and gene products, categorising gene functions into three hierarchical levels: molecular function, cellular component and biological process. The Kyoto Encyclopaedia of Genes and Genomes (KEGG) is a database resource for understanding biological systems' functions (http://www.genome.jp/kegg/). The clusterProfiler R package (V.3.14.3) was used to perform GO enrichment analysis and KEGG pathway enrichment analysis of differentially expressed genes (DEGs). GO terms and KEGG pathways with a p value less than 0.05 were considered significantly enriched for DEGs.

To analyse the interactions between inflammation-related DEGs or inflammasome-related DEGs and classic inflammatory factors using bioinformatics tools, we searched for the keywords 'inflam' and 'inflammasome' in GSEA (https://www.gsea-msigdb.org/gsea/msigdb). We integrated the results and defined them as the inflammation and inflammasome dataset, identifying 994 inflammation-related genes and 67 inflammasome-related genes. Next, we used protein interaction data and their degree values from STRING to build a protein–protein interaction (PPI) network using Cytoscape V.3.9.0 from the National Resource for Network Biology.[28] This network displayed the interactions between inflammation-related DEGs or inflammasome-related DEGs and classic inflammatory factors of the interleukin family.

Flow cytometry

Blood was collected from mice sedated with isoflurane and placed in tubes containing heparin-saline anticoagulant 72 hours after reperfusion. The mice were then perfused with 0.9% NaCl at 4°C, and their ipsilateral ischaemic brains were extracted. Leucocytes obtained from these brains were processed according to previously published research.[26] The ipsilateral brain tissues were homogenised and passed through a 70 µm cell strainer (Fisherbrand). After centrifugation, the brain cell solution was resuspended with 60% Percoll and overlaid with 30% Percoll. The brain cell suspension was centrifuged for 30 min at 2000 rpm and 4°C. Interphase brain cells were carefully harvested for further labelling. Antibodies from Biolegend (San Diego, California, USA), including fluorescein isothiocyanate (FITC) anti-mouse CD3 (100203,1:100), PE anti-mouse CD8a (100707,1:100), allophycocyanin (APC) anti-mouse CD4 (100412,1:100), PerCP/Cy5.5 anti-mouse CD45(157208,1:50), APC/Cy7 anti-mouse CD11b(101226,1:100), PE anti-mouse Ly-6c(128008,1:100), PE/Cy7 anti-mouse Ly-6G(127618,1:100) were used to stain cellular populations. CD3+CD4+ cells were classified as CD4+T cells, CD3+CD8+ cells as CD8+T cells, Ly-6C^{high}CD11b+cells as monocytes, and Ly-6G+CD11b+cells as neutrophils. Cells were analysed using a BD LSRII flow cytometer and BD FACSDiva TM Software (V.8.0), with results represented as a percentage of the total number of cells assessed.

Inflammatory cytokine measurement

72 hours following reperfusion, the ipsilateral ischaemic hemisphere and blood were collected from anaesthetised mice. Brain tissues were homogenised in ice-cold phosphate-buffered solution (10 mg/mL), and plasma samples were prepared, as described.[24] Protein levels of IL-6, IL-1β, IFN-γ and IL-4 in the brain homogenates and peripheral plasma were measured in triplicate using ProcartaPlex multiplex immunoassays, according to the manufacturer's instructions.

Western blotting

Western blotting was conducted as described previously.[24] The brains of mice were homogenised using RIPA lysis solution, and the protein content was determined with a BCA kit. Subsequently, 50 µg of total protein were electrophoresed on a polyacrylamide gel containing 12% sodium dodecyl sulfate and transferred onto polyvinylidene difluoride membranes. After blocking the membranes with 10% non-fat milk in Tris-buffered saline containing 0.5% Tween-20 (TBST) for 1 hour, they were incubated overnight at 4°C with primary antibodies (1:1000). The membranes were then incubated with a horseradish peroxidase-conjugated secondary antibody (1:5000) for 1 hour at room temperature. The bands were analysed using an enhanced chemiluminescence kit and quantified using Image-Pro Plus software V.5.0, following the manufacturer's instructions. The average expression level of each protein in the sham group was assigned a value of 1. Actin served as the internal control, and all antibodies were purchased from Cell Signaling Technology (CST) including NLRP3 (#15101), p-ERK (#4370), ERK (#4695), HMGB1 (#6893), Cleaved Caspase-1 (#89332) and Actin (#4970)

Statistical analysis

All continuous variables were normally distributed as determined by the Shapiro-Wilk normality test and presented as mean±SEM using the GraphPad Prism V.7.0 program (Graphpad Software, San Diego, California, USA). Comparisons between two groups were performed using the Student's t-test. For multiple comparisons, one-way analysis of variance followed by the Bonferroni post

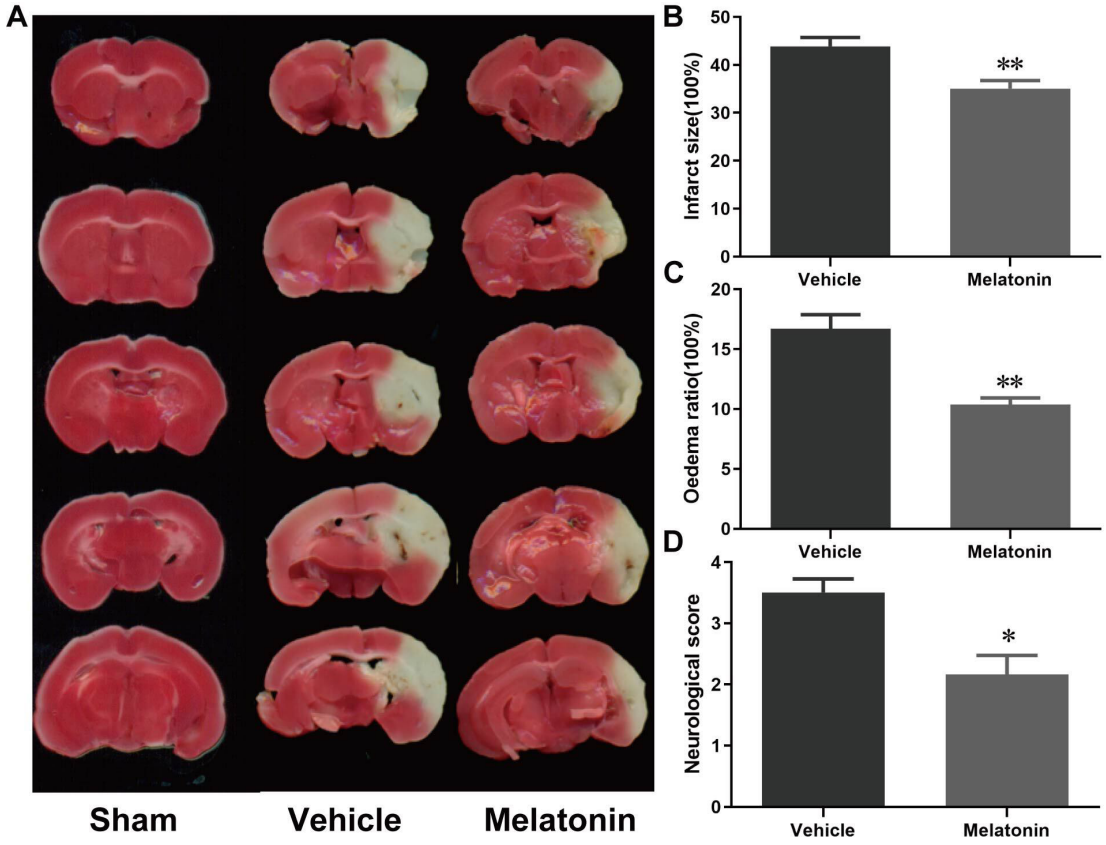

Figure 1 Melatonin ameliorates brain damage and lowers neurological scores in mice with T1DM at 3 days after stroke. (A) Representative photographs of TTC-stained brain sections. (B) Infarct size quantification. (C) Oedema ratio computation. (D) Neurological score analysis. *p<0.05, **p<0.01 compared with vehicle at 3 days postreperfusion. n=6 per group. MCAO, middle cerebral artery occlusion; Melatonin, MCAO plus melatonin therapy; Sham, sham surgery; T1DM, type 1 diabetes mellitus; TTC, 2,3,5-triphenyltetrazolium chloride; Vehicle, MCAO plus vehicle.

hoc test was employed. A significance level of p<0.05 was used for all analyses.

RESULTS

Melatonin reduced ischaemic brain damage in diabetic type 1 mice after stroke

Following 45 min of ischaemia and 72 hours of reperfusion, we examined whether poststroke melatonin therapy protects mice with T1DM from ischaemic brain damage. The results show that melatonin significantly reduced infarct sizes and improved cerebral oedema compared with the vehicle group (figure 1A–C). Additionally, neurological scores were significantly improved by melatonin administration 72 hours after reperfusion (figure 1D).

Gene sequencing and flow cytometry reveal that melatonin inhibits inflammatory responses in ischaemic brain tissue of diabetic mice

To investigate the protective mechanism of melatonin in the brain tissue of diabetic mice, we collected the cerebral cortex for RNA sequencing 24 hours after ischaemic stroke following melatonin treatment. As shown in figure 2A,B we identified 802 significant DEGs caused by melatonin (|FC|>1.2, p<0.05), with the majority being downregulated. The top 20 upregulated and downregulated DEGs showed significant changes caused by melatonin delivery compared with the vehicle (figure 2C). KEGG analysis revealed that the downregulated DEGs are associated with immune cell activation and migration, including leucocyte transendothelial migration, while the upregulated DEGs are predominantly enriched in neuronal signalling transduction pathways, such as neurotransmitter receptor interactions. GO enrichment analysis suggests that melatonin downregulates immune responses to external stimuli, such as immune system processes, while upregulating cell membrane-related components, such as intrinsic components of the cell membrane (figure 2D,E).

To further understand how melatonin administration affects the infiltration of peripheral immune cells into the brain tissue after stroke, we used flow cytometry to analyse the immune cell population in the ipsilateral ischaemic brain hemisphere (figure 3). MCAO increased the number of CD4+T cells, CD8+T cells and neutrophils infiltrating the brain, but melatonin reduced the number of CD8+T cells and neutrophils (figure 3A,B,D). Neither the vehicle nor melatonin groups exhibited a significant change in the infiltration of monocytes (figure 3C).

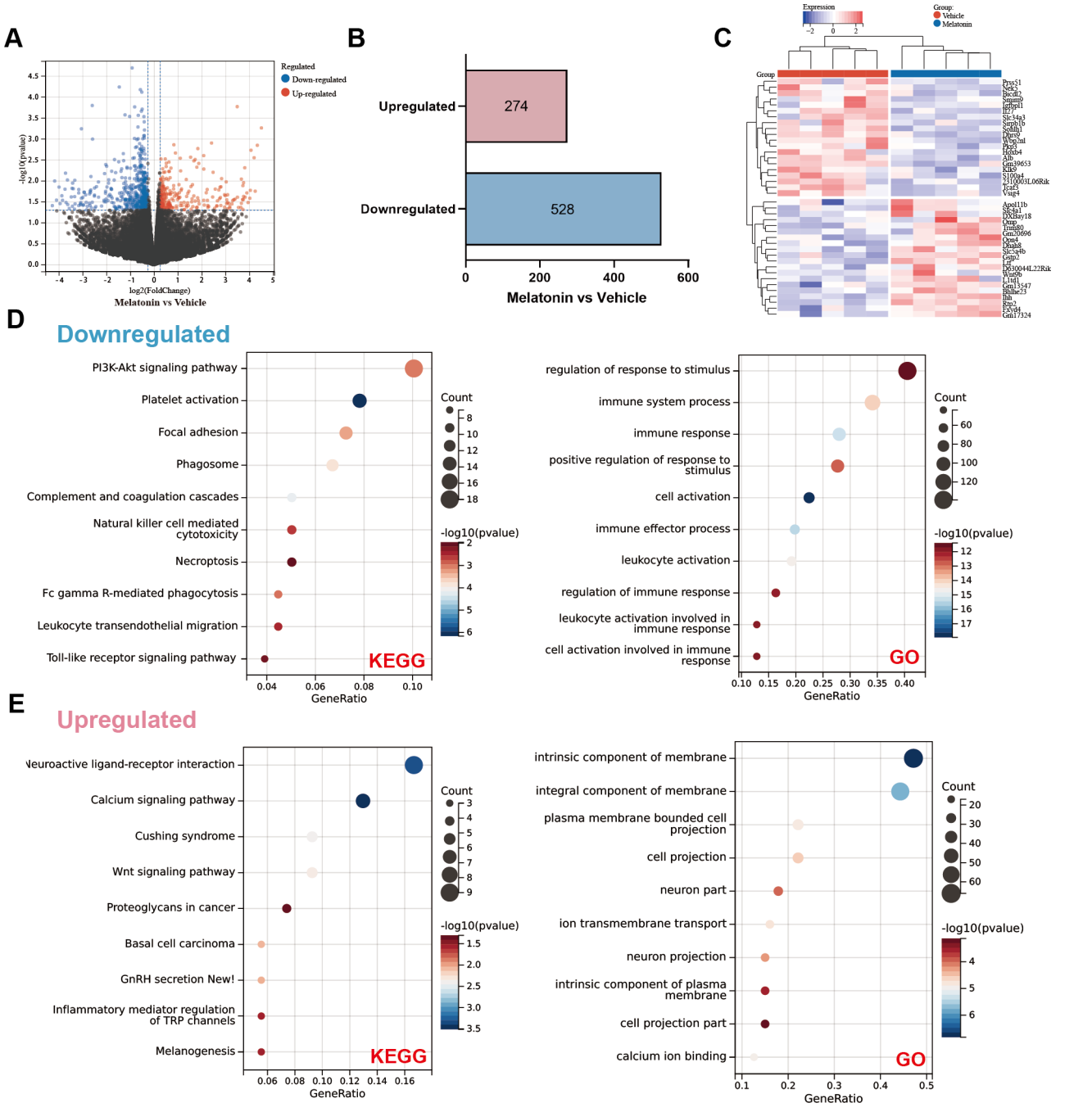

Figure 2 RNA sequencing reveals the impact of melatonin treatment on transcriptome changes in brain tissue of mice with T1DM following stroke. (A) Volcano plot of differentially expressed genes (DEGs) after melatonin treatment compared with vehicle (|FC|>1.2, p<0.05), n=5 per group. (B) Bar chart quantifying upregulated and downregulated DEGs. (C) Heat map of the top 20 upregulated and downregulated genes. (D) Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathway and Gene Ontology (GO) term enrichment analysis of downregulated DEGs, showing involvement in leucocyte activation and inflammatory responses. (E) Enrichment analysis of upregulated DEGs, associated with cell survival, highlighting the protective effects of melatonin on ischaemic brain tissue. FC, fold change; T1DM, type 1 diabetes mellitus.

Melatonin reverses stroke-induced inflammation and inflammasome activation in diabetic mouse brain

We delved deeper into how melatonin treatment affected the inflammatory response in the brains of diabetic mice. Using a previously described gene set, we discovered 62 DEGs related to inflammation, which were upregulated after stroke and reversed by melatonin (figure 4A,C). Figure 4B illustrates significant interactions between the interleukins Il6, Il1b, Ifng and Il4, and the 62 inflammation-related DEGs. Changes in the expression of inflammatory factors are closely related to the activation of inflammasomes. From the selected inflammasome gene set, we delineated 11 DEGs associated with inflammasomes (figure 4D). Our enrichment analysis revealed

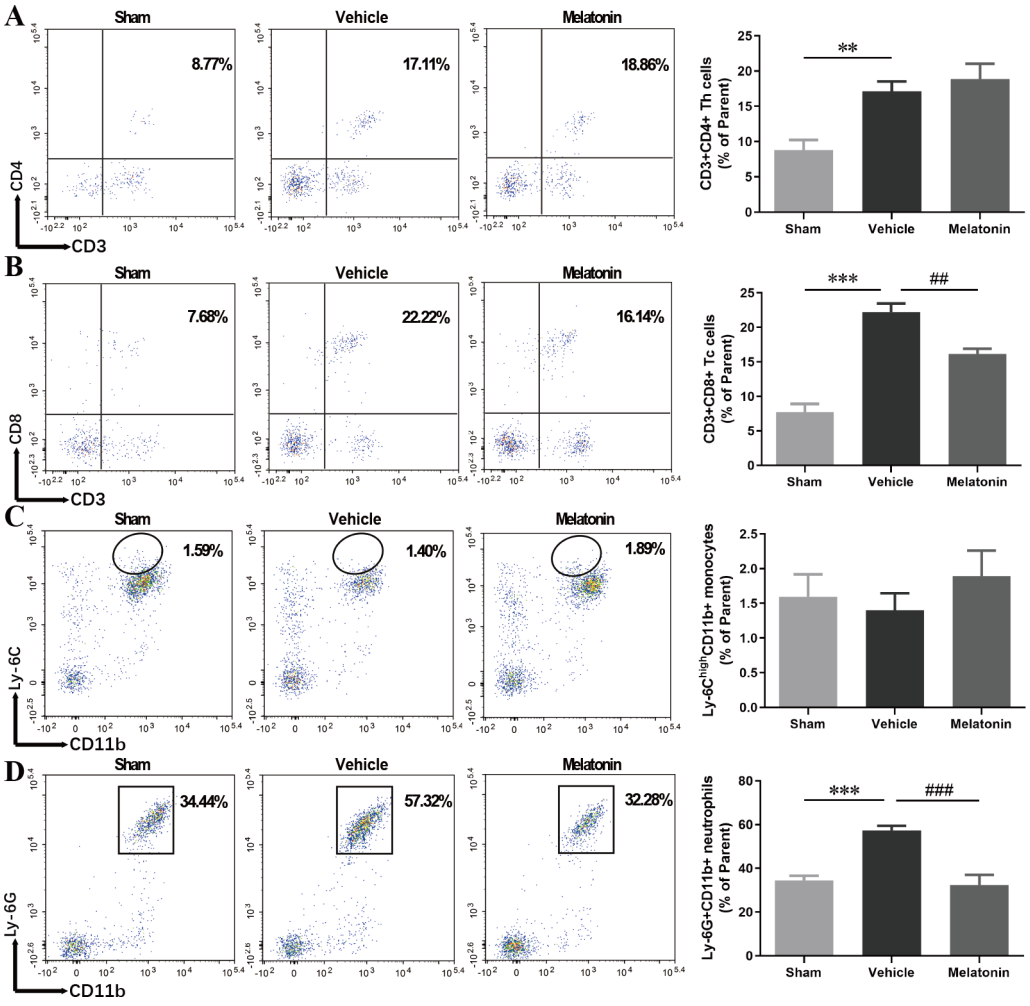

Figure 3 Effects of melatonin on immune cell infiltration in the brains of mice with T1DM after stroke. (A–D) Representative flow cytometry gating images and statistical data of CD4+T cells, CD8+T cells, monocytes and neutrophils in the ischaemic brain after melatonin administration. n=5 per group. **p<0.01, ***p<0.001 compared with Sham; ##p<0.01, ###p<0.001 compared with vehicle. T1DM, type 1 diabetes mellitus.

that the inflammasome-related DEGs were involved in the production and secretion of interleukin-1 and interleukin-1β, as well as the assembly of the NLRP3 inflammasome complex (figure 4E). Melatonin administration significantly reduced the expression levels of Casp1, Tlr4 and Gsdmd, which are closely linked to NLRP3 inflammasome activation and function. These genes were previously upregulated after stroke (figure 4F).

We then used multiplex cytokine assay and Western Blotting to examine inflammatory markers in the ipsilateral ischaemic brain after stroke. After stroke, proinflammatory factors IL-6 and IL-1β and anti-inflammatory factors IL-4 were elevated in the brain tissue of mice with T1DM. Notably, their levels were decreased in part by melatonin administration. The IFN-γ level did not change substantially in the vehicle and melatonin groups' brains (figure 5A). After a stroke, it is known that HMGB1-induced NLRP3 activities play a significant role in brain damage. Using western blotting, we thus investigated whether melatonin influenced NLRP3 activities in the ischaemic brains of type 1 diabetic mice after stroke. In mice with T1DM, stroke reduced p-ERK expression while increasing NLRP3, HMGB1 and Cleaved Caspase-1 expression (figure 5B). After stroke, therapy with melatonin dramatically reduced the expression of NLRP3, HMGB1 and Cleaved Caspase-1 in the brains of mice with T1DM.

Melatonin modulates gene expression and immune cell proportions in peripheral blood of diabetic mice poststroke

After studying melatonin effects in the ischaemic brain, we examined its impact on the blood. First, we conducted RNA sequencing on PBMCs from diabetic mice 24 hours after ischaemic stroke. Differential expression analysis between the melatonin and vehicle groups revealed 939 DEGs (|FC|>1.2, p<0.05), including 252 upregulated and 687 downregulated genes (figure 6A,B). The top 20 upregulated and downregulated DEGs are shown in the heatmap (figure 6C). KEGG pathway analysis indicated that melatonin treatment predominantly downregulated genes in metabolic pathways. In contrast, upregulated DEGs were associated with the Jak-STAT signalling

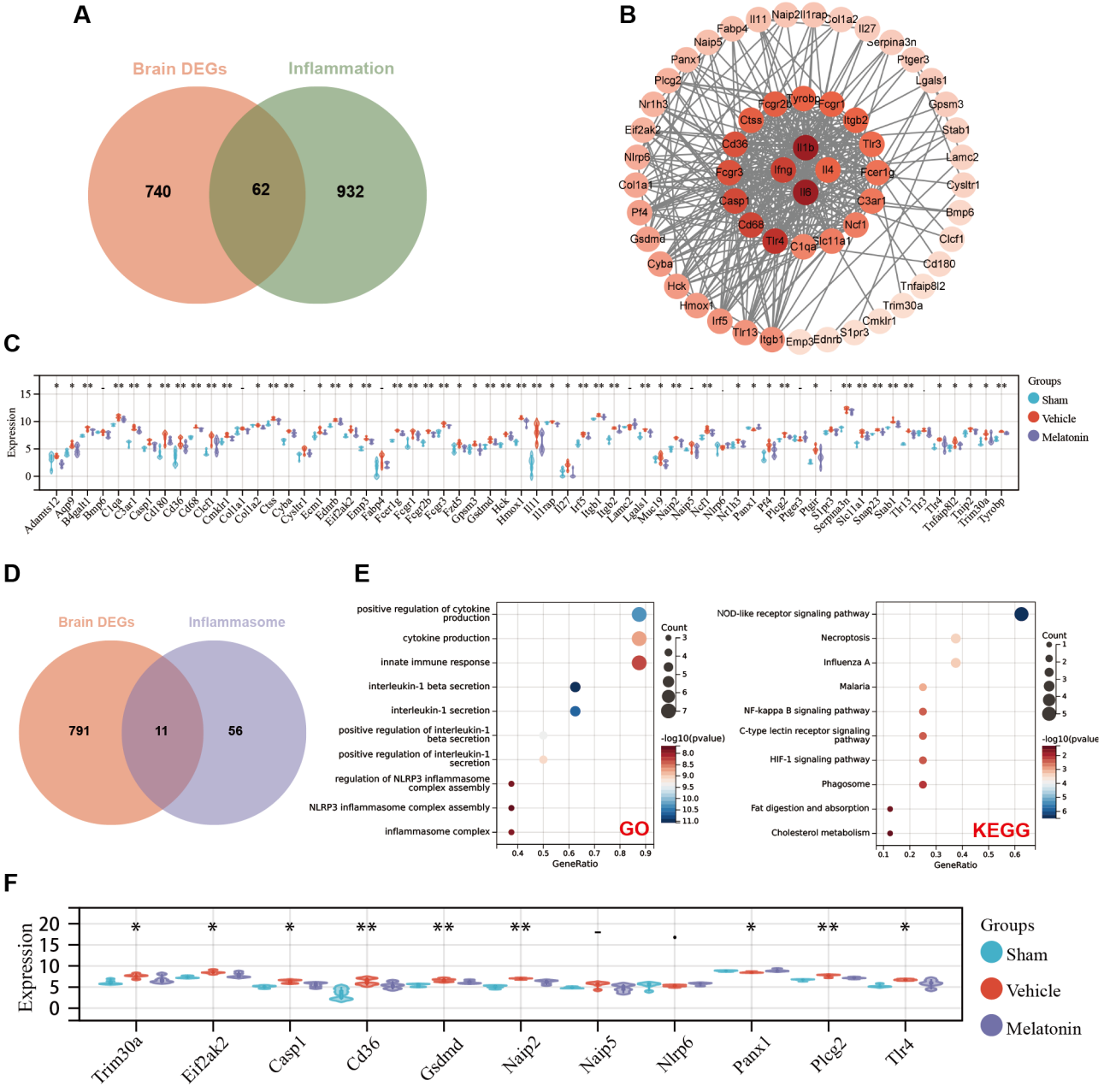

Figure 4 Bioinformatics analysis revealed that melatonin inhibits inflammation in the brain tissue of mice with T1DM after stroke. (A) Venn diagram showing the intersection of differentially expressed genes (DEGs) in melatonin-treated brains versus vehicle-treated brains and genes associated with inflammation. (B) PPI network illustrating relationships between 62 inflammation-related DEGs and key inflammation genes (Il6, Il1b, Ifng and Il4). Colour intensity represents the protein's importance in the network. (C) Box plot demonstrating expression levels of the 62 inflammation-related DEGs with melatonin treatment. (D) Venn diagram showing the intersection between DEGs induced by melatonin treatment and genes related to the inflammasome. (E) GO and KEGG enrichment analyses of 11 inflammasome-related genes, predominantly enriched in pathways associated with the NLRP3 inflammasome. (F) Box plot showing expression levels of the 11 inflammasome-related DEGs with melatonin treatment, highlighting statistical significance with *p<0.05, **p<0.01. GO, Gene Ontology; PPI, protein–protein interaction; T1DM, type 1 diabetes mellitus.

pathway. GO enrichment analysis revealed downregulated DEGs were enriched in cytosol, while upregulated DEGs were related to macromolecule modification (figure 6D,E).

Melatonin treatment in PBMCs led to the differential expression of 38 inflammation-associated genes (figure 6F). The PPI network analysis showed these genes were closely linked to Il6, Il1b, Ifng and Il4 (figure 6G). The violin plot (figure 6H) illustrates changes in expression levels of these genes.

Using flow cytometry, we analysed peripheral immune cell proportions in the blood of type 1 diabetic mice poststroke. Percentages of CD4+T cells, CD8+T cells, monocytes and neutrophils decreased after stroke.

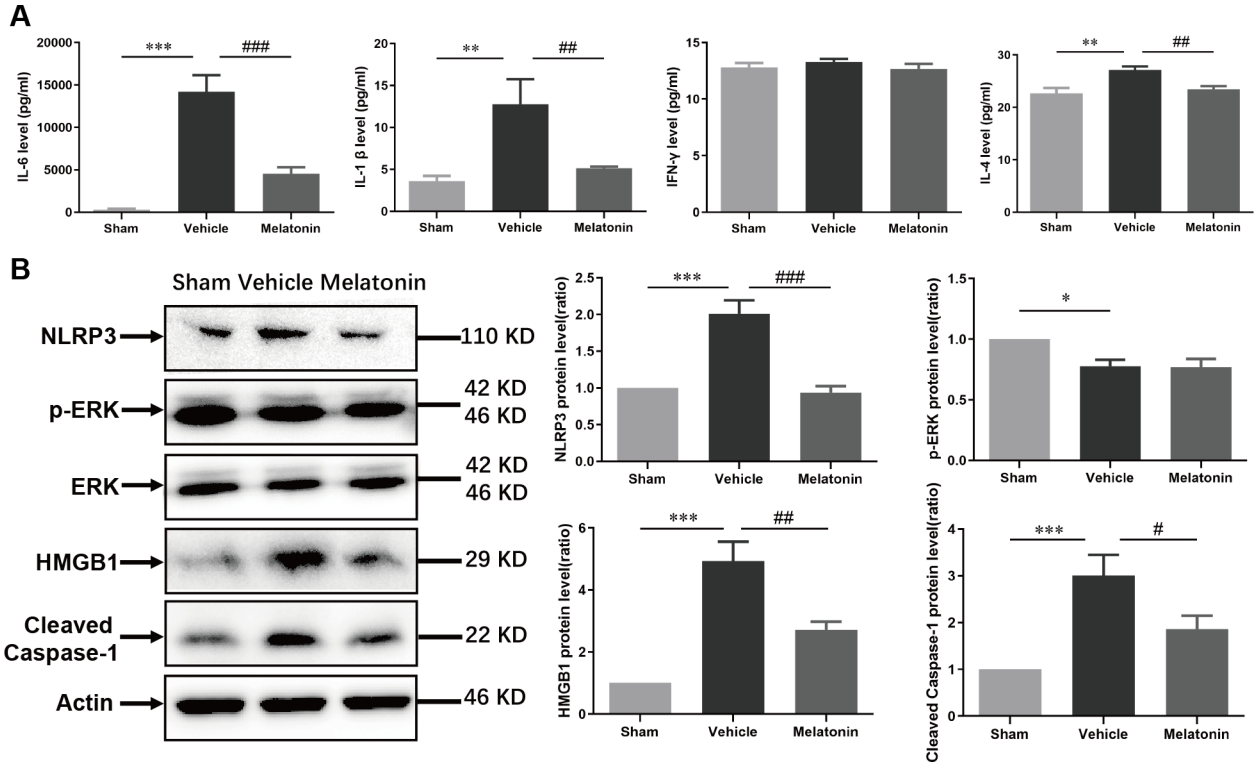

Figure 5 Melatonin therapy reduces inflammatory responses in the ischaemic brains of mice with T1DM. (A) Protein levels of inflammatory factors (IL-6, IL-1β, IFN-γ, IL-4) in the ischaemic brain following melatonin administration, measured using a multiplex cytokine assay. n=5 per group. **p<0.01, ***p<0.001 compared with Sham; ##p<0.01, ###p<0.001 compared with vehicle. (B) Representative images and statistical data of protein levels of NLRP3, p-ERK, HMGB1 and cleaved caspase-1 in the ischaemic brain. n=4–6 per group. *p<0.05, ***p<0.001 compared with Sham; #p<0.05, ##p<0.01, ###p<0.001 compared with vehicle. T1DM, type 1 diabetes mellitus.

Melatonin significantly increased the percentages of CD8+T cells, monocytes and neutrophils in peripheral blood (figure 7A).

To validate the effects of melatonin on peripheral inflammation, we measured inflammatory markers in the peripheral blood. IL-6 and IFN-γ protein levels were elevated poststroke, but melatonin therapy significantly decreased these levels. IL-1β and IL-4 levels were not substantially altered by MCAO or melatonin administration (figure 7B).

DISCUSSION

Despite previous studies that have investigated melatonin's protective effects against brain injury after stroke, few have examined its protective mechanisms implicated in both the brain and peripheral blood in diabetic animals. Therefore, our study is innovative in its comprehensive investigation of melatonin's effects on both the brain and the PBMCs from a systemic perspective. Our study demonstrates that melatonin treatment poststroke significantly reduces infarct sizes and improves neurological function in diabetic mice by modulating both central and peripheral inflammatory responses. In the brain, melatonin downregulated inflammatory factors, reduced the infiltration of CD8+T cells and neutrophils, and decreased levels of IL-6, IL-1β and IL-4. RNA sequencing and bioinformatics analyses identified key DEGs related to inflammation and inflammasomes. In the PBMC, melatonin treatment resulted in significant changes in gene expression, including downregulating genes involved in metabolic pathways and upregulating in the Jak-STAT signalling pathway. Flow cytometry revealed increased percentages of CD8+T cells, monocytes and neutrophils, and multiplex cytokine assay confirmed reduced IL-6 and IFN-γ levels. These findings highlight the potential interaction between brain and peripheral immune responses, suggesting that melatonin's dual modulation of inflammation supports its neuroprotective effects, thereby making it a promising therapeutic agent for managing stroke in diabetic conditions.

The interaction between the brain and the peripheral immune system plays a crucial role in the pathophysiology of stroke and recovery. After a stroke, inflammatory signals from the brain can trigger systemic immune responses, leading to changes in peripheral immune cell dynamics and cytokine levels.[29 30] These peripheral changes can, in turn, influence the brain's inflammatory environment and impact recovery outcomes.[31] Melatonin's ability to modulate central and peripheral inflammation highlights its protective effects. By reducing inflammatory cytokines and immune cell infiltration in the brain, melatonin helps mitigate direct neuronal damage and supports

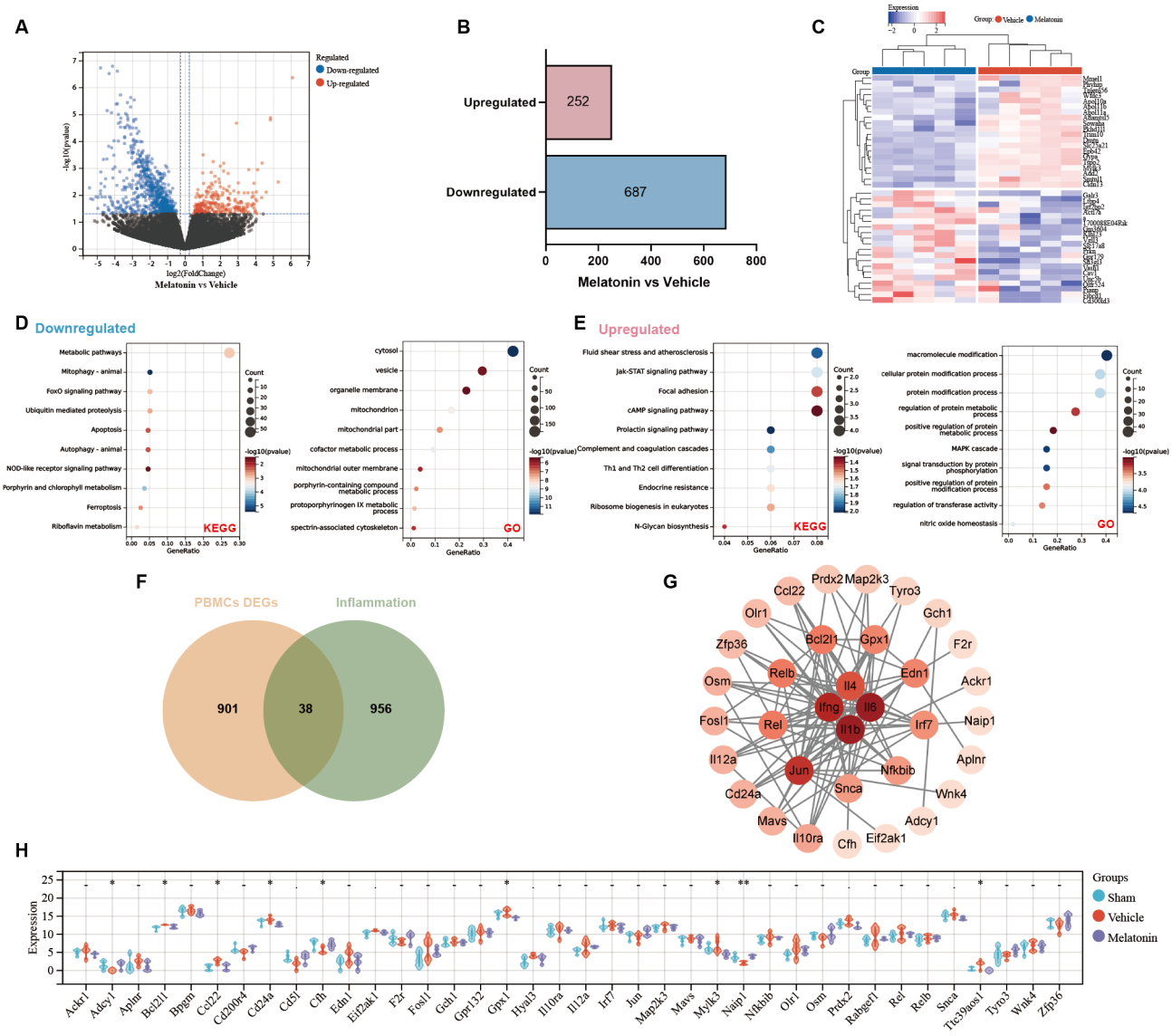

Figure 6 Melatonin administration downregulated inflammation-related genes in the PBMCs of mice with T1DM poststroke, demonstrating by RNA sequencing analysis. (A) A volcano plot illustrates the differentially expressed genes (DEGs) in PBMCs following melatonin treatment, compared with the vehicle group (|FC|>1.2, p<0.05). (B) A bar chart depicts the upregulated and downregulated DEGs induced by melatonin compared with the vehicle. (C) The heat map illustrates the top 20 genes that are upregulated and downregulated. (D) The KEGG and GO enrichment analysis of downregulated DEGs indicate pathways significant related to melatonin action. (E) Enrichment analysis elucidates the key pathways through which melatonin exerts its protective effects in PBMCs. (F) A Venn diagram shows the overlap between DEGs in PBMCs treated with melatonin versus vehicle and genes linked to inflammation. (G) The protein–protein interactions (PPI) network displays the connections among 38 DEGs related to inflammation and key inflammation genes (Il6, Il1b, Ifng and Il4). (H) A box plot exhibits the expression levels of the 38 inflammation-related DEGs following melatonin treatment. GO, Gene Ontology; KEGG, Kyoto Encyclopaedia of Genes and Genomes; PBMCs, peripheral blood mononuclear cells; T1DM, type 1 diabetes mellitus.

neuroprotection.[32] Concurrently, melatonin's impact on peripheral blood[33] shows its role in regulating systemic inflammation, which can help reduce the overall inflammatory burden and promote a more favourable environment for brain recovery. These findings suggest that melatonin's dual action on both the brain and peripheral immune responses is crucial for its neuroprotective effects in diabetic mice poststroke. The modulation of gene expression and immune cell dynamics by melatonin underscores its potential as a therapeutic agent in managing stroke-induced inflammation and improving functional recovery.

Our current study suggests that in diabetic mice poststroke, melatonin therapy significantly reduced the elevated expression of HMGB1 in the brain. HMGB1 is a highly conserved, DNA-binding nuclear protein that may be produced by microglia or passively released by damaged neurons.[34 35] Increasing evidence suggests that HMGB1 may trigger NLRP3 activities following stroke.[36–38] It has been discovered

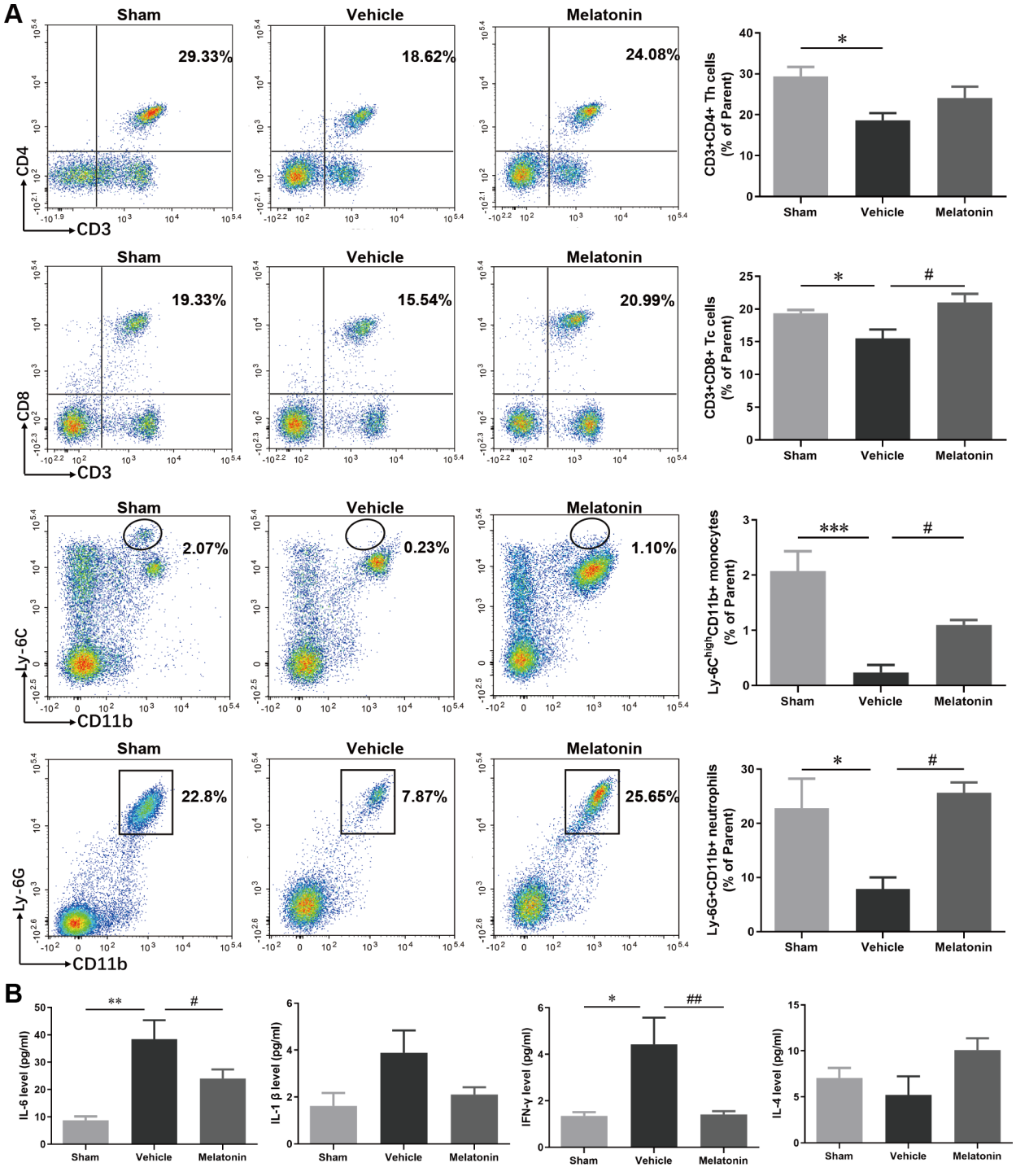

Figure 7 Melatonin treatment alleviates immunosuppression and reduces inflammatory response in peripheral blood of mice with T1DM after stroke. (A) Flow cytometry gating images and statistical data of CD4+T cells, CD8+T cells, monocytes and neutrophils. (B) Levels of inflammatory factor proteins. *p<0.05; **p<0.01,***p<0.001 compared with Sham; #p<0.05; ##p<0.01 compared with vehicle; n=5 per group. T1DM, type 1 diabetes mellitus.

that melatonin reduces poststroke neuroinflammation by decreasing HMGB1 and its subsequent signalling axis.[39] According to another piece of research, melatonin's regulation of 7nAchR alleviates ischaemia and reperfusion in part via blocking HMGB1-mediated microglia activation.[40] Increased HMGB1 levels have been known to be initially associated with higher NLRP3 inflammasome activity and elevated Cleaved Caspase-1.[41] Thus, we assume that melatonin's reduction of HMGB1 led to decreased NLRP3 inflammasome activity and suppression of its downstream signaling pathway. These results suggest that melatonin's neuroprotective effects may partly be due to its ability to lower HMGB1 levels, thereby mitigating

inflammation and brain injury following ischaemic stroke in diabetic conditions.

Our findings reveal that melatonin selectively inhibits the infiltration of neutrophils and T lymphocytes into the ischaemic brain without affecting monocyte migration. This differential effect likely arises from melatonin's modulation of IL-6 and IL-1β, which are crucial for the recruitment of neutrophils and T cells but may not significantly impact monocyte chemotaxis. Instead, monocyte recruitment may be more closely associated with CCL2 (MCP-1), a chemokine not strongly targeted by melatonin.[42] Additionally, while IL-6 and IL-1β are known to drive monocyte transformation into proinflammatory macrophages,[43] melatonin's reduction of these cytokines may limit this transformation, promoting a more reparative phenotype. This selective modulation allows melatonin to inhibit potentially damaging immune cell infiltration while preserving monocyte activity that may aid in tissue repair. These findings suggest that melatonin's anti-inflammatory effects are not merely suppressive but may strategically support recovery by fostering a balanced immune response. Further investigation into the specific signalling pathways involved could enhance our understanding of melatonin's therapeutic potential in stroke, particularly in diabetic conditions.

Studying the protective effects of melatonin in diabetic stroke models is crucial due to the unique pathophysiological challenges of stroke patients, involving several key issues such as blood–brain barrier permeability, microglial function and comparisons with non-diabetic models. Melatonin's ability to cross the blood–brain barrier, as documented,[44 45] is essential for its neuroprotective effects. Microglia, the first responders to inflammation poststroke, play a critical role in the brain's immune response. Melatonin has been shown to modulate microglial polarisation towards an anti-inflammatory phenotype, reducing their neurotoxic effects on hypoxic neurons.[46 47] While our current study did not investigate melatonin's specific impact on microglia in diabetic mice poststroke, future research will address this. Additionally, melatonin's efficacy in non-diabetic stroke models has been demonstrated,[16 47] highlighting its broad neuroprotective potential. Comparative studies between diabetic and non-diabetic stroke models are necessary to determine any differential efficacy of melatonin, as diabetes exacerbates oxidative stress and inflammation. These studies will help ascertain whether melatonin offers specific advantages in diabetic stroke. Further investigations are warranted to fully understand melatonin's protective mechanisms in diabetic and non-diabetic stroke contexts.

This study has several limitations. First, bulk RNAseq analysis of PBMCs has some limitations. PBMCs include lymphocytes, monocytes and dendritic cells but exclude neutrophils, which are significant in ischaemia/reperfusion injury. We combined bulk RNAseq with other complementary techniques such as flow cytometry and multiplex cytokine assay to address these limitations. Flow cytometry allowed us to precisely assess different cell populations, including neutrophils, while multiplex cytokine assay provided detailed measurements of cytokine levels. This multifaceted approach gave us a comprehensive understanding of melatonin's anti-inflammatory effects across various cell types and cytokines. We did not use specific cell transfer or depletion techniques to investigate the roles of different cell populations in melatonin's neuroprotection. Additionally, the absence of HMGB1 or NLRP3 inhibitors to test their roles in melatonin's effects were not conclusively confirmed. Furthermore, the study focused on short-term effects and did not evaluate the long-term impact of melatonin treatment on stroke recovery. While several genes and pathways were identified, not all possible mechanistic pathways were thoroughly explored. Lastly, the study's findings in mice with T1DM may not be directly applicable to humans or other types of diabetes, limiting the generalisability of the results.

In future studies, we plan to use specific cell transfer or depletion techniques to investigate the roles of different cell populations in melatonin's neuroprotection. Additionally, we will employ HMGB1 or NLRP3 inhibitors to confirm their roles in the anti-inflammatory effects of melatonin. This approach will help to understand better the mechanisms by which melatonin exerts its protective effects in diabetic stroke models and potentially translate these findings into clinical practice.

CONCLUSION

Melatonin treatment poststroke significantly reduces infarct sizes and improves neurological function in diabetic mice. It modulates inflammatory responses in both the brain and peripheral blood, reduces immune cell infiltration and inhibits NLRP3 inflammasome activity. These findings suggest melatonin's potential as a therapeutic agent for managing stroke in diabetic conditions. Further research is needed to explore its long-term effects and broader applicability.

Contributors CL and HZ designed and supervised the whole project. CL, JG and LG carried out the whole experiment. CL and JG wrote the paper. CL, JG, JL, BX and HZ participated in revision. HZ is the guarantor.

Funding This study was funded by National Natural Science Foundation of China (81701154).

Competing interests None declared.

Patient consent for publication Not applicable.

Provenance and peer review Not commissioned; externally peer reviewed.

Data availability statement No data are available.

ORCID iDs

Cuiying Liu http://orcid.org/0000-0003-4162-543X
Junfa Li http://orcid.org/0000-0002-1930-9724
Baohui Xu http://orcid.org/0000-0002-2857-7464
Heng Zhao http://orcid.org/0009-0004-4722-5634

REFERENCES

1 Kumari R, Willing LB, Patel SD, *et al*. Increased cerebral matrix metalloprotease-9 activity is associated with compromised recovery in the diabetic db/db mouse following a stroke. *J Neurochem* 2011;119:1029–40.
2 Liu L, Cao Q, Gao W, *et al*. Melatonin ameliorates cerebral ischemia-reperfusion injury in diabetic mice by enhancing autophagy via the SIRT1-BMAL1 pathway. *The FASEB Journal* 2021;35:e22040.
3 Venkat P, Chopp M, Chen J. Cell-Based and Exosome Therapy in Diabetic Stroke. *Stem Cells Transl Med* 2018;7:451–5.
4 Alghamdi BS. The neuroprotective role of melatonin in neurological disorders. *J of Neuroscience Research* 2018;96:1136–49.
5 Tamtaji OR, Mirhosseini N, Reiter RJ, *et al*. Melatonin, a calpain inhibitor in the central nervous system: Current status and future perspectives. *J Cell Physiol* 2019;234:1001–7.
6 Sadanandan N, Cozene B, Cho J, *et al*. Melatonin—A Potent Therapeutic for Stroke and Stroke-Related Dementia. *Antioxidants (Basel)* 2020;9:672.
7 Yang B, Zang L-E, Cui J-W, *et al*. Melatonin Plays a Protective Role by Regulating miR-26a-5p-NRSF and JAK2-STAT3 Pathway to Improve Autophagy, Inflammation and Oxidative Stress of Cerebral Ischemia-Reperfusion Injury. *Drug Des Devel Ther* 2020;14:3177–88.
8 Wang X. The antiapoptotic activity of melatonin in neurodegenerative diseases. *CNS Neurosci Ther* 2009;15:345–57.
9 Wang J, Gao S, Lenahan C, *et al*. Melatonin as an Antioxidant Agent in Stroke: An Updated Review. *Aging Dis* 2022;13:1823.
10 Chitimus DM, Popescu MR, Voiculescu SE, *et al*. Melatonin's Impact on Antioxidative and Anti-Inflammatory Reprogramming in Homeostasis and Disease. *Biomolecules* 2020;10:1211.
11 Gao Y, Li Z-T, Jin L, *et al*. Melatonin attenuates hepatic ischemia-reperfusion injury in rats by inhibiting NF-κB signaling pathway. *Hepatobiliary Pancreat Dis Int* 2021;20:551–60.
12 Qin T, Feng D, Zhou B, *et al*. Melatonin Suppresses LPS-Induced Oxidative Stress in Dendritic Cells for Inflammatory Regulation via the Nrf2/HO-1 Axis. *Antioxidants (Basel)* 2022;11:10.
13 Li Y, Guo Y, Fan Y, *et al*. Melatonin Enhances Autophagy and Reduces Apoptosis to Promote Locomotor Recovery in Spinal Cord Injury via the PI3K/AKT/mTOR Signaling Pathway. *Neurochem Res* 2019;44:2007–19.
14 Cui Y, Yang M, Wang Y, *et al*. Melatonin prevents diabetes-associated cognitive dysfunction from microglia-mediated neuroinflammation by activating autophagy via TLR4/Akt/mTOR pathway. *The FASEB Journal* 2021;35:e21485.
15 Yawoot N, Govitrapong P, Tocharus C, *et al*. Ischemic stroke, obesity, and the anti-inflammatory role of melatonin. *Biofactors* 2021;47:41–58.
16 Yu X, Wang S, Wang X, *et al*. Melatonin improves stroke by inhibiting autophagy-dependent ferroptosis mediated by NCOA4 binding to FTH1. *Exp Neurol* 2024;379:114868.
17 Sun X, Chen L, Gui Q-J. The Potential of Melatonin to Treat Atherosclerosis by Targeting Mitochondria. *Curr Top Med Chem* 2023;23:848–59.
18 Xu Q, Cheung RTF. Melatonin mitigates type 1 diabetes-aggravated cerebral ischemia-reperfusion injury through anti-inflammatory and anti-apoptotic effects. *Brain Behav* 2023;13:e3118.
19 Liu L, Cao Q, Gao W, *et al*. Melatonin protects against focal cerebral ischemia-reperfusion injury in diabetic mice by ameliorating mitochondrial impairments: involvement of the Akt-SIRT3-SOD2 signaling pathway. *Aging (Milano)* 2021;13:16105–23.
20 Ding Y, DeGracia D, Geng X, *et al*. Perspectives on effect of spleen in ischemic stroke. *Brain Circ* 2022;8:117–20.
21 Mamtilahun M, Jiang L, Song Y, *et al*. Plasma from healthy donors protects blood-brain barrier integrity via FGF21 and improves the recovery in a mouse model of cerebral ischaemia. *Stroke Vasc Neurol* 2021;6:561–71.
22 Liu C, Zhang C, Du H, *et al*. Remote ischemic preconditioning protects against ischemic stroke in streptozotocin-induced diabetic mice via anti-inflammatory response and anti-apoptosis. *Brain Res* 2019;1724:146429.
23 Liu C, Yang J, Zhang C, *et al*. Analysis of long non-coding RNA expression profiles following focal cerebral ischemia in mice. *Neurosci Lett* 2018;665:123–9.
24 Liu C, Yang J, Zhang C, *et al*. Remote ischemic conditioning reduced cerebral ischemic injury by modulating inflammatory responses and ERK activity in type 2 diabetic mice. *Neurochem Int* 2020;135:104690.
25 Gan X, Luo Y, Ling F, *et al*. Outcome in acute stroke with different intra-arterial infusion rate of urokinase on thrombolysis. *Interv Neuroradiol* 2010;16:290–6.
26 Joo SP, Xie W, Xiong X, *et al*. Ischemic postconditioning protects against focal cerebral ischemia by inhibiting brain inflammation while attenuating peripheral lymphopenia in mice. *Neuroscience* 2013;243:149–57.
27 Liu C, Zhao L, Han S, *et al*. Identification and Functional Analysis of MicroRNAs in Mice following Focal Cerebral Ischemia Injury. *IJMS* 2015;16:24302–18.
28 Barua JD, Omit SBS, Rana HK, *et al*. Bioinformatics and System Biological Approaches for the Identification of Genetic Risk Factors in the Progression of Cardiovascular Disease. *Cardiovasc Ther* 2022;2022:1–14.
29 Sekhon MS, Stukas S, Hirsch-Reinshagen V, *et al*. Neuroinflammation and the immune system in hypoxic ischaemic brain injury pathophysiology after cardiac arrest. *J Physiol (Lond)* 2024;602:5731–44.
30 Simats A, Liesz A. Systemic inflammation after stroke: implications for post-stroke comorbidities. *EMBO Mol Med* 2022;14:e16269.
31 Liu F, Cheng X, Zhong S, *et al*. Communications Between Peripheral and the Brain-Resident Immune System in Neuronal Regeneration After Stroke. *Front Immunol* 2020;11.
32 Won E, Na K-S, Kim Y-K. Associations between Melatonin, Neuroinflammation, and Brain Alterations in Depression. *Int J Mol Sci* 2021;23:305.
33 Liu Y, Chen X-Q, Wang F, *et al*. Melatonin relieves Th17/CD4−CD8− T cells inflammatory responses via nuclear-receptor dependent manner in peripheral blood of primary Sjögren's syndrome. *Int Immunopharmacol* 2022;109:108778.
34 An C, Shi Y, Li P, *et al*. Molecular dialogs between the ischemic brain and the peripheral immune system: Dualistic roles in injury and repair. *Prog Neurobiol* 2014;115:6–24.
35 Ye Y, Zeng Z, Jin T, *et al*. The Role of High Mobility Group Box 1 in Ischemic Stroke. *Front Cell Neurosci* 2019;13:127.
36 Wang M, Liu Z, Hu S, *et al*. Taohong Siwu Decoction Ameliorates Ischemic Stroke Injury Via Suppressing Pyroptosis. *Front Pharmacol* 2020;11:590453.
37 Liang Y, Song P, Chen W, *et al*. Inhibition of Caspase-1 Ameliorates Ischemia-Associated Blood-Brain Barrier Dysfunction and Integrity by Suppressing Pyroptosis Activation. *Front Cell Neurosci* 2020;14:540669.
38 Fann DY-W, Lee SY, Manzanero S, *et al*. Intravenous immunoglobulin suppresses NLRP1 and NLRP3 inflammasome-mediated neuronal death in ischemic stroke. *Cell Death Dis* 2013;4:e790.
39 Chibaatar E, Le K, Abdoulaye IA, *et al*. Melatonin Ameliorates Lipopolysaccharide-Induced Microglial Inflammation via Triggering SIRT1/HMGB1 Signaling Axis. *J Mol Neurosci* 2021;71:691–701.
40 Chen S, Sun Y, Li F, *et al*. Modulation of α7nAchR by Melatonin Alleviates Ischemia and Reperfusion-Compromised Integrity of Blood-Brain Barrier Through Inhibiting HMGB1-Mediated Microglia Activation and CRTC1-Mediated Neuronal Loss. *Cell Mol Neurobiol* 2022;42:2407–22.
41 Tan S-W, Zhao Y, Li P, *et al*. HMGB1 mediates cognitive impairment caused by the NLRP3 inflammasome in the late stage of traumatic brain injury. *J Neuroinflammation* 2021;18:241.
42 Park HJ, Kim HJ, Ra J, *et al*. Melatonin inhibits lipopolysaccharide-induced CC chemokine subfamily gene expression in human peripheral blood mononuclear cells in a microarray analysis. *J Pineal Res* 2007;43:121–9.
43 Dayan V, Yannarelli G, Billia F, *et al*. Mesenchymal stromal cells mediate a switch to alternatively activated monocytes/macrophages after acute myocardial infarction. *Basic Res Cardiol* 2011;106:1299–310.
44 Wang X, Figueroa BE, Stavrovskaya IG, *et al*. Methazolamide and melatonin inhibit mitochondrial cytochrome C release and are neuroprotective in experimental models of ischemic injury. *Stroke* 2009;40:1877–85.
45 Lin H-W, Lee EJ. Effects of melatonin in experimental stroke models in acute, sub-acute, and chronic stages. *Neuropsychiatr Dis Treat* 2009;5:157–62.
46 Liu Z-J, Ran Y-Y, Qie S-Y, *et al*. Melatonin protects against ischemic stroke by modulating microglia/macrophage polarization toward anti-inflammatory phenotype through STAT3 pathway. *CNS Neurosci Ther* 2019;25:1353–62.
47 Li D, He T, Zhang Y, *et al*. Melatonin regulates microglial polarization and protects against ischemic stroke-induced brain injury in mice. *Exp Neurol* 2023;367:114464.