Adipose-derived mesenchymal stem cell-derived HCAR1 regulates immune response in the attenuation of sepsis

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Abstract. Sepsis serves as a leading cause of admission to and death of patients in the intensive care unit (ICU) and is described as a systemic inflammatory response syndrome caused by abnormal host response to infection. Adipose-derived mesenchymal stem cells (ADSCs) have exhibited reliable and promising clinical application potential in multiple disorders. However, the function and the mechanism of ADSCs in sepsis remain elusive. In the present study, the crucial inhibitory effect of ADSC-derived hydroxy-carboxylic acid receptor 1 (HCAR1) on sepsis was identified. Reverse transcription quantitative-PCR determined that the mRNA expression of HCAR1 was reduced while the mRNA expression of Toll-like receptor 4 (TLR4), major histocompatibility complex class II (MHC II), NOD-like receptor family pyrin domain containing 3 (NLRP3), and the levels of interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), interleukin-10 (IL-10), and interleukin-18 (IL-18) were enhanced in the peripheral blood of patients with sepsis. The expression of HCAR1 was negatively correlated with TLR4 (r=-0.666), MHC II (r=-0.587), and NLRP3 (r=-0.621) expression and the expression of TLR4 was positively correlated with NLRP3 (r=0.641), IL-1β (r=0.666), TNF-α (r=0.606), and IL-18 (r=0.624) levels in the samples. Receiver operating characteristic (ROC) curve analysis revealed that the area under the ROC curve (AUC) of HCAR1, TLR4, MHC II and NLRP3 mRNA expression was 0.830, 0.853, 0.735 and 0.945, respectively, in which NLRP3 exhibited the highest diagnostic value, and the AUC values of IL-1β, IL-18, TNF-α, and IL-10 were 0.751, 0.841, 0.924 and 0.729, respectively, in which TNF-α exhibited the highest diagnostic value. A sepsis rat model was established by injecting lipopolysaccharide (LPS) and the rats were randomly divided into 5 groups, including a normal control group (NC group; n=6), a sepsis model group (LPS group; n=6), an ADSC transplantation group (L + M group; n=6), a combined HCAR1 receptor agonist group [L + HCAR1 inducer (Gi) + M group; n=6], and a combined HCAR1 receptor inhibitor group [L + HCAR1 blocker (Gk) + M group; n=6]. Hematoxylin and eosin staining determined that ADSCs attenuated the lung injury of septic rats and ADSC-derived HCAR1 enhanced the effect of ADSCs. The expression of HCAR1, TLR4, MHC II, NLRP3, IL-1β, IL-18 and TNF-α levels were suppressed by ADSCs and the effect was further induced by ADSC-derived HCAR1. However, ADSC-derived HCAR1 induced the levels of anti-inflammatory factor IL-10. The negative correlation of HCAR1 expression with TLR4, MHC II, and NLRP3 expression in the peripheral blood and lung tissues of the rats was then identified. It is thus concluded that ADSC-derived HCAR1 regulates immune response in the attenuation of sepsis. ADSC-derived HCAR1 may be a promising therapeutic strategy for sepsis.

Introduction

Sepsis is a major cause of admission to and death of patients in the intensive care unit (ICU), and defined as a systemic inflammatory response syndrome caused by abnormal host response to infection (1). Studies have indicated a high incidence and mortality of sepsis, accounting for almost 20% of global deaths (2,3). Notably, even patients who have survived sepsis, may still suffer from severe sequelae, including long-term physical, psychological and cognitive disabilities (4,5). Sepsis is usually accompanied with abnormal immune response and leads to sustained immunosuppression following recovery (6). In addition to immune suppression, sepsis also causes other severe symptoms such as acute lung injury (7), and myocardial injury (8,9). When sepsis occurs, a dysregulated immune response, including activated pro-inflammatory and anti-inflammatory responses develop into an inflammatory cytokine storm, extensive endothelial injury, subsequently sustained immunosuppression, and even death in some

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cases (6). Confining the immunosuppression and regulating the inflammatory response during sepsis are therefore the main therapeutic methods for sepsis. Moreover, mesenchymal stem cells (MSCs) are mobile in an inflammatory environment and modulate inflammatory response, hence they are regarded as a promising therapeutic approach for sepsis (10).

MSCs are derived from multipotent progenitor cells of the mesoderm lineage during blast cytogenesis and may be isolated from several tissues such as the bone marrow, adipose, and umbilical cord (11,12). MSCs are characterized by plasticity, adhesion, and the ability to differentiate into osteocytes, adipocytes, and chondrocytes (13). MSCs are also regarded as a potential new approach of immune modulatory therapy for diseases (14,15). Adipose-derived MSCs (ADSCs), have also shown reliable and promising clinical application potential (16,17). In a lipopolysaccharide (LPS)-induced acute respiratory distress syndrome (ARDS) model, transplant of ADSCs decreased inflammatory neutrophil infiltration, collagen deposition and lung injury, possibly by suppressing classic inflammatory Toll-like receptor 4 (TLR4) signaling (18,19). However, allogeneic immune rejection is a great challenge for MSC transplantation. During immune regulation, major histocompatibility complex class II (MHC II), the immunogenicity-related gene, is essential for antigen presentation to $CD4^+$ Th cells, affects the selection, immune tolerance of T cells, and the production of antibodies (20). Therefore, handling the survival of MSCs and immune rejection have become the primary issues of stem cell transplantation.

A class of G protein-coupled receptors (GPCRs) exist on the surface of antigen-presenting immune cells, namely the hydroxy-carboxylic acid (HCA) receptor, among which hydroxy-carboxylic acid receptor 1 (HCAR1) functions as the receptor of lactate, and play important roles during the development and differentiation of adipocytes, adipose metabolism, and inflammatory response (21). Hoque *et al*, found that lactate-HCAR1 signaling inhibited TLR4-mediated inflammatory response (22). In addition, HCAR1 was revealed to induce downregulation of MHC II activity and to promote immunosuppression via lactate/HCAR1/ERK/STAT3/Arg-1 signaling, protecting patients from intense inflammatory response-caused high mortality in the early phase of sepsis (23).

The aim of the present study was to determine the specific role of HCAR1 during immunoreaction following MSC transplantation. An *in vivo* sepsis model was established and the model rats were treated with an agonist or an antagonist and then the levels of inflammatory factors and MHC II were detected. Lung tissue damage was also observed. The present study provided a novel approach for MSC transplantation-treated sepsis.

Materials and methods

Clinical samples. Patients diagnosed with sepsis (n=20) who were hospitalized at the Third Affiliated Hospital of Inner Mongolia Medical University (Baotou, China) from April 2014 to December 2019, and healthy donors (n=20) who underwent a physical exam, were recruited for the present study. Each group included 10 females and 10 males, aged 55 to 70 years old. Blood samples (2 ml) were collected and stored at -80°C. All participants provided signed informed consent.

All experiments were performed under the authorization and guidelines of Ethics Committee of the Third Affiliated Hospital of Inner Mongolia Medical University. The standard for diagnosis of sepsis was according to the Sequential Organ Failure Assessment (SOFA) score (24) guidelines of Sepsis 3.0 (Table I). The exclusion criteria were as follows: i) aged <18 or >80 years old; ii) patients with liver and kidney failure; iii) pregnant or breastfeeding women; iv) malignant tumors or blood system diseases; v) granulocytes $<0.5 \times 10^9/l$; vi) immunodeficient patients; and vii) participants who quit. The use of humans/human tissues followed the guidelines of The Declaration of Helsinki.

Adipocyte-derived mesenchymal stem cell (ADMSC) culture and transfection. Rat ADMSCs were purchased from Procell Life Science & Technology Co., Ltd. and the cells were transplanted into a 25-cm^2 plate with DMEM/F12 medium (Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (Thermo Fisher Scientific, Inc.), 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in an incubator filled with 5% CO_2. LVC-GFP lentivirus vectors were co-transfected with packaging vector psPAX2 (0.1 μg; Addgene, Inc.) and envelope vector pMD2.G (0.9 μg; Addgene, Inc.) (lentiviral plasmid:packaging vector:envelope vector, 10:1:9) into 3rd generation 293T (CRL-3216; ATCC) cells using Lipofectamine® 2000 (cat. no. 11668-019; Invitrogen; Thermo Fisher Scientific, Inc.). Following transfection at 25°C for 48 h, the lentiviral particles were collected via ultracentrifugation at 55,000 x g, at 4°C for 2.5 h. After 24 h of transfection, the cells were cultured for 24 h with serum-free transfer solution as the complete medium. Cells were plated in 6-well plates (1x10^6 cells/ml) and incubated with LVC-GFP lentivirus (MOI=10) under Polybrene (Thermo Fisher Scientific, Inc.) (5 μg/ml) using Lipofectamine 2000 at 37°C for 24 h. The LVC-GFP lentivirus was synthesized and purchased from Shanghai Genechem Co., Ltd. The medium was then replaced by fresh medium and cultured for another 48 h.

Animal experiments. Sprague-Dawley (SD) male rats weighing 100-150 g (n=30) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. Rats were randomly divided into five groups: i) The normal control that received regular feeding without any treatment; ii) the LPS group (25) which was the sepsis group that received intraperitoneal injection of LPS (L; 5 mg/kg) (26,27); iii) the L + M group in which rats received ADMSCs (1x10^6) transplanted 30 min following LPS treatment; iv) the L + Gi (HCAR1 receptor agonist) + M group, in which rats received intraperitoneal injection of the HCAR1 receptor agonist (30 mg/kg, 3-chloro-5-hydroxy BA; APeXBIO Technology LLC) 30 min after LPS treatment, followed by intravenous injection of ADMSCs 1 h later; and v) the L + Gk (HCAR1 receptor antagonist) + M group, in which rats received intraperitoneal injection of HCAR1 receptor antagonist (30 mg/kg, [(R)-3-hydroxybutaboic acid]; MedChemExpress) 30 min after LPS treatment, followed by intravenous injection of ADMSCs 1 h later. HCAR1 is a GPCR, and the agonist was named according to the 'inducer' and the antagonist according to the 'blocker' in the initial application. For ADMSC transplant, ADMSCs transfected with LVC-GFP lentivirus were collected, suspended in

Table I. SOFA score.

System or organ	Variables	Score 0	1	2	3	4
Respiratory	PaO$_2$/FiO$_2$ (mmHg)	>400	301-400	<201-300	101-200	≤100
Respiratory	Respiratory support (yes/no)				Yes	Yes
Blood coagulation	Platelets (x10^9/l)	>150	101-150	51-100	21-50	≤20
Liver	Bilirubin (μmol/l)	<20	20-32	33-101	102-204	>204
Circulatory	Mean arterial pressure (mmHg)	>70	<70			
Circulatory	Dopamine (μg/kg/min)			≤5.0 or	>5 or	>15 or
Circulatory	Adrenaline (μg/kg/min)				≤0.1 or	>0.1 or
Circulatory	Noradrenaline (μg/kg/min)				≤0.1	>0.1
Circulatory	Dobutamine (yes/no)			Yes		
Central nervous system	GCS score	15	13-14	10-12	6-9	<6
Kidney	Creatinine (μmol/l)	<110	110-170	171-299	300-440	>440
Kidney	Urine amount (ml/d)				201-500	<200

SOFA, sepsis-related organ failure; GCS, Glasgow coma scale.

saline (1x10^6 cell/ml) and were intravenously injected into the tail vein of rats. To identify the successful inoculation of ADMSCs in rats, 72 h after injection, rats were anesthetized with phenobarbital sodium (50 ml/kg), and observed on an *in vivo* optical two-dimensional imaging system (Lumina II; PerkinElmer, Inc.). After 5 days of ADMSC injection, the rats were euthanized with an overdose of pentobarbital sodium (150 mg/kg, intravenous injection). To investigate the mechanism, the NF-κB inhibitor antioxidant pyrrolidine dithiocarbamate (PDTC; MilliporeSigma) was used (intragastrical injection, 100 mg/kg) 30 min after LPS treatment There were six rats per group (n=6/group). Animal care and method procedures were authorized by the Animal Ethics Committee (approval no. 2020-0618-37) of the Third Affiliated Hospital of Inner Mongolia Medical University.

Tissue collection and histological analysis. After establishment of a rat model, the rats were anesthetized by an overdose of pentobarbital sodium. In brief, the abdomen of rats was opened to expose the abdominal aorta, then the blood was collected (2 ml/rat) and centrifuged at 2,000 x g at 4˚C for 15 min to obtain serum. The serum was stored at -20˚C. Subsequently, the chest of rats was opened to isolate the lungs. The lung tissues were partially stored at -80˚C for RNA and protein analysis. For hematoxylin and eosin (H&E) staining and fluorescence imaging, lung tissues were embedded in optimal cutting temperature (OCT) gel (Agar Scientific, Ltd.), and cut into 5-μm slices at -20˚C For H&E staining, the slices were fixed with 4% paraformaldehyde (PFA) overnight at 4˚C, stained with H&E (hematoxylin staining at 25˚C for 3 min and eosin staining at 25˚C for 3 min) and observed under an optical microscope. For fluorescence imaging, the nucleus was stained with DAPI for 10 min at 4˚C, and the GFP and DAPI were observed under LSM 710 NLO/7 MP confocal microscope.

Reverse transcription quantitative-PCR (RT-qPCR). Total RNA of peripheral blood (1 ml) and lung tissue (0.2 g) was extracted using TRIzol reagent (Thermo Fisher Scientific, Inc.), reversed to cDNA using ReverTra Ace PCR RT (Toyobo Life Science) according to the manufacturer's instructions. Gene expression was quantified using THUNDERBIRD SYBR qPCR Mix (Toyobo Life Science) according to the manufacturer's instructions, following the 2$^{-\Delta\Delta Cq}$ method (8). The following thermocycling conditions were used for qPCR: Initial denaturation at 95˚C for 30 sec; followed by 39 cycles at 95˚C for 5 sec and 60˚C for 30 sec; and a final extension at 72˚C for 5 min. GAPDH was used as an internal control. Primers were synthesized by Takara Bio, Inc., and the sequences are listed in Table II.

Enzyme-linked immunosorbent assay (ELISA). The levels of interleukin (IL)-1β (IL-1β ELISA kit; cat. no. E-EL-R0012), IL-10 (IL-10 ELISA kit; cat. no. E-EL-R0016; Elabscience Biotechnology, Inc.), tumor necrosis factor-α (TNF-α; TNF-α ELISA kit; cat. no. E-EL-R2856), and IL-18 (IL-18 ELISA Kit; cat. no. E-EL-R0567), in blood were determined by ELISA using commercial kits (all from Elabscience Biotechnology, Inc.) following the manufacturer's instructions. In brief, the blood samples were centrifuged at 2,000 x g at 4˚C for 15 min, and the supernatants were added to test plates and incubated for 1 h at 37˚C. Biotin-labeled antibodies (1:100; cat. nos. A18821, PA1-29608 and A27035; Thermo Fisher Scientific, Inc.) were then added, followed by incubation for 1 h at 4˚C. The absorbance values at 450 nm were detected on a microplate reader (Beijing Liuyi Biotechnology Co., Ltd.).

Immunofluorescence assay. The identification of ADMSCs was performed using immunofluorescence assays. In brief, cells (1x10^6) were plated on coverslips, fixed with 4% PFA overnight at 4˚C, penetrated with 0.5% Triton X-100, blocked with 10% goat serum (Thermo Fisher Scientific, Inc.) for 2 h at 4˚C, followed by incubation with specific primary antibodies against CD44 (cat. no. bs-2507R), CD90 (cat. no. bs-0778R),

Table II. Primers for reverse transcription quantitative-PCR.

Primer	Sequence
Human-HCAR1	Forward, 5'-AGAACCATCTCTGCGTGCAA-3'
	Reverse, 5'-GCGACCGAGGTTCGAAATTG-3'
Human-MHC II (HLA-DRB1)	Forward, 5'-CTCCTGCATGGCAGTTCTGA-3'
	Reverse, 5'-ATTGTGGATCAGGCCTGTGG-3'
Human-TLR4	Forward, 5'-GGTGCCTCCATTTCAGCTCT-3'
	Reverse, 5'-ACTGCCAGGTCTGAGCAATC-3'
Human-NLRP3	Forward, 5'-CACTTCCAGTTTTTGCCGGG-3'
	Reverse, 5'-CAAGGCATTCTCCCCCACAT-3'
Human-GAPDH	Forward, 5'-GAGAAGGCTGGGGCTCATTT-3'
	Reverse, 5'-AGTGATGGCATGGACTGTGG-3'
Rat-HCAR1	Forward, 5'-GTGTTGGCGAGGCTCTACTT-3'
	Reverse, 5'-AACACACTTGGAGACCCCAC-3'
Rat-MHC II (RT1-Db2)	Forward, 5'-TCCGGAATGGTGAGGAGGAA-3'
	Reverse, 5'-GTAGATGAACAGCCCCACCG-3'
Rat-TLR4	Forward, 5'-TCAGCTTTGGTCAGTTGGCT-3'
	Reverse, 5'-GTCCTTGACCCACTGCAAGA-3'
Rat-NLRP3	Forward,5'-ACGGCAAGTTCGAAAAAGGC-3'
	Reverse, 5'- AGACCTCGGCAGAAGCTAGA-3'
Rat-GAPDH	Forward, 5'-GCGAGATCCCGCTAACATCA-3'
	Reverse, 5'-CTCGTGGTTCACACCCATCA-3'

TLR4, Toll-like receptor 4; MHC II, major histocompatibility complex class II; NLRP3, NOD-like receptor family pyrin domain containing 3; HCAR1, hydroxy-carboxylic acid receptor 1.

CD34 (cat. no. bs-0646R) and CD45 (cat. no. bs-0522R; all from BIOSS) at 4˚C overnight. The following day, the cells were incubated with Cy2- (Cy™2 AffiniPure; 1:50; code no. 111-225-144) or Cy3-conjugated secondary anti-rabbit antibodies (Cy™3 AffiniPure; 1:50; code no. 111-165-003; both from Jackson ImmunoResearch Laboratories, Inc.) at 25˚C for 1 h in dark. The nucleus was stained with DAPI (Beyotime Institute of Biotechnology) at 25˚C for 5 min. The images were obtained under a fluorescence microscope (Nikon Corporation).

Western blotting. Proteins were extracted from lung tissues using ice-cold RIPA lysis buffer, and quantified by BCA kit (both from Beyotime Institute of Biotechnology). An equal amount of protein (2 μg) was separated on 10% gels using SDS-PAGE and then transferred to PVDF membranes. The membranes were blocked with 5% skimmed milk for 1 h at 25˚C, followed by incubation with primary antibodies, including HCAR1 (1:1,000; product code ab106942), MHC II (1:1,000; product code ab55152), TLR4 (1:1,000; product code ab13556), NLRP3 (1:1,000; product code ab263899), GFP (1:1,000; product code ab290), and GAPDH (1:1,000; product code ab8245; all from Abcam) at 4˚C overnight. The following day, the membranes were incubated with HRP-conjugated secondary antibodies (1:1,000; product codes ab150077, ab150113 and ab6741; all Abcam) at 25˚C for 1 h, and visualized using ECL reagent (Millipore; Merck KGaA). Images were obtained on a gel imaging system (Bio-Rad Laboratories, Inc.). ALL antibodies were purchased from Abcam and were used according to the manufacturer's protocols. The densitometric analysis was performed using ImageJ (1.8.0; National Institutes of Health).

Statistical analysis. Statistical analysis was conducted by using SPSS 21.0 software (IBM Corp.). Differences between two groups were analyzed using unpaired Student's t-test and differences among multiple groups were analyzed using one-way ANOVA followed by Tukey's post hoc test. Correlation analysis was performed using Pearson's correlation analysis. The diagnostic value of HCAR1, TLR4, MHC II, NLRP3, IL-1β, TNF-α, IL-10, and IL-18 in patients with sepsis was assessed using receiver operating characteristic (ROC) curves. In this method, a perfect biomarker has 100% sensitivity, shows no false positives (100% specificity), and produces an area under the curve (AUC) of 1.0, while a biomarker with no diagnostic value has an AUC of 0.5. Youden's index with the highest sum of sensitivity and specificity was used to determine the optimal cut-off value for differentiation. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Expression levels of HCAR1, TLR4, MHC II, NLRP3, and inflammatory factors in the peripheral blood of patients with sepsis. The expression levels of HCAR1, TLR4, MHC II, NLRP3, and inflammatory factors in the peripheral blood of patients with sepsis were first evaluated. The results revealed that the mRNA expression of HCAR1 was reduced
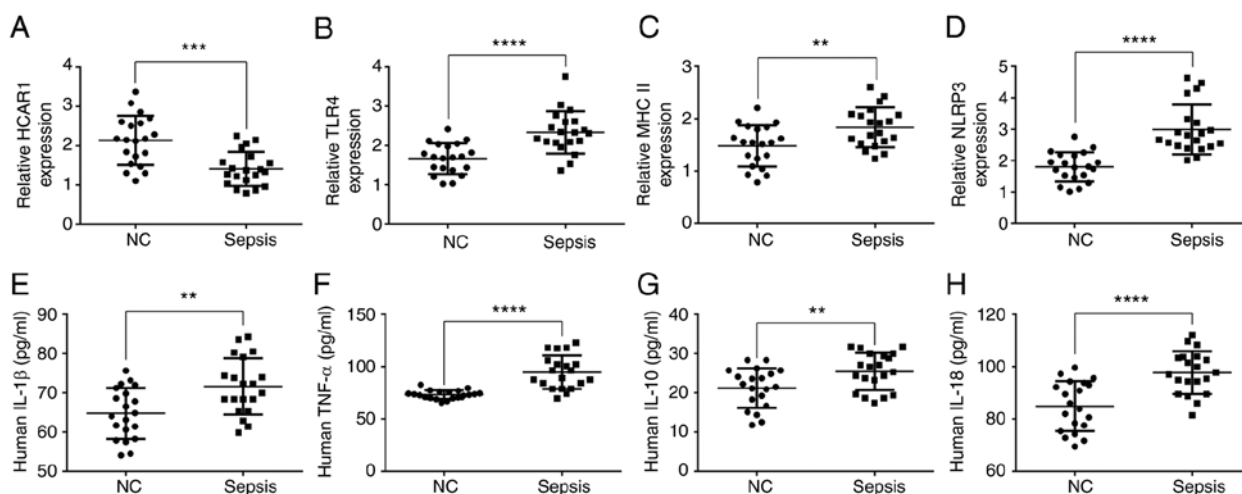
Figure 1. Expression levels of HCAR1, TLR4, MHC II, NLRP3, and inflammatory factors (IL-1β, TNF-α, IL-10, and IL-18) in the peripheral blood of patients with sepsis. The levels of HCAR1 (A), TLR4 (B) MHC II (C), and (D) NLRP3 were assessed by quantitative PCR in the peripheral blood of patients with sepsis and healthy controls. The levels of (E) IL-1β, (F) TNF-α, (G) IL-10, and (H) IL-18 were detected by ELISA assays. **P<0.01, ***P<0.001 and ****P<0.0001. HCAR1, hydroxy-carboxylic acid receptor 1; TLR4, Toll-like receptor 4; MHC II, major histocompatibility complex class II; NLRP3, NOD-like receptor family pyrin domain containing 3; IL, interleukin; TNF-α, tumor necrosis factor-α; NC, normal control.
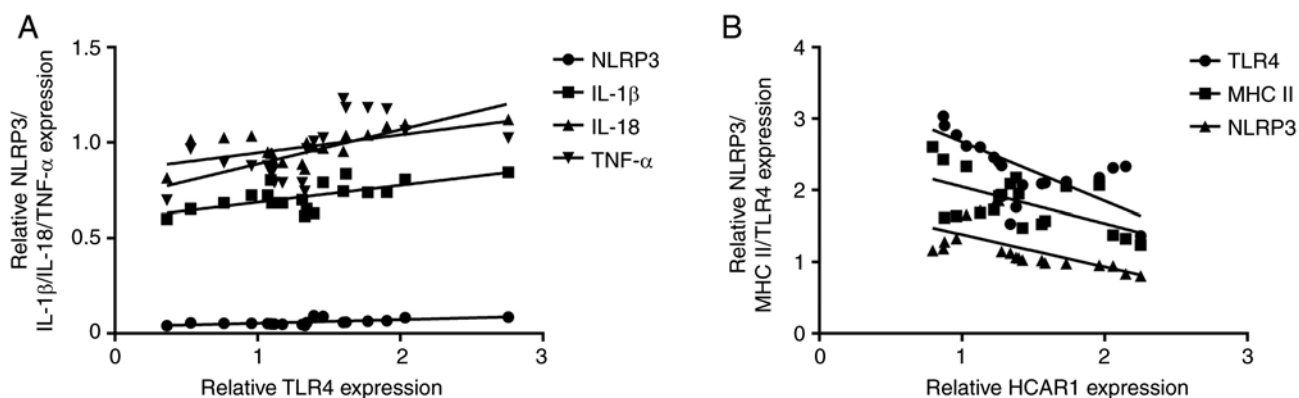

Figure 2. Correlation of the expression levels of HCAR1, TLR4, MHC II, NLRP3, and inflammatory factors (IL-1β, TNF-α, IL-10, and IL-18) in the peripheral blood of patients with sepsis. (A and B) The correlation of the expression levels of HCAR1, TLR4, MHC II, NLRP3, IL-1β, TNF-α, IL-10, and IL-18 were analyzed in peripheral blood of patients with sepsis. HCAR1, hydroxy-carboxylic acid receptor 1; TLR4, Toll-like receptor 4; MHC II, major histocompatibility complex class II; NLRP3, NOD-like receptor family pyrin domain containing 3; IL, interleukin; TNF-α, tumor necrosis factor-α.

while the mRNA expression levels of TLR4, MHC II, and NLRP3 were enhanced in the peripheral blood of patients with sepsis compared with those in the healthy controls (P<0.01, P<0.001 and P<0.0001; Fig. 1A-D and Table I). In addition, the levels of IL-1β, TNF-α, IL-10, and IL-18 were elevated in the peripheral blood of patients with sepsis compared with those in the healthy controls (P<0.01 and P<0.0001; Fig. 1E-H).

Correlation of the expression levels of HCAR1, TLR4, MHC II, NLRP3, and inflammatory factors in the peripheral blood of patients with sepsis. Next, the correlation of HCAR1, TLR4, MHC II, NLRP3, and inflammatory factors in the peripheral blood of patients with sepsis was evaluated. It was observed that the expression of TLR4 was positively associated with NLRP3 (r=0.641), IL-1β (r=0.666), TNF-α (r=0.606), and IL-18 (r=0.624) levels in the peripheral blood of patients with sepsis (Fig. 2A). In addition, it was further observed that the expression of HCAR1 was negatively correlated with TLR4 (r=-0.666),

MHC II (r=-0.587), and NLRP3 (r=-0.621) expression in the peripheral blood of patients with sepsis (Fig. 2B).

Diagnostic value of HCAR1, TLR4, MHC II and NLRP3 mRNA expression and IL-1β, IL-18, TNF-α and IL-10 levels for predicting sepsis. HCAR1, TLR4, MHC II and NLRP3 mRNA expression and IL-1β, IL-18, TNF-α and IL-10 levels were then assessed to determine their diagnostic value for predicting sepsis. The AUC values of IL-1β, IL-18, TNF-α, and IL-10 were 0.751, 0.841, 0.924 and 0.729, respectively, in which TNF-α exhibited the highest diagnostic value (Fig. 3A). ROC curve analysis revealed that the AUC values of HCAR1, TLR4, MHC II and NLRP3 mRNA expression were 0.830, 0.853, 0.735 and 0.945, respectively, in which NLRP3 exhibited the highest diagnostic value (Fig. 3B).

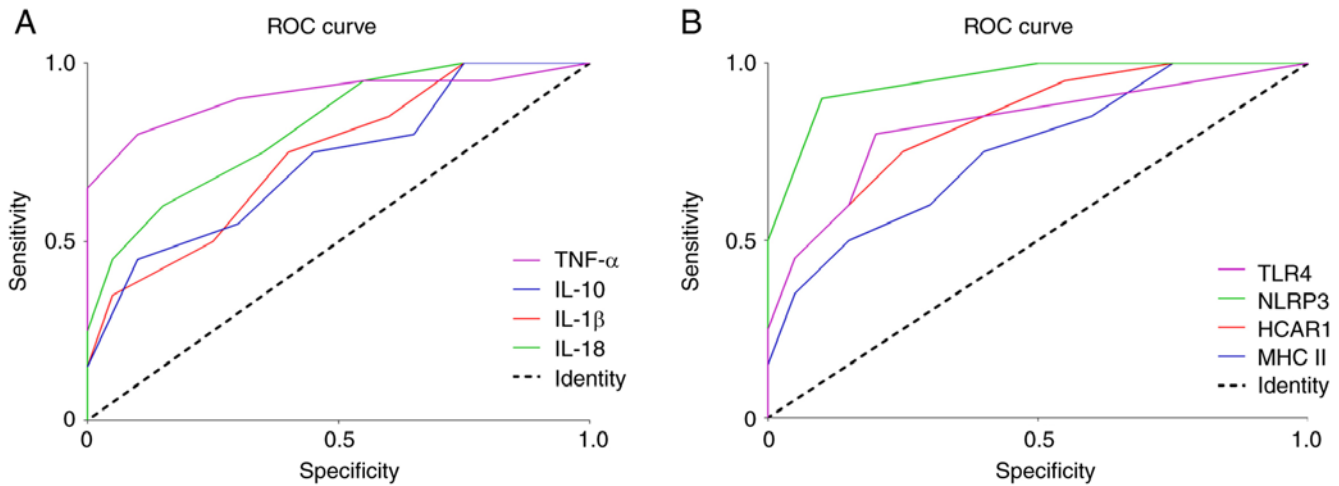*Isolation and identification of ADSCs.* In order to analyze the effect of ADSC-derived HCAR1 on sepsis *in vivo*, the

Figure 3. Diagnostic value of HCAR1, TLR4, MHC II and NLRP3 mRNA expression and IL-1β, IL-18, TNF-α and IL-10 levels for predicting sepsis. (A and B) The diagnostic value of HCAR1, TLR4, MHC II and NLRP3 mRNA expression and IL-1β, IL-18, TNF-α and IL-10 levels for predicting sepsis was revealed using ROC curve analysis. HCAR1, hydroxy-carboxylic acid receptor 1; TLR4, Toll-like receptor 4; MHC II, major histocompatibility complex class II; NLRP3, NOD-like receptor family pyrin domain containing 3; IL, interleukin; TNF-α, tumor necrosis factor-α; ROC, receiver operating characteristic.

ADSCs were isolated and the morphology of ADSCs was observed (Fig. 4A). The majority of the adherent cells were fibroblast-like cells, but showed heterogenic morphology, including spindle-shaped or small round cells (Fig. 4A). In addition, the ADSCs were identified by the positive markers, including CD90 and CD44, while the negative markers, such as CD34 and CD45, were undetectable (Fig. 4B). Notably, the GFP-labeled ADSCs were validated (Fig. 4C) and GFP-labeled ADSCs were identified in the rats by *in vivo* fluorescence tracing (Fig. 4D), suggesting that ADSCs can be colonized in the lungs through blood circulation after transplantation.

Effect of ADSC-derived HCAR1 on lung injury of rats with sepsis. Next, the rat model of sepsis was established by injecting LPS and the rats were randomly divided into 5 groups, including the normal control group (NC group; n=6), the sepsis model group (LPS group; n=6), the ADSC transplantation group (L + M group; n=6), the combined HCAR1 receptor agonist group (L + Gi + M group; n=6), and the combined HCAR1 receptor inhibitor group (L + Gk + M group; n=6). Using H&E staining, the comparison between the L + M group and the LPS group revealed that there were still neutrophils and inflammatory cell infiltrates in the L + M group, but congestion and edema were reduced and the alveolar cavity and interstitial structures were partially intact. In the L + Gi + M group inflammatory cell infiltrates were markedly reduced in comparison with the L + M and LPS groups, with no obvious congestion and edema and clear alveolar and interstitial structures (Fig. 5A). The L + Gk + M group compared with the L + Gi + M and L + M groups revealed a marked increase of inflammatory cell infiltration, with visible congestion and edema, and partially clear alveoli and interstitial structures, but the degree of pathological damage was weaker than that of the LPS group (Fig. 5A). In addition, it was determined that the L + M group exhibited an increase in the number of ADSCs in the lung tissue compared with the LPS group (Fig. 5B). The L + Gi + M group showed an

enhanced number of ADSCs compared with the L + M group (Fig. 5B). The L + Gk + M group showed a marked decrease in the number and distribution of ADSCs compared with the L + Gi + M and L + M groups (Fig. 5B). Notably, it was identified that the L + Gk + M group demonstrated a marked increase of inflammatory cell infiltration, with visible congestion and edema, and partially clear alveoli and interstitial structures, but the degree of pathological damage was weaker than that of the LPS group, while the treatment with the NF-κB inhibitor PDTC could reverse the effect, implying that ADSC-derived HCAR1 may regulate sepsis through NF-κB signaling (Fig. 5C).

Effect of ADSC-derived HCAR1 on the expression levels of HCAR1, TLR4, MHC II, NLRP3, and inflammatory factors in rats with sepsis. Furthermore, it was observed that the expression levels of HCAR1 were reduced while those of TLR4, MHC II, and NLRP3 were enhanced in the rats with sepsis, while the L + M treatment produced the opposite results. In addition, the treatment of L + Gi + M further enforced the effect but L + Gk + M treatment rescued the effect in the system (P<0.05; Fig. 6A-E). In addition, the expression of IL-1β, IL-18 and TNF-α in the peripheral blood of the rats was significantly higher in the LPS group compared with the NC group. The expression of IL-1β, IL-18 and TNF-α in the L + M group was significantly lower compared with the LPS group. The L + Gi + M group exhibited decreased expression compared with the L + M and LPS groups, but still exhibited increased expression compared with the NC group. In addition, the L + Gk + M group exhibited significantly higher expression compared with the L + Gi + M, L + M groups. The expression of IL-10 was significantly higher in the LPS group compared with the NC group, in the L + M group compared with the LPS group, and in the L + Gi + M group compared with both the L + M and NC groups. The L + Gk + M group exhibited a significant decrease in expression of IL-10 compared with the L + Gi + M, L + M groups, but still higher than the LPS group (P<0.05; Fig. 6F-I).
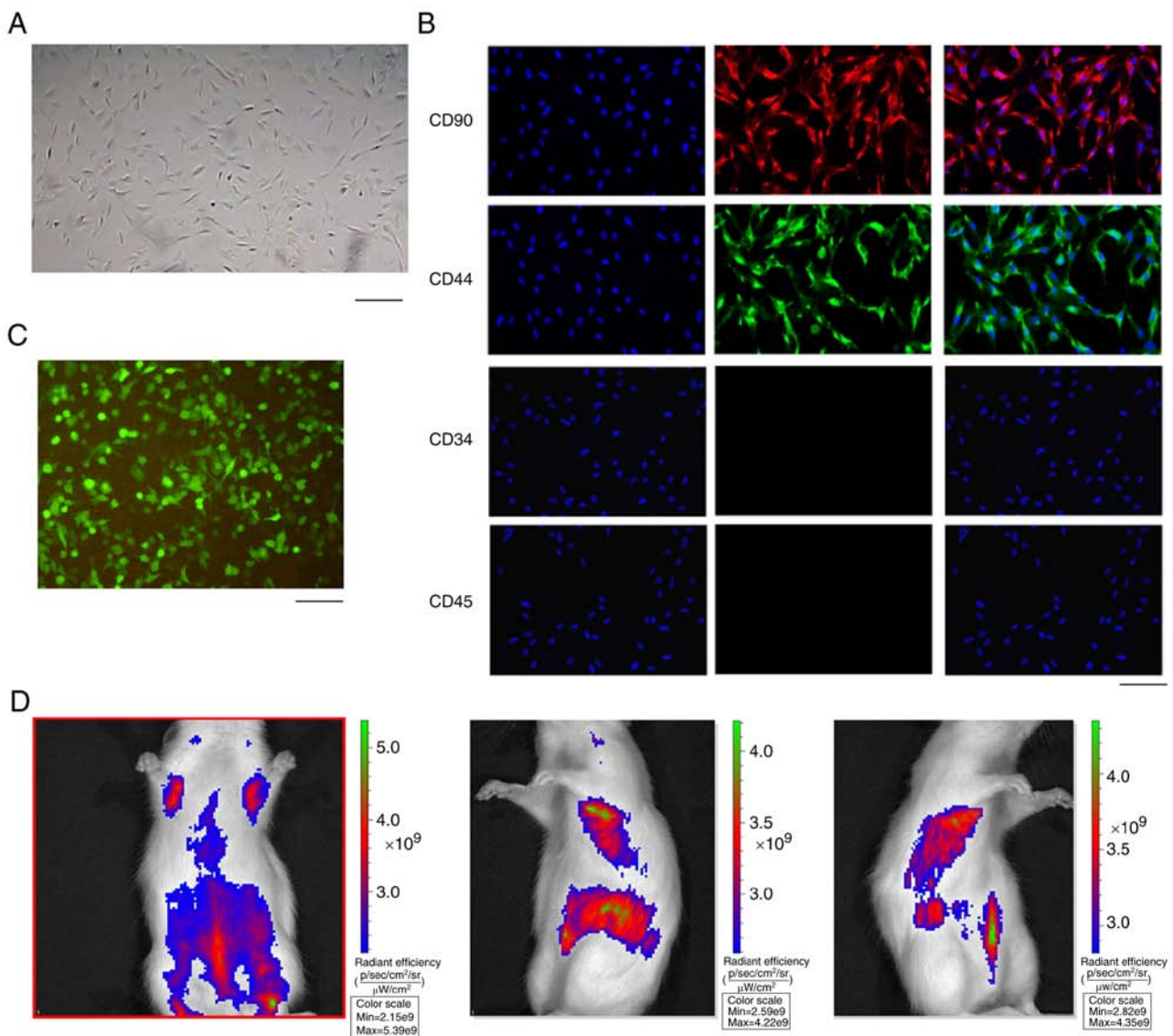
Figure 4. Isolation and identification of ADSCs. (A) The morphology of ADSCs is presented. Scale bar, 50 μm. (B) The ADSCs were identified by the positive and negative markers, including CD90, CD44, CD34, and CD45, using immunofluorescence analysis. Scale bar, 50 μm. (C and D) The GFP-labeled ADSCs was identified by *in vivo* fluorescence tracing. Scale bar, 50 μm. ADSCs, adipose-derived mesenchymal stem cells.

Discussion

Sepsis is a life-threatening disease in which the body normally produces an organismic immune response to bacterial infection, which can cause tissue and organ damage when the immune response is too intense (28). As a key receptor molecule in the classical inflammatory signaling pathway, TLR4 can be activated and activate downstream NLRP3 inflammatory vesicles to participate in the inflammatory response, and TLR-4 receptors are activated by exogenous signaling molecules to cause the secretion of cytokines such as IL-1β, IL-18 and TNF-α (29). In the present study, the expression levels of HCAR1, TLR4, MHC II, NLRP3, and inflammatory factors were assessed in the peripheral blood of patients with sepsis. It was determined that the mRNA expression of HCAR1 was reduced while the mRNA expression levels of TLR4, MHC II, and NLRP3, and the levels of IL-1β, TNF-α, IL-10, and IL-18 were enhanced in the peripheral blood of patients with sepsis compared with those in the healthy controls. These data indicated that the

tissue cell damage caused by sepsis is the result of an intense inflammatory response involving multiple inflammatory cytokines activated by TLR4/NLRP3. This is similar to what was aforementioned in previous research.

HCAR1 is a receptor for lactate and has an important role in the regulation of inflammatory responses (30). It has been demonstrated that in RAW264.7 cells, lactate inhibits TLR4-mediated NF-κB activation and suppresses pro-IL-1β, NLRP3 and CASP1 gene expression (31). It has also been reported that lactate can promote immunosuppression by macrophage M2-type polarization through signaling pathways such as HCAR1/PI3K/AKT/CEBPβ/IL-10 and HCAR1/ERK/STAT3/Arg1, thus the discovery of HCAR1, makes lactate no longer an inert molecule but a bioactive molecule (32,33). In the present study, it was found that the expression levels of HCAR1 in clinical peripheral blood and animal peripheral blood and lung tissues were significantly negatively associated with the expression of TLR4, MHC II and NLRP3 in both the clinical sepsis group and the LPS group
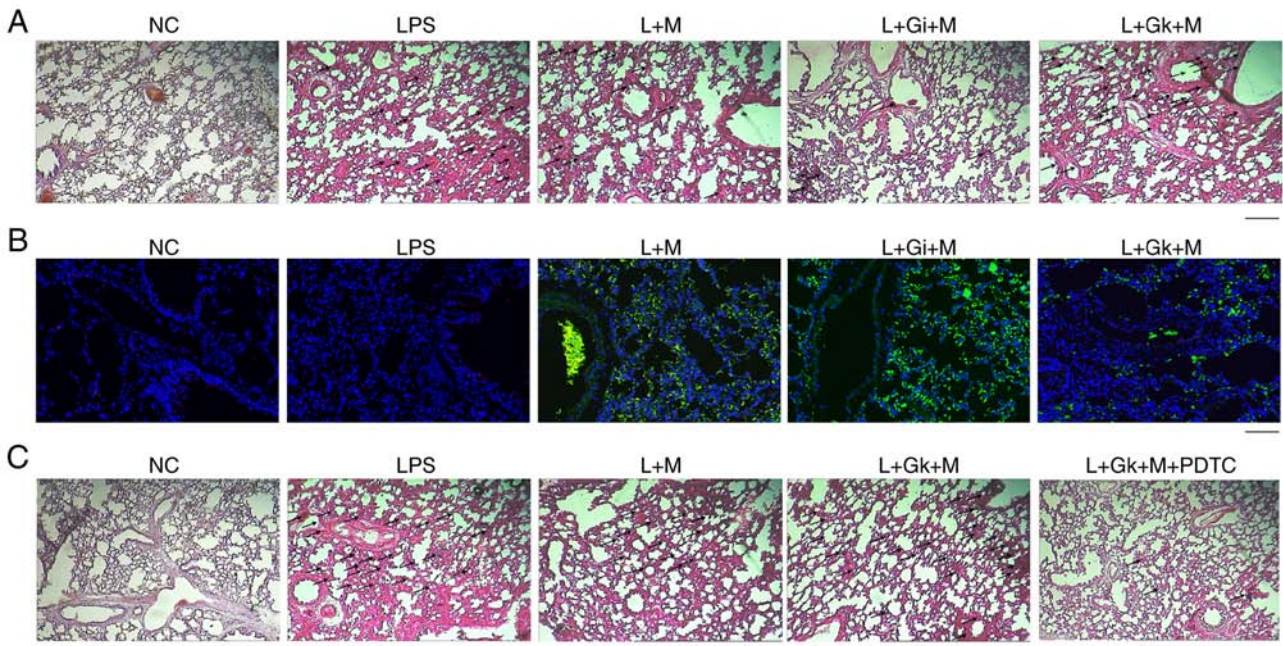
Figure 5. Effect of ADSC-derived HCAR1 on lung injury of sepsis rats. (A) The lung injury was analyzed using H&E staining in the rats with sepsis. Scale bar, 50 μm. (B) The number and distribution of ADSCs in the lung tissues of rats with sepsis was detected by immunofluorescence analysis. Scale bar, 50 μm. (C) The lung injury was analyzed using H&E staining in the rats with sepsis. The following groups were observed: The normal control group (NC), the sepsis model group (LPS), the adipocyte mesenchymal stem cell transplantation group (L + M), the combined HCAR1 receptor agonist group (L + Gi + M), the combined HCAR1 receptor inhibitor group (L + Gk + M), and the combined PDTC (NF-κB inhibitor group; L + Gk + M + PDTC); n=6 per group. The inflammatory cell infiltration was identified by pathologists and indicated by black arrows. Scale bar, 50 μm. ADSC, adipose-derived mesenchymal stem cell; HCAR1, hydroxy-carboxylic acid receptor 1; H&E, hematoxylin and eosin; NC, normal control; LPS, lipopolysaccharide.

of the animal model, indicating that HCAR1 expression was suppressed in the septic state, attenuating the inhibitory effect on TLR4/NLRP3 signaling activation with upregulation of MHC II expression and promoting the inflammatory response.

It has been reported that in the respiratory system, allogeneic and allogeneic MSCs effectively protected the lung from ischemia-reperfusion injury by downregulating inflammatory response signaling pathways in rats, which involved the inhibition of TLR4/MyD88/TAK1/IKK/I-κB/NF-κB signaling pathways by adipose-derived MSCs, resulting in a reduction in Cox-2, TNF-α and IL-1. TNF-α, IL-1β as well as other cytokines as a result of the aforementioned studies suggest that MSC transplantation therapy is used in life science research (33,34). As for the treatment of acute lung injury due to sepsis, it has been shown that bone marrow MSC transplantation can reduce the functional impairment of organs caused by inflammation in rats (35). In the present study, the crucial effect of ADSC-derived HCAR1 on lung injury of rats with sepsis was identified. ADSCs can reduce the inflammatory response and improve lung tissue injury, similar to the results of the aforementioned study. Moreover, it has been reported that miR-17 is inhibited in sepsis and MSC-derived extracellular vesicles transfer miR-17 to attenuate LPS-induced sepsis (36). In the present study, it was also revealed that the mRNA expression of HCAR1 was reduced in sepsis and that ADSC-derived HCAR1 regulated the immune response in the attenuation of sepsis. The data of the present is consistent with the previous aforementioned study. Some negative feedback mechanism may be involved in the decrease of the expression level of HCAR1 in sepsis and should be explored in future investigations. However, in the present study, it was revealed

that ADSCs failed to completely repair the damaged lung tissues after transplantation and did not achieve the expected results. The immune rejection involved is mainly related to the antigen presentation-related molecule MHC II on the surface of APCs, which, on the one hand, upregulates the expression of inflammatory factors such as IL-6, IL-12 and TNF-α, and on the other hand promotes the TLR4/MyD88/NF-κB signaling pathway and increases the production of inflammatory factors, while it also presents alloantigens. It can also present allogeneic antigens to T lymphocytes, which promote cellular immunity to produce a large number of cytokines and chemokines, thus promoting humoral immunity to remove allogeneic substances (37,38). In the present study, it was determined that activation of the lactate receptor HCAR1 inhibited the maturation of antigen-presenting peripheral blood dendritic cells (DC) and also promoted the polarization of antigen-presenting macrophages to the M2 type in tissues, both of which were characterized by a downregulation of the expression of MHC II molecules on the cell surface. Thus, in the experimental results, it was found that in the septic state, the results of genetic testing of peripheral blood and lung tissue of clinical patients and model rats showed that HCAR1 expression was significantly downregulated in the sepsis and LPS groups compared with the normal and NC groups, whereas MHC II expression was significantly increased, which is involved in the septic inflammatory response.

There are still some limitations in the present study. Further in vitro experiments are needed to verify the modulatory effect of HCAR1 on this immune rejection of MHC II and its effect on stem cell transplantation survival. In addition, the function of HCAR1 derived from ADSCs, in the attenuation
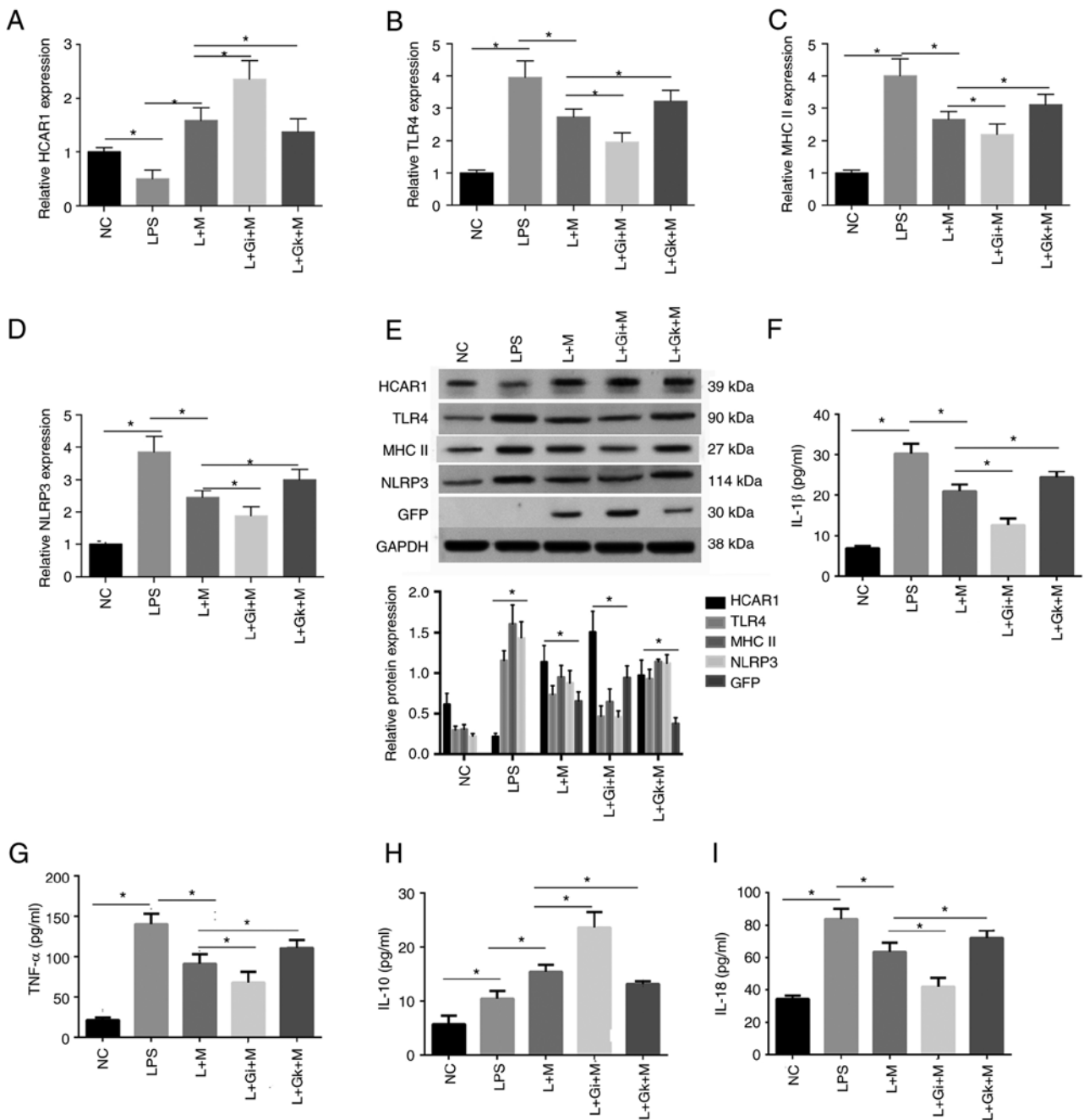
Figure 6. Effect of ADSC-derived HCAR1 on the expression levels of HCAR1, TLR4, MHC II, NLRP3, and inflammatory factors (IL-1β, TNF-α, IL-10, and IL-18) in rats with sepsis. (A-D) The levels of HCAR1 (A), TLR4 (B), MHC II (C), and NLRP3 (D) were assessed by quantitative PCR in the peripheral blood and lung tissues of rats with sepsis. (E) The protein levels of HCAR1, TLR4, MHC II and NLRP3 were analyzed by western blotting in the lung tissues of rats with sepsis. (F-I) The levels of IL-1β, TNF-α, IL-10, and IL-18 were detected by ELISA assays. The following groups were assessed: The normal control group (NC), the sepsis model group (LPS), the adipocyte mesenchymal stem cell transplantation group (L + M group), the combined HCAR1 receptor agonist group (L + Gi + M group), the combined HCAR1 receptor inhibitor group (L + Gk + M group); n=6 per group. Results were analyzed using ANOVA; $^{*}P<0.05$. ADSC, adipose-derived mesenchymal stem cell; HCAR1, hydroxy-carboxylic acid receptor 1; TLR4, Toll-like receptor 4; MHC II, major histocompatibility complex class II; NLRP3, NOD-like receptor family pyrin domain containing 3; IL, interleukin; TNF-α, tumor necrosis factor-α; NC, normal control.

of LPS-induced lung injury in sepsis was identified. However, which types of HCAR are present in ADSCs and further investigation with regard to HCAR1 protein expression in ADSCs should be performed to characterize the receptor of interest. In addition, the sample size for the ROC analysis was small and in future a larger sample size is warranted. In addition, the experimental results did not reveal a dose-effect or time-effect association, and there were some contradictions in the experimental results. Furthermore, the investigation into

the mechanism showed that the treatment of NF-κB inhibitor PDTC could reverse the effect of L + Gk + M on sepsis, indicating that ADSC-derived HCAR1 may regulate sepsis through NF-κB signaling. The specific mechanism should be confirmed with more studies in the future.

In summary, it was concluded that ADSC-derived HCAR1 regulates the immune response in the attenuation of sepsis. ADSC-derived HCAR1 may be a promising therapeutic strategy for sepsis.

Availability of data and materials

The datasets used during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

HW, PX and LQ designed the study. HW, PX, HT, XH, JY and XX performed the experiments in Figs. 1-6. HW, PX, JPY and LQ wrote the manuscript. XX and LQ confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All experiments were performed under the authorization and guidelines of the Ethics Committee of the Third Affiliated Hospital of Inner Mongolia Medical University (Baotou, China). All participants provided signed informed consent. Animal care and method procedures were authorized by the Animal Ethics Committee (approval no. 2020-0618-37) of the Third Affiliated Hospital of Inner Mongolia Medical University.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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