Journal of Integrative Neuroscience

J. Integr. Neurosci. **2023**; 22(2): 44
https://doi.org/10.31083/j.jin2202044

Original Research

Identification of Key Genes and Pathways in the Hippocampus after Traumatic Brain Injury: Bioinformatics Analysis and Experimental Validation

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Academic Editor: Raji Paul Grewal
Submitted: 25 June 2022 Revised: 27 September 2022 Accepted: 30 September 2022 Published: 20 February 2023

Abstract

Background: Traumatic brain injury (TBI) is a common brain injury with a high morbidity and mortality. The complex injury cascade triggered by TBI can result in permanent neurological dysfunction such as cognitive impairment. In order to provide new insights for elucidating the underlying molecular mechanisms of TBI, this study systematically analyzed the transcriptome data of the rat hippocampus in the subacute phase of TBI. **Methods**: Two datasets (GSE111452 and GSE173975) were downloaded from the Gene Expression Omnibus (GEO) database. Systematic bioinformatics analyses were performed, including differentially expressed genes (DEGs) analysis, gene set enrichment analysis (GSEA), Gene Ontology (GO) enrichment analysis, and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis, protein-protein interaction (PPI) network construction, and hub gene identification. In addition, hematoxylin and eosin (HE), Nissl, and immunohistochemical staining were performed to assess the injured hippocampus in a TBI rat model. The hub genes identified by bioinformatics analyses were verified at the mRNA expression level. **Results**: A total of 56 DEGs were shared in the two datasets. GSEA results suggested significant enrichment in the MAPK and PI3K/Akt pathways, focal adhesion, and cellular senescence. GO and KEGG analyses showed that the common DEGs were predominantly related to immune and inflammatory processes, including antigen processing and presentation, leukocyte-mediated immunity, adaptive immune response, lymphocyte-mediated immunity, phagosome, lysosome, and complement and coagulation cascades. A PPI network of the common DEGs was constructed, and 15 hub genes were identified. In the shared DEGs, we identified two transcription co-factors and 15 immune-related genes. The results of GO analysis indicated that these immune-related DEGs were mainly enriched in biological processes associated with the activation of multiple cells such as microglia, astrocytes, and macrophages. HE and Nissl staining results demonstrated overt hippocampal neuronal damage. Immunohistochemical staining revealed a marked increase in the number of Iba1-positive cells in the injured hippocampus. The mRNA expression levels of the hub genes were consistent with the transcriptome data. **Conclusions**: This study highlighted the potential pathological processes in TBI-related hippocampal impairment. The crucial genes identified in this study may serve as novel biomarkers and therapeutic targets, accelerating the pace of developing effective treatments for TBI-related hippocampal impairment.

Keywords: traumatic brain injury; hippocampus; bioinformatics analysis; hub gene; neuroinflammation

1. Introduction

Traumatic brain injury (TBI) is a lethal and disabling injury and is a major challenge to public health and social development worldwide [1]. The global incidence of TBI has been continuously increasing over the past several decades [1,2]. In 2016, there were more than 27 million new cases of TBI, and the total number of prevalent cases exceeded 55 million [2]. TBI is caused by the application of an external mechanical force to the head, followed by a series of primary and secondary pathological mechanisms, such as cell death in the brain parenchyma, disruption of the neurovascular unit, axonal injury, neuroinflammation, and neurodegeneration [3]. Due to the complex pathological mechanisms of TBI, developing effective neuroprotective therapeutics for afflicted patients has been challenging [3]. In recent years, increasing emphasis has been placed on the progressive and chronic consequences of TBI. Many preclinical and clinical studies have suggested that TBI is a significant risk factor for neuropsychiatric and cognitive disorders such as posttraumatic stress disorder (PTSD), chronic traumatic encephalopathy (CTE), and Alzheimer's disease (AD) [4–7]. The hippocampus is the main locus responsible for cognitive processing involving learning and memory [6,8]. It has been shown that hippocampus dysfunction is associated with cognitive deficits following a TBI [6,8,9]. Existing treatments for TBI primarily focus on immediate neurosurgical procedures and long-term behavioral rehabilitation, but few treatments are sufficient to improve TBI-induced cognitive deficits [10,11]. TBI differs from other diseases in that it involves different periods after the injury. However, subacute phase studies of TBI functional recovery have received substantially less attention than acute

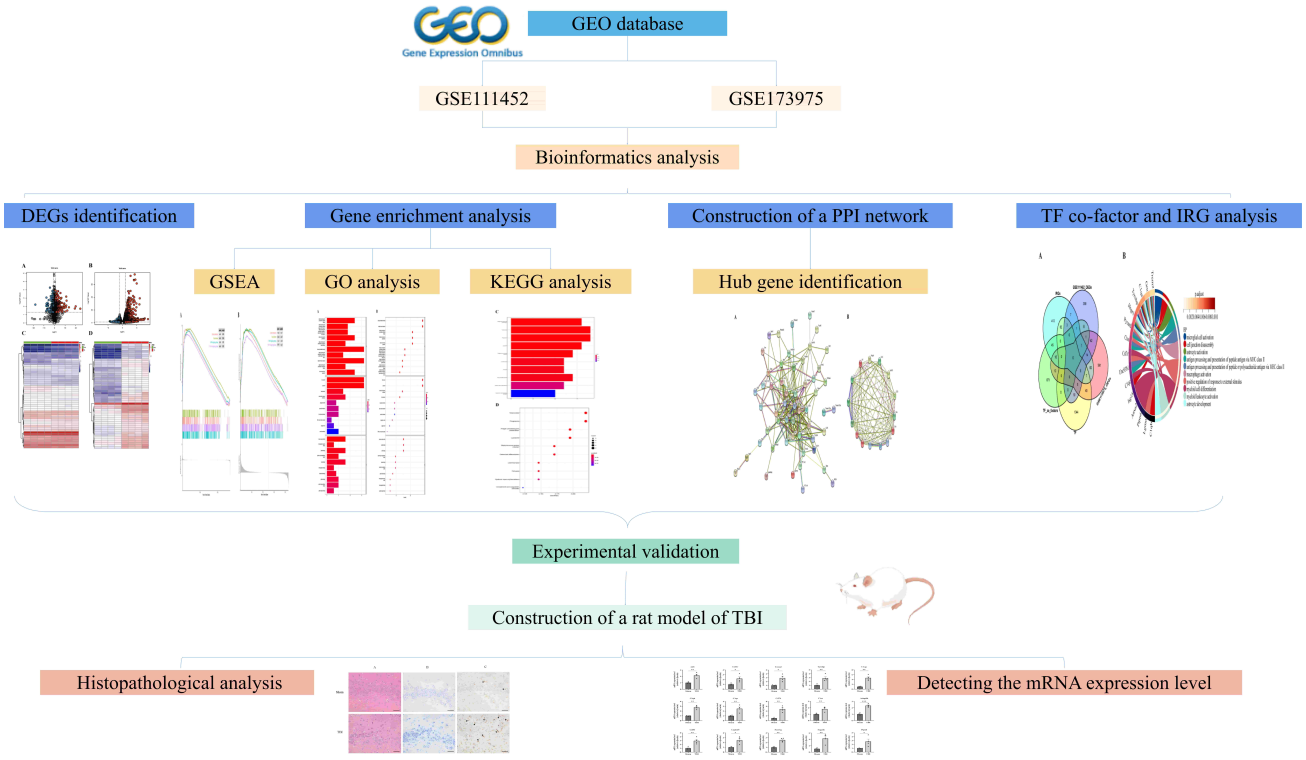

Fig. 1. Schematic illustration of bioinformatics analyses and experimental validation in the study.

phase studies [12,13]. Since effective therapeutic strategies for the subacute phase of TBI are limited, it is essential to explore optimal therapeutic targets at this stage [12,13].

Understanding the molecular mechanisms underlying pathological processes is a critical step toward developing effective treatments for TBI. Based on the systematic bioinformatics analysis, microarray and high throughput sequencing techniques have emerged as essential tools for exploring the pathogenesis of complex human diseases [14,15]. Many bioinformatics studies have been conducted in the field of oncology and some non-tumor diseases, but relatively few have focused explicitly on traumatic nerve injuries such as TBI [16]. The Gene Expression Omnibus (GEO) is a publicly available database consisting of an invaluable resource of mass gene expression data that can be systematically analyzed for biomarker or therapeutic target discovery [17]. In this study, we used two datasets from the GEO database to screen the differentially expressed genes (DEGs) of the hippocampus in the subacute phase of TBI. Gene functional enrichment analyses, protein-protein interaction (PPI) network construction, and hub gene identification were also performed. Furthermore, we observed the main histopathological features of the injured hippocampus and selected several DEGs for verification *in vivo*. The schematic workflow of our study is presented in Fig. 1. This study may provide new insight into potential biomarkers and therapeutic targets for cognitive deficits induced by TBI.

2. Materials and Methods

2.1 Dataset Source

We downloaded two mRNA expression datasets (GSE111452 and GSE173975) from the GEO database (https://www.ncbi.nlm.nih.gov/geo/) [18,19]. In two experiments, adult male Sprague-Dawley (SD) rats were used as the experimental models. The rats were anesthetized by inhaling 1.5–3.0% isoflurane; then, a circular craniotomy was made on the right side of the parietal bone. The rats in the model group were subjected to a fluid percussion injury (FPI). The fluid pressure pulses were 2.3 and 2.12 atm in the GSE111452 and GSE173975 datasets. The rats in the sham group underwent a similar surgical preparation except for the FPI. The rat hippocampus tissues on the side of the lesion were harvested on the 14th day (subacute phase) after TBI modeling. Each dataset contained four separate hippocampus tissue samples in the sham and TBI groups. Detailed information of the two datasets can be found in **Supplementary Table 1**.

2.2 DEGs Identification

We utilized the “limma” and “edgeR” packages to identify DEGs between the sham and TBI groups in the GSE111452 and GSE173975 datasets, respectively. The criterion for defining differential gene expression was a p-value < 0.05 and a $|\log2 \text{ (fold change)}| > 1.00$. The common DEGs in the two datasets were identified using the “VennDiagram” package.

2.3 Gene Enrichment Analysis

Gene set enrichment analysis (GSEA), Gene Ontology (GO) enrichment analysis, and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis were conducted by the "clusterProfiler" package [20]. For GSEA, a |standardized enrichment score (NES)| >1.00 and an adjusted p-value < 0.05 were considered as the criteria for significant enrichment. For the GO and KEGG analyses, the significant threshold was set as an adjusted p-value < 0.05. The GO enrichment analysis contained three categories: biological process (BP), cellular components (CC), and molecular function (MF).

2.4 Construction of a PPI Network and Identification of Hub Genes

A PPI network was drawn by the Search Tool for the Retrieval of Interacting Genes (STRING) database (http://string-db.org/). The threshold for the minimum interaction score was set to 0.4. The PPI network was plotted in Cytoscape. The hub genes were identified using the MCC algorithm of the CytoHubba plug-in.

2.5 Transcription Factor (TF) and Co-Factor Analysis

The list of TF and co-factor of rats were downloaded from the AnimalTFDB database [21]. The differentially expressed TF and co-factors shared in common between the two datasets were identified using the "VennDiagram" package.

2.6 Immune-Related Genes (IRGs) Analysis

The list of IRGs was obtained from the InnateDB database [22,23]. The overlapped differentially expressed IRGs in the two datasets were identified using the "VennDiagram" package.

2.7 Animal Experiment

Specific pathogen-free (SPF) Male SD rats (7 weeks old, 220–240 g body weight) were purchased from Beijing Huafukang Bioscience (Beijing, China). All animals were housed in a standard environment (12-h light/dark cycle, room temperature: 23–25 °C, relative humidity: 50%–60%) with water and food ad libitum. After adaptive feeding for ten days, fourteen rats were randomly allocated into the sham and TBI groups (n = seven per group). The experimental procedures were approved by the Laboratory Animal Ethics Committee of Jinan University (ethical number: 14075) and followed the Principles of Laboratory Animal Care.

2.7.1 TBI Model

TBI model was produced with a controlled cortical impact (CCI) device (YHC199, Wuhan Yihong Science & Technology Co., Ltd., Wuhan, Hubei, China). Briefly, rats were anesthetized with 2% pentobarbital sodium (40 mg/kg) with an intraperitoneal injection. Then, they were fixed in a stereotaxic frame, and a skin incision was made on the scalp to expose the skull. A craniotomy (diameter of 5.0 mm) was performed over the right parietal bone (2.0 mm lateral to the sagittal suture and 1.0 mm posterior to the bregma) to expose the dura and cerebral cortex. A flat-tipped impactor (diameter of 4 mm) was utilized to impact the exposed dura (impact parameters: depth of 2.5 mm, dwell time of 250 ms, and impact velocity of 3.5 m/s) [24]. After surgery, scalp incisions were sutured, and the rats were moved into clean cages with heated pads for recovery. The rats in the sham group were subjected to the same procedures without the CCI.

2.7.2 Tissue Collection and Preparation

To collect the hippocampal tissue, on the 14th day after TBI, four rats in each group were anesthetized with 2% pentobarbital sodium (40 mg/kg) with an intraperitoneal injection, followed by transcardial perfusion with ice-cold 0.9% normal saline. Then, the brain was removed, and the hippocampal tissue in the ipsilateral (lesion) side was rapidly removed. The collected samples were flash-frozen in liquid nitrogen and stored at –80 °C for subsequent analysis. For histopathological detection, three rats in each group were anesthetized in the same manner; then, the brain was harvested after transcardial perfusion with ice-cold 0.9% normal saline followed by 4% paraformaldehyde. The tissue blocks were embedded in paraffin after fixation in 4% paraformaldehyde for 24 hours.

2.8 Hematoxylin and Eosin (HE) Staining

HE staining was conducted using a HE staining kit (Servicebio, Wuhan, Hubei, China) according to the instruction manual. Brain sections (5 um) were subjected to deparaffinization with dimethylbenzene, ethanol hydration, hematoxylin staining, eosin staining, and ethanol dehydration. Finally, the sections were sealed with neutral resin. Tissue sections were observed by a light microscope (Olympus, Tokyo, Japan).

2.9 Nissl Staining

Nissl staining was performed using Nissl staining solution (Servicebio, Wuhan, Hubei, China) according to the manufacturer's instructions. After deparaffinization and hydration, brain slices were stained in Nissl staining solution and then differentiated in 0.1% glacial acetic acid. After sealing the slices, the staining was visualized under a light microscope (Olympus, Tokyo, Japan).

2.10 Immunohistochemical Staining

After deparaffinization and hydration, brain sections underwent high-temperature antigen repair using citrate buffer (pH 6.0). The sections were cooled naturally after antigen retrieval. To block endogenous peroxidases, 3% hydrogen peroxide solution was applied to the sections for 25 minutes, and nonspecific bindings were blocked with

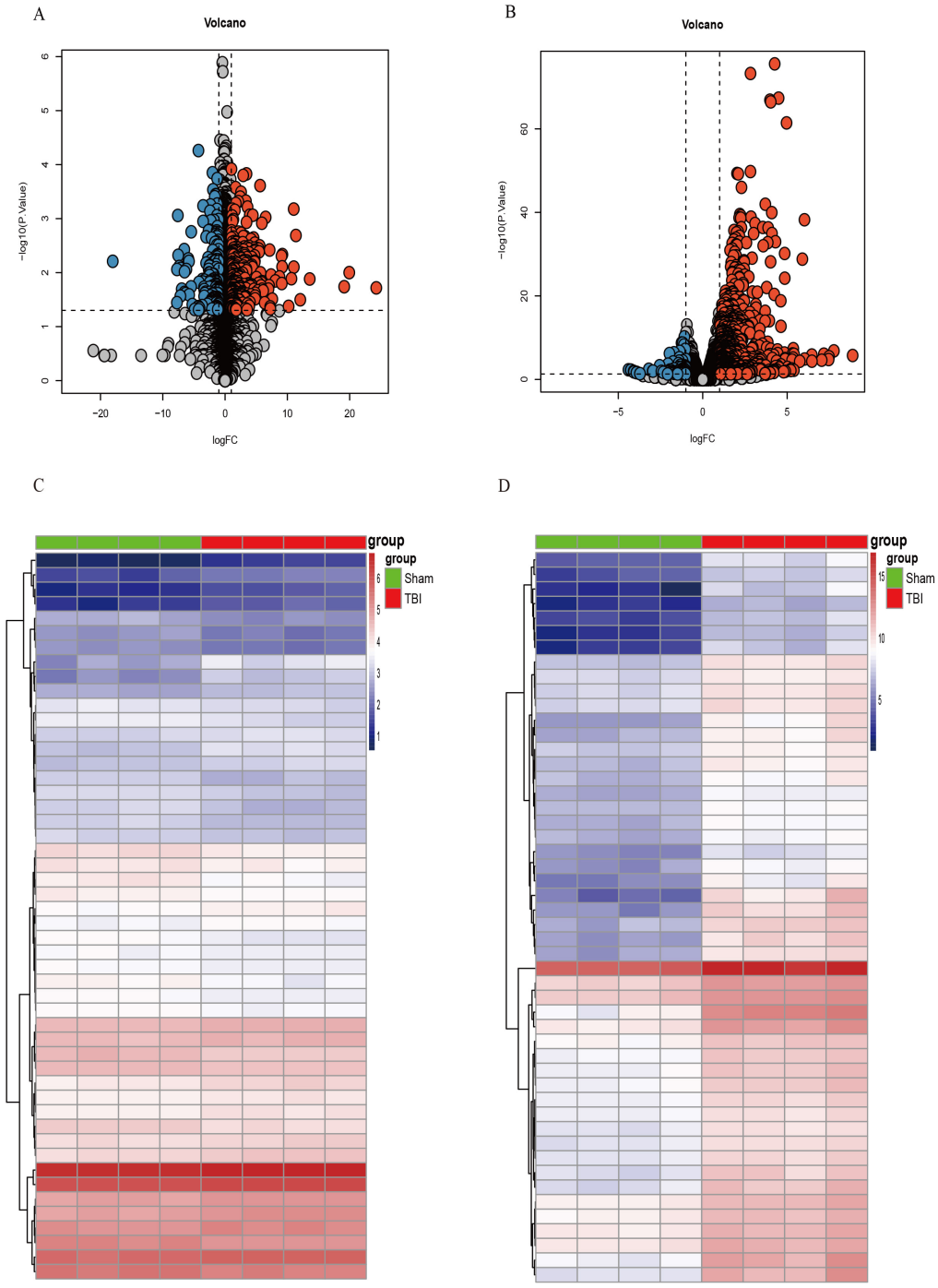

Fig. 2. The DEGs between the sham and TBI groups in the GSE111452 and GSE173975 datasets. (A) Volcano plot of the DEGs in the GSE111452 dataset. (B) Volcano plot of the DEGs in the GSE173975 dataset. Red dots represent upregulated genes, and blue dots represent downregulated genes. (C) Heatmap of the top 50 DEGs in the GSE111452 dataset. (D) Heatmap of the top 50 DEGs in the GSE173975 dataset.

3% bovine serum albumin (BSA) solution for 30 minutes. Then, the sections were incubated with primary antibody mouse anti-Iba1 (1:500, Servicebio, Wuhan, Hubei, China) overnight at 4 °C. After removing the primary antibody, the sections were incubated in anti-mouse HRP-labeled secondary antibody (1:500, Servicebio, Wuhan, Hubei, China) at room temperature for one hour. Diaminobenzidine (DAB) staining was performed using the DAB chromogenic agent (Servicebio, Wuhan, Hubei, China). The sections were sealed with coverslips after nuclear counterstaining and dehydration. The positive staining cells were observed using a light microscope (Olympus, Tokyo, Japan).

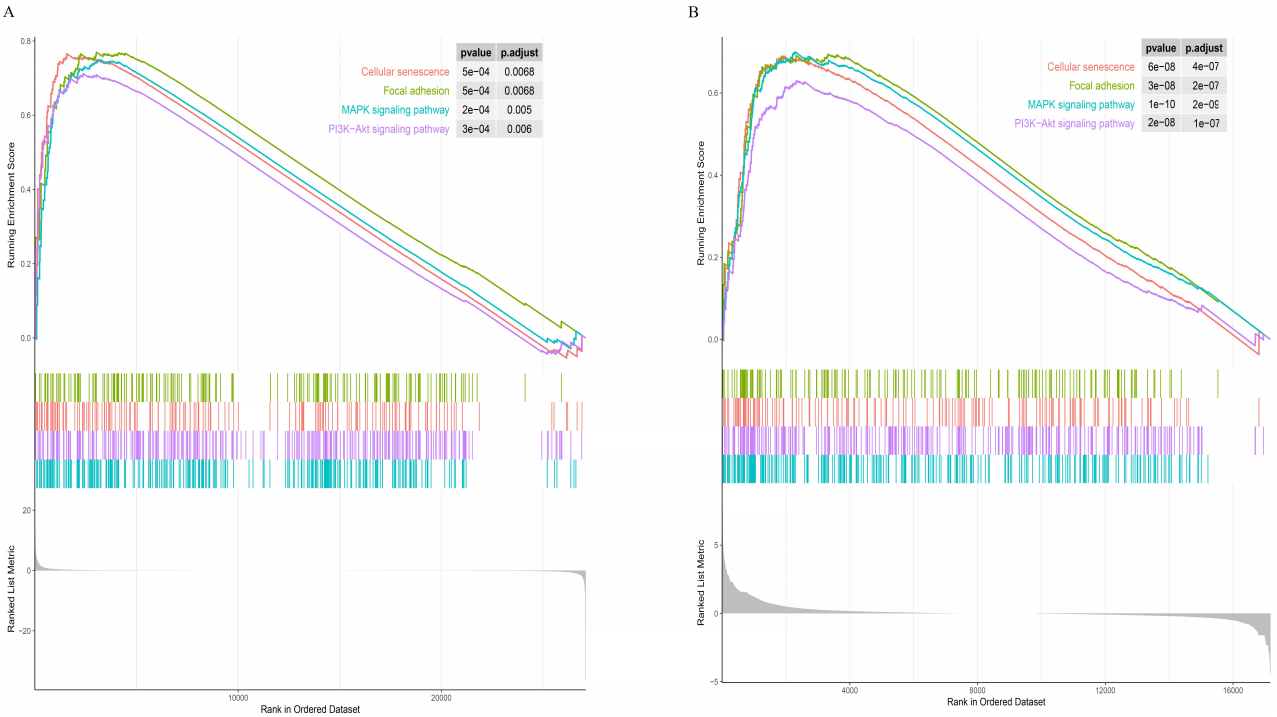

Fig. 3. The main results of GSEA in the GSE111452 (A) and GSE173975 (B) datasets.

2.11 Quantitative Real-Time PCR (RT-qPCR)

Total RNA was extracted from the hippocampal tissues using RNAiso Plus (Takara, Ohtsu, Japan). A NanoDrop spectrophotometer (Thermo Scientific, Waltham, MA, USA) was used to measure RNA concentration and purity. RT-qPCR was performed using the SYBR Green RT-qPCR kit (Takara, Ohtsu, Japan) and the CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). We used β-actin as a reference gene and calculated the relative mRNA expression level by the $2^{-\Delta\Delta Ct}$ method. Reactions were performed in triplicate. The sequences of primers used are provided in **Supplementary Table 2**.

2.12 Statistical Analysis

All data were presented as mean $\pm$ standard error of the mean (SEM). Two groups of data were compared using Student's *t*-test. Statistical analyses were performed with SPSS (version 23.0, IBM Corp., Chicago, IL, USA) software. Plots were generated using GraphPad Prism (version 6.0, GraphPad Software Inc., San Diego, CA, USA) and R (version 4.1.1, the R Foundation, Vienna, Austria) softwares. A p-value < 0.05 indicated statistical significance.

3. Results

3.1 Bioinformatics Analyses of the GEO Datasets

3.1.1 Identification of DEGs

A total of 445 genes were differentially expressed between the sham and TBI groups in the GSE111452 dataset, including 276 upregulated genes and 169 downregulated genes (Fig. 2A,C). In the GSE173975 dataset, 802 DEGs were screened between the sham and TBI groups, including 718 upregulated genes and 84 downregulated genes (Fig. 2B,D). The Venn diagram was utilized to identify the intersections of DEGs from the GSE111452 and GSE173975 datasets. Overall, 56 upregulated DEGs were shared in common between the two datasets.

3.1.2 GSEA Analysis

To avoid missing some genes with crucial biological relevance during the screening of DEGs, GSEA was conducted based on all gene expression data of the two datasets. We extracted four main pathways from the overlapped pathways in the two datasets, including the mitogen-activated protein kinase (MAPK) signaling pathway, phosphatidylinositol 3-kinases/protein kinase B (PI3K/Akt) signaling pathway, focal adhesion, and cellular senescence. The enrichment plots of the GSEA results are shown in Fig. 3.

3.1.3 GO and KEGG Analyses of DEGs

For the BP terms, the DEGs were mainly involved in antigen processing and presentation, leukocyte-mediated immunity, adaptive immune response, and lymphocyte-mediated immunity.

For the CC terms, the DEGs were primarily enriched in lytic vacuole, lysosome, late endosome, external side of plasma membrane, phagocytic vesicle, lysosomal membrane, lytic vacuole membrane, MHC class II protein complex, collagen trimer, and vacuolar membrane.

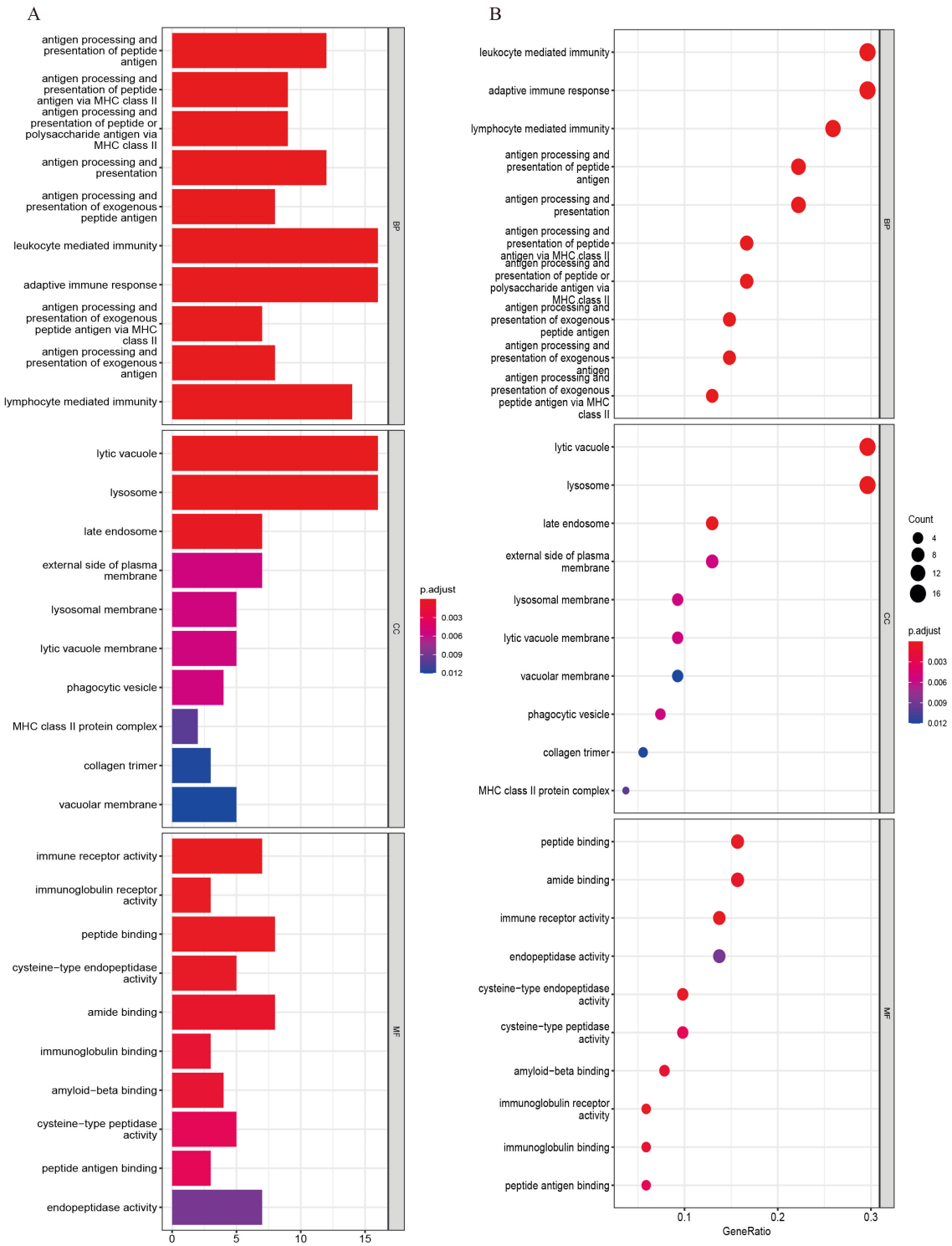

Fig. 4. GO analyses of the common DEGs. (A) The bar plot of the top 10 BP, CC, and MF terms. (B) The bubble plot of the top 10 BP, CC, and MF terms.

For the MF terms, the DEGs were significantly enriched in immune receptor activity, immunoglobulin receptor activity, peptide binding, cysteine-type endopeptidase activity, amide binding, immunoglobulin binding, amyloid-beta binding, cysteine-type peptidase activity, peptide antigen binding, and endopeptidase activity.

KEGG pathway enrichment analysis found that the DEGs were mainly involved in antigen processing and presentation, phagosome, lysosome, and complement and coagulation cascades, etc. The results of the GO and KEGG analyses are shown in Figs. 4,5.

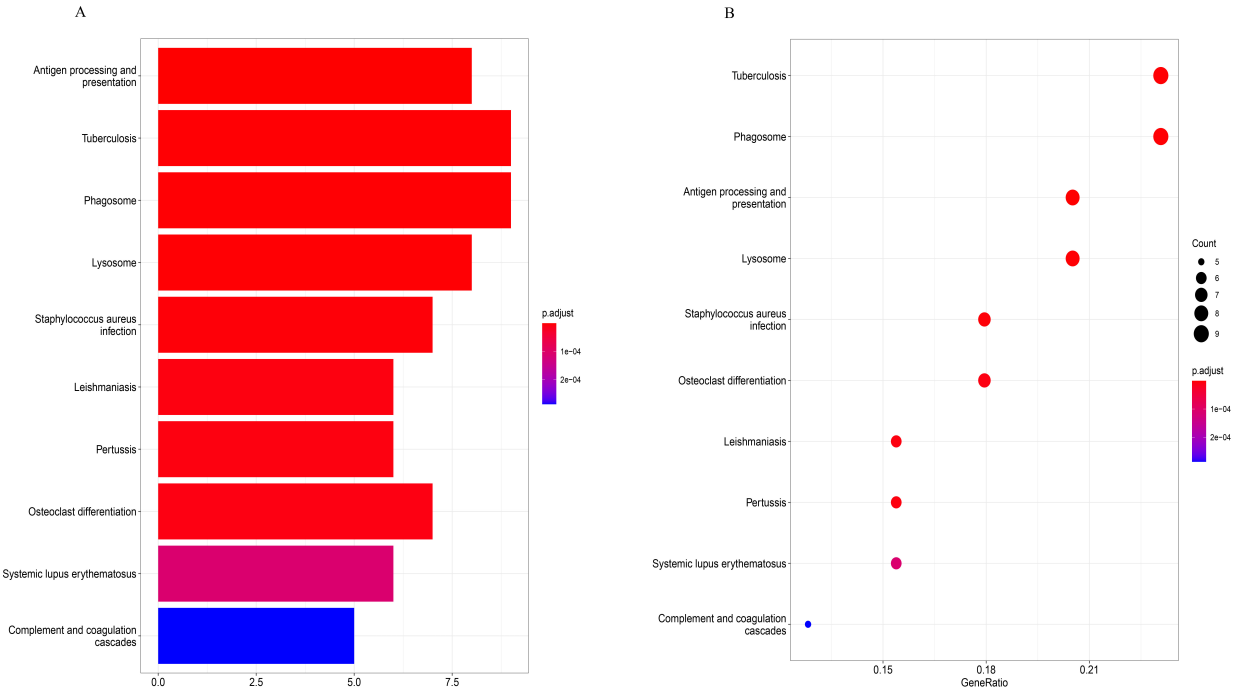

Fig. 5. KEGG analyses of the common DEGs. (A) The bar plot of the top 10 KEGG pathways. (B) The bubble plot of the top 10 KEGG pathways.

3.1.4 PPI Analysis and Hub Gene Identification

The PPI network was constructed to reveal the potential connection among the DEGs. As shown in Fig. 6A, the PPI network consisted of 52 nodes and 220 linkages. We further identified the top 15 hub genes according to the MCC method. Next, we performed K-means clustering and grouped these hub genes into four distinct clusters as follows: cluster 1: allograft inflammatory factor 1 (*Aif1*), colony-stimulating factor 1 receptor (*Csf1r*), triggering receptor expressed on myeloid cells 2 (*Trem2*), and TYRO protein tyrosine kinase-binding protein (*Tyrobp*); cluster 2: *C1qa*, *C1qb*, *C1qc*, *Cd74*, and Cathepsin S (*Ctss*); cluster 3: Rho GDP dissociation inhibitor-β (*Arhgdib*), *Cd53*, lysosomal-associated protein transmembrane 5 (*Laptm5*); and cluster 4: Fc epsilon receptor Ig (*Fcer1g*), Fc gamma receptor IIb (*Fcgr2b*), and protein tyrosine phosphatase non-receptor type 6 (*Ptpn6*) (Fig. 6B).

3.1.5 TF, TF Co-Factors, and IRGs Analysis

We identified two overlapped TF co-factors among the DEGs shared in the two datasets (Fig. 7A). They were adipocyte enhancer-binding protein 1 (*Aebp1*) and PYD and CARD domain containing (*Pycard*) genes. Furthermore, we also found 15 immune-related DEGs, including *C1qa*, interferon γ receptor 1 (*Ifngr1*), *Csf1r*, Unc-93 homolog B1 (*Unc93b1*), *Ctss*, *C1qc*, *Ptpn6*, *C1qb*, legumain (*Lgmn*), *Tyrobp*, *Trem2*, granulin precursor (*Grn*), annexin A2 (*Anxa2*), interferon-stimulated gene 15 (*Isg15*), and *Pycard*. The GO enrichment analysis of these immune-related DEGs indicated that the significant BP terms included microglial cell activation, cell junction disassembly, astrocyte activation, antigen processing and presentation, macrophage activation, positive regulation of response to external stimulus, myeloid cell differentiation, myeloid leukocyte activation, and astrocyte development (Fig. 7B).

3.2 Experimental Validation in a Rat Model of TBI

3.2.1 Histopathological Features

HE staining was used to observe the cell morphology of neurons. In the sham group, neurons in the hippocampus were arranged regularly and showed clear and intact cellular structure (Fig. 8A). However, the TBI group showed distinct morphological alterations, nuclear pyknosis, and neuronal necrosis (Fig. 8A). Nissl staining was used to detect neuronal loss. The results of Nissl staining also demonstrated severe structural damage in the neurons and apparent reductions in the number of intact neurons in the TBI group (Fig. 8B). No such pathological abnormalities were observed in the sham group (Fig. 8B). Moreover, microglia was visualized by staining with anti-Iba1 antibody. The results of Iba1 staining suggested that the number of Iba1-positive cells was markedly increased in the TBI group (Fig. 8C).

3.2.2 The mRNA Expression Levels of the Hub Genes

To validate the transcriptome data, we selected the 15 hub genes (*Aif1*, *Csf1r*, *Trem2*, *Tyrobp*, *C1qa*, *C1qb*, *C1qc*, *Cd74*, *Ctss*, *Arhgdib*, *Cd53*, *Laptm5*, *Fcer1g*, *Fcgr2b*, and *Ptpn6*) for RT-qPCR (Fig. 9). Compared with the sham group, the mRNA expression levels of these genes were sig-

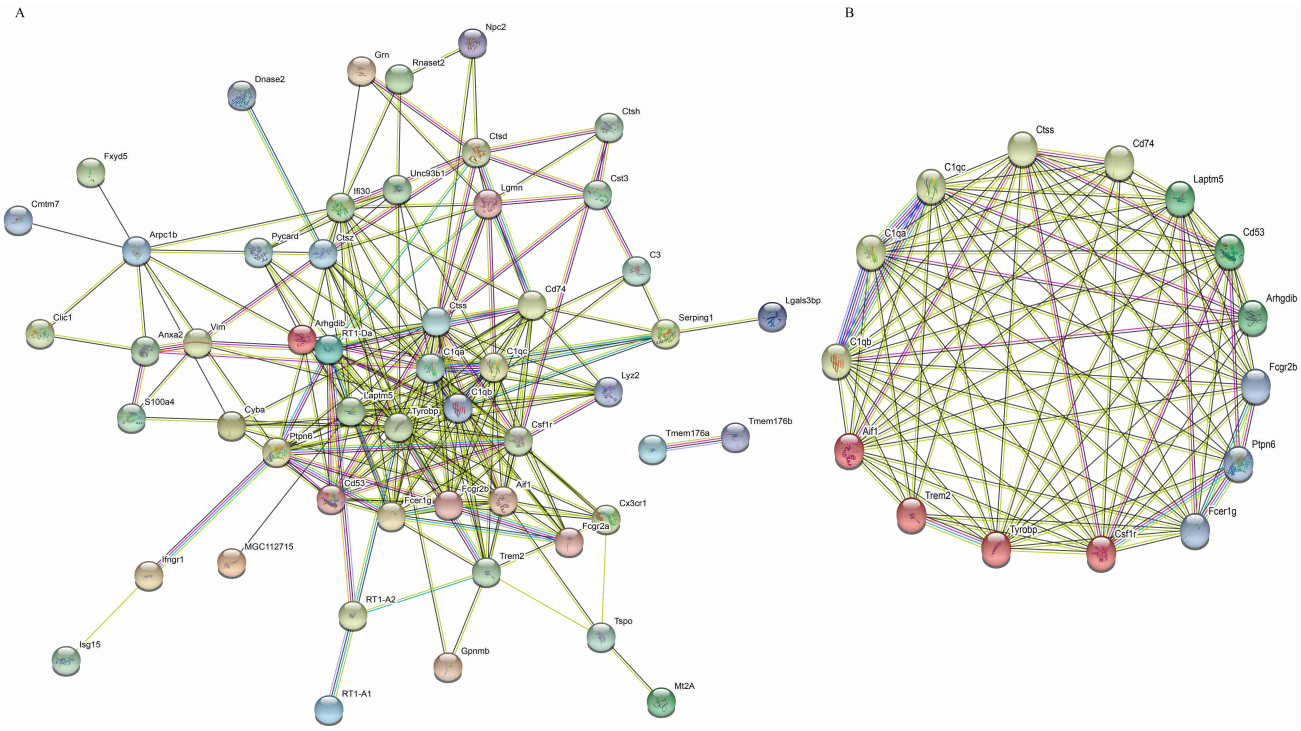

Fig. 6. Construction of the PPI networks of the DEGs (A) and the top 15 hub genes (B).

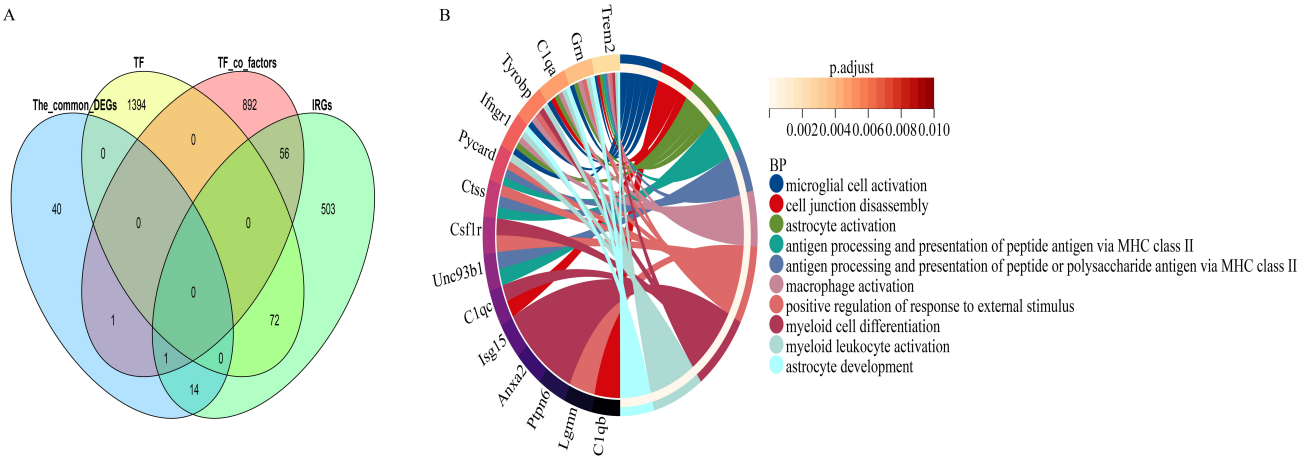

Fig. 7. Identification of differentially expressed TF, TF co-factors, and IRGs. (A) The Venn diagram presenting overlapped genes among the common DEGs, TF, TF co-factors, and IRGs. (B) The circle plot of the top 10 BP terms of the immune-related DEGs.

nificantly higher in the TBI group ($p < 0.05$). Overall, these RT-qPCR results were in accordance with the transcriptome data.

4. Discussion

TBI is one of the most common traumatic injuries which can cause long-lasting cerebral damage and cognitive deficits [25]. Elucidating the potential molecular mechanisms and therapeutic targets for improving cognitive function helps to improve the clinical prognosis and alleviate the burden on patients suffering from TBI [26]. Transcriptomic profiling is a powerful tool that can identify the genes differentially expressed in specific physiological and pathological states and discover new diagnostic or therapeutic targets [27]. In this study, we conducted a systematic bioinformatics analysis of the two GEO datasets (GSE111452 and GSE173975) to reveal the potential molecular mechanisms responsible for hippocampal damage in rats in the subacute phase of TBI.

Initially, we performed GSEA on the basis of all transcriptome data of the two GEO datasets, separately. The GSEA results revealed notable enrichment in the MAPK signaling pathway, PI3K/Akt signaling pathway, focal adhesion, and cellular senescence. The MAPK family in mammals consists of c-Jun N-terminal kinase, p38 MAPK,

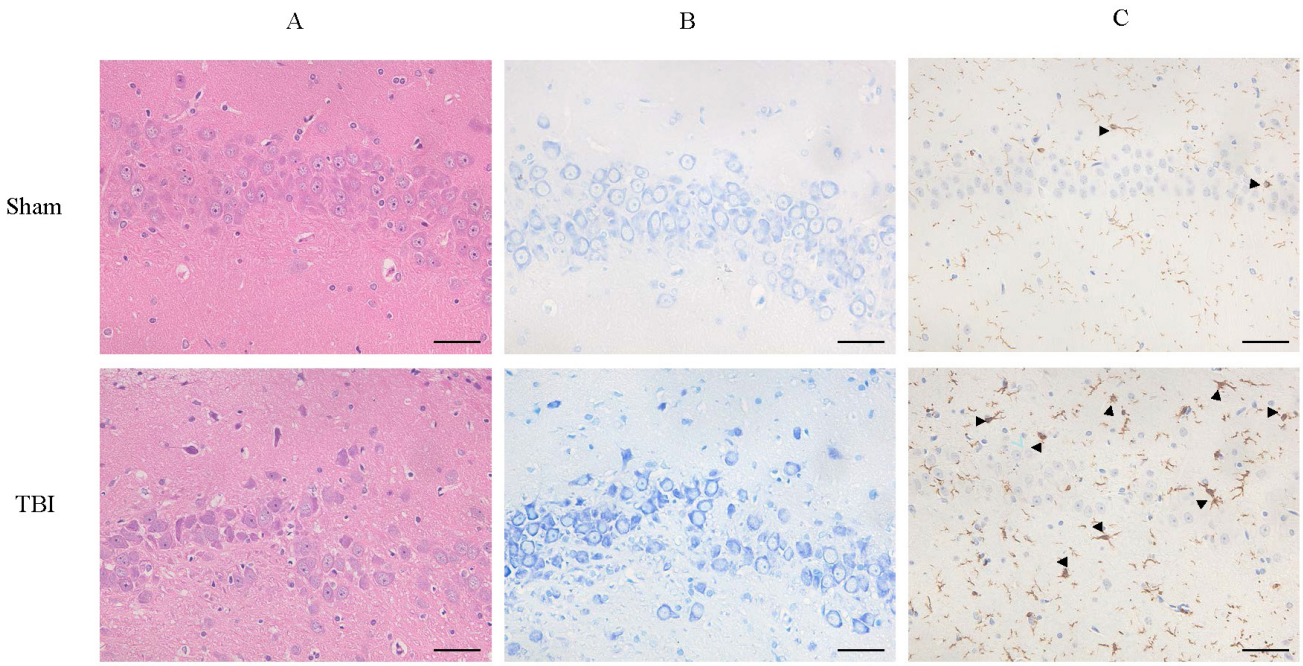

Fig. 8. Representative histopathological images of the hippocampus from the sham and TBI groups (scale bar = 50 um). (A) HE staining. (B) Nissl staining. (C) Immunohistochemical staining for Iba1. Arrows point to Iba1-positive cells.

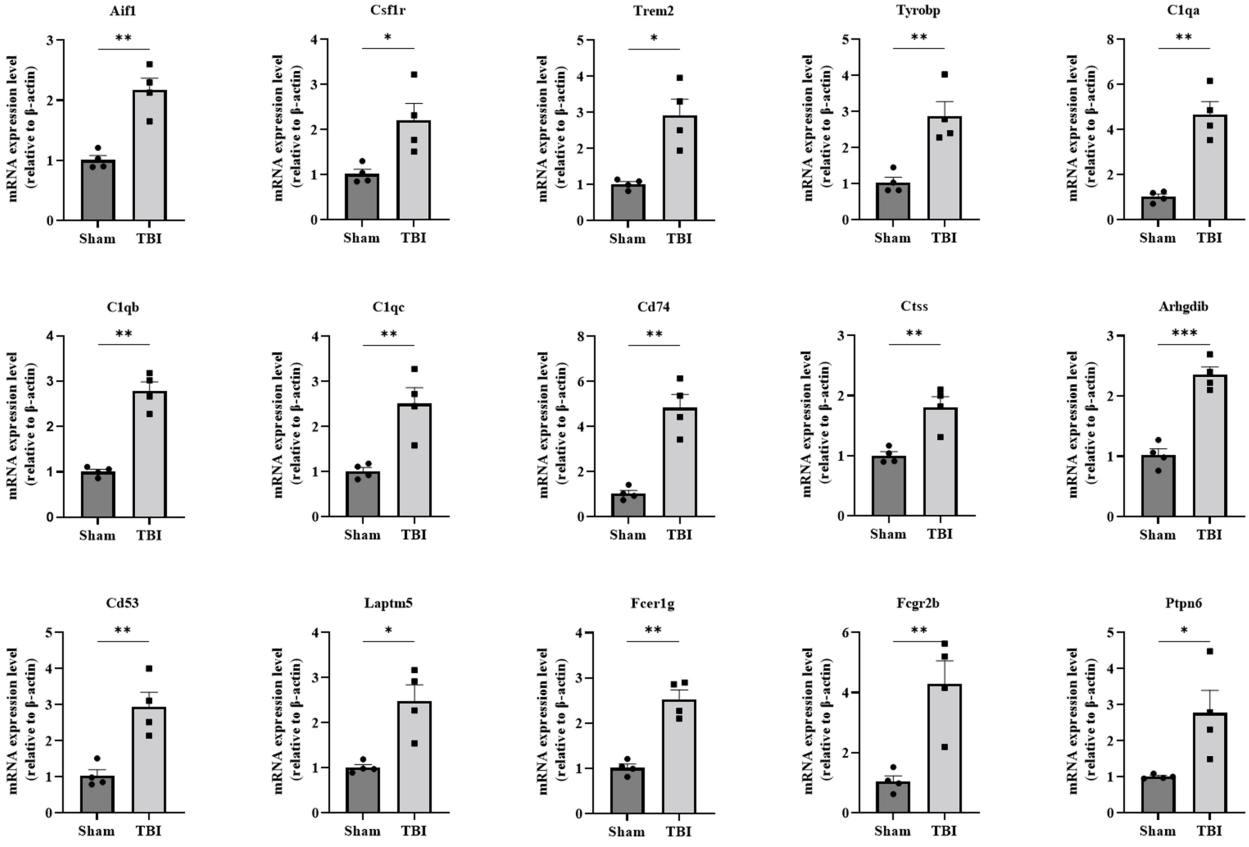

Fig. 9. Validation of the 15 hub genes at the mRNA level between the sham and TBI groups. n = four per group, data were presented as mean ± SEM. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

and extracellular signal-regulated kinase [28]. Previous findings suggested that inhibiting the activation of the MAPK signaling pathway might produce neuroprotective effects in TBI [29,30]. As a critical regulator of organismal growth and multiple cellular processes, the PI3K/Akt signaling pathway plays an important role under various pathophysiological conditions [31]. Preclinical studies of TBI revealed that the PI3K/Akt signaling pathway exerted positive effects on neural repair by regulating neuronal apoptosis and neurogenesis [32,33]. Focal adhesions are protein complexes that mediate cell adhesion by linking the extracellular matrix to the cytoskeleton [34]. A recent *in vitro* study demonstrated that inhibiting focal adhesion kinase phosphorylation could mitigate astrocyte activation induced by mechano-stimulation [35]. Cellular senescence refers to a cell state triggered by various stress factors and some specific physiological conditions [36]. It is characterized by proliferative arrest, secretory phenotype, macromolecular damage, and alternation in metabolism [36,37]. Several studies suggested that DNA damage-induced cellular senescence was a possible driver of TBI-associated sequelae such as cognitive impairment [38–40]. Among the common DEGs, several genes might play a role in the response to DNA damage. Du *et al*. [41] found that the activation of complement C3 contributed to retinal DNA damage and C3 deficiency alleviated alkylation-induced retinal degeneration in mice. Raso *et al*. [42] reported that Isg15 upregulation promoted DNA replication fork progression, leading to extensive DNA damage and chromosomal aberrations in a human osteosarcoma cell line. Xie *et al*. [43] found that downregulating the expression of CX3C chemokine receptor 1 (Cx3cr1) accelerated double-strand DNA damage in irradiated ovarian cancer cells. These experiments were primarily in the field of oncology, and the studies that investigated how these genes regulated DNA damage response to impact the progression of TBI are still limited.

To further explore potential biological functions and signaling pathways involved in hippocampal damage, we performed GO and KEGG analyses based on the common DEGs of the two datasets. We found that the DEGs were mainly involved in an array of critical immune and inflammatory processes, including antigen processing and presentation, leukocyte and lymphocyte mediated immunity, adaptive immune response, phagosome, lysosome, and complement and coagulation cascades. Following the initial insult triggered by TBI, peripheral leukocytes and lymphocytes were successively recruited to the injury site [44]. Antigen processing and presentation by antigen-presenting cells promote the transition between innate and adaptive immune responses [45]. Moderate immune responses promote the removal of cellular debris, repair, and regeneration in the central nervous system (CNS) post-TBI, but excessive immune responses result in reactions causing destructive neuroinflammation and maladaptive secondary injuries [3,44]. In view of the enrichment of phagosome- and lysosome-related genes after TBI, it is reasonable to speculate that active phagocytosis plays a critical role in neural injury and repair [46]. Microglia act as important phagocytes in the brain and can rapidly remove cellular and myelin debris after brain injury [47]. Moderate microglial phagocytosis is beneficial for remodeling the brain microenvironment and alleviating secondary injury [47,48], whereas excessive phagocytosis by microglia of neurons, synapses, or myelin may impede tissue and functional recovery [49,50]. Current evidence indicates extensive crosstalk between the complement and coagulation systems, which have fundamental clinical implications for inflammation, immunity, and tissue damage [51]. A previous proteomics study suggested that complement and coagulation cascades were activated during the acute and subacute phases of TBI, which was consistent with our findings [52].

In addition to the functional enrichment and pathway analyses described above, we proceeded to construct the PPI network of the DEGs and then identified the top 15 hub genes which were upregulated in both datasets. Among the hub genes, we found several microglia signature genes such as *Csf1r*, *Aif1*, and *Trem2*. Csf1r is a crucial regulator of myeloid lineage cells and is also necessary for maintaining the cell viability of microglia [53]. Several experiments adopted the method of Csf1r inhibition to attenuate microglia-mediated neuroinflammation in rodent models of TBI [54,55]. However, given that microglia plays a key role in maintaining homeostasis and normal function of the brain [56], inhibiting Csf1r may increase the risk of failure in clinical translation. In contrast, the infiltrating peripheral macrophages in the brain after TBI may be promising targets for developing potential treatment strategies [57]. Aif1 is a calcium-binding protein that regulates immune and inflammatory responses [58,59]. Increased expression of Aif1 is an indication of microglia and macrophage activation in response to brain trauma [60,61]. A previous experimental study identified *Trem2* and *Tyrobp* as significant hub genes in TBI mice expressing human APOE [62]. Trem2 is an innate immune receptor mainly expressed on various tissue macrophages, such as microglia in the brain [63]. It transmits intracellular signals through binding with the adaptor protein Tyrobp, which in turn regulates many crucial biological processes, including phagocytosis, chemotaxis, inflammatory responses, and lipid metabolism [56,63,64]. It was reported that Trem2 deficiency alleviated the acute peripheral macrophage infiltration and attenuated chronic hippocampal atrophy and cognitive deficits in a murine TBI model [65]. Given the vital role of Trem2 in the progression of neurodegeneration, it was worthwhile to explore it as a potential therapeutic target in managing TBI-mediated neurodegenerative pathologies [56,57,65].

Ctss is a cysteine protease expressed by diverse immune cells that is responsible for cleaving certain extracellular matrix proteins and cell adhesion molecules to pro-

mote immune cell motility [66,67]. In the acute phase after TBI, the upregulation of Ctss expression was mainly observed in microglia; inhibiting Ctss could reduce the level of inflammatory factors, alleviate brain edema, and improve neurobehavioral function [68]. The Cd74 molecule is the cognate receptor of macrophage migration inhibitory factor that is recognized to be a pleiotropic inflammatory cytokine [69,70]. Tobin *et al.* [45] found that TBI-induced neurodegeneration depended on antigen processing and presentation that required Cd74. Cd74 deficiency could decrease peripheral lymphocyte activation and neurodegeneration following TBI, suggesting that targeting the switch between innate and adaptive immunity might be a potential therapeutic strategy [45]. C1qa, C1qb, and C1qc are three distinct subunits of complement component C1q, which is a crucial protein in the complement cascade within the innate immune system [71,72]. One recent clinical study showed that elevated serum C1q levels were significantly correlated with traumatic severity and could serve as an independent prognostic factor for long-term outcomes after TBI [73]. After TBI, microglia and neurons were the primary source of C1q [74,75]. The increased expression of C1q might lead to neuron loss and chronic neuroinflammation and correlate with sleep spindle loss and epileptic spikes. Blocking C1q was beneficial to counteract these effects, indicating that C1q might be a potential target for treating the devastating long-term outcomes of TBI [75].

We also discovered two upregulated Fc receptor genes. It was reported that Fcer1g might be a potential microglial biomarker related to aging and neurodegeneration [72,76]. Among the Fc receptors, Fcgr2b could serve as one marker of the pro-inflammatory phenotype of microglia after TBI [77–79]. There was some evidence that Laptm5 had pro-inflammatory properties in macrophages [80]. In a chronic constriction injury in rats, *Laptm5* was identified as a significant upregulated neuroinflammation-related gene that influenced neuropathic pain behavior [81]. However, due to the lack of relevant literature, it is unknown whether Laptm5 plays a role in the pathological processes of TBI.

TF co-factors play an essential role in diverse biological processes by interacting with TFs to suppress or activate gene transcription [21,82]. Among the DEGs shared in common between the GSE111452 and GSE173975 datasets, we found two inflammation-related TF co-factors (Aebp1 and Pycard). Aebp1 has been shown to promote inflammatory processes in macrophages by activating the nuclear factor kappa B [83,84]. Shijo *et al.* [85] found that Aebp1 was highly expressed in hippocampal neurons and glial cells in AD patients, implying its potential role in the progression of AD pathology. The *Pycard* gene encodes a critical adaptor protein in activating inflammasomes [86]. Recent research has shown that inflammasome proteins such as Pycard are elevated in the blood of TBI patients and are closely associated with injury severity and pathological outcomes [87]. Experimental inhibition of inflammasomes exerted anti-inflammatory and neuroprotective effects in rodent models of TBI, implying that developing inflammasome-targeting therapeutics might be a potential direction for future research [88–90].

CTE refers to a progressive Tau-dependent neurodegenerative disease that remains relatively understudied [91,92]. In several animal models of TBI, the immunocytochemical signature for CTE has been presented, which is in agreement with clinical studies [4,7]. Among the common DEGs identified in this study, several genes (e.g., *Trem2*, *Tyrobp*, and *Cx3cr1*) might be involved in the progression of tauopathy based on the current literature. Jiang *et al.* [93] found that Trem2 attenuated tau kinase activity by inhibiting neuroinflammation and Trem2 deficiency exacerbated tau pathology and neurodegeneration in P301S tau transgenic mice. Another study reported that Trem2 deletion enhanced tau dispersion through microglia exosomes [94]. In a tauopathy mouse model, silencing of Tyrobp reduced C1q levels and improved learning behavior and electrophysiological properties despite increasing tau phosphorylation [95]. Cx3cr1 was deemed as a potential target for treating tauopathy, since its deficiency impaired the phagocytosis and internalization of extracellular tau by microglia [96,97].

Recent evidence suggested that endoplasmic reticulum (ER) stress resulted in extensive behavioral changes related to a CTE-like phenotype after TBI [91,98]. Inhibiting ER stress ameliorated cognitive deficits by reducing tau hyperphosphorylation in rodent models of CTE [91,98]. Several genes within the common DEGs might be involved in ER stress response. It was reported that Glycoprotein nonmetastatic melanoma protein B (Gpnmb) increased the expression of chaperone protein BiP to increase the protein-folding capacity of the ER and attenuate cell death caused by ER stress [99]. In a cerebral ischemia mouse model, the neuronal damage of Gpnmb-transgenic mice was significantly alleviated compared with wild-type mice [99]. Kam *et al.* [100] found that Fcgr2b mediated amyloid-β neurotoxicity by activating ER stress and caspase-12. It has been reported that the translocator protein (Tspo) is a potential biomarker in multiple inflammatory and neurodegenerative diseases [101,102]. Loss of Tspo in hepatocytes caused free cholesterol accumulation, which subsequently induced ER stress [103]. More importantly, recent clinical studies suggested that PET imaging with tracers binding to Tspo could be applied to detect the neuroinflammatory changes in subjects with CTE [102,104]. Interestingly, a multi-tracer PET Study showed that tau deposition and increased Tspo expression were co-localized in the cortex and hippocampus in a tau transgenic mouse model; however, the complex interaction between tauopathy and Tspo expression remains to be further studied [105]. Neuroimaging modalities could be conducted longitudinally at various time points on the same subject, showing an advantage in observing dynamic change during the progression of CTE

[92]. In future studies, molecular imaging through PET technology such as Tspo-PET may be used as a novel tool to study CTE on preclinical models and help to elucidate the complex pathophysiological mechanisms leading from TBI to symptomatic CTE [102,104]. In addition, Gouna and colleagues [106] demonstrated that Trem2 deficiency in microglia/macrophages resulted in ER stress due to impaired lipid droplet formation. The potential critical role of these genes in both tauopathy progression and ER stress implied that they might be of considerable value in the field of CTE modeling, although the specific molecular mechanisms remain to be determined in future studies. Tau transgenic rodent models combined with targeted gene deletion might be instrumental for the study of CTE modeling and help to reveal the potential pathophysiological processes of CTE.

In summary, our research was a comprehensive bioinformatics study analyzing the transcriptome data of rat hippocampus in the subacute phase of TBI. We used multiple tools for enrichment analysis to reveal the underlying biological mechanisms involved in TBI-related hippocampal impairment. The enrichment analysis showed that TBI might result in a significant dysregulation of multiple inflammation and immune processes. We further identified the hub genes, TF co-factors, and IRGs, which likely play a critical role in the progression of hippocampal impairment.

In the validation experiment, we found some apparent histopathologic changes in the hippocampus during the subacute phase of TBI, including neuronal necrosis and reductions in the number of intact neurons. A previous study supports the pathological changes described in our model [107]. Furthermore, we used immunohistochemical staining to observe the number of microglia. The staining results suggested that the level of microglia activation was markedly elevated in the hippocampus of rats in the TBI group when compared with the sham group. Through immunofluorescence staining analysis, Luo *et al*. [52] also found that the number of microglia was significantly increased in the hippocampus during the subacute phase of TBI, which was consistent with our results. Similarly, the immune-related DEGs identified by bioinformatic analyses were significantly enriched in BP terms associated with the activation of microglia and macrophages. Microglia is the major type of glial cells involved in neuroinflammation in the mammalian CNS [108]. The peripheral immune cells (especially macrophages) are also essential responders to tissue injuries in the CNS [3,109]. These findings suggest that these inflammatory cells might be valuable targets in the therapy of hippocampal impairment in the subacute phase after TBI. Moreover, we also detected the mRNA expression levels of the 15 hub genes identified by bioinformatics analyses. Overall, the RT-qPCR results were consistent with the transcriptome data, indicating that the transcriptome data were reliable. Overall, traumatic homogeneity between the datasets and our validation experiment was relatively high. Both the datasets and our experiment used adult male SD rats as the experimental objects, and the location and extent of the brain injury were similar. In addition, the tissue type and disease stage were consistent.

However, there were several limitations of this study. First, it specifically focused on the hippocampus rather than other brain regions. The principal reason was that hippocampal impairment acts as a key player in the pathogenesis of TBI-induced cognitive decline [6,8,9], which was one of the most dominant clinical manifestations in the subacute phase [110–112]. Developing effective treatments to promote cognitive rehabilitation in TBI patients during the subacute phase is of high clinical importance [110]. The bioinformatics analyses and experimental validation performed in this study mainly focused on the subacute phase (14 days), which was important for assessing cognitive function in the rodent model of TBI [13,113]. Notably, there was some evidence that several potential biomarkers were significantly upregulated in the acute phase. For instance, Saber *et al*. [65] found that both the protein and mRNA expressions of Trem2 were significantly increased in the ipsilateral cortex in mice on the third day after experimental TBI. The study by Luo and colleagues [52] revealed that the multiple proteins (e.g., C1qa, Pycard, Aif1, Ptpn6, and Fcer1g) in the ipsilateral hippocampus were markedly upregulated in a rat model of TBI on the third day. In addition, Wang *et al*. [54] reported that the gene expression levels of C1qa, Aif1, and Csf1r were prominently increased in the perilesional area on the fifth day post injury in TBI mice. Therefore, these biomarker genes might become important therapeutic targets during the early phase of TBI. Further study on these crucial genes might provide useful insight for treating TBI-induced cognitive deficits in fundamental, translational, and clinical research.

Second, in this study, only male rats were used in the experiments. Previous observations have suggested that there may be a link between sex and clinical outcomes after TBI [114,115]. Some studies demonstrated that females had a lower risk of mortality and cognitive dysfunction than males, while others failed to find such advantages [114,115]. Given the predominant role of microglia in posttraumatic neuroinflammation, it is worthwhile to determine whether sex differences exist in microglial activation after TBI [114]. Several studies have revealed that male rodents may exhibit more significant microglial activation than female rodents [114,116,117]. However, there has also been conflicting evidence about the influence of sex on microglial activation in rodent models of TBI [79,114]. Regrettably, there is currently an apparent male bias in the field of TBI research, both in preclinical rodent studies and clinical trials [116,118]. Thus, future studies are needed before drawing definite conclusions about the role of sex in TBI. Finally, traditional microarray and mRNA sequencing techniques mainly investigate the global gene expression patterns of the same tissues. Single-cell mRNA sequenc-

ing techniques may help to reveal the exact role of specific cell types in the complex pathophysiological mechanisms underlying TBI.

5. Conclusions

In conclusion, this study identified several critical genes and key signaling pathways involved in hippocampal impairment induced by TBI. Several genes involved in neuroinflammation (e.g., *Trem2*, *C1q*, and *Pycard*) might represent potential targets for treating TBI-induced cognitive deficits.

Abbreviations

TBI, Traumatic brain injury; PTSD, posttraumatic stress disorder; CTE, chronic traumatic encephalopathy; AD, Alzheimer's disease; GEO, Gene Expression Omnibus; DEGs, differentially expressed genes; PPI, protein-protein interaction; FPI, fluid percussion injury; SD, Sprague-Dawley; GSEA, gene set enrichment analysis; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; NES, standardized enrichment score; BP, biological process; CC, cellular components; MF, molecular function; STRING, Search Tool for the Retrieval of Interacting Genes; TF, Transcription factor; IRGs, immune-related genes; SPF, specific pathogen-free; CCI, controlled cortical impact; HE, hematoxylin and eosin; BSA, bovine serum albumin; DAB, Diaminobenzidine; RT-qPCR, quantitative Real-Time PCR; SEM, standard error of the mean; MAPK, mitogen-activated protein kinase; PI3K/Akt, phosphatidylinositol 3-kinases/protein kinase B; Ctss, Cathepsin S; Aif1, allograft inflammatory factor 1; Csf1r, colony-stimulating factor 1 receptor; Trem2, triggering receptor expressed on myeloid cells 2; Tyrobp, TYRO protein tyrosine kinase-binding protein; Arhgdib, Rho GDP dissociation inhibitor-β; Laptm5, lysosomal-associated protein transmembrane 5; Fcer1g, Fc epsilon receptor Ig; Fcgr2b, Fc gamma receptor IIb; Ptpn6, protein tyrosine phosphatase non-receptor type 6; Aebp1, adipocyte enhancer-binding protein 1; Pycard, PYD and CARD domain containing; Ifngr1, interferon γ receptor 1; Unc93b1, Unc-93 homolog B1; Lgmn, legumain; Grn, granulin precursor; Anxa2, annexin A2; Isg15, interferon-stimulated gene 15; Cx3cr1, CX3C chemokine receptor 1; CNS, central nervous system; ER, endoplasmic reticulum; Gpnmb, Glycoprotein nonmetastatic melanoma protein B; Tspo, translocator protein.

Availability of Data and Materials

The transcriptomic datasets are available in the GEO database https://www.ncbi.nlm.nih.gov/geo/.

Author Contributions

HZ and YZ contributed to the study design. SZ, SW, and ZP participated in data collation and statistical analysis. HZ, SZ, SW, and ZP finished the experimental validation. HZ and LC wrote the manuscript. YZ and SW revised the manuscript. All authors read and approved the final manuscript.

Ethics Approval and Consent to Participate

The animal experiment was approved by the Laboratory Animal Ethics Committee of Jinan University (ethical number: 14075).

Acknowledgment

The authors appreciate the GEO database and contributors for providing their meaningful datasets.

Funding

This study was supported by the National Natural Science Foundation of China (nos. 81873362, 82174483, and 82205249), the Natural Science Foundation of Guangdong Province (nos. 2021A1515011219 and 2021A1515110146), China Postdoctoral Science Foundation (2022M710912), and the Administration of Traditional Chinese Medicine of Guangdong Province (no. 20231086).

Conflict of Interest

The authors declare no conflict of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.31083/j.jin2202044.

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