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Original Article

# Serum from Acute-on-chronic Liver Failure Patients May Affect Mesenchymal Stem Cells Transplantation by Impairing the Immunosuppressive Function of Cells

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## Abstract

***Background and Aims:*** The safety and efficacy of mesenchymal stem cells (MSCs) in the treatment of acute-on-chronic liver failure (ACLF) have been validated. However, the impact of the pathological ACLF microenvironment on MSCs is less well understood. This study was designed to explore the changes in the functional properties of MSCs exposed to ACLF serum. ***Methods:*** MSCs were cultured in the presence of 10%, 30% and 50% serum concentrations from ACLF patients and healthy volunteers. Then, the cell morphology, phenotype, apoptosis and proliferation of MSCs were evaluated, including the immunosuppressive effects. Subsequently, mRNA sequencing analysis was used to identify the molecules and pathways involved in MSC functional changes in the context of ACLF. ***Results:*** In the presence of ACLF serum, MSC morphology significantly changed but phenotype did not. Besides, MSC proliferation activity was weakened, while the apoptosis rate was lightly increased. Most importantly, the immunosuppressive function of MSCs was enhanced in a low-concentration serum environment but transformed into a proinflammatory response in a high-concentration serum environment. RNA sequencing indicated that 10% serum concentration from ACLF patients mediated the PI3K-Akt pathway to enhance the anti-inflammatory effect of MSCs, while the 50% serum concentration from ACLF patients promoted the conversion of MSCs into a proinflammatory function by affecting the cell cycle. ***Conclusions:*** The 50% ACLF serum concentration is more similar to the environment in the human body, which means that direct peripheral blood intravenous infusion of MSCs may reduce the effect of transplantation. Combining treatments of plasma exchange to reduce harmful substances in serum may promote MSCs to exert a stronger anti-inflammatory effect.

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## Introduction

Acute-on-chronic liver failure (ACLF) is a distinct clinical syndrome, characterized by liver failure due to acute hepatic injury and underlying chronic liver disease; it has a high 28-day mortality. Liver transplantation is the only treatment that has proven beneficial, but the rapid disease progression and lack of donors limit the application of this treatment.[1,2] The infusion of mesenchymal stem cells to treat liver failure has been verified as safe and effective in clinical trials[3,4] as well as by animal experiments based upon acute liver failure models.[5–7] In clinical practice, the treatment of patients with ACLF with infusions of mesenchymal stem cells (MSCs) significantly increased the 24-week survival rate by improving liver function and decreasing the incidence of severe infections.[4] In the treatment of fulminant hepatic failure in large animal (pig) models, immediate intraportal transplantation of MSCs quickly participated in liver regeneration via proliferation and transdifferentiation into hepatocytes.[5] MSCs harbor anti-inflammatory, immunomodulatory, antiapoptotic and proliferative properties and hold great promise in the treatment of both acute and chronic liver diseases.[2] However, studies generally have focused on the effectiveness and mechanism of MSCs in the treatment of liver failure, while the impact of the pathological ACLF microenvironment on MSCs has received little attention thus far.

The impact of the pathological microenvironment on MSC

**Keywords:** Mesenchymal stem cells; Acute-on-chronic liver failure; Immunomodulatory; Cell therapy; Microenvironment.

**Abbreviations:** ACLF, acute-on-chronic liver failure; AP, ACLF patient; DEGs, differentially expressed genes; GO, Gene Ontology; GSEA, Gene Set Enrichment Analysis; HC, healthy control; KDA, Key driver analysis; KEGG, Kyoto Encyclopedia of Genes and Genomes; MSCs, mesenchymal stem cells; PPI, protein-protein interaction.

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**Table 1. Clinical characteristics of the participants enrolled in the study**

| Group | HBV-ACLF, *n*=20 | HC, *n*=20 |
|---|---|---|
| Male sex | 18 | 14 |
| Age in years | 35.10±6.03 | 36.6±7.42 |
| WBC as $10^9$/L | 5.88±2.81 | 6.17±1.46 |
| PLT as $10^9$/L | 120.35±64.65 | 240.55±37.03 |
| ALT in U/L | 171.10±214.34 | 19.05±13.55 |
| AST in U/L | 130.65±192.93 | 19±5.53 |
| PTA, % | 32.2±5.03 | N.D. |
| INR | 2.42±0.43 | N.D. |
| Albumin in g/L | 36.96±5.39 | N.D. |
| TBil in μmol/L | 311.48±132.24 | N.D. |
| CR in μmol/L | 63.94±12.16 | 70.23±14.24 |
| Na in μmol/L | 137.10±4.06 | N.D. |
| HBeAg-positive | 4 | 0 |
| Complication | 11 | 0 |
| Ascites | 6 | 0 |
| SBP | 5 | 0 |
| Hepatic encephalopathy | 0 | 0 |
| Hepatorenal syndrome | 0 | 0 |
| UGB | 0 | 0 |
| MELD score | 23.49±3.74 | N.D. |
| MELD-Na score | 20.15±8.36 | N.D. |
| COSSH-ACLF Grade | 1 | N.D. |

Data are shown as means±standard deviations. ACLF, acute-on-chronic liver failure; ALT, alanine aminotransferase; AST, aspartate aminotransferase; COSSH-ACLF, Chinese Group on the Study of Severe Hepatitis B-ACLF; CR, creatinine; HBeAg, hepatitis B e antigen; HC, healthy control; INR, international normalized ratio; MELD, model for end-stage liver disease; N.D., not determined; PLT, platelet; PTA, prothrombin time activity; SBP, spontaneous bacterial peritonitis; TBil, total bilirubin; UGB, upper gastrointestinal bleeding; WBC, white blood cell.

function is extremely important. In the treatment of inflammatory diseases, the therapeutic effect of MSCs is mainly the result of immunomodulation and this function is mediated by the inflammatory microenvironment, which means that these cells have immunoregulatory plasticity. In response to different amounts and kinds of inflammatory mediators, MSCs produce ample amounts of immunoregulatory factors, cell-mobilization factors and growth factors, thereby facilitating tissue repair by tissue-resident stem cells.[8,9] In organismal aging, hormonal, immunologic, and metabolic factors are the critical microenvironmental signals that trigger MSC dysfunction, particularly the shift in differentiation from osteoblasts to adipocytes that occurs following the activation of key signaling pathways, such as intracellular oxidative stress and posttranscriptional regulation.[10] In addition, in systemic sclerosis, patient serum mediates oxidative stress effects on MSC function, such as increasing the apoptosis rate and osteoblastic/adipogenic potential, whereas the immunosuppressive function of MSCs becomes reduced.[11] Although the influence of the pathological ACLF microenvironment on MSCs has never been reported, our previous studies have examined the changes in the functional properties of heterologous umbilical cord (UC)-MSCs exposed to ACLF serum and aimed to simulate the pathological microenvironment *in vitro*, as well as to determine the molecular mechanisms of MSC plasticity.

## Methods

### *Human serum sample collection*

Serum was collected from 20 patients and 20 healthy volunteers, who were included as healthy controls (HCs). The clinical and biological characteristics of the participants are shown in Table 1. The inclusion criterion and the exclusion criteria of ACLF were based on the Asian Pacific Association for the Study of the Liver.[12] The ACLF grade was based on Chinese Group on the Study of Severe Hepatitis B-ACLF.[13] Blood from ACLF patients and healthy volunteers was centrifuged at 2,000 *g* for 15 m and the serum samples were stored at −80°C. The isolation and culture of UC-MSCs were performed according to Good Manufacturing Practice (referred to as GMP) protocols in our GMP laboratory, as previously described.[14] In addition, human peripheral blood mononuclear cells from healthy donors were isolated by Ficoll, according to standard procedures. The samples were obtained with written informed consent from all subjects, in accordance with the Declaration of Helsinki. This study was carried out in accordance with the

recommendations of the ethics committee of our hospital (Third Affiliated Hospital of Sun Yat-sen University, Guangzhou, China).

### *Serum pretreatment scheme for MSCs*

Before analyzing the functional characteristics of MSCs (except for proliferation assay), different concentrations of ACLF patient (AP) sera and HC sera were added to normal MSC culture medium for 48 h. According to the treatment concentrations (10%, 30% and 50%), the groups were named AP10, AP30, AP50, HC10, HC30, and HC50, and the normal cultured MSC group was a blank control.

### *Surface marker expression*

When the MSCs had grown to 80% confluence, the cells from different groups were harvested, and the positive and negative expression of the surface markers (CD14, CD34, CD45, HLA-DR, CD29, CD44, CD105 and CD166; all relevant monoclonal antibodies were purchased from Biolegend, San Diego, CA, USA) was examined. The staining was carried out according to the manufacturer's instructions, and the data were analyzed with Kaluza software (Beckman Coulter, Brea, CA, USA) and FlowJo 7.5 (Treestar, Ashland, OR, USA).

### *Proliferation assay*

MSCs were plated at 2,000 cells/well in 96-well plates and then treated with different serum concentrations from ACLF patients and HC volunteers. Ten microliters of cell counting kit 8 (CCK-8) solution (Dojindo, Kumamoto, Japan) was added at the indicated time points on days 1, 3, 5, and 7. Proliferation was measured using the IncuCyte HD imaging system (Essen BioScience, Tokyo, Japan) by measuring the optical density value at 450 nm. The results are expressed as the percentage of proliferation±standard error of the mean and normalized to 100% as the initial number of cells plated.

### *Apoptosis assay*

MSCs were plated at $1\times10^6$ cells/well in six-well plates. After adding different concentrations of serum to stimulate the cells for 48 h, the number of apoptotic cells was evaluated by annexin V and propidium iodide (BD Biosciences, Franklin Lakes, NJ, USA) labeling, according to the manufacturer's instructions. The labeled cells were analyzed using Kaluza software (Beckman Coulter) and FlowJo 7.5 (Treestar). The results are expressed as the percentage of annexin V+ cells.

### *T lymphocyte proliferation assay*

For cell sorting, peripheral blood mononuclear cells were stained with human CD3 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany), according to the manufacturer's instructions, and then sorted by a Miltenyi magnetic bead sorter to harvest CD3+ T cells. The purified CD3+ T cells were stained with 5 mM 5-(and-6)-carboxyfluorescein diacetate succinimidyl ester (Cell Trace; Invitrogen, Carlsbad, CA, USA). MSCs ($1\times10^5$ cells/well) were seeded in 24-well flat-bottom plates and incubated for 24 h. After serum pretreatment, T cells were added at a MSC/T cell ratio of 1:10 and were cocultured with MSCs for 5 days in RPMI-1640 containing 10% (v/v) fetal bovine serum, 50 U/mL penicillin and 50 U/mL streptomycin; a 5 μg/mL aliquot of phytohemagglutinin (Sigma-Aldrich, St. Louis, MO, USA) was added to activate T cell proliferation. The results are expressed as the percentage of proliferation±standard error of the mean.

### *RNA sequencing*

The total RNA from MSCs, which had been pretreated with serum from each group, were isolated by Trizol (Invitrogen), following the manufacturer's protocol. The transcripts were sequenced using the BGISEQ-500 sequencing platform (BGI Tech Company, Guangdong, China). Essentially, differential expression analysis was performed using the DESeq2 (v1.4.5) with Q value <0.05. To obtain insight into the change of phenotype, Gene Ontology (GO; http://www.geneontology.org/) and Kyoto Encyclopedia of Genes and Genomes (KEGG; https://www.kegg.jp/) and Gene Set Enrichment Analysis (GSEA) of annotated different expression gene was performed by Phyper (https://en.wikipedia.org/wiki/Hypergeometric_distribution) based on hypergeometric test. The significant levels of terms and pathways were corrected by Q value with a rigorous threshold (Q value <0.05) by Bonferroni. The protein-protein interaction (PPI) analysis was conducted by DIAMOND and STRING. Key driver analysis (KDA) was performed according to Tran's methods. All the analyses were conducted with the online bioinformatic platform Dr. Tom (biosys.bgi.com/) provided by BGI.[15]

### *Statistical analysis*

The results are presented as the means±standard deviations of the independent experiments. Comparisons were made using a two-tailed *t*-tests (between two groups), one-way ANOVA (for multigroup comparisons) or Kruskal-Wallis and Mann-Whitney *U* tests (for nonnormally distributed data). A $p$ value of <0.05 was considered to represent a significant difference. The statistical analyses were performed using SPSS v. 22.0 (IBM Corp., Armonk, NY, USA). Graphing was performed using Prism 6.01 software (GraphPad, San Diego, CA, USA).

## Results

### *ACLF serum pretreatment significantly changed MSC morphology but not phenotype*

First, we observed the morphology of MSCs under the microscope after serum pretreatment and then detected their phenotypes. Microscopic analysis (Fig. 1A) showed that the cells were no longer arranged in a spindle-like manner in the AP groups, as compared with that of the MSC group in normal culture conditions and the HC group. With increasing serum concentrations in the AP groups, the gaps between cells became larger and many coarse particles appeared around the nuclei. In the HC group, when the serum concentration increased to 50%, the cell morphology also changed slightly. Second, we analyzed the phenotypes of MSCs treated with different types of serum and found no large changes (Fig. 1B). It suggested that when MSCs entered patients with ACLF, the cells might be adversely affected, although the phenotypes

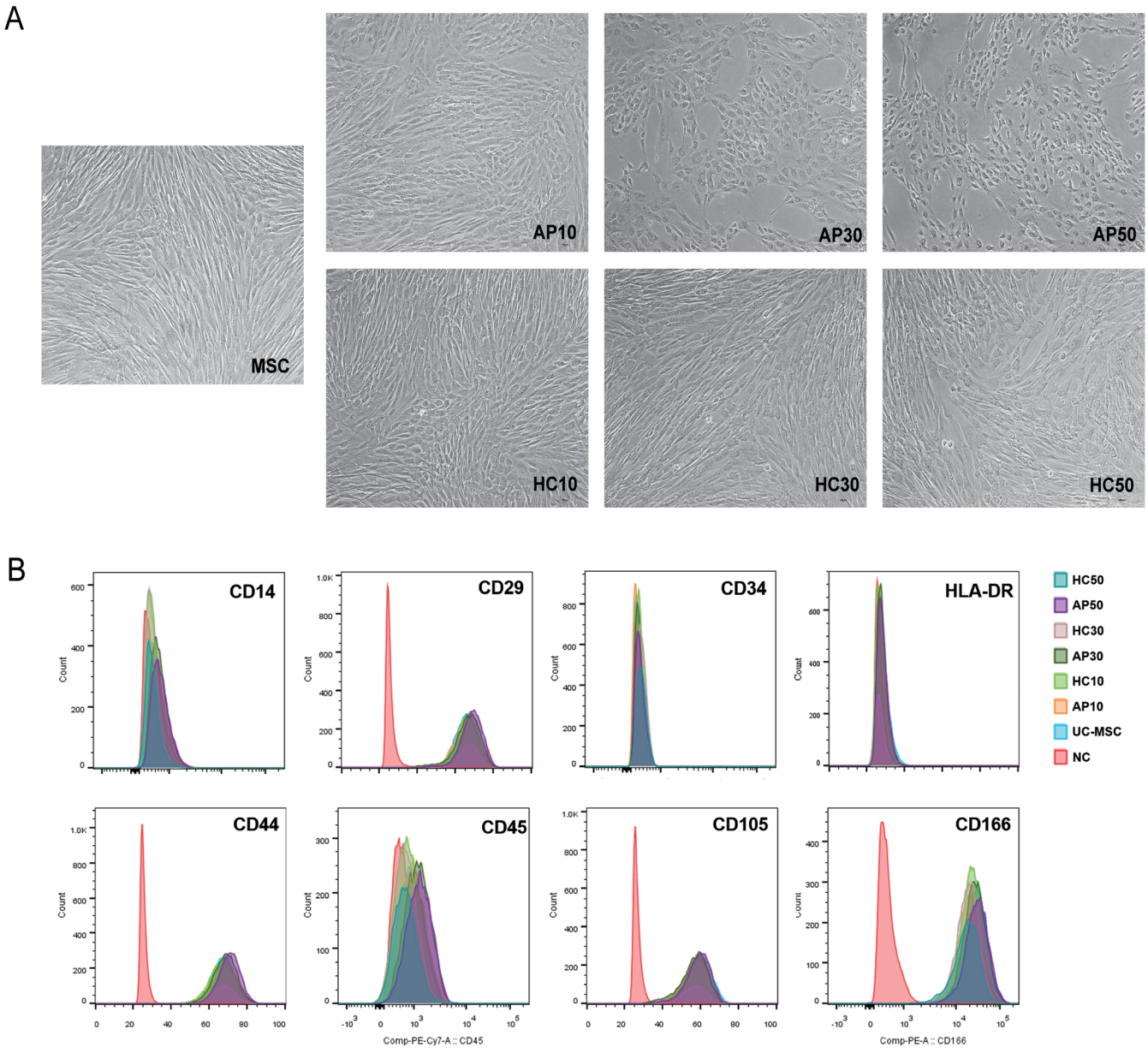


**Fig. 1. ACLF serum pretreatment significantly changed MSC morphology but not phenotype.** (A) Representative photographs of MSCs cultured after serum treatment for 48 h in different groups. Scale bars, 200 μm. (B) UC-MSC phenotype showed no differences after serum pretreatment among groups. Flow cytometry analysis showed that the cultured UC-MSCs were positive for CD29, CD105, CD166 and CD44 but negative for CD34, CD45, CD14, CD164 and HLA-DR. ACLF, acute-on-chronic liver failure; AP, ACLF patient; HC, healthy control; MSCs, mesenchymal stem cells.

were still maintained.

### *ACLF serum dose-dependently decreased the proliferation rate of MSCs but significantly induced apoptosis only at 50% concentration*

Since ACLF serum pretreatment significantly changed the morphology of MSCs, we wondered whether the proliferation and apoptosis of MSCs would be affected. Therefore, we evaluated the proliferation rate of MSCs cultured for 7 days and the apoptosis level after serum pretreatment. In AP groups, only 10% serum significantly reduced MSC proliferation, as compared to that of the MSC group and HC group (Fig. 2A). In addition, as the serum concentration increased, the MSC proliferation rate remained sluggish (Fig. 2B). However, we found that it did not cause obvious apoptosis, regardless of whether the serum was from HC donors or ACLF patients, when the pretreatment concentration was 10% (Fig. 2C–D). In the AP group, only when the serum pretreatment concentration was 50%, the percentage of apoptotic MSCs increased. To our surprise, with increase in serum concentration in the HC group, the proportion of early and late apoptotic cells increased, and there was a significant difference (Fig. 2D). Altogether, the data indicated that there might be certain harmful substances existing in the serum of ACLF patients, which could inhibit the proliferation of MSCs

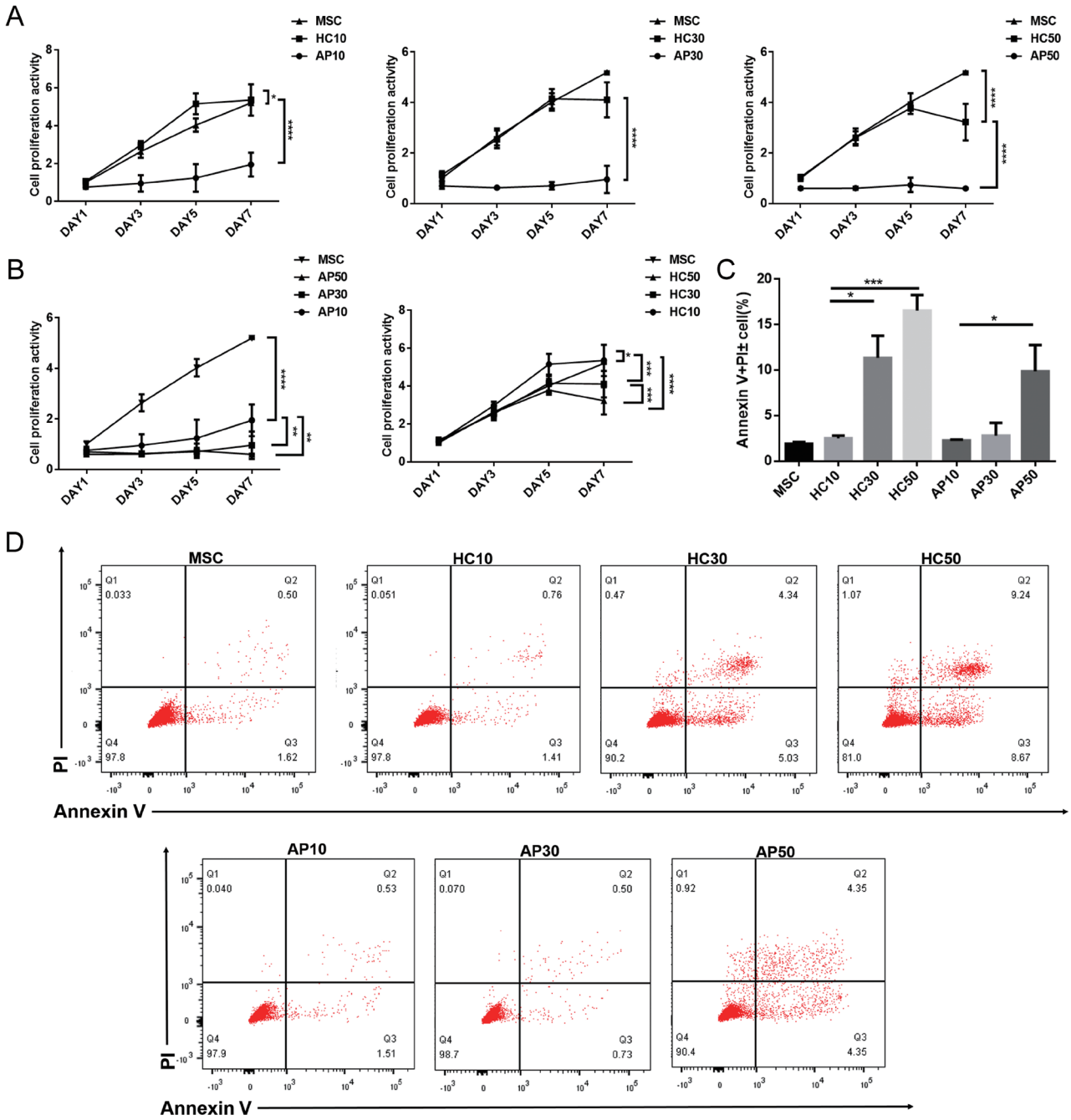


**Fig. 2. Serum pretreatment obviously decreased MSC proliferation in the AP group and increased apoptosis only in the AP50 group.** (A) MSC proliferation in the AP group compared with the MSC group and HC group at the same serum pretreatment concentration. (B) In the AP group or HC group, MSC proliferation activity decreased compared with that of the MSC group after pretreatment with different serum concentrations. The data were normalized to MSCs plated on day 1 without serum treatment. (C) Percentage of annexin V+ apoptotic MSCs in each group. (D) Proportion of early and late apoptotic cells in different groups. The graphs indicate the means±standard deviations, with statistically significant differences indicated as follows: *$p$<0.05, **$p$<0.01, ***$p$<0.001, ****$p$<0.0001; $n$=5. AP, ACLF patient; HC, healthy control; MSCs, mesenchymal stem cells.

and induce apoptosis when accumulated to a certain degree.

### *ACLF serum dose-dependently regulated the immunosuppressive effects of MSCs*

In cell transplantation therapy, the immunomodulatory effect of MSCs is extremely important for the improvement of liver failure. Thus, we investigated the immunosuppressive potential of MSCs after serum pretreatment in each group (Fig. 3). The sorted CD3+ T lymphocytes cultured alone proliferated after adding stimulants, such as phytohemagglutinin, while MSCs in normal culture inhibited the proliferation of these activated T cells to exert an immuno-

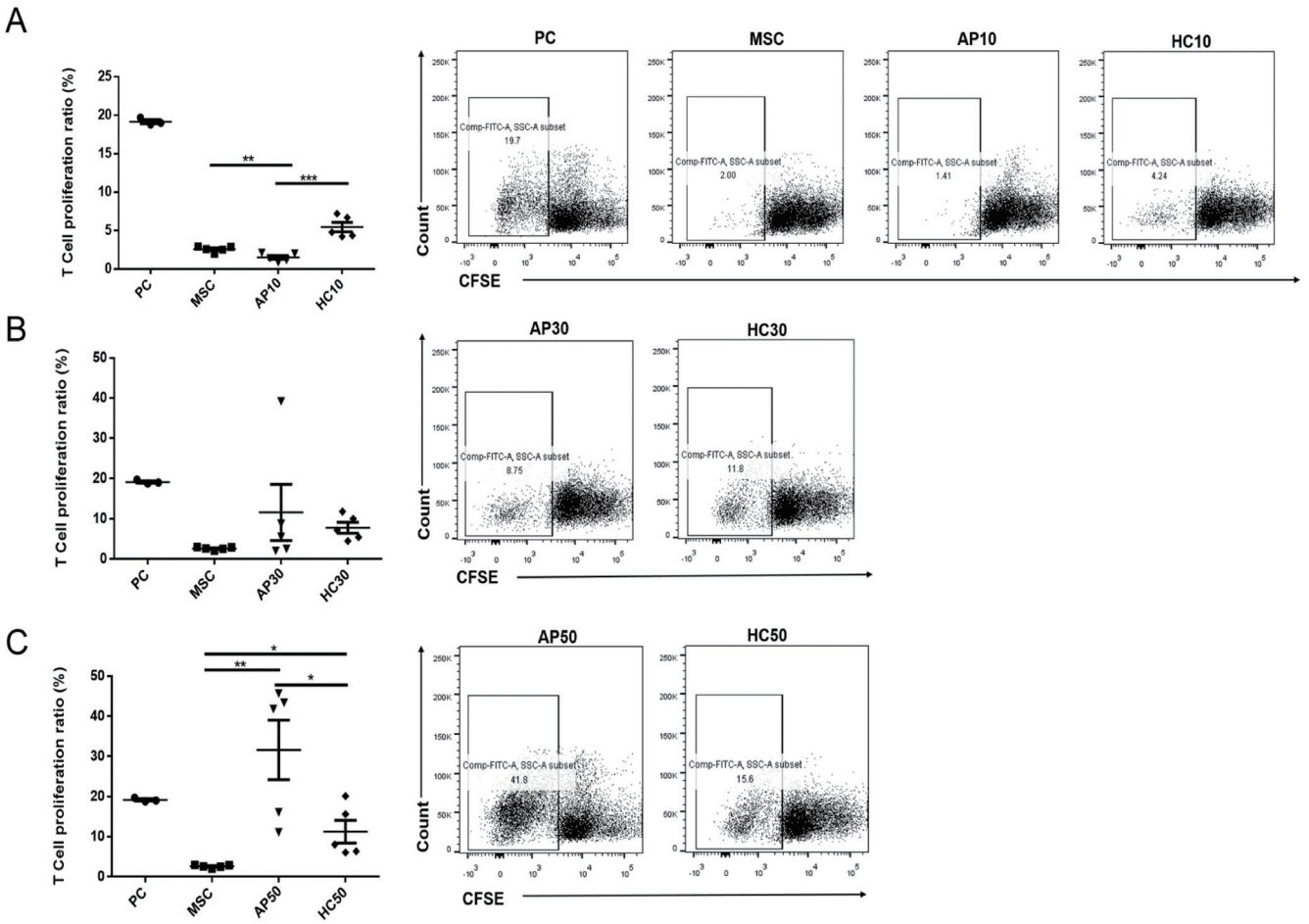


**Fig. 3. MSCs exerted a stronger anti-inflammatory effect under ACLF serum pretreatment at 10% concentration but transformed into proinflammatory function at 50% concentration.** The percentage of T lymphocyte proliferation after culture alone as the positive control (PC) or culturing at a 1:10 MSC:T lymphocyte ratio with MSCs that were pretreated for 48 h with human serum from each group. (A) MSCs from the AP10 group markedly inhibited the proliferation and activation of naive CD3+ T cells compared with those from the MSC group and HC group. (B–C) MSCs from the AP group increased the proliferation and activation of naive CD3+ T cells compared with those from the MSC group and HC group when the serum concentration was increased to 30% and 50%. The bar graphs indicate the means±standard deviations, statistically significant differences are indicated as follows: *$p$<0.05, **$p$<0.01, ***$p$<0.001; $n$=5. AP, ACLF patient; HC, healthy control; MSCs, mesenchymal stem cells.

suppressive effect. When the serum pretreatment concentration was 10%, the proliferation rate of T cells in the AP10 group was the least, which indicated MSCs in this group exhibited significantly enhanced anti-inflammatory effects, while the effect on MSCs in the HC group was not significantly different (Fig. 3A). When the serum concentration was 30%, MSCs did not exhibit stronger immunosuppressive potential, even though the anti-inflammatory effect of MSCs shifted to a proinflammatory effect when the concentration was 50%; although, there may have been individual differences (Fig. 3B–C). Overall, MSCs that were pretreated with 50% ACLF serum promoted the proliferation of activated T lymphocytes. All the data suggest that the pathological ACLF microenvironment may have an adverse effect on the infused MSCs. After all, the 50% serum concentration *in vitro* is closer to the environment in the human body.

### *Molecular pathways by which ACLF serum enhanced MSC immunosuppressive functions in the AP10 group*

In order to investigate the mechanism by which ACLF serum pretreatment at 10% concentration enhanced the immunosuppressive function of MSCs and the key driver genes, thereby suggesting some upstream molecules, we collected samples of serum-treated MSCs and performed mRNA sequencing analysis according to the experiment workflow (Fig. 4). The volcano maps show the differentially expressed genes (DEGs) between the AP10 group and HC10 group (Fig. 5A). Then, 1,221 up-regulated genes and 1,641 down-regulated genes were analyzed by KEGG, GO and GSEA to identify the pathways of interest (the screening conditions were log2-fold change (referred to herein as log2FC) >1 and Q value <0.05). Therefore, in the KEGG analysis of down-regulated genes (Fig. 5B), we selected immune-related pathways (shown in red boxes) and performed PPI network analysis on the DEGs involved in these pathways (Fig. 5C). PPI analysis indicated that the proteins expressed by the genes in the blue circle affected each other, which were more likely to be in the same pathway. Moreover, KDA was used to screen the key genes (denoted by the black arrow) that affected these pathways (Fig. 5D). In addition, the expression cluster heatmap clearly showed the differences in the expression of these core genes in each group (Fig. 5E). Furthermore, we performed re-enrichment of these KDA

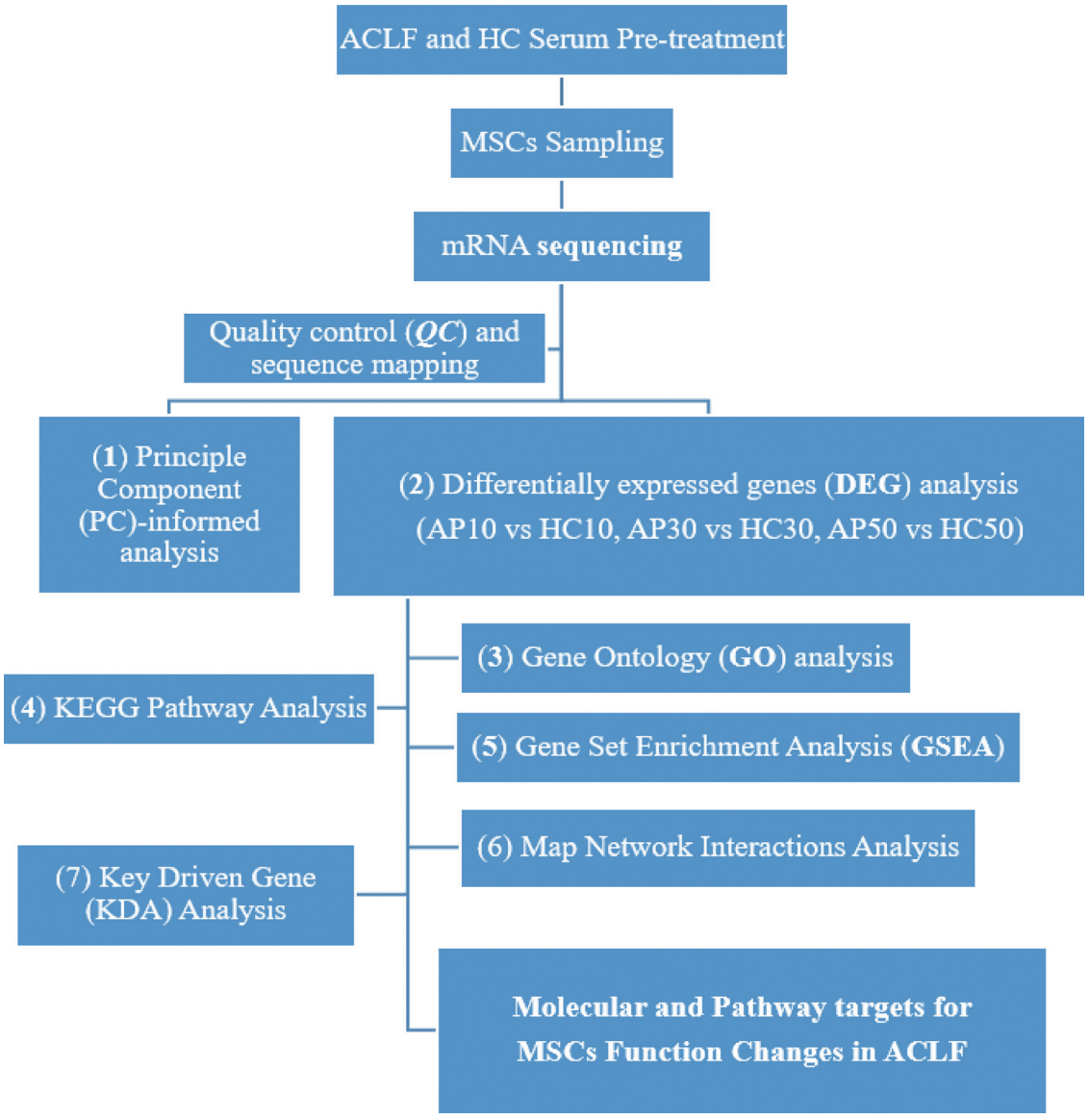


**Fig. 4. RNA sequencing experiment workflow.** The workflow of serum pretreatment, MSC sample collection, mRNA sequencing data analysis and interpretation is presented. More information on the detailed methods is provided in the Materials and Methods section. Since the AP10 group and AP50 group exhibited significantly different effects on the immunoregulatory function of MSCs, subsequent sequencing analysis should focus on these two groups. AP, ACLF patient; MSCs, mesenchymal stem cells.

genes and found that the PI3K-Akt signaling pathway might play a major role in the serum-mediated MSCs' exerting stronger immunosuppressive function.

### *Molecular pathways by which serum transformed MSCs into proinflammatory cells in the AP50 group*

According to the same analysis workflow, we performed GO enrichment analysis of up-regulated DEGs between the AP50 group and HC50 group (Fig. 6A). In fact, we found that immune-related pathways were rarely enriched in various enrichment analyses. However, the pathways related to cell cycle, cell division, cell proliferation and apoptotic process were significantly enriched. For the DEGs involved in these pathways, we also performed PPI analysis and KDA. Similarly, the expression cluster heatmap (Fig. 6B) and the histogram based on Fragments per kilobase of exon model per million mapped fragments (FPKM) (Fig. 6C) clearly showed the differences in the expression of these core genes between the AP50 group and HC50 group. The re-enrichment results of the KDA genes (Fig. 6D) suggested that ACLF serum at 50% concentration might affect the cell cycle and threaten the basic metabolic activities, leading to the transformation of MSCs into a proinflammatory function.

## Discussion

Our research idea was originally derived from the longitudinal comparison of two clinical studies of the use of MSCs in the treatment of ACLF, which were performed in our depart-

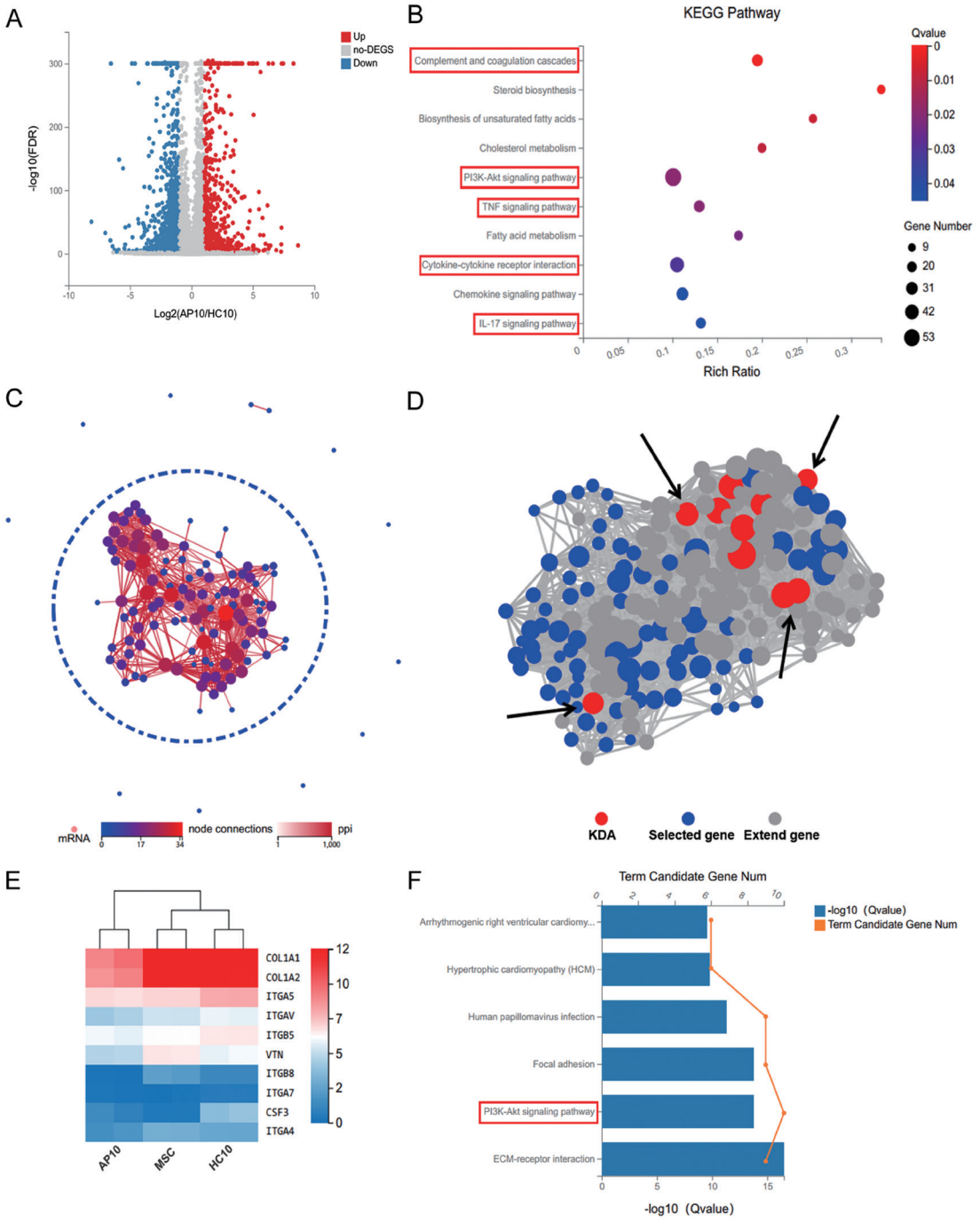


**Fig. 5. Molecular pathways by which ACLF serum enhanced the immunosuppressive effects of MSCs in the AP10 group compared with the HC10 group.** (A) The DEGs between the AP10 group and HC10 group are shown by volcano plots. The X-axis represents the difference multiplied after the log2 conversion, and the Y-axis represents the significance value after the log10 conversion. The red dots represent up-regulated DEGs, the blue dots represent down-regulated DEGs, and the gray dots represents non-DEGs. log2FC≥1 and Q value < 0.05. The Q value is the calibration value of the P value. (B) Bubble diagram showing KEGG pathway enrichment analysis of down-regulated DEGs. The X-axis is the enrichment ratio and the Y-axis is the KEGG pathway. The size of each bubble represents the number of genes annotated to the KEGG pathway, while the color represents the enrichment Q value and darker color represents smaller Q values. The red box encloses the pathways of interest. (C) PPI map showing how genes in our pathway of interest interact, are coexpressed or regulate relationships. Each circle represents a gene; the larger the circle, the more highly the gene is connected to other genes. The line represents the interaction among genes. (D) KDA showed which genes are major regulators in the PPI map (denoted by the black arrow). (E) Expression cluster analysis was conducted on the FPKM value among the AP10 group, HC10 group and MSC group, to show 10 KDA genes. The thermogram showing the log2 (FPKM+1) of the sample, which is represented by the horizontal axis, and the gene is represented by the vertical axis. Under default color-matching, the redder the color of the block, the higher the expression level, and the bluer the color, the lower the expression level. (F) Histogram diagram showing the KEGG re-enrichment of KDA genes between AP10 group and HC10 group. Y-axis represents the KEGG enrichment pathway; the bar chart length represents the number of genes annotated to a KEGG pathway. ACLF, acute-on-chronic liver failure; AP, ACLF patient; DEGs, differentially expressed genes; KDA, Key driver analysis; KEGG, Kyoto Encyclopedia of Genes and Genomes; MSCs, mesenchymal stem cells; PPI, protein-protein interaction.

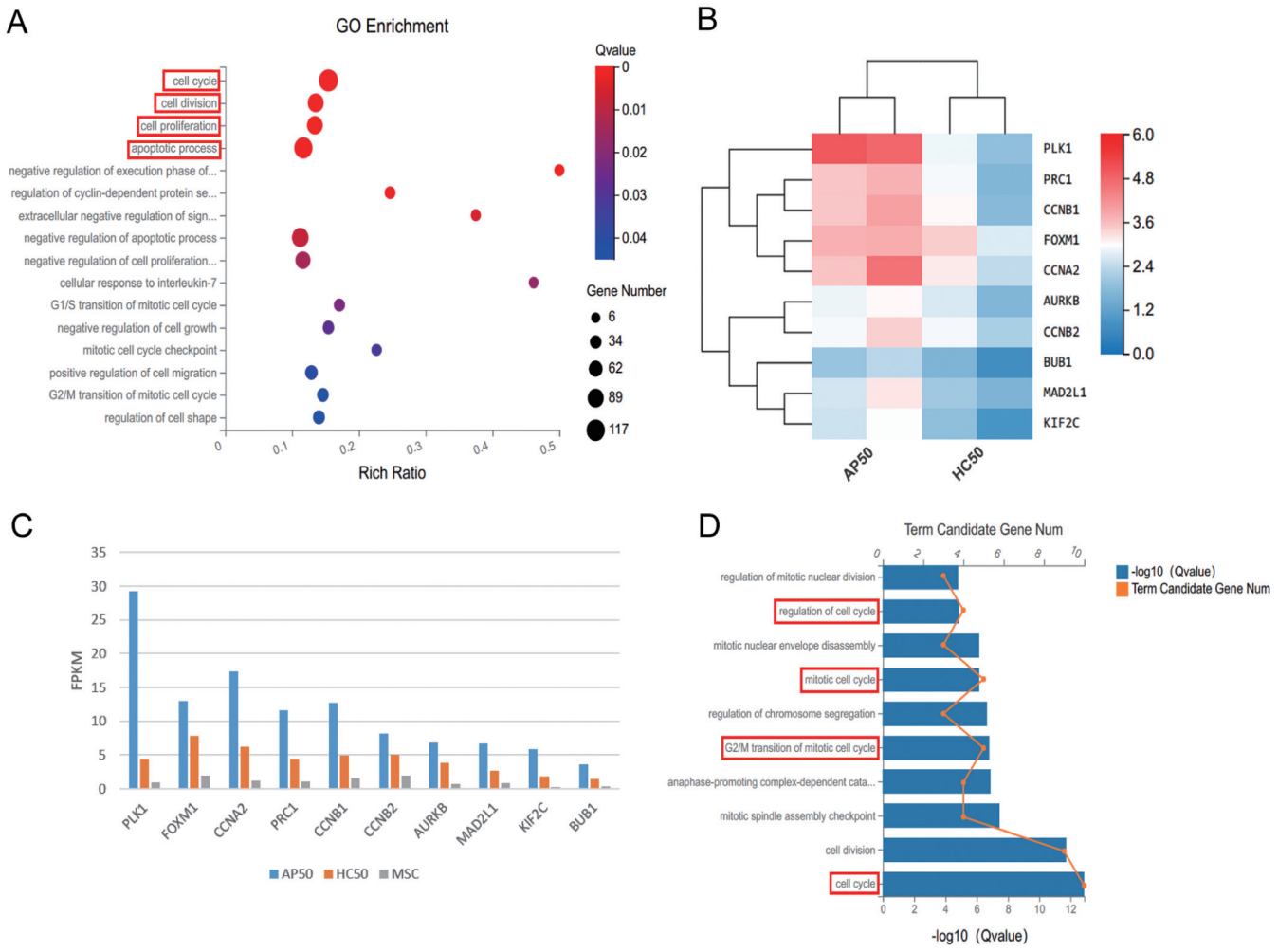


**Fig. 6. Molecular pathways target how ACLF serum transformed MSCs into a proinflammatory effect in the AP50 group compared with the HC50 group, following the same sequencing analysis workflow.** (A) Bubble diagram showing GO enrichment analysis of up-regulated DEGs. The red boxes enclose pathways of interest that are significantly enriched. (B–C) The expression cluster heatmap and the histogram based on FPKM of 10 KDA genes after PPI analysis and KDA. (D) The GO re-enrichment results of KDA genes between AP50 group and HC50 group. ACLF, acute-on-chronic liver failure; AP, ACLF patient; DEGs, differentially expressed genes; GO, Gene Ontology; HC, healthy control; KDA, Key driver analysis; MSCs, mesenchymal stem cells.

ment in 2011 and 2017 respectively.[3,4] There were many differences, such as the cell source, generation, infusion volume and infusion methods, between the two studies. Nonetheless, we were concerned about why a small group of patients did not respond well to MSC treatment. Moreover, it was reported that the infusion of MSCs via a peripheral vein did not rescue acute liver failure pigs, while most of the acute liver failure pigs survived more than 6 months after the transplantation of MSCs via the portal vein.[16,17] We wondered whether MSCs were more susceptible to the adverse effects of the pathological microenvironment in the body when they were administered via intravenous infusion compared with *in situ* infusion, rendering these cells unable to exert a beneficial therapeutic effect. Thus, this study aimed to investigate MSC properties in the specific context of allogeneic transplantation and the molecular and mechanism pathways that affect the plasticity of MSCs.

In this study, we observed that pretreatment of MSCs with ACLF serum reduced proliferation but did not obviously increase the level of apoptosis at 10% concentration. Besides, the immunosuppressive function of these cells was significantly enhanced at 10% concentration, while becoming shifted to a proinflammatory state at 50% concentration. In another study, Fonteneau *et al*.[11] found that in the oxidative environment of systemic sclerosis patient serum, MSCs retained their proliferative potential, with increased apoptosis rate occurring at day 10. In addition, the immunosuppressive function of these cells was slightly decreased. Although systemic sclerosis and ACLF have different disease backgrounds, the phenomena we observed in terms of proliferation and apoptosis were the same, while the immunosuppressive functions were not exactly the same. Moreover, several studies have reported that apoptotic cells could modulate immune responses.[18–20] Galleu *et al*.[21] used a murine model of graft-versus-host disease to demonstrate that MSCs were actively induced to undergo perforin-independent apoptosis by recipient cytotoxic cells and that this process was essential for initiating MSC-induced immunosuppression. In addition, it was reported that MSCs could be shifted from a suppressive to supportive phenotype when exposed to defective immune cells, since MSCs are very sensitive to their environment. This immune activating effect may be due to MSC prestimulation.[22]

In this study, although ACLF serum pretreatment concen-

tration of 50% caused obvious apoptosis, the MSCs did not exert a stronger immunosuppressive effect. According to the mRNA sequencing results of the AP50 group, the pathways related to cell cycle, cell division, cell proliferation and apoptotic process were significantly enriched. We have to suspect that some unfavorable factors in the serum seriously affect the basic metabolism of MSCs, and even exceed the role of some inflammatory factors that can empower MSCs, causing MSCs to exhibit proinflammatory effects. Interestingly, the proliferative activity of MSCs pretreated with HC serum was not significantly weakened, whereas the level of apoptosis was significantly increased and the immunosuppressive function was not significantly enhanced. We wondered whether when the apoptosis rate exceeded a certain threshold or if the lack of some inflammatory factors that stimulate MSCs in HC serum prevented pretreatment with healthy donor serum from enhancing the immunosuppressive function of MSCs.

Human serum accounts for approximately 50% of the total blood volume. Compared with ACLF serum pretreatment of 10% concentration, the 50% concentration may be more similar to the environment in the human body. Thus, MSCs enter the pathological ACLF microenvironment and may be negatively affected and unable to exert a beneficial therapeutic effect. However, combining treatments of plasma exchange or double plasma molecular adsorption system to reduce harmful substances in serum may promote MSCs to exert a stronger anti-inflammatory effect. According to our mRNA sequencing results of the MSCs pretreated with ACLF serum at 10% concentration, the PI3K-Akt signaling pathway might play a major role in the serum-mediated MSCs exerting stronger immunosuppressive function. As we know, the PI3K-Akt pathway is indispensable in immunologic defense mechanisms and acts in part as a compensatory mechanism in response to the activation of intracellular proinflammatory signaling pathways.[23–25] We suspected that ACLF serum pretreatment may down-regulate the PI3K-Akt pathway, thereby stimulating cascade reactions and driving MSCs to exert stronger immunoregulatory effects.

Our study also has many shortcomings. First, our experimental design cannot fully simulate the internal environment of ACLF, since it is an extremely complex disease condition. Indeed, no specific factor can play a completely different role in the immunomodulation of MSCs at different concentrations. Second, we did not verify the mRNA sequencing results by measuring transcription or protein levels. Considering that simple verification can only show that the corresponding pathway was affected, it cannot indicate whether the affected pathway is the main reason for the alteration in MSC functional characteristics. Therefore, we plan to explore the importance of the KDA genes in the involved pathways and their influence on MSCs in subsequent experiments.

Presently, most clinical studies on MSC treatment of diseases generally use intravenous infusion due to safety considerations. However, this also means that compared to the short and direct infusion route of *in situ* infusion, MSCs are likely to be affected by the environment during the lengthy internal circulation. To determine which substances in the serum would adversely affect the MSCs and filter out these harmful substances by plasma exchange or double plasma molecular adsorption system may further improve the efficiency of MSCs transplantation as well as suggest the reasons for the poor response of some patients to treatment. The above is also the content of our subsequent research.

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## Conflict of interest

The authors have no conflict of interests related to this publication.

## Author contributions

Design of the study (YZ, CX, LP), acquisition of data (YZ, SZ, XZ, WX, XL, JL), data analysis (YZ, SZ), manuscript preparation (YZ, SZ, CX, LP). All authors reviewed the manuscript and gave final approval for the work.

## Data sharing statement

No additional data are available.

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