

Potential roles of aromatic hydrocarbon receptors and tryptophan metabolism in alleviating liver inflammation and immune imbalance in ACLF rats

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Article

Keywords: Tryptophan metabolism, Aryl hydrocarbon receptors, Acute-on-chronic liver failure model, Th17/Treg balance

Posted Date: October 5th, 2026

DOI: <https://doi.org/10.21203/rs.3.rs-11096461/v1>

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Additional Declarations: No competing interests reported.

Abstract

BACKGROUND

The imbalance between immunity and inflammation plays a crucial role in the progression of ACLF, and the value of the tryptophan-AhR pathway, which has significant regulatory effects, remains unclear.

AIM

To investigate the regulatory role and underlying mechanism of tryptophan metabolism-activated aryl hydrocarbon receptor (AhR) signaling in liver inflammation and immunity during acute-on-chronic liver failure (ACLF).

METHODS

An ACLF Wistar rat model was established. Rats were treated with intraperitoneal injection of 1-MT (IDO inhibitor) (3 mg/kg) or gavage administration of Leflunomide (AhR agonist) (10 mg/kg). Serum liver function parameters were measured using a biochemical analyzer; liver histopathological damage was observed using HE staining and Masson staining; serum tryptophan and its metabolites were detected using targeted metabolomic assays; the proportion of Treg and Th17 cells in liver was detected by flow cytometry; CD4 and SOCS3 co-expression in liver and lymphoid tissue were detected using fluorescence double staining; and AhR expression level in liver was detected by immunohistochemistry.

RESULTS

Compared with normal rats, ACLF rats exhibited impaired liver function, significant pathological damage in liver tissue, an increased Th17/Treg ratio, and decreased AhR and SOCS3 expression. Intervention with IDO inhibitor exacerbated these changes, while intervention with AhR agonist attenuated them. In addition, targeted metabolomics analysis in each group showed that differentially expressed metabolites were concentrated in the tryptophan metabolic pathway.

CONCLUSION

IDO inhibitors can aggravate liver injury and inflammatory response in ACLF rats. Meanwhile, AhR agonists are associated with the increase in SOCS3 expression and can affect the Th17/Treg balance, inhibiting the inflammatory response, thereby alleviating the inflammation and immune imbalance in ACLF rats.

Introduction

Acute-on-chronic liver failure (ACLF) is a severe clinical syndrome that occurs on the basis of chronic liver disease. Its core pathological feature is extensive hepatocellular necrosis, mainly manifested as acute jaundice and coagulation dysfunction, and often complicated by multiple organ dysfunction syndrome^[1]. Its short-term mortality rate can reach up to 74%^[2–4], which is the main cause of death in patients with cirrhosis^[4, 5]. At present, the specific mechanism leading to the occurrence of ACLF has not been fully elucidated, and there are no drugs or interventions that can effectively control its development. Therefore, elucidating the pathogenesis of ACLF and finding new therapeutic drugs have become the top priorities of research.

The main characteristic of ACLF is single or multiple organ failure^[6], and systemic inflammatory response and oxidative stress play a key role in this process^[7]. Studies have found that ACLF patients will have a systemic inflammatory response^[8], which can further aggravate ACLF^[9], and the inflammatory response is one of the core mechanisms of ACLF development. Systemic inflammatory response not only affects metabolic processes, but is also related to changes in the metabolism of various bioactive small molecules. Among them, the process of tryptophan being broken down into tryptophan-kynurenine derivatives through the kynurenine metabolic pathway has attracted much attention in various liver diseases^[10].

In a systemic inflammatory response, tryptophan metabolites kynurenine and kynuric acid bind to aryl hydrocarbon receptors (AhR) and G protein-coupled receptor 35 in immune cells, exerting immunomodulatory effects^[11]. In recent years, multiple studies have suggested that tryptophan metabolism plays an important role in the pathological process of ACLF. For example, clinical observations have found that after receiving plasma exchange therapy, the levels of phenylalanine and tryptophan metabolism-related products in ACLF patients increased with the duration of treatment^[12]. In addition, in patients with hepatitis B virus-associated ACLF, key nodes of the tryptophan metabolic pathway and cofactor biosynthesis network underwent significant remodeling^[13]. Therefore, tryptophan metabolism disorder may be involved in the occurrence and development of ACLF.

In this study, we investigated the effects of tryptophan metabolism on ACLF and its underlying mechanisms by constructing an ACLF rat model and intervening it with a tryptophan metabolism inhibitor or activator. The aim of this study is to investigate the regulatory role and mechanism of tryptophan metabolism-activated AhR signaling in liver inflammation and immunity during ACLF.

Materials and methods

1.1 Experimental animals and grouping

Twenty-five 8-week-old male SPF-grade Wistar rats (Sibef (Beijing) Biotechnology Co., Ltd., License No.: SCXK (Beijing) 2024-0001) were housed in an SPF-level animal room with a temperature of 20–26 °C, a relative humidity of 40%–70%, and a 12 h light/12 h dark cycle. They had free access to food and water

during this period. The sample size was determined based on the results of the previous pre-experiment and literature reports. According to the differences in the main outcome indicators (serum ALT, TBIL) between the ACLF model group and the normal control group in the pre-experiment, while strictly following the 3R principles (replacement, reduction, optimization), it was finally determined that each group would have 5 rats, totaling 5 groups of 25 rats. After 7 days of adaptive feeding, the 25 rats were randomly divided into 5 groups (5 rats per group, 1 rat per cage, 5 cages per group) using a computer-generated random number table: 1) Normal control group, 2) Acute-on-chronic liver failure (ACLF) group, 3) ACLF + IDO inhibitor group, 4) ACLF + AhR agonist group, 5) ACLF + IDO inhibitor + AhR agonist group. The random allocation sequence was generated and sealed by researchers who were not involved in the experimental operation and data analysis. 1-MT solution was prepared using PBS, and Leflunomide solution was prepared using the solvent (10% DMSO + 40% PEG300 + 5% Tween-80 + 45% saline). LPS and D-gal were prepared using physiological saline. For the 2nd, 3rd, 4th, and 5th groups of rats, intraperitoneal injection of porcine serum (20241019, Solarbio) was performed (0.5 mL per rat), with a duration of 11 weeks, twice a week. Chronic liver failure was constructed. The 3rd group was intraperitoneally injected with IDO inhibitor L-1-MT (S26893-1g, Shanghai Youye) at a dose of 3 mg/kg; the 4th group was given AhR agonist Leflunomide (H24J9Z66497, Shanghai Youye) at a dose of 10 mg/kg; the 5th group received both 1-MT and Leflunomide intervention, with the control group and experimental group receiving the same volume of physiological saline, for 1 week, once a day. After 30 minutes of tail vein injection of 50 µg/mL LPS (1001164401, Sigma-Aldrich) solution (50 µg/kg), 60 mg/mL D-gal (JS260131, Shanghai Youye) solution was intraperitoneally injected at a dose of 10 mL/kg for 5 hours after the acute attack. 5 hours after the acute attack, the rats were anesthetized by intravenous injection of Shaitai (12.5 mg/kg) combined with Suimianxin (6.25 mg/kg), and after the rats completely lost the righting reflex and pain reflex, blood was drawn from the abdominal aorta for euthanasia. Subsequently, tissue sampling was performed. During the experiment, observations and records were made at least twice a day (9:00 a.m. and 3:00 p.m.), including general condition (mental state, coat, posture), activity level, diet and water intake, weight changes (weighing twice a week), and any abnormal secretions or bleeding. During the chronic liver injury modeling period (11 weeks), liver dysfunction manifestations such as ascites and jaundice were observed weekly. The predetermined humane endpoint: If any of the following conditions occurred during the experiment, euthanasia would be carried out prematurely: weight loss: rapid loss of the original weight by 15–20%; or growing animals without weight gain continuously; without monitoring weight but the animals showed cachexia and continuous muscle wasting. Loss of appetite: small rodents did not eat for 24–36 hours. Weakness: Unable to feed or drink by oneself. Personnel need to first determine whether it is the recovery period after anesthesia, and then assess whether the animal is weak due to diseases or experimental factors. Organ infections: Presenting physical indicators and abnormal blood test values, showing no good response to drug treatment and continuously evolving into systemic diseases. Others: Organ failure, no response to treatment, or assessed by the institution's veterinarian as having an extremely poor prognosis. This study adopts a single-blind design. During the modeling, administration, and sample collection stages, since the administration schemes for each group are different (intraperitoneal injection, gavage), the operators cannot be blinded. However, in the subsequent index detection stages

(including serum biochemical testing, liver tissue pathological scoring, immunostaining analysis, etc.), all the testing personnel are blinded to the group information. All the above experimental operations were approved by the Experimental Animal Ethics Committee of Jiangxi Zhonghong Bohuangyuan Biotechnology Co., Ltd. (Approval Number: LL-202411110002).

1.2 Immunofluorescence double staining

After baking, dewaxing, and hydration, spleen, liver, and lymphoid tissue sections were subjected to antigen retrieval using citrate buffer. The sections were then transferred to a humidified chamber and incubated at room temperature for 25 min with 3% hydrogen peroxide to remove endogenous peroxidase. After blocking with 5% BSA at 37°C for 30 min, primary antibody rabbit anti-CD4 (1:200, BS-0766R, BIOSS) was added to the slides, and incubated overnight at 4°C. At the following day, after the slides were brought to room temperature, they were washed with PBS and horseradish enzyme-labeled goat anti-rabbit IgG (H + L) (1:200, ZB-2301, ZSGB-Bio) was added. The slides were incubated at room temperature for 50 min, washed with PBS, and TSA (1:1500, G1223, Servicebio) was added. The slides were incubated at room temperature in the dark for 10 min. The tissue sections were placed in a slide holder, and antigen retrieval solution was added for heat retrieval. The sections were then transferred to a humidified chamber, and 0.5% Triton X-100 was added for permeation at room temperature for 10 min. After blocking with 5% BSA at 37°C for 30 min, primary antibody against rabbit anti-SOCS3 (1:100, ABclonal, A0694) was added to the slides, and incubated overnight at 4°C. The following day, after the slides were brought to room temperature, they were washed with PBS, goat anti-rabbit IgG/488 (1:100, ZF-0511, ZSGB-Bio) was added, and the slides were incubated at 37°C for 45 min. Then the slides were incubated with DAPI in the dark for 2 min, rinsed with tap water for 1 min, mounted on a slide containing antifade reagent, and observed under a fluorescence microscope (BX53, OLYMPUS). For each sample, three sections were collected and three non-overlapping fields of view were obtained for each section. The average fluorescence intensity of CD4 + and SOCS3 + staining was analyzed using ImageJ software. All quantitative analyses were independently completed by researchers who were unaware of the experimental groups.

1.3 Biochemical detection of the liver function

Following the kit instructions, the serum levels of aspartate aminotransferase (AST) (701138, Biobase); alanine aminotransferase (ALT) (70111, Biobase); and total bilirubin (TBIL) (70182, Biobase) were detected using a fully automated biochemical analyzer (BK-600, Biobase).

1.4 Hematoxylin-eosin staining

After paraffin sectioning of rat liver tissue, it was subjected to dehydration, rehydration, and staining with hematoxylin solution for 3–5 minutes. After rinsing with water, it was differentiated with 1% hydrochloric acid alcohol, then stained with bluing solution, and stained with eosin for 3–5 minutes. The sections were dehydrated, sealed, and observed under a microscope (CX43, OLYMPUS). Three images were collected for each sample. The collected images were scored using Knodell HAI, and all scores were independently completed by researchers who were unaware of the experimental groups.

1.5 Masson staining

After baking, dewaxing, and hydration, paraffin sections of the rat liver tissue were immersed in Masson's trichrome staining solution (G1006, Servicebio) overnight at room temperature according to the instructions. The next day, the sections were incubated in Masson's trichrome staining solution at 65°C for 30 min, followed by rinsing with tap water for 30 s until the yellow color faded. Simultaneously, Masson's D and F solutions were preheated at 65°C. Equal volumes of Masson's B and C solutions were mixed, and the sections were immersed in the mixture for 1 min, followed by a brief rinse with running water. The sections were differentiated in 1% hydrochloric acid alcohol for approximately 1 min, until the cell nuclei turned grayish-black and the background was almost colorless or pale gray. The sections were rinsed with tap water, excess water was drained, and the sections were immersed in Masson's D solution for 6 min. The sections were slightly drained (not completely dry) and immersed in Masson's E solution for approximately 1 min. The sections were then slightly drained from the Masson's E solution, and without rinsing with water, directly immerse in Masson F solution for staining for 2–30 seconds. Then, the sections were dehydrated, cleared with xylene, mounted with neutral resin, and observed under a microscope (CX43, OLYMPUS). Three images were collected for each sample. The collected images were scored using the Ishak method, and all the scores were independently completed by researchers who were unaware of the experimental groups.

1.6 Flow cytometry detection

Take the rat liver, cut it up, grind it, collect the cell suspension, centrifuge at 1500 rpm for 5 minutes, discard the supernatant, wash twice with PBS. Add the flow cytometry antibody CD4 (201509, Biolegend, 1:100) to the PBS solution, keep it in the dark. Add the antibody mixture to the cell suspension and incubate for 20–60 minutes, centrifuge at 1500 rpm for 5 minutes, discard the supernatant, add 100 µL Fixation Buffer for fixation, keep it in the dark for 20–60 minutes, resuspend the cell precipitate with 2 mL 1× Permeabilization Buffer, centrifuge at 400×g for 5 minutes at room temperature. Discard the supernatant. Prepare the antibody mixture tube: Add the flow cytometry antibody IL-17A (25-7177-82, Invitrogen, 1:100) and FOXP3 (320008, Biolegend, 1:100) to the PBS solution, add the antibody mixture to the cell suspension, incubate for 20–60 minutes. Add 1 mL PBS, centrifuge at 1500 rpm for 5 minutes, discard the supernatant. Resuspend the cells with 500 µL PBS and mix well before loading onto the flow cytometer (NovoCyte 2060R, EISSEN BIOTECHNOLOGY (Hangzhou) CO., LTD) for detection. Process the data after the machine is turned off in FlowJo software: Draw the density graph with FSC-H and SSC-H, remove the cell debris, circle the main cell population; Draw the density graph with CD4 and IL-17A, circle the CD4 + IL-17A+ Th17 cells according to the negative control (untreated); Draw the density graph with CD4 and FOXP3, circle the CD4 + FOXP3+ Treg cells according to the negative control (untreated).

1.7 Immunohistochemistry

After baking, dewaxing, and hydration, paraffin sections of liver tissue were subjected to antigen retrieval with citrate buffer. The sections were then transferred to a humidified chamber and incubated at room temperature for 10 min with freshly prepared 3% hydrogen peroxide to remove endogenous peroxidase.

Permeabilization with 0.5% Triton X-100 was performed at room temperature for 20 min, followed by blocking with 5% BSA at 37°C for 30 min. Primary antibody AhR (1:100, A1451, ABclonal) was added to the slides, and incubated overnight at 4°C. The next day, the slides were brought to room temperature, washed with PBS, added with horseradish enzyme-labeled goat anti-rabbit IgG (H + L) (1:100, ZB-2301, ZSGB-Bio) and incubated at 37°C for 30 min. Then, the slides were developed with DAB (CW0125M, CWBIO) for 3–5 min, rinsed with tap water for 1 min, and counterstained with hematoxylin for 3 min. They were then subjected to hydrochloric acid alcohol differentiation, bluing, rinsing with tap water, dehydration, xylene clearing, neutral resin mounting, and observation under a microscope (CX43, OLYMPUS). For each sample, three sections were collected, and three non-overlapping fields of view were taken for each section. The average fluorescence intensity of AhR positive staining was analyzed using ImageJ software. All quantitative analyses were independently completed by researchers who were unaware of the experimental groups.

1.8 Targeted metabolomics

Sample Pretreatment: After thawing serum samples at -20°C, operation was performed on ice. 50 µL of sample was weighed into a centrifuge tube, 250 µL of internal standard methanol solution was added, vortexed for 3 min, incubated at -20°C for 30 min, and were centrifuged at 12,000 rpm for 10 min at 4°C. Then, 300 µL of supernatant was transferred to another correspondingly numbered centrifuge tube, and recentrifuged at 12000 rpm for 10 min at 4°C. Subsequently, 150 µL of supernatant was transferred to a new centrifuge tube, recentrifuged, and 100 µL of supernatant was then transferred to the corresponding sample vial liner tube, and readied for LC-MS/MS analysis.

The chromatographic conditions were as follows: a Waters ACQUITY UPLC HSS T3 C18 column (1.8 µm, 2.1 mm x 100 mm) was used as the column; mobile phase A was 0.1% formic acid aqueous solution, and mobile phase B was 0.1% formic acid acetonitrile solution; the instrument column temperature was set to 40°C; the flow rate was 0.35 mL/min; and the injection volume was 2 µL. The specific process of targeted metabolomics detection and analysis is detailed in the supplementary methods file.

1.9 Statistical analysis

All experiments were repeated 3 times or more. All data were presented as the mean ± standard deviation (SD). Graphpad Prism 9.0 software was used for graphing and statistical analysis. Before conducting the statistical analysis, the Shapiro-Wilk test was used to assess the normality of the data, and the Levene test was used to detect the homogeneity of variance. For data that did not meet these assumptions, non-parametric alternatives (Kruskal-Wallis and Dunn's post-hoc) were applied. One-way analysis of variance was performed between multiple groups, followed by Tukey's post-hoc test. A P value < 0.05 indicated a significant difference.

Results

2.1 Effects of tryptophan metabolism on liver function in ACLF rats

Figure 1A is a schematic diagram showing the establishment of the ACLF rat model. To construct the ACLF model, the rats were acclimated and then intraperitoneally injected with porcine serum for 11 weeks. Subsequently, the rats were intravenously injected with LPS solution, and 30 minutes later, they were intraperitoneally injected with D-gal solution to induce acute liver injury. The biochemical analyzer detected AST, ALT, and TBIL in the rat serum. The results showed that compared with the Control group, the contents of ALT, AST, and TBIL in the ACLF group significantly increased. After intervention with an IDO inhibitor, the contents of ALT and AST continued to increase; intervention with an AhR agonist could reduce the levels of ALT, AST, and TBIL; and after intervention with both IDO inhibitor and AhR agonist, the levels of ALT, AST, and TBIL were similar to those in the ACLF group (Fig. 1B), indicating that 1-MT can exacerbate liver function damage in ACLF rats, while Leflunomide can alleviate liver function damage in ACLF rats.

2.2 Tryptophan metabolism affects the pathological damage in ACLF rats

HE staining was performed on liver tissues of rats in each group to observe the pathological changes, and liver damage was scored according to the Knodell Histology Activity Index (HAI, total score 0–22). Results showed that compared with the Control group, the ACLF group had more disordered liver tissue structure, increased number of hepatocytes undergoing lysis and necrosis, increased inflammatory cell infiltration, and a higher HAI score. After treatment with IDO inhibitor, a large number of hepatocytes with nuclear lysis were observed in the liver, with inflammatory cells clustered together and interstitial hemorrhage, resulting in a higher HAI score compared with the ACLF group. However, after treatment with AhR agonist, inflammatory cell infiltration decreased, and the HAI score decreased compared with the ACLF group. Liver damage in the ACLF + IDO inhibitor + AhR agonist group was between that of the two treatment groups alone (Fig. 2A). Masson staining and Ishak fibrosis scores also showed the same trend; no significant collagen deposition was observed in the Control group. A large amount of collagen deposition was observed around the blood vessels in the ACLF group, and the Ishak score increased. After treatment with IDO inhibitor, perivascular collagen deposition increased further, fibers thickened, and the Ishak score continued to rise; while treatment with AhR agonist reduced the degree of fibrosis to stage 1 (mild) (Fig. 2B). The results showed that 1-MT could aggravate liver injury and collagen deposition in ACLF rats, while Leflunomide could alleviate liver injury and fibrosis in these rats.

2.3 Tryptophan metabolism can regulate the Th17/Treg balance in ACLF rats

Flow cytometry analysis of the proportion of Th17 and Treg cells in rat liver tissue revealed that, compared with the Control group, the proportion of Th17 (CD4⁺IL-17A) cells was significantly increased

and the proportion of Treg (CD4⁺FOXP3) cells was significantly decreased in the ACLF group. Treatment with IDO inhibitor continued to increase the proportion of Th17 cells and decrease the proportion of Treg cells; however, treatment with AhR agonist had the opposite effect. The proportions of Th17 and Treg cells after simultaneous treatment with both IDO inhibitor and AhR agonist were between that of the two treatment groups alone (Fig. 3A). Immunohistochemical analysis of AhR expression in rat liver showed that, compared with the Control group, AhR expression was significantly decreased in the ACLF group; AhR expression continued to decrease after IDO inhibitor treatment; treatment with AhR agonist increased AhR expression; and the AhR expression in the ACLF + IDO inhibitor + AhR agonist group was between that of the two treatment groups alone (Fig. 3B). The results show that 1-MT can exacerbate the Th17/Treg imbalance in ACLF rats, while LFD can alleviate this imbalance in ACLF rats.

2.4 Tryptophan metabolism affects SOCS3 expression in ACLF rats

Immunofluorescence detection of SOCS3 expression in CD4⁺ T cells of rat liver and lymphoid tissue revealed that SOCS3 expression in CD4⁺ T cells of the ACLF group was significantly decreased compared with the Control group. IDO inhibitor treatment significantly decreased SOCS3 expression in CD4⁺ T cells, while AhR agonist treatment increased SOCS3 expression. SOCS3 expression in CD4⁺ T cells of the ACLF + IDO inhibitor + AhR agonist group was between that of the two treatment groups alone (Fig. 4). The results showed that 1-MT could reduce the expression of SOCS3, while after treatment with Leflunomide, the opposite effect occurred.

2.5 Targeted metabolomics in rat serum

The results of the targeted metabolism assays in rat serum showed that, compared with the Control group, the ACLF group showed significant upregulation of tryptophan metabolites indole-3-acetamide, indoxylsulfate, and N-acetylserotonin. Compared with the ACLF group, the ACLF + IDO inhibitor group showed significant upregulation of 3-hydroxykynurenine and significant downregulation of indoxylsulfate; the ACLF + AhR agonist group showed significant downregulation of indoxylsulfate; and the ACLF + IDO inhibitor + AhR agonist group showed significant upregulation of N-acetylserotonin and significant downregulation of 5-hydroxyindoleacetic acid, 3-indoleacrylic acid, and indoxylsulfate (Fig. 5A).

KEGG signaling pathway enrichment analysis revealed that differentially metabolites primarily function in tryptophan metabolism, metabolic pathways, biosynthesis of cofactors, and neuroactive ligand-receptor interaction (Fig. 5B). These results indicate that the differential metabolites in ACLF rats are enriched in the tryptophan metabolic pathway, and 1-MT and leflunomide can affect the expression of these metabolites.

Discussion

ACLF is a clinical syndrome associated with organ failure based on severe liver dysfunction, and its severity is closely related to systemic inflammatory response^[14]. ACLF can lead to high mortality and a surge in medical resource consumption. Studies have shown that tryptophan metabolism is involved in the regulation of inflammatory and immune responses. During systemic inflammatory responses, tryptophan can produce immunomodulatory metabolites through the kynurenine pathway^[15]. For example, kynurenine pathway metabolites can act on AhR to exert immunosuppressive effects and promote the differentiation of regulatory T cells (Tregs)^[16]. Although tryptophan metabolism plays a regulatory role in a variety of diseases caused by excessive inflammatory responses^[17, 18], the effect of tryptophan metabolism on ACLF remains unclear. More noteworthy is that different studies have presented contrasting views on the effects of tryptophan metabolism on liver injury. One clinical study found that the kynurenine pathway was activated in patients with acute decompensated cirrhosis, reaching its peak in patients with ACLF; and the content of kynurenine metabolites in the patient's serum was related to the patient's mortality, suggesting that tryptophan metabolism activation promotes ACLF development^[19]; while another study showed that tryptophan metabolism and IDO activation can promote the differentiation of regulatory T cells into Treg cells, thereby preventing the Th17/Treg imbalance induced by the inflammatory response in ACLF patients^[20].

To gain a clear understanding of the roles of 1-MT and Leflunomide in the progression of ACLF, this study established a rat ACLF model and intervened with 1-MT and Leflunomide. It was found that the liver of ACLF rats showed significant damage, liver function declined, and there was an imbalance in Th17/Treg. After 1-MT treatment, it was observed that the severity of ACLF in rats increased, while Leflunomide treatment could alleviate this change. Targeted metabolic profiling of the serum of each group of rats revealed that the differential metabolites were concentrated in the tryptophan metabolic process. This study elucidated the role of tryptophan metabolism in the progression of ACLF, and found that leflunomide could upregulate the expression of AhR and SCOS3, inhibit inflammatory responses, alleviate liver damage in the ACLF model, and affect the Th17/Treg balance.

Previous studies have found that IDO and AhR are abnormally expressed in ACLF patients. Yu et al. found that IDO expression increased in patients with hepatitis B virus-related ACLF (HBV-ACLF) and that it can regulate T cell proliferation and function, thereby affecting liver function^[21]. In this study, it was found that ALT, AST, and TBIL increased more significantly in ACLF rats after intervention with IDO inhibitors, and there were more inflammatory cells and collagen deposition in the liver tissue. However, the above indicators of ACLF rats showed the opposite changes after intervention with AhR agonists. Some studies have shown that AhR can be activated by tryptophan metabolites catalyzed by IDO, thereby inhibiting the inflammatory response^[16]. In a clinical study of HBV-ACLF, serum tryptophan levels were significantly reduced in the deceased group, suggesting that tryptophan was broken down in large quantities. Tryptophan metabolism is considered to be related to the occurrence of ACLF^[22]. In our targeted metabolomics study on ACLF rats, we also found that after intervention with IDO inhibitors and AhR agonists, the differential metabolites in the rats were enriched in tryptophan metabolism, cofactor biosynthesis, and metabolic pathways, indicating that tryptophan metabolism plays an important

regulatory role in the occurrence of ACLF. This further suggests that the tryptophan metabolism regulated by 1-MT and Leflunomide is closely related to the progression of ACLF.

Tryptophan metabolism plays an important role in inflammatory responses. Cells expressing IDO can regulate innate and adaptive immune responses to inflammatory stimuli by producing a variety of metabolites through the breakdown of tryptophan. Tryptophan enters the kynurenine pathway via IDO catalysis and is degraded to produce a variety of metabolites. These metabolites can act as ligands for AhR's immune activity, promote Treg cell differentiation, and exhibit immunosuppressive functions^[16]. The Th17/Treg balance is crucial in immune regulation. Multiple studies have shown that the IDO pathway and downstream kynurenine metabolites can specifically inhibit pro-inflammatory Th17 cells, thereby promoting a shift in Th17/Treg balance towards Treg cells. IDO inhibitors or IDO gene knockout will hinder the normal differentiation of Treg cells^[23]. AhR can bind to tryptophan metabolites to regulate immune and inflammatory responses in inflammatory diseases^[24]. The results of this study show that when tryptophan metabolism is inhibited, the proportion of Th17 cells in the liver of ACLF rats increases, the proportion of Treg cells decreases, and the decrease in AhR expression is more pronounced. Activating tryptophan metabolism can improve this change. This suggests that IDO may activate AhR's immunomodulatory function through tryptophan metabolism, and AhR may inhibit the inflammatory response in ACLF rats by regulating the Th17/Treg balance. This indicates that 1-MT treatment downregulates AhR expression, promoting the imbalance of Th17/Treg, while Leflunomide treatment upregulates AhR expression, maintaining the balance of Th17/Treg and inhibiting the inflammatory response.

However, the mechanism by which AhR participates in the regulation of the Th17/Treg balance in ACLF remains unknown. Previous studies have shown that classical suppressor of cytokine signaling (SOCSs) have negative inflammatory and immune regulatory functions in inflammatory responses, among which SOCS3 plays an important role^[25]. Some studies have reported that SOCS3 can be used as a prognostic indicator for liver failure in acute and chronic hepatitis B, and is negatively correlated with the degree of inflammation in patients with HBV-ACLF^[26]. Some studies have found that AhR can upregulate SOCS3 expression, and promoter analysis has identified SOCS3 as a novel transcriptional target of AhR^[27]. In our study, we found that SOCS3 expression decreased in the liver and lymphoid tissue of ACLF rats, and the decrease in SOCS3 was more pronounced after inhibiting tryptophan metabolism. SOCS3 expression increased after AhR activation, indicating that classical SOCS3 signaling also plays an important role in this inflammatory and immune process. Related studies have also shown that SOCS3 plays a key regulatory role in the Th17/Treg balance. SOCS3 can regulate the JAK-STAT signaling pathway through negative feedback, inhibit Th17 cell differentiation, and maintain the Th17/Treg balance in the inflammatory response^[28]. Therefore, we speculate that the possible restoration of the Th17/Treg balance after AhR agonist treatment might be achieved by upregulating SOCS3.

Although this study demonstrated that tryptophan metabolism can affect the progression of ACLF by regulating the Th17/Treg balance, this study still has limitations. First, all the results in this study were

obtained from male animal models. It is still unclear whether this regulatory mechanism exists in ACLF patients, and whether there are gender differences in this regulatory mechanism is also unclear. In future studies, we will try to collect clinical samples from ACLF patients or further verify this regulatory mechanism in female animals. Second, we did not use survival rate as the endpoint of this study. Given the characteristics of ACLF, which has a high short-term mortality rate, survival data will provide the most direct evidence of therapeutic benefits. Therefore, our conclusion is limited to the attenuation of biochemical and histological markers of liver injury, and cannot be inferred as an increase in survival rate. Thirdly, in this study, an IDO inhibitor was used, but no expression or activity of IDO was detected. In future studies, we will focus on the changes in IDO expression. Fourthly, the leflunomide used in this study is not an ideal agonist for AhR, and whether it can pass through the AhR-SOCS3 signaling pathway still needs to be further determined. At the same time, no solvent group was set to exclude the influence of the solvent of leflunomide on the animals. Later, we will further study the role of AhR through genetic methods (such as AhR knockout models) or more selective pharmacological tools. Finally, this study suggests that AhR may regulate the Th17/Treg balance through SOCS3, but the mediating role of SOCS3 has not been further proved through retrospective experiments. In future studies, we will regulate the expression of SOCS3 and further explore the effect of SOCS3 on the Th17/Treg balance in ACLF rats.

In conclusion, this study found that in the ACLF environment, Leflunomide is associated with the upregulation of SOCS3 expression by AhR, and is related to the alleviation of liver inflammation and immunity, as well as the improvement of liver tissue damage. On the contrary, 1-MT has the opposite effect. This study proposed the possible mechanisms of 1-MT and leflunomide in the progression of ACLF (Fig. 6), providing new ideas for exploring new treatment methods for ACLF.

Conclusion

In conclusion, this study found that in the ACLF environment, Leflunomide can upregulate the expression of SOCS3 by activating AhR, while simultaneously reducing liver inflammation and immunity, and improving liver tissue damage. On the contrary, 1-MT has the opposite effect. This study suggests the possible mechanisms of 1-MT and Leflunomide in the progression of ACLF (Fig. 6), it provides new insights into the pathogenesis and intervention measures of ACLF.

Declarations

Funding

This work was supported by the Priority Union Foundation of Yunnan Provincial Science and Technology Department and Kunming Medical University (202401AY070001-245); Yunnan Revitalization Talent Support Program; Yunnan Key Laboratory of Organ Transplantation (No. 202449CE340016).

Competing Interests

The authors have no conflicts of interest related to this publication.

Author contributions

Conception and design: HEL, GZ, JL. Acquisition of data: GZ, JL, RYZ, QL JLD, ALW, YJQ, WRW. Writing - original draft: GZ, JL, and Critical revision and final approval: all the authors.

Ethics Approval

The animal experiments were approved by the Experimental Animal Ethics Committee of Jiangxi Zhonghong Bohuan Biotechnology Co., Ltd. (Approval Number: LL-202411110002), and were conducted in accordance with the guidelines for animal care and utilization institutions.

Consent for Publication

All the authors consent to the publication of this manuscript.

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Figures

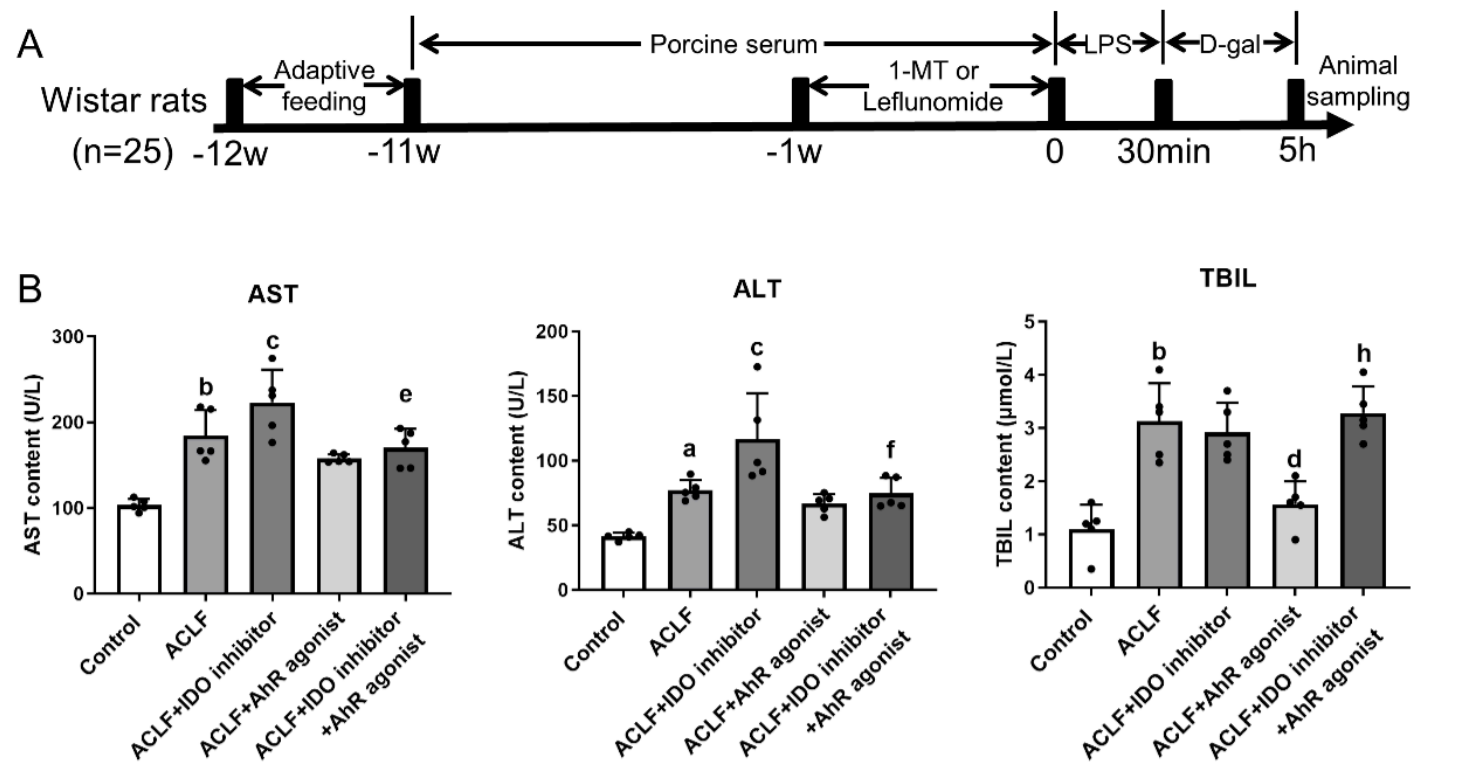


Figure 1

The effect of tryptophan metabolism on liver function in ACLF rats. (A) Construction of the ACLF animal model and the administration time schedule, (B) Biochemical detection of ALT, AST, and TBIL contents in the rat serum ($n = 5$). Compared with the Control group, ^a $P < 0.05$, ^b $P < 0.01$; compared with the ACLF group, ^c $P < 0.05$, ^d $P < 0.01$; compared with the ACLF + IDO inhibitor group, ^e $P < 0.05$, ^f $P < 0.01$; compared with the ACLF + AhR agonist group, ^h $P < 0.01$.

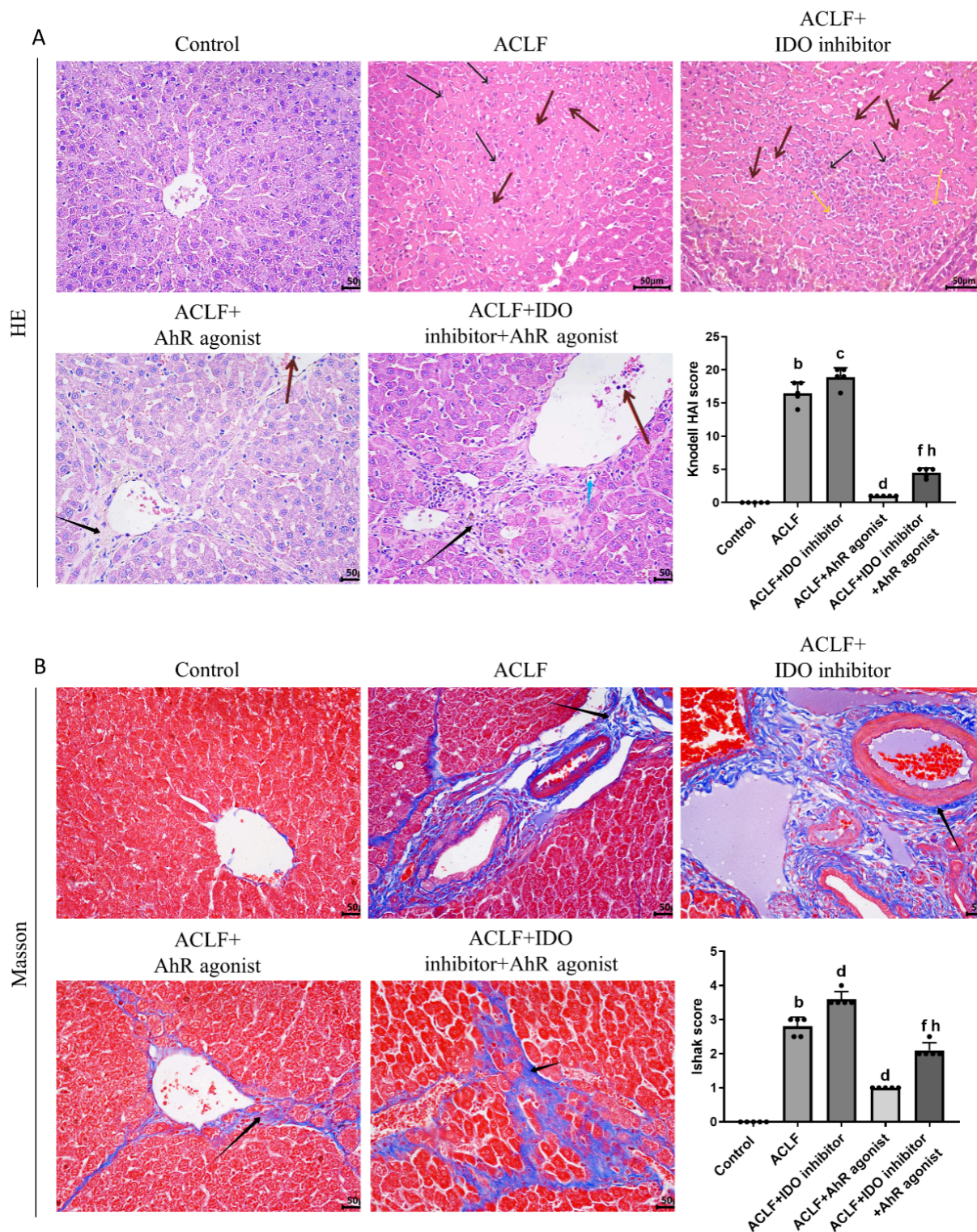


Figure 2

The metabolism of tryptophan affects liver injury and collagen deposition in ACLF rats. (A) Results of HE staining of rat liver (scale = 50 μ m, n = 5). (B) Results of Masson staining of rat liver (scale = 50 μ m, n = 5). Compared with the Control group, ^bP < 0.01; compared with the ACLF group, ^cP < 0.05, ^dP < 0.01; compared with the ACLF + IDO inhibitor group, ^fP < 0.01, and compared with the ACLF + AhR agonist group, ^hP < 0.01.

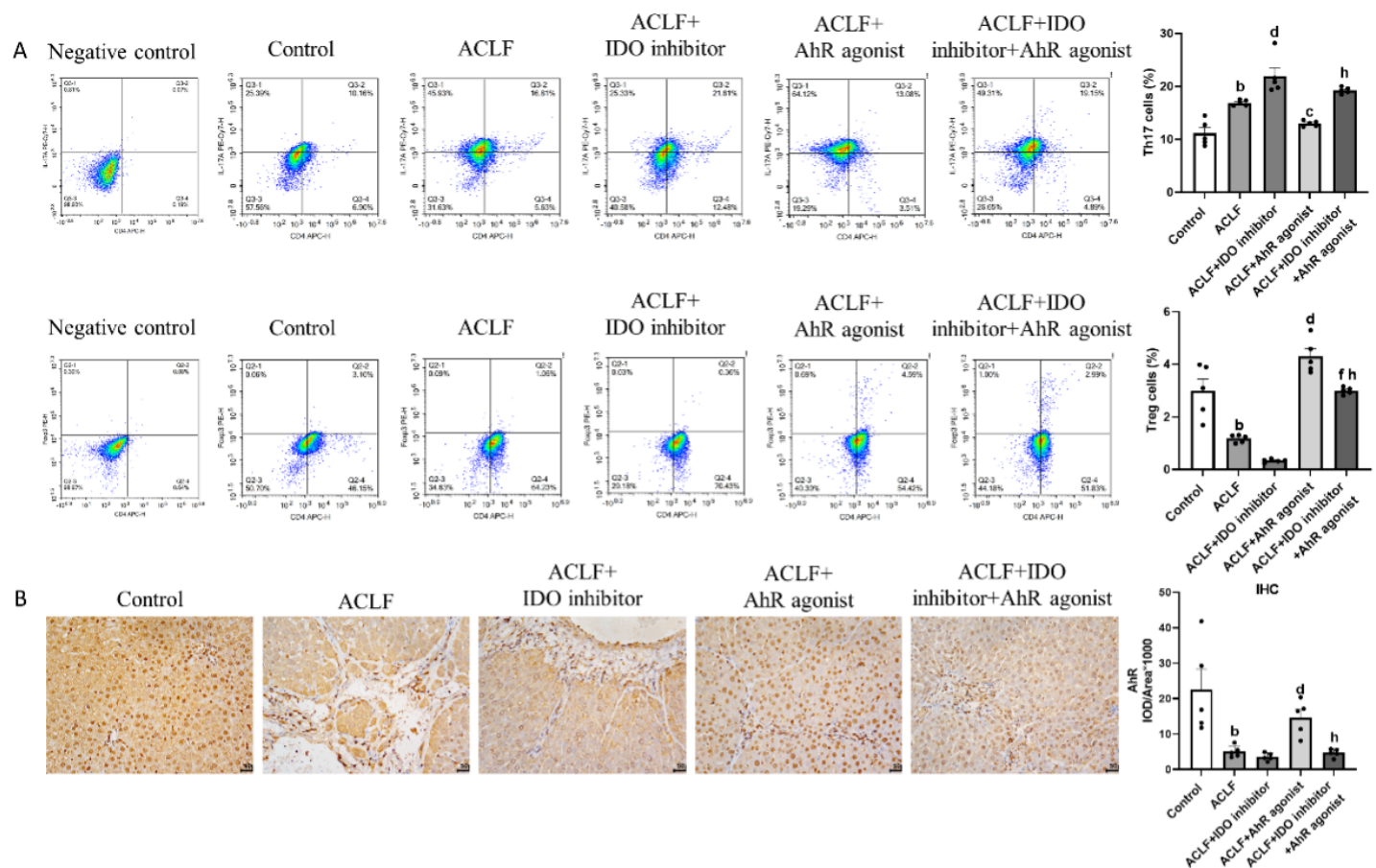


Figure 3

The effect of tryptophan metabolism on the Th17/Treg balance in ACLF rats. (A) Flow cytometry analysis of the proportion of Th17 and Treg cells in the liver of rats ($n = 5$). (B) Immunohistochemical detection of AhR expression in the liver of rats (scale = 50 μ m, $n = 5$). Compared with the Control group, ^b $P < 0.01$; compared with the ACLF group, ^c $P < 0.05$, ^d $P < 0.01$; compared with the ACLF + IDO inhibitor group, ^f $P < 0.01$; compared with the ACLF + AhR agonist group, ^h $P < 0.01$.

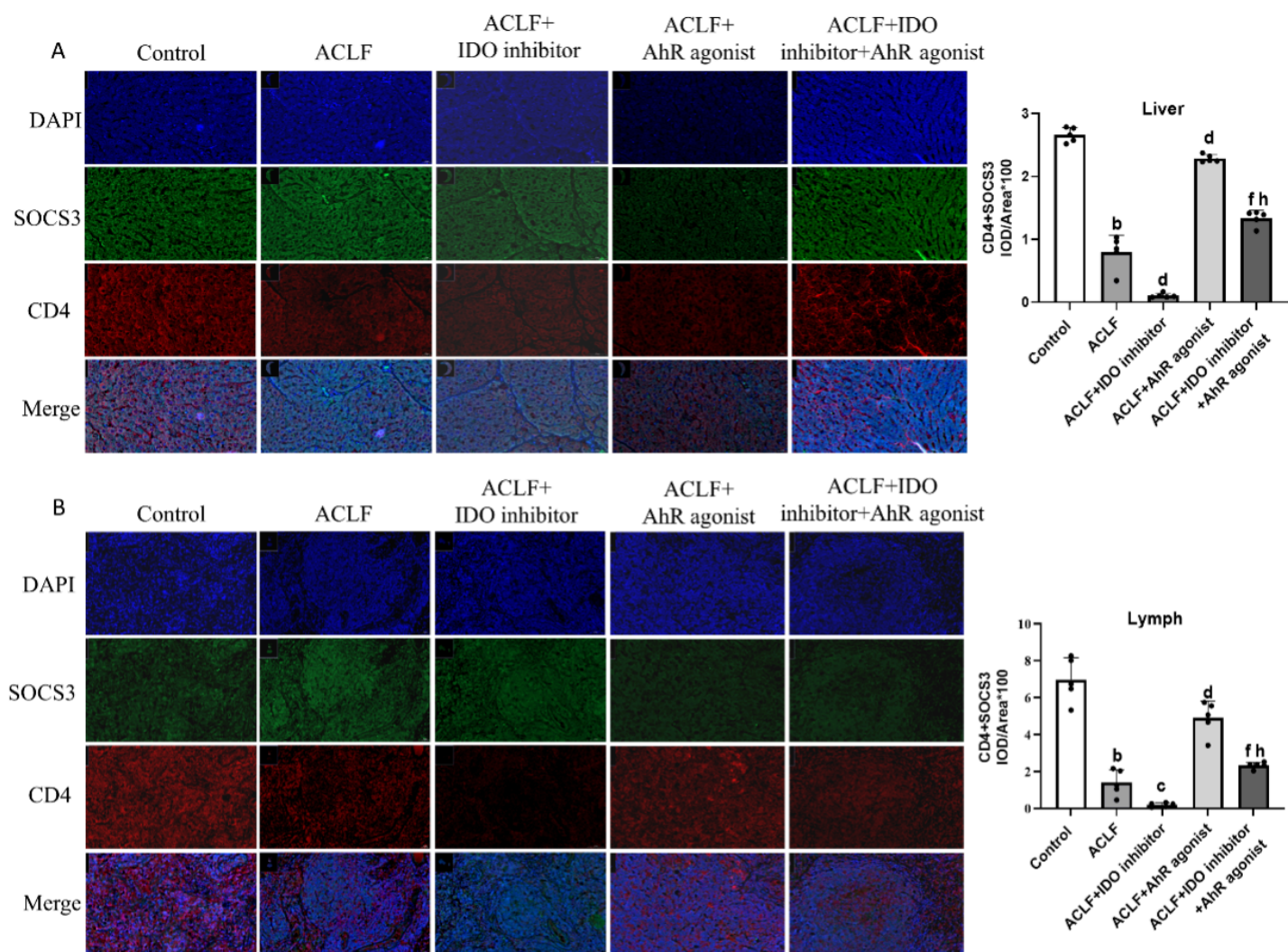


Figure 4

The influence of tryptophan metabolism on the expression of SOCS3 in CD4+ T cells. (A) Double immunofluorescence staining results of CD4 and SOCS3 in rat liver tissues (scale = 50 μ m, n = 5). (B) Double immunofluorescence staining results of CD4 and SOCS3 in rat lymphoid tissues (scale = 50 μ m, n = 5). Compared with the Control group, ^bP < 0.01; compared with the ACLF group, ^cP < 0.05, ^dP < 0.01; compared with the ACLF + IDO inhibitor group, ^fP < 0.01; compared with the ACLF + AhR agonist group, ^hP < 0.01.

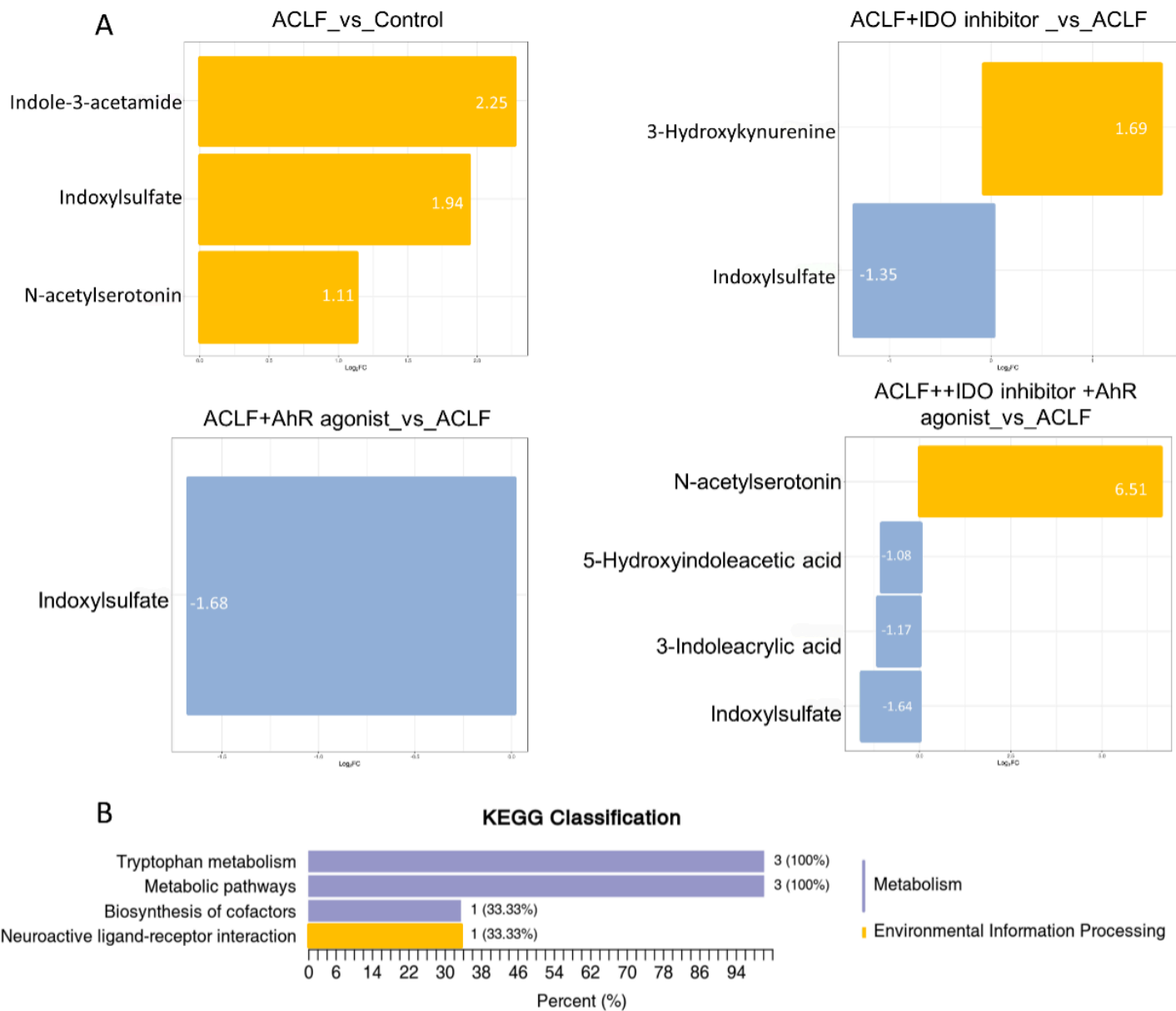


Figure 5

The results of targeted metabolomics in rat serum ($n = 5$).(A) Bar chart of differentially expressed metabolites among each comparison group (yellow represents up-regulated metabolites, light blue represents down-regulated metabolites). (B) KEGG classification diagram of differentially expressed metabolites.

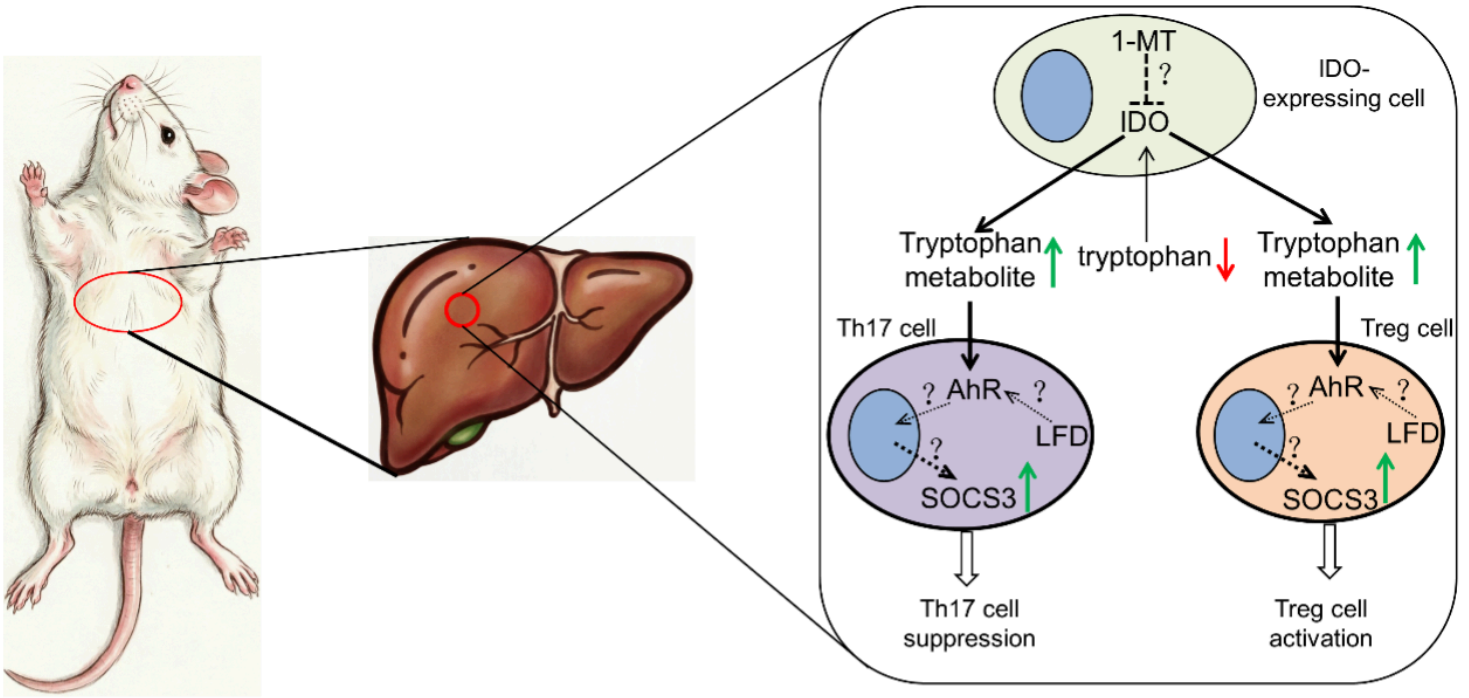


Figure 6

The working model proposed in this study for the mechanism by which tryptophan metabolism regulates ACLF. In the liver of ACLF rats, IDO activates immune cells through the production of tryptophan metabolites, which in turn activates AhR in the nucleus and promotes the expression of SOCS3, thereby down-regulating immune and inflammatory responses. (The solid arrows represent the associations supported by the experiment; the dashed arrows represent the proposed connections that require further investigation.)