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Original Article

Regulatory Effect of JAK2/STAT3 on the Immune Function of Endotoxin-tolerant Dendritic Cells and its Involvement in Acute Liver Failure

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Abstract

Background and Aims: Acute liver failure (ALF) is a potentially fatal clinical syndrome with no effective treatment. This study aimed to explore the role of Janus kinase 2/signal transducer and activator of transcription 3 (JAK2/STAT3) pathway in modulating the phenotype and immune function of endotoxin-tolerant dendritic cells (ETDCs). In addition, we explored the use of EDTCs in an experimental model of ALF and investigated the associated mechanisms. ***Methods:*** In the *in vitro* experiment, ETDCs were transfected with adenovirus to induce SOCS1$^{+/+}$ETDCs and SOCS1$^{-/-}$ETDCs. Thereafter, costimulatory molecules and mixed lymphocyte reaction were assessed. Experimental mice were randomly divided into normal control, ALF, ALF+mock-ETDCs, ALF+SOCS1$^{+/+}$ETDCs, ALF+AG490, and ALF+AG490+SOCS1$^{+/+}$ETDCs groups. We examined the therapeutic effect of adoptive cellular immunotherapy by tail-vein injection of target ETDCs 12 h before ALF modeling. AG490, a JAK2/STAT3 inhibitor, was used in the *in vivo* experiment to further explore the protective mechanism of SOCS1$^{+/+}$ETDCs. ***Results:*** Compared with control ETDCs, SOCS1$^{+/+}$ETDCs had lower expression of costimulatory molecules, weaker allostimulatory ability, lower levels of IL-6 and TNF-α expression and higher IL-10 secretion. SOCS1$^{-/-}$ETDCs showed the opposite results. In the *in vivo* experiments, the ALF+SOCS1$^{+/+}$ETDCs and ALF+AG490+SOCS1$^{+/+}$ETDCs groups showed less pathological damage and suppressed activation of JAK2/STAT3 pathway. The changes were more pronounced in the ALF+AG490+SOCS1$^{+/+}$ETDCs group. Infusion of SOCS1$^{+/+}$ETDCs had a protective effect against ALF possibly via inhibition of JAK2 and STAT3 phosphorylation. ***Conclusions:*** The *SOCS1* gene had an important role in induction of endotoxin tolerance. SOCS1$^{+/+}$ETDCs alleviated lipopolysaccharide/D-galactosamine-induced ALF by downregulating the JAK2/STAT3 signaling pathway.

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Introduction

Acute liver failure (ALF) is a life-threating syndrome caused by massive necrosis of liver cells and rapid impairment of hepatic function. The key clinical features include coagulopathy, jaundice, hepatic encephalopathy, and ascites.[1,2] Despite significant advances in management and therapy, there is a paucity of effective treatment modalities for ALF. Currently, the only suitable available treatment is liver transplantation. However, the high cost of liver transplantation, shortage of donors, and the risk of immune rejection are key barriers to its wider use.[1,2] Immune injury has an important role in the progression of ALF. Therefore, development of strategies for suppressing the excessive immune response in the setting of ALF is a key imperative.[2,3]

Dendritic cells (DCs) are antigen-presenting cells (APCs) that play a critical role in the initiation and orchestration of the immune response.[4] As a special class of immune cells bridging innate and adaptive immunity, DCs are currently an immunologic and biomedical research hotspot. As APCs, the crucial role of DCs is to capture, process, and present antigens to T cells and initiate the immune response.[5] Adoptive cell therapy (ACT) employs specific immune cells

Keywords: Liver failure; Acute; Janus kinase 2/signal transducers and activators of transcription 3 (JAK2/STAT3); Endotoxin tolerance; Dendritic cells.

Abbreviations: ACT, adoptive cell therapy; Ad-mock, negative-control adenovirus; ALF, acute liver failure; ALT, alanine aminotransferase; APC, allophycocyanin; APCs, antigen-presenting cells; AST, aspartate aminotransferase; Ad-SOCS1, adenovirus encoding SOCS1; DCs, dendritic cells; EAE, experimental autoimmune encephalomyelitis; ECL, electrochemiluminescence; ET, endotoxin tolerance; FBS, fetal bovine serum; G$^-$, Gram-negative; GM-CSF, granulocyte-macrophage colony stimulating factor; HCMV, human cytomegalovirus; HE, hematoxylin-eosin; IRAK, interleukin-1 receptor-associated kinase; JAK2/STAT3, Janus kinase 2/signal transducer and activator of transcription 3; LPS, lipopolysaccharide; NF-κB, nuclear factor kappa B; PE, Phycoerythrin; PVDF, polyvinylidene fluoride; qRT-PCR, quantitative real-time reverse transcription-polymerase chain reaction; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; shRNA, small hairpin RNA; SOCS1, suppressor of cytokine signaling 1; TNF-α, tumor necrosis factor-alpha; Treg, regulatory T cell.

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that are induced, modified, and expanded *in vitro* prior to their infusion into the patient.[6,7] As the ideal immune cell type for ACT, DCs with specific genetic modifications have been widely applied in the fields of autoimmune disease and carcinoma.[8,9]

Endotoxin tolerance (ET) is a condition wherein organisms or cells become less responsive to a lethal dose of lipopolysaccharide (LPS) after successive primary exposures to low-dose LPS.[10–12] Endotoxin, an LPS-protein component found in the outer membrane of Gram-negative (G^-) bacteria, is the main activator of innate immunity.[13,14] ET is a self-protective mechanism involving the inhibition of toll-like receptors, interleukin-1 receptor-associated kinase (IRAK) 1 and 4, and nuclear factor kappa B (NF-κB) signaling.[15,16] Studies have demonstrated the phenomenon of ET not only in the context of infectious diseases but also in noninfectious diseases.[10,17] Endotoxin-tolerant dendritic cells (ETDCs) have been shown to suppress the differentiation of TH17 cells and inhibit the secretion of inflammatory cytokines and differentiation of $CD4^+$ T regulatory cells.[18] Studies have shown the therapeutic effect of adoptive transfer of ETDCs in experimental models of chronic kidney disease and autoimmune diseases such as experimental autoimmune encephalomyelitis (EAE).[19,20] However, the underlying mechanism of ET is not fully elucidated.

The suppressor of cytokine signaling 1 (SOCS1), acting as a negative feedback loop, is a critical regulator of the Janus kinase 2/signal transducer and activator of transcription 3 (JAK2/STAT3) signaling pathway.[21] Cytokines such as interleukin (IL) 6, IL10, and tumor necrosis factor-alpha (TNF-α) were shown to promote SOCS1 expression. Previous studies have shown the functions of SOCS1 in the context of liver injury, anti-human cytomegalovirus (HCMV) treatment, and ischemic stroke/neuroinflammation.[22–24] Ag490 is an inhibitor of the cytokine receptor-mediated signaling pathways that can specifically inhibit the JAK2/STAT3 signal pathway. It has been widely used in the study of inflammation, tumors, and autoimmune diseases.[25] In this study, we investigated the effect of overexpression and silencing of the *SOCS1* gene on the phenotype and function of ETDCs *in vitro*. In an *in vivo* experiment, we explored the therapeutic effect of $SOCS1^{+/+}$ETDCs in ALF mice and investigated whether the JAK2/STAT3 pathway had a major role in the *in vitro* results. Herein, we offer a potential strategy that may facilitate the clinical use of adoptive transfer of ETDCs in the treatment of ALF.

Methods

Animals

Male BALB/C and C57BL/6 mice 6–7 weeks of age were purchased from the Shanghai Laboratory Animal Center (Shanghai, China). All animals were maintained in an air-conditioned laminar flow facility at 22±2°C on a 12 h light/dark cycle. All animal experiments were carried out in accord with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Animal Ethics Committee of Wenzhou Medical University. All surgical procedures were performed under sodium pentobarbital anesthesia, and all efforts were made to minimize animal suffering.

Reagents

IL4 and granulocyte-macrophage colony stimulating factor (GM-CSF) were purchased from PeproTech (London, UK). Phycoerythrin (PE)- or allophycocyanin (APC)-conjugated mAbs to CD86, CD80 and CD11c were purchased from eBioscience (San Diego, CA, USA). Ag490 and mitomycin-C were purchased from MedChemExpress (Monmouth Junction, NJ, USA). D-GalN (G0500-5g) and lipopolysaccharide (LPS, L-2880-10 mg) were purchased from Sigma (St. Louis, MO, USA). Mouse Th17 and mouse regulatory T Cell (Treg) ELISA staining kit were purchased from MultiSciences (Hangzhou, China). Adenovirus encoding SOCS1 (Ad-SOCS1), negative-control adenovirus (Ad-mock), small hairpin RNA (shRNA)-encoding SOCS1 (Ad-shSOCS1), and negative-control adenovirus (Ad-shmock) were constructed by Hanbio (Shanghai, China); the adenoviral vector was pDC315-EGFP and pDC311-U6-MCMV-EGFP, respectively.

Preparation of ETDCs

Bone marrow mesenchymal stem cells were extracted from the tibia and femur of male BALB/C mice. Red blood cells were removed with lysis buffer and neutralized with fresh medium containing 5% fetal bovine serum (FBS; Gibco, Waltham, MA, USA). Then, the cells were transferred to 6-well plates and cultured in RPMI 1640 medium (Gibco, USA) containing 10% FBS and 10 ng/mL IL4 and 20 ng/mL GM-CSF to induce bone marrow-derived dendritic cells. The cell supernatant was replaced with fresh medium on day 2. On day 4, cells were cocultured with a low dose of LPS (100 ng/mL) for 48 h to induce ETDCs.

Adenovirus transfection

ETDCs were transfected with Ad-SOCS1, Ad-mock, Ad-shSOCS1 and Ad-shmock at a fit multiplicity of infection for 7 h on day 5. The resulting ETDCs were labeled as ETDCs, ETDCs + LPS, $SOCS1^{+/+}$ETDCs, mock-ETDCs, $SOCS1^{-/-}$ETDCs, and shmock-ETDCs. On day 9, except for those in the ETDCs group, the cells stimulated with high-dose LPS (10 μg/mL) to induce maturation of DCs. On the next day, the ETDCs were collected for subsequent experiments.

Assay of T cells proliferation

$CD4^+$ T cells were isolated from the spleens of C57BL/6 mice and plated in triplicate wells in a 96-well plates. Allogeneic ETDCs were pretreated with mitomycin-c (25 μg/mL) for 30 m and then cocultured with $CD4^+$ T cells at different densities (1:15, 1:10). After 4 days of cocultivation, the cells were treated with CCK-8 solution for 4 h in normal culture conditions, and the absorbance was measured at 450 nm to assess proliferation.

Animal model

Seven-week-old male BALB/C mice were randomly assigned to normal control, ALF, ALF+mock-ETDCs, ALF+$SOCS1^{+/+}$ETDCs, ALF+AG490, and ALF+AG490+$SOCS1^{+/+}$ETDCs groups of 10 each. The ALF model was constructed by intraperitoneal injection of LPS (10 mg/kg) and D-GalN (600 mg/kg) dissolved in neutral saline solution. Mice in the normal control group received intraperitoneal injection of the same volume of neutral saline. Mice in the ALF+AG490 group and ALF+AG490+$SOCS1^{+/+}$ETDCs group received once-daily intraperitoneal injections of AG490 (25 mg/kg) for 5 days before model construction. Mice in the other groups received once-daily injections of an equal volume of phos-

phate buffered saline. Twelve hours before ALF induction, mice in the ALF+mock-ETDCs, ALF+SOCS1$^{+/+}$ETDCs, and ALF+AG490+SOCS1$^{+/+}$ETDCs groups received tail vein injections of mock-ETDCs or SOCS1$^{+/+}$ETDCs (1×10^6 cells per mouse) respectively. Mice in the control, ALF, and ALF+AG490 groups received injections of an equal volume of NS. Mice in each group were sacrificed for subsequent experiments by intraperitoneal injection of pentobarbital sodium after 12 h of ALF modeling.

Quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR)

RNA was isolated from cells and murine livers using Trizol. *SOCS1* gene expression was assayed by qRT-PCR. Reported values were relative to those of GAPDH expression and were calculated by the $2^{-\Delta\Delta Ct}$ method. The primers. were forward 5′-ATAGAAGCCGCAGGCGTCCAG-3′andreverse5′-CAGCCGACAATGCGATCTCCC-3′.

Flow cytometry

The expression of costimulatory molecules of ETDCs was assayed by flow cytometry. Cells were harvested and stained with APC- or PE-conjugated CD86, CD11c, and CD80 for 30 m following the manufacturer's protocols. After staining, cells were washed and then fixed with 4% paraformaldehyde before analysis. Splenic lymphocytes were isolated from fresh spleen samples of mice using lymphocyte separation medium and incubated with the combination of PMA/ionomycin and PMB/monensin mixture for 6 h, then stained with CD4-FITC and IL-17-PE. For Treg cell analysis, T cells were first stained with anti-mouse CD4-FITC and CD25-APC followed by incubation with fixation/permeabilization reagent for 45 m and then stained with anti-mouse Foxp3-PE. Th-17 cells presented as CD4$^+$IL-17$^+$ T cells, and Treg cells presented as CD4$^+$CD25$^+$Foxp3$^+$ T cells. Cells labeled with fluorescence-conjugated antibodies were assayed by flow cytometry, and ratios of Th17 or Treg cells to total CD4+ T cells were determined by Flowjo software.

Western blot assays

Liver tissue was lysed, and the protein lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes. The membranes were incubated in blocking buffer for 90 m at room temperature and incubated overnight with primary antibodies at 4°C and then incubated with secondary antibodies for 1 h at room temperature. Protein expression was visualized with a commercial electrochemiluminescence (ECL) system.

Hepatic pathological analysis

Liver tissues were harvested, fixed in 4% paraformaldehyde, embedded in paraffin, cut into 5 μm sections, and stained with hematoxylin-eosin (HE).

Cytokine and biomarker analysis

Levels of TNF-α, IL-6, and IL-10 in cell supernatants and serum of mice in each group were analyzed using ELISA kits according to the manufacturer's protocol. Serum levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were assessed using kits obtained from Nanjing Jiancheng Bioengineering.

Statistical analysis

Between-group differences were assessed using Student's *t*-test or analysis of variance. Data were reported as means±standard deviation. *P*-values ≤0.05 were considered statistically significant. Data analysis was performed using SPSS 20.0 (IBM Corp., Armonk, NY. USA)

Results

Successful transduction of ETDCs with Ad-SOCS1 and Ad-shSOCS1 and the effect of SOCS1 expression on allostimulatory ability

Figure 1A and D show the specific dendritic bulges on the surface of each group of ETDCs observed by light microscopy and the efficient transfection of adenovirus (approximately 90%) with an inverted fluorescence microscope. The qRT-PCR assay showed a significant increase in the expression of SOCS1 after stimulation with high-dose LPS compared with control cells. Moreover, SOCS1 mRNA expression in SOCS1$^{+/+}$ETDCs was significantly greater than that in ETDCs and mock-ETDCs ($p<0.05$, Fig. 1B); on the contrary, SOCS1 gene expression in SOCS1$^{-/-}$ETDCs was significantly less than that in ETDCs and shmock-ETDCs ($p<0.05$, Fig. 1E). To evaluate the effect of SOCS1 gene expression on the immune reaction induced by ETDCs, we assessed the proliferation of T lymphocytes cocultured with ETDCs. We observed that LPS stimulation triggered T lymphocyte proliferation (Fig. 1C). In addition, the rate of proliferation of T lymphocytes in the group cocultured with SOCS1$^{+/+}$ETDCs was lower than that in the group cocultured with ETDCs and mock-ETDCs (Fig. 1C). In contrast, the T lymphocyte proliferation rate in the SOCS1$^{-/-}$ETDCs group was much higher than that in ETDCs and shmock-ETDCs (Fig. 1F). The findings indicated a weaker allostimulatory ability of SOCS1$^{+/+}$ETDCs.

Effect of the SOCS1 gene on costimulatory molecules and the cytokine profile of ETDCs

CD11c, CD80, and CD86 levels were significantly downregulated in SOCS1$^{+/+}$ETDCs and significantly up-regulated in SOCS1$^{-/-}$ETDCs compared with ETDCs and mock-ETDCs. In addition, the expression levels of costimulatory molecules did not significantly differ between ETDCs and mock-ETDCs, suggesting that the adenovirus vector itself had no effect on the phenotype (Fig. 2A, B). As shown in Figure 3A, B, LPS stimulation induced maturation of DCs, and ETDCs that overexpressed SOCS1 had lower levels of costimulatory molecules. Collectively, the findings indicate that SOCS1 gene expression played an important role in promoting LPS-induced maturation of DCs.

The ELISA results showed an obvious increase in the secretion of TNF-α, IL-6, and IL-10 after LPS stimulation. Compared with ETDCs and mock-ETDCs, SOCS1$^{+/+}$ETDCs showed higher IL10 levels and lower IL6 and TNF-α levels (Fig. 3C). SOCS1$^{-/-}$ETDCs had lower IL10 and higher IL6 and TNF-α levels (Fig. 2C). The results indicated that the *SOCS1* gene specifically increased the production of anti-inflammatory cytokines and decreased the production of proinflammatory cytokines by ETDCs.

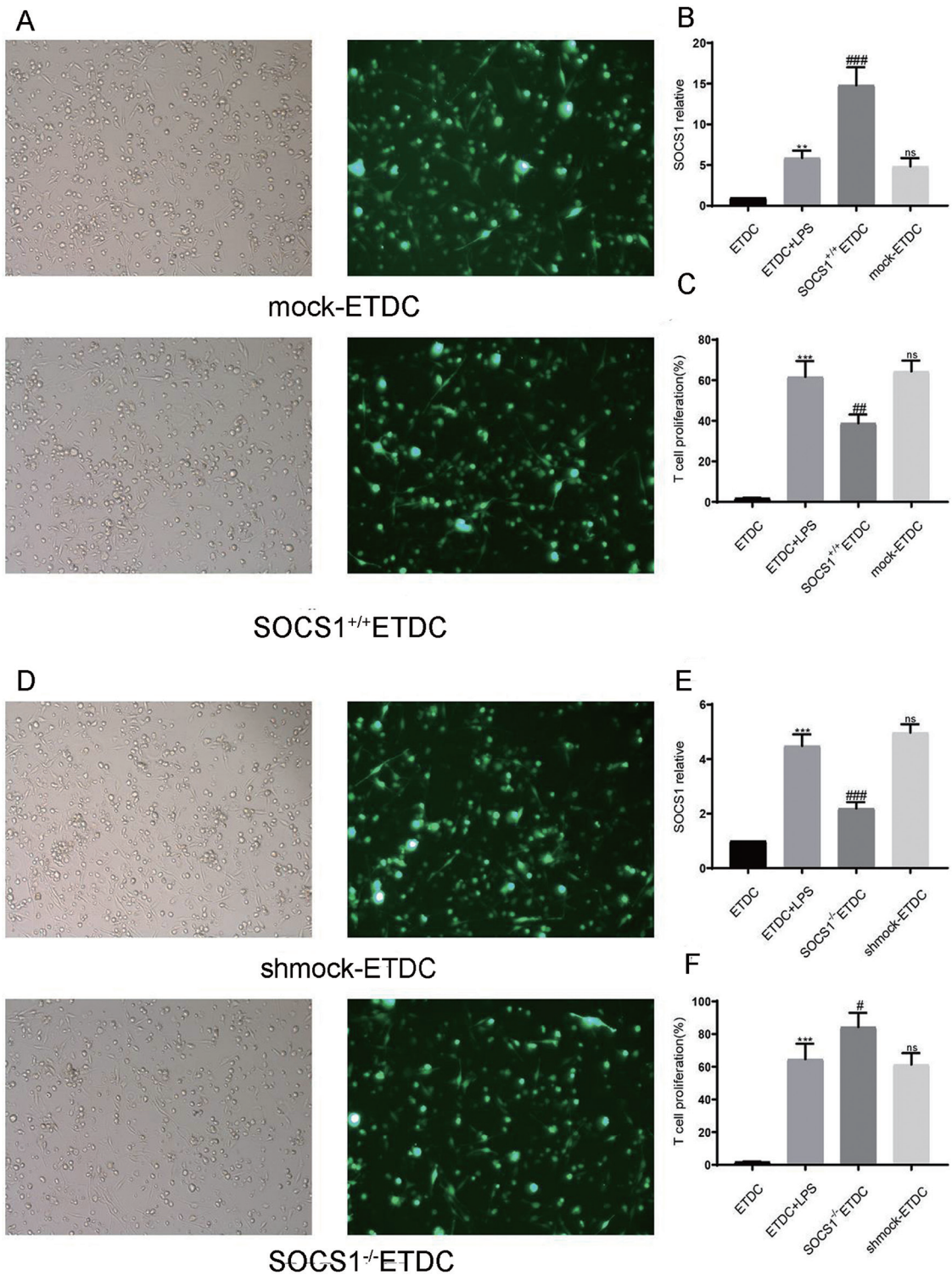

Fig. 1. Successful transduction of Ad-SOCS1 and Ad-shSOCS1, and the effect of SOCS1 expression on the allostimulatory ability of ETDCs. (A) Characteristic dendritic protrusions and adenovirus transfection efficiency of SOCS1$^{+/+}$ETDCs and mock-ETDCs. (B) qRT-PCR assay of SOCS1 expression. Data are fold-change relative to the ETDCs group. (C) Proliferation of T cells after cocultivation in each group. Mean±standard deviation of three independent assays; ns, not significant. *p<0.05, **p<0.01, ***p <0.001 vs. ETDCs group, and $^{\#}p$<0.05, $^{\#\#}p$<0.01, $^{\#\#\#}p$<0.001 vs. ETDCs+LPS group. (D) Characteristic dendritic protrusions and adenovirus transfection efficiency of SOCS1$^{-/-}$ETDCs and shmock-ETDCs. (E) qRT-PCR assay of SOCS1 expression. Data are fold-change relative to the ETDCs group. (F) T cell proliferation of after cocultivation in each group. Mean±standard deviation of three independent assays; ns, not significant. *p<0.05, **p<0.01, ***p<0.001 vs. ETDCs group, and $^{\#}p$<0.05, $^{\#\#}p$<0.01, $^{\#\#\#}p$<0.001 vs. ETDCs+LPS group (Ad-SOCS1, Adenovirus encoding SOCS1; Ad-shSOCS1, small hairpin RNA (shRNA)-encoding SOCS1; ETDCs, endotoxin-tolerant dendritic cells; qRT-PCR, quantitative real-time reverse transcription-polymerase chain reaction; SOCS1, suppressor of cytokine signaling 1).

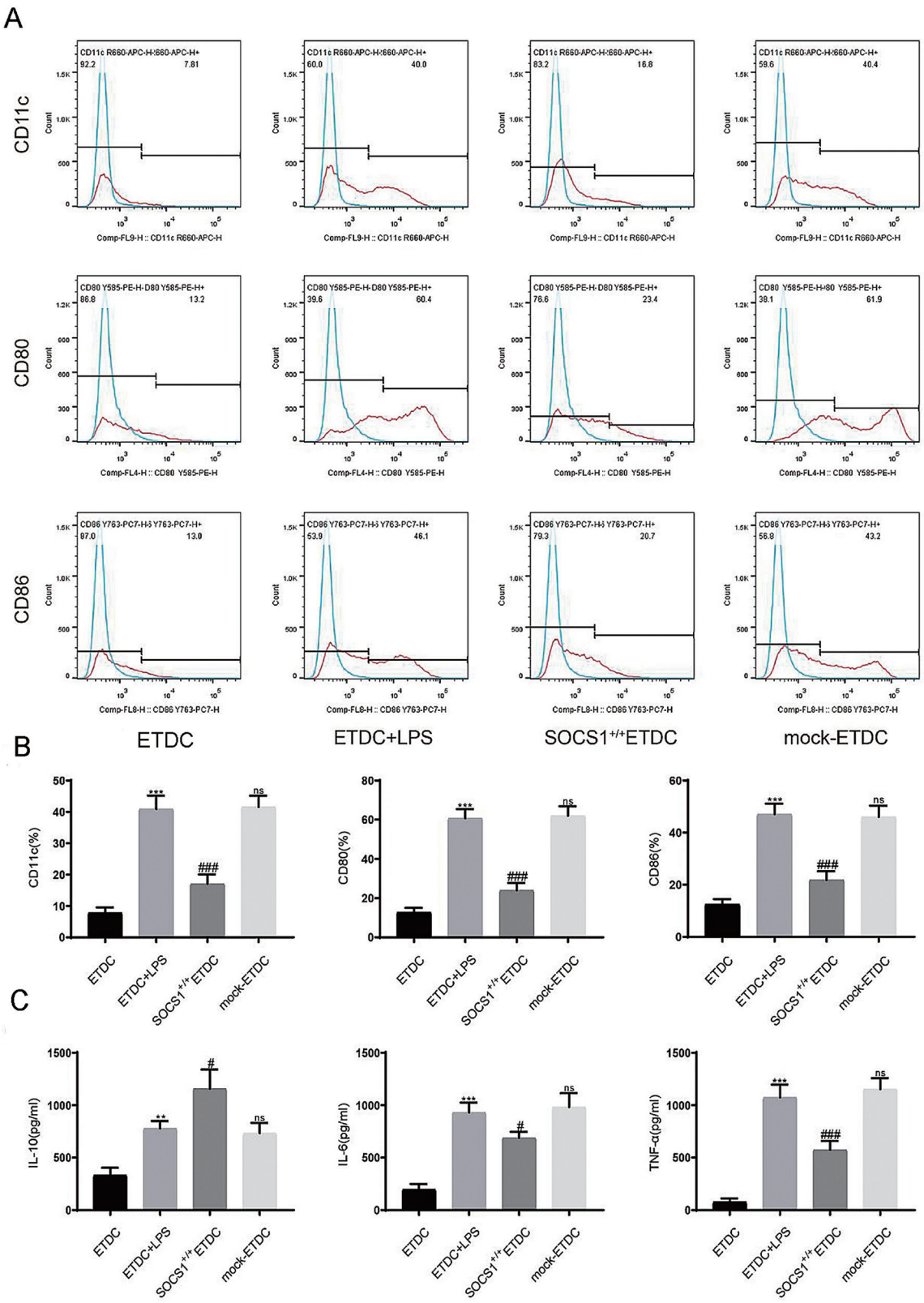

Fig. 2. Silencing of SOCS1 modulates the function of ETDCs. (A, B) Flow cytometry analysis of cell costimulatory molecules CD11c, CD80, and CD86 of ETDCs. (C) ELISA results showing IL10, TNF-α, and IL6 levels in the supernatant. Mean±standard deviation of three independent experiments; ns, not significant. *p<0.05, **p<0.01, ***p<0.001 vs. ETDCs group, and $^{\#}p$<0.05, $^{\#\#}p$<0.01, $^{\#\#\#}p$<0.001 vs. ETDC+LPS group.

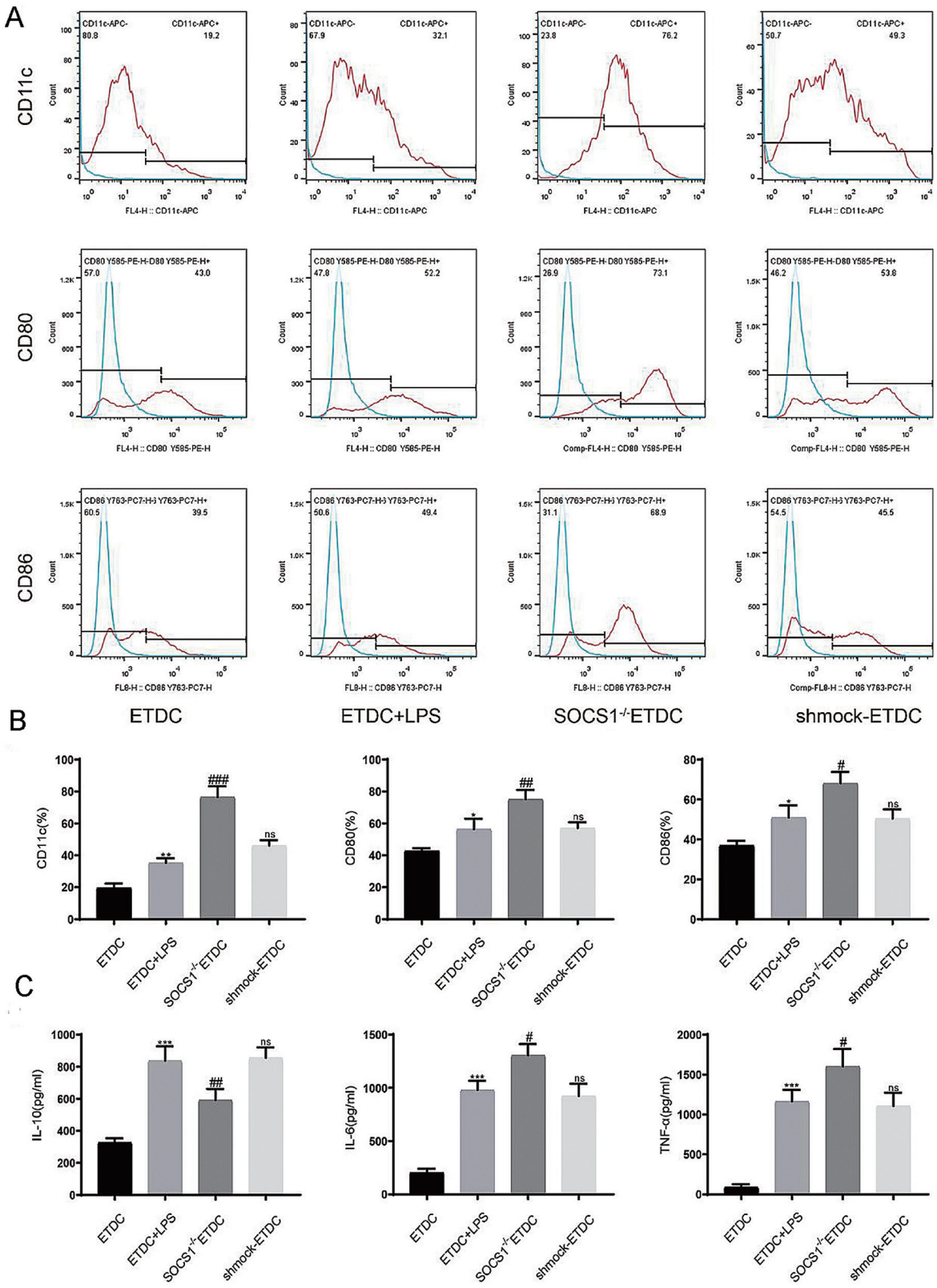

Fig. 3. Overexpression of SOCS1 modulates the function of ETDCs. (A, B) Flow cytometry analysis of cell costimulatory molecules CD11c, CD80, and CD86 of ETDCs. (C) ELISA results showing IL10, TNF-α, and IL6 levels in the supernatant. Mean±standard deviation of three independent assays; ns: not significant. *p<0.05, **p<0.01, ***p<0.001 vs. ETDC group, and $^{\#}p$<0.05, $^{\#\#}p$<0.01, $^{\#\#\#}p$<0.001 vs. ETDC+LPS group.

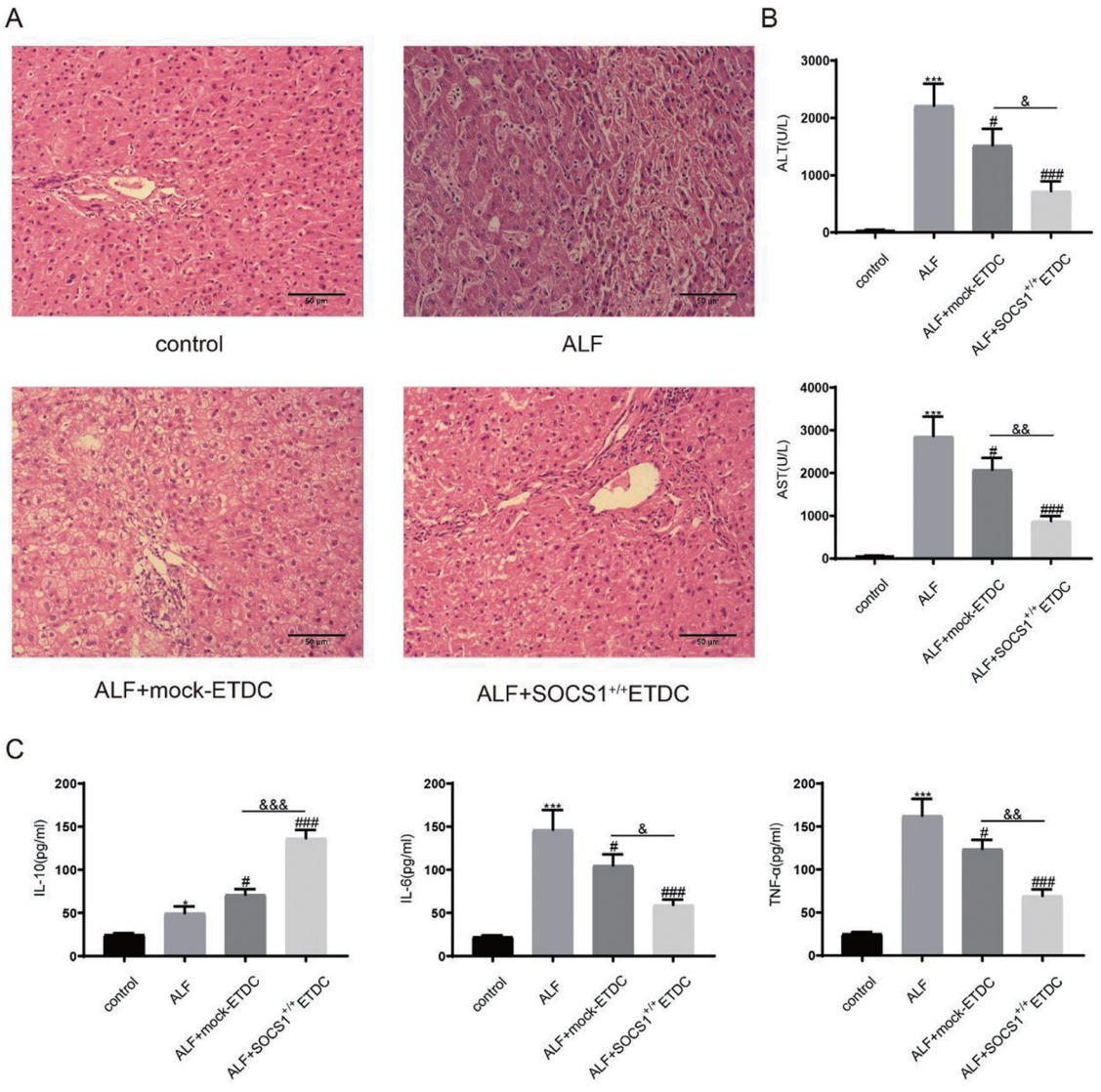

Fig. 4. SOCS1$^{+/+}$ETDCs protect against liver injury induced by LPS/D-GalN and affect the production of inflammatory cytokines in serum. (A) Hematoxylin and eosin-stained liver sections, 100×. (B) Serum AST and ALT levels. (C) ELISA results showing serum IL10, TNF-α, and IL6 levels. Mean±standard deviation of three independent assays. *p<0.05, **p<0.01, ***p<0.001 vs. control group, $^{\#}p$<0.05, $^{\#\#}p$<0.01, $^{\#\#\#}p$<0.001 vs. ALF group, $^{\&}p$<0.05, $^{\&\&}p$<0.01, $^{\&\&\&}p$<0.001 (ALT, alanine aminotransferase; AST, aspartate aminotransferase).

Effect of SOCS1$^{+/+}$ETDCs on liver damage and inflammatory cytokine secretion in ALF mic

HE staining revealed massive hepatocyte swelling/necrosis, disordered hepatic cords, and infiltration of inflammatory cells in the ALF group (Fig. 4A). In the ALF+mock-ETDCs group, we observed slight alleviation of liver damage compared with the ALF group. Importantly, the ALF+ SOCS1$^{+/+}$ETDCs group showed significant alleviation of pathological damage (Fig. 4A).

Serum ALT and AST are indicators of liver damage. AST and ALT levels in the ALF group were significantly higher than those in the control group (Fig. 4B). The levels of both AST and ALT in ALF+ mock-ETDCs group were slightly lower than those in the ALF group (Fig. 4B). However, the ALF+SOCS1$^{+/+}$ETDCs group showed a marked decrease in the degree of elevation. The results revealed that SOCS1$^{+/+}$ETDCs alleviated hepatic damage in the LPS/D-GalN-induced ALF model.

As shown in Figure 5C, TNF-α, IL6, and IL10 significantly increased after ALF induction. Both ALF+mock-ETDCs and ALF+SOCS1$^{+/+}$ETDCs groups had decreased IL6 and TNF-α levels and increased IL10 levels. The changes were more pronounced in the ALF+ SOCS1$^{+/+}$ETDCs group (Fig. 4C).

Effect of SOCS1$^{+/+}$ETDCs on the JAK2/STAT3 signaling pathway

To investigate the mechanism of the potential therapeutic effect of SOCS1$^{+/+}$ETDCs in ALF, we assessed the expression of SOCS1, JAK2, p-JAK2, STAT3, and p-STAT3 proteins in liver tissues obtained from each group (Fig. 5A). The phosphorylation of JAK2 and STAT3 proteins was significantly higher in the ALF group than in the control group. Moreover, the phosphorylation of JAK2 and STAT3 proteins

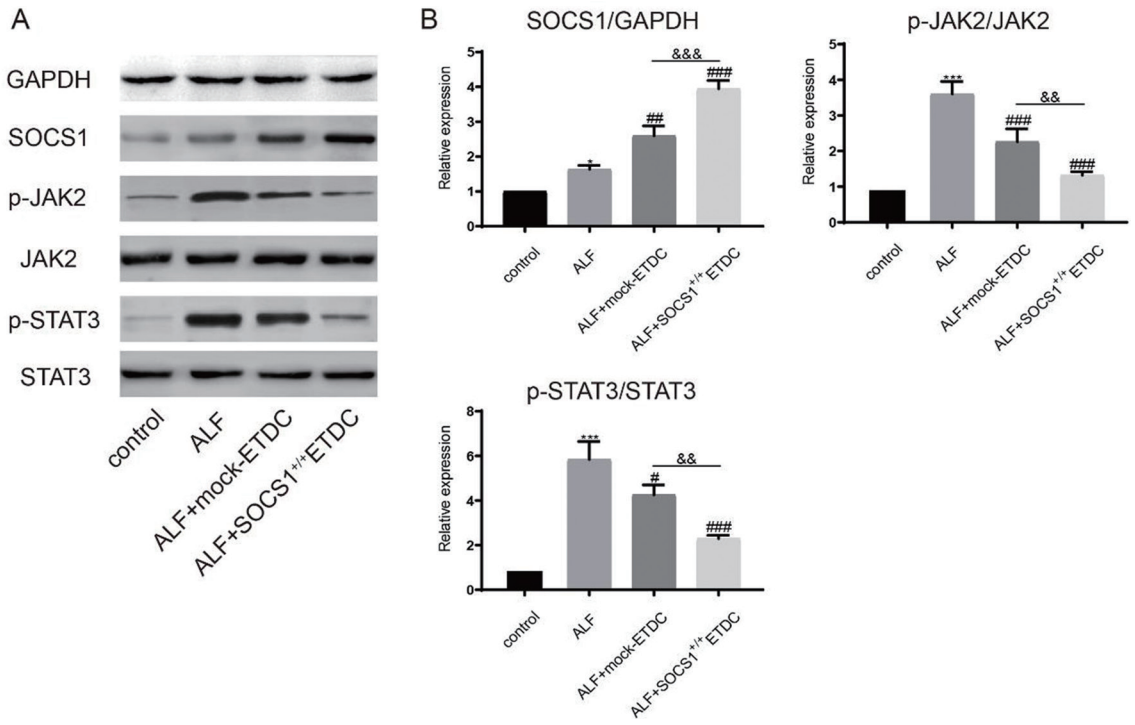

Fig. 5. SOCS1$^{+/+}$ETDCs inhibit JAK2/STAT3 signaling. (A, B) Western blot assays of the relative expression of SOCS1, JAK2, STAT3, p-JAK2, and p-STAT3 proteins. Mean±standard deviation of three independent assays. *p<0.05, **p<0.01, ***p<0.001 vs. control group, and $^{\#}p$<0.05, $^{\#\#}p$<0.01, $^{\#\#\#}p$<0.001 vs. ALF group, $^{\&}p$<0.05, $^{\&\&}p$<0.01, $^{\&\&\&}p$<0.001 (JAK2/STAT3, Janus kinase 2/signal transducer and activator of transcription 3).

in the ALF+ SOCS1$^{+/+}$ETDCs group were obviously lower than those in both ALF and ALF+mock-ETDCs groups and the level of SOCS1 protein was much higher (Fig. 5A, B). After infusion of SOCS1$^{+/+}$ETDCs, there was an obvious decrease in the phosphorylation of JAK2 and STAT3 proteins in the liver tissues of ALF mice.

Effect of SOCS1$^{+/+}$ETDCs on the proportion of Th17 and Treg cells

To further evaluate whether the therapeutic effect of SOCS1$^{+/+}$ETDCs in ALF mice was related to the differentiation of Treg and Th17 cells, we evaluated the percentage of Treg and Th17 cells in the spleen by flow cytometry. The proportion of Th17 cells in the ALF group was higher than that in the control group, and the percentage was reduced after treatment with ALF+mock-ETDCs, ALF+490, and ALF+SOCS1$^{+/+}$ETDCs. Moreover, the percentage of Th17 cells in the ALF+ mock-ETDCs group was higher than that in the ALF+ SOCS1$^{+/+}$ETDCs group (Fig. 6A, B). The percentage of Treg cells in spleen lymphocytes was markedly enhanced after ALF modeling compared with the normal group. ALF+mock-ETDCs, ALF+490, and ALF+ SOCS1$^{+/+}$ETDCs treatment further elevated the percentage of Treg cells compared with the ALF group, and the increase in the ALF+ SOCS1$^{+/+}$ETDCs group was significant compared with the ALF+ mock-ETDCs group (Fig. 6A, B).

Effect of JAK2/STAT3 inhibition on prognosis of ALF

AG490, a selective inhibitor of JAK2/STAT3, was employed to explore the effect of JAK2/STAT3 inhibition on ALF. HE-stained sections revealed massive hepatocyte swelling/necrosis, disordered arrangement of hepatic cords, and infiltration of inflammatory cells in the ALF group (Fig. 4A). As shown in Figure 7A, the pathological changes were alleviated in the ALF+AG490 and ALF+AG490+ SOCS1$^{+/+}$ETDCs group. In addition, the pathological damage in the ALF+AG490+ SOCS1$^{+/+}$ETDCs group was a bit more severe compared to that in the ALF+ SOCS1$^{+/+}$ETDCs group (Fig. 7A).

Serum AST and ALT levels in all ALF groups were significantly higher than those in the control group (Fig. 7B). ALF+AG490, ALF+SOCS1$^{+/+}$ETDCs, and ALF+AG490+ SOCS1$^{+/+}$ETDCs groups had markedly decreased serum levels of AST and ALT, even though there was no significant difference among them. AST and ALT levels in the ALF+AG490+ SOCS1$^{+/+}$ETDCs group were much lower than those in the ALF+AG490 and ALF+SOCS1$^{+/+}$ETDCs groups.

The levels of IL6 and TNF-α in the ALF+AG490, ALF+ SOCS1$^{+/+}$ETDCs, and ALF+ AG490+SOCS1$^{+/+}$ETDCs groups were significantly lower, and the level of IL-10 was significantly higher compared with the ALF group. Even though the differences between the ALF+AG490, ALF+SOCS1$^{+/+}$ETDCs, and ALF+ AG490+SOCS1$^{+/+}$ETDCs groups were not significant, the ALF+AG490+ SOCS1$^{+/+}$ETDCs group had the highest IL10, and lowest IL6 and TNF-α levels (Fig. 7C).

As shown in Figure 8, the phosphorylation of JAK2 and STAT3 proteins in the ALF+AG490, ALF+SOCS1$^{+/+}$ETDCs and ALF+AG490+SOCS1$^{+/+}$ETDCs groups as observably decreased compared with the ALF group. SOCS1 level and JAK2 and STAT3 phosphorylation in the ALF+SOCS1$^{+/+}$ETDCs group were not significantly different from those in the ALF+AG490 or ALF+AG490+SOCS1$^{+/+}$ETDCs groups. However, JAK2 and STAT3 phosphorylation in the ALF+AG490+SOCS1$^{+/+}$ETDCs group was much lower than that in the ALF+AG490 and ALF+SOCS1$^{+/+}$ETDCs group. The results indicate that SOCS1$^{+/+}$ETDCs may play a protective role in ALF by inhibition of the JAK2/STAT3 pathway.

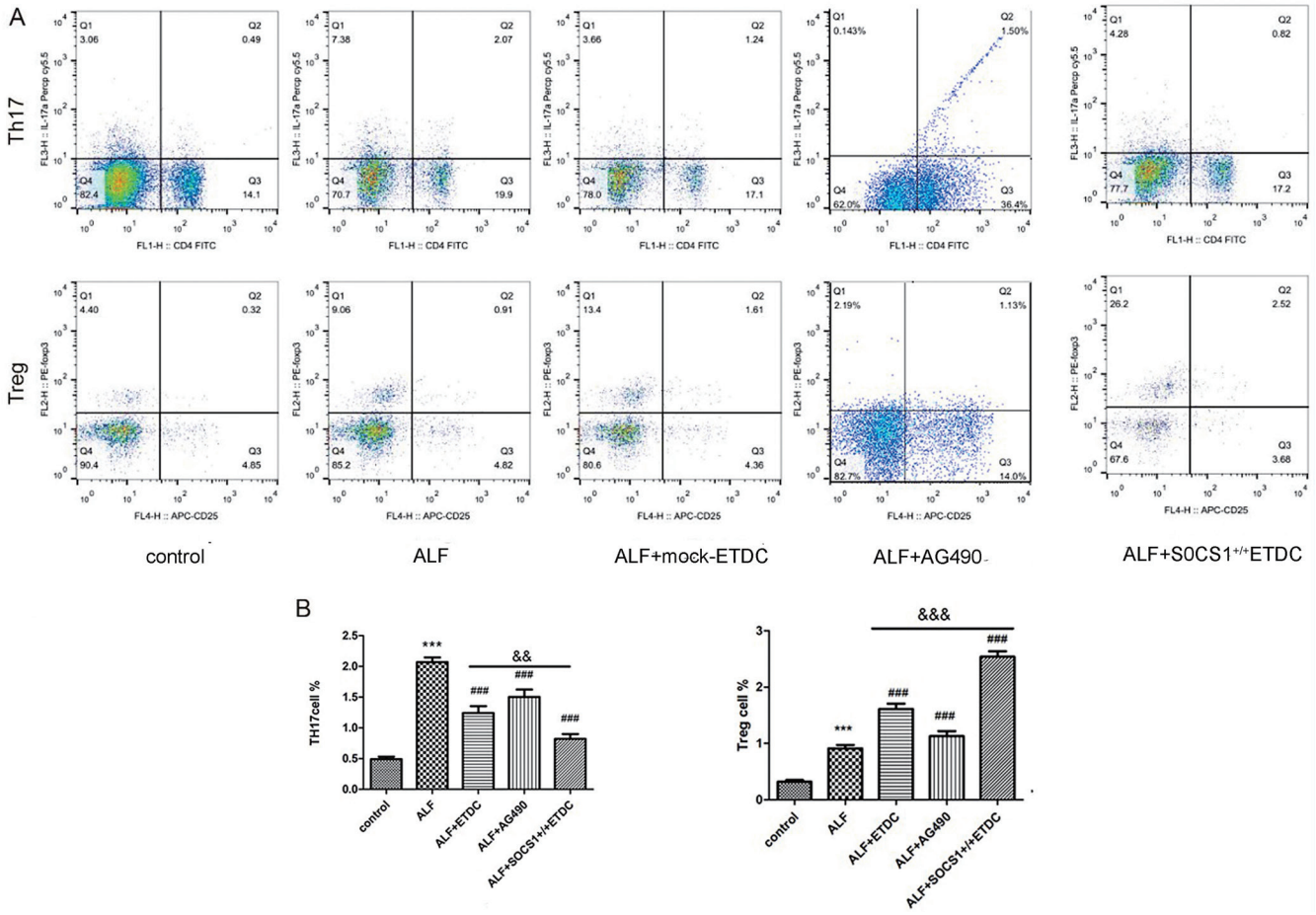

Fig. 6. SOCS1$^{+/+}$ETDCs regulate the percentage of Th17 and Treg among spleen lymphocytes in liver injury caused by LPS/D-GalN. (A) Flow cytometry assay of Th17 and Treg cells in spleen. (B) The percentages of Th17 and Treg cells in spleens from each group. Mean±standard deviation of three independent assays. *p<0.05, **p<0.01, ***p<0.001 vs. control group, and $^{\#}p$<0.05, $^{\#\#}p$<0.01, $^{\#\#\#}p$<0.001 vs. ALF group, $^{\&}p$<0.05, $^{\&\&}p$<0.01, $^{\&\&\&}p$<0.001 (Treg, regulatory T Cell).

Discussion

ET is a vital self-protective mechanism against inflammatory disorders. The protective effect of ET against endotoxin-related diseases has been widely reported.[17,26] SOCS proteins are a family of intracellular regulatory factors that inhibit the negative feedback attenuating cytokine signaling and play a major role in the maintenance of immune responses.[27] SOCS1, one of the eight members of the SOCS family, participates in multiple cytokine signaling and the differentiation of immunocytes.[28] In our previous studies, ET and DCs overexpressing SOCS1 had a protective effect in mice with ALF.[29,30] However, the underlying mechanism by which SOCS1 affects the ETDCs phenotype and the effect of SOCS1$^{+/+}$ETDCs on ALF in mice have not been demonstrated. Adenovirus has been widely employed in experimental studies and clinical trials.[31] We constructed SOCS1$^{+/+}$ETDCs and SOCS1$^{-/-}$ETDCs by adenovirus transfection, and used them to assess the effects of SOCS1 on the phenotype and function of ETDCs. In the *in vitro* experiments, the RT-PCR results showed overexpression of SOCS1 mRNA in the SOCS1$^{+/+}$ETDCs and downregulation in the SOCS1$^{-/-}$ETDCs groups. We also found that SOCS1$^{+/+}$ETDCs had an immature phenotype, with downregulation of CD11c and the expression of CD80 and CD86 costimulatory molecules. In addition, they had a poor ability to induce the proliferation of T cells because of the reduced expression of surface markers. The interaction between T cell stimulation and surface molecules of DCs is consistent with recent data.[32] In addition to the expression of costimulatory molecules and communication between each group of ETDCs and T cells, we also investigated the production of soluble cytokines. With decreased TNF-α and IL6 and increased IL10 secretion, we found that SOCS1$^{+/+}$ETDCs played a protective role by suppressing inflammation induced by high-dose LPS. IL10 is an anti-inflammatory factor that plays an important role in immune homeostasis. IL10 can be produced by DCs, and in turn inhibits the maturation of DCs.[33] TNF-α and IL6 are proinflammatory factors involved in the activation of inflammatory cells and pathogen clearance.[34,35] Adenovirus-infected DCs were previously shown to have enhanced expression of proinflammatory cytokines. However, our results showed no significant influence on these cytokines. Collectively, we speculate that SOCS1 participates in the development and regulation of ET, and SOCS1 overexpression enhances the protective function of ET while SOCS1 silencing weakens that function.

ALF is caused by massive hepatocellular injury resulting from an inflammation-mediated process. It is a life-threatening clinical syndrome with uncontrollable inflammation and extensive hepatocyte death.[2] LPS/D-GalN-induced ALF is a classical experimental liver injury model in which immune disorder is the main pathological process.[1,36] Our

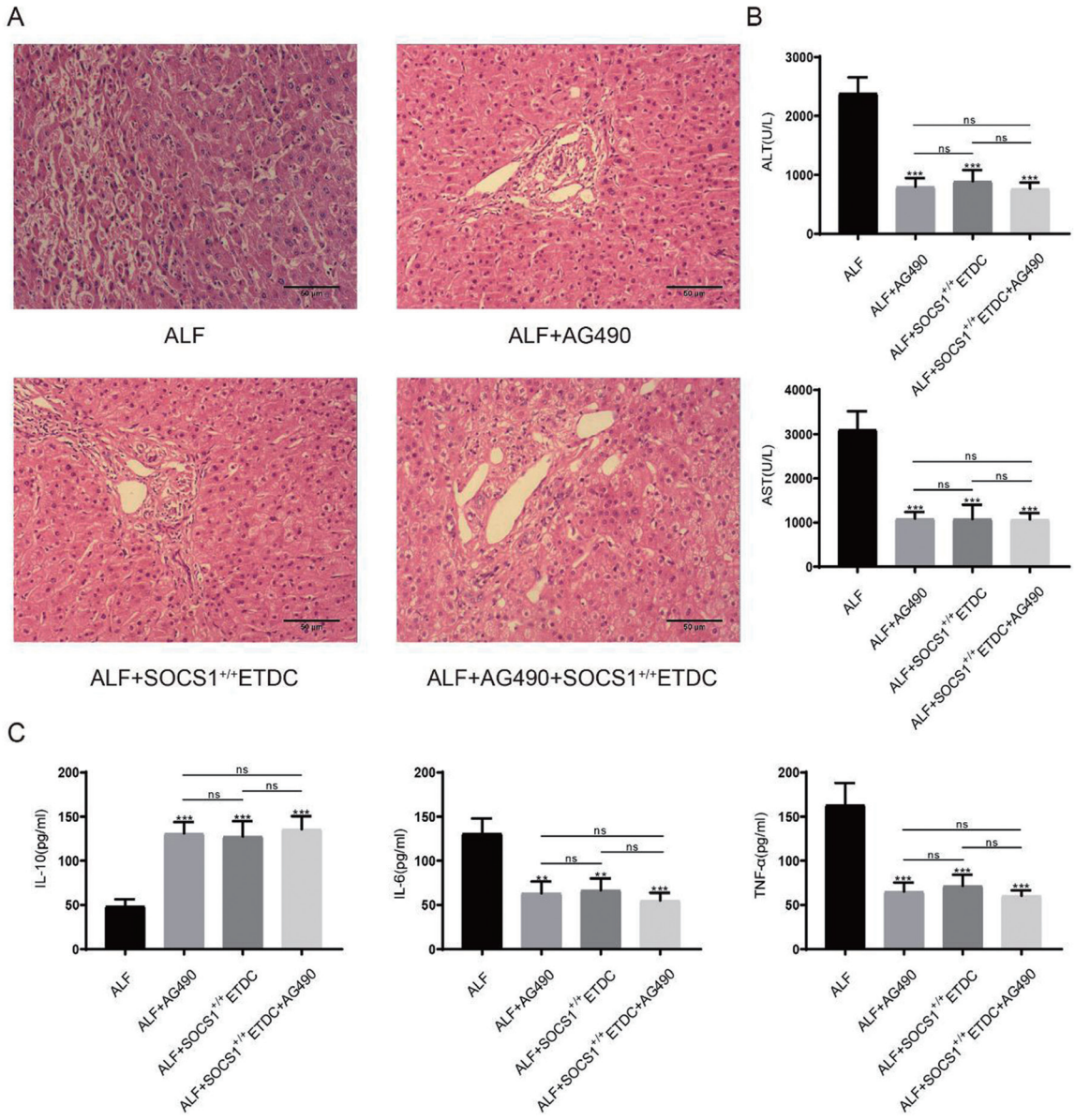

Fig. 7. AG490 or SOCS1$^{+/+}$ETDCs protect against liver injury induced by LPS/D-GalN and affect serum levels of inflammatory cytokines. (A) Hematoxylin and eosin-stained liver sections 100× (B) Serum ALT and AST levels. (C) ELISA results of IL10, TNF-α, and IL6 serum levels. Mean±standard deviation of three independent assays; ns, not significant. *p<0.05, **p<0.01, ***p<0.001 vs. ALF group.

previous study demonstrated the therapeutic effect of SOCS1$^{+/+}$DCs on ALF. In this study, we assessed the therapeutic effect of SOCS1$^{+/+}$ETDCs on ALF. Results included abnormal increases in the levels of ALT, AST, and proinflammatory cytokines (TNF-α, IL6) and severe histological damage in the ALF group. Notably, we observed higher serum IL10 and lower serum ALT, AST, TNF-α and IL6 levels in both the ALF+ mock-ETDCs and ALF+SOCS1$^{+/+}$ETDCs groups compared with the in the ALF group. Moreover, the outcome of the SOCS1$^{+/+}$ETDCs group was significantly better than that of the mock-ETDCs. Treg and Th17 cells have opposing roles during regulation of the immune response.[37] Th17 cells secrete IL17, thereby producing TNF-α and IL6 and initiating inflammation.[38] Treg cells produce IL10 and TGF-β and then inhibit immune responses.[39] The balance of Th17 and Treg plays a critical role in autoimmune and inflammatory diseases such as rheumatoid arthritis, inflammatory bowel disease, and liver failure.[40,41] Based on our results, we speculate that SOCS1$^{+/+}$ETDCs ameliorate LPS/D-GalN-induced ALF by regulating inflammatory cytokine production and Th17/Treg cells differentiation.

The JAK/STAT signaling pathway is evolutionarily conserved pathway and can be activated by several inflammatory cytokines. The pathway is involved in diverse processes such as immunity, cell growth, differentiation, and cell survival.[42,43] Previous reports have shown an intrinsic interaction between JAK2/STAT3 signaling pathway and the inflammatory reaction. The western blot results showed significant increases in the phosphorylation levels of JAK2 and STAT3 after ALF modeling compared with control group. We found that SOCS1$^{+/+}$ETDCs significantly decreased the increment in the phosphorylation of JAK2 and STAT3. To further clarify

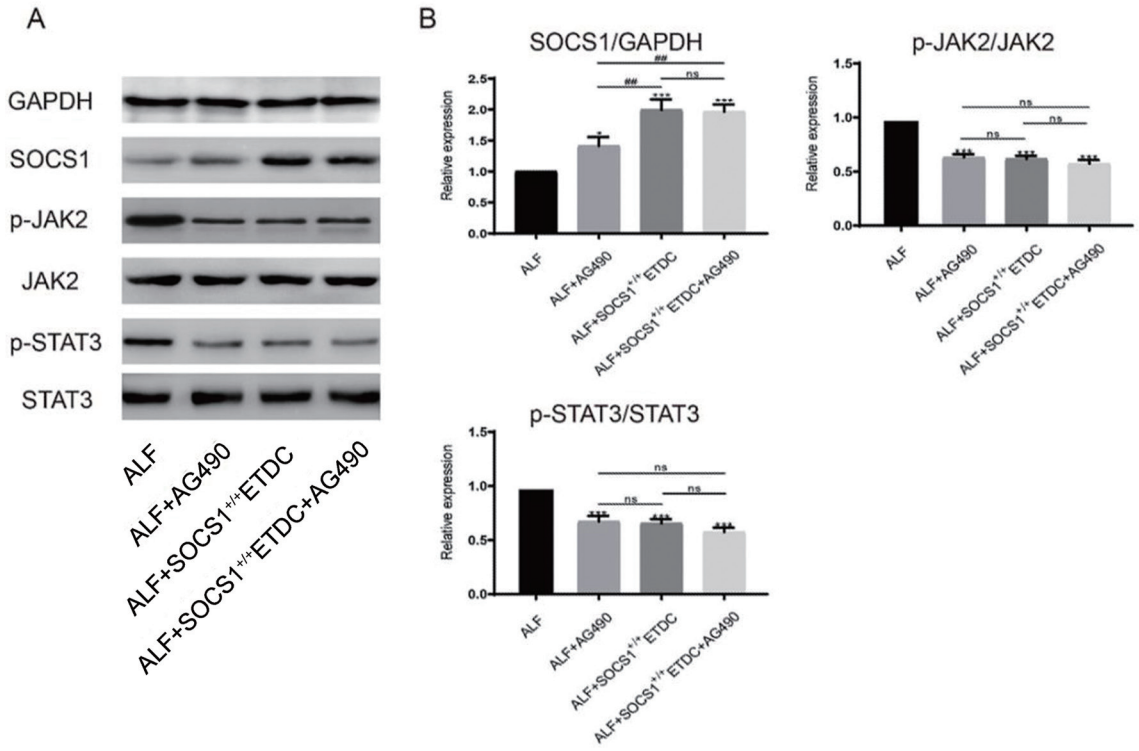

Fig. 8. AG490 or SOCS1$^{+/+}$ETDCs inhibit JAK2/STAT3 signaling. (A, B) Western blot assays of the relative expression of JAK2, STAT3, SOCS1, p-JAK2, and p-STAT3 proteins. Mean±standard deviation of three independent assays; ns, not significant. *p<0.05, **p<0.01, ***p<0.001 vs. ALF group.

the correlation between the effect of SOCS1$^{+/+}$ETDCs and the JAK2/STAT3 signaling pathway, mice were pretreated with AG490 and SOCS1$^{+/+}$ETDCs+AG490. We found that both AG490 and SOCS1$^{+/+}$ETDCs+AG490 significantly suppressed the phosphorylation process of JAK2 and STAT3, there was no significant difference between the ALF+SOCS1$^{+/+}$ETDCs group and ALF+AG490+SOCS1$^{+/+}$ETDCs group with respect to liver tissue histopathology and inflammatory cytokine expression, the AG490+ SOCS1$^{+/+}$ETDCs group had a stronger protective effect. Recent studies have reported that STAT3 undergoes phosphorylation following activation of JAK2.[44] Several studies have suggested that inhibition of activation of the JAK2/STAT3 signaling pathway may protect against ischemia/reperfusion-induced liver injury.[45,46] Zheng *et al*.[47] reported that liver injury induced by concanavalin A was attenuated by reducing STAT3 phosphorylation. Sobowale *et al*.[48] also found that downregulation of the JAK2/STAT3 signaling pathway reduced inflammation caused by liver damage. On the other hand, an accumulating body of evidence suggests that SOCS1 can suppress the JAK2/STAT3 pathway, which is consistent with our results.[49,50] In aggregate, in this preliminary study, we demonstrated that SOCS1$^{+/+}$ETDCs downregulated the JAK2/STAT3 pathway by suppressing phosphorylation of JAK2 and STAT3, thereby alleviating LPS/D-GalN-induced liver injury.

In conclusion, our study indicates that the *SOCS1* gene may play an important role in ET induction and that *SOCS1* gene overexpression may augment ET. SOCS1$^{+/+}$ETDCs exerted a protective effect against aggressive inflammation by downregulating of the JAK2/STAT3 signaling pathway, and regulation of Th17 and Treg cell differentiation and their associated cytokines. Thus, SOCS1$^{+/+}$ETDCs may represent a novel treatment modality for ALF. However, further studies are required for in-depth characterization of the underlying mechanisms.

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Conflict of interest

ML has been an editorial board member of *Journal of Clinical and Translational Hepatology* since 2019. Other authors have no conflict of interests related to this publication.

Author contributions

Study design (ML, SL), performance of experiments (YkC, CH, DK), analysis and interpretation of data (YkC, ML), manuscript writing (YY, YrC), critical revision (SL, ML), statistical analysis (YkC, YJ, SZ), critical funding (ML), administration (ML), and technical or material support (ML).

Data sharing statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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