miR-505-5p alleviates acute rejection of liver transplantation by inhibiting Myd88 and inducing M2 polarization of Kupffer cells

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Original Article

miR-505-5p alleviates acute rejection of liver transplantation by inhibiting Myd88 and inducing M2 polarization of Kupffer cells

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Abstract

The occurrence of acute rejection after liver transplantation seriously impairs the prognosis of patients. miRNA is involved in many physiological and pathological processes of the body, but the mechanism of miRNA action in liver transplantation is not completely clear. In this study, we discuss the role of miR-505-5p in acute rejection after liver transplantation and its putative regulating mechanism. We construct an allogeneic rat liver transplantation model, observe the morphological and pathological changes in liver tissue, detect the expression levels of Myd88, miR-505-5p, IL-10, and TNF-α, and confirm that Myd88 is one of the direct targets of miR-505. The effects of miR-505-5p on the Myd88/TRAf6/NF-κB and MAPK pathways are detected both in vitro and in vivo, and the standard markers of Kupffer cell M1/M2 polarization are also detected. The results of qRT-PCR experiments show
that miR-505-5p has a downward trend in rats with acute rejection. Western blot analysis reveals that overexpression of miR-505-5p induces the reduction of NF-κB and MAPK pathways both in vitro and in vivo. The role of miR-505-5p in alleviating acute rejection after transplantation may be accomplished by inducing M2-type polarization of Kupffer cells. In conclusion, we find that miR-505-5p alleviates acute rejection of liver transplantation by inducing M2 polarization of macrophages via the Myd88/TRAF6 axis, which suggests a potential strategy based on miRNAs in the follow-up treatment of liver transplantation.

**Keywords:** immune tolerance, liver transplantation, macrophages, miR-505-5p, Myd88

**Introduction**
Liver transplantation is an effective treatment for end-stage liver disease. Acute rejection is a serious complication after liver transplantation and an important reason for reducing the long-term survival rate of transplanted livers. Mechanism exploration of immune tolerance to identify a more ideal treatment has always been a research priority to improve rejection [1]. Macrophages are critical with phagocytosis and digestion functions and play an important role in the adaptive immunity as well as innate immunity. Several studies showed that macrophages are closely related to immune rejection after organ transplantation [2]. Under stimulation of different external factors, macrophages could be further differentiated into the classically-activated M1 type and alternatively-activated M2 type [3]. M1-type macrophages have a strong antigen-presenting function after transplantation and play a pivotal role in the immune response, immune surveillance and acute rejection after transplantation. Under some other stimuli, macrophages are polarized into the M2 type, which promote the repair of damaged tissues, reduce the inflammatory response, and weaken the immune response.

Kupffer cells (KCs) are macrophages located in the liver and responsible for liver immunity. KCs account for 80%–90% of the resident macrophages in the whole body, and exhibit a strong phagocytic function and secrete disparate cytokines. KCs are indispensable in immune rejection after liver transplantation and other liver diseases [4,5]. In liver transplantation, activated KCs also exert their immunoregulatory functions by activating the NF-κB signaling pathway, secreting
cytokines and expressing related immune adhesion molecules [6]. NF-κB is an important nuclear transcription factor that is ubiquitous in the cytoplasm and is located in the center of the downstream signaling pathway of TLR4 [7]. The mammalian Toll-like receptor (TLR) family consists of 13 members and recognizes specific patterns of microbial components called pathogen-associated molecular patterns (PAMPs) [8]. TLRs are primarily expressed on dendritic cells and macrophages, including epithelial and endothelial cells. TLRs signal via specific adaptor proteins, such as Myd88, to activate the NF-κB/MAPK signaling pathway, which results in KC polarization and the secretion of specific cytokines to promote T cell differentiation.

MicroRNAs (miRNAs) are small endogenous noncoding RNAs that interact with the 3'-untranslated region (3'-UTR) of target mRNA to inhibit translation or promote mRNA degradation. Shaked et al. [9] collected 318 serum samples from liver transplant recipients and found that the expression levels of several miRNAs correlated with acute rejection, suggesting that miRNAs interfere with acute rejection and play a certain role in reducing postoperative acute rejection. Previous studies have shown that miR-505-5p is an anti-inflammatory regulator targeting HMGB1 to inhibit lipoendometritis [10] and induces M2-type polarization of mouse bone marrow stromal cells via transmembrane protein 229B [11]. In addition, Li et al. [12] performed liver transplantation on rats and screened for differentially expressed miRNAs in acute rejection and rejection. These studies indicated that miR-505-5p may play an important role in immune tolerance. However, the role and mechanism of miR-505-5p in acute liver transplantation have not been fully elucidated.

In this study, we found that the expression of miR-505-5p is significantly reduced in acute rejection rats and miR-505-5p may be involved in immune tolerance after liver transplantation.

Materials and Methods

Animals and liver transplantation models
Male Lewis (LEW) rats and Brown Norway (BN) rats (12 weeks old, body weight 200–220 g) were purchased from Weitong Lihua Laboratory Animal Technology Co., Ltd (Beijing, China). All animal experiments were performed in accordance with the guidelines approved by the Animal Ethics Committee of Chongqing Medical University. The “double-cuff method” was used to establish the orthotopic liver
transplantation model of LEW-BN rats (LEW rats were used as donors, and BN rats were used as recipients) [13]. After the experiment, the normal saline (NS)-treated group was injected with 1 mL of sterile normal saline through the portal vein. For the experiment groups, the rats were injected with agomiR-505-5p or agomiR NC (10 µg/kg, diluted in siRNA-mate) (GenePharma, Shanghai, China) through the portal vein. The sequence of agomiR-505-5p is: 5′-GGGAGCCAGGAUGAUGAUU-3′, the sequence of agomiR NC is: 5′-UUCUCCGAACGUGUCACGUTT-3′. In addition, in order to eliminate the influence of postoperative hepatitis, the control group was treated only by laparotomy, the sham group was the experimental control group, and the BN to BN syngeneic transplantation was used in the control group, and the rest were allogeneic transplantation groups. At 1, 3, 5, 7, and 9 days after the surgery, blood samples were collected and rats were sacrificed to obtain liver tissue. Part of the liver tissue was used to extract KCs and detect mRNA and protein expressions, and part of the liver tissue was fixed with 4% paraformaldehyde for subsequent experiments.

Liver pathology observation
The livers were cut into 0.5-cm pieces, fixed with paraformaldehyde, and stained with hematoxylin&eosin (H&E) (Bopei, Chongqing, China). Pathological changes were observed under a light microscope. Acute rejection was graded according to the Banff International Panel consensus document [14].

Liver Kupffer cell isolation
After the rats were anesthetized and opened, D-Hanks buffer (Solarbio, Beijing, China) was injected through the portal vein until the liver became khaki, and then Hanks buffer (Beyotime, Shanghai, China) containing 0.5 mg/mL type IV collagenase (Beyotime) was injected through the portal vein. The perihepatic blood vessels were clamped after the liver was inflated, and the blood vessels were opened after 5 min of digestion. The above procedure was repeated several times until the surface of the liver appeared gritty. The liver was removed, fully crushed and placed at 37°C in 0.1 mg/mL type IV collagenase for further digestion for 30 min. The Kupffer cells were isolated via differential centrifugation using Percoll solution. The cells were reselected with 10 mL of DMEM (Gibco, Grand Island, USA) and seeded in a plastic Petri dish. After 2 h, the surface of the culture dish was rinsed with PBS, and the
adherent cells were KCs.

**Cell culture and transfection**

Cells were cultured in DMEM supplemented with 5% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Cells were grown at 37°C in a humidified atmosphere with 5% CO₂ to 50%–70% confluence for subsequent experimental treatments. KCs were stimulated with 500 ng/mL LPS (Cell Signaling Technology, Danvers, USA) for 3, 6, 12, 24, 36 and 48 h according to the reagent instructions, and then with concentrations of 50, 100, 200, 500 and 1000 ng/mL LPS stimulated KCs for 24 h to find the optimal experimental conditions for the *in vitro* inflammation model. The miR-505-5p mimic (5'-GGGAGCCAGGAAGUAUUGAUGUU-3') and negative control miRNA (5'-UUCUCCGAACGUACGUACGUUTNC-3') (GenePharma) were transected into cells using Lipo8000 (Beyotime) separately following the manufacturer’s instructions. Myd88 adenovirus (adv-Myd88) or NC adenovirus (adv-NC) (VectorBuilder, Guangzhou, China) was transfected into cells according to the manufacturer's instructions.

**Quantitative real-time polymerase chain reaction**

Total RNA was extracted from cells using TRIzol Reagent (Invitrogen, Carlsbad, USA). cDNA was reverse transcribed using a PrimeScript RT reagent Kit (TaKaRa, Tokyo, Japan). The expressions of genes were detected by qRT-PCR using the SYBR PrimeScript RT-PCR kit (ToyoBo, Osaka, Japan). The PCR program is 95°C for 5 min, followed by 40 cycles of 95°C for 15 s and 60°C for 60 s. GAPDH was used as an internal normalisation control for mRNA levels, and *U6* was used as an internal control for normalisation of miRNA levels. Primers were purchased from GenePharma and primer sequences are shown in Table 1. Calculation of mRNA levels was fulfilled by 2^ΔΔCt comprising triplicate results.

**Transfection and luciferase reporting assay**

The wild-type 3'-UTR (WT-pSi-Check2/Myd88 3'-UTR) luciferase reporter vector (Hanbio Biotechnology, Shanghai, China) was constructed by restriction endonuclease (Thermo Scientific, Waltham, USA) digestion, which was confirmed by sequencing. In addition, we synthesized the Myd88 3'-UTR MUT vector in which the binding site of miR-505-5p was mutated. Plasmids containing the WT or MUT of
the 3’-UTR sequence of Myd88 were cotransfected with negative control mimics or miR-505-5p mimics in 293T cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. Cells were assayed 48 h later using the Promega Dual-Luciferase system (Promega, Madison, USA).

**Western blot analysis**

Total protein was extracted from treated cells using RIPA lysis buffer (Beyotime), and subject to 10% SDS-PAGE, followed by electrotransfer onto PVDF membranes (Millipore, Boston, USA). The membranes were blocked with 5% nonfat milk for 1 hour, incubated with the primary antibody at 4°C overnight, and incubated with the HRP-conjugated secondary antibody for 1 h at room temperature. Protein bands were visualized using enhanced chemiluminescence (ECL) kit (New England Biolabs, Beverly, USA). Antibodies used are shown in Table 2.

**Immunofluorescence microscopy**

Cells were plated into 35 mm confocal dishes (Biosharp, Hefei, China), fixed with 100 μL of 4% paraformaldehyde for 15 min, permeabilized with 0.1% Triton X-100, and blocked with 10% goat serum. Fixed cells were incubated with primary antibodies overnight at 4°C, followed by incubation with the fluorescent secondary antibody in the dark for 1 h. DAPI was used to stain the cell nuclei after incubation. Cells were examined and images were captured with a fluorescence microscope (ZEISS, Jena, Germany). Antibodies used are shown in Table 3.

**ELISA**

Blood samples were collected from the abdominal aorta of rats, and the serum concentrations of TNF-α, IL-10, TGF-β1, and ALT and AST were measured using the corresponding ELISA kits (Meike, Shanghai, China) according to the manufacturer’s instructions.

**Statistical analysis**

Data are presented as the mean ± SD. The independent samples t test was used to analyze significant differences between two groups, and one-way analysis of variance (ANOVA) was used to compare multiple groups. In vitro experiments were repeated at least three times. The Kaplan-Meier method with the log-rank test was used to
analyze survival. \( P < 0.05 \) was considered to be statistically significant.

**Results**

**miR-505-5p is negatively correlated with the expression of Myd88 after liver transplantation**

Acute rejection occurred after liver transplantation in rats, and the acute rejection gradually aggravated with time. We extracted primary KCs from the transplanted liver and detected the expression levels of miR-505-5p and Myd88. In order to eliminate the influence of postoperative hepatitis, we first detected the relative expression of mRNA in the control group, sham group and AR group 5 days after operation (Figure 1A). It was found that there was no significant difference between sham group and control group, which proved that the influence of postoperative hepatitis was negligible in this experiment. For the accuracy of the experiment, we used the sham operation group of syngeneic transplantation as the control experiment. Compared to that in the sham group, the expression of Myd88 showed a continuous upward trend with the progression of acute rejection (Figure 1B). MiR-505-5p showed the opposite trend (Figure 1C). Pearson correlation analysis was used to determine the correlation between the levels of miR-505-5p and Myd88 mRNA in each group (Figure 1D). After liver transplantation, the serum liver enzymes of rats also continued to rise (Figure 1E). In addition, the protein levels showed the same trend for Myd88 and its downstream proteins, such as TRAF6, IKK, p65 and p38, as revealed by western blot analysis (Figure 1F,G). HE staining of the liver tissue revealed the infiltration of a large number of inflammatory cells in the portal area and central vein, damage to the hepatic lobular structure, a partially necrotic liver parenchyma, and some fibrous connective tissue hyperplasia (Figure 1H). These data indicated that Myd88 is a key molecule involved in the immune response after liver transplantation in rats, and its expression level is higher with the aggravation of postoperative inflammation. In contrast, miR-505-5p has lower expression in the inflammatory environment and is negatively correlated with Myd88.

**miR-505-5p targets Myd88 and negatively regulates its expression**

The TargetScan database predicted Myd88 as a candidate target gene of miR-505-5p, which is a conserved miRNA complementary to the 3'-UTR sequence of Myd88 (Figure 2A). Luciferase reporter gene analysis revealed that over-expression of
miR-505-5p inhibited the luciferase activity of the wild-type Myd88 3'-UTR structure, but the mutant Myd88 3'-UTR structure was not affected. These luciferase reporter experiments suggest that Myd88 binds directly to miR-505-5p (Figure 2B). The mRNA and protein expression levels of Myd88 in cells transfected with miR-505-5p mimic over-expression were also detected (Figure 2C). These data indicated that we can alter the expression of Myd88 by regulating miR-505-5p, and then regulate the cells and the in vivo environment to some extent, and finally make it possible to alleviate the acute rejection after liver transplantation.

**miR-505-5p over-expression suppresses Myd88 expression and the downstream NF-κB and MAPK pathways following LPS treatment**

We speculated that the overexpression of miR-505-5p would inhibit the expression of Myd88 and the expressions of its downstream inflammatory factors, such as TRAF6, p-p65, p-p38 and other related pathways. The cells were stimulated with LPS to simulate the inflammatory environment in vitro, and miR-505-5p mimics and mimic NCs were added. From our experimental data, in vitro experiments were performed after LPS (200 ng/ml) stimulation for 24 h (Figure 3A,B). Using qRT-PCR analysis, we found that over-expression of miR-505-5p reduced the mRNA expressions of Myd88 and TNF-α in cells stimulated by LPS, while the expressions of IL-10 and TGF-β1 were increased (Figure 4A). Protein levels were analyzed by western blot analysis. The data showed that the expressions of Myd88 and TRAF6 were increased significantly under LPS stimulation (Figure 4B), the levels of p-IKK and p-p65 in the NF-κB signaling pathway were increased (Figure 4B), and the levels of p-ERK, p-JNK, and p-p38 in the MAPK signaling pathway were also increased significantly (Figure 4C), which proved that the inflammatory pathways downstream of Myd88 were activated. However, the addition of miR-505-5p mimics significantly reduced the inflammatory factors (Figure 4D). The enzyme-linked immunosorbent assay results showed that TNF-α in the cell supernatant was significantly increased under LPS stimulation, but decreased after over-expression of miR-505-5p (Figure 4E). The results of IL-10 and TGF-β1 were opposite (Figure 4F,G). The immunofluorescence results also confirmed these experimental results (Figure 4H,E). After overexpression of miR-505-5p, the nuclear import of p-p65 was reduced, which proved that miR-505-5p has a certain inhibitory effect on the nuclear import of p-p65. All of the above data confirmed that over-expression of miR-505-5p significantly inhibits the
expression of Myd88 and reduces the release of inflammatory factors.

Adv-Myd88 reverses the effects of miR-505-5p over-expression

Our previous experiments demonstrated that miR-505-5p can inhibit the expression of Myd88 in vitro to reduce the release of cellular inflammatory factors; however, after transfection with adv-Myd88, we obtained opposite results. Inflammatory factors were significantly increased in the adv-Myd88-transfected group compared with the miR-505-5p overexpression group. Western blot analysis results showed that downstream pathways were reactivated (Figure 5A,B) and additional inflammatory factors, such as TNF-α, were released (Figure 5C-E). These results indicate that the addition of adv-Myd88 reversed the inhibitory effect of miR-505-5p on inflammation.

miR-505-5p reduces inflammation by promoting M2 polarization of macrophages

Macrophages play an important role in rejection after liver transplantation, therefore we examined the changes of miR-505-5p in macrophages in a simulated inflammatory environment in vitro. The marker of M1 polarization, TNF-α, was significantly increased in the inflammatory environment (Figure 6A,B). In contrast, the marker of M1 polarization was significantly decreased under the action of miR-505-5p. The expression of M2-type polarization marker Arg1 was increased (Figure 6A,B). These results suggest that the anti-inflammatory effect of miR-505-5p on the body is accomplished via promotion of the M2-type polarization of macrophages.

Over-expression of miR-505-5p reduces acute rejection after liver transplantation in rats

After liver transplantation in rats, miR-505-5p agomiR and agomiR NC were injected into the rats through the portal vein. Primary KCs were isolated, and inflammatory factors in the NF-κB and MAPK pathways were detected. The miR-505-5p over-expression group showed the same effect of inhibiting inflammation in vivo and in vitro (Figure 7A-F). The serum of each group was taken 7 days after the operation to detect serum liver enzymes, and the AST and ALT in the miR-505-5p overexpression group were significantly decreased (Figure 7G). The expressions of M2-type polarization markers Arg-1 and IL-10 were also detected, and the results showed that over-expression of miR-505-5p induced M2 polarization of macrophages.
**Discussion**

Liver transplant patients often experience a variety of immune impairments after surgery, and acute rejection seriously affects the prognosis of patients [1]. Long-term use of high-dose immunosuppressive agents is generally used to treat acute rejection of liver transplantation, but it is accompanied by many adverse complications. Therefore, the identification of a new treatment method or target has become an urgent need in the field of liver transplantation.

KCs participate in various immune tolerance reactions in the body, including liver transplantation [15, 16]. Many factors lead to the activation of KCs after liver transplantation. For example, cold ischemia after transplantation leads to the impairment of ATP synthesis in the liver, imbalance in the intestinal flora and the release of a large amount of toxic substances during the anhepatic period. After the blood supply is restored to the liver, these substances activate KCs to promote their immune function [17]. Alloantigens in grafts are also an important factor in activating immune rejection. Previous studies found that more pro-inflammatory factors are released when KCs undergo M1-type polarization, which aggravates acute rejection after liver transplantation. When M2-type polarization occurs, KCs exhibit stronger anti-inflammatory effects and induce the formation of immune tolerance after liver transplantation [18].

TLR4 is a pattern recognition receptor, and multiple downstream signaling pathways are involved in the regulation of inflammation in the body [8]. LPS is a component of the outer wall of gram-negative bacterial cell walls which are composed of lipids and polysaccharides, and can act as a ligand to bind to TLR4 to activate...
various downstream signaling pathways [19]. When cells are stimulated by external inflammatory factors, such as LPS or proinflammatory ILs, TLRs are activated, and through signal transduction, Myd88 and multiple downstream signaling pathways are activated [20]. Myd88 myeloid differentiation factor is one of the key nodes in the TLR4 pathway. Several studies have shown that TLR4 participates in the occurrence of inflammation in the body, and some studies have shown that the loss of innate Myd88 signaling induces immune tolerance in allografts [21]. Therefore, we investigated additional possibilities for the regulation of the Myd88 gene. We found that miRNAs, as immune regulators, may govern the expression of genes relevant to allograft rejection, tolerance introduction and posttransplant infection in recipients of organ transplants [22]. We found the potential clinical application of miR-505-5p in previous studies; in addition to the roles mentioned in the introduction, miR-505-5p can be used as a potential marker of imatinib response in chronic myeloid leukemia patients and may be applied for the detection of early breast cancer [23]. There is also evidence that miR-505-5p can inhibit the proliferation and migration of bladder cancer cells by targeting PLK1 expression [24]. The above studies prove that miR-505-5p plays a regulatory role in various diseases in the body. Our continuous exploration of the mechanism by which miR-505-5p regulates the immune response will provide new ideas for the diagnosis and treatment of tumors, immune tolerance after organ transplantation and other diseases. Among our investigations, the expression of miR-505-5p is significantly decreased at an early stage in rats with acute rejection after liver transplantation, which is followed by a small recovery. This change in miR-505-5p negatively correlates with the expression of Myd88. These experimental results suggest that a negative feedback mechanism controls Myd88 level. To verify our hypothesis, we used dual luciferase and mutation experiments to confirm that Myd88 is a target of miR-505-5p. We used qRT-PCR and western blot analysis in vitro and confirmed that miR-505-5p inhibits the expression of Myd88 in KCs at both mRNA and protein levels, respectively. The over-expression of miR-505-5p after rat allogeneic liver transplantation reduces the expression of Myd88 in KCs. These experiments demonstrated that Myd88 is a direct target of miR-505-5p, and miR-505-5p regulates its expression in vivo and in vitro. In light of the important role of macrophage polarization in the pathogenesis of acute rejection after liver transplantation [4], we demonstrated marked alterations in M1 and M2-specific markers, including TNF-α, iNOS and arg-1. We found that over-expression of
miR-505-5p in vivo and in vitro significantly inhibited the expressions of M1 polarization markers TNF-α and iNOS, but did not inhibit the expressions of M2 polarization markers, which indicated that miR-505-5p significantly inhibits M1 polarization in KCs via reducing the inflammatory response. In addition, many studies have pointed out that miRNAs can induce the secretion of different cytokines by regulating the M1/M2 polarization of KCs, thereby changing the immune environment of the body [25,26]. Similar to our results, it has been proved that miRNAs are involved in immune rejection by regulating the polarization of KCs [27], which is a feasible and potential research direction.

The use of LPS to activate Myd88 led to the activation of TRAF6 and downstream NF-κB and MAPK signaling pathways, which led to the polarization of KCs to the M1 type and the release of more inflammatory factors. These factors induce the differentiation of T cells and aggravate the inflammatory response [28,29]. Overexpression of miR-505-5p inhibited the activation of the Myd88/TRAF6/NF-κB and MAPK signaling pathways in vitro and was found to significantly attenuate the nuclear translocation of p-p65, as revealed by immunofluorescence microscopy. We overexpressed miR-505-5p in an allogeneic rat liver transplantation model and reached the same conclusion. In addition, during the overexpression of miR-505-5p, we found that the secretion of the anti-inflammatory cytokines IL-10 and TGF-β1 was significantly upregulated, demonstrating that upregulation of miR-505-5p can effectively induce macrophages to secrete anti-inflammatory factors after inflammation. The above results are consistent with the decreased inflammatory cell infiltration observed by HE staining of recipient livers.

At present, the clinical application of miRNA in organ transplantation mainly judges the postoperative situation by detecting some miRNAs with significant differential expression in the peripheral blood of postoperative patients and provides a reference for optimizing the use of immunosuppressants. Some studies have also found that high expression of miRNA in donors is related to the functional recovery of the graft. After regulation of miRNA, the elderly may also become an important source of liver donors [30]. In addition, the use of miRNA therapy will inevitably have off-target effects. By optimizing the design of miRNA, the possibility of off-target effects can be reduced [31], and a minimum effective dose can also significantly reduce off-target effects [32].

Nevertheless, there are some limitations in this study. Myd88 is regulated by
multiple TLR receptors; unfortunately, we have no direct evidence that the upstream
target of Myd88 is TLR4 or others. In addition, miR-505-5p can target RASSF8 to
inhibit the proliferation of osteosarcoma and can also target HMGB1 to inhibit
endometritis. We cannot rule out that miR-505-5p may be involved and affect the
immune system through other pathways, which will require extensive follow-up
studies.

In summary, our results indicate that the role of miR-505-5p in alleviating acute
liver rejection after liver transplantation is directly related to Myd88, primarily via
inhibiting the activation of its downstream NF-κB and MAPK signaling pathways and
promoting KCs to an M2-type polarization. Further in-depth study of miRNAs in the
field of liver transplantation and the results of our experiments will provide new
treatment ideas for the future preoperative diagnosis of liver transplantation,
postoperative follow-up, and promotion of immune tolerance after transplantation.

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**Conflict of interest**

Authors declare that they have no conflict of interest

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**Figure Legends**

**Figure 1. The expression of MyD88 in rat KCs after liver transplantation is negatively correlated with the expression of miR-505-5p**  
(A) The mRNA levels of MyD88 and mir-505-5p in control group, sham group and acute rejection group (AR group) were detected on the 5th day after operation. (B-C) The expression levels of miR-505-5p and MyD88 in 1d-14d KCs after liver transplantation were detected by qRT-PCR. (D) Pearson correlation analysis was used to determine the correlation between the levels of miR-505-5p and MyD88 mRNA in each group. (E) Changes of ALT and AST in the serum 7 days after liver transplantation were detected by ELISA. (F-G) The protein expressions of MyD88, TRAF6, IKK, p65, JNK, and p38 in primary KCs after liver transplantation in rats changed with the increase of days after transplantation. (H) Liver tissue sections of control group and transplantation group of rats, stained with HE under light microscope. Scale bar=100 μm and 200 μm. Data are presented as the mean ± SD (n = 3). *P < 0.05, **P < 0.01.

**Figure 2. MyD88 is molecular target of miR-505-5p**  
(A) TargetScan predicted the
binding site of miR-505-5p to MyD88 3’UTR. (B) HEK 293T cells were co-transfected with luciferase reporter construct containing wild-type (WT) or mutant (MUT) MyD88 3’-UTR fragment with negative control (NC) mimic or miR-505-5p mimic. The luciferase activity was measured at 48 h after transfection. The results are expressed as relative to NC transfection (n = 3). (C) KCs were transfected with miR-505-5p mimic or negative control (NC) for 24 h, and the mRNA level of MyD88 was detected by qRT-PCR. Data are presented as the mean ± SD (n = 3). *P < 0.05, **P < 0.01, ***P < 0.001.

Figure 3. Optimal processing time and concentration for in vitro experiments
(A) KCs were stimulated with LPS (500 ng/mL), and the expression levels of miR-505-5p was detected at 3, 6, 12, 24, 36 and 48 h. (B) Expression levels of miR-505-5p after KCs were stimulated with LPS at concentrations of 50, 100, 200, 500 and 1000 ng/mL for 24 h. Data are presented as the mean ± SD (n = 3). *P < 0.05.

Figure 4. miR-505-5p inhibits MyD88 and NF-κB/MAPK inflammatory pathway in vitro
(A) The expressions of various mRNAs, including miR-505-5p, MyD88, IL-1β, TNF-α, IL-10, and TGF-β1, in KCs after stimulation with LPS were detected by qRT-PCR. (B-D) Western blot analysis was used to measure the protein level changes on MyD88, TRAF6 and downstream pathways following upregulation of miR-505-5p in KCs. (E-G) The levels of cytokines TNF-α, IL-10β and TGF-β1 in the supernatant were detected by ELISA. (H-I) The expression of MyD88 and the nuclear translocation of the p65 subunit were assessed by immunofluorescence staining. F4/80 was used as a functional marker of mature macrophages. Scale bar = 50 μm. Data are presented as the mean ± SD (n = 3). *P < 0.05, **P < 0.01.

Figure 5. adv-MyD88 rescued the effect of over-expression of miR-505-5p
(A,B) The protein levels of NF-κB and MAPK pathways were detected by western blot analysis after adv-MyD88 was added in vitro. (C-E) Cell treatment is similar to (A), the levels of cytokines TNF-α, IL-10 and TGF-β1 in the supernatant were detected by ELISA. Data are presented as the mean ± SD (n = 3). *P < 0.05, **P < 0.01.

Figure 6. Over-expression of miR-505-5p induces M2 polarization in LPS-treated
KCs  (A) The protein levels of Arg-1 and iNOS showed an opposite trend under the
stimulation of LPS and miR-505-5p, as detected by western blot analysis. (B) Cell
treatment is similar to (A), the mRNA levels of Arg-1 and iNOS were detected by
qRT-PCR. Data are presented as the mean ± SD (n = 3). *P < 0.05, **P < 0.01.

Figure 7. Over-expression of miR-505-5p inhibits acute rejection after liver
transplantation in vivo  (A,B) Western blot analysis of MyD88, TRAF6, NF-κB
and MAPK pathway protein levels in KCs after over-expression of miR-505-5p in
vivo. (C-F) In the same group as (A), the mRNA expression levels of MyD88, Arg-1,
iNOS, and TNF-α in KCs. (G,H) The release of liver enzymes, cytokines and
chemokines in rat serum was detected 7 days after liver transplantation. (I)
Pathological changes of transplanted liver in different treatment groups 7 days after
liver transplantation under a light microscope. (J) Nuclear translocation of p-p65 was
detected by immunofluorescence microscopy. F4/80 was used as a functional marker
of mature macrophages. Scale bar = 50 μm. (K) RAI scores of different treatment
groups 7 days after liver transplantation. (L) Postoperative survival rate of recipient
rats (n = 9 per group) in different treatment groups were analyzed by Kaplan-Meier.
NS-treated group VS NC agomiR-treated group, P > 0.05; NS-treated group vs
miR-505-5p agomiR-treated group, **P < 0.01. Data in (A-K) are shown as the mean
± SD (n = 3). *P < 0.05, **P<0.01, ***P<0.001.
A

SV40 promoter

Luciferase

Myd88-3UTR

miR-505-5p

3′...UUGUAGUUAUGAAGGACCGAGGG...5′

Myd88-3UTR-wt

5′...UCCUGUCUGCAUCUUUGGCUCCC...3′

Myd88-3UTR-mu

5′...UCCUGUCUGCAUCUUAGUCACAC...3′

B

Relative activity: RLuc/Fluc

C

Relative Myd88 mRNA level

NC mimic + Myd88-3UTR-wt

miR-505-5p + Myd88-3UTR-wt

NC mimic + Myd88-3UTR-mu

miR-505-5p + Myd88-3UTR-mu

***

**
<table>
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<tr>
<th></th>
<th>LPS</th>
<th>+</th>
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<tbody>
<tr>
<td>miR-905-5p mimic</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Adv-MyD88</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
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</table>

**A**

- MyD88
- TRAF6
- p-IKK
- IKK
- p-p65
- p65
- p-JNK
- JNK
- p-p38
- p38
- β-actin

**B**

1. LPS
2. LPS+miR-905-5p mimic
3. LPS+miR-905-5p mimic+Adv-MyD88

**C**

- MyD88
- TRAF6
- p-IKK
- p-p65
- p-JNK
- p-p38

**D**

- MyD88
- TRAF6
- p-IKK
- p-p65
- p-JNK
- p-p38

**E**

- MyD88
- TRAF6
- p-IKK
- p-p65
- p-JNK
- p-p38
170x41mm (300 x 300 DPI)
### Table 1. Sequences of primers used in this study

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<tr>
<th>Gene</th>
<th>Primer sequence (5′→3′)</th>
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<tr>
<td>Myd88</td>
<td>Forward: AGCAGAACCAGGAGTCCGAGAAG</td>
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<tr>
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<td>Reverse: GGCAGTAGCAGATAAAGGGCATCG</td>
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<td>Arg-1</td>
<td>Forward: CATATCTGCCAAGACATCGTG</td>
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<tr>
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<td>Reverse: GACATCAAAGGCTAGGTGAATC</td>
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<td>TNF-α</td>
<td>Forward: CAGGCGGTGCCTATGTCTC</td>
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<td>Reverse: CGATACCCCGAAGTTCAGTAG</td>
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<tr>
<td>TGF-β1</td>
<td>Forward: TTGCTTCAGCTCCACAGA</td>
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<tr>
<td></td>
<td>Reverse: TGGTTGAGGGCAAGGAC</td>
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<td>iNOS</td>
<td>Forward: ACTCAGCCAAAGCCTACCTAC</td>
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<td></td>
<td>Reverse: TCAAATCTCTGCTATCCGTCCTG</td>
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<td>IL-10</td>
<td>Forward: TGCTATGTTGCCTGCTTTACTG</td>
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<tr>
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<td>Reverse: TCAAATGCTCCTTGATTTCCTG</td>
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<td>miR-505-5p</td>
<td>Forward: TGGCCGCTCAACACTTGCT</td>
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<td>Reverse: CCAGTGAGGGTCGAGGT</td>
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<td>U6</td>
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<td>Reverse: GTGCTCGCTCGGACGCACAT</td>
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<td>GAPDH</td>
<td>Forward: CACCATCTCCAGGAGCGAG</td>
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<tr>
<td></td>
<td>Reverse: GGGGCCATCCACAGTCTTC</td>
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### Table 2. Antibodies and concentrations used in western blot analysis

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Dilution ratio</th>
<th>Brand</th>
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<tbody>
<tr>
<td>IKK, p-IKK, p65, p-P65, ERK, p-ERK, JNK, p-JNK, p38, p-p38, Arg-1</td>
<td>1:1000</td>
<td>CST</td>
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<tr>
<td>Myd88, TRAF6, iNOS</td>
<td>1:1500</td>
<td>Bioss</td>
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<tr>
<td>β-Actin</td>
<td>1:5000</td>
<td>ZENBIO</td>
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</table>

### Table 3. Antibodies and concentrations used Immunofluorescence analysis

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Dilution ratio</th>
<th>Brand</th>
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</thead>
<tbody>
<tr>
<td>Myd88</td>
<td>1:200</td>
<td>Bioss</td>
</tr>
<tr>
<td>p-p65</td>
<td>1:500</td>
<td>CST</td>
</tr>
<tr>
<td>Alexa Fluor 488, Alexa Fluor 564</td>
<td>1:200</td>
<td>ThermoFisher</td>
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**Highlight**

Acute rejection after liver transplantation is one of the most important causes of postoperative survival in patients. Here, we find that overexpression of miR-505-5p can target and inhibit Myd88, which ultimately effectively attenuates acute rejection after liver transplantation, and most likely by inducing hepatic Kupffer cells to polarize toward M2.

- Expression of miR-505-5p is significantly reduced in the event of acute rejection after liver transplantation.
- miR-505-5p has a direct action relationship with Myd88, and overexpression of miR-505-5p effectively attenuates the release of inflammatory factors in *vitro* and in *vivo*.
- Overexpression of miR-505-5p induces Kupffer cells to polarize toward M2 type and promotes immune tolerance in the organism.