



Identification of potential biomarkers associated with CD4⁺ T cell infiltration in myocardial ischemia-reperfusion injury using bioinformation analysis

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Background: Myocardial ischemia-reperfusion injury (MIRI) is often part of clinical events such as cardiac arrest, resuscitation, and reperfusion after coronary artery occlusion. Recently, more and more studies have shown that the immune microenvironment is an integral part of ischemia-reperfusion injury (IRI), and CD4⁺ T-cell infiltration plays an important role, but there are no relevant molecular targets for clinical diagnosis and treatment.

Methods: The transcriptome data and matched group information were retrieved from the Gene Expression Omnibus (GEO) database. The ImmuCellAI-mouse (Immune Cell Abundance Identifier for mouse) algorithm was used to calculate each symbol's CD4⁺ T cell infiltration score. The time period with the greatest change in the degree of CD4⁺ T cell infiltration [ischemia-reperfusion 6 hours (IR6h)-ischemia-reperfusion 24 hours (IR24h)] was selected for the next analysis. Weighted gene co-expression network analysis (WGCNA) and differential expression analysis were performed to screen out CD4⁺ T cell-related genes and from which the gene *CLEC5A* was screened for the highest correlation with CD4⁺ T cell infiltration. The potential regulatory mechanism of CD4⁺ T cells in MIRI was discussed through various enrichment analysis. Finally, we analyzed the expression and molecular function (MF) of *CLEC5A* and its related genes in MIRI.

Results: A total of 406 CD4⁺ T cell-related genes were obtained by intersecting the results of WGCNA and differential expression analysis. Functional enrichment analysis indicated that the CD4⁺ T cell-related genes were mainly involved in chemokine signaling pathway and cell cycle. By constructing a protein-protein interaction (PPI) network, a total of 12 hub genes were identified as candidate genes for further analysis. Through the correlation analysis between the 12 candidate genes found in the PPI network and CD4⁺ T cell infiltration fraction, we determined the core gene *CLEC5A*. Finally, a gene interaction network was constructed to decipher the biological functions of *CLEC5A* using GeneMANIA.

Conclusions: In this study, RNA sequencing (RNA-Seq) data at different time points after reperfusion were subjected to a series of bioinformatics methods such as PPI network, WGCNA module, etc., and *CLEC5A*, a pivotal gene associated with CD4⁺ T-cells, was found, which may serve as a new target for diagnosis or treatment.

Keywords: Myocardial ischemia-reperfusion injury (MIRI); CD4⁺ T cell; weighted gene co-expression network analysis (WGCNA); C-type lectin; immune microenvironment

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Introduction

At present, there is no effective treatment for myocardial ischemia-reperfusion injury (MIRI), which exacerbates energy metabolism disorder, myocardial structure damage, and functional injury (1,2). After coronary recanalization in the infarcted area, arrhythmia, decline of cardiac function, and sudden cardiac death often occur clinically. The pathogenesis of MIRI is complex, involving oxidative damage (3,4), inflammatory response (5,6), calcium overload (7,8), and other processes. Many mechanisms of MIRI are unclear, especially the role of the immune microenvironment in MIRI remains to be further explored. Previous studies have shown that CD4⁺ T cells are involved in this pathophysiological process (9,10). Further study on the mechanism of T cells can provide new ideas for the treatment of ischemia-reperfusion injury (IRI).

Adaptive immune responses, particularly those involving CD4⁺ T cells, play an important role in ischemia-reperfusion. Several studies have used CD4⁺ T cell-deficient mice and demonstrated a critical role for CD4⁺ T cells in IRI and infarct healing (11,12). However, there is a lack of suitable biomarkers for predicting the extent of CD4⁺ T cell infiltration in ischemia-reperfusion. The use of biosignature analysis to solve challenging biological problems will not only help advance basic research, but may also provide new breakthroughs in biomedical applications.

C-type lectins are the largest and most diverse family of mammalian carbohydrate-binding proteins. In previous

studies, they have been shown to interact with various endogenous and exogenous ligands and participate in important physiological processes such as immune defense, immune stability, and immune monitoring, so as to maintain bodily homeostasis (13,14). *CLEC5A*, a type II transmembrane protein belonging to the type C lectin family, can recognize endogenous ligands, such as self-carbohydrates, proteins, or lipids, to control homeostasis and tissue damage. *CLEC5A* is associated with the progression of a variety of acute and chronic inflammatory diseases. However, the detailed mechanism of *CLEC5A* and CD4⁺ T cell infiltration in MIRI has not been determined.

In this study, we assessed the immune microenvironment of each sample. Based on the increase in CD4⁺ T-cell infiltration after 6 hours of reperfusion, a series of bioinformatics analyses were performed using the available data, providing a reliable basis for exploring the molecular mechanisms of CD4⁺ T-cell infiltration in MIRI. We present this article in accordance with the STREGA reporting checklist (available at <https://jtd.amegroups.com/article/view/10.21037/jtd-23-1335/rc>).

Methods

Data collection

In this study, we compared the gene expression profiles of the myocardial tissue of mice treated with reperfusion after ischemia at 0, 6, 24, and 72 hours of ischemia/reperfusion (I/R) from the Gene Expression Omnibus (GEO; n=4 per group) and downloaded and collected high-throughput gene expression dataset (No. GSE160516). The gene probe was transformed into gene symbols according to the annotation information for GPL23038 [Clariom_S_Mouse] Affymetrix Clariom S Assay (Affymetrix, Santa Clara, CA, USA), Mouse (includes Pico assay) and normalized for differentiation analysis.

Analysis of infiltrating immune cells

ImmuCellAI-mouse (Immune Cell Abundance Identifier for mouse) is a tool to estimate the abundance of 36 immune cells based on gene expression profile from RNA sequencing (RNA-Seq) or microarray data (15). To ensure the prediction accuracy, the abundance of cells in 3 layers was predicted separately by calculating the single-sample gene set enrichment analysis (ssGSEA) enrichment score of the expression deviation profile per cell type.

Highlight box

Key findings

- *CLEC5A* is a hub gene associated with CD4⁺ T cells in myocardial ischemia-reperfusion injury.

What is known and what is new?

- Immune cell infiltration plays an important role in myocardial ischemia-reperfusion injury. Infiltration of CD4⁺ T cells can lead to increased myocardial damage.
- Our analysis identified a potential association between *CLEC5A* expression and CD4⁺ T cell infiltration in myocardial ischemia-reperfusion injury.

What is the implication, and what should change now?

- *CLEC5A* is closely associated with CD4⁺ T cell recruitment in ischemia-reperfusion injury. These findings will shed light on the clarification of the immunological mechanism of ischemia-reperfusion injury and provide new targets for subsequent clinical treatment.

Besides, ImmuCellAI-mouse can be applied to estimate the difference of immune cell infiltration in diverse groups.

Co-expression network construction by WGCNA

We used WGCNA to define the specific gene modules representing each experimental group and the CD4⁺ T cell compositions. First, samples were clustered to assess the presence of any obvious outliers. Second, the automatic network construction function was used to construct the co-expression network. To make the co-expression network align with the distribution of scale-free network, a soft-thresholding power was computed with the pickSoftThreshold function. Third, hierarchical clustering and the dynamic tree cut function were employed to identify modules. Subsequently, a dynamic tree-cutting algorithm was utilized to establish module assignments. Module eigengenes (MEs) were computed using the MEs function. Pearson correlation coefficients between ME and each experimental group and the CD4⁺ T cell compositions were evaluated. After co-expression network construction by WGCNA, MEturquoise genes were selected for further analysis.

Differential expression analyses of CD4⁺ T cell-related genes

By Wilcoxon test in the R language (R Foundation for statistical Computing, Vienna, Austria), the differentially expressed genes (DEGs) between the IR6h and IR24h groups were identified. The cut-off criteria for the differential gene are adjusted P value (adj. P) <0.01 and false discovery rate (FDR) <1. We acquired 578 CD4⁺ T cell-related genes differential expression between IR6h and IR24h group. The intersection of the CD4⁺ T cell-related DEGs and MEturquoise genes, as visualized in a Venn diagram, were selected for further analysis.

Construction of a protein-protein interaction (PPI) network

We used the Metascape database (<https://metascape.org>) to establish a PPI network to visualize the connections between the target genes. Metascape provides a rather unique PPI network analysis capability. To infer more biologically interpretable results, Metascape applies a mature complex identification algorithm called Molecular Complex Detection (MCODE) to automatically extract protein complexes embedded in such large network. The min

overlap =3, P value cutoff =0.01, min enrichment =1.5 were regarded as standard.

Functional enrichment analysis

To identify pathways significantly enriched between IR6h and IR24h, we performed Gene Ontology (GO) enrichment and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses. Meanwhile, we performed ssGSEA to derive the enrichment score of each immune-related term according to the “clusterprofiler” package. P value <0.05 was considered statistically significant.

Statistical analysis

The data was processed using the PERL programming language (Version 5.32.0; <http://www.perl.org>). All statistical analyses were performed using the R software (version 4.1.2; <https://www.r-project.org/>). P value <0.05 was considered to indicate statistical significance, if not otherwise stated.

Results

CD4⁺ T cell-associated module identification by WGCNA

Previous studies have found that CD4⁺ T cells are involved in reperfusion injury after myocardial ischemia (9,10). The changes of CD4⁺ T cells infiltration with the time of reperfusion are shown in *Figure 1A*. We noticed that CD8⁺ T cells did not play a significant role in this process (*Figure 1B*). At the same time, at the transcriptome level, caspase family members also showed differences at different time points after reperfusion (*Figure 1C*). The immune infiltrating abundance of 36 immune cells were predicted using ImmuCellAI-mouse. In the R package WGCNA, we constructed co-expression networks with reperfusion time and CD4⁺ T cells infiltration score (*Figure 1D-1I*). CD4⁺ T cells, which presented statistical significance between IR6h and IR24h, were used to construct a co-expression network with 12 as the soft thresholding power β . The changes of gene expression and T cell infiltration in each module with reperfusion time were observed and compared, and the MEturquoise module were selected for further analysis.

Identification of CD4⁺ T cell-related genes and construction of a PPI network

By Wilcoxon test in the R language, the 578 DEGs between

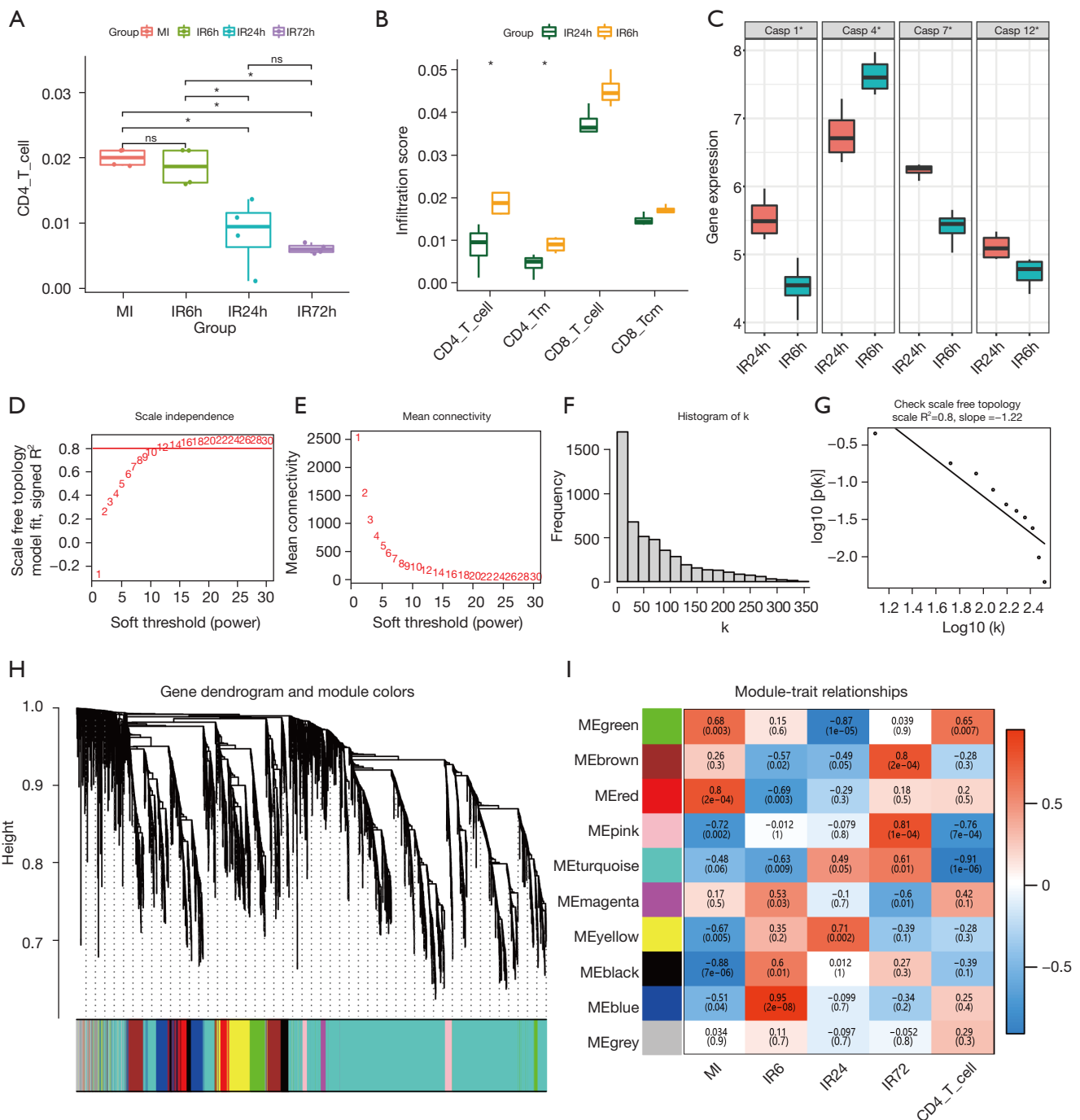


Figure 1 Recognition of hub genes related to CD4⁺ T cells. (A) The box shows the changes of T cell infiltration at different time points. (B) The infiltration of 4 types of T cells at 6 hours and 24 hours were compared. (C) Transcriptional level analysis of caspase family members. (D,E) Checking the scale free topology when $\beta=12$. (F) Analysis of the scale-free index for various soft-threshold powers (β). (G) Analysis of the mean connectivity for various soft-threshold powers. (H) Dendrogram of WGCNA module. (I) Heat map of the correlation between modular characteristic genes and myocardial ischemia-reperfusion time and T cell infiltration characteristics. We selected MEturquoise level blocks for subsequent analysis. (A,B) *, $P < 0.05$; ns, not significant. (C) *, $P < 0.05$. MI, myocardial infarction; IR6h, ischemia-reperfusion 6 hours; IR24h, ischemia-reperfusion 24 hours; IR72h, ischemia-reperfusion 72 hours; ME, module eigengene; WGCNA, weighted gene co-expression network analysis.

IR6h and IR24h samples were identified (Figure 2A,2B). The 406 intersecting genes of DEGs and MEturquoise module genes were shown by Venn diagram (Figure 2C). Then, the PPI network was constructed by using these intersecting genes, because these DEGs have been shown to relate to the interacting proteins (Figure 2D). The Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) furnishes original reliable protein data for consequent analysis. Through the PPI network, a total of 12 hub genes were identified as candidate genes for further analysis.

Functional enrichment analysis of intersecting genes

To further comprehend the latent functions of the 406 intersecting genes in MIRI, we performed GO, gene set variation analysis (GSVA), gene set enrichment analysis (GSEA), and KEGG enrichment analyses. KEGG pathway analysis demonstrated that the intersection genes were mainly involved in chemokine signaling pathway and cell cycle (Figure 3A,3B). In the results of GSVA and GSEA analysis, hypoxia and immune checkpoints were in a conspicuous position (Figure 3C,3D). The top 10 components of biological process (BP), cellular component (CC), and molecular function (MF) are illustrated in Figure 3E. Interestingly, we discovered that the cell activation involved in immune response and reactive oxygen species metabolic process also appeared in the results.

CLEC5A is closely related to CD4⁺ T cell infiltration after MIRI

Through the correlation analysis between the 12 hub genes found in the PPI network and CD4⁺ T cell infiltration fraction, we identified the core gene *CLEC5A* (Figure 4A). Moreover, the change of *CLEC5* expression after reperfusion was shown to have a very high correlation with the degree of T cell infiltration (Figure 4B). Since we found the important role of immune checkpoints through enrichment analysis, we paid more attention to the role of *CLEC5A* in immunity. According to the expression of *CLEC5A*, we divided all samples into two groups and analyzed the infiltration of all immune cells (Figure 4C). In addition to CD4⁺ T cells, *CLEC5A* is also associated with macrophages and neutrophils. At the same time, we found that *CLEC5A* was also associated with a variety of chemokines, which reasonably explained the correlation between *CLEC5A* and immune infiltration (Figure 4D).

Functional analysis of CLEC5A and its related genes

GeneMANIA was used to identify the top 20 *CLEC5A*-related genes of the hub gene and construct a gene interaction network (Figure 5A). At the same time, the PPI network was constructed (Figure 5B). We then observed the expression of these genes at different times after ischemia. We used IR6h and IR24h to analyze the difference with the MIRI group (Figure 5C,5D). There were significant differences in some genes after reperfusion. Then, the 20 genes were analyzed by functional enrichment using the GeneMANIA website. The results showed that it was mainly related to C-type lectin (Figure 5E).

Discussion

IRI is often a component of clinical events such as cardiac arrest, resuscitation, and reperfusion after coronary artery occlusion (16). In clinical practice, it has been found that restoring blood perfusion may cause more serious damage than ischemia, and this phenomenon exists in different species (17). This study shows that CD4⁺ T cells are involved in MIRI in mice, but not in the early MI/R (MI/R for 6 h, IR6h). In order to screen the genes most closely related to T cell immunity, we further combined with the immune infiltration of mice, and screened *CLEC5A* as the key target gene through WGCNA and gene expression difference analysis. Previous studies on *CLEC5A* have mostly been related to the immune response caused by microbial virus or tumor, but research on the immune effect of MIRI has been neglected (18,19).

The pathophysiological mechanisms of myocardial ischemia-reperfusion injury are complex, but progress is being made in this area of research to explore more effective preventive and therapeutic approaches to achieve the goal of mitigating the impact of myocardial reperfusion injury on heart health. Existing research findings are mainly related to inflammatory responses, oxidative stress, and calcium overload (20-22). Researchers are also currently exploring new approaches such as the use of antioxidants, inhibition of inflammatory responses, stem cell therapy or gene therapy to protect cardiomyocytes (23-25). This will hopefully improve the quality of life and prognosis of heart disease patients.

Adaptive immune responses, especially those involving CD4⁺ T cells, are important for wound healing. In recent years, many robust evidences have been yielded from animal models that CD4⁺ T cells play an important role

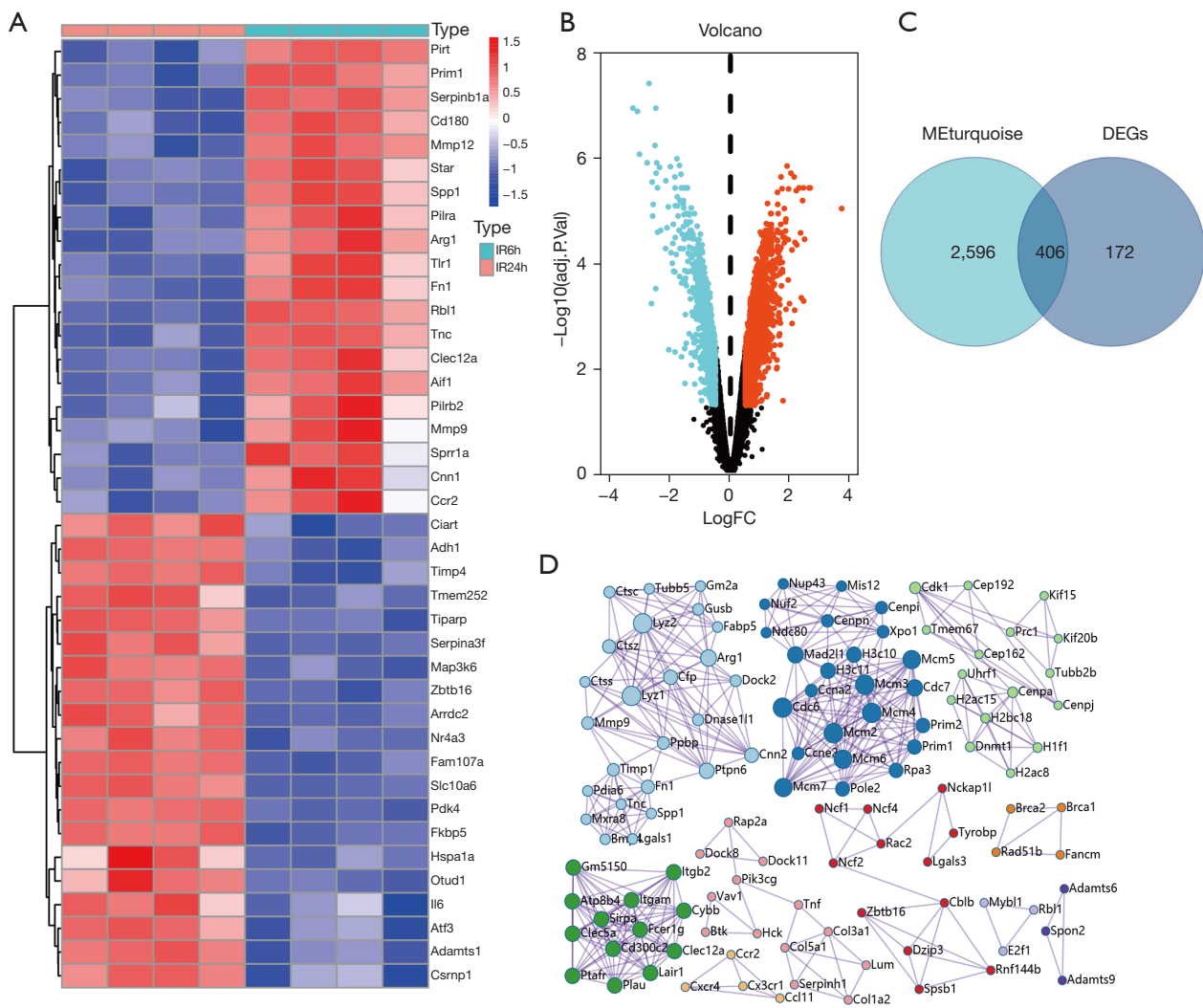


Figure 2 Identification of the candidate CD4⁺ T cell-related genes. (A) Heat map shows the expression of differentially expressed genes in IR6h and IR24h. (B) Volcano plot shows the expression of differentially expressed genes in IR6h and IR24h. The red dots represent significantly up-regulated genes with at least 1.5-fold change, while the green dots represent significantly down-regulated genes with at least 1.5-fold change. (C) Venn diagram to identify intersection genes of DEGs and METurquoise genes. (D) PPI network shows links of the candidate CD4⁺ T cell-related genes. IR6h, ischemia-reperfusion 6 hours; IR24h, ischemia-reperfusion 24 hours; FC, fold change; ME, module eigengene; DEGs, differentially expressed genes; PPI, protein-protein interaction.

in ischemia-reperfusion (26,27). CD4⁺ T cells are widely involved in reperfusion injury, not limited to myocardial tissue (28). CD4⁺ T cells mediate hepatic neutrophil recruitment and liver injury during hepatic ischemia-reperfusion (29). Previous studies have suggested that CD4⁺ lymphocytes are rapidly recruited to the injured site after ischemia-reperfusion, and promote the subsequent neutrophil recruitment through an interleukin-17 (IL-17) dependent mechanism (30,31). Similarly, CD4⁺ T cells also

play a role in renal and intestinal IRI through their immune characteristics (32,33).

CLEC5A, a type II transmembrane protein belonging to the type C lectin family, can recognize endogenous ligands, such as self-carbohydrates, proteins, or lipids, to control homeostasis and tissue damage (34). *CLEC5A* is associated with the progression of a variety of acute and chronic inflammatory diseases, including dengue fever (35,36), fatal shock (37,38), Crohn's disease (39),

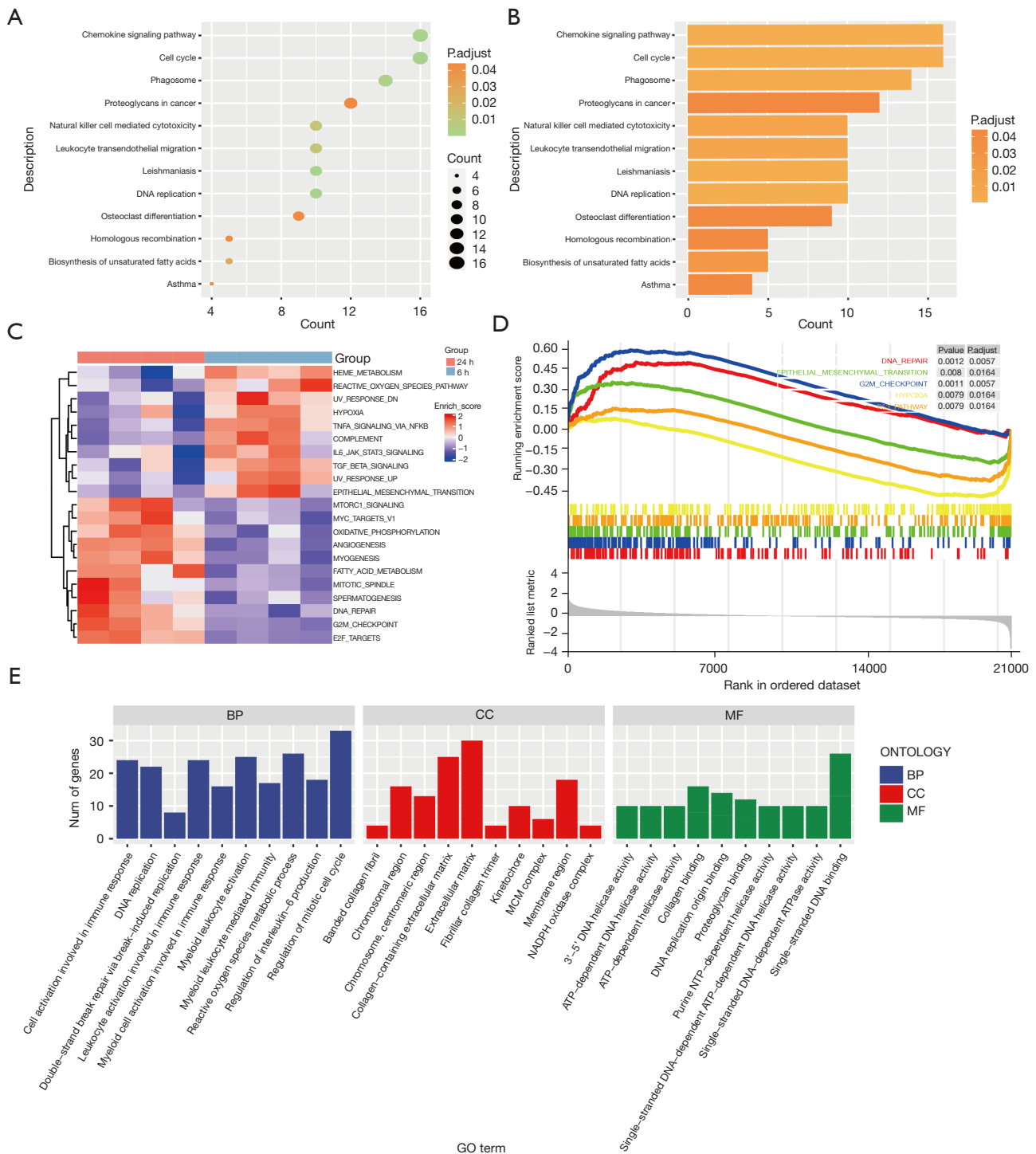


Figure 3 Functional enrichment analyses of the candidate CD4⁺ T cell-related genes. (A,B) KEGG pathway analysis results of CD4⁺ T cell-related genes. (C) GSEA analysis results of CD4⁺ T cell-related genes. (D) The GSEA analysis of CD4⁺ T cell-related genes. (E) GO analysis results of CD4⁺ T cell-related genes. BP, biological process; CC, cellular component; MF, molecular function; MCM, minichromosome maintenance; NADPH, nicotinamide adenine dinucleotide phosphate hydrogen; NTP, nucleotide triphosphate; ATP, adenine nucleoside triphosphate; KEGG, Kyoto Encyclopedia of Genes and Genomes; GSEA, gene set variation analysis; GSEA, gene set enrichment analysis; GO, Gene Ontology.

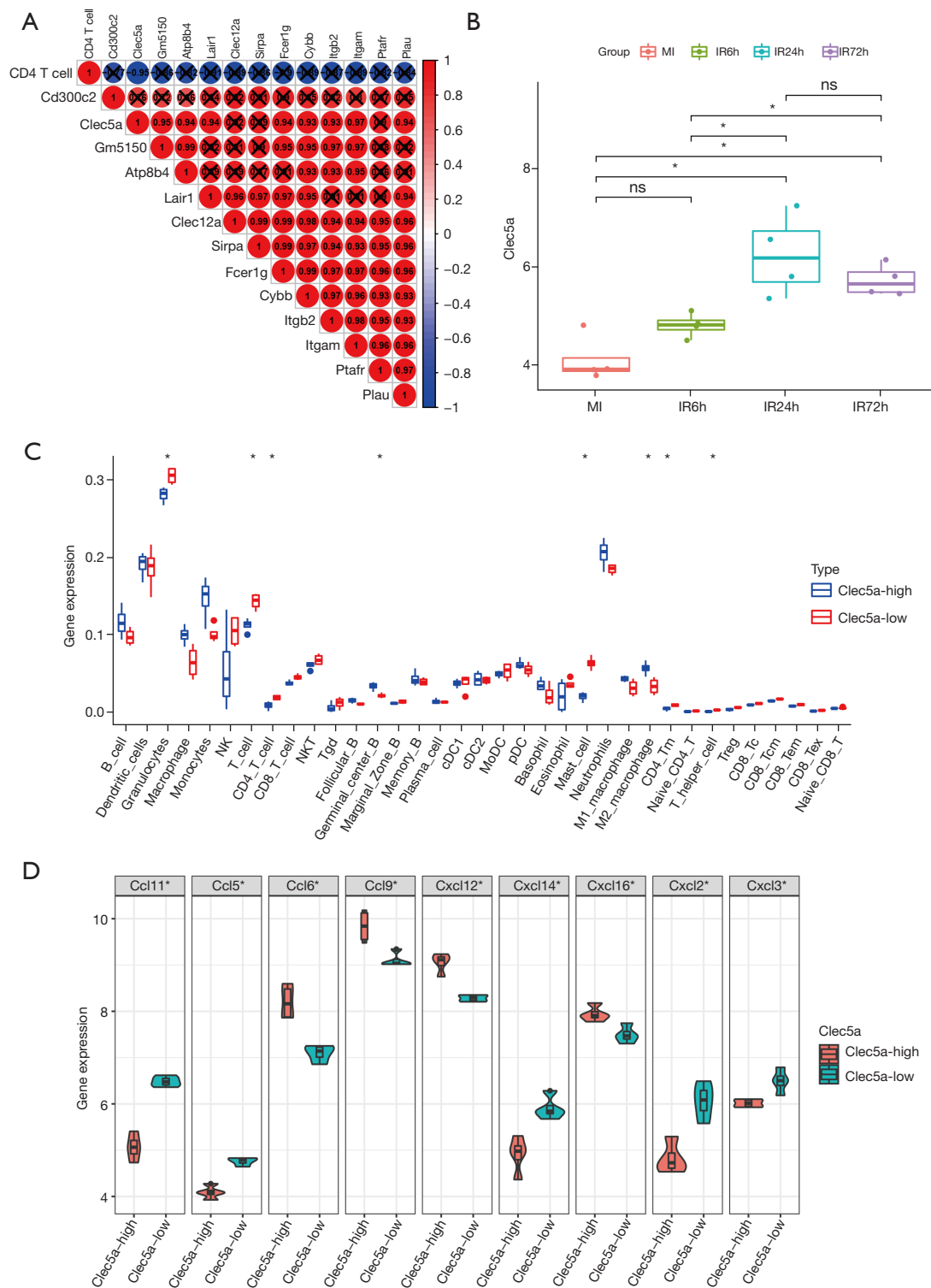


Figure 4 *CLEC5A* is the core gene affecting CD4⁺ T cell infiltration. (A) Correlation analysis between hub genes in PPI network and T cell infiltration. (B) The expression of *CLEC5A* at different reperfusion time points. (C) Correlation analysis between *CLEC5A* expression and various immune cell infiltration. (D) Correlation analysis between *CLEC5A* and multiple chemokines. (A) ×, $P \geq 0.05$. (B,C) *, $P < 0.05$; ns, not significant. (D) *, $P < 0.05$. MI, myocardial infarction; IR6h, ischemia-reperfusion 6 hours; IR24h, ischemia-reperfusion 24 hours; IR72h, ischemia-reperfusion 72 hours; PPI, protein-protein interaction.

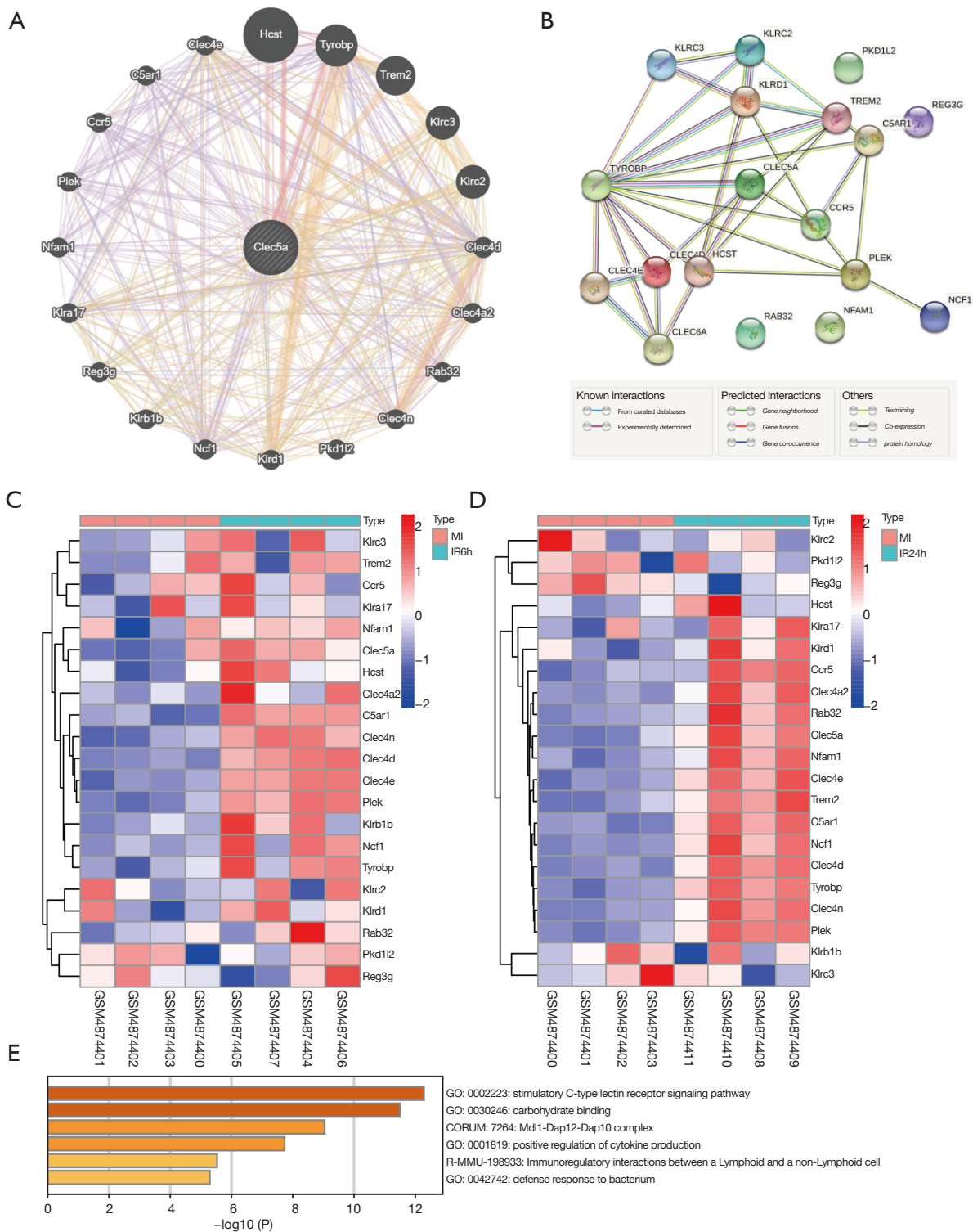


Figure 5 Function Prediction for *CLEC5A*. (A) PPI network (geneMANIA) of *CLEC5A*. (B) PPI network of *CLEC5A*-related genes. (C,D) The heat map showed the difference of *CLEC5A*-related genes expression at 6 h and 24 h after myocardial reperfusion. (E) Functional enrichment analyses of *CLEC5A* related genes. MI, myocardial infarction; IR6h, ischemia-reperfusion 6 hours; IR24h, ischemia-reperfusion 24 hours; GO, Gene Ontology; PPI, protein-protein interaction.

and autoimmune arthritis (40). In addition, some studies have shown that *CLEC5A* may act as a biomarker in the field of cancer (41,42). A previous study found that *CLEC5A* knockdown prevents myocardial infarction (MI)-induced cardiac insufficiency by regulating macrophage polarization, NLRP3 inflammation, and cell death in the left ventricle of mice (43). It has been reported that lectin plays an important role in local and remote tissue injury related to gastrointestinal ischemia-reperfusion (GI/R), and expounded the correlation between lectin and complement pathway (44). Similarly, macrophage-inducible C-type lectin has been shown to induce persistent aseptic inflammation after acute renal IRI in mice, aggravating tissue injury and subsequent renal atrophy (45). However, the detailed mechanism of *CLEC5A* and CD4⁺ T cell infiltration needs to be further studied.

Admittedly, there were limitations to this study. Above all, our study included a small sample size. Most importantly, we cannot completely deny the role of other genes in recruiting or inhibiting CD4⁺ T cells (46). In addition, large differences were observed between the values of individual tissue samples. Secondly, for the mouse models in the data set, many experimental details were not provided in detail, and there was a lack of evaluation of the mouse MIRI model, such as the degree of myocardial injury, infarct size, ejection function, and so on. However, this does not affect the reliability of our results and conclusions, as well as the method of data analysis. Thirdly, there were relatively few studies on C-type lectins and CD4⁺ T cells, strong experimental evidence is lacking, and this area requires further research.

Conclusions

This study involved a bioinformatics analysis of RNA-Seq data at different time points after reperfusion. Then, a series of bioinformatics methods including PPI network and WGCNA module were used to find the hub gene related to CD4⁺ T cells. The correlation between C-type lectin and IRI was found by enrichment analysis. These findings will shed light on the clarification of the immunological mechanism of IRI and provide new targets for subsequent clinical treatment.

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Footnote

Reporting Checklist: The authors have completed the STREGA reporting checklist. Available at <https://jtd.amegroups.com/article/view/10.21037/jtd-23-1335/rc>

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Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at <https://jtd.amegroups.com/article/view/10.21037/jtd-23-1335/coif>). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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