Inducible microRNA-590-5p inhibits host antiviral response by targeting the soluble interleukin-6 (IL6) receptor

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MicroRNA (miR)-590-5p has been identified as an important regulator of some signaling pathways such as cell proliferation and tumorigenesis. However, little is known about its role during viral infection. Here, we report that miR-590-5p was significantly induced by various viruses and effectively potentiated virus replication in different viral infection systems. Furthermore, miR-590-5p substantially attenuated the virus-induced expression of type I and type III interferons (IFNs) and inflammatory cytokines, resulting in impaired downstream antiviral signaling. Interleukin-6 receptor (IL6R) was identified as a target of miR-590-5p. Interestingly, the role of miR-590-5p in virus-triggered signaling was abolished in IL6R knockout cells, and this could be rescued by restoring the expression of the soluble IL6R (sIL6R) but not the membrane-bound IL6R (mIL6R), suggesting that sIL6R is indispensable for miR-590-5p in modulating the host antiviral response. Furthermore, miR-590-5p down-regulated endogenous sIL6R and mIL6R expression through a translational repression mechanism. These findings thus uncover a previously uncharacterized role and the underlying mechanism of miR-590-5p in the innate immune response to viral infection.

MicroRNAs (miRNAs) belong to the category of single-stranded small (19–25-nucleotide) RNAs that can typically mediate mRNA cleavage or translational repression via binding to complementary sequences of the target mRNAs (1–4). Following the discovery of functional microRNAs in the early 1990s (5, 6), the study of microRNA biology has attracted remarkable attention, resulting in rapid advances. It is well supported that microRNAs play important roles in the regulation of cellular processes through down-regulating their targets, including cell differentiation, cell apoptosis, tumor metastasis, and circadian behavior (7–10). Viral infection triggers host responses that engage signaling networks, which have a fundamental role in the antiviral response. Previous studies have identified numerous microRNAs involved in this process. For example, miRNA-548 down-regulates the host antiviral response via direct targeting of IFN-α1 (11), whereas miR-130 families repress essential hepatitis C virus cofactors, thus restricting viral infection at multiple stages (12). Up to now, more cellular microRNAs have been identified to affect virus infection in a positive or negative manner.

The mir-590 gene is located on the proximal end of the long arm of chromosome 7 in the human genome (13, 14). miR-590-5p, processed from the 5′-arm of mir-590, has been reported to be an important regulator of signaling pathways involved in cell proliferation, cell differentiation, tumorigenesis, and angiogenesis (15–18). Research showed that miR-590-5p modulated opioid-induced immunosuppression in monocytes by targeting cAMP-response element binding protein 1/5 (CREB1/CREB5) and NF-κB signaling pathway (19), suggesting it may play a role in immune regulation. MicroRNA PCR arrays revealed that miR-590-5p was up-regulated in the serum samples from dengue virus type 1 (DENV-1)–infected patients compared with healthy controls (20), but the underlying mechanism has not been elucidated. Whether and how miR-590-5p is involved in viral infection–triggered host immune responses remain unclear.

The soluble interleukin-6 receptor (sIL6R) and the membrane-bound IL6 receptor (mIL6R) are two types of receptors mediating IL6 activation (21). The sIL6R, generated by either limited proteolysis of the membrane-bound protein or translation from an alternatively spliced mRNA (22), is readily detected in the circulation and at sites of inflammation (23). In addition to its role in inflammation diseases, sIL6R has gained increasing attention for its antiviral effects. Lymphocytic choriomeningitis virus delivered intravenously or intragastrically to rhesus macaques induces high levels of sIL6R (24). The sIL6R induced by viral infection exhibits extensive antiviral activity against DNA and RNA viruses by elevating the expression of type I and type III IFNs (25, 26). Several published studies about microRNA and sIL6R have focused on the dysregulation of IL6 trans-signaling, which is mediated by the sIL6R–IL6 complex. Previous research demonstrated that miR-34a targeted sIL6R,
namely, IL6 trans-signaling, to promote epithelial to mesenchymal transition, invasion, and metastasis of colorectal cancer (27). However, a direct effect of microRNA on virus infection via sIL6R has never been explored.

Here, we identified miR-590-5p as a mediator of the host immune response during viral infection by directly targeting sIL6R. It was observed that viral infection elevated miR-590-5p expression, and increased miR-590-5p impaired the antiviral pathway, which in turn promoted virus replication. Conversely, inhibition of miR-590-5p repressed virus replication. Furthermore, miR-590-5p targeted IL6R and showed no ability to regulate virus-triggered signaling in IL6R knockout cells, but this was restored by the reconstitution of sIL6R. Further investigation demonstrated that miR-590-5p down-regulated endogenous sIL6R and mIL6R expression through a translational repression mechanism. The collective findings support the targeting of miR-590-5p as a promising therapeutic strategy for virus infection.

Results

Viral infection up-regulates miR-590-5p expression

Some microRNAs were reported as candidate regulators of host genes that are important for influenza virus replication because their levels were significantly altered after virus infection (28–32). We first detected the expression levels of nine microRNAs in human embryonic lung diploid fibroblast MRC-5 cells that were infected with influenza A virus (IAV) (multiplicity of infection (m.o.i.) = 1) for 4 h or left uninfected. miR-590-5p was found to be significantly up-regulated during IAV infection as determined by quantitative real-time PCR (qRT-PCR) (Fig. 1A). To test whether the induction of miR-590-5p is IAV-specific, the expression of miR-590-5p was investigated in several virus infection models. Human lung epithelial A549 cells were infected with herpes simplex virus 1 (HSV-1), sendai virus (SeV), vesicular stomatitis virus (VSV), IAV, human enterovirus 71 (EV71), and Zika virus (ZIKV) or transfected with poly(I:C). Significant increases of miR-590-5p were observed in the presence of those viruses or poly(I:C) (Fig. 1B). To further explore miR-590-5p expression in viral infection systems, IAV-infected A549 cells, EV71-infected human rhabdomyosarcoma RD cells, and VSV-infected A549 cells were utilized to perform time-course experiments. miR-590-5p increased sharply after viral infection and reached a maximum at 4 h (Fig. 1C). These data suggest that miR-590-5p is widely induced during viral infection.

miR-590-5p promotes virus replication

Considering that miR-590-5p expression is regulated during viral infection, we next investigated the role of miR-590-5p in virus replication using different viral infection systems, includ-
MicroRNA-590-5p inhibits host antiviral response

Results from qRT-PCR assays showed that the transfection of A549 or RD cells with different doses of miR-590-5p mimics for 12 h up-regulated the miR-590-5p level compared with that of mimic control, and the level of endogenous miR-590-5p declined after transfection of miR-590-5p inhibitor in A549 or RD cells for 12 h compared with that of miRNA inhibitor control (Fig. 2, A and C). In this study, 20 nM was used as the selective concentration of miR-590-5p in the following experiments. To study the cytotoxic effect, A549 and RD cells were transfected with mimic control or miR-590-5p mimics (0.2, 2, 20, and 40 nM), and the cell viability was determined by MTS assays. No significant effect on cell viability was observed with the dose increase of miR-590-5p mimics (Fig. 2, B and D). A549 cells were transfected with mimic control or

![Image](image_url)

**Fig. 2**

A549 and RD cells were transfected with different doses of miR-590-5p mimics or inhibitors. **A** and **C**: miR-590-5p level in A549 and RD cells were measured by qRT-PCR. **B** and **D**: Cell viability was determined by MTS assays. **E** and **F**: VSV and **G** and **H**: EV71 infection titers were measured using VSV-GFP and EV71-GFP infection assays. **I** and **J**: IAV infection titers were measured using rRNA, vRNA, and mRNA levels were measured by qRT-PCR. **K** and **L**: IAV infection titers were measured using rRNA, vRNA, and mRNA levels were measured by qRT-PCR.
miR-590-5p mimics for 12 h and infected with GFP-tagged VSV (m.o.i. = 5) for 24 h. Enhanced replication of VSV-GFP was observed in A549 cells transfected with miR-590-5p mimics compared with the control cells as measured by both fluorescence microscopy and flow cytometry analysis (Fig. 2E). Similar experiments were performed in RD cells infected with GFP-tagged EV71 (m.o.i. = 5), and miR-590-5p was also found to effectively enhance EV71-GFP replication (Fig. 2G). Subsequently, the supernatants of A549 cells transfected with mimic control or miR-590-5p mimics and infected with VSV (m.o.i. = 1) for 24 h were examined by plaque analysis. The results showed that miR-590-5p significantly potentiated VSV replication (Fig. 2F). Consistent with the results from EV71-GFP replication analysis, EV71 VP1 RNA levels in RD cells transfected with miR-590-5p mimics were considerably higher than that in the control cells (Fig. 2H). Next, the IAV nucleoprotein (NP) gene expression levels based on mRNA, cRNA, and vRNA in A549 cells were measured using qRT-PCR. The results showed that the expression of miR-590-5p mimics strongly promoted IAV replication compared with the mimic control (Fig. 2I). Consistently, the 50% tissue culture infective dose (TCID50) of IAV in harvested supernatants of A549 cells, calculated according to the Reed–Muench method, also showed that IAV titers in A549 cells transfected with miR-590-5p mimics were much higher than that in the control cells (Fig. 2J). Conversely, miR-590-5p inhibitor exhibited inhibitory effects on IAV replication by analysis of the three types of vRNA (Fig. 2K) or TCID50 of IAV in cell culture supernatants (Fig. 2L). These data suggest that miR-590-5p strongly potentiates virus replication.

miR-590-5p impairs virus-triggered signaling

Because miR-590-5p negatively regulates the cellular antiviral responses against multiple viruses, broadly implicating its role in host defense, we next examined whether miR-590-5p plays a role in virus-triggered innate immune signaling. In reporter assays, the luciferase activities of IL10 and IL22 promoters were not affected by the transfection of miR-590-5p mimics or miR-590-5p inhibitor. However, miR-590-5p inhibited the virus-induced activation of IFN-β, NF-κB, IFN-stimulated response element (ISRE), and IL6 promoters, and miR-590-5p inhibitor had the opposite effect (Fig. 3, A and B), indicating that miR-590-5p is involved in inactivating NF-κB, ISRE signaling, and the acute inflammatory response to viral infection. Next, IFN-β mRNA levels in A549 cells cotransfected with poly(I:C) and mimic control or miR-590-5p mimics were examined. The results showed that the induction of IFN-β was significantly dampened by miR-590-5p for the indicated time (Fig. 3C). Considering that miR-590-5p functions in different virus infection systems, the expression levels of IFN-β, IFN-α, IL-1β, IL6, and IL32 were measured in A549 cells infected with HSV-1, IAV, SeV, or VSV and those that remained uninfected. Similar inhibition was observed in the cells transfected with miR-590-5p mimics (Fig. 3, D–I). NF-κB and IRF3 are two transcription factors that are essential for virus-induced expression of downstream genes. The results from immunoblot analysis showed that SeV-induced phosphorylation of IκBα and IRF3 was substantially impaired in cells transfected with miR-590-5p mimics compared with the control cells (Fig. 3I). These results suggest that miR-590-5p negatively regulates the virus-induced immune signaling and subsequent induction of antiviral and inflammatory genes.

IL6R is a target of miR-590-5p

Signaling of IL6 via the sIL6R promotes proinflammatory activities (33). Recent studies have found that sIL6R expression up-regulates the levels of its own ligand IL6 as well as the proinflammatory cytokine IL32 (34), and the circulating sIL6R elicited extensive antiviral activity through the induction of type I and type III IFNs (25, 26). Furthermore, sIL6R was found to facilitate the nuclear translocation of IRF3 and NF-κB (25). Interestingly, miR-590-5p was predicted to target the IL6R 3′-UTR by analysis with programs for miRNA target prediction, including PicTar, TargetScan, and miRDB. In addition, recent studies have found that miR-21, which shares the seed sequence with miR-590-5p, targeted IL6R (35). To examine whether miR-590-5p affects the function of IL6R 3′-UTR, we performed luciferase reporter assays in which several reported miRNAs were used as a control. Intriguingly, the results showed that miR-590-5p did inhibit the IL6R 3′-UTR activity (Fig. 4A). To verify the predicted targeting site on the 3′-UTR, a mutant construct was generated from the IL6R 3′-UTR reporter plasmid, and miR-590-5p mutant mimics were synthesized (Fig. 4B). To test the dependence of the IL6R 3′-UTR activity on the targeting site, luciferase reporter assays were performed in A549 or 293T cells transfected with WT or mutant 3′-UTR along with miR-590-5p mimics, miR-590-5p mutant mimics, or miR-590-5p inhibitor. The results indicated that the activity of the IL6R 3′-UTR was effectively reduced by miR-590-5p but stimulated by its inhibitor, whereas the mutant 3′-UTR was not affected (Fig. 4, C and D). What’s more, miR-
Figure 3. miR-590-5p impairs virus-triggered signaling. A and B, luciferase assays of A549 cells cotransfected with IFN-β, NF-κB, ISRE, IL6, IL10, and IL22 luciferase reporter constructs and pRL-TK together with mimic control (miR-Ctrl) or miR-590-5p mimics (miR-590-5p) or with miRNA inhibitor control or miR-590-5p inhibitor (miR-590-5p Inhibitor) and then infected with SeV (m.o.i. = 1) for 12 h. pRL-TK was used as an internal control. Rel. Lucif. Act., relative luciferase activity. C, qRT-PCR analysis of IFN-β mRNA levels in A549 cells that were transfected with mimic control or miR-590-5p mimics for 4 h and then treated with poly(I:C) (2.5 μg) for the indicated time. D–H, qRT-PCR analysis of IFN-β, IFN-α, IL1β, IL6, and IL32 mRNA levels in A549 cells transfected with mimic control or miR-590-5p mimics and infected with HSV-1, IAV, SeV, or VSV or left uninfected (Mock). I, immunoblot analysis of total and p-IκBα, IRF3, or total β-actin in A549 cells transfected with mimic control or miR-590-5p mimics and infected with SeV for 0–10 h (left panel). The intensities (Rel. Intensit.) of p-IκBα and p-IRF3 were normalized to β-actin (right panel). All experiments were repeated at least three times with similar results. Error bars represent S.D. *, p < 0.05; **, p < 0.01; ***, p < 0.001; n.s., not significant (analysis of two-way ANOVA followed by Bonferroni post-test).
MicroRNA-590-5p inhibits host antiviral response

590-5p mutant mimics inhibited the luciferase activity controlled by the mutant IL6R 3′-UTR but not the WT IL6R 3′-UTR (Fig. 4E). These data indicate that miR-590-5p could suppress IL6R expression by sequence-specific binding to its 3′-UTR.

miR-590-5p regulates antiviral signaling through sIL6R

Because IL6R is regulated by miR-590-5p and sIL6R has been identified as a functional antiviral cytokine (25), we hypothesized that miR-590-5p modulated virus-triggered signaling mainly by targeting sIL6R. To test this hypothesis, luciferase reporter assays and qRT-PCR assays were performed in an IL6R knockout A549 cell line, which was produced using the CRISPR-Cas9 system as described previously (26). Results showed that the luciferase activities of IFN-β, IFN-α, NF-κB and ISRE promoters (Fig. 5A) and the mRNA expression levels of IFN-α, IFN-β, and IFN-α1 in IL6R−/− A549 cells were not affected by the transfection of miR-590-5p mimics (Fig. 5B). Because it has been reported that IAV-induced sIL6R is a truncated protein produced by a differential splicing variant of the IL6R mRNA (DS-sIL6R) and is not shed from the cell surface by proteolytic cleavage of the mIL6R (PC-sIL6R) (25, 34), sIL6R and mIL6R expression plasmids were transfected into IL6R−/− A549 cells, respectively, to restore their expression, which was confirmed by immunoblot analysis (Fig. 5C). Interestingly, reconstitution of sIL6R into IL6R−/− A549 cells restored the inhibition of IFN-β, IFN-α1, and IFN-β expression by miR-590-5p, whereas the reconstitution of the empty vector or mIL6R did not (Fig. 5, D–F). Consistent with these observations, the enhanced replication of IAV was restored in IL6R−/− A549 cells transfected with miR-590-5p mimics and reconstituted with sIL6R but not in those reconstituted with the empty vector or mIL6R (Fig. 5G). In addition, miR-590-5p inhibitor impaired IAV replication in IL6R−/− A549 cells reconstituted with sIL6R but not the empty vector or mIL6R (Fig. 5H). These data collectively suggest that sIL6R is indispensable for miR-590-5p in regulating virus-triggered signaling and promoting virus replication.

miR-590-5p down-regulates endogenous sIL6R and mIL6R expression

Because miR-590-5p targeted the IL6R 3′-UTR, we hypothesized that both endogenous sIL6R and mIL6R expression were
affected by miR-590-5p mimics and miR-590-5p inhibitor. To test this hypothesis, A549 cells were transfected with miR-590-5p mimics or miR-590-5p inhibitor followed by infection with IAV for 0–10 h, and the mRNA and protein levels of sIL6R and mIL6R were examined. miR-590-5p mimics were found to have no effect on sIL6R and mIL6R mRNA expression for the indicated time compared with mimic control (Fig. 6, A and B). However, ELISA analysis of sIL6R protein in the cell culture supernatants and immunoblot analysis of intracellular sIL6R and mIL6R together showed that the protein levels of sIL6R and mIL6R could be efficiently diminished by miR-590-5p mimics (Fig. 6, C and D). Similarly, the mRNA expressions of sIL6R and mIL6R were not affected by miR-590-5p inhibitor (Fig. 6, E and F), whereas the protein levels of sIL6R

Figure 5. miR-590-5p regulates antiviral signaling through sIL6R. A, luciferase assays of IL6R−/− A549 cells cotransfected with IFN-β, IFN-λ1, NF-κB, and ISRE luciferase reporter constructs and pRL-TK together with mimic control (miR-Ctrl) or miR-590-5p mimics and infected with SeV (m.o.i. = 1) for 12 h. Rel. Lucif. Act., relative luciferase activity. B, qRT-PCR analysis of IFN-α, IFN-β, and IFN-λ1 mRNA levels in IL6R−/− A549 cells transfected with mimic control or miR-590-5p mimics and then infected with SeV for 8 h. C, immunoblot analysis of mIL6R and sIL6R in IL6R−/− and IL6R+/+ A549 cells reconstituted with empty vector, sIL6R, mIL6R, or sIL6R and mIL6R together. The intensities of mIL6R and sIL6R were normalized to β-actin. D–F, qRT-PCR analysis of IFN-β (D), IFN-λ1 (E), and IL6 (F) mRNA levels in IL6R−/− A549 cells reconstituted with empty vector, sIL6R, mIL6R, or sIL6R and mIL6R together; transfected with mimic control or miR-590-5p mimics; and then infected with SeV for 8 h. G and H, IL6R−/− A549 cells reconstituted with empty vector, sIL6R, mIL6R, or sIL6R and mIL6R together were transfected with miR-590-5p mimics (G), miR-590-5p inhibitor (H), or their controls and then infected with IAV (m.o.i. = 1) for 12 h. Relative levels of NP-specific mRNA, cRNA, and vRNA were measured by qRT-PCR. All experiments were repeated at least three times with similar results. Error bars represent S.D. **, p < 0.01; ***, p < 0.001; n.s., not significant (analysis of two-way ANOVA followed by Bonferroni post-test).
and mIL6R could be up-regulated by miR-590-5p inhibitor (Fig. 6, G and H). Taken together, these data suggest that miR-590-5p inhibits sIL6R and mIL6R expression mainly through the translational repression mechanism.

Discussion

Previous studies of miR-590-5p have focused more on the occurrence and development of tumors (16, 18, 36, 37). Although the up-regulated expression of miR-590-5p has been observed in the sera of dengue-infected patients, suggesting its close association with dengue infection (20), no further research has been carried out. In this study, we investigated a novel function and the underlying mechanism of miR-590-5p in the immune response to viral infection. The results showed that miR-590-5p was significantly and quickly induced by various viruses, including IAV, SeV, VSV, HSV-1, EV71, and ZIKV, which inspired us to investigate whether miR-590-5p plays a role in virus replication. Fluorescence microscopy and flow cytometry analysis confirmed the enhanced VSV-GFP or EV71-GFP replication in cells transfected with miR-590-5p.
**MicroRNA-590-5p inhibits host antiviral response**

mimics. These results were underpinned by the plaque analysis of VSV and qRT-PCR analysis of EV71 VP1 mRNA. In addition, overexpressed miR-590-5p facilitated IAV replication, and inhibition of miR-590-5p dampened IAV replication. Taken together, these results confirmed the correlation of miR-590-5p with virus infection and revealed a positive role of virus-induced miR-590-5p in regulating the replication of various viruses.

To investigate the underlying mechanism of the newfound effect, we examined whether miR-590-5p affected host antiviral signaling. It has been demonstrated that miR-590 down-regulated proinflammatory cytokine secretion in oxidized low-density lipoprotein–treated human THP-1 macrophages (14). In our study, miR-590-5p exerted strong inhibition on the activation of IFNs and inflammatory cytokines after viral infection, which reaffirmed miR-590-5p as a mediator of immunosuppression. Interestingly, Long et al. (19) demonstrated that miR-590-5p targeted CREB1/CREB5 to promote NF-kB activity in morphine-stimulated monocytes, but we observed impaired NF-kB signaling in the presence of miR-590-5p, implying that miR-590-5p has complex and diverse functions in different cellular processes.

The sIL6R was originally identified as a receptor involved in IL6 trans-signaling and later characterized as an antiviral protein independent of IL6 (21, 25, 26). Here, we found that IL6R was a target of miR-590-5p. miR-590-5p binds to the 3’-UTR of IL6R and down-regulates sIL6R expression through a translational repression mechanism. Furthermore, we determined that sIL6R, but not mIL6R, is indispensable for miR-590-5p in modulating the host immune response during viral infection. It was reported that IAV-induced release of sIL6R in A549 cells is independent of mIL6R (25, 34). Few studies of microRNAs have distinguished between sIL6R and mIL6R because sIL6R has a full-length 3’-UTR in which the 3’-UTR of mIL6R is included. Utilizing an IL6R−/− A549 cell line, we observed that the role of miR-590-5p was abolished in antiviral signaling in the absence of IL6R. However, the reconstitution of sIL6R, but not mIL6R, into IL6R−/− A549 cells restored the regulatory function of miR-590-5p. These results together add to the growing evidence for the specific and independent antiviral role for sIL6R and expand our knowledge of the different biological functions between the two forms of IL6 receptors.

It is generally accepted that microRNAs targeting IL6R regulate both sIL6R and mIL6R expressions. For example, miL6R is part of a positive feedback loop involving miR-34a and STAT3 (38), and the subsequent investigation showed that the sIL6R is a direct target of miR-34a (27). In addition to the regulation of antiviral signaling, it is conceivable that miR-590-5p may affect IL6 trans-signaling, which is mediated via sIL6R, and classical IL6 signaling involving mIL6R. Previous studies have shown that microRNAs targeting sIL6R or IL6 trans-signaling play a role in tumorigenesis, tumor proliferation, immunoregulation, and diabetic nephropathy (39–42), suggesting that miR-590-5p is likely to function in those processes. This presumption may lead to interesting future work to reveal precisely how miR-590-5p functions in different ways to regulate the immune responses and tumorigenesis.
MicroRNA-590-5p inhibits host antiviral response

A549 cells were grown in 12-well plates, transfected with the indicated microRNA mimics for 24 h, and infected with VSV at an m.o.i. of 1. After 1 h, the cells were washed with warm PBS, and fresh medium was added. After 24 h, the supernatants were harvested; diluted to $10^{-6}$, $10^{-5}$, $10^{-4}$, $10^{-3}$, and $10^{-2}$; and used to infect confluent Vero cells cultured on 24-well plates. At 1 h postinfection, the supernatants were removed, and a mixture of warm 3% low-melting-point agarose and fresh medium was overlaid. At 72 h postinfection, the cells were stained with 0.2% crystal violet for 2 h, and the overlay was removed. The number of plaques was counted, averaged, and multiplied by the dilution factor to determine the viral titer (plaque-forming units/ml).

Flow cytometry

Cells seeded into 24-well plates (2 x 10⁵ cells/well) were infected with VSV-eGFP or EV71-GFP. Two hours later, the supernatants were removed, and the cells were washed with prewarmed PBS (1 ml) twice followed by culture in full medium for 24 h. Viral replication was analyzed by flow cytometry.

Quantitative real-time PCR analysis

The total RNA was isolated using TRIzol reagent (Invitrogen). For quantitative mature miRNA detection, total RNA (2 μg) was reverse transcribed with Bulge-Loop miRNA-specific reverse transcription primers (GenePharma, Shanghai, China). The miRNA-specific qRT-PCR primers were also purchased from GenePharma. U6 was used as the normalization control and was reverse transcribed with the following primer: 5'-GTGGATATCCAGTGGGTGTCG-3' (antisense); for IL6, 5'-AGCAGCCACTCTCCATTCCAG-3' (sense) and 5'-TTCTTGTGCGAACGAGAC-3' (antisense); for IFN-α, 5'-TTCTTGCAGCCATTCCAC-3' (antisense); and 5'-GGTCATCTGGCTCGAACCAC-3' (antisense). The cellular RNA samples were reverse transcribed with oligo(dT) and random primers, and quantitative PCR assays were performed using a Bio-Rad CFX Connect system by a fast two-step amplification program with iTaq™ Universal SYBR® Green Supermix (Bio-Rad). GAPDH was used as an endogenous control to normalize the amount of total mRNA in each sample. The following primer pairs were used: for GAPDH, 5'-AAGGCTGTTGGGCAAGG-3' (sense) and 5'-TGGAGGTGTTGTCG-3' (antisense); for IL6, 5'-AGCAGCCACTCTCCATTCCAG-3' (sense) and 5'-TTCTTGTGCGAACGAGAC-3' (antisense); for IFN-α, 5'-TTCTTGCAGCCATTCCAC-3' (antisense); and 5'-GGTCATCTGGCTCGAACCAC-3' (antisense).
MicroRNA-590-5p inhibits host antiviral response

GTGTTAGAGATT-3’ (sense) and 5’-CACCAGTTGT-TAATGGAG-3’ (antisense); for siL6R, 5’-CGACAAGGCTC-CAGGTGTTCA-3’ (sense) and 5’-GTCGCCACCCGAGCCGC-TATC-3’ (antisense); for miL6R, 5’-CTCTCTCATTGAGC-ATTGT-3’ (sense) and 5’-TGGTGCTGAGGATTGTCA-3’ (antisense); and for U6, 5’-GCTCGGCAAGACATA-TCAAAT-3’ (sense) and 5’-CGCTCCAGGAATTTGC-GTGCT-3’ (antisense).

Immunoblot analysis

Cells were lysed in Nonidet P-40 lysis buffer containing 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, and 1% protease and phosphatase inhibitor mixture (Biotool). The protein concentration was determined by the Bradford assay (Bio-Rad). Cell lysates were subjected to SDS-PAGE, and immunoblot analysis was performed with the appropriate antibodies.

Statistical analysis

Differences between the experimental and control groups were tested using Student’s t test or two-way ANOVA with Bonferroni’s post-test. p values <0.05 were considered statistically significant.

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