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Inducible microRNA-155 Feedback Promotes Type I IFN Signaling in Antiviral Innate Immunity by Targeting Suppressor of Cytokine Signaling 1

Pin Wang,^{*1} Jin Hou,^{*1} Li Lin,[†] Chunmei Wang,^{*} Xingguang Liu,^{*} Dong Li,[†] Feng Ma,[†] Zhugang Wang,[‡] and Xuetao Cao^{*†}

Effective recognition of viral infection and subsequent triggering of antiviral innate immune responses are essential for the host antiviral defense, which is tightly regulated by multiple regulators, including microRNAs. Our previous study showed that a panel of microRNAs, including miR-155, was markedly upregulated in macrophages upon vesicular stomatitis virus infection; however, the biological function of miR-155 during viral infection remains unknown. In this paper, we show that RNA virus infection induces miR-155 expression in macrophages via TLR/MyD88-independent but retinoic acid-inducible gene I/JNK/NF- κ B-dependent pathway. And the inducible miR-155 feedback promotes type I IFN signaling, thus suppressing viral replication. Furthermore, *suppressor of cytokine signaling 1 (SOCS1)*, a canonical negative regulator of type I IFN signaling, is targeted by miR-155 in macrophages, and SOCS1 knockdown mediates the enhancing effect of miR-155 on type I IFN-mediated antiviral response. Therefore, we demonstrate that inducible miR-155 feedback positively regulates host antiviral innate immune response by promoting type I IFN signaling via targeting *SOCS1*. *The Journal of Immunology*, 2010, 185: 6226–6233.

Upon viral infection, host innate immunity is the first line of antiviral defense, designed to recognize viral components and produce proinflammatory cytokines and type I IFN (1–3). Type I IFN plays critical roles in antiviral immune response mainly through induction of cellular resistance to viral infection and apoptosis of virus-infected cells. Hence, type I IFN is extensively applied to the therapy of viral infection. However, the outcome of IFN therapy varies with different individuals depending on different disease stages and the factors influencing host responses to IFN. Although IFN signaling pathways have been investigated extensively so far, the underlying mechanisms for the regulation of this pathway are still not fully characterized. JAK/STAT cascade, the well-established cardinal pathway transmitting IFN signals, was found to be tightly regulated by several mechanisms. Among them, some molecules, such as suppressor

of cytokine signaling (SOCS) family members, can negatively regulate type I IFN signaling (4, 5). However, there are few reports about the regulation of type I IFN signaling and subsequent antiviral innate immunity by microRNAs (miRNAs) to date.

miRNAs are an abundant class of highly conserved small non-coding RNAs. They function mainly through suppressing target genes expression by binding to the 3'-untranslational region (UTR) of target mRNAs to induce degradation or suppress translation. It has been demonstrated that miRNAs participate in various biological processes, including innate and adaptive immune responses (6–8). Using genetic approaches, miR-155 has been demonstrated to have an indispensable role in humeral and cellular immunity (9–15). Regarding innate immunity and inflammatory response, miR-155 expression is induced by TLR signals, such as TLR2, TLR3, TLR4, and TLR9, or stimulations from cytokines, such as IL-1, TNF- α , and IFN- β , indicating the participation of miR-155 in these immune responses (13–15). Although the fact that miR-155 expression is induced by stimulation of poly(I:C) or IFN- β implies the involvement of miR-155 in antiviral responses, there is no report by now about the regulation and underlying mechanism of type I IFN signaling and subsequent type I IFN-mediated antiviral innate immunity by the inducible miR-155.

On the basis of our previous observation that a panel of miRNAs including miR-146a and miR-155 were upregulated in murine peritoneal macrophages upon vesicular stomatitis virus (VSV) challenge (16), in this study, we found that the induction of miR-155 by VSV infection was through a TLR/MyD88-independent but retinoic acid-inducible gene I (RIG-I)/JNK/NF- κ B-dependent mechanism. Up-regulated miR-155 suppressed SOCS1 expression in macrophages and subsequently enhanced type I IFN effector gene expression and type I IFN-mediated antiviral response, thus suppressing viral replication. We for the first time, to our knowledge, demonstrate that the inducible miR-155, upregulated upon RNA virus infection, acts as a feedback positive regulator of type I IFN signaling in antiviral immunity by targeting *SOCS1*.

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Abbreviations used in this paper: BMDC, bone marrow-derived dendritic cell; IP-10, inflammatory protein 10; ISG, IFN-stimulated gene; miRNA, microRNA; MOI, multiplicity of infection; N.D., not detected; PDTIC, pyrrolidinedicarboxylic acid; RIG-I, retinoic acid-inducible gene I; SeV, Sendai virus; siRNA, small interfering RNA; SOCS, suppressor of cytokine signaling; TCID₅₀, 50% tissue culture infectious dose; UTR, untranslational region; VSV, vesicular stomatitis virus.

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Materials and Methods

Mice and reagents

C57BL/6 mice (6–8 wk) were purchased from Joint Ventures Sipper BK Experimental Animal Company (Shanghai, China). *TLR3* knockout mice were described previously (16). *TLR4*, *TLR9*, and *MyD88* knockout mice were provided by Prof. S. Akira (Osaka University, Osaka, Japan). *RIG-I* knockout mice were generated as described previously (17). All animal experiments were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, with the approval of the Scientific Investigation Board of Second Military Medical University (Shanghai, China). VSV was a gift from Prof. W. Pan (Second Military Medical University, Shanghai, China), and Sendai virus (SeV) was from Prof. B. Sun (Chinese Academy of Sciences, Shanghai, China). Pyrrolidinedithioic acid, an inhibitor of NF- κ B, SB203580, an inhibitor of p38, PD98059, a MEK/ERK inhibitor, and SP600125, a JNK inhibitor, were from Calbiochem (San Diego, CA). Murine rIFN- β was described previously (16). Abs specific to SOCS1 and HRP-coupled secondary Abs were from Santa Cruz Biotechnology (Santa Cruz, CA). Abs specific to STAT1, JAK1, phosphorylated STAT1, and phosphorylated JAK1 were from Cell Signaling Technology (Danvers, MA). Abs specific to STAT2 and phosphorylated STAT2 were from Abcam (Cambridge, U.K.). Ab specific to β -actin was from Sigma-Aldrich (St. Louis, MO).

Cell culture and transfection

Murine macrophage cell line RAW264.7 was obtained from the American Type Culture Collection (Manassas, VA) and cultured as described previously (18). Thioglycolate-elicited murine peritoneal macrophages were prepared and cultured as described previously (19, 20). A total of $0.5 \text{ ml } 2 \times 10^5$ cells were seeded into each well of 24-well plates, or a total of $3 \text{ ml } 1 \times 10^6$ cells were seeded into each well of 6-well plates and incubated overnight and then transfected as described previously (16). Bone marrow-derived dendritic cells (BMDCs) and splenocytes were generated as described previously (21). SOCS1 stably overexpressed RAW264.7 cell clones were selected in $600 \mu\text{g/ml}$ G418 for 3–4 wk and then confirmed by Western blot analysis for the expression of SOCS1.

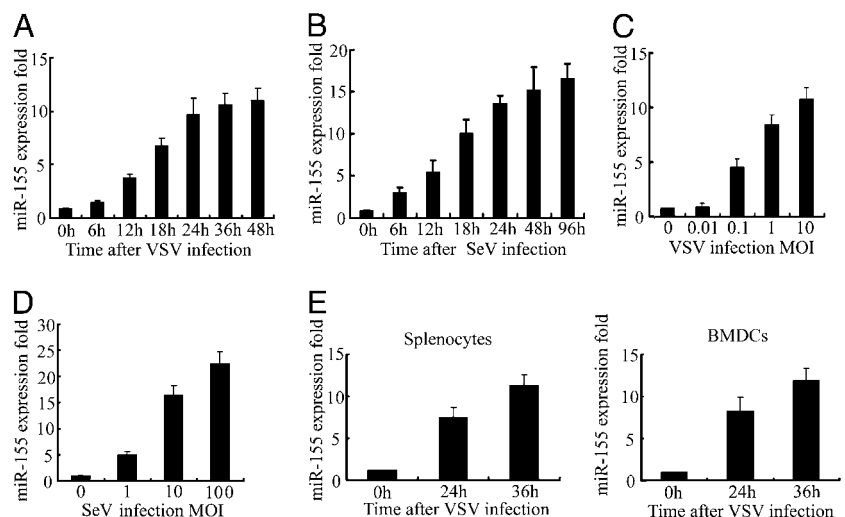
Prediction of miR-155 targeting sites in VSV RNA in silico

RNA22 miRNA target (http://cbcsrv.watson.ibm.com/rna22_targets.html) detection (22) was used to predict miR-155 target site in VSV RNA sequences with settings as follows. Maximum number of allowed unpaired bases: 0 in seed/nucleus of 6 nt; minimum number of paired-up bases in heteroduplex: 12; and maximum folding energy for heteroduplex (Kcal/mol): -25 .

miR-155 mimic and inhibitor

miR-155 mimic (dsRNA oligonucleotides) and miR-155 inhibitor (single-stranded chemically modified oligonucleotides) from GenePharma (Shanghai, China) were used for the overexpression and inhibition of miR-155 in murine macrophages, respectively. Macrophages described above were transfected as previously described (16) at a final concentration of 10 nM. Negative control mimic or inhibitor (GenePharma) was transfected as their matched controls.

FIGURE 1. RNA virus infection induces significant upregulation of miR-155 in murine macrophages and dendritic cells. *A* and *B*, Murine peritoneal macrophages were infected with VSV at MOI 1 (*A*) or SeV at MOI 10 (*B*) for indicated time. Expressions of miR-155 was measured by quantitative RT-PCR and normalized to that of U6 in each sample. *C* and *D*, Murine peritoneal macrophages were infected with VSV (*C*) or SeV (*D*) at indicated MOI for 36 h. Expression of miR-155 was measured as in *A*. *E*, Murine splenocytes (*left panel*) and BMDCs (*right panel*) were infected with VSV at MOI 1 for indicated time, and miR-155 expression was measured as described in *A*. Data are shown as mean \pm SD ($n = 3$) of one representative experiment. Similar results were obtained in three independent experiments. MOI, multiplicity of infection.



RNA interference

The SOCS1-specific small interfering RNA (siRNA) (siSOCS1) was 5'-CUA CCU GAG UUC CUU CCC CTT-3' (sense) and 5'-GGG GAA GGA ACU CAG GUA GTT-3' (antisense). The scrambled control RNA sequences were described previously (16). siRNA duplexes were transfected into murine peritoneal macrophages at a final concentration of 10 nM as described previously (18).

RNA quantification

Total RNA, containing miRNA, was extracted, reverse-transcribed, and real-time PCR amplified as described previously (16). For miRNA analysis, reverse transcription primers for miR-155 were 5'-GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACC CCC TA-3'. Quantitative PCR primers were 5'-CTC GTG GTT AAT GCT AAT TGT GA-3' (forward) and 5'-GTG CAG GGT CCG AGG T-3' (reverse). The relative expression level of miRNA was obtained as described previously (16). For murine β -actin, IFN- β , and IFN- α mRNA analysis, the primers were described previously (19, 20); for murine *SOCS1*, the primers were 5'-CTG CGG CTT CTA TTG GGG AC-3' (forward) and 5'-AAA AGG CAG TCG AAG GTC TCG-3' (reverse); for murine inflammatory protein 10 (*IP-10*), the primers were 5'-CCA AGT GCT GCC GTC ATT TTC-3' (forward) and 5'-GGC TCG CAG GGA TGA TTT CAA-3' (reverse); for murine IFN-stimulated gene 15 (*ISG15*), the primers were 5'-GGT GTC CGT GAC TAA CTC CAT-3' (forward) and 5'-TGG AAA GGG TAA GAC CGT CCT-3' (reverse); for VSV Indiana serotype, the primers were described previously (16). Data were normalized to the level of β -actin expression in each sample.

ELISA

A total of 2×10^5 cells in 0.5 ml culture medium were seeded into each well of 24-well plates and incubated overnight and then transfected as described above. After 48 h, the cells were infected with VSV or SeV for the indicated time periods. The concentration of IFN- β in culture supernatants was measured as described previously (16).

Western blot analysis

The cells were washed twice with cold PBS and lysed with cell lysis buffer (Cell Signaling Technology) supplemented with protease inhibitor mixture (Calbiochem). Protein concentrations of the cell lysis extracts were measured with BCA assay (Pierce, Rockford, IL) and equalized with the extraction reagent. Equal amount of the extracts were loaded and subjected to SDS-PAGE, transferred onto nitrocellulose membranes, and then blotted as described previously (19, 20).

VSV yield qualification

Macrophages were transfected as described above and infected by VSV as indicated. A total of 0.1 ml cultural supernatants were serially diluted on the monolayer of BHK21 cells, which were obtained from the American Type Culture Collection, and 1×10^4 cells were seeded into 96-well plates a day before measurement. Fifty percent tissue culture infectious dose (TCID_{50}) was measured 3 d later as described previously (16).

Statistical analysis

Statistical significance was determined by Student *t* test, with $p < 0.05$ considered to be statistically significant.

Results

RNA virus infection upregulates miR-155 expression in macrophages and dendritic cells

We previously reported that a panel of miRNAs was markedly upregulated upon VSV infection in macrophages, and miR-155 was one of the mostly upregulated (16). However, the role of inducible miR-155 expression in antiviral immune response is still not known. The kinetic induction of mature miR-155 following VSV challenge indicated that miR-155 was a VSV infection responsive gene in macrophages, and its induction reached the peak at ~24 h after VSV challenge (Fig. 1A). SeV, another RNA virus, also induced miR-155 expression in macrophages with similar kinetics (Fig. 1B). Furthermore, these RNA viruses both induced miR-155 expression in a dose-dependent manner (Fig. 1C, 1D). Also, miR-

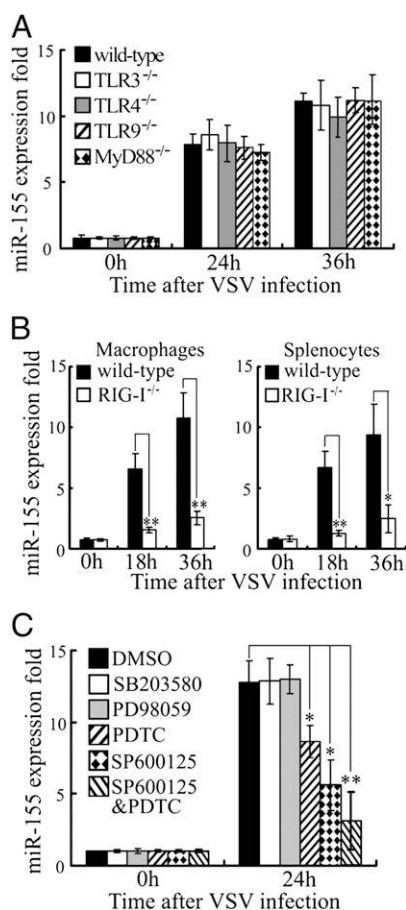


FIGURE 2. VSV infection induces miR-155 expression in macrophages via RIG-I/JNK/NF- κ B but not TLR/MyD88 pathway. **A**, Peritoneal macrophages from wild-type, TLR3^{-/-}, TLR4^{-/-}, TLR9^{-/-}, or MyD88^{-/-} mice were infected with VSV at MOI 10 for indicated time. Expression of miR-155 was measured as in **A**. **B**, Murine peritoneal macrophages or splenocytes from wild-type or RIG-I^{-/-} mice were infected with VSV at MOI 10 for indicated time, and miR-155 expression was measured as in **A**. **C**, Murine peritoneal macrophages were pretreated with DMSO, SB203580 (10 μ M), PD98059 (10 μ M), PDTC (100 μ M), or SP600125 (10 μ M) as indicated for 30 min and then infected with VSV at MOI 10 for indicated time. miR-155 expression was measured as in **A**. Data are shown as mean \pm SD ($n = 3$) of one representative experiment. Similar results were obtained in three independent experiments. ** $p < 0.01$; * $p < 0.05$. PDTC, pyrrolidinedicarboxylic acid.

155 expression was upregulated by VSV infection in murine splenocytes and BMDCs (Fig. 1E). So, expression of miR-155 can be upregulated in APCs in response to RNA virus infection.

RNA virus infection upregulates miR-155 expression via RIG-I/JNK/NF- κ B but not TLR/MyD88 pathway

We next investigated the underlying mechanism by which miR-155 was induced. As it was reported that signals from TLR2, TLR3, TLR4, and TLR9 induced miR-155 expression through MyD88- or TRIF-dependent signaling pathways (13), we exclude the possibility that activation of TLR signals contribute to VSV-induced miR-155 expression. Peritoneal macrophages from TLR3^{-/-}, TLR4^{-/-}, TLR9^{-/-}, or MyD88-deficient mice showed identical induced miR-155 expression following VSV infection as compared with wild-type macrophages (Fig. 2A). However, VSV-induced miR-155 expression was markedly impaired in RIG-I-deficient macrophages and splenocytes (Fig. 2B), suggesting that inducible miR-155 expression was dependent on RIG-I signaling. As control, LPS-induced miR-155 expression was impaired by TLR4 deficiency but not significantly influenced by RIG-I deficiency (Supplemental Fig. 1).

Because induction of miR-155 was reported to be JNK dependent (13), we further investigated the effect of JNK inhibitor on VSV-induced miR-155 expression in macrophages. As shown in Fig. 2C, inhibition of JNK efficiently impaired VSV-induced miR-155 expression. In addition, inhibition of NF- κ B also partially impaired VSV-induced miR-155 upregulation, and combined inhibition of JNK and NF- κ B suppressed VSV-induced miR-155 expression more significantly. However, inhibition of p38 or ERK had little effect on the induction of miR-155 (Fig. 2C). Taken together, these results suggest that VSV infection upregulates miR-155 expression in macrophages mainly through RIG-I/JNK/NF- κ B-dependent pathway.

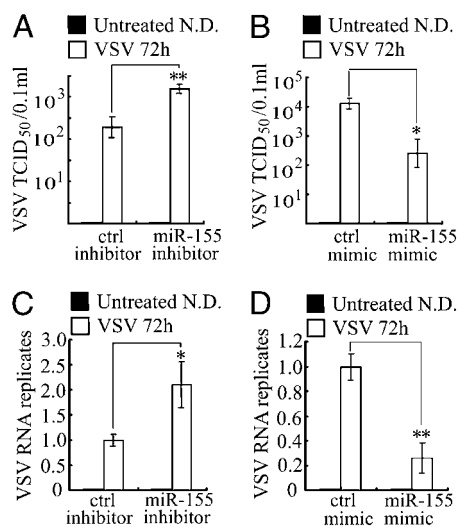


FIGURE 3. miR-155 attenuates VSV replication in macrophages. **A** and **B**, Murine peritoneal macrophages transfected with control inhibitor or miR-155 inhibitor (**A**) or with control mimic or miR-155 mimic (**B**) were infected by VSV at MOI 10 for 1 h and washed, then added with fresh medium. After 72 h, VSV TCID₅₀ in cultural supernatants were measured. **C** and **D**, Murine peritoneal macrophages were treated as in **A**, and intracellular VSV RNA replicates were qualified using quantitative RT-PCR and normalized to that of β -actin in each sample. Data are shown as mean \pm SD ($n = 3$) of one representative experiment. Similar results were obtained in three independent experiments. ** $p < 0.01$; * $p < 0.05$. N.D., not detected.

miR-155 feedback attenuates the viral replication in macrophages

To investigate the biological significance of upregulated miR-155 during viral infection, we further examined the effect of miR-155 on VSV replication in macrophages. By measuring VSV TCID₅₀ in the cultural supernatants of the infected macrophages, we found that inhibition of induced miR-155 facilitated VSV replication, whereas overexpression of miR-155 suppressed VSV replication (Fig. 3A, 3B). Consistent with the data of VSV TCID₅₀ assay in cultural supernatants, intracellular VSV RNA replicates were also increased by miR-155 inhibition and decreased by miR-155 overexpression (Fig. 3C, 3D). Thus, we conclude that induced miR-155 expression in turn, as a positive feedback, attenuates VSV replication in VSV-infected macrophages.

miR-155 attenuates viral propagation mainly through enhancing type I IFN signaling in macrophages

To gain an insight into the mechanism of how miR-155 induction attenuated viral propagation, we analyzed in silico with RNA22 miRNA target detection (22) using both VSV sense and antisense RNA sequences but found no potential target sites in VSV RNA (data not shown). Hence, miR-155 is less likely to target VSV RNA directly, as miR-24 and miR-93 have been shown to do so

(23). We next examined type I IFN production in VSV-infected macrophages and found no significant difference in either mRNA or protein level of IFN-β when miR-155 was inhibited or overexpressed (Fig. 4A, 4B), although there was a slight increase in IFN-β production when miR-155 was overexpressed. Similar results were also obtained when IFN-4α mRNA level was examined (Fig. 4C). Finally, we focused on the effect of miR-155 on type I IFN downstream signaling. After type I IFN binds to their receptors, IFN-αβ receptors, JAK/STAT pathway is activated, and the STATs are phosphorylated and translocated into nucleus, resulting in transcription of IFN-stimulated genes. The kinetic phosphorylation of STAT1 was detected after VSV challenge in macrophages transfected with miR-155 mimic or miR-155 inhibitor. As shown in Fig. 4D, VSV-induced STAT1 phosphorylation was promoted by miR-155 overexpression while inhibited by miR-155 inhibitor. We also examined expression of IFN-induced immune regulatory genes, such as *ISG15* and *IP-10*. In macrophages treated with rIFN-β, we found that induced expressions of *ISG15* and *IP-10* were increased by miR-155 overexpression and decreased by miR-155 inhibition (Fig. 4E). These results suggest that inducible miR-155 in response to RNA virus infection promotes type I IFN signaling but does not significantly affect type I IFN production.

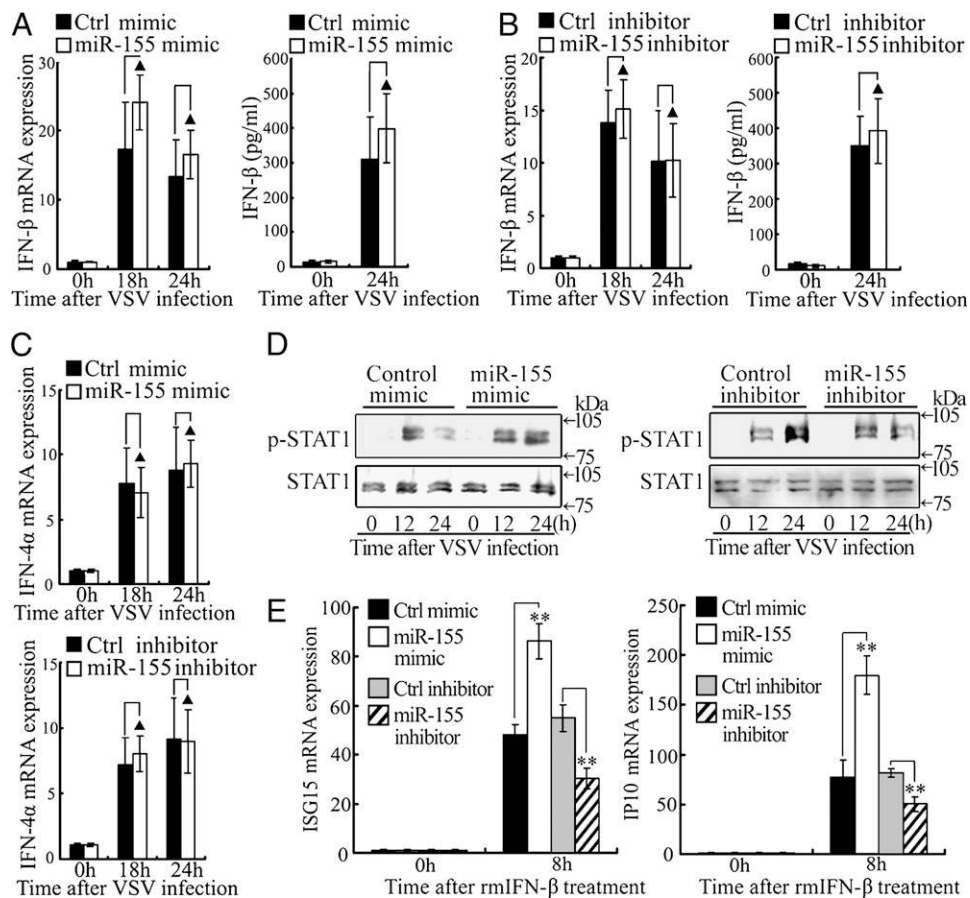


FIGURE 4. miR-155 promotes type I IFN signaling rather than enhances type I IFN production. *A* and *B*, A total of 0.5 ml 2×10^5 murine peritoneal macrophages were transfected with control mimic or miR-155 mimic (*A*) or control inhibitor or miR-155 inhibitor (*B*) as indicated. After 48 h, cells were infected by VSV at MOI 10 for indicated time. IFN-β mRNA expression (*left panel*) was measured by quantitative RT-PCR and normalized to that of β-actin in each sample. IFN-β in supernatants (*right panel*) was measured by ELISA. *C*, Murine peritoneal macrophages were transfected and infected as in *A* and *B*, and IFN-4α mRNA expression was measured as in *A* and *B*. *D*, Murine peritoneal macrophages were transfected as in *A* and *B* and then infected by VSV at MOI 10. Phosphorylated STAT1 was detected by immunoblot at the indicated time and total STAT1 as an input control. *E*, Macrophages were transfected as in *A* and *B*. After 48 h, recombinant murine IFN-β (100 U/ml) was added for indicated time, and ISG15 and IP-10 mRNA expression were measured as in *A* and *B*. Data are shown as mean ± SD ($n = 3$) of one representative experiment. Similar results were obtained in three independent experiments. ** $p < 0.01$; ▲ $p > 0.05$.

miR-155 targets *SOCS1* in virus-infected macrophages

We next investigated what was the major target of miR-155 that could modulate type I IFN signaling. We found that miR-155 was one of the broadly conserved miRNAs that putatively targets conserved sites of murine *SOCS1*'s 3'-UTR by computational prediction via TargetScan (www.targetscan.org) (Supplemental Fig. 2). *SOCS1* has been identified to negatively regulate various immune responses and signaling pathways, including type I IFN signaling (24). *SOCS1* mRNA level accumulates sharply in no more than 24 h after VSV challenge (Fig. 5A), but its protein level hardly changes or even slightly decreases over a time course of 48 h (Fig. 5B), suggesting that the expression of *SOCS1* in macrophages is regulated by a posttranscriptional mechanism, presumably miRNA inhibition. Hence, we hypothesized that miR-155 might suppress *SOCS1* translation in macrophages after VSV challenge.

Then we examined whether the protein level of *SOCS1* in macrophages was targeted and regulated by miR-155. As expected, *SOCS1* expression was increased by miR-155 inhibition and decreased by miR-155 overexpression (Fig. 5C). To present direct evidence that inducible miR-155 during VSV infection suppressed *SOCS1* protein expression, we examined the kinetic level of *SOCS1* protein and mRNA after VSV challenge when inducible miR-155 was inhibited. As shown in Fig. 5D and 5E, miR-155 inhibitor rescued *SOCS1* mRNA translation upon VSV challenge, and *SOCS1* mRNA kinetic level was not affected significantly. As *SOCS1* was reported to be recruited to the JAKs and in turn block JAKs' tyrosine kinase activity and downstream STAT phosphorylation (25, 26), so we further proved that miR-155 regulated

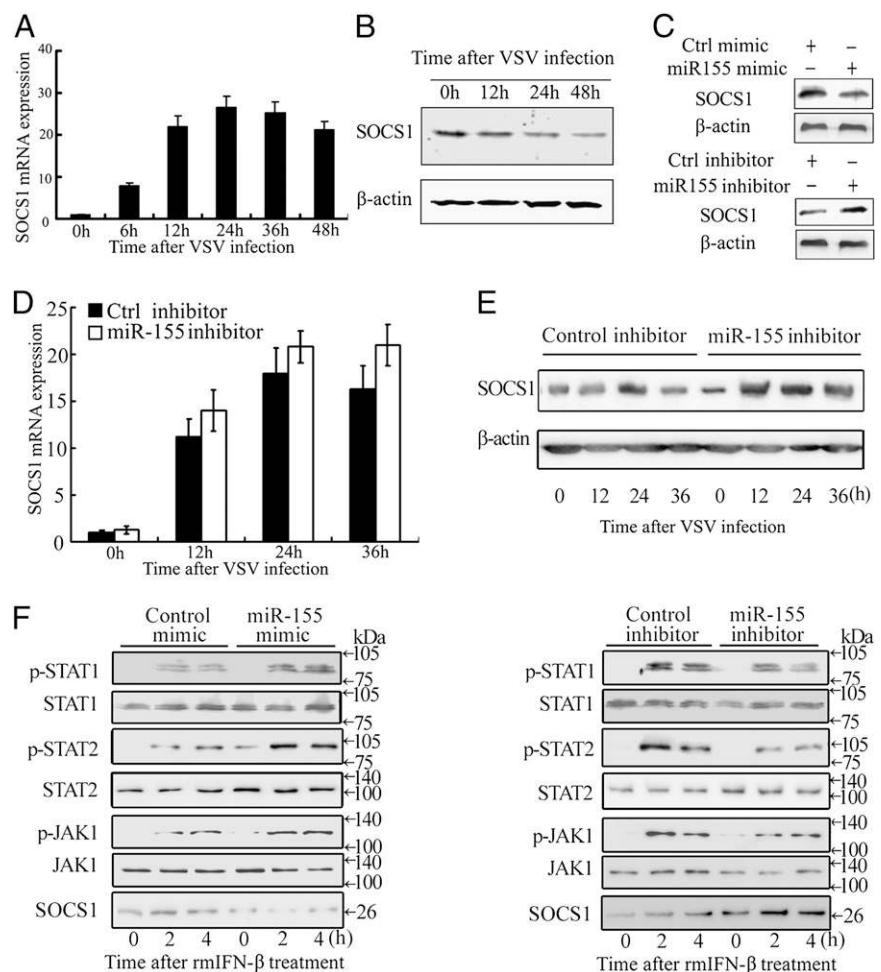
type I IFN signaling via *SOCS1* by examining kinetic phosphorylation of JAK1 and STAT1/2 in response to rmIFN- β . As shown in Fig. 5F, inhibition of inducible miR-155 suppressed STAT1/2 and JAK1 phosphorylation, whereas overexpression of miR-155 enhanced STAT1/2 and JAK1 phosphorylation, which is consistent with the regulatory mechanism of *SOCS1* (27, 28). These results were consistent with a recent report showing that miR-155 directly targeted the potential conserved target site in *SOCS1* mRNA 3'-UTR through reporter gene assay (29). Taken together, these data show that endogenous *SOCS1* is targeted and directly regulated by miR-155 in macrophages.

Antiviral function of miR-155 is mainly through targeting *SOCS1*

To demonstrate the role of targeting *SOCS1* in the antiviral function of miR-155, we carried out experiments of *SOCS1* RNA interference knockdown and overexpression. And we confirmed that *SOCS1* siRNA effectively inhibited its expression in murine peritoneal macrophages (Fig. 6A), whereas *SOCS1*-stably overexpressed cell clone of RAW264.7 macrophages, which expressed *SOCS1* mRNA without its 3'-UTR sequence, had significantly elevated *SOCS1* protein expression (Fig. 6B).

In murine peritoneal macrophages, *SOCS1* knockdown attenuated VSV replication, which resembled the effect of miR-155 overexpression (Fig. 6C). And VSV replication increased by miR-155 inhibition was rescued by *SOCS1* knockdown (Fig. 6D). Also, rIFN- β -induced *ISG15* expression is reduced by miR-155 inhibition and further rescued by *SOCS1* knockdown (Fig. 6E).

FIGURE 5. miR-155 targets murine *SOCS1* in VSV-infected macrophages. **A** and **B**, Murine peritoneal macrophages were infected with VSV at MOI 10 for indicated time. *SOCS1* mRNA expression was measured as in Fig. 4A (**A**), and *SOCS1* protein level and β -actin were detected by immunoblot (**B**). **C**, Murine peritoneal macrophages were transfected with control mimic or miR-155 mimic (*top panel*), or control inhibitor or miR-155 inhibitor (*bottom panel*) as indicated. After 48 h, *SOCS1* was detected by immunoblot as in **B**. **D** and **E**, Murine peritoneal macrophages were transfected with control inhibitor or miR-155 inhibitor as indicated; 12 h after transfection, cells were infected with VSV at MOI 10 for indicated time. *SOCS1* mRNA expression was measured as in Fig. 4A (**D**), and *SOCS1* protein level and β -actin were detected by immunoblot (**E**). **F**, Macrophages were transfected as in Fig. 4A and 4B. After 48 h, recombinant murine IFN- β (100 U/ml) was added, and *SOCS1* and tyrosine phosphorylation of STAT1, STAT2, and JAK1 were detected by immunoblot at indicated time and total STAT1, STAT2, and JAK1 as input controls. Data are shown as mean \pm SD ($n = 3$) of one representative experiment. Similar results were obtained in at least three independent experiments.



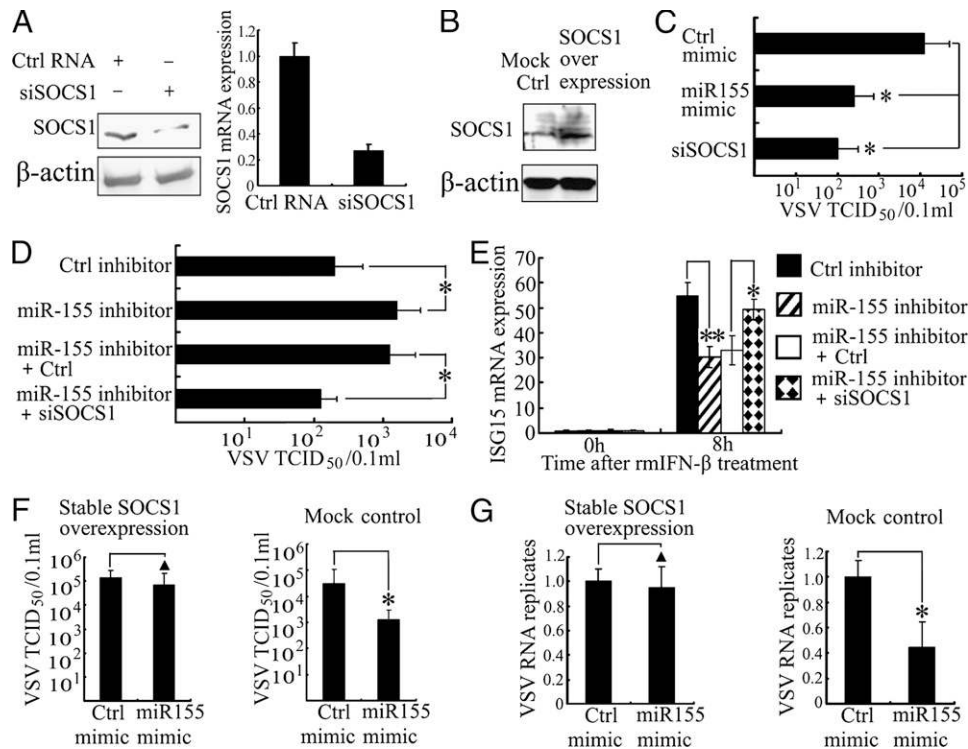


FIGURE 6. Antiviral effect of miR-155 is mainly through targeting *SOCS1*. *A*, Murine peritoneal macrophages were transfected with control RNA *SOCS1* siRNA as indicated. After 24 h, *SOCS1* mRNA expression (right panel) was measured as in Fig. 4*A*, and after 48 h, *SOCS1* protein level and β -actin (left panel) were detected by immunoblot. *B*, *SOCS1* protein level in *SOCS1*-stably overexpressed RAW264.7 cell clones and mock control cell clones were detected by immunoblot. *C*, Murine peritoneal macrophages were transfected with control RNA, miR-155 mimic, or *SOCS1* siRNA as indicated. After 48 h, macrophages were treated, infected, and detected as in Fig. 3*A*. *D*, Murine peritoneal macrophages were transfected with control inhibitor, miR-155 inhibitor, miR-155 inhibitor combined with control RNA, or miR-155 inhibitor combined with *SOCS1* siRNA, respectively, as indicated. After 48 h, macrophages were treated, infected, and detected as in Fig. 3*A*. *E*, Murine peritoneal macrophages were transfected as in *D*. After 48 h, recombinant murine IFN- β (100 U/ml) was added for indicated time, and ISG15 mRNA expression was measured as in Fig. 4*A*. *F* and *G*, *SOCS1*-stably overexpressed RAW267.4 cells or mock control cells transfected with control mimic or miR-155 mimic were infected as in Fig. 3*A*. Virus in supernatants (*F*) and intracellular VSV RNA replicates (*G*) were detected as in Fig. 3*A* and 3*B*. Data are shown as mean \pm SD ($n = 3$) of one representative experiment. Similar results were obtained in at least three independent experiments. ** $p < 0.01$; * $p < 0.05$; $\blacktriangle p > 0.05$.

These results suggest that *SOCS1* knockdown phenocopied the antiviral effect of induced miR-155 and counteracted the effect of miR-155 inhibition.

Because the *SOCS1*-stably overexpressed RAW264.7 cell clones transcribed *SOCS1* mRNA without its 3'-UTR, miR-155 should no longer target *SOCS1* expression in these cells. We did not find that miR-155 overexpression could influence VSV proliferation in *SOCS1*-stably overexpressed RAW264.7 cells, documented by both measuring VSV TCID₅₀ in the cultural supernatants (Fig. 6*F*) and intracellular VSV RNA replicates (Fig. 6*G*). So, we concluded that induced miR-155 upon viral infection exhibits its antiviral function mainly through suppressing endogenous *SOCS1* expression and subsequently promoting type I IFN signaling.

Discussion

miR-155 is generally believed to be a multifunctional miRNA (9, 10). It is transcribed from a noncoding gene named *BIC* gene, which is highly conserved in many species and broadly expressed in various organs, tissues, and cell types, indicating its versatile functions in various biological processes. It has been demonstrated that miR-155 plays important roles in physiological conditions (e.g., circulation, hematopoiesis, immunity, and inflammation), as well as pathological conditions (e.g., neoplastic diseases and cardiovascular disorders). Up until now, there were only two reports about the roles of miR-155 in viral infection: one was about the miR-155 ortholog named miR-K12-11 encoded by Kaposi's sarcoma-associated herpes virus (30); the other was the induced miR-155

expression during EBV infection (31). Both of the viruses belong to the herpesvirus family, DNA virus. The induced miR-155 and the viral ortholog miR-K12-11 were both found to participate in the biological process of the indicated herpesvirus infection. In this paper, we show that miR-155, as a positive feedback regulator, participates in antiviral immune responses once induced in murine macrophages upon RNA virus challenge. Our data broaden and deepen the understanding of the roles of miR-155 in the interaction between host and virus.

For IFN- β stimulation-induced downstream signaling, we found that miR-155 could not significantly influence JAK1 phosphorylation at short time points (0, 15, and 30 min poststimulation), but STAT1/2 phosphorylation is influenced by miR-155 expression more obviously (Supplemental Fig. 3). This discrepancy may be due to the expression level of endogenous *SOCS1* in peritoneal macrophages. Considering that *SOCS1* is expressed at a relative low level in peritoneal macrophages (32) and its expression could be induced upon IFN stimulation in 2 h (Supplemental Fig. 4), it is plausible that lower expression of *SOCS1* preferentially inhibits STAT1/2 phosphorylation, whereas the enhanced expression of *SOCS1* inhibits both JAK1 and STAT1/2 phosphorylation as detected at longer time points in Fig. 5*F*. This presumption is consistent with previous reports showing that *SOCS1* could inhibit STAT1 activation at a very low protein expression level in vivo (33), phosphorylation of JAKs is required for the binding of *SOCS1* (34), and further forced *SOCS1* overexpression inhibits both JAKs autophosphorylation and STAT1/2 phosphorylation in

vitro (27, 28). However, the detailed regulatory mechanism mediating this discrepancy remains to be investigated in the future.

Up until now, several genes have been identified as targets of miR-155, through which the function of miR-155 is revealed. However, the target profile of miR-155 does not seem to end so far. The major targets of one specific miRNA always changes in different physical or pathological conditions, or different cell types, to implement different functions accordingly. miR-155 has been suggested to participate in different biological processes by targeting different genes expression, such as *PU.1* (11, 35), *AID* (36, 37), *SHIP1* (38), and *SMAD5* (39). In this paper, we show that miR-155 directly targets *SOCS1* expression in VSV-infected macrophages to strengthen IFN effects, which is consistent with a recent report revealing that miR-155 expression is required for regulatory T cell activity also via targeting *SOCS1* expression (29). It seems that the essential roles of miR-155 elucidated by targeting *SOCS1* expression are not confined to regulatory T cells or macrophages. Because *SOCS1* is a general regulator of cell signaling downstream lots of cytokines, such as IL-2, IL-4, IL-6, IFN- γ , growth hormone, and erythropoietin, thus modulating various biological functions of immune cells (4), the finding that miR-155 targeting *SOCS1* may explain, to some extent, some of the pathological phenotypes in miR-155 knockout mice or miR-155 transgenic mice [e.g., impaired hematopoietic homeostasis (40), lymphoblastic leukemia (41), increased lung airway remodeling (10), and unbalanced cytokine production (12)]. Also, miR-155 targeting *SOCS1* may contribute to the pathogenesis of many human hemopoietic tumors with overexpressed miR-155 (42–44). In addition, overexpressed *SOCS1* lacking miR-155 suppression in incompetent immunocytes is probably the explanation for immune tolerance or immunodeficiency in patients with poor response to IFN antiviral or antitumor therapy. Predictably, upregulation of miR-155 expression in activated immunocytes may facilitate antitumor responses, whereas, in contrast, enhanced reactivity of immunocytes to proliferative cytokines mediated by miR-155 overexpression may otherwise contribute to the increased risk of suffering hemopoietic tumors. These predictions need to be validated further through extensive experiments in the future.

Induced cytokine production is required for the elimination of pathogen infection; nevertheless, it is clarified that their overproduction or overreaction may result in local or systematic pathology. For instance, solid evidence reveals that host immune responses contribute to the pathogenesis of human seasonal influenza A virus disease (45). Hence, the suppression of *SOCS1* by inducible miR-155 strengthens antiviral immunity and elicits more effective immune responses as a positive aspect. In contrast, miR-155 overexpression may also probably initiate excessive antiviral responses and contribute to viral infection-induced tissue damage, which is supported by studies demonstrating that *SOCS1* negatively regulates innate immune responses triggered by influenza A virus (46) and *SOCS1* protects mice against lethal poxvirus infection (47) and *Chlamydia pneumoniae* infection (48). Induced miR-155 expression upon viral infection may be a double-edged sword, because it may participate in both physiological antiviral immune response and pathological viral infection-induced tissue damage. And maybe this is the reason why miR-155 is moderately, neither vigorously nor slightly, induced in macrophages and other APCs upon RNA virus challenge, and deregulation of miR-155 probably contributes to the pathogenesis of viral infection-induced pathological responses.

Our previous work documented that miR-146a, upregulated upon VSV challenge, inhibited RIG-I-dependent type I IFN production as a negative feedback regulator in antiviral immunity (16), whereas in this study, the inducible miR-155 promoted type I

IFN signaling as a positive feedback regulator. Taking these works together, we further confirmed that antiviral immune response was under accurate and sophisticated regulation by multiple regulators, both negatively and positively, like miR-146a and miR-155. It is probable that we are far from revealing the last miRNA that modulates antiviral immunity. And each miRNA may act as a counterbalance to the other, so the overall effect of all these miRNAs on IFN-mediated antiviral response may be not apparent. This may be the reason why Dicer-deficient macrophages, which leading to deficiency of all miRNAs, showed no obviously altered IFN-mediated antiviral response (23). In addition, besides these significantly induced miRNAs after VSV challenge, we could not exclude the possibility that the less markedly altered miRNAs also influence host antiviral response via the cumulative impact of all or most of them, if single one has little effect.

Host favorable defense against viral infection requires not only appropriate induction of antiviral cytokines but also appropriate cellular responses to them. We show in this study that the inducible miR-155 promotes host cell response to type I IFN in antiviral immunity by targeting *SOCS1*, further indicating that different host miR-155 expression levels may contribute to different responses to IFN therapy of viral infections among individuals. Hence, miR-155 may be a potential therapeutic target in viral infections or diseases subjected to type I IFN therapy, which needs to be identified in the future.

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Disclosures

The authors have no financial conflicts of interest.

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