Inhibition of microRNA-155 relieves sepsis-induced liver injury through inactivating the JAK/STAT pathway

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Abstract. The present study aimed to investigate whether the microRNA (miR)-155 inhibitor has an anti-inflammatory effect on sepsis-associated liver injury and whether this effect is associated with the activity of the janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathway. BALB/c mice were randomly divided into three groups (n=40 per group): Control, lipopolysaccharide (LPS) and miR-155 inhibitor plus LPS groups. Liver injury was induced by injection of LPS (20 mg kg^{-1}). In the inhibitor plus LPS group, LPS was administered after injecting the miR-155 inhibitor (80 mg kg^{-1}) for 3 days. Liver tissues were collected at 6, 12, 24 and 48 h after LPS exposure. Hematoxylin and eosin was used to identify the histological changes in the liver. The expression levels of miR-155, suppressor of cytokine signaling 1 (SOCS1) and STAT1 were determined by reverse transcription-quantitative polymerase chain reaction. The protein expression of tumor necrosis factor (TNF)-α and interleukin (IL)-10 were detected by ELISA. miR-155 inhibitor pretreatment alleviated the symptoms of LPS-exposed mice, and reduced LPS-induced mortality and liver injury. Compared with the LPS group, expression of miR-155 was significantly reduced in the miR-155 inhibitor plus LPS group at 6 h (P<0.05). SOCS1 expression was significantly increased in miR-155 inhibitor plus LPS group compared with the control and the LPS group at 12 h (P<0.05). There was a lower level of STAT1 in the miR-155 inhibitor plus LPS group compared with the LPS group (P<0.05). In addition, TNF-α and IL-10 were significantly decreased in the miR-155 inhibitor plus LPS group compared with the LPS group (P<0.05). In conclusion, the miR-155 inhibitor relieves liver injury by enhancing the expression of SOCS1 and inactivating JAK/STAT signaling.

Introduction

On an annual basis, sepsis is diagnosed in ~600,000 patients in North America, with a mortality rate ranging between 30 and 50% (1), thereby resulting in an expensive medical problem. Moreover, sepsis is a leading cause of mortality among patients in intensive care units (2). Although sepsis can be brought under control with the use of comprehensive treatment (3), specific therapeutic interventions for this disease have not been identified, posing a serious threat to human health.

It is well-known that sepsis can induce systemic inflammatory response syndrome, frequently leading to multiple organ failure, including liver injury (4). Although liver dysfunction occurs frequently in cases of sepsis (5), the comprehensive understanding of sepsis-associated liver injury remains limited. The liver may directly function in the development of inflammation in response to sepsis-induced injury, leading to the further promotion of sepsis (6), in addition to its vital role in metabolism. Therefore, liver injury may be positively associated with sepsis, suggesting that sepsis may be controlled to an extent through relieving liver injury.

Recently, invading microorganisms have been identified as a major cause of sepsis (7). Lipopolysaccharide (LPS), a major component of the outer membrane of Gram-negative bacteria (8), may uncontrollably activate the innate immune system, resulting in the production of inflammatory mediators that may be a cause of septic shock (9). This event is caused by activation of toll-like receptor 4, a conventional pattern-recognition receptor, which recognizes LPS and results in the triggering of downstream signaling cascades and production of chemokines, such as tumor necrosis factor (TNF)-α and interleukin (IL)-10 (10,11). The latter binds to receptors and then activates a major second messenger pathway, the Janus activated kinase (JAK)/signal transducer and activator of transcription (STAT) signaling pathway (12). Although the continuous activation of the JAK/STAT pathway contributing to inflammation and sepsis has been identified (13), the correlation between the JAK/STAT signaling pathway and sepsis-associated liver injury remain largely unknown.

MicroRNAs (miRNA), single-stranded noncoding small RNA molecules ~19-24 nucleotides in length, are

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posttranscriptional regulators of gene expression and exhibit their effects by imperfect base pairing to target mRNAs for degradation or translational repression (14). Studies have demonstrated that circulating miRNAs were identified in the serum and plasma as biomarkers of sepsis (15,16). Specifically, LPS, an activator of sepsis and the JAK/STAT signaling pathway, increases the production of miR-155 (17). miR-155 inhibits the negative feedback loop of the JAK/STAT signaling pathway via posttranscriptional silencing of suppressor of cytokine signaling 1 (SOCS1) and potentiates the inflammatory signaling of STAT3 (18). Given the important role of JAK/STAT signaling in sepsis, it is rational to suggest that miR-155 may be a major contributor to sepsis and inhibition of miR-155 may suppress sepsis through inactivating JAK/STAT signaling. However, the role of miR-155 in sepsis-associated liver injury has not yet been defined. The present study aimed to investigate the expression of miR-155 in the liver of LPS-exposed mice and to determined whether inhibition of miR-155 may relieve sepsis-induced liver injury through inactivating the JAK/STAT pathway.

Materials and methods

Experimental animals and study design. A total of 120 male BALB/c mice (weight, 20±2 g) were obtained from the Department of Animal science of Fudan University in Shanghai (Shanghai, China). The mice were kept in cages with a 12 h light-dark cycle and access to dry pellets and sterile water *ad libitum*. The mice were randomly divided into three groups (n=40 per group): Control, LPS and miR-155 inhibitor plus LPS groups. After anesthetizing intraperitoneally (i.p.) with pentobarbital (0.3 mg kg⁻¹), the control and LPS groups were injected with sterile saline i.p., and the miR-155 inhibitor plus LPS group was intravenously injected with 80 mg kg⁻¹ miR-155 inhibitor (Shanghai GenePharma Co., Ltd., Shangai, China) for 3 days through the tail vein, twice per day. Subsequently, the LPS and miR-155 inhibitor plus LPS group were injected with 20 mg kg⁻¹ LPS (*E. coli* 0111:B4, Sigma-Aldrich, St. Louis, MO, USA), and the control group was i.p. injected with an equal volume of sterile saline at the same time. Mice in these three groups were sacrificed by decapitation at four time points, 6, 12, 24 and 48 h after LPS exposure (n=10 per time point), and livers were removed surgically for the follow-up experiments. All experiments were conducted in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The present study was approved by the ethics committee of the Children's Hospital Affiliated to Shanghai Jiaotong University (Shanghai, China).

Histology and ELISA analyses. Prior to paraffin embedding, livers were fixed in 4% paraformaldehyde overnight at room temperature and then transferred to 70% ethanol. Subsequently, organs were embedded and frozen using liquid nitrogen-cooled isopentane, and then paraffin-embedded samples were sectioned at 4-μm thickness. For pathological analysis, paraffin sections were stained with hematoxylin and eosin. The sections were observed under an optical microscope (CKX31SF; Olympus Corporation, Tokyo, Japan).

Table I. Gene-specific primers for miR155, SOCS1, STAT1, GAPDH and 5S in RT-qPCR.

Gene	Primer
miR-155	F: 5'-CGGCGGTTAATGCTAATTGTGAT-3'
	R: 5'-GTGCAGGGTCCGAGGT-3'
SOCS1	F: 5'-TCCGATTACCGGCGCATCACG-3'
	R: 5'-CTCCAGCAGCTCGAAAAGGCA-3'
STAT1	F: 5'-ATTTCTCCTTCTGGCCTTG-3'
	R: 5'-AGGAACGTCCCTGGCTG-3'
GAPDH	F: 5'-TGCACCACCAACTGCTTAGC-3'
	R: 5'-GCATGGACTGTGGTCATGAG-3'
5S	F: 5'-TCGTCTGATCTCGGAAGCTA-3'
	R: 5'-AAGCCTACAGCACCCGGTAT-3'

RT-qPCR, reverse transcription-quantitative polymerase chain reaction; miR, microRNA; F, forward; R, reverse; SOCS1, suppressor of cytokine signaling 1; STAT1, signal transducer and activator of transcription.

Prior to ELISA analysis, liver samples were mechanically homogenized in protease-inhibitor (Sigma-Aldrich) containing phosphate-buffered saline, and the homogenates were centrifuged at 11,330 x g for 30 min at 4°C. The protein concentrations of the supernatant fraction was measured according to a bicinchoninic acid (BCA) protein measurement kit (R&D Systems, Minneapolis, MN, USA). TNF-α and IL-10 ELISA kits were purchased from R&D Systems; sheep anti-mouse polyclonal antibody (1:200) was used to detect TNF-α, and mouse monoclonal antibody (1:1,000) was used to detect IL-10.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was isolated from livers (50 mg) using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. First-strand cDNAs were generated from 3 μg of total RNA using commercially available kits (Applied Biosystems, Foster City, CA, USA). All subsequent PCR reactions were performed using the Universal PCR Master mix (Applied Biosystems). Primers of miR-155, STAT1 and SOCS1 used for amplification are shown in Table I. Amplification conditions were as follows: 95°C for 10 min, followed by 40 cycles of 15 sec at 95°C, 1 min at 60°C. The melting curves were acquired by heating the samples to 95°C for 1 min, cooling to 55°C for 1 min and then slowly increasing the temperature from 65 to 95°C at a rate of 0.5°C/30 sec. Thermal cycling and fluorescence detection of mRNA were analyzed by the 7500 real-time PCR system (Applied Biosystems). To normalize mRNA concentrations, transcriptional levels of 5S or GAPDH mRNA were identified in parallel for each sample, and the relative transcriptional level of miR-155 was adjusted by standardization based on the 5S mRNA levels and, SOCS1 and STAT1 were adjusted by standardization based on the GAPDH mRNA levels. Samples for each experimental condition were run in triplicate.
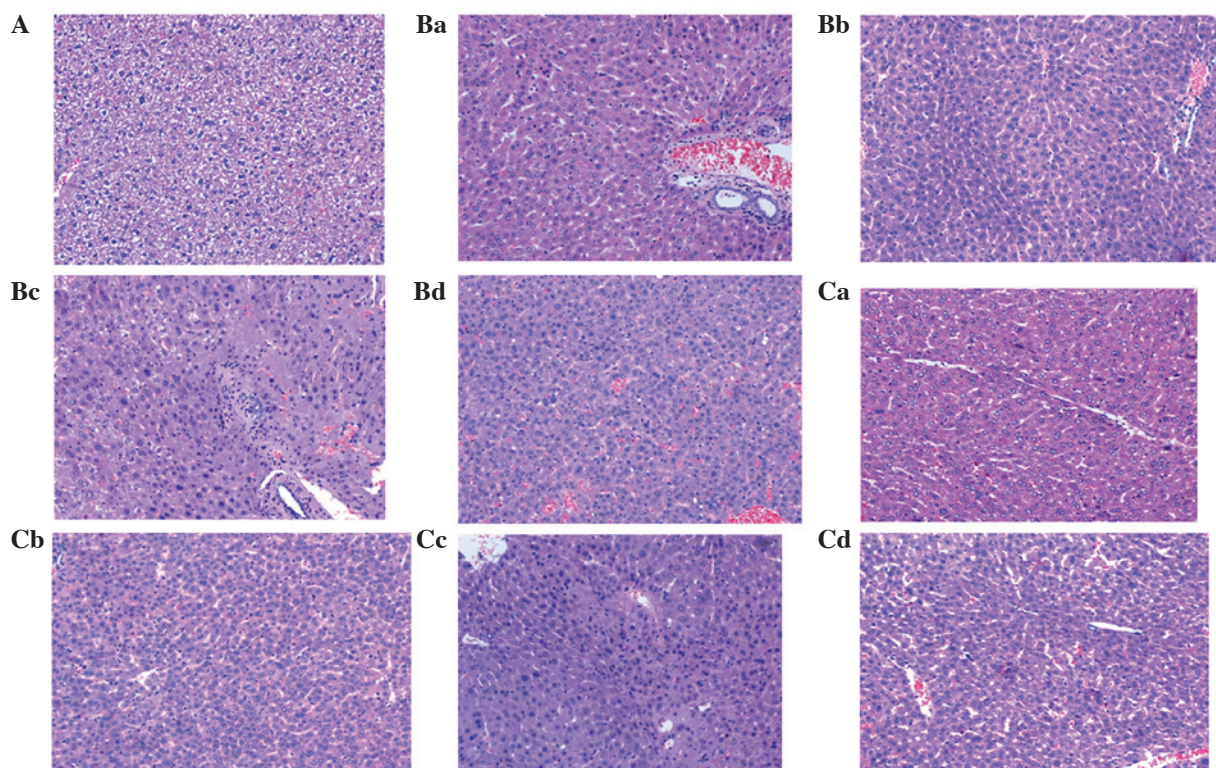
Figure 1. Light microscopy analysis shows that the miR-155 inhibitor relieves sepsis-associated liver injury induced by LPS administration (hematoxylin and eosin staining, x200). (A) Liver section from the control mouse. Liver sections from mice treated with LPS at (Ba) 6, (Bb) 12, (Bc) 24 and (Bd) 48 h. Liver sections from the miR-155 inhibitor-pretreated mice induced by LPS at four sequential time points (Ca) 6, (Cb) 12, (Cc) 24 and (Cd) 48 h. miR, microRNA; LPS, lipopolysacharide.

Statistical analysis. All data were subjected to assessment of the treatment effects using Student's t-test with SPSS 13.0 software (SPSS Inc., Chicago, IL, USA). P<0.05 and 0.01 were considered to indicate a statistically significant difference. The results are presented as the mean ± standard deviation.

Results

miR-155 inhibitor alleviates the symptoms of LPS-exposed mice. The ethological changes after LPS treatment in miR-155 inhibitor-pretreated mice were first surveyed. As expected, LPS administration lead to fatigue and increased heart rate, and significantly reduced activity and food intake as compared with the saline-treated control group. Although mice pretreated with the miR-155 inhibitor for 3 days also exhibited these symptoms in contrast to the LPS group, they were less severe. In addition, the mortality of the LPS group and miR-155 inhibitor plus LPS group was 12.5% and 5%, respectively. In the LPS group, 7.5% of mice died within 12 h and 5% of mice died between 12 and 24 h. However, 5% of mice receiving miR-155 inhibitor died within 12 h after LPS challenge, and no mice died between 12 and 24 h. These results demonstrate that the miR-155 inhibitor contributes to the reduction of LPS-induced mortality.

miR-155 inhibitor relieves LPS-induced liver injury. Next to investigate the effects of the miR-155 inhibitor on liver injury, the histological changes following LPS treatment in miR-155 inhibitor-pretreated mice were determined. In the control group, liver tissue had a normal structure (Fig. 1A). By contrast, when mice were injected with LPS, characteristics of liver injury, such as infiltration of inflammatory cells and acidophilic and vacuolar degeneration, were first observed 6 h after LPS treatment, and the symptoms became more severe in a time-dependent manner (Fig. 1B). Furthermore, abnormal characteristics, including karyopyknosis, nodular necrosis, cytoplasm rarefaction, infiltration of neutrophils into the hepatic sinusoid and portal area, and disorder of liver structure were first observed in mice treated with LPS for 24 h and then became more severe at 48 h. In the miR-155 inhibitor plus LPS group, miR-155 inhibitor pretreatment markedly relieved LPS-induced pathological changes in the liver (Fig. 1C). The infiltration of inflammatory cells was observed at 24 h after LPS treatment, and only slight infiltration of inflammatory cells and acidophilic degeneration was observed in the following time-points. Moreover, disorder of liver structure and nodular necrosis were not observed at any time point. These data suggest that the miR-155 inhibitor protected mice against LPS-induced liver injury.

miR-155 inhibitor reduces the expression of LPS-induced miR-115. To investigate whether expression of miR-155 induced by LPS can be suppressed by miR-155 inhibitor, expression of miR-155 in the mouse liver was detected by RT-qPCR, as shown in Fig. 2. The mouse livers had a low basal level of miR-155 expression in the control group. The expression of miR-155 was significantly increased when treated with LPS for 6 h (P<0.05), which suggests that LPS is able to improve miR-155 expression in mouse liver, and this effect was inversely dependent on the time of LPS exposure. miR-155
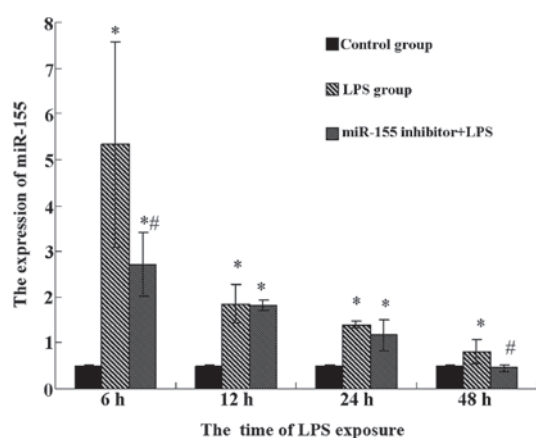
Figure 2. miR-155 inhibitor reduces the expression of miR-155 in mouse liver, as detected by reverse transcription-quantitative polymerase chain reaction. *P<0.05, compared with the control group and #P<0.05, compared with the LPS group. miR, microRNA; LPS, lipopolysaccharide.
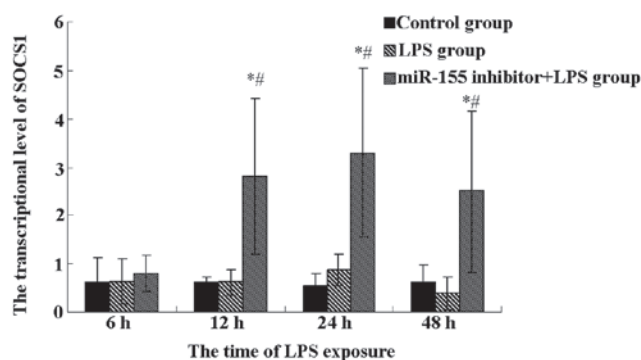

Figure 3. Transcriptional levels of SOCS1 can be enhanced by the miR-155 inhibitor, as detected by reverse transcription-quantitative polymerase chain reaction. *P<0.05, compared with the control group and #P<0.05, compared with the LPS group. miR, microRNA; LPS, lipopolysaccharide; SOCS1, suppressor of cytokine signaling 1.
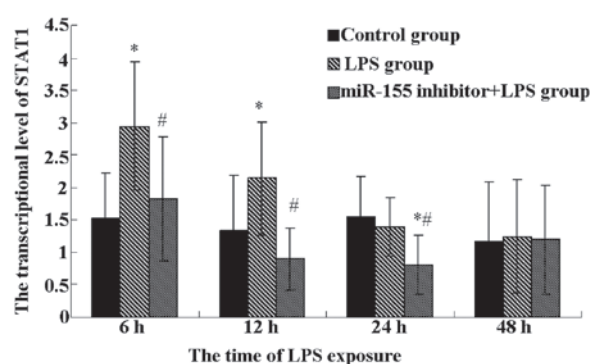
Figure 4. miR-155 inhibitor decreases the expression of STAT1 at the mRNA level, as detected by reverse transcription-quantitative polymerase chain reaction. *P<0.05, compared with the control group and #P<0.05, compared with the LPS group. miR, microRNA; LPS, lipopolysaccharide; STAT1, signal transducer and activator of transcription.

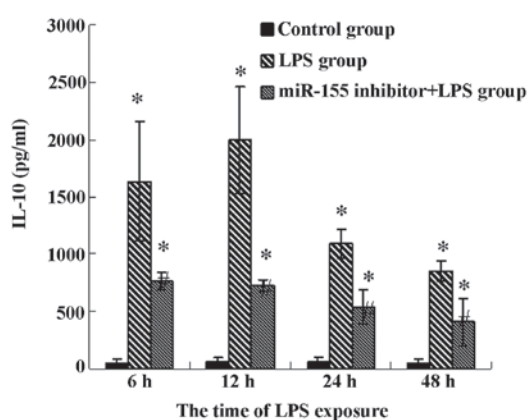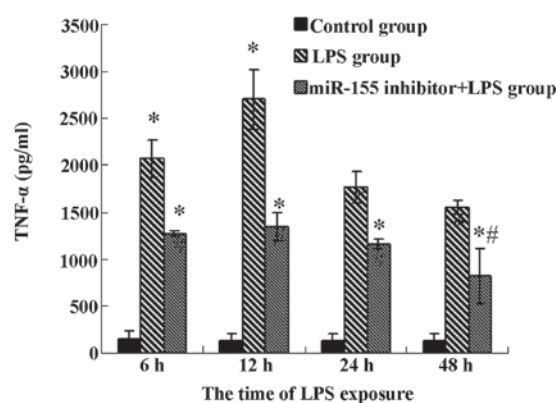

Figure 5. Production of TNF-α and IL-10 enhanced by LPS administration can be partly neutralized by pretreament with the miR-155 inhibitor, as detected by ELISA. *P<0.05, compared with the control group; #P<0.05, compared with the LPS group. TNF, tumor necrosis factor; IL interleukin; LPS, lipopolysaccharide; miR, microRNA.

inhibitor-pretreated mice incubated with LPS showed significantly lower expression of miR-155 than saline-pretreated mice incubated with LPS at 6 and 48 h respectively (P<0.05). However, no significant difference was identified between these two groups at 12 or 24 h. Collectively, these findings suggest that LPS induces miR-155, which is inhibited by the miR-155 inhibitor.

miR-155 inhibitor inactivates JAK/STAT1 signaling by elevating SOCS1 expression. It has been described that miR-155 potentiates the inflammatory signaling of JAK/STAT through targeting SOCS1 protein (18,19). Therefore, it was primarily assessed whether the miR-155 inhibitor can regulate the expression of SOCS1 in the mouse liver. As shown in Fig. 3, as expected, the transcriptional level of SOCS1 was detected to show differential expression in the miR-155 inhibitor plus LPS group. Although identical expression was found among these three groups at 6 h, the SOCS1 expression was significantly increased in the miR-155 inhibitor plus LPS group compared with the control and LPS groups after 12 h treatment (P<0.05), indicating that the miR-155 inhibitor enhances the SOCS1 expression.

It was then determined whether SOCS1 expression enhanced by the miR-155 inhibitor results in the inhibition of JAK/STAT signaling. STAT1 expression was observed at the mRNA level, as shown in Fig. 4. Results showed that the LPS group highly expressed STAT1 at 6 and 12 h, while the expression of STAT1 was markedly reduced in the miR-155 plus LPS group at the corresponding times (P<0.05). Additionally, the miR-155 inhibitor plus LPS group also reduced the expression of STAT1 at 24 h in contrast to the control and LPS groups

(P<0.05), although there was no significant difference between the control and LPS group. These observations indicate that the miR-155 inhibitor inactivates JAK/STAT1 signaling by elevating SOCS1 expression.

Production of TNF-α and IL-10 are decreased by the miR-155 inhibitor. Given that LPS results in the production of cytokines, including TNF-α and IL-10, which are involved in the process of sepsis, it was then investigated whether the production of these two cytokines is altered by treatment with LPS in miR-155 inhibitor-pretreated mouse liver. Notably, the TNF-α production was higher when mice were treated with LPS (P<0.05), however, the TNF-α production was significantly reduced when miR-155 inhibitor-pretreated mice were treated with LPS (P<0.05, Fig. 5). Similarly, the production of IL-10 was significantly increased in the LPS treated group compared with the saline treated control group (P<0.05), and this increase can be partly neutralized by pretreatment with the miR-155 inhibitor (P<0.05). These results reveal that LPS induces the production of TNF-α and IL-10, which is reversed partially by the miR-155 inhibitor.

Discussion

In the present study, LPS administration was used to investigate sepsis-associated liver injury. It was demonstrated that the miR-155 inhibitor exerts potent anti-inflammatory and immunomodulatory actions in LPS-exposed mouse liver, as evidenced by markedly reducing the mortality of LPS-exposed mice and the significant decreases in liver injury. Previous studies have demonstrated that overexpression of miRNA let-7e in macrophage results in sensitivity to LPS in cell culture and animal models (20), and downregulated miR-125b upon LPS challenge probably contributes to an increase in the production and secretion of TNF-α (21). Previous studies, together with the results from the present study give strong indication that miRNA maybe central in LPS-induced sepsis and highlights the significance and relevance of miRNAs, such as miR-155, as potential downstream biomarkers for therapeutic intervention in sepsis-associated liver injury.

miR-155, is the most extensively investigated miRNA in innate immune cells (22), and mediates the immune response through targeting a number of genes for translational repression. A previous study demonstrated that miR-155 most likely directly targets transcripts coding for several proteins involved in LPS signaling, such as Fas-associated death domain protein, IκB kinase ε and the receptor (TNFR superfamily)-interacting serine-threonine kinase 1, thereby enhancing TNF-α translation (21). In addition, miR-155 regulates human dendritic cell development and IL-12 production through targeting Kip1 ubiquitination-promoting complex 1 and SOCS1, respectively (23). Foxp3-dependent miR-155 confers competitive fitness to regulatory T cells by inducing SOCS1 downregulation (19). Given the results in previous studies, administration of the miR-155 inhibitor may lead to the inactivation of above-mentioned genes and reduced production of cytokines, such as TNF-α. This is consistent with the current results that miR-155 inhibitor-pretreated mice induced by LPS not only show the observably upregulated expression of SOCS1 but also exhibit a significant decrease in the secretion of TNF-α.

SOCS1, a pivotal downregulating factor for LPS signal pathways (24), possesses a negative regulatory role in the JAK/STAT signal cascade (25), thereby acting as an essential negative regulatory molecule in innate immune responses. Thus, it was hypothesized that expression of SOCS1 enhanced by the miR-155 inhibitor may inhibit the JAK/STAT signaling pathway. Consistent with this, follow-up experiments identified a member of the STAT family, STAT1, which exhibited lower expression levels following treatment with LPS in miR-155 inhibitor-pretreated mice compared with treatment with LPS in saline-pretreated mice. It is therefore rational to conclude that the ability of the miR-155 inhibitor to protect against LPS-induced liver injury occurs through enhancing the expression of SOCS1 and in turn inhibiting JAK/STAT signaling.

Additionally, besides the decrease in the secretion of TNF-α, IL-10, is also attenuated in miR-155 inhibitor-pretreated mice induced by LPS. Given that excessive cytokine-mediated inflammation was hypothesized to be crucial in the development of other tissue injuries (26), decreases in the levels of cytokines, such as TNF-α and IL-10, may alleviate the inflammatory reaction and then ameliorate the sepsis-associated liver injury.

In conclusion, the present study identified that the miR-155 inhibitor enhanced the expression of SOCS1 and significantly relieved the liver injury induced by LPS through inactivating the JAK/STAT signaling pathway. These findings emphasize the correlation between miRNA and sepsis-associated liver injury, and suggest that miR-155 may be a potential target for treating sepsis-associated liver injury in the future.

Acknowledgements

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