Am J Transl Res 2022;14(11):7689-7704
www.ajtr.org /ISSN:1943-8141/AJTR0145349

Original Article

Inducible downregulation of miR-93 feedback promotes innate responses against RNA virus by amplifying interferon signaling

Yuanyu Zhao[1*], Fang Liu[3*], Junfeng Dong[1*], Hong Fu[1], Jianhua Luo[4], Junsong Ji[1], Xiaogang Gao[2], Wenyuan Guo[1]

[1]Institute of Organ Transplantation, Changzheng Hospital, Navy Medical University, Shanghai, China; [2]Institute of Organ Transplantation, Changhai Hospital, Navy Medical University, Shanghai, China; [3] Experimental Teaching Center, College of Basic Medical Sciences, Naval Medical University, Shanghai, China; [4]National Key Laboratory of Medical Immunology & Institute of Immunology, Navy Medical University, Shanghai, China. [*]Equal contributors.

Received July 14, 2022; Accepted September 30, 2022; Epub November 15, 2022; Published November 30, 2022

Abstract: Type I interferons (IFN) and their downstream effector signaling pathways play critical roles in the innate antiviral response. The underlying mechanisms that regulate IFN production and their effector signaling, especially by microRNAs, are well understood. We found that the expression of miR-93 was significantly downregulated by RNA virus infection in innate cells. miR-93 expression was also downregulated in influenza virus-infected patients. Furthermore, we showed that JAK1 is targeted by miR-93 to inhibit type I IFN's antiviral activity. Functionally, antagomir of miR-93 markedly reduced influenza virus replication in mice in vivo and prevented their death. Therefore, hosts recognize the invading RNA virus infection and activate RIG-I/JNK pathways to decrease miR-93 expression. The reduction of miR-93 feedback enhances the antiviral innate immune response by activating the IFN-JAK-STAT effectors type I, indicating miR-93 as a possible therapeutic target for infection with RNA viruses.

Keywords: microRNA, innate immunity, RNA virus, influenza A, type I interferon, IFN-JAK-STAT pathway

Introduction

The innate immune response is the body's first line of defense against viruses [1]. One of the major functions of the innate immune system is to recognize viral components as well as initiate the production of proinflammatory cytokines and type I interferons (IFN) [2]. Type I IFNs, including IFN-β and many IFN-α species, play critical roles in the innate antiviral immune response mainly through inducing cellular resistance to viral infection and apoptosis of virally infected cells [3]. Hence, IFN has been widely used as a treatment for viral infection. When IFN binds to its cell surface receptors, it activates the downstream Janus kinase (JAK)/signal transducer and activator of transcription (STAT) signaling cascade, which induces IFN-induced transcription of a set of genes known as IFN-stimulated genes (ISGs) [2]. Despite extensive investigations of IFN signaling pathways, the mechanisms regulating these pathways are still largely unknown. Coding genes and non-coding RNAs are both important regulators of the IFN-JAK/STAT pathway, and altering their expression could influence not only viral replication but also the outcome of viral infection in the host [4]. Additionally, to survive in host cells, viruses have developed strategies to subvert the antiviral IFN-JAK/STAT pathway [5]. From this viewpoint, the interaction between viruses and hosts is a never-ending war, and a complicated network of molecules regulates these interactions.

Many eukaryotic species express microRNA (miRNA), a non-coding RNA molecule with short single-strands [6]. miRNAs bind imperfectly to sites located in the 3' untranslated region (3'-UTR) of target mRNA sequences and downregulate the expression of target genes by repressing their translation or directly degrading their mRNA molecules. Numerous studies have revealed that miRNAs play crucial roles in sev-

eral biologic processes, including development, infection, immune responses, and carcinogenesis [7]. Previously, we reported that upregulated miR-146a negatively regulates myeloid derived growth factor production by targeting TRAF6, IRAK1, and IR by targeting miRNAs modulating host antiviral innate immune responses [8]. On the other hand, miR-155 promotes IFN effector signaling that is critical for the antiviral innate immunity of the host [9]. Furthermore, miR-93 is downregulated upon virus infection, but its role in the host antiviral immune response remains unclear.

miR-93 belongs to the miR-106b-25 cluster and has recently been reported to participate in tumor-growth pathogenesis, angiogenesis, and aging [10-12]. In addition, host miR-93 has been demonstrated to directly target the vesicular stomatitis virus (VSV) RNA sequence to inhibit VSV replication. However, since viruses can also interact with the host at the miRNA level in several ways, it remains unclear whether VSV infection can alter host miR-93 expression. Moreover, how miR-93 regulates host signaling pathways may be involved in the antiviral innate immune response remains unclear.

Our microarray analysis showed that a series of miRNAs were upregulated in murine peritoneal macrophages. Instead, we chose to investigate the role of miR-93, which is downregulated by viral infection. MiR-93 expression in macrophages was profoundly reduced upon VSV infection. More importantly, miR-93 expression was also downregulated in influenza virus-infected patients. In our *in vivo* experiments, the feedback from miR-93 downregulation attenuated viral replication. Furthermore, we established that miR-93 targeted JAK1 expression and suppressed activation of the JAK/STAT signaling pathway downstream of IFN signaling. In mice challenged with VSV, these miR-93-mediated effects could easily translate into consistent *in vivo* outcomes. Furthermore, miR-93 antagomir suppressed influenza virus infection and prevented the related death. Therefore, our data suggest that miR-93 feedback downregulation inhibits RNA virus replication by upregulating the IFN-JAK/STAT pathway in response to RNA virus infection.

Materials and methods

Patients and specimens

Blood and sputum samples were obtained from patients diagnosed with influenza A viral infection at the Second Affiliated Hospital of Navy Medical University. Sputum samples were subsequently subjected to real-time PCR analysis to confirm the diagnosis according to standard protocol [37]. A complete collection of peripheral blood mononuclear cells (PBMCs) was also performed.

Mice

6-8 weeks old C57BL/6 mice were provided by Company Sipper BK Experimental Animals Joint Ventures (Shanghai, China). We obtained Toll-like receptor 3 (TLR3) knockout mice from The Jackson Laboratory (Bar Harbor, ME), and TLR4, TLR9, and MyD88 knockout mice from Dr. S. Akir. RIG-I$^{-/-}$ mice were previous obtained [38]. All knockout mice were C57/B6 background. Guide for the Care and Use of Laboratory Animals at the National Institutes of Health was followed in all animal experiments. We are grateful for the kindness of Dr. Jiahua Xu (Navy Medical University, Shanghai, China) for providing VSV M4-mutations [14]. Influenza A/PR/8 (PR8) virus (H1N1 subtype) was grown in the chorioallantoic cavities of 10-day-old embryonated hen eggs for three days at 35°C. The infected allantoic fluids were clarified by centrifugation at 1000×*g* for 20 min and stored in small portions at -80°C as a virus stock solution ($TCID_{50}/\mu L = 10^{-8.5}$). Four different inhibitors were obtained from Calbiochem (San Diego, CA) including PDTC, SB203580, PD98059, and SP600125, a JNK inhibitor. Cell Signaling Technology (Danvers, MA) provided antibodies specific for JAK1, STAT1, phosphorylated JAK1, and phosphorylated STAT1. Sigma-Aldrich (St. Louis, MO) offered an antibody specific to actin.

Cell culture and transfection

We obtained the murine macrophage cell line RAW264.7 from ATCC [39]. Thioglycolate-elicited macrophages were cultured [40]. After plating, an overnight incubation followed by transfection was conducted on cells [13]. One billion HEK293 cell lines are seeded in each well of 96-well plates overnight. For cotransfection of plasmids and RNAs, Polyplus transfection reagents (New York, NY) were used. BMDDCs were generated by differentiation from bone marrow cells [41]. RAW264.7 cell clones stably overexpressing JAK1 were selected in 600 mg/mL G418 for 3-4 weeks, and overexpression was confirmed by western-blot analysis for JAK1 expression.

ChIP assay

DNA was analyzed by ChIP using a kit (Upstate Biotechnology, USA) [22]. In this study, chromatin was fragmented with ultrasound to produce 100-200 bp DNA using an Ab against H3K4me3 and H3K27me3. In ChIP assays, the primer pairs were used: F1: 5'-AGCAGTGGAAAGTCGGAGAA-3' and R1: 5'-TGTTTCCCACAGACGGTACA-3' (antisense); F2: 5'-TGTACCGTCTGT GGGAAACA-3' and R2: 5'-CCCACTACAGGTGCTTTGGT-3'. Varying quantities of ChIP sample were initially used with varying PCR cycle numbers.

Bioinformatic analysis

Predicting miR-93 target sites with TargetScan software was achieved. The Starbase (http://starbase.sysu.edu.cn) database was searched for any potential pathways.

MiR-93 mimics and inhibitor

We used miR-93 mimics and inhibitors to overexpress/inhibit miR-93 in murine macrophages [9]. Negative control mimics or inhibitors were transfected into cells as the respective controls.

RNA interference

Here are the sequences of JAK1-specific siRNAs used in the experiments: siJAK1-1638: 5'-GGUCCAAUCUGCACAGAAUTT-3' (sense) and 5'-AUUCUGUGCAGAUUGGACCTT-3' (antisense); siJAK1-1952: 5'-GGUCCAAUCUGCACAGAAUTT-3' (sense) and 5'-AUUGGAGAUUUCUCG AGGCTT-3' (antisense). Previously, we described scrambled control RNA sequences [13]. We transfected 20 nM concentrations of siRNA duplexes into murine peritoneal macrophages [39].

Quantification of RNA

miRNAs were extracted from total RNA, reverse transcribed, and amplified by real-time PCR [13, 42]. For miRNA analysis, the reverse transcription primer for miR-93 was 5'-GTCGTATCCAGTGCAGGGTCCG AGGTATTCGCACTGGATACGACCTACCT-3'. Quantitative PCR primers were 5'-ATCAAAGTGCTGTTCGTGC-3' (forward) and 5'-GTGCAGGGTCCGAGGT-3' (reverse). According to the previous description, miRNA expression was calculated as a ratio [13]. For VSV Indiana serotype, the primers were 5'-ACGGCGTACTTCCA GATGG-3' (forward) and 5'-ACGGCGTACTTCCAGATGG-3' (reverse). For SeV, the primers were 5'-GCCAAGAGAGGCATAGCACTCA-3' (forward) and 5'-CCTGGG GCATATGTAGGTCAAC-3' (reverse). For influenza virus A, the primers were described previously [37]. Primers for each target gene are as follows: GAPDH (F: 5'-TGACCACAGTCCATGCCATC-3'; R: 5'-GACGGACACATTGGGGGTA G-3'); inflammatory protein 10 (IP-10) (F: 5'-CCTGCCCACGTGTTGAGAT-3'; R: 5'-TGATGGTCTTAGATTCCGGATTC-3'); IFN-stimulated gene 15 (ISG15) (F: 5'-GGTGTCCGTGACTAACTCCAT-3'; R: 5'-TGGAAAGGGTAA GACCGTCCT-3'); IFN-β (F: 5'-ATGGACGCTGATGGCAATACC-3'; R: 5'-TCCCCATTCACGTCTCCCA-3'); IL-6 (F: 5'-ACAACCACGGCCTTCCC TACTT-3'; R: 5'-CACGATTTCCCAGAGAACATGTG-3'); TNF-α (F: 5'-AAGCCTGTAGCCCACGTCGTA-3'; R: 5'-GGCACCACTAGTTGGTTGT CTTTG-3'); JAK1 (F: 5'-AGGTTCTACTTTACCAACTGGCA-3'; R: 5'-GAACTGGCATCAAGGAGTGGG-3').

Luciferase analysis

The mouse JAK1 mRNA 3'-UTR sequence was cloned into the pGL3-promoter construct. The pRL-TK-Renilla-luciferase plasmid, and siRNAs were co-transfected. Luciferase activities were measured after 24 h.

Western-blot

Two washes of cold PBS were performed before cells were lysed with Cell Signaling Technology's lysis buffer (Calbiochem) supplemented with protease inhibitor mixture. Using an extraction reagent, the total protein content was equalized and quantified using the BCA test. Extracts were loaded onto 10% SDS-PAGE gels, and transferred to membranes [43].

In vivo assay of miR-93 in the innate response against RNA virus infection

To address the effect of miR-93 *in vivo*, mice were intravenously (i.v.) injected with 5 nmol miR-93 agomir or negative control agomir. After 24 h, mice were inoculated intraperitoneally (i.p.) with 50 μL of $10^{-6.5}$ $TCID_{50}$/μL VSV M4 or 50 μL of 10^{-7} $TCID_{50}$/μL influenza A H1N1. Otherwise, mice were i.v. injected with 100 nmol micrOFF™mmu-miR-93-5p antagomir or negative control antagomir 24 h before viral infection. After another 24 h, the mice's lungs,

spleen, and liver were harvested. Lungs were embedded in paraffin after being fixed in 10% formalin. Total RNA was extracted from the spleen and liver as described previously [44]. Virus burden in the spleen and liver was measured by qPCR analysis.

To address the effects of miR-93 on virus infection, mice were anesthetized with isofluorane inhalation and anesthetized with isofluorane (OptiFlo, Abbott Laboratories). After 12 h, miR-93, NC agomirs (5 nmol), or antagomirs (100 nmol) were injected. Survival of the mice was observed for 14 days.

Statistical analysis

Data from multiple groups were analyzed using one-way ANOVA with post-hoc Bonferroni's correction (GraphPad Prism 5.0; GraphPad Software, San Diego, CA). In addition, data derived from two groups were analyzed using an unpaired Student's *t*-test or a Mann-Whitney test. All data are expressed as mean ± SD. P = 0.05 was considered significant for all tests.

Results

miR-93 expression in innate immune cells is downregulated in response to RNA virus infection

We previously reported the miRNA expression profiles that occur in murine macrophages upon VSV infection from data obtained using an array-based miRNA profiling method [13]. While our previous studies established the involvement of most highly upregulated miRNAs in host antiviral responses [9, 13], we focused on investigating the downregulated miRNAs in this study and miR-93 in particular. Intrigued by a potential link between the function of markedly downregulated miRNAs and the innate antiviral immune response, we explored the role of miR-93 on host response and the mechanism of how miR-93 accomplishes this regulation. First, we confirmed the microarray results by qPCR analysis of isolated murine peritoneal macrophages challenged with VSV infection in vitro. As expected, RNA virus infection dramatically downregulated miR-93 expression dose-dependent (**Figure 1A**), and its expression reached a trough level at 36 h after the VSV challenge (**Figure 1B**). As miR-93 has target gens within the VSV sequence itself that interferes with viral replication [14], we repeated the above experiment using the VSV M4 virus, mutations in the miR-93 target site in VSV mutant, which was used in our following experiments to exclude the impact of this effect in our system. Indeed, we observed similar miR-93 expression kinetics as the wild-type VSV strain (**Figure 1C**, **1D**). MiR-93 was also decreased in VSV-infected BMDCs (**Figure 1E**) and splenocytes (**Figure 1F**). Furthermore, infection of influenza A virus also downregulated its expression in macrophages in a dose- (**Figure 1G**) and time-dependent manner (**Figure 1H**).

Notably, miR-93 was downregulated in human PBMCs harvested from influenza virus-infected patients during active infection (**Figure 1I**). In support of these findings, infection with RNA virus SeV downregulated miR-93 expression (Supplementary Figure 1). Therefore, these results suggest that the level of miR-93 in immune cells can be downregulated in vitro and in vivo by infection with an RNA virus.

MiR-93 expression is downregulated by RNA virus infection through pathways dependent upon RIG-I/JNK

Although TLR signaling interaction with miRNA expression [15] and to signal upstream of miR-93 are not yet known, we explored miR-93's underlying mechanism. Since miR-93 expression in TLR3-, TLR4-, TLR9-, or MyD88-deficient peritoneal macrophages were similar to each other following the VSV challenge as compared to their wild-type counterparts (**Figure 2A**), we excluded the possibility that alterations in miR-93 expression could be attributed to TLR signal activation. However, the observed downregulation of miR-93 expression by VSV challenge was abolished in RIG-I-deficient macrophages from mice (**Figure 2B**), suggesting regulation of miR-93 following VSV infection likely depends on RIG-I signaling.

To find the targets of miR-93, we analyzed the downstream genes following RIG-I activation. As displayed in **Figure 2C**, miR-93 expression was not altered after NF-κB, p38, or ERK inhibition. In contrast, inhibition of JNK significantly impaired VSV-induced miR-93 downregulation. Because epigenetic modification is vital in regulating the expression of many miRNAs, we were curious whether histone methylation affected

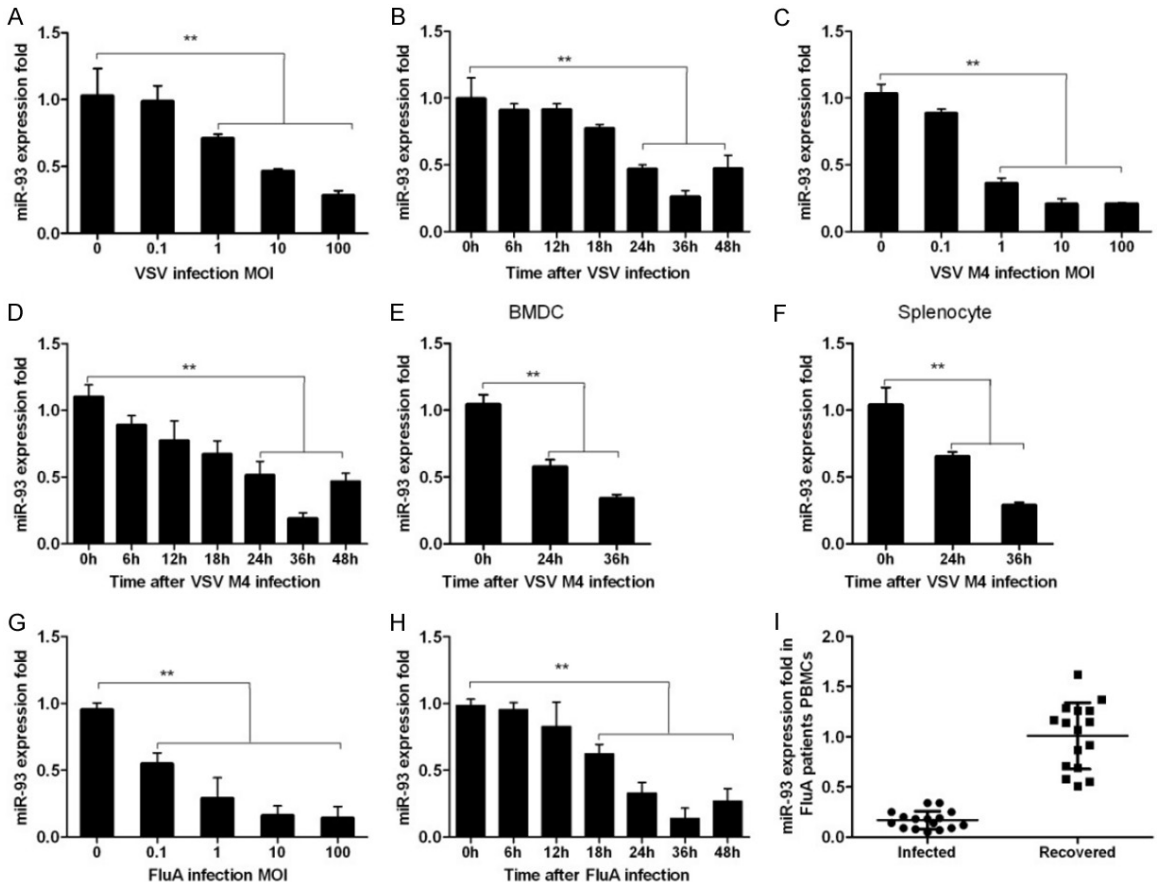


Figure 1. RNA virus infection decreases miR-93 expression. (A, B) Murine peritoneal macrophages challenged with VSV for 36 h at different MOIs for 36 h expressed miR-93. U6 gene was used as a control. MOI, Multiplicity of Infection. (C, D) The expression of miR-93 in murine macrophages challenged with VSV M4 for 36 h and for 10 h at different MOIs (C) or at different MOIs for different durations (D). (E, F) Amounts of miR-93 expressed in murine BMDCs (E) and splenocytes (F) infected with VSV at MOI 10 for the indicated periods. (G, H) Fluent murine macrophage expression of miR-93 after 36 hours of infection with influenza A virus at different MOIs (G) or at MOI 10 for different times (H). (I) PBMCs were collected from influenza A virus-infected patients during an active infection, and miR-93 expression was determined using qRT-PCR. After recovering from the infection, PBMCs from the same individuals served as the control samples. Each point represents a specific patient. **P<0.01.

miR-93 expression. VSV infection decreased miR-93 promoter H3K4 methylation levels and upregulated H3K27 levels. In line with these findings, inhibition of JNK could rescue these effects (**Figure 2D**). Together, these results implied that VSV infection decreased miR-93 expression throught the RIG-I/JNK signaling pathway and histone methylation was involved in this process.

Inhibition of miR-93 attenuates RNA viral replication

To determine miR-93's biologic significance in macrophages, we assessed its role in VSV replication. miR-93 mimics or inhibitors were transfected into macrophages, and VSV $TCID_{50}$ in culture supernatants was measured after VSV infection. miR-93 mimic transfection increased the level of miR-93, whereas the inhibitor decreased its expression (Supplementary Figure 2). miR-93 mimics significantly promoted VSV replication, whereas miR-93 inhibitors suppressed VSV replication (**Figure 3A**, **3B**). VSV $TCID_{50}$ was increased by miR-93 overexpression but suppressed by miR-93 knockdown (**Figure 3C**, **3D**). In addition, similar results were obtained by challenging macrophages with SeV (Supplementary Figure 3). We also observed the influenza A replication in macrophages after transfection in miR-93 mimics or inhibitors. Following VSV infection, miR-93 mimics promoted influenza A replication at the RNA level (**Figure 3E**), while miR-93 inhibitors

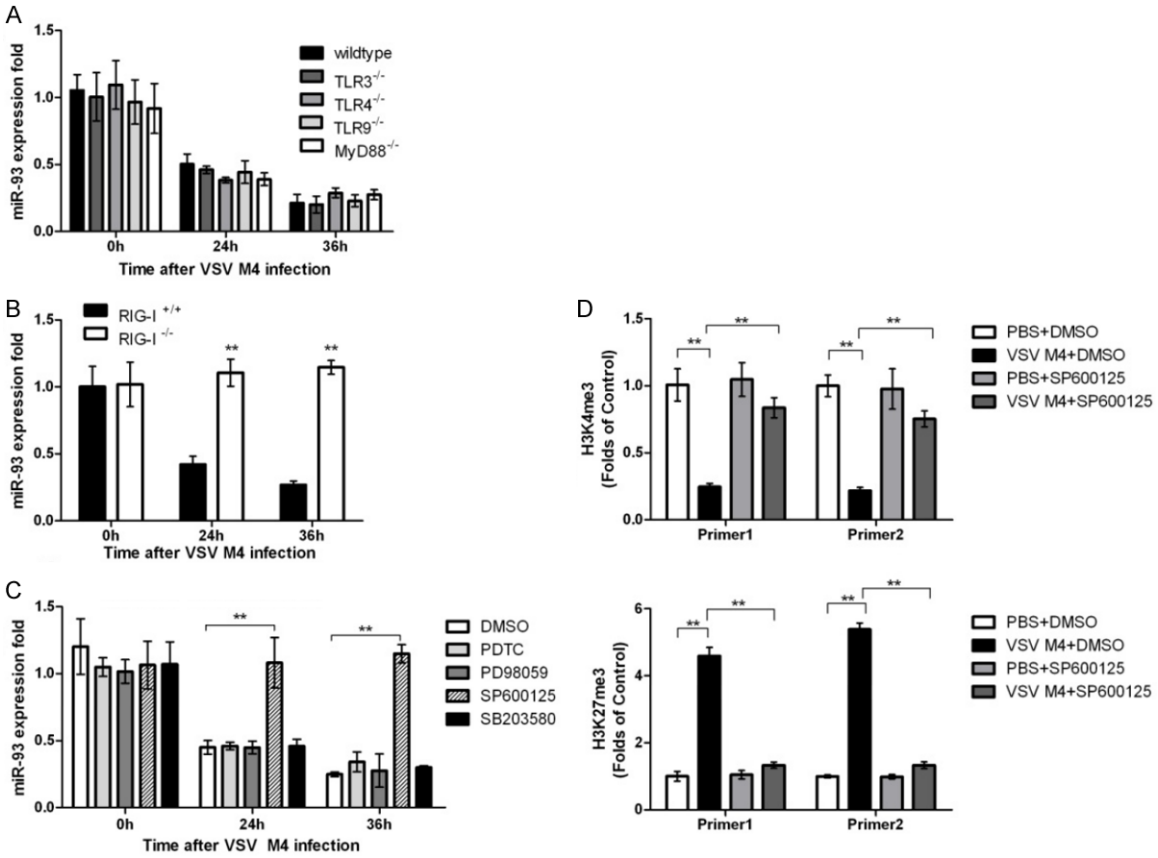


Figure 2. VSV infection suppresses miR-93 expression in macrophages by targeting RIG-I/JNK. A. Various mice with TLR3, TLR4, and TLR9 defects and those with MyD88 deficiency were infected with VSV, and miR-93 expression was analyzed in the peritoneal macrophages. B. miR-93 expression was measured for the indicated time period following inoculation by VSV at MOI 10. C. After treating murine macrophages with DMSO, SB203580, PD98059, PDTC, or SP600125 as mentioned for 30 minutes, VSV was infected at MOI 10 MG. D. VSV was infected into mice's peritoneal macrophages for the indicated duration after being pre-treated with DMSO or SP600125. The methylation levels of H3K4 and H3K27 were measured by qRT-PCR. Data are shown as the mean ± SD (n = 3) from one representative experiment. Similar results were obtained in three independent experiments. **P<0.01. PDTC, Pyrrolidinecarbodithoic Acid; Me3, Trimethylation.

impaired its replication (**Figure 3F**). Influenza A $TCID_{50}$ was also increased by miR-93 overexpression but decreased by miR-93 knockdown (**Figure 3G**, **3H**). The downregulation of miR-93 in macrophages infected with RNA viruses may facilitate viral replication.

Activation of JAK/STAT is suppressed by miR-93

The overexpression of miR-93 did not affect VSV-triggered type I IFN production at either the mRNA or protein levels; however, RNA virus infection did not appear to affect miR-93-induced IFN production (**Figure 4A**). Next, we examined the phosphorylation status of nuclear factors that regulate IFN production. As displayed in **Figure 4B** and **4C**, we did not observe any significant differences in MAPK, NF-κB, or IRF3/7 phosphorylation, and IL-6 or TNF-α production after miR-93 overexpression or knockdown.

Next, we focused on whether miR-93 impacted signals downstream of IFN signaling. When type I IFN binds to its receptor, the JAK/STAT pathway is activated to phosphorylate STAT1. Phosphorylated STAT1 then translocates into the nucleus, resulting in ISG transcription. Therefore, the kinetics of STAT1 phosphorylation was studied in macrophages transfected with miR-93 mimics or inhibitors after the VSV challenge. STAT1 phosphorylation was reduced by miR-93 overexpression during VSV infection (**Figure 4D**). In line with this, the expression of ISGs, including IP-10 and ISG15, was sup-

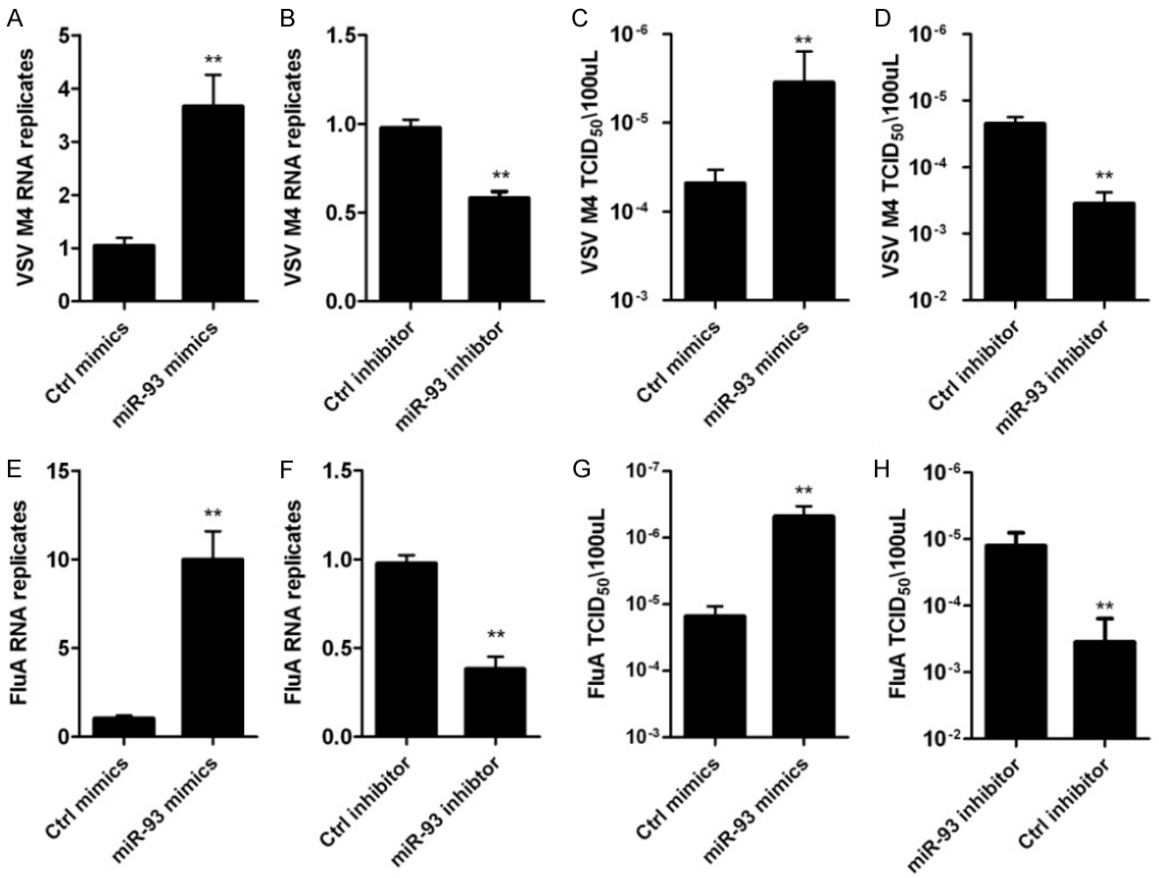


Figure 3. Inhibition of miR-93 attenuates replication of VSV and influenza A in macrophages. (A, B) MiR-93 mimics (A) and miR-93 inhibitors (B) were transfected into mouse peritoneal macrophages, and VSV at MOI 100 was infected with the cells for 1 hour. After 72 h, qRT-PCR was used to quantify intracellular VSV RNA replicates. (C, D) VSV $TCID_{50}$ in cultural supernatants was measured in mouse peritoneal macrophages treated as in (A). (E, F) The peritoneal macrophages of mice were transfected with miR-93 mimics (E), or miR-93 inhibitors (F), then infected with influenza A. Quantification of intracellular influenza A RNA replicates was performed using quantitative real-time PCR. (G, H) The influenza A $TCID_{50}$ in culture supernatants was determined as in (E) using peritoneal macrophages from mice. Data are displayed as the mean ± SD (n = 3) from one representative experiment. Similar results were obtained in three independent experiments. **P<0.01.

pressed upon miR-93 overexpression or promoted upon miR-93 knockdown (**Figure 4E**). The results show that miR-93 appears to negatively regulate the activation of JAK/STAT and the production of type I IFN in macrophages following RNA virus infection.

MiR-93 targets JAK1

Since miRNAs mainly function by inhibiting target genes, we searched for the target of miR-93. To do this, we not only performed computational prediction analysis using TargetScan software (http://www.targetscan.org) but also manually compared sequences in various signaling molecules involved IFN-JAK/STAT pathway. Our combined method revealed that miR-93 is a potential target for JAK1 (**Figure 5A**). To verify this finding experimentally, our study of HEK293 cells using a dual-luciferase reporting system revealed that miR-93 inhibits the expression of a reporter containing the 3'-UTR of JAK1 (**Figure 5B**). Furthermore, miR-93 mimics transfected into macrophages downregulated the expression of JAK1 at protein and mRNA levels (**Figure 5C**, **5D**), indicating that miR-93 could likely inhibit JAK1 expression by translational inhibition and mRNA degradation. Therefore, miR-93 directly regulates macrophage JAK1.

JAK1 is mainly targeted by miR-93 to facilitate viral replication

To confirm that miR-93 promotes VSV replication by targeting JAK1, we performed overex-

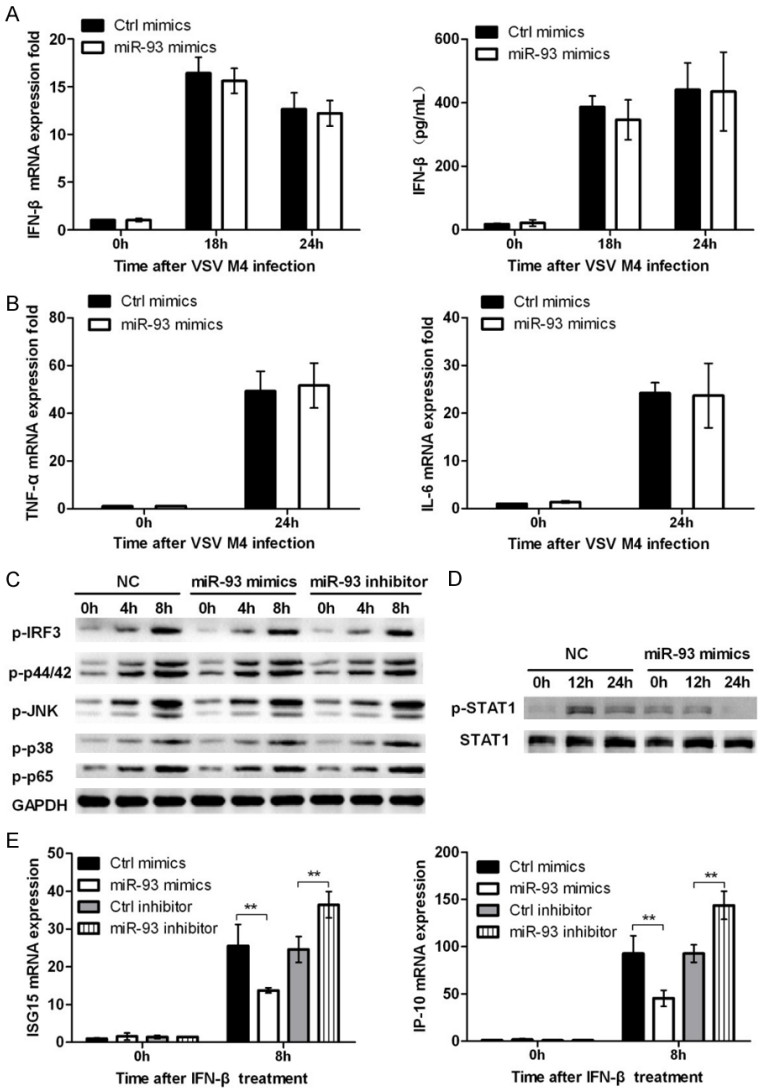


Figure 4. A miR-93 knockdown suppresses the activation of the JAK/STAT pathway without affecting the production of type I IFN. A. Macrophages were transfected with miR-93 mimics or control in 0.5 mL and challenged with VSV. The expression of IFN-β was measured using qRT-PCR. The level of IFN-β was determined using ELISA. B. In mice, peritoneal macrophages were transfected with miR-93 mimics, control, or si-JAK1 as indicated. Following 48 h, they were challenged with VSV at MOI 10 for the indicated timeframe. Protein levels of p-IRF3, p-p38, p-JNK, p-p42/44, and p-p65 were measured using western blot, and GAPDH was employed as input control. NC, Negative Control. C. QRT-PCR was used to assess the expression of TNF-α and IL-6 mRNA. D. Infected mouse peritoneal macrophages were transfected with miR-93 mimics or control, and infected with VSV. E. The cells were treated with miR-93 mimics or inhibitors. Recombinant murine IFN-(100 U/mL) was added after 48 hours, and the amount of IP-10 and ISG15 mRNA was determined using qRT-PCR. **P<0.01.

pression and knockdown experiments on JAK1. We constructed two different JAK1 siRNAs at nucleotide sites 1638 and 1952 using siDIGAN, and both qPCR and western blot analysis indicated that the latter siRNA worked better (Supplementary Figure 4).

Using these tools, we investigated the effect of miR-93 on VSV replication and JAK/STAT pathway activation in murine macrophages with overexpressed or knocked-down JAK1. JAK1 knockdown led to VSV replication, as expected from miR-93 overexpression (**Figure 6A**). The effect of miR-93 inhibitors on VSV replication was further diminished by knocking down JAK1 (**Figure 6B**, **6C**), suggesting that miR-93 function relied on JAK1 activation when VSV infection.

To inhibit miR-93's effects on JAK1, we created a clone of overexpressed JAK1 RAW 264.7 macrophages. Furthermore, WB analysis also confirmed that this cell clone expressed significantly more JAK1 protein (**Figure 6D**). As RAW 264.7 cells stably overexpressing JAK1 without its 3'-UTR, miR-93 mimics was not altered JAK1. In addition, miR-93 mimics did not alter VSV replication in these cells via analysis of VSV $TCID_{50}$ in supernatants and VSV RNA replications (**Figure 6E**, **6F**). Therefore, miR-93 downregulates the expression of JAK1 and suppresses JAK/STAT signaling pathway during viral replication.

Viral virus and influenza A infection in mice can be suppressed by miR-93 inhibition in vivo

Since we observed that miR-93 overexpression *in vitro* enhanced VSV replication in macrophages, miR-93 may affect host resistance to VSV infection *in vivo*. Mice were first pretreated with miR-93 agomirs, antagomirs, or their controls to address this. miR-93 expression was most abundant in the liver, kidney, and thymus (**Figure 7A**) after miR-93 agomir treatment. Accordingly, in vivo miR-93 treatment signifi-

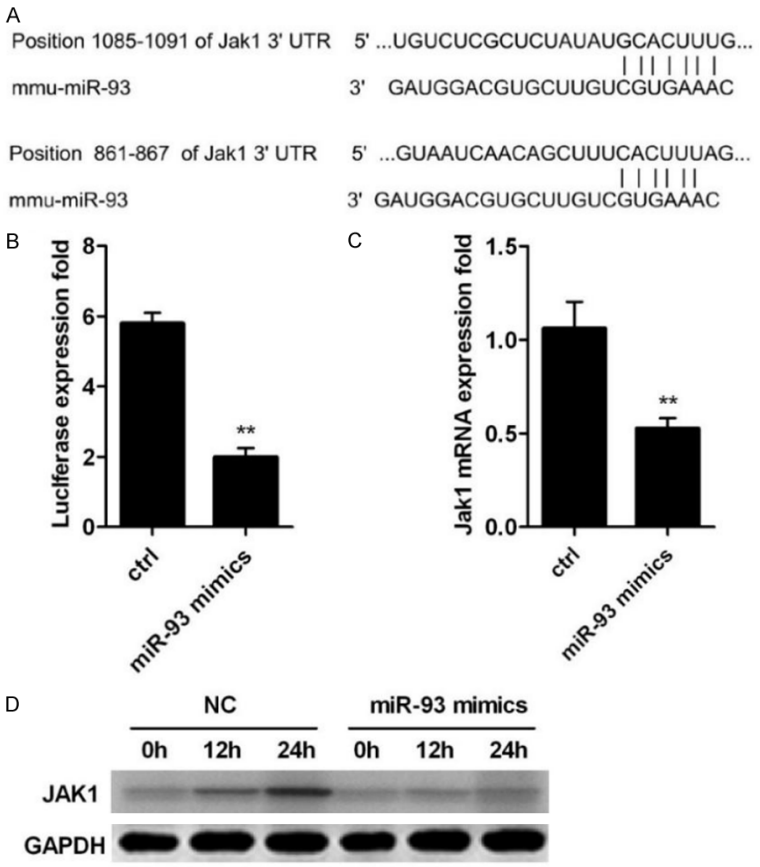


Figure 5. The miR-93 target is JAK1. (A) Based on TargetScan results, miR-93 may target JAK1. (B) HEK293 cells (1×10^4) were co-transfected with 80 ng of pMIR-JAK1 3'-UTR luciferase reporter plasmids and 40 ng of pTK-Renilla-luciferase reporter plasmids combined with control (ctrl) mimics, miR-93 mimics, control inhibitor, or miR-93 inhibitor (final concentration: 20 nM), as appropriate. Luciferase activity was measured. Mouse peritoneal macrophages (1×10^6) were transfected as described in **Figure 4E**. In both (C and D), The expression of JAK1 were detected 48 h after qRT-PCR (C) and western blot (D), respectively. **P<0.01.

cantly reduced JAK1 protein expression in the lung, liver, and spleen (**Figure 7B** upper). In addition, we used miR-93 antagomir to suppress its expression and function *in vivo* and found that repression of miR-93 expression was only seen in PBMC, not in any other organs in mice (Supplementary Figure 5). However, miR-93 antagomir increased JAK1 expression in the liver, spleen, and lung (**Figure 7B** bottom). Mice were infected with VSV to determine the function of miR-93 in regulating antiviral immune responses. Since the lung is the main organ impacted by VSV-induced pathology, lungs were harvested and subjected to pathological examination 24 h after infection. **Figure 7C**, **7D** illustrate moderate alveolar destruction and inflammatory infiltration in lung tissues harvested from VSV-infected, control agomir/antagomir-treated mice. In contrast, alveolar structures remained intact with no inflammatory infiltration in non-infected, control agomir/antagomir-treated mice. In comparison to control mice, miR-93 agomir administration attenuated the host's resistance to VSV infection by causing greater alveolar destruction and inflammatory infiltration (**Figure 7C**); whereas miR-93 antagomir inhibited lung injury (**Figure 7D**). Consistent with these findings, miR-93-treated mice had a higher viral burden in the spleen and liver than Ctrl-treated mice, whereas miR-93 inhibition suppressed viral replication (**Figure 7E**), with similar serum IFN-β levels as control groups (**Figure 7F**). These results confirmed that inhibition of miR-93 suppressed viral infection *in vivo*.

To further determine the therapeutic potential of miR-93 inhibition in viral infection, we used the mouse model with influenza A virus infection and treated the mice with miR-93 agomir or antagomir after influenza A infection. As demonstrated in **Figure 7G**, miR-93 agomir promoted lung injury, whereas administration of miR-93 antagomir markedly suppressed lung injury. Consistently, replication of influenza A virus in the lung was also increased by miR-93 overexpression (**Figure 7H**). As a result, miR-93 agomir promoted the death of influenza-infected mice (**Figure 7I**), with all mice dying within four days after infection. Mice infected with influenza A were much less likely to die after receiving miR-93 antagomir (**Figure 7I**), with 90% surviving over 14 days. Meanwhile, all the mice died within seven days in the control group. Taken together, we demonstrated that the downregulated miR-93 feedback attenuates virus replication by activating the JAK/STAT signaling pathway (**Figure 8**), suggesting that downregu-

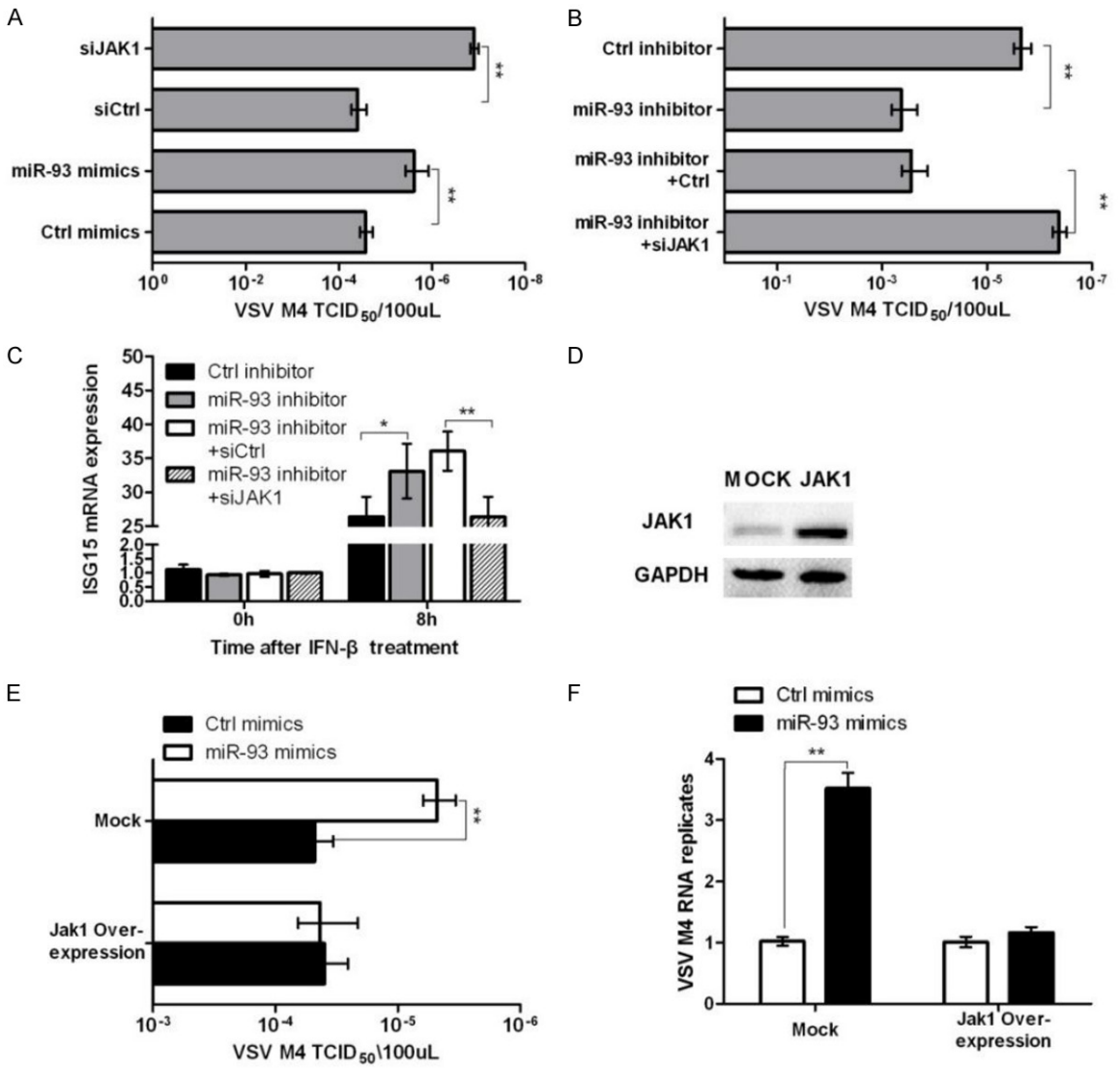
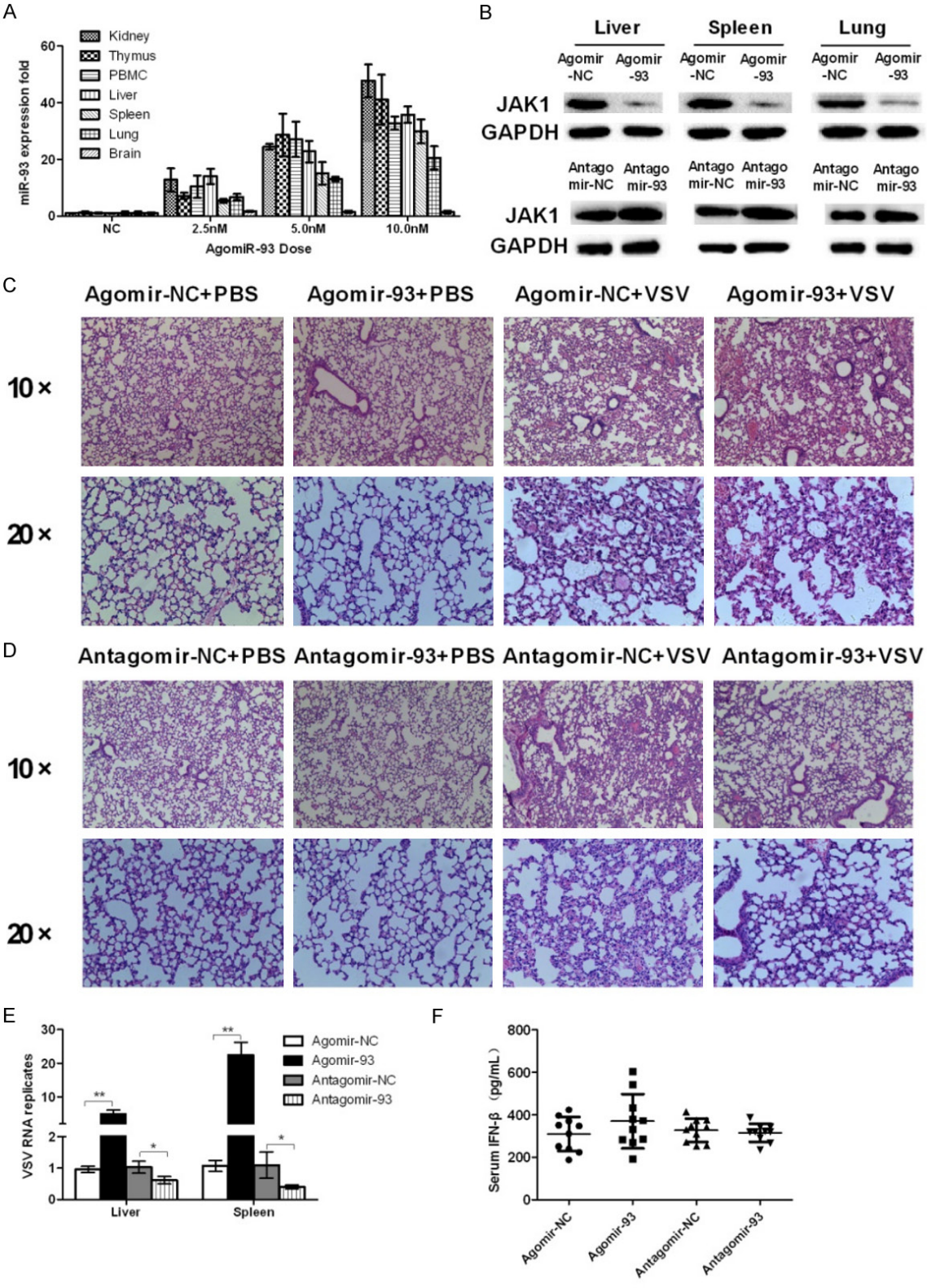


Figure 6. MiR-93 promotes RNA viral replication by targeting JAK1. (A) Macrophages were transfected with si-JAK1, miR-93 mimics, or control. Macrophages were treated and infected with VSV after 48 h, and VSV $TCID_{50}$ was determined in supernatants. (B) MiR-93 inhibitor, control siRNA, or JAK1 siRNA were transfected into macrophages. After 48 h, macrophages were infected by VSV, and VSV $TCID_{50}$ of culture supernatants was determined. (C) Following transfection as in (B), IFN-β was added in cells, and ISG15 expression was measured. (D) Cell line RAW 267.4 overexpressing JAK1 as determined by western blot. (E, F) The JAK1 overexpressing group (left panel) or control (right panel) was transfected with control or miR-93 mimics. *P<0.05, **P<0.01.

lation of miR-93 may significantly improve the effect of influenza or RNA viral infection.

Discussion

Recently, immune responses against viral infections were found to be regulated by several miRNAs. Therefore, the microRNA system is likely another layer of regulation in which viruses and hosts can interact [16, 17]. MiRNA microarray analysis revealed that the expression of 28 miRNAs was significantly regulated in response to VSV infection in a baby hamster kidney (BHK-21) cell line [18]. As macrophage activation is crucial for host defense against many intracellular pathogens, our colleagues have previously demonstrated that miR-155 promotes antiviral immune responses against IFN through SOCS1 activation [9]. MiR-146a feedback negatively regulated IFN production in VSV infection by targeting IRAK2 [13]. In this study, we show that RNA virus infection increas-


A
Kidney
Thymus
PBMC
Liver
Spleen
Lung
Brain
miR-93 expression fold
60
40
20
0
NC
2.5nM
5.0nM
10.0nM
AgomiR-93 Dose
B
Liver
Spleen
Lung
Agomir -NC
Agomir -93
JAK1
GAPDH
Antago mir-NC
Antago mir-93
C
Agomir-NC+PBS
Agomir-93+PBS
Agomir-NC+VSV
Agomir-93+VSV
10×
20×
D
Antagomir-NC+PBS
Antagomir-93+PBS
Antagomir-NC+VSV
Antagomir-93+VSV
E
VSV RNA replicates
Agomir-NC
Agomir-93
Antagomir-NC
Antagomir-93
Liver
Spleen
F
Serum IFN-β (pg/mL)
800
600
400
200
0
Agomir-NC
Agomir-93
Antagomir-NC
Antagomir-93

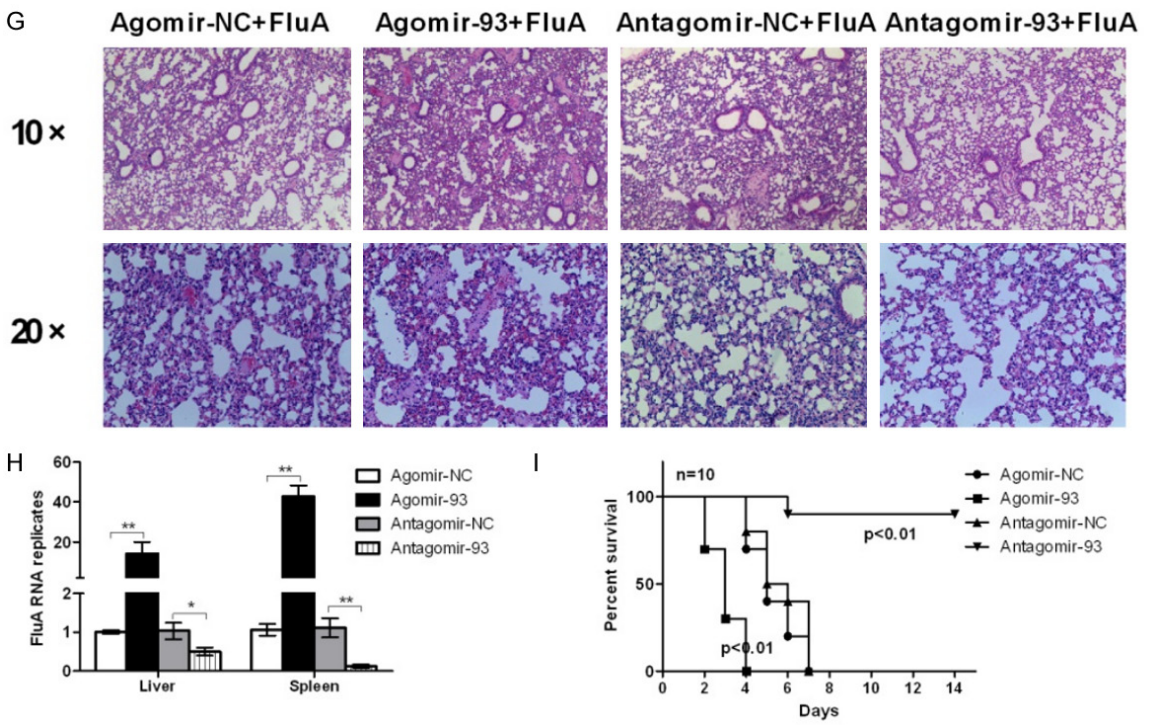


Figure 7. MiR-93 attenuates resistance to VSV and influenza A infection in mice. (A) Mice were i.v. injected with miR-93 or NC agomirs in different doses. At 24 h later, the level of miR-93 was analyzed. (B) Mice were i.v. injected with miR-93, NC agomirs (5 nmol) or antagomirs (100 nmol), and i.p. inoculated with VSV. After 24 h, the protein level of JAK1 was detected by western blot in the liver, spleen, and lung. (C, D) Mice were pretreated with miR-93 agomirs (C) or antagomirs (D), and after 24 h infected with VSV i.p., analysis of the H&E stain was performed for pathological examination. (E, F) The viral burden of VSV in liver and spleen (E) or serum IFN-β levels (F) of mice in (C, D) was measured. (G, H) Mice were pretreated with miR-93 agomirs or antagomirs as in (C, D). After 24 h infected by influenza A *via* retropharyngeal instillation, H&E staining was done for histology (G) and influenza A RNA virus burden was determined by qPCR (H). (I) Mice were infected with influenza A. After 12 h, miR-93, NC agomirs or antagomirs were i.v. injected, and mice survival was evaluated for 14 days (Mann-Whitney test, n = 10). *P<0.05, **P<0.01.

es IFN production by downregulating miR-93 after RNA virus infection, identifying miR-93 as a key microRNA during RNA viruses-induced innate immune response.

Pattern recognition receptor (PRR) families, especially TLRs and RIG-I-like receptors (RLRs), play a key role in host innate immune responses by recognizing invading viruses [19-21]. This innate recognition may lead to the induction of type-I IFNs, which initiate cellular antiviral immune responses [22-24]. Additionally, these signals tightly regulate several other mediators, including miRNAs, to ensure an outcome that is beneficial to the host when dealing with foreign invaders [25]. Regarding TLR signaling, a series of adaptor proteins are coupled to downstream protein kinases that, when activated, ultimately result in transcription factor activation. Neither TLR3-, TLR4-, TLR9-, nor MyD88-deficient macrophages were affected by VSV-induced miR-93 downregulation, nor was it affected by inhibiting nuclear factors downstream of TLR signaling, indicating that miR-93 expression was regulated independently of TLR signals. Since RLRs are another critical component of the host defense system responsible for sensing invading viruses and triggering innate immunity [26], they have recently garnered much attention as potential antiviral agents [24, 26, 27]. MiR-93 downregulation was abolished either in macrophages lacking RIG-I or inhibiting of JNK, demonstrating that this miR-93 regulation is a RIG-I/JNK dependent, but TLR/MyD88-independent, pathway following RNA virus infection.

Recent studies have revealed a close relationship between miRNA expression and epigenetic modification [28]. It is widely accepted that miRNAs can regulate the expression of critical epigenetic modifiers by targeting relevant

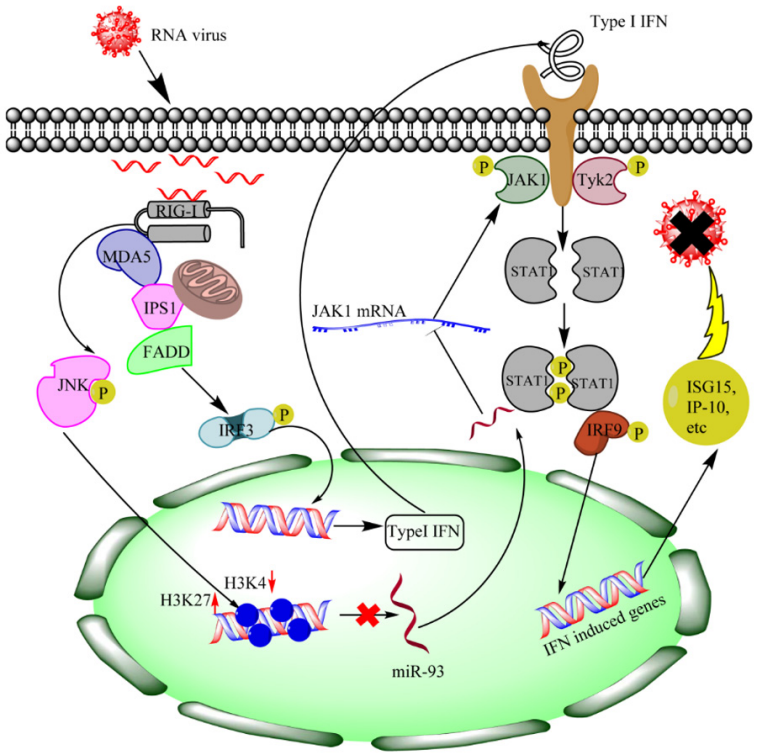


Figure 8. Mechanism of miR-93 in RNA virus infection by regulating type I IFN-JAK/STAT pathway. RNA viruses are recognized by innate immune cells. After activating the JAK/STAT pathway, IFN is induced and released from immunocytes after increased IFN-induced genes (ISGs) and eliminates invading viruses. RNA virus-infected macrophages also downregulate miR-93 expression through the RIG-I/JNK signaling pathway. The miR-93 inhibits JAK1 and suppresses the RNA viruses-induced innate response. The downregulation of miR-93 feedback induced by RNA virus infection increases JAK/STAT pathway activity in innate cells, thus accelerating and promoting antiviral immunity in the host.

genes [29]. Conversely, epigenetic mechanisms, including DNA and histone modification, can regulate miRNA expression [30]. We report evidence that miR-93 is modified by miRNA during RNA virus infection. After VSV infection, H3K4me3 was downregulated and rescued by inhibition of JNK. These results serve as further evidence to support our previous result suggesting that regulation of miR-93 is JNK dependent. Additionally, these findings may enhance the molecular mechanism of miRNAs and epigenetic pathways.

miR-93 has been demonstrated through the identification of several genes to function as a target of miR-393. Prior studies showed that miR-93 target integrin-beta-8 can promote tumor growth and angiogenesis [31] and that targeting large tumor suppressor kinase 2 (LATS2) may account for its role in enhancing tumor angiogenesis and metastasis [32]. These studies primarily focused on the role of miR-93 in oncology. However, miRNAs have been thought to play a role in various biologic or pathologic processes by targeting multiple mRNAs, referred to as the targetome, to exert their regulatory effect on gene expression [33]. In the present study, we revealed that manipulating JAK1 expression by siRNA significantly promoted RNA virus replication, whereas taking JAK1 down phenocopies the function of miR-93 and counteracts the miR-93 inhibitors on antiviral effects. Alternatively, miR-93 prevented targeting JAK1 in cells due to the deletion of 3'-UTR regions, thus, it cannot influence the replication of VSVs. According to these findings, those results facilitate the host's antiviral innate immunity against RNA viruses.

To eliminate invading pathogens, cytokine production must be stimulated. JAK/STAT signaling may be a major pathway for inducing ISGs in response to viral infection [34]. Among these ISGs, ISG15 is the most strongly induced protein and plays a central role in host antiviral responses [35]. Several viruses, however, have developed strategies to invert or subvert the activation of JAK/STAT signaling to complete self-replication [36]. Therefore, JAK/STAT signaling is undoubtedly a stronghold that viruses and hosts fight over to control for their benefit. In the present study, we showed that miR-93 decreased ISG15 expression by targeting JAK1, suggesting that miR-93 normally suppresses activation of the JAK/STAT pathway. Thus, these results further elucidate the molecular mechanisms underlying the involvement of miR-93 in antiviral immunity and shed new light on understanding a novel molecular mechanism that contributes to the ongoing battle between host and pathogen.

Our in vivo results showed miR-93's role in host resistance were consistent with our in vitro data. We demonstrated that VSV infection was less resistant to mice treated with miR-93. Given that the lung is the main site where viral replication and inflammation occurs and that viruses may be carried to the liver and spleen by various phagocytes after VSV infection, we examined these organs in our mouse model after administering a miR-93 agomir. There was more severe alveolar destruction, a greater lung inflammatory infiltration, and a higher viral burden in the liver and spleen. The expression of miR-93 in human influenza virus-infected patients was also downregulated. In addition, miR-93 antagomir inhibited the replication of influenza virus, resulting in 90% of the mice surviving after infection with influenza A. This suggests that miR-93 may represent an effective therapeutic target in the treatment of viral infections since its function can readily be translated into in vivo effects.

Despite this paper's inspiring results, some questions remain and will be the focus of future investigations. First, since miRNAs have a high conservation rate, current technical limitations do not allow us to distinguish whether miR-93 is generated by the virus or the host cell. Second, the result reported in previous studies shows that miR-93 directly targets large viral protein and phosphoprotein genes to suppress virus replication [14], which somewhat contradicts the data presented here. Finally, while it is not surprising that systems exist to balance a specific biological process on a global scale, it is unusual for one individual molecule to have two opposing biological functions. One possible explanation is that miR-93 itself serves only as a modifier to relay signals, where appropriate expression can facilitate IFN production to eliminate intruding pathogens on the one hand and protect the host from sustaining damage due to IFN overreaction-induced systemic inflammation on the other. However, the fact that miR-93 can also target VSV directly could be a total coincidence, and this presumption may raise interest in further investigating the mechanisms underlying the precise roles of miR-93 on antiviral immune response.

Conclusions

miR-93 is decreased in antigen-presenting cells by the RIG-I/JNK-pathway upon RNA virus infection. Activation of RIG-I/JNK pathways then alters histone methylation patterns in the promoter region of miR-93. Downregulation of miR-93 feedback, in turn, inhibits viral replication by activating of JAK/STAT pathway. The findings shed new light on how miRNAs mediate antiviral innate immunity. Additionally, strategies targeting miR-93 expression may lead to the development of new and effective antiviral drugs.

Acknowledgements

This work was supported by grants from the National Natural Science Foundation of China (No. 31670907, No. 81971503).

Disclosure of conflict of interest

None.

Abbreviations

IFN, Interferon; ISGs, IFN-Stimulated Genes; JAK-1, Janus kinase-1; MiR-93, MicroRNAs MiRNA-93; STAT-1, Signal Transducer And Activator Of Transcription-1.

Address correspondence to: Wenyuan Guo and Junsong Ji, Institute of Organ Transplantation, Changzheng Hospital, Navy Medical University, No. 415, Fengyang Road, Huangpu District, Shanghai 200003, China. E-mail: guowenyuan@smmu.edu.cn (WYG); jijunsong@smmu.edu.cn (JSJ); Xiaogang Gao, Institute of Organ Transplantation, Changhai Hospital, Navy Medical University, No. 168, Changhai Road, Yangpu District, Shanghai 200433, China. E-mail: shieldgun@sina.com

References

[1] Brubaker SW, Bonham KS, Zanoni I and Kagan JC. Innate immune pattern recognition: a cell biological perspective. Annu Rev Immunol 2015; 33: 257-290.
[2] Schneider WM, Chevillotte MD and Rice CM. Interferon-stimulated genes: a complex web of host defenses. Annu Rev Immunol 2014; 32: 513-545.
[3] Mesev EV, LeDesma RA and Ploss A. Decoding type I and III interferon signalling during viral infection. Nat Microbiol 2019; 4: 914-924.
[4] Trobaugh DW and Klimstra WB. MicroRNA regulation of RNA virus replication and pathogenesis. Trends Mol Med 2017; 23: 80-93.
[5] Zhao J, Qin C, Liu Y, Rao Y and Feng P. Herpes simplex virus and pattern recognition recep-

tors: an arms race. Front Immunol 2020; 11: 613799.
[6] Momen-Heravi F and Bala S. MiRNA regulation of innate immunity. J Leukoc Biol 2018; 103: 1205-1217.
[7] Rupaimoole R and Slack FJ. MicroRNA therapeutics: towards a new era for the management of cancer and other diseases. Nat Rev Drug Discov 2017; 16: 203-222.
[8] Zhao X, Sun L, Mu T, Yi J, Ma C, Xie H, Liu M and Tang H. An HBV-encoded miRNA activates innate immunity to restrict HBV replication. J Mol Cell Biol 2020; 12: 263-276.
[9] Wang P, Hou J, Lin L, Wang C, Liu X, Li D, Ma F, Wang Z and Cao X. Inducible microRNA-155 feedback promotes type I IFN signaling in antiviral innate immunity by targeting suppressor of cytokine signaling 1. J Immunol 2010; 185: 6226-6233.
[10] Montanini L, Lasagna L, Barili V, Jonstrup SP, Murgia A, Pazzaglia L, Conti A, Novello C, Kjems J, Perris R and Benassi MS. MicroRNA cloning and sequencing in osteosarcoma cell lines: differential role of miR-93. Cell Oncol (Dordr) 2012; 35: 29-41.
[11] Li N, Muthusamy S, Liang R, Sarojini H and Wang E. Increased expression of miR-34a and miR-93 in rat liver during aging, and their impact on the expression of Mgst1 and Sirt1. Mech Ageing Dev 2011; 132: 75-85.
[12] Feng S, Gao L, Zhang D, Tian X, Kong L, Shi H, Wu L, Huang Z, Du B, Liang C, Zhang Y and Yao R. MiR-93 regulates vascular smooth muscle cell proliferation, and neointimal formation through targeting Mfn2. Int J Biol Sci 2019; 15: 2615-2626.
[13] Hou J, Wang P, Lin L, Liu X, Ma F, An H, Wang Z and Cao X. MicroRNA-146a feedback inhibits RIG-I-dependent type I IFN production in macrophages by targeting TRAF6, IRAK1, and IRAK2. J Immunol 2009; 183: 2150-2158.
[14] Otsuka M, Jing Q, Georgel P, New L, Chen J, Mols J, Kang YJ, Jiang Z, Du X, Cook R, Das SC, Pattnaik AK, Beutler B and Han J. Hypersusceptibility to vesicular stomatitis virus infection in Dicer1-deficient mice is due to impaired miR24 and miR93 expression. Immunity 2007; 27: 123-134.
[15] O'Neill LA, Sheedy FJ and Mccoy CE. MicroRNAs: the fine-tuners of Toll-like receptor signalling. Nat Rev Immunol 2011; 11: 163-175.
[16] Roberts A and Jopling CL. Targeting viral infection by microRNA inhibition. Genome Biol 2010; 11: 201.
[17] Dong G, Liu S, Liu J, Dong X, Jin P, Lu X and Mei D. Upregulation of miR-155 contributes to the suppression of inflammatory responses by targeting Socs1 in LPS-induced acute lung injury. Int J Clin Exp Patho 2016; 9: 7010-7019.
[18] Lian H, Liu W, Liu Q, Jin H, Sun Y, Li J, Xia Z and Gao H. A laboratory-attenuated vesicular stomatitis virus induces apoptosis and alters the cellular microRNA expression profile in BHK cells. Arch Virol 2010; 155: 1643-1653.
[19] Brennan K and Bowie AG. Activation of host pattern recognition receptors by viruses. Curr Opin Microbiol 2010; 13: 503-507.
[20] Takeuchi O and Akira S. Pattern recognition receptors and inflammation. Cell 2010; 140: 805-820.
[21] Hoffmann J and Akira S. Innate immunity. Curr Opin Immunol 2013; 25: 1-3.
[22] Chen W, Han C, Xie B, Hu X, Yu Q, Shi L, Wang Q, Li D, Wang J, Zheng P, Liu Y and Cao X. Induction of Siglec-G by RNA viruses inhibits the innate immune response by promoting RIG-I degradation. Cell 2013; 152: 467-478.
[23] Zhang SY, Jouanguy E, Sancho-Shimizu V, von Bernuth H, Yang K, Abel L, Picard C, Puel A and Casanova JL. Human toll-like receptor-dependent induction of interferons in protective immunity to viruses. Immunol Rev 2007; 220: 225-236.
[24] Bruns AM and Horvath CM. Activation of RIG-I-like receptor signal transduction. Crit Rev Biochem Mol Biol 2012; 47: 194-206.
[25] Sedger LM. MicroRNA control of interferons and interferon induced antiviral activity. Mol Immunol 2013; 56: 781-793.
[26] Ireton RC and Gale M. RIG-I like receptors in antiviral immunity and therapeutic applications. Viruses 2011; 3: 906-919.
[27] Connolly DJ and O'Neill LA. New developments in Toll-like receptor targeted therapeutics. Curr Opin Pharmacol 2012; 12: 510-518.
[28] Moutinho C and Esteller M. MicroRNAs and epigenetics. Adv Cancer Res 2017; 135: 189-220.
[29] Hou J, Lin L, Zhou W, Wang Z, Ding G, Dong Q, Qin L, Wu X, Zheng Y, Yang Y, Tian W, Zhang Q, Wang C, Zhang Q, Zhuang SM, Zheng L, Liang A, Tao W and Cao X. Identification of miRNomes in human liver and hepatocellular carcinoma reveals miR-199a/b-3p as therapeutic target for hepatocellular carcinoma. Cancer Cell 2011; 19: 232-243.
[30] Saito Y, Liang G, Egger G, Friedman JM, Chuang JC, Coetzee GA and Jones PA. Specific activation of microRNA-127 with downregulation of the proto-oncogene BCL6 by chromatin-modifying drugs in human cancer cells. Cancer Cell 2006; 9: 435-443.
[31] Fang L, Deng Z, Shatseva T, Yang J and Yang BB. MicroRNA miR-93 promotes tumor growth and angiogenesis by targeting integrin-beta 8. Oncogene 2010; 30: 806.
[32] Fang L, Du WW, Yang W, Rutnam ZJ, Peng C, Li H, O'Malley YQ, Askeland RW, Sugg S, Liu M,

Mehta T, Deng Z and Yang BB. MiR-93 enhances angiogenesis and metastasis by targeting LATS2. Cell Cycle 2012; 11: 4352-4365.

[33] Skalsky RL, Corcoran DL, Gottwein E, Frank CL, Kang D, Hafner M, Nusbaum JD, Feederle R, Delecluse HJ, Luftig MA, Tuschl T, Ohler U and Cullen BR. The viral and cellular microRNA targetome in lymphoblastoid cell lines. PLoS Pathog 2012; 8: e1002484.

[34] Takaoka A and Yanai H. Interferon signalling network in innate defence. Cell Microbiol 2006; 8: 907-922.

[35] Skaug B and Chen ZJ. Emerging role of ISG15 in antiviral immunity. Cell 2010; 143: 187-190.

[36] Fros JJ, Wen JL, Prow NA, Geertsema C and Pijlman GP. Chikungunya virus nonstructural protein 2 inhibits type I/II interferon-stimulated JAK-STAT signaling. J Virol 2010; 84: 10877-10887.

[37] Wenzel JJ, Panning M, Kaul KL, Mangold KA, Revell PA, Luna RA, Zepeda H, Perea L, Vazquez-Perez JA, Young S, Rodic-Polic B, Eickmann M, Drosten C, Jilg W and Reischl U. Analytical performance determination and clinical validation of the novel Roche RealTime ready influenza A/H1N1 detection set. J Clin Microbiol 2010; 48: 3088-3094.

[38] Wang Y, Zhang HX, Sun YP, Liu ZX, Liu XS, Wang L, Lu SY, Kong H, Liu QL, Li XH, Lu ZY, Chen SJ, Chen Z, Bao SS, Dai W and Wang ZG. RIG-I-/- mice develop colitis associated with downregulation of G alpha i2. Cell Res 2017; 17: 858-868.

[39] Wang C, Chen T, Zhang J, Yang M, Li N, Xu X and Cao X. The E3 ubiquitin ligase Nrdp1 'preferentially' promotes TLR-mediated production of type I interferon. Nat Immunol 2009; 10: 744-752.

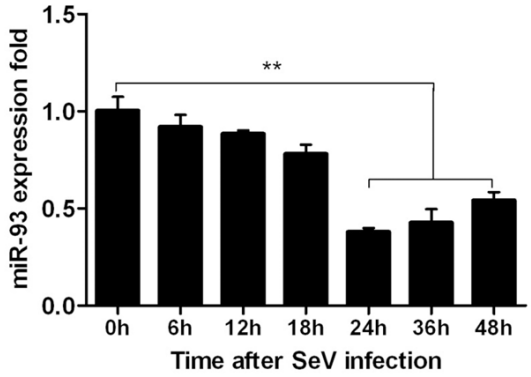
[40] Liu X, Zhan Z, Xu L, Ma F, Li D, Guo Z, Li N and Cao X. MicroRNA-148/152 impair innate response and antigen presentation of TLR-triggered dendritic cells by targeting CaMKIIα. J Immunol 2010; 185: 7244-7251.

[41] Xia S, Guo Z, Xu X, Hai Y and Cao X. Hepatic microenvironment programs hematopoietic progenitor differentiation into regulatory dendritic cells, maintaining liver tolerance. Blood 2008; 112: 3175-3185.

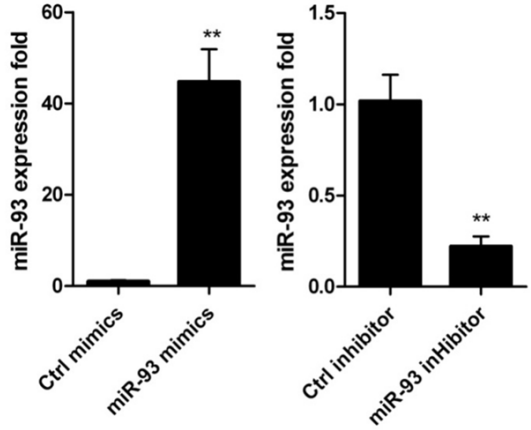
[42] Wang P, Gu Y, Zhang Q, Han Y, Hou J, Lin L, Wu C, Bao Y, Su X, Jiang M, Wang Q, Li N and Cao X. Identification of resting and type I IFN-activated human NK cell miRNomes reveals microRNA-378 and microRNA-30e as negative regulators of NK cell cytotoxicity. J Immunol 2012; 189: 211.

[43] Xu S, Liu X, Bao Y, Zhu X, Han C, Zhang P, Zhang X, Li W and Cao X. Constitutive MHC class I molecules negatively regulate TLR-triggered inflammatory responses via the Fps-SHP-2 pathway. Nat Immunol 2012; 13: 551-559.

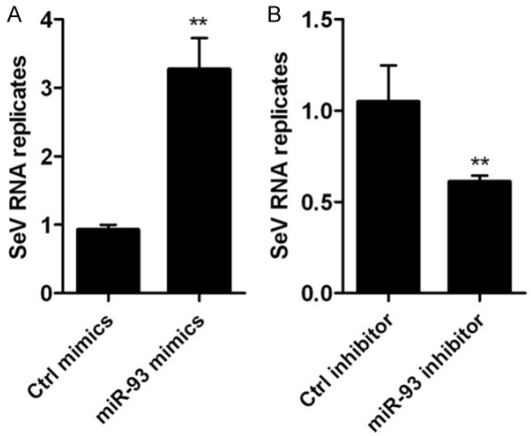
[44] Lin L, Hou J, Ma F, Wang P, Liu X, Li N, Wang J, Wang Q and Cao X. Type I IFN inhibits innate IL-10 production in macrophages through histone deacetylase 11 by downregulating microRNA-145. J Immunol 2013; 191: 3896-3904.



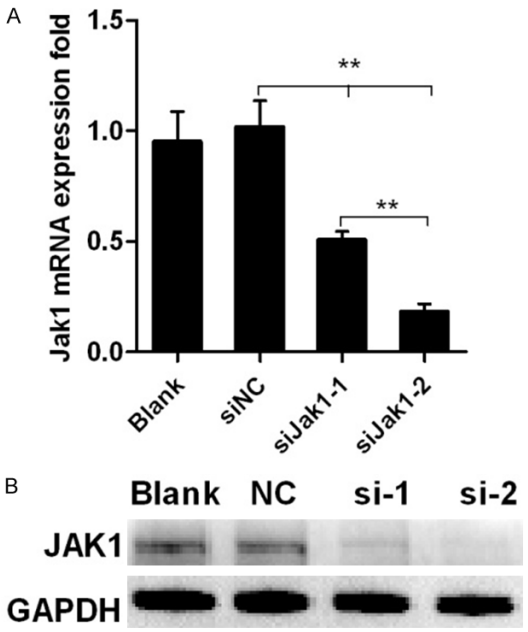
Supplementary Figure 1. SeV infection downregulates miR-93 expression in murine macrophages. Murine peritoneal macrophages were challenged with SeV at MOI 10 for the indicated time. miR-93 expression was measured as in **Figure 1A**. Data are shown as the mean ± s.d. (n=3) from one representative experiment. Similar results were obtained in three independent experiments. **p<0.01.



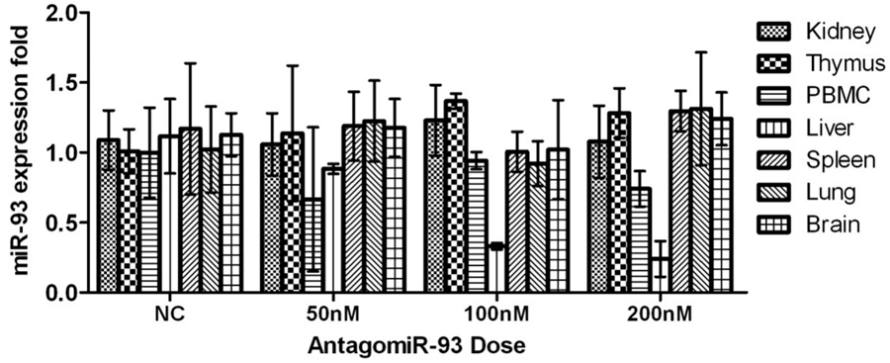
Supplementary Figure 2. The transfection efficiency of miR-93. Murine peritoneal macrophages were transfected with control or miR-93 mimics (left panel), or with control or miR-93 inhibitors (right panel) for 72 h. miR-93 expression was measured. Data are shown as the mean ± s.d. from one representative experiment. Similar results were obtained in three independent experiments. **p<0.01.



Supplementary Figure 3. Inhibition of miR-93 attenuates SeV replication. Murine peritoneal macrophages were transfected with control or miR-93 mimics (A), or with control or miR-93 inhibitors (B). These macrophages were then infected with SeV at MOI 10 for 1 h, washed, and fresh medium was added. After 72 h, intracellular SeV RNA replicates were measured. Data are shown as the mean ± s.d. (n=3) from one representative experiment. Similar results were obtained in three independent experiments. **p<0.01.



Supplementary Figure 4. Selection of si-JAK1. Murine peritoneal macrophages were transfected with control RNA, two separate JAK1 siRNAs at the sites of 1638 (siJAK1-1) and 1952 (siJAK1-2) respectively as indicated. After 24 h, JAK1 mRNA expression was measured by RT-PCR (A) and western blot (B). Data are shown as mean ± s.d. (A) or one representative experiment (B) obtained in three independent experiments with similar results.



Supplementary Figure 5. miR-93 expression *in vivo* after injected with AntagomirmiR-93. C57 mice were intravenously injected with miR-93 or NC antagomir in different dose. After 24 h, expressions of miR-93 in concerned tissues were measured by qRT-PCR. Data are shown as mean ± s.d. of one representative experiment. Similar results were obtained in three independent experiments.