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ORIGINAL ARTICLE

MicroRNA-155 deficiency attenuates ischemia–reperfusion injury after liver transplantation in mice

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Conflicts of interest
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Summary

Liver ischemia–reperfusion injury (IRI) is a major cause of morbidity and mortality after resection surgery, liver transplantation, and hemorrhagic and septic shock. Mir-155 is upregulated by a broad range of inflammatory mediators, and it has been demonstrated to be involved in both innate and adaptive immune responses. However, the role of mir-155 in liver IRI has never been investigated. In this study, mir-155 deficiency protected mice from liver IRI, as shown by lower serum alanine aminotransferase (ALT) levels and Suzuki scores. Mir-155 deficiency results in the development of M2 macrophages, which respond to IR-induced innate immune stimulation by producing a regulatory inflammatory response with higher level of IL-10, but lower levels of TNF-α, IL-6, and IL-12p40. Mir-155 deficiency suppresses IL-17 expression, which contributes to the liver IRI development. In our further *in vitro* study, the results show that the Th17 differentiation is inhibited by SOCS1 overexpression and the promoted M2 macrophage development induced by mir-155 deficiency is abolished by SOCS1 knockdown. In conclusion, mir-155 deficiency attenuates liver IRI through upregulation of SOCS1, and this was associated with promoted M2 macrophage and inhibited Th17 differentiation.

Introduction

Liver ischemia–reperfusion injury (IRI) is a major cause of morbidity and mortality after resection surgery, liver transplantation, and hemorrhagic and septic shock [1]. The pathophysiology of liver IRI includes direct cellular damage as the result of the ischemic insult as well as delayed dysfunction and damages that result from activation of inflammatory pathways. Clinical and experimental data have established that up to 10% early graft dysfunction and higher incidence of both acute and chronic rejection are associated with IRI, and therefore, it dampens the long-term graft survival [2].

A novel small noncoding RNA species known as microRNAs (mir) is involved in biological control at multiple levels. They regulate gene expression essential for cell development and function through mRNA degradation or translational inhibition. Evidences indicated that miRNAs played important roles in immune system. Mir-155 has been found apparently upregulated in several activated immune cells, including T lymphocytes, B lymphocytes, macrophages, and dendritic cells (DCs). Mir-155 is upregulated by a broad range of inflammatory mediators [3,4], and it has been demonstrated to be involved in both innate and adaptive immune responses [5,6]. However, the role of mir-155 in liver IRI has never been investigated.

In this study, we demonstrate a crucial role for mir-155 in regulating macrophage and Th17 differentiation in liver IRI. Mir-155 deficiency results in the development of M2 macrophages, which respond to IR-induced innate immune stimulation by producing higher level of IL-10, but lower levels of TNF-α, IL-6, and IL-12p40. Mir-155 deficiency suppresses IL-17 expression, which contributes to the liver IRI development. Further study indicates that mir-155 deficiency regulates macrophage and Th17 differentiation via upregulation of SOCS1.

Materials and methods

Mouse liver IRI model

Animal facilities and protocols were approved by the Laboratory Animal Care and Use Committee of Guilin Medical University. Three-month-old male wild-type C57 mice were from Guilin Medical University, and mir-155$^{-/-}$ mice were from Jackson Laboratory. In our principal model, donor livers were subjected to cold preservation followed by orthotopic liver transplantation [7]. Livers stored for 20 h at 4 °C in UW solution were transplanted orthotopically to syngeneic hosts. The arterial segment from the donor liver was end-to-side anastomosed to the infrarenal aortic artery of the recipient. The inclusion criteria of recipient's condition after transplantation were no anastomotic blood leak and liver graft becoming red when the liver blood supply was recovered. The exclusion criteria were the recipients died with 24 h after liver transplantation. The success rate of the mouse liver transplantation model in this study was 88%. The phenomena in this study are only proved by long-term stored liver transplantation model. The data may be not consistent with simple inflow block of liver or with transplantation of freshly harvested liver.

For neutralization of endogenous IL-17, 200 μg of neutralizing rabbit anti-mouse IL-17 (Biolegend, New York, NY, USA) was administered i.v. 5 min prior to reperfusion. For inhibition of macrophages, mice were injected i.v. with $GdCl_3$ at 10 μg/g (Sigma-Aldrich, St. Louis, MO, USA) 5 min prior to reperfusion.

Serum alanine aminotransferase levels

Serum alanine aminotransferase (ALT) levels were measured with an autoanalyzer by ANTECH Diagnostics (Los Angeles, CA, USA).

Immunohistochemistry

Part of the liver was fixed in 10% buffered formalin and embedded in paraffin. Liver sections (4 μM) were stained with H&E. The severity of liver IRI was analyzed with Suzuki's criteria [8]. Liver m2 macrophages were detected using primary rat anti-mouse Arg mAb. The secondary, biotinylated goat anti-rat IgG was incubated with immunoperoxidase (ABC Kit; from Vector, Burlingame, CA, USA).

Myeloperoxidase assay

Liver myeloperoxidase (MPO) activities were measured. Samples were homogenized in hexadecyltrimethyl ammonium bromide (Sigma-Aldrich) and dissolved in potassium phosphate. After centrifugation, supernatants were collected and mixed with o-dianisidine dihydrochloride (Sigma-Aldrich) and H_2O_2 in phosphate buffer. The activity of MPO was measured spectrophotometrically at 655 nm using microplate reader (ELx800, Bio-Tek Instruments, Houston, TX, USA). One unit of MPO activity was defined as the quantity of enzyme degrading 1 μmol H_2O_2/min at 25 °C/g of tissue.

Real-time PCR

Total RNA was extracted from cultured cells or tissues using TRIzol (Invitrogen, Carlsbad, CA, USA) and reverse-transcribed into cDNA using the PrimeScript RT reagent kit (Takara Biotechnology, Dalian, China) according to the manufacturer's instructions. mRNA levels of target genes were quantified using SYBR Green Master Mix (Takara Biotechnology) with ABI PRISM 7900 Sequence Detector system (Applied Biosystems, Foster City, CA, USA).

Cell cultures

Cells were derived from mir-155$^{-/-}$ mice and wild-type mice after euthanasia (pentobarbital 100 mg/kg, i.p.). Peritoneal macrophages were generated by the injection of 1 ml 2% Bio-Gel P-100 (Bio-Rad, Hercules, CA, USA) into the mouse peritoneal cavity, followed by peritoneal lavage with sterile PBS 4 days later.

Kupffer cells (KCs) were isolated as previously described [9]. Briefly, livers were perfused *in situ* via the portal vein with calcium- and magnesium-free HBSS supplemented with 2% heat-inactivated FBS, followed by 0.27% collagenase IV. Perfused livers were dissected and teased through 70-μm nylon mesh cell strainers. Nonparenchymal cells (NPCs) were separated from hepatocytes by centrifuging at 50 *g* for 2 min three times. NPCs were suspended in HBSS and layered onto a 50%/25% two-step Percoll gradient in a 50-ml conical centrifuge tube and centrifuged at 1800 *g* at 4 °C for 15 min. KCs in the middle layer were collected and allowed to attach onto cell culture plates in DMEM with 10% FBS, 10 mM HEPES, 2 mM GlutaMax, 100 U/ml penicillin, and 100 μg/ml streptomycin for 15 min at 37 °C. Nonadherent cells were removed by replacing the culture medium.

Spleen macrophages were isolated using an immunomagnetic CD11b Positive Selection Kit (STEMCELL Technologies, Vancouver, BC, Canada), according to the manufacturer's manual. Macrophages or KCs were cultured overnight prior to the start of stimulations with TLR ligands: 100 ng/ml LPS-EK, 1 μg/ml LAM-MS, or 1 μg/ml polyinosinic–polycytidylic acid. Cells or culture supernatants were harvested after various lengths of stimulation for RNA, protein, and ELISA assays.

Bone marrow-derived macrophages (BMMs) were obtained from mir-155$^{-/-}$ and WT mice as described [10]. Cells were transfected with Socs1-siRNA or Control-siRNA (Ambion, Beijing, China) by electroporation and then cultured in the presence of phorbol-12-myristate-13-acetate (PMA, 100 ng/ml; Sigma, St. Louis, MO, USA) for 48 h [11]. Then, the cells were harvested for mRNA analysis.

ELISA

Cytokine secretion in cell culture supernatants or serum was measured by ELISA, according to the manufacturer's protocol (eBioscience, San Diego, CA, USA).

Nucleofection

Nucleofection was performed with Mouse T Cell Nucleofector® Kit and Nucleofector device (Amaxa, Koelin, Germany). First, 1×10^7 naive CD4$^+$ T cells were resuspended in 100 μl Nucleofector® solution. 2.5 μg pmaxGFP® Vector or 100 pmol oligonucleotides (including SOCS1-TP$^{miR\text{-}155}$ and control-TP$^{miR\text{-}155}$) were added into the solution and mixed gently. Then, the mixtures were gently transferred to electroporation cuvettes and placed in the Nucleofector device. Cells were nucleofected in the X-01 program. Finally, transfected cells were transferred to a 12-well plate with 1.5 ml prepared Mouse T Cell Nucleofector® Medium in each plate and incubated in a humidified 37 °C/5% CO_2 incubator until analysis. SOCS1-TP$^{miR\text{-}155}$ from Gene Tools, LLC (Corvallis, OR, USA) was used to interfere miR-155 and suppressors of cytokine signaling1 (SOCS1) interaction, with control-TP$^{miR\text{-}155}$ as the matched control.

CD4$^+$ T-cell activation and polarization

Four hours after nucleofection, CD4$^+$ T cells were activated by 5 μg/ml plate-bound anti-CD3 and 2 μg/ml soluble anti-CD28. For propagation under Th17 condition, 2.5 ng/ml rTGF-β1 and 30 ng/ml rIL-6 were provided. All antibodies used were purchased from eBioscience. All cytokines used were purchased from Peprotech (Rocky Hill, NJ, USA).

FACS analysis

For the detection of the number of liver infiltrating cells, isolated cells were stained using the following fluorochrome-labeled Abs: anti-Ly6G and F4/80. The entire Abs used for flow cytometry was obtained from eBioscience or BD Biosciences (San Jose, CA, USA). Flow cytometry was performed on a FACSCalibur and analyzed using CellQuest (BD Pharmingen) or WINMDI 2.8 software.

For Th17 cell analysis *in vitro*, 4 days after transfection and activation, cells were collected and used for detecting Th17 cells differentiation. Cells were stimulated for 4 h with 25 ng/ml phorbol myristate acetate (PMA) and 1 μg/ml ionomycin in the presence of 2 μM monensin in the last 2 h. Cells were collected and stained with FITC-labeled anti-mouse CD4 for 30 min at 4 °C. Then, cells were fixed and permeabilized according to the manufacturer's protocol. Next, cells were incubated with PE-labeled anti-mouse IL-17A in the dark for 20 min. Finally, stained cells were resuspended in 200 μl washing buffer and analyzed by FACSCalibur Flow Cytometer (BD Biosciences). All reagents used were purchased from eBioscience.

Western blotting

The protein levels were determined by Western blotting. Protein extracted from cells or tissue was separated on 10% SDS-polyacrylamide electrophoresis gels and transferred to nitrocellulose membranes (Pierce, Rockford, IL, USA). After being blocked with 5% nonfat milk in TBS for 3 h, the membranes were incubated with indicated primary antibodies (0.2 μg/ml) at 4 °C overnight, followed by incubation with HRP-conjugated secondary antibody (1:5000) for 3 h. β-actin was used as a loading control for comparison between samples.

Statistics

Data are presented as means ± standard deviation (SD). Differences were evaluated using unpaired Student's *t*-test between two groups and one-way ANOVA for multiple comparisons, followed by a Student–Newmann–Keuls *post hoc* test. All analyses were performed using SPSS 13.0 (SPSS, Chicago, IL, USA), and statistical significance was set at $P < 0.05$.

Results

Mir-155 deficiency protects livers from IRI

The degree of liver injury after reperfusion was determined by the serum ALT levels and Suzuki scores. Mir-155 deficiency protected mice from liver IRI, as shown by lower ALT levels and Suzuki scores, at 6 h after reperfusion

(Fig. 1a and b). To determine whether this effect was donor or recipient specific, we cross-transplanted livers between WT and mir-155$^{-/-}$ mice. Indeed, well-preserved hepatocellular function detected in WT→mir-155$^{-/-}$ mice but not in mir-155$^{-/-}$→WT mice (Fig. 1a). The ALT levels correlated with Suzuki scores of liver IRI at 6 h post-transplant (Fig. 1b). This indicated the protective function of mir-155 in recipient. Liver MPO activities also were reduced in livers of mir-155$^{-/-}$ mice at both 6 and 24 h of reperfusion (Fig. 1c). Next, we compared myeloid cell compositions between WT and mir-155 deficiency mice. We found that the number of Ly6G^{+} neutrophils in mir-155$^{-/-}$ mice was lower than that in WT mice at 6 and 12 h after reperfusion. However, the number of F4/80^{+} macrophages in mir-155$^{-/-}$ mice was higher than that in WT mice at 0, 6, and 12 h after reperfusion (Fig. 1d). The liver proinflammatory immune response against IRI was also regulated by mir-155, as shown by lower induction of proinflammatory genes, including TNF-α, IL-6, and IL-17, and higher induction of anti-inflammatory IL-10 genes (Fig. 1e).

Mir-155 deficiency promotes M2 macrophage differentiation

Previous studies revealed a very high degree of plasticity among macrophages and their quick adjustment in response to changed in the microenvironment. In particular, macrophages display two different phenotypes with different functions, namely classically (M1) and alternatively (M2) activated macrophages [12,13]. To identify M1 and M2 macrophages, we measured their presence in livers using M2 and M1 makers, respectively (arginase (Arg) and mannose receptor (Mrc) for M2; nitric oxide synthase 2 (Nos2) for M1). Our results showed that the number of Arg-positive cells in mir-155$^{-/-}$ mice was significantly higher than that in WT mice at 6 h after reperfusion (Fig. 2a). Mir-155$^{-/-}$ mice also indicated higher levels of Arg and Mrc and lower level of Nos2 when compared with that in WT mice (Fig. 2b and c).

Next, we investigated the function of mir-155 on m2 macrophage differentiation *in vitro*. KCs and splenic macrophages were isolated from liver of both WT and mir-155$^{-/-}$ mice and stimulated with LPS. ELISA results showed that mir-155$^{-/-}$ KCs and spleen macrophages produced significantly less TNF-α and more IL-10 in response to LPS stimulation compared with those from WT controls (Fig. 3a). Next, we measured the expression of macrophage differentiation markers in KCs and peritoneal macrophages by Q-PCR. Clearly, mir-155$^{-/-}$ cells expressed constitutively higher levels of Arg and Mrc but lower levels of Nos2 gene (Fig. 3b). Functionally, the mir-155$^{-/-}$ macrophages produced significantly less TNF-α, IL-6, and IL-12p40, but

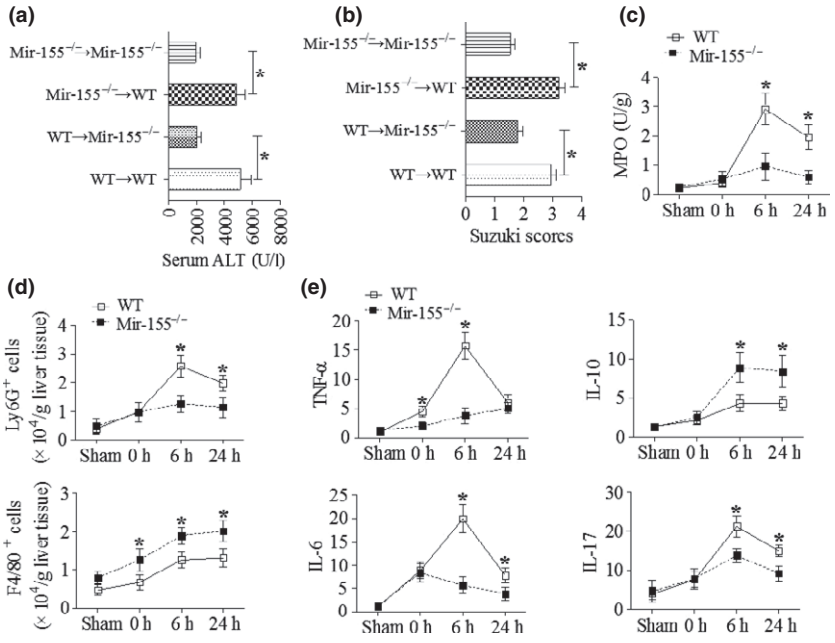

Figure 1 Mir-155 deficiency protects livers from ischemia–reperfusion injury (IRI). (a) Alanine aminotransferase (ALT) levels at 6 h after reperfusion; (b) Suzuki's scores of liver histological analysis at 6 h after reperfusion; (c) myeloperoxidase (MPO) activity was measured in sham or ischemic liver tissues harvested from WT or mir-155$^{-/-}$ mice at 0, 6, or 24 h after reperfusion; (d) F4/80^{+} or Ly6G^{+} cells infiltration in sham and ischemic livers after 0, 6, or 24 h of reperfusion were analyzed by FACS; (e) Quantitative RT-PCR analysis of inflammatory gene expression in sham-operated and ischemic livers after 0, 6, or 24 h of reperfusion. The ratios of target gene/HPRT were plotted against different experiment groups. *$P < 0.05$. $n = 6$. WT indicates WT→WT group, mir-155$^{-/-}$ indicated mir-155$^{-/-}$ →mir-155$^{-/-}$ group.

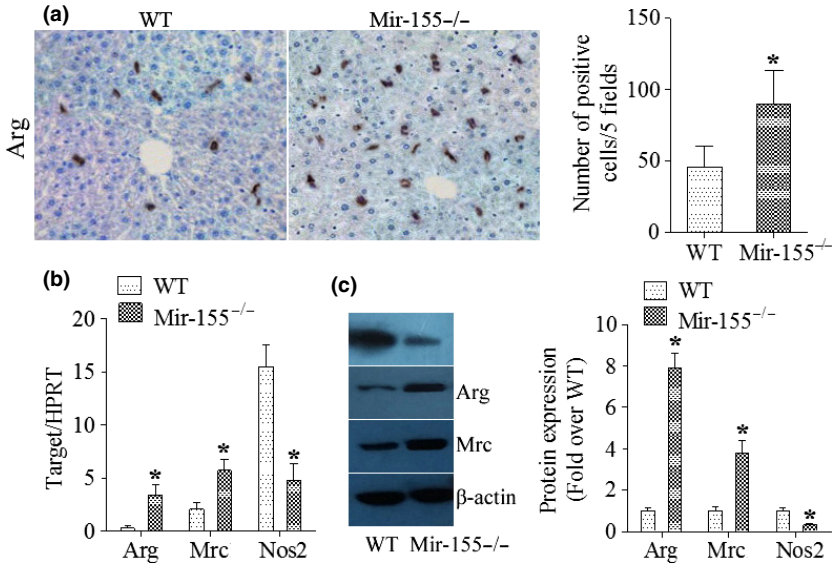

Figure 2 Mir-155 deficiency results in M2 macrophage development *in vivo*. (a) M2 marker Arg expression was analyzed by immunohistological method. (b) M2 marker gene expression was measured by quantitative RT-PCR. (c) M2 marker protein expression was measured by Western blotting. *$P < 0.05$. $n = 6$. WT indicates WT→WT group, mir-155$^{-/-}$ indicated mir-155$^{-/-}$ →mir-155$^{-/-}$ group.

more IL-10, than did WT cells in response to LPS. Additionally, their response to other TLR ligands also became less proinflammatory: less TNF-α, IL-6, and IL-12p40 against the TLR2 and less TNF-α and IL-12p40, but more IL-10, against the TLR3 ligand (Fig. 3c).

Mir-155-deficient KCs protects livers against IRI

To test potential immune-regulatory functions of mir-155$^{-/-}$ macrophages *in vivo* in response to IRI, $GdCl_3$ was used to inactivate these cells prior to the start of liver ischemia. $GdCl_3$-treated WT mice had decreased levels of ALT and lower Suzuki scores at 6 h after reperfusion (Fig. 4a and b). Liver inflammatory gene induction, including TNF-α, IL-6, IL-12p40, and IL-10, also was decreased compared with their vehicle-treated controls (Fig. 4c). In contrast, $GdCl_3$-treated mir-155$^{-/-}$ mice had increased levels of ALT and Suzuki scores (Fig. 4a and b). Liver proinflammatory genes, including TNF-α, IL-6, and IL-12p40, were increased compared with vehicle-treated mir-155$^{-/-}$ mice. However, the IL-10 gene level was downregulated by $GdCl_3$ treatment in mir-155$^{-/-}$ mice (Fig. 4c).

Mir-155 deficiency suppresses Th17 cell differentiation

Previous results have indicated lower IL-17 expression in mir-155$^{-/-}$ mice with liver IRI. To determine whether the suppressed T-cell activity in mir-155$^{-/-}$ mice was a result of intrinsic differences in CD4$^+$ T cells, we compared the cytokine profiles of WT and mir-155$^{-/-}$ total CD4$^+$ T cells in response to *ex vivo* stimulation with anti-CD3 and anti-CD28. We found lower production of IL-17 by mir-155$^{-/-}$ CD4$^+$ T cells than WT CD4$^+$ T cells (Fig. 5a). To further determine the role of mir-155 on Th17 differentiation, WT and mir-155$^{-/-}$ naïve CD4$^+$ T cells were differentiated into Th17 cells. IL-17 production in mir-155$^{-/-}$ T cells was significantly reduced compared with WT-T cells (Fig. 5b). Polarization of T cells to Th1, Th2, or Th17 phenotypes is a critical feature of cell-mediated immunity and is influenced by production of cytokines by DC. Therefore, we tested whether mir-155 modulates the expression of Th17-polarizing cytokine by DCs. For this, we stimulated bone marrow-derived DCs from WT and mir-155$^{-/-}$ mice with the TLR ligand LPS and analyzed cytokine expression. In mir-155$^{-/-}$ DCs, decreased levels of Th17-polarizing cytokines were observed in response to LPS stimulation (Fig. 5c). In our further DC-T-cell coculture system, mir-155 deficiency in DCs resulted in significantly less IL-17 production from T cells (Fig. 5d).

To further investigate the role of IL-17 in liver IRI, WT mice were treated with anti-IL-17 antibody. Results showed that IL-17 neutralization leaded to lower Suzuki scores and decreased levels of ALT (Fig. 6a and b). The numbers of Ly6G$^+$ neutrophils and liver MPO activities were also markedly decreased in IL-17 ab-treated mice compared with that in control mice (Fig. 6c and d).

Mir-155 deficiency regulates SOCS1 expression

Three transcripts, including SOCS1, SMAD2, and SMAD5, had been reported to be targets of mir-155. But we found

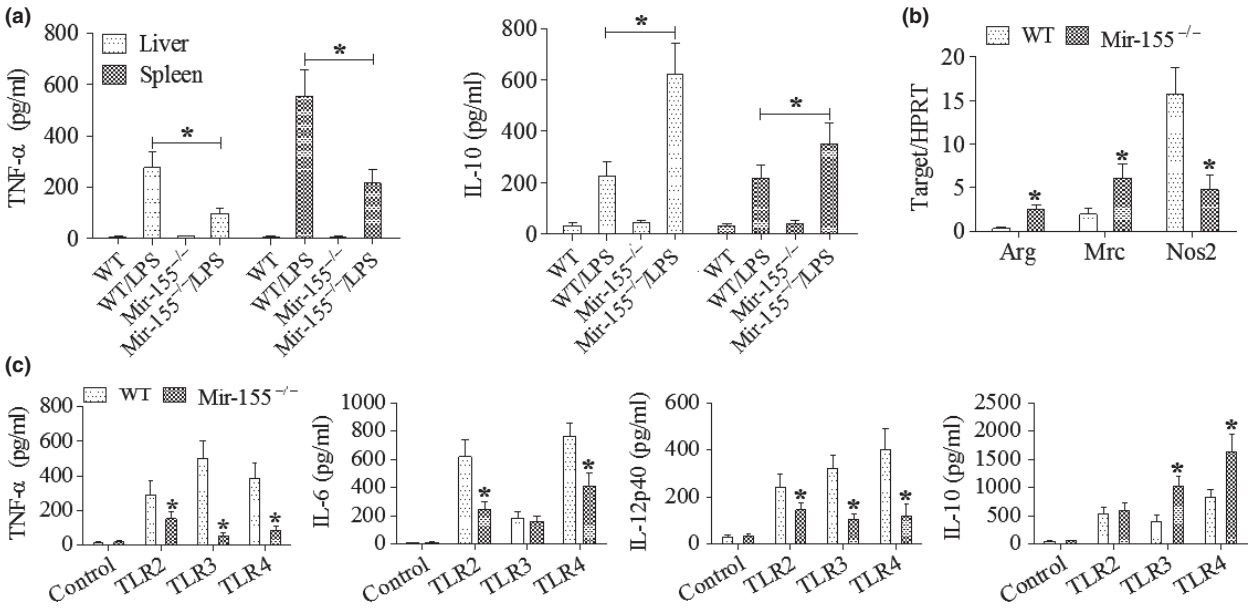

Figure 3 Mir-155 deficiency results in m2 macrophage development *in vitro*. (a) Cytokine production by KC and splenic macrophages from WT and mir-155$^{-/-}$ mice. Liver and spleen macrophages were isolated from WT and mir-155$^{-/-}$ mice and cultured *in vitro* with or without LPS stimulation for 24 h. TNF-α and IL-10 levels in culture supernatants were measured by ELISA. (b) Peritoneal macrophages were isolated from WT and mir-155$^{-/-}$ mice, and M1/M2 marker gene expression was measured by quantitative RT-PCR. (c) Inflammatory cytokine production by peritoneal macrophages. Cells were cultured *in vitro* for 24 h before TLR stimulation. Cytokines in culture supernatants after 24 h of stimulation were measured by ELISA. *$P < 0.05$. $n = 6$.

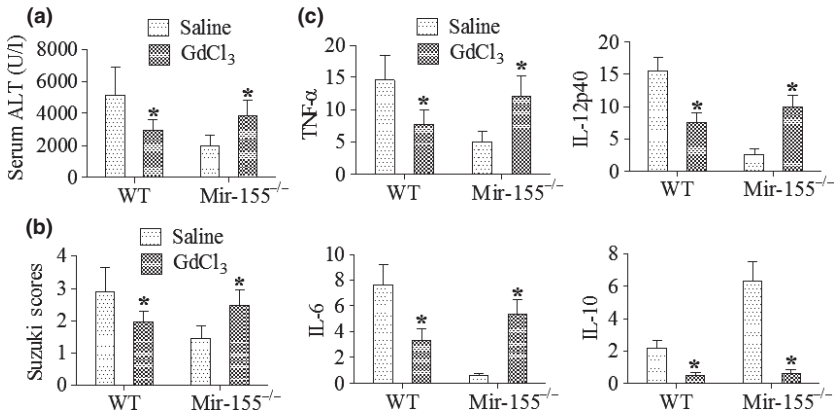

Figure 4 Mir-155 deficiency macrophages protect livers from ischemia–reperfusion injury (IRI). Saline or $GdCl_3$ were administrated 24 h prior to the start of liver ischemia. Liver IRI was evaluated at 6 h postreperfusion by measuring alanine aminotransferase (ALT) (a) and assessing Suzuki scores (b). (c) Liver immune response against IR was determined by quantitative RT-PCR analysis of inflammatory gene expression in IR livers. *$P < 0.05$. $n = 6$. WT indicates WT→WT group, mir-155$^{-/-}$ indicated mir-155$^{-/-}$ →mir-155$^{-/-}$ group.

that SOCS1 was the only one that was enhanced by mir-155 deficiency (Fig. 7a). To further investigate the role of SOCS1 in the regulated differentiation of Th17, target protector (TP) was used. SOCS1-TP$^{mir\text{-}155}$ interfere mir-155-SOCS1 interaction by binding to the binding site of mir-155 in the 3′-untranslated region of SOCS1, without interfering mir-155 interaction with other target mRNAs. So, when SOCS1-TP$^{mir\text{-}155}$ was transfected, a dramatic increase in SOCS1 protein was observed (Fig. 7b). Furthermore, the percentages of Th17 cells in CD4$^+$ T cells as well as the expression of IL-17A in cell culture supernatant were decreased when SOCS1-TP$^{mir\text{-}155}$ was transfected (Fig. 7c). Next, we also studied the role of SOCS1 in the promoted M2 differentiation; we prevented the function of SOCS1 in mir-155 deficiency macrophages with siRNAs. SOCS1 knock-down in macrophages largely abolished the promoted effect on M2 macrophages differentiation induced by mir-155 deficiency (Fig 7d).

Discussion

Our current study provides strong evidence that mir-155 is involved directly in liver innate immune response against IR by regulating macrophage and Th17 differentiation. Mir-155 deficiency results in the development of M2 macrophages, which respond to IR-induced innate immune stimulation by producing a regulatory inflammatory response with higher levels of IL-10, but lower levels of TNF-α, IL-6, and IL-12p40. Mir-155 deficiency suppresses expression of IL-17, which contributes to liver IRI development. Further study indicates that mir-155 deficiency regulates macrophage and Th17 differentiation via upregulation of SOCS1. These findings establish an innate immune-regulatory role for mir-155 *in vivo* in a relevant clinical disease model and provide the rationale for selectively targeting this signaling pathway as a novel therapeutic strategy to ameliorate tissue inflammatory diseases.

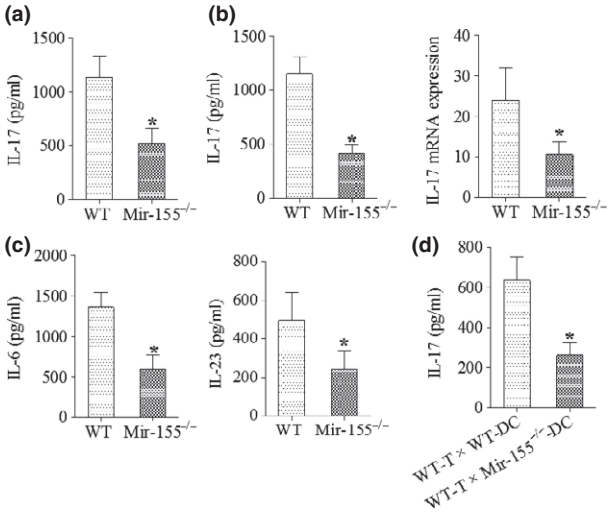

Figure 5 Mir-155 deficiency suppresses Th17 differentiation *in vitro*. (a) Total CD4$^+$ T cells were isolated from the spleens of WT and mir-155$^{-/-}$ mice and stimulated with anti-CD3 and anti-CD28. Supernatants from culture were harvested 72 h after initiation of cultures and analyzed for IL-17; (b) Naive CD4$^+$ T cells were isolated from the spleens of WT and mir-155$^{-/-}$ mice and stimulated with anti-CD3 and anti-CD28 under Th17-skewing condition. Supernatants from culture were harvested 72 h after initiation of cultures and analyzed for IL-17 by ELISA and Q-PCR; (c) Bone marrow-derived dendritic cells (DCs) were isolated from WT and mir-155$^{-/-}$ mice and were cultured with 100 ng/ml LPS. Cell-free supernatants were measured for Th17-polarizing cytokine by ELISA. (d) CD4$^+$ T cells isolated from WT mice were cultured with CD11c$^+$ DCs derived from WT and mir-155$^{-/-}$ mice. Supernatants from cultures were harvested 72 h after initiation of cultures and assayed by ELISA for IL-17. Data are representative of three to five independent experiments. *$P < 0.05$.

Macrophages are involved in the innate and adaptive immunity and played a key role in the initiation and effector phases of the immune response [14,15]. Liver macrophages are the major responding cells to IR and are responsible for triggering tissue inflammation, leading to neutrophil activation and infiltration. Functionally, M1 seems to be indispensable for an effective immune response against some pathogens known to have an excessive destructive response on tissues [16]. In contrast, an M2 subset of macrophages has well-established role in tissue homeostasis and repair with its potent anti-inflammatory properties [17,18]. M2 macrophages were shown to protect kidneys, brain, and hindlimbs from ischemia injuries by promoting either the resolution of tissue inflammation or healing/recovery [19–21]. Our study showed an upregulation of the M2 numbers in liver in mir-155 deficiency mice. Inhibition of the macrophage proinflammatory response by mir-155 deficiency would result in diminished neutrophil infiltration/activation. In fact, we found that mir-155 deficiency macrophages became the anti-inflammatory M2 type. Thus, neutrophils might have been inactivated by the regulatory immune response generated by mir-155

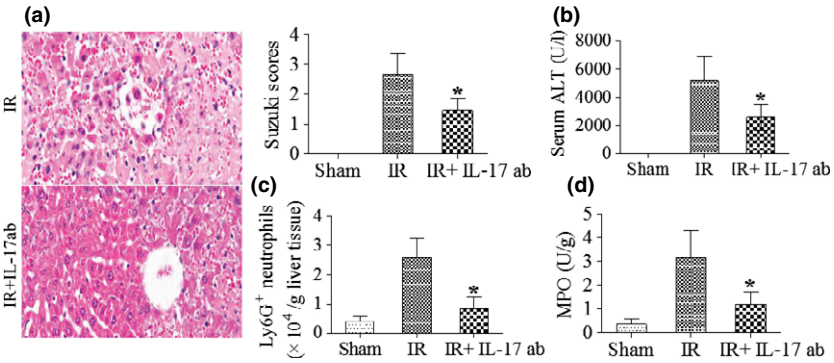

Figure 6 IL-17 neutralization protects liver from ischemia–reperfusion injury (IRI). Liver IRI was evaluated at 6 h postreperfusion by assessing Suzuki scores (a) and measuring alanine aminotransferase (ALT) (b). (c) Ly6G$^+$ cells infiltration in sham or ischemic liver tissues harvested from different groups 6 h after reperfusion were analyzed by FACS. (d) Myeloperoxidase (MPO) activity was measured in sham or ischemic liver tissues harvested from different groups 6 h after reperfusion. *$P < 0.05$. $n = 6$.

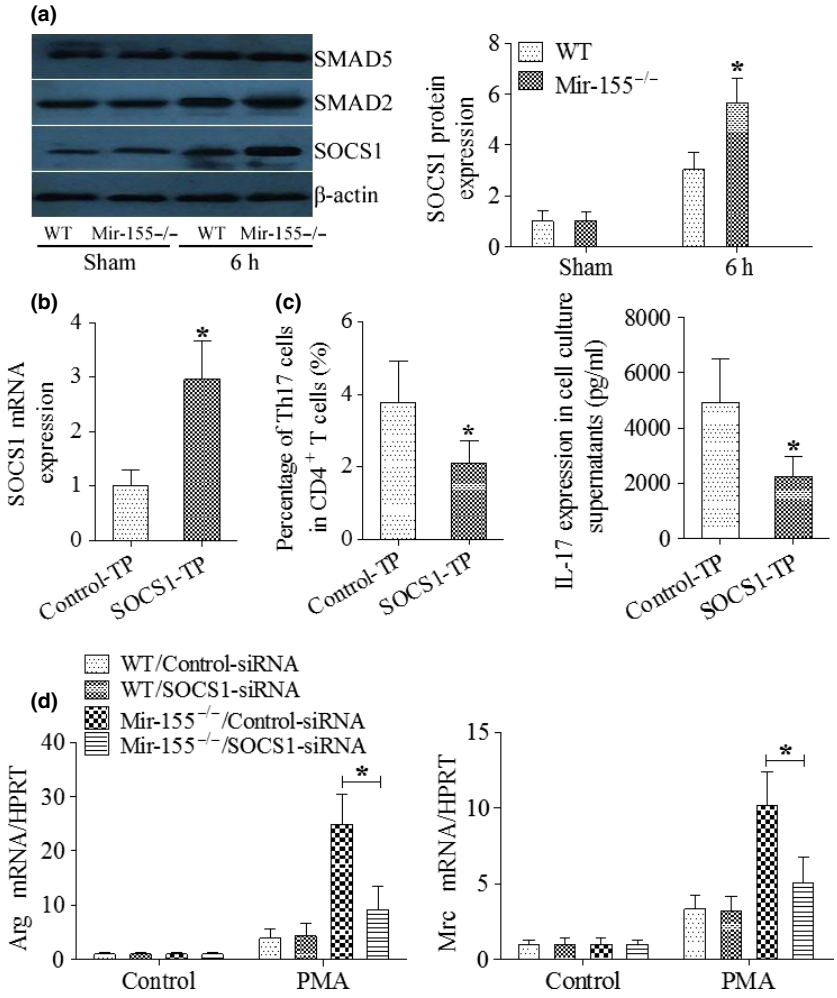

Figure 7 Mir-155 deficiency suppressed Th17 cell differentiation via upregulation of SOCS1. (a) SOCS1 protein expression in regions surrounding the injury site of WT and mir-155$^{-/-}$ mice 42 days after SCI was analyzed by Western blot; (b) 4 days after SOCS1-TP$^{miR\text{-}155}$ and control-TP$^{miR\text{-}155}$ were transfected, the levels of the transcripts involved in Th17 cells differentiation were detected by Q-PCR; (c) 4 days after SOCS1-TP$^{miR\text{-}155}$ and control-TP$^{miR\text{-}155}$ were transfected, the percentages of Th17 were analyzed by flow cytometry and the level of IL-17A in cell culture supernatant was quantified by ELISA; (d) bone marrow-derived macrophages (BMMs) obtained from mir-155$^{-/-}$ and WT mice were transfected with Socs1-siRNA or Control-siRNA (Ambion) and then cultured in the absence or presence of phorbol myristate acetate (PMA) (100 ng/ml; Sigma) for 48 h. Then, the cells were harvested for mRNA analysis. *$P < 0.05$. $n = 6$.

deficiency macrophages. This was supported by our finding that $GdCl_3$, which inactivates KCs/macrophages, reversed the nature of the liver immune response and recreated IRI in mir-155 deficiency mice.

Interleukin (IL)-17A is a member of the IL-17 family, which includes six structurally related isoforms: IL-17A, IL-17B, IL-17C, IL-17D, IL-17E, and IL-17F. IL-17A was secreted by different cells, including Th17 cells, γδ T cells, NK cells, NKT cells, and neutrophils. IL-17A has been shown to play a critical role in inflammation as well as host immune defense [22,23]. Indeed, IL-17A is upregulated in a variety of inflammatory conditions, including rheumatoid arthritis, systemic lupus erythematosus, and sepsis [24,25]. Furthermore, IL-17A induces expression of several additional proinflammatory genes, including IL-8, TNF-α, and IL-1 [25]. In our experiment, mir-155 deficiency alleviates Th17 response and limits Th17 differentiation, and inhibition of IL-17 with anti-IL-17 antibody attenuates liver IRI. All of these demonstrated that decreased IL-17 expression contributes to the alleviated liver IRI induced by mir-155 deficiency.

Many direct targets of mir-155 in DCs and T cells have been identified. The essential role of mir-155 elucidated by targeting SOCS1 expression is not only confined to Treg cells, but also macrophages and DCs. Recently, it has been shown that IL-12 production by DCs is regulated by mir-155-mediated targeting of SOCS1 [26]. Consistent with previous studies, we found reduced expression of

Th17-polarizing cytokines in DCs from mir-155$^{-/-}$ mice with liver IRI. Altered expression of Th17-polarizing cytokine expression in DCs that influence the differentiation of Th17 responses could be an additional mechanism by which mir-15$^{-/-}$ mice are resistant to liver IRI.

SOCS1 is a negative regulator of Janus kinase (JAK)/STAT signaling pathway. Recently, the role of SOCS1 in Th17 cell differentiation and function was clarified by Johnson and his group by characterizing a mimetic of SOCS1, namely novel tyrosine kinase inhibitor peptide (Tkip). They found that Tkip blocked IL-6-induced activation of STAT3 and inhibited the development of Th17 and the production of IL-17A [27–29]. Meanwhile, recent study uncovered SOCS1 as a critical molecular switch that tunes key signaling pathway to effectively program different sides of the macrophage balance [30]. In our current study, we showed that mir-155 is a critical player in driving Th17 cell differentiation and enhancing Th17 cell function by directly inhibiting SOCS1. And SOCS1 knock-down in macrophages largely abolished the promoted effect on M2 macrophages differentiation induced by mir-155 deficiency. These were in accordance with recent publication indicating the important role of SOCS1 in the mir-155 regulation [31,32].

In summary, our data document an innate immune-regulatory role for mir-155 in the liver inflammatory response against IR. Mir-155 deficiency promotes M2 macrophages development and suppresses IL-17 expression via upregulation of SOCS1. Although further investigations are needed to fully clarify the precise molecular and cellular mechanism involved in the immunoregulation, mir-155 may be a novel therapeutic target to protect liver IRI.

Authorship

BT, ZW and SH: designed the study. BT, ZW, GQ, SY, SY and BL: performed the study. YW, QH and RZ: collected the data and wrote the paper.

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