Proteomics and bioinformatics reveal insights into neuroinflammation in the acute to subacute phases in rat models of spinal cord contusion injury

Xin-Qiang Yao1 | Zhong-Yuan Liu1 | Jia-Ying Chen2 | Zu-Cheng Huang1 | Jun-Hao Liu1,3 | Bai-Hui Sun4 | Qing-An Zhu1 | Ruo-Ting Ding1 | Jian-Ting Chen1

1Division of Spine Surgery, Department of Orthopaedics, Nanfang Hospital, Southern Medical University, Guangzhou, China
2Department of Comprehensive Medical Treatment Ward, Nanfang Hospital, Southern Medical University, Guangzhou, China
3Division of Spine Surgery, Department of Orthopaedics, Guangzhou First People's Hospital, School of Medicine, South China University of Technology, Guangzhou, China
4Department of General Surgery, Nanfang Hospital, Southern Medical University, Guangzhou, China

Abstract
Neuroinflammation is recognized as a hallmark of spinal cord injury (SCI). Although neuroinflammation is an important pathogenic factor that leads to secondary injuries after SCI, neuroprotective anti-inflammatory treatments remain ineffective in the management of SCI. Moreover, the molecular signatures involved in the pathophysiological changes that occur during the course of SCI remain ambiguous. The current study investigated the proteins and pathways involved in C5 spinal cord hemi-contusion injury using a rat model by means of 4-D label-free proteomic analysis. Furthermore, two Gene Expression Omnibus (GEO) transcriptomic datasets, Western blot assays, and immunofluorescent staining were used to validate the expression levels and localization of dysregulated proteins. The present study observed that the rat models of SCI were associated with the enrichment of proteins related to the complement and coagulation cascades, cholesterol metabolism, and lysosome pathway throughout the acute and subacute phases of injury. Intriguingly, the current study also observed that 75 genes were significantly altered in both the GEO datasets,

Correspondence
Ruo-Ting Ding and Jian-Ting Chen,
Division of Spine Surgery, Department of Orthopaedics, Nanfang hospital, Southern Medical University, 1838 North Guangzhou Avenue, Guangzhou, Guangdong 510515, China.
Email: ding_ruoting@163.com (R.-T. D.) and chenjt@smu.edu.cn (J.-T. C.)

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Abbreviations: A2M, alpha-2-macroglobulin; ABCA1, ATP-binding cassette subfamily A member 1; ANXA1, annexin A1; APOA4, apolipoprotein A-IV; APOE, apolipoprotein E; ARPC1B, actin-related protein 2/3 complex subunit 1B; ATP5F1D, ATP synthase subunit delta, mitochondrial; C1QC, complement C1q subcomponent subunit C; C3, complement C3; CFD, complement factor D; CNS, central nervous system; CORO1A, coronin-1A; CTSB, cathepsin B; CTSC, cathepsin C; CTSZ, cathepsin Z; DEGs, differentially expressed genes; DEPs, differentially expressed proteins; DNASE2, deoxyribonuclease-2-alpha; dpi, days post-injury; GBP2, guanylate binding protein 2; GEO, Gene Expression Omnibus; GM2A, GM2 ganglioside activator; GO, Gene Ontology; GPNB, transmembrane glycoprotein NMB; GPX3, glutathione peroxidase; GSEA, gene set enrichment analysis; ITGAM, integrin alpha M; ITGB2, integrin beta; KEGG, Kyoto Encyclopedia of Genes and Genomes; LC-MS/MS, liquid chromatography tandem mass spectrometry; NPC2, NPC intracellular cholesterol transporter 2; PASEF, parallel accumulation serial fragmentation; PLIN2, perilipin; PYCARD, PYD and CARD domain-containing; SCI, spinal cord injury; SERPING1, plasma protease C1 inhibitor; SLC25A5, ADP/ATP translocase 2; SYK, tyrosine-protein kinase; TCIRG1, V-type proton ATPase subunit a.
Introduction

Spinal cord injury (SCI) impairs the neurological functions and is associated with a high rate of mortality and disability. The initial trauma results in the death of neurons and glial cells and initiates a secondary injury cascade that includes neuroinflammation, demyelination, and astrocyte scar formation in the following weeks. Although neuroinflammation can lead to secondary injuries, it also contributes to the removal of debris, remyelination and axonal regeneration. Hence, neuroinflammation has the potential to aggravate and relieve neural damage. Neuroprotective anti-inflammatory strategies in the treatment of SCI have been largely unsuccessful. The scenario urgently warrants insights regarding the signaling pathways that regulate neuroinflammation after SCI for the development of effective treatments.

High-throughput assays have recently emerged as important tools in the investigation of the molecular pathways involved in disease progression, thereby providing new opportunities for the development of drugs. A previous study by Squair et al that involved integrated systems analysis showed that the module of coexpressed genes enriched for markers of microglia, inflammatory response, and response to wounding was correlated with the severity of SCI. The scenario urgently warrants insights regarding the signaling pathways that regulate neuroinflammation after SCI for the development of effective treatments.

In the present study, the cervical spinal cord hemi-contusion injury was induced according to the technique described in previous literature. The rats were briefly anesthetized using isoflurane (3.0% for induction and 2.0% for maintenance) in oxygen by means of a small animal anesthesia machine (VP1000, Matrix, United States). The spinal cord was exposed after removing the left side of the C5 laminae and contused with a metal impact of diameter 1.5 mm with a preset displacement of 2.0 mm at 500 mm/s, controlled by an electromagnetic servo material testing machine (Instron E1000, Instron, United States). Sham surgery was performed by way of the same surgical procedure without the induction of spinal cord contusion.
2.3 | Tissue preparation

At three, seven, and fourteen days post-injury (dpi), the animals were deeply anesthetized using sodium pentobarbital (50 mg/kg, i.p.) to harvest tissue samples. The spinal cord samples of the sham group were collected 14 days after sham surgery. In order to conduct the proteomic analysis (n = 3/time point) and Western blot assays (n = 3/time point), the animals underwent transcardial perfusion with phosphate-buffered saline alone. The spinal cord tissue (5 mm rostral to 5 mm caudal to the epicenter) was removed and stored at –80°C for processing. In order to perform immunofluorescent staining, the animals from the sham group and the 7-dpi group (sham group, n = 5; 7-dpi group, n = 5) underwent perfusion with phosphate-buffered saline followed by ice-cold 4% paraformaldehyde (104005, Merck, Germany). The spinal cord tissue samples were dissected, post-fixed with paraformaldehyde overnight, and dehydrated in 12%, 18%, and 24% sucrose solution at 4°C. Subsequently, the tissue samples were embedded in optimal cutting temperature compound (Tissue-Tek, 4583, Sakura), sliced transversely into 20 µm sections using a cryostat (Leica CM1950, Germany), and stored at –80°C.

2.4 | Protein extraction

Each spinal cord tissue sample was converted into protein lysates and transferred into centrifuge tubes (5 mL in volume),
in order to perform the proteomic analysis. Subsequently, four volumes of the lysis buffer (8 M urea, 1% protease inhibitor cocktail) was added to the centrifugal tube. Finally, the supernatant was collected after centrifugation at 12 000g at 4°C for 10 minutes, and the concentration of each sample was evaluated using a bicinchoninic acid assay kit, in accordance with the manufacturer’s instructions.

2.5 | Trypsin digestion

The protein solution was reduced using 5 mM dithiothreitol at 56°C for 30 minutes, followed by alkylation in darkness with 11 mM iodoacetamide at room temperature for 15 minutes for the purpose of digestion. Successively, the protein sample was diluted by the addition of 100 mM triethylammonium bicarbonate to a urea concentration less than 2 M. Finally, trypsin was added at an enzyme-to-protein ratio of 1:50 for the initial, overnight digestion and at an enzyme-to-protein ratio of 1:100 for the second, four-hour digestion.

2.6 | Liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis

The LC–MS/MS analysis was conducted using a timsTOF Pro (Bruker) mass spectrometry coupled to a NanoElute UPLC system. The tryptic peptides were dissolved in solvent A (0.1% formic acid, 2% acetonitrile) and further separated using a homemade reversed-phase analytical column at a flow rate of 450 nL/min with a 90 minutes gradient: (1) 6% to 22% solvent B (0.1% formic acid in acetonitrile) over 70 minutes; (2) 22% to 32% in 14 minutes; (3) 32% to 80% in 3 minutes; (4) and 80% (vol/vol) solvent in 87-90 minutes. The peptides were subjected to a capillary source and acquired in a parallel accumulation serial fragmentation (PASEF) mode with 30 seconds of dynamic exclusion. The precursors and fragments were analyzed using the TOF detector with an MS/MS scan range of 100 to 1700 m/z. In the precursors with charge states ranging from 0 to 5, one MS scan was followed by 10 PASEF-MS/MS scans.

2.7 | Database search

The raw MS data were analyzed and searched using MaxQuant v.1.6.6.0 against the Rattus_norvegicus_10116_PR_20191115 (29938 sequences) concatenated with a reverse decoy database. Trypsin/P was specified as a cleavage enzyme, allowing up to two missed cleavages and a minimum length of seven amino acids. The mass tolerance for precursor ions was set to 20 ppm in the first and main searches and the mass tolerance for fragment ions was set to 0.02 Da. Carbamidomethyl on Cys was specified as a fixed modification, and acetylation on protein N-terminal and oxidation on Met were specified as variable modifications. The maximum false discovery rate was set to 1%.

2.8 | Bioinformatic analysis

The proteomic analysis was performed using “DEP” package version 1.8.0 within R version 3.6.3. This package provides an integrated analysis workflow for the robust and reproducible analysis of MS datasets generated by MaxQuant, in order to identify differentially expressed proteins (DEPs). Prior to normalization, the MS dataset was filtered for proteins that were quantified in at least two-thirds of the samples, followed by background correction and normalization by variance stabilizing transformation (vsn). The residual missing values in the dataset were imputed using the “min” method for each group of samples. The analysis of DEPs was based on the protein-wise linear models combined with the empirical Bayes statistics using the limma package. Finally, the DEPs were defined by the cut-off values (P < .05, fold change > 2 or < −2). The principal component analysis involved the average protein abundance of the top 800 variable proteins. A heatmap was plotted using the k-means clustering, in order to obtain an overview of all the significant proteins in all the samples. The Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis was performed using the “clusterProfiler” package version 3.14.3, in order to examine the biological pathways pertaining to the DEPs.

2.9 | Validation of the expression level in two transcriptomic datasets from GEO database

In order to identify the consensus changes in the RNA sequencing and microarray studies of SCI in rats, two GEO datasets, ie, GSE115067 and GSE45006, were used. The mRNA expression profile data concerning GSE115067 (GPL25029 NextSeq 550) and GSE45006 (Affymetrix Rat Genome 230 2.0 Array) was obtained from the GEO database (https://www.ncbi.nlm.nih.gov/geo/). The GSE115067 dataset included the data regarding fifteen SCI samples of different severities obtained seven days post-contusion. The Bioconductor packages of edgeR, limma, and DESeq2 were used to identify the differentially expressed genes (DEGs) (fold change >4 or < −4, P < .05). The intersection of the DEGs identified in each method served as the significant genes. The GSE45006 dataset included twenty samples with clip impact-compression injury at five different time point and four control rat spinal cords. The raw counts, processed microarray data, and associated clinical information were downloaded. Subsequently, the probe-level gene expression
profiles were converted to the official gene symbol levels. Genes with multiple probes were represented by the median expression value. The DEGs were screened using the “limma” package version 3.42.2 by way of the contrasts of every sample versus the control. The genes with \( P < .05 \) and fold change >4 or < −4 were selected as the significant genes.

### 2.10 Identifying time dependent protein expression programs

The proteins that were identified were categorized using the fuzzy c-mean clustering in “Mfuzz” package version 2.46.0, in order to further understand the dynamic changes at the protein expression level in relation to the progression of injury.\(^{11}\) First, the average expression of each protein in relation to each time point during the course of progression was calculated. Successively, the analysis workflow was followed using the “filter.std (min.std = 0),” “standardise (.),” and “mestimate ()” functions. Finally, the proteins were clustered into 10 different expression profiles, among which, the cluster 8 showed distinctive and remarkable patterns. Furthermore, the present study performed Gene Ontology (GO) and KEGG pathway enrichment analysis, in order to identify the function of DEPs in the cluster 8.

### 2.11 Gene set enrichment analysis (GSEA)

The current study performed GSEA to determine the biological pathways and functions of all the proteins in cluster 8. This method was implemented using the “GSVA” version 1.34.0 Bioconductor R package,\(^{12}\) together with the version 7.2 annotated gene sets (c2 KEGG, c5 GO biological processes, and c8 all cell type signature) downloaded from the MsigDB database. Prior to the commencement of GSEA, the statistical significance was calculated using the Student’s t test for the comparison of fold changes pertaining to each time point after SCI versus the sham group. The analysis was conducted using 1000 permutations and gene sets range from 10 to 500. The top 10 pathways or gene sets were shown as heatmaps using the pheatmap R package.

### 2.12 Western blot assays

The spinal cord tissue samples were homogenized and lysed in ice-cold RIPA buffer with 1 mM PMSF, protease, and phosphatase inhibitors. Lysates were cleared by centrifugation (12 000 rpm, 4°C, 15 minutes) and denatured by boiling in the loading buffer. The extracted proteins were loaded in equal quantities in each well, separated with 10% SDS-PAGE gel, and transferred to PVDF membranes (Millipore, MA, USA). The membranes were blocked in 5% milk at room temperature and incubated overnight at 4°C with the following primary antibodies: anti-C3 (1:2000, Abcam ab200999), anti-ITGAM (1:1000, Abcam ab133357), anti-CTSB (1:1000, Abcam ab214428), anti-CTSZ (1:1000, Abcam ab182575), anti-GAPDH (1:5000, Bioworld AP0063), and anti-NPC2 (1:2000, Abcam ab218192). After incubation with the secondary antibody (1:5000 dilution) at room temperature for one hour, the bands were visualized using an ECL chemiluminescent substrate luminescent solution (1 705 061, Bio-Rad, United States) on a luminescent imaging workstation (Tanon 6600, Shanghai Tanon, China).

### 2.13 Immunofluorescent staining

The slices were washed once in PBS. Subsequently, they were blocked in 0.01 M of PBS with 0.1% Triton X-100 with 10% normal donkey serum for 30 minutes. The slices were incubated overnight at room temperature with the following primary antibodies: anti-F4/80 (1:200, Cell Signaling Technology 71299S), anti-GFAP (1:800, Cell Signaling Technology 3670S), anti-CD68 (1:200, Abcam ab125212), anti-C3 (1:200, Abcam ab200999), anti-ITGAM (1:200, Abcam ab133357), anti-CTSB (1:400, Abcam ab214428), anti-CTSZ (1:400, Abcam ab182575), and anti-NPC2 (1:400, Abcam ab218192). After the incubation with primary antibodies, the slices were washed thrice in PBS and incubated by secondary antibodies (donkey anti-mouse Alexa Fluor 488, donkey anti-rabbit Alexa Fluor 555, donkey anti-rat Alexa Fluor 647, 1:200, Abcam) diluted using PBS with 0.1% Triton X-100 at room temperature for two hours. Subsequently, the slices were washed thrice in PBS and mounted with Fluoromount-G (0100-20, Southern Biotech). The image acquisition and analysis were performed using a Zeiss confocal microscope (LSM980, Zeiss, Germany) and ZEISS ZEN 3.3 (blue edition) software.

### 2.14 Statistical analyses

In the present study, statistical analyses were performed using the software GraphPad Prism version 8.2.1 for Mac OS (GraphPad Software, San Diego, California USA). One-way analysis of variance and subsequent Bonferroni post hoc tests were used to analyze the protein expression. \( P < .05 \) was considered to be statistically significant.
3 | RESULTS

3.1 | Biomechanical parameters of the model and neuroinflammatory response after SCI

The current study developed a rat model of SCI in accordance with previously established protocols, in order to investigate the novel disease pathways associated with SCI pathogenesis.6,7 A typical example of the changes in biomechanical parameters during injury is shown in Figure 1B. The mean contusion displacement was 2.00 ± 0.17 mm, the mean contusion speed was 501.03 ± 1.64 mm/s, and the mean peak force was 1.61 ± 0.23 N (Figure 1C-E). These biomechanical parameters were consistent with the SCI model. The immunofluorescent staining of the spinal cord sections obtained seven dpi showed a robust cellular neuroinflammatory response after SCI. The CD68+ macrophages/microglia infiltrated the lesion’s epicenter, surrounded by GFAP + reactive astrocytes in the lateral region, ipsilateral to the site of impact (Figure 1F).

3.2 | Proteomic analysis at three different time points following injury

The spinal cord tissue samples collected from the uninjured rats and the rats with SCI at three, seven, and fourteen dpi were subjected to LC-MS/MS analysis through a 4-D label-free strategy to obtain their proteome profiles. A total of 6615 proteins were successfully identified, among which, 4720 proteins were retained to perform further differential expression analysis. The current study initially examined the proteomic data quality, which showed that the injured and uninjured spinal cord tissue samples were distinguishable by means of the principal component analysis of the entire proteomic datasets (Figure 2B). Subsequently, k-means clustering was employed to obtain an overview of all significant proteins in all the samples (Figure 2A) and the protein expression levels at three different time points after SCI were compared with the uninjured tissue samples. Consequently, 738 proteins (397 upregulated and 341 downregulated) in the 3-dpi group versus sham group, 611 proteins (341 upregulated and 270 downregulated) in the 7-dpi group versus sham group, and 434 proteins (301 upregulated and 133 downregulated) in the 14-dpi group versus sham group were identified as the DEPs. Moreover, 301 DEPs overlapped at all the three time points of analysis after injury (Figure 2C).

The present study performed KEGG enrichment analysis, in order to gain insight into the pathways associated with the 301 overlapping DEPs. The upregulated proteins were mainly involved in the complement and coagulation cascades, cholesterol metabolism, and lysosome pathway, whereas the downregulated proteins were mainly involved in the pathways of neurodegeneration-multiple diseases, and oxidative phosphorylation (Figure 2D). Regulated proteins in the complement and coagulation cascades included A2M, C1QC, C3, ITGAM, and ITGB2. The complement cascade has been reported to mediate inflammatory response and repair after SCI.13 Interestingly, the proteins linked to cholesterol metabolism (eg, APOE, NPC2, ABCA1 and APOA4) were significantly upregulated at the three time points of analysis after SCI. Among these proteins, ABCA1 can regulate cholesterol efflux and phenotypic changes in macrophages in the injured spinal cord and APOE contributes to the preservation of the spinal cord-blood barrier and the reduction of inflammatory response after SCI.14,15 Furthermore, the lysosome pathway was significantly enriched and the representative genes included CTSB, CTSC, CTSZ, GM2A and NPC2. CTSB secretion following lipid overload and lysosomal damage has a neurotoxic effect and mediates neurodegeneration.16 Recently, CTsz was recognized as an important inflammatory mediator in neuroinflammation.17 In addition, the increased number of downregulated proteins, including GPX3, SLC25A5 and ATP5F1D, was associated with multiple neurodegenerative diseases, such as Huntington disease, amytrophic lateral sclerosis, Parkinson disease, and Alzheimer disease.

3.3 | The proteome profiling was consistent with the data at the transcriptome levels

The current study compared the proteome results with two independent transcriptome studies, in order to evaluate the reliability and reproducibility of the 301 DEPs at the transcriptomic expression levels. The GSE115067 dataset introduced a contusive SCI model with an impactor tip (2.0 mm) and an impact of 0 (control), 100, or 200 kdyn of pre-defined force. The DEGs in the 200 kdyn and control groups were analyzed. A total of 102 overlapping genes were observed in relation to the proteomic data pertaining to the present study and the 200 kdyn samples (Figure 3A). The overlapping genes included the genes that are known to be involved in the lysosome pathway (eg, CTSB, CTSC, and TCIRG1), complement and coagulation cascades (eg, A2M, C1QC, and SERPING1), cholesterol metabolism (eg, APOE, NPC2, and ABCA1), and Fc gamma R-mediated phagocytosis (eg, ARPC1B and SYK) (Figure 3B,C). All these genes in the four pathways were significantly upregulated, which is consistent with the proteome profiling in the present study.

The GSE45006 dataset used an aneurysm clip impact-compression injury model and analyzed the gene expression in the acute, subacute, and chronic stages of injury using a microarray gene chip approach. The DEGs in the samples obtained seven dpi and sham samples were compared. In total, 90 overlapping genes were observed in relation to the proteomic data and the genome-wide gene expression profiling (Figure 4A). These genes are known to have functional
roles in the activation of immune response (e.g., C1QC and PYCARD), phagocytosis (e.g., ANXA1, ABCA1, and SYK), regulation of neuronal death (e.g., CTSZ and GPNMB), lipid transport (e.g., PLIN2 and GM2A), lysosome organization (e.g., ABCA1 and CORO1A), and complement activation (e.g., C1QC and CFD) (Figure 4B). Most of these overlapping genes showed an increasing trend in the spinal cord samples after SCI, which was concurrent with the results of the current proteomic analysis. Moreover, the KEGG pathway analysis highlighted the involvement of lysosome, phagosome, complement and coagulation cascades, NOD-like receptor signaling, HIF-1 signaling, Fc gamma R-mediated phagocytosis, and cholesterol metabolism (Figure 4C).

Interestingly, 75 genes (e.g., ANXA1, C1QC, CTSZ, GM2A, GPNMB and PYCARD) were significantly altered in both the GEO datasets. Notably, ANXA1 was identified as a candidate biomarker for different severity of SCI in an integrated systems analysis. A recent study has reported that the GPNMB, a marker for lipid-associated neuroinflammatory signals, was observed to be upregulated in the brains of the patients with Parkinson's disease. These findings indicate that the proteomic data pertaining to the present study are consistent with the transcriptomic data from the GEO database.

3.4 Temporal clustering analysis of the spinal cord proteomes in SCI

The present study investigated the dynamic protein expressions over the course of the progression of SCI. A total of 10
YAO et al. time-dependent expression patterns were identified, among which, cluster 8 displayed distinctive and remarkable patterns (Figure 5A). The cluster 8 contained 541 proteins and the expression levels of these proteins were observed to be significantly increased at three dpi and remained elevated at seven and fourteen dpi. Subsequently, these proteins were intersected with the 301 overlapping DEPs at the three time points after SCI and 135 proteins were obtained. The GO and KEGG pathway enrichment analyses were conducted (Figure 5B-E), in order to obtain an overview of the functions of these significant proteins. These proteins were significantly enriched in the biological processes associated with the activation of immune response, phagocytosis, lipid transport, and antigen processing and presentation. Actin binding was the most enriched molecular function. Cellular component analysis revealed enrichment of the proteins related to the actin cytoskeleton, lysosome, phagocytic vesicles, and membrane microdomains. The KEGG pathway analysis showed that these proteins were significantly enriched in the lysosome, antigen processing and presentation. Fc gamma
R-mediated phagocytosis, complement and coagulation cascades, NOD-like receptor signaling pathway, apoptosis, and cholesterol metabolism pathways. These results were consistent with the enrichment results associated with the previous step and indicated that the immune response was activated in both the acute and subacute stages and that it might be associated with the lipid transport and lysosome.

The present study performed GSEA using the fold changes of all the proteins in cluster 8, in order to investigate the biological significance of the proteins in the cluster. The top 10 pathways were identified at different time points after injury and the overlapping pathways were merged in the heatmaps (Figure 5F). Cluster 8 was mainly enriched for markers of myeloid cells, such as microglia, macrophages, and biological processes, such as adaptive immune response and lipid localization. The top KEGG pathway was related to the lysosome pathway. The cell type-specificity analysis revealed that most of the pathway enrichment scores were the highest at seven dpi. At this time point, the proteins associated with adaptive immune response (eg, TCIRG1 and SYK), lipid transport (eg, GM2A, PLIN2, APOA4, and ABCA1), and lysosomes (eg, GM2A, TCIRG1, and CTSZ) were ranked high (Figure 5G). The results of the temporal clustering analysis and GSEA collectively indicate the importance of myeloid cell activation at the acute and subacute phases of injury, and the importance of the functions associated with immune response, lipid regulation, and lysosome.

3.5 | Western blot analyses and immunofluorescent staining to validate the results of MS

Finally, the current study used Western blot assays and immunofluorescent staining to confirm the results of MS (Figure 6F-J). The complement component C3 was observed to be significantly upregulated at all the three time points. Moreover, ITGAM which is one of the receptors of
the aforementioned complement component, was also signifi-
cantly upregulated at three and seven dpi. The abovemen-
tioned results indicate the involvement of the complement
pathway in SCI. CTSB is a lysosomal cysteine protease
with both endopeptidase and exopeptidase activity. CTSZ is
also a cysteine enzyme, which is mainly expressed by the
antigen-presenting cells and is associated with neuroinflam-
matory disorders. Both the aforementioned enzymes were
significantly upregulated in response to the SCI. NPC2, which
regulates the transport of cholesterol through the late
endosomal/lysosomal system, was significantly upregulated
at all the three time points after SCI. Hence, the results of the
Western blot assays were concurrent with the proteomic pro-
file analysis. In addition, immunofluorescent staining showed
that these proteins coexisted with F4/80+ reactive microglia and
infiltrating macrophages, which is consistent with the GSEA
results that indicated that the functions of immune response,
lipid regulation, and lysosome were associated with activated
myeloid cells (Figure 6A-E).

4 | DISCUSSION

Neuroinflammation has multiple effects on the response
to central nervous system (CNS) injuries. It is destructive
to the neurons, axons, and myelin sheaths through the
generation of toxic cytokines, proteases, and free
radicals. Conversely, it also has beneficial effects on the
injured CNS, such as the production of neurotrophic fac-
tors, phagocytosis of cellular debris, and the production of
proteases to clear inhibitory molecules for remyelination. However, the temporal occurrence of these functions and the pathways and molecular signatures that mediate these functions remain ambiguous. In the present study, an integrated analysis of the proteome and transcriptome in rat models of SCI was used to investigate the dysregulated genes, proteins, and pathways in the acute and subacute phases of SCI. The present study identified 301 shared DEPs at the following three time points: three, seven, and fourteen dpi. Among the aforementioned DEPs, the upregulated proteins were mainly involved in the pathways of complement and coagulation cascades, cholesterol metabolism, and lysosome pathways. Subsequently, the results were verified at the mRNA level using two GEO datasets and similar results were observed. Finally, the cluster positively associated with the progressive course of SCI.
demonstrated the important role of proteins involved in the markers of myeloid cells, immune response, lipid metabolism, and lysosome.

The present temporal clustering analysis showed that the cluster with continuously upregulated proteins was associated with the immune cells. Furthermore, most of the datasets displayed the highest enrichment score at seven dpi and the lowest enrichment score at fourteen dpi. Damage-associated molecular patterns released immediately after the injury initiate innate and adaptive immune responses. Beck et al showed that the neutrophil count, macrophages/microglia, and the T cells peaked on the first, seventh, and ninth dpi, respectively, in the early phase of a rat spinal contusion model. Microglia and macrophages are important modulators of CNS inflammation, regeneration, and repair. The centripetal migration of the macrophage towards the epicenter of the lesion has an important role in spontaneous recovery. Microglia and macrophages also contribute to corralling and wound compaction, in order to provide protection from secondary tissue injury. After the injury, the tissues self-repair to a certain extent and the different phenotypes of microglial and macrophage populations occur at different stages of the injury. The present data showed increased levels of several proteins associated with phagocytosis (eg, CORO1A, ABCA1 and SYK) at the three time points after injury. Phagocytosis plays a clear role in the removal of cellular and myelin debris, and inhibitory molecules in the lesions. Consequently, there has been an interest in the therapeutic modulation of phagocytosis.

Complement cascades are essential for the microglial clearance of tissue debris after acute CNS injury. The present study observed significantly upregulated proteins associated with the complement and coagulation cascades, including A2M, C1QC, C3, ITGAM and SERPING1. Moreover, the Western blot assays revealed increasing amounts of C3 and ITGAM after the injury. A previous study has reported that the complement proteins were upregulated within one day after SCI and chronic persistence was observed. Recruited inflammatory cells and resident CNS cells are the main sources of complement proteins and these proteins mediate immune cell infiltration, cytokine generation, myelin clearance, and cell death by way of interactions with the microenvironment of injury. For instance, complement receptor 3 plays a role in myelin clearance by means of binding to myelin. C1Q can also bind to myelin and modulate the neurite outgrowth after SCI. Regardless of these beneficial functions associated with the complement system, excessive activation of the complement cascades may result in neuronal death. Mallah et al suggested that the inhibition of the complement system is a potential neuroprotective strategy for the management of CNS injuries. However, it is notable that appropriate timing of intervention using the complement-targeted therapeutics needs to be explored, in order to alleviate the tissue damage safely, without hampering the tissue repair.

A previous study that involved cell type-specific macrophage transcriptional profiling after SCI reported lipid catalysis as the primary function at seven dpi and an amenable therapeutic target for SCI. The present results imply that several proteins related to lipid transport, lipid localization, and cholesterol metabolism were significantly upregulated, such as APOE, NPC2, ABCA1, APOA4, GM2A, and PLIN2. These data are consistent with the results reported by previous analyses, which showed that the SCI exhibits increased levels of proteins involved in lipid and cholesterol metabolism. These alterations could be attributed to the formation of foamy macrophages after SCI. There is a growing body of evidence that shows that cellular lipid accumulation is associated with neuroinflammation and plays a critical role in the pathogenesis of neurodegeneration. For instance, cholesterol accumulation induced lysosomal rupture and inflammasome activation in a demyelination model in aged mice as a result of excessive phagocytosis of myelin debris. In addition, lipid accumulation could induce lysosomal damage and increased CTSB release in a mouse model of Niemann–Pick disease type C. However, the exact mechanism of lipid metabolism in the progression of SCI remains ambiguous and is worthy of further research.

The current proteomic data showed a significant increase in the expression of proteins responsible for the lysosome pathway, such as cathepsins (CTSB, CTSC, and CTSZ), GM2A, NPC2, TCIRG1, and DNASE2. Furthermore, the present study observed significantly upregulated proteins associated with the NOD-like receptor signaling pathway, such as CSTB, GBP2, and PYCARD, which is consistent with our previous findings. Cathespins include more than 10 proteases with potent endopeptidase activity. Traumatic CNS injury can lead to increased levels of cathepsins and aggravate inflammation. In addition, CTSB expressed by the microglia has been demonstrated as a primary driver of neuroinflammation. CTSZ is also indicated as a pathogenic factor and a potential therapeutic target for the management of neuroinflammation. However, cathepsins expressed by axonal growth cones play an important role in neuronal plasticity. Hence, the scenario warrants further understanding regarding the temporal-spatial distribution of cathepsins after SCI. Recent reports have shown that the microvascular endothelial cells degrade the engulfed myelin debris through the autophagy-lysosome pathway after neural injury. Further studies are required to understand the underlying mechanism concerning the association between the lysosome pathway and SCI.

The present study has several limitations. First, the current analysis can only represent the data concerning rodent models of contusive SCI and not humans. Despite the differences in relation to humans, the rodent models of contusive
SCI created by impactors have become useful models in the simulation of major pathophysiological changes in traumatic human SCI.\(^{3,0,41}\) Second, the present study mainly focused on the changes in the acute and subacute phases and did not investigate the chronic phase of injury, which is an important time point and warrants further research. Third, the present study could not use inhibition experiments to investigate the exact pathophysiological mechanism that transpires after the SCI. The present study provides a direction for future research regarding the same.

In conclusion, the proteomic data pertaining to the present study and the two transcriptome datasets indicate that the complement and coagulation cascades, cholesterol metabolism, and the lysosome pathway are some of the main pathways involved in the progression of SCI. Moreover, the present study observed that the protein module continuously upregulated at the acute and subacute time points after SCI was associated with the markers of myeloid cells and functions of immune response, lipid regulation, and lysosome, which may be utilized to modulate neuroinflammation after SCI.

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CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial associations that might pose a conflict of interest.

AUTHOR CONTRIBUTIONS

XQ Yao, QA Zhu, RT Ding and JT Chen conceived and designed the research. XQ Yao, ZY Liu, ZC Huang and JH Liu performed the experiments. XQ Yao, JY Chen and BH Sun analyzed the data. XQ Yao, ZY Liu, RT Ding and JT Chen wrote the paper. All authors had read and reviewed the final manuscript.

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**SUPPORTING INFORMATION**

Additional Supporting Information may be found online in the Supporting Information section.

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