Down-regulation of miR-155 inhibits inflammatory response in human pulmonary microvascular endothelial cells infected with influenza A virus by targeting sphingosine-1-phosphate receptor 1

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Abstract

Background: Endothelial cells play a key role in the cytokine storm caused by influenza A virus. MicroRNA-155 (miR-155) is an important regulator in inflammation. Its role in the inflammatory response to influenza A infection, however, has yet to be elucidated. In this study, we explored the role as well as the underlying mechanism of miR-155 in the cytokine production in influenza A-infected endothelial cells.

Methods: Human pulmonary microvascular endothelial cells (HPMECs) were infected with the influenza A virus strain H1N1. The efficiency of H1N1 infection was confirmed by immuno-fluorescence. The expression levels of proinflammatory cytokines and miR-155 were determined using real-time polymerase chain reaction. A dual-luciferase reporter assay characterized the interaction between miR-155 and sphingosine-1-phosphate receptor 1 (S1PR1). Changes in the target protein levels were determined using Western blot analysis.

Results: MiR-155 was elevated in response to the H1N1 infection in HPMECs (24 h post-infection vs. 0 h post-infection, 3.875 ± 0.062 vs. 1.043 ± 0.013, P = 0.001). Over-expression of miR-155 enhanced inflammatory cytokine production (miR-155 mimic vs. negative control, all P < 0.05 in regard of cytokine levels) and activation of nuclear factor kappa B in infected HPMECs (miR-155 mimic vs. negative control, P = 0.004), and down-regulation of miR-155 had the opposite effect. In addition, S1PR1 was a direct target of miR-155 in the HPMECs. Inhibition of miR-155 enhanced the expression of the S1PR1 protein. Down-regulation of S1PR1 decreased the inhibitory effect of the miR-155 blockade on H1N1-induced cytokine production and nuclear factor kappa B activation in HPMECs.

Conclusion: MiR-155 maybe modulate influenza A-induced inflammatory response by targeting S1PR1.

Keywords: MicroRNA-155; Sphingosine 1-phosphate receptor 1; Influenza A virus; Endothelial cells

Introduction

Influenza A virus is a respiratory pathogen that infects both humans and animals. It is a major threat to human health because it is highly contagious and variable in nature.1,2 A mild influenza A viral infection causes self-limited upper respiratory tract disease, and in severe cases, the disease can progress to acute respiratory distress syndrome (ARDS), which can be fatal.3-5 Although the molecular mechanism is not fully known, accumulating evidence suggests that aberrant proinflammatory cytokine production, also known as a cytokine storm, is a key contributor to the development of severe disease caused by influenza A.6,7 Conversely, modulation of the influenza A virus-mediated inflammatory response may represent a valid therapeutic strategy.

Sphingosine-1-phosphate receptor 1 (S1PR1) is a G protein-coupled receptor of sphingosine-1-phosphate implicated in the regulation of several physiologic processes, including cell trafficking and the immune response. S1PR1 is abundantly expressed on vascular endothelial cells.8 In our previous study, it was demonstrated that S1PR1 was a negative regulator of cytokine production and nuclear factor kappa B (NF-κB) activation induced by influenza A virus in human pulmonary microvascular endothelial cells (HPMECs).9 However, the specific mechanism of this negative regulation remains unclear. Recent studies have highlighted the importance of microRNAs (miRNAs) in
the pathogenesis of infectious diseases.\(^\text{[10]}\) miRNA alterations have been reported after various viral infections, including hepatitis B, enterovirus 71, coxsackievirus 16, and influenza virus.\(^\text{[11-13]}\) Whether miRNAs provided upstream regulation of S1PR1 in the modulation of influenza A-induced inflammatory response is intriguing, but unknown.

MiRNAs are small non-coding RNAs composed of 19 to 22 nucleotides. They play crucial roles in various biologic and pathologic processes as post-transcriptional regulators of gene expression.\(^\text{[14]}\) MiR-155 expression has been shown to be elevated following influenza A viral infection using both in vivo and in vitro microarrays.\(^\text{[15,16]}\) In addition, miR-155 has been shown to be remarkably upregulated during vesicular stomatitis viral infection, and it promoted type I interferon signaling, which is potentially pathogenic in influenza infection according to a former study.\(^\text{[11,18]}\) In autoimmune diseases, miR-155 has been well characterized as a promotor of inflammation.\(^\text{[12,19]}\) To date, however, little is known about the functional role of miR-155 in the pathogenesis of influenza A. By utilizing the prediction produced by bioinformatics tools, miR-155 was also identified as a potential upstream regulator of S1PR1. Therefore, miR-155 is focused on in this study, and this study aims to determine the role of miR-155 in influenza A-infected endothelial cells.

**Methods**

**Ethical approval**

This experiment proposal was reviewed and approved by the Ethics Committee of Jinling Hospital, Nanjing University School of Medicine (approval number: JLYY: 2013021).

**Cells and reagents**

Primary HPMECs were obtained from ScienCell (San Diego, CA, USA) and cultured in endothelial cell medium using the recommended supplements from the supplier, and they were used in passages 3 to 5. The Madin-Darby canine kidney cell line was purchased from the American Type Culture Collection (ATCC, VA, USA). The embryonic kidneys were cultured in Dulbecco Modified Eagle’s medium (Thermo Fisher Scientific, Waltham, MA, USA) containing 10% fetal bovine serum and 1% penicillin-streptomycin in a humidified incubator at 37°C using both 5% CO₂.

Anti-S1PR1 antibody and fluorescein isothiocyanate-conjugated anti-influenza A virus nucleoprotein (NP) antibody were obtained from Abcam (Cambridge, UK). Antibodies against the p65 subunit of NF-kB and phospho-p65 were obtained from Cell Signaling Technology (CST, Danvers, MA, USA). The anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody was obtained from Bioworld (St. Louis Park, MN, USA).

**Virus and viral infection**

The previously described influenza virus strain A/Nanjing/108/2009 (hereafter referred to as H1N1) was employed in this study.\(^\text{[20]}\) The viruses were propagated and tittered as described previously.\(^\text{[9]}\) HPEMCs were seeded onto 6-cm Petri dishes at 5 × 10⁴ cells per plate 12 h before infection. The cells were then infected with H1N1 at a multiplicity of infection (MOI) of 1. After 1 h of absorption, the viral inoculum was removed and added to each culture plate using 3 mL of endothelial cell medium with 0.3% bovine serum albumin and 1 μg/mL of N-tosyl-l-phenylalanine chloromethyl ketone-treated trypsin (Sigma-Aldrich, St. Louis, MO, USA). The infected HPMECs cells were cultured at 37°C with 5% CO₂ for different periods.

**Real-time polymerase chain reaction**

The total RNA from the cultured HPMECs was isolated using the Trizol reagent (Thermo Fisher Scientific). A total of 2 μg of total RNA was reverse transcribed using the Prime script RT reagent kit (TaKaRa, Shiga, Japan). The cDNA products were subjected to real-time polymerase chain reaction (PCR) assay using the SYBR premix Ex Taq II kit (TaKaRa) following the manufacturer’s instructions. Real-time PCR analysis for tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, IL-8, CC motif chemokine ligand (CCL) 2, CCL5, interferon (IFN)-β, and the housekeeping gene, GAPDH, was performed using the SYBR Green Master Mix (Thermo Fisher) and analyzed using Viia™7 software (Applied Biosystems, Foster City, CA, USA).

To detect the miR-155 expression, 1 μg of total RNA was reverse transcribed using the Bulge-Loop™ miRNA qRT-PCR Starter kit (RiboBio, Guangzhou, China) followed by real-time analysis for miR-155 and U6 with the same kit according to the supplier’s instructions. The fold changes in expression of each gene were calculated using the $2^{-ΔΔCT}$ method with GAPDH or U6 used as an internal control. The sequences of the primers used in the real-time PCR are listed in Supplementary Table 1, http://links.lww.com/CM9/A283.

**Western blot analysis**

The total cell protein was extracted according to previously described procedures.\(^\text{[9]}\) Protein concentrations were determined using a bicinchoninic acid assay commercial kit (Thermo Fisher). The samples were denatured at 95°C for 5 min before being subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). The membranes were blocked using 5% skim milk for 2 h at room temperature and then incubated with respective primary antibodies overnight at 4°C. After washing, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. The bands were visualized using a Tanon Imaging System (Tanon, Shanghai, China). Densitometry analysis was performed using Image J software (http://imagej.nih.gov/ij/).

**Immunofluorescence staining and microscopy**

The H1N1-infected HPMECs following infection were grown on coverslips for 12 h and then washed and fixed.
Tukey multiple comparison post-test. The reported data
did not show remarkable differences between multiple groups were
previously estimated using analysis of variance, with a
statistically significant difference (P < 0.05) between two groups (IBM SPSS, Armonk, NY, USA).
Significant differences between multiple groups were estimated using analysis of variance, with a post hoc Tukey multiple comparison post-test. The reported data are the mean of three independent experiments ± standard error. A P value of less than 0.05 was considered statistically significant.

Results

MIR-155 expression in the HPMECs induced by H1N1 infection

Previously, it was identified that H1N1 could infect HPMECs effectively, and this was found by testing the influenza matrix (M) gene. In this study, this was further confirmed using immunofluorescence. The HPMECs were exposed to H1N1 or the vehicle for 1 h and then cultured for different periods. As shown in Figure 1A, the intracellular expression of the influenza virus NP was detected at 8 h post-infection (p.i.). Importantly, in virus-infected HPMECs, the alteration of MIR-155 expression level was remarkable (P = 0.001) [Figure 1B]. Compared with 0 h p.i., the fold change of miR-155 was significantly increased at 16 h p.i. (16 h p.i. vs. 0 h p.i., 1.923 ± 0.034 vs. 1.043 ± 0.013, P = 0.0002) and 24 h p.i. (24 h p.i. vs. 0 h p.i., 3.875 ± 0.062 vs. 1.043 ± 0.013, P = 0.001) in infected cells.

Proinflammatory cytokine production is regulated by MIR-155

To investigate the role of miR-155 in the inflammatory response induced by influenza A virus infection, HPMECs were transfected with miR-155 mimic or miR-155 inhibitor and corresponding NCs. The high up-regulation of MIR-155 expression following miRNA transfection (MIR-155 mimic vs. NC, 185.8 ± 9.676 folds, t = 19.09, P = 0.003), as well as down-regulation by miR-155 inhibitor (miR-155 inhibitor vs. NC, 0.35 ± 0.011 folds, t = 56.29, P = 0.0003), compared with control group, was confirmed by real-time PCR. Transfected cells were exposed to H1N1 at an MOI of 1. In the H1N1-infected HPMECs, over-expression of miR-155 significantly increased the mRNA expression of multiple cytokines, including IL-1β, IL-6, IL-8, CCL2, CCL5, TNF-α, and IFN-β (miR-155 mimic vs. NC, P < 0.001 in IL-1β; P = 0.03 in IL-6; P = 0.029 in IL-8; P = 0.025 in CCL2; P = 0.014 in CCL5; P = 0.032 in TNF-α; P = 0.027 in IFN-β). In contrast, in the virus infected cells, inhibition of miR-155 decreased the levels of proinflammatory cytokines (miR-155 inhibitor vs. NC, P < 0.005 in IL-1β; P = 0.03 in IL-6; P = 0.049 in IL-8; P = 0.012 in CCL2; P = 0.007 in CCL5; P = 0.007 in TNF-α; P = 0.038 in IFN-β) [Figure 2]. Above findings suggest a pro-inflammatory role of miR-155 in H1N1-infected HPMECs.

MIR-155 regulates cytokine expression by targeting S1PR1

MiR-155 was discovered to be broadly conserved by computational prediction using TargetScan and targeted the 3'-UTR of S1PR1. Our previous study discovered that S1PR1 negatively regulated inflammatory responses in vitro and in vivo.9,21 Further, we synthesized the 3'-UTR of S1PR1 containing the putative miR-155 binding sites without (WT) or with (mutant [MT]) mutations, then inserted them into luciferase reporter vectors. After co-transfecting pmir-S1PR1-WT or pmir-S1PR1-MT along with miR-155 mimic or NC. As shown in Figure 3A, miR-155 significantly decreased the luciferase activity of the pmir-S1PR1-WT (NC vs. Mimic, P = 0.003), but did not affect the luciferase activity of the pmir-S1PR1-MT in HPMECs, suggesting S1PR1 is a downstream target of the miRNA. Consistent with this, the S1PR1 expression was decreased by miR-155 over-expression and was enhanced by miR-155 inhibition. Compared with the control group, remarkable changes in the protein levels of S1PR1 were observed in both miR-155 mimic and miR-155 inhibitor-treated groups (NC vs. miR-155 mimic, P = 0.001; NC vs.
miR-155 inhibitor, \( P = 0.002 \) [Figure 3B]. Additionally, the S1PR1 protein levels were notably up-regulated in miR-155-deficient cells following H1N1 infection [control (Ctl) inhibitor vs. miR-155 inhibitor, \( P = 0.003 \)] [Figure 3C]. Finally, the reduction of S1PR1 significantly decreased the inhibitory effect of the miR-155 blockade on H1N1-mediated inflammation [Ctl inhibitor + Si-S1PR1 vs. miR-155 inhibitor + Si-S1PR1, \( P = 0.376 \) in IL-1\( \beta \); \( P = 0.162 \) in IL-6; \( P = 0.044 \) in IL-8; \( P = 0.051 \) in CCL2; \( P = 0.088 \) in CCL5; \( P = 0.093 \) in TNF-\( \alpha \); \( P = 0.223 \) in IFN-\( \beta \)] [Figure 3D].

**Down-regulation of miR-155 inhibits H1N1-driven NF-\( \kappa \)B activation**

Previous studies have shown that NF-\( \kappa \)B activation is involved in the proinflammatory response to influenza.\(^{[9,22]}\) It has been found that the over-expression/activation of S1PR1 inhibited NF-\( \kappa \)B signaling in H1N1-infected HPMEC cells. As shown in Figure 4A, over-expression of miR-155 in H1N1-challenged HPMECs increased the phospho-p65 expression (miR-155 mimic vs. NC, \( P = 0.004 \)) as a marker of NF-\( \kappa \)B activation, while treatment with miR-155 inhibitor led to a decrease in phospho-p65 (miR-155 inhibitor vs. NC, \( P = 0.008 \)). To gain further insight, S1PR1 transcript and protein knockdown in the target-specific siRNA-treated HPMECs was confirmed. The results showed that both mRNA (\( P = 0.005 \)) and protein (\( P = 0.004 \)) levels of S1PR1 declined dramatically in S1PR1 siRNA-treated group relative to corresponding control group [Figure 4B]. Next, phospho-p65 expression was determined in HPMECs with or without viral infection following co-transfecting miR-155 inhibitor or NC together with S1PR1 siRNA or NC. As shown in Figure 4C, in the presence of S1PR1 siRNA, there was no significant difference between miR-155 down-regulated group and control group in regard of phospho-p65 expression level (Si-S1PR1 + NC vs. Si-S1PR1 + miR-155 inhibitor, \( P = 0.992 \)). On the contrary, phospho-p65 protein level was attenuated in the miR-155 inhibitor-only treated virus-infected HPMECs compared with cells treated concurrently with an miR-155 inhibitor and S1PR1 siRNA (Si-S1PR1 + miR-155 inhibitor vs. SiRNA Ctl + miR-155 inhibitor, \( P = 0.001 \)). Together, these findings suggested that the function of miR-155 in H1N1-infected HPMECs was mediated by S1PR1.

**Discussion**

ARDS is a fatal complication of influenza infection. Currently, treatment for influenza-induced ARDS is non-specific supportive management to relieve the symptoms. However, effective therapy is still lacking, and this leads to a high mortality rate (over 40%).\(^{[5]}\) Given the morbidity and mortality of the last influenza outbreak and the potential for novel strain influenza to cause pandemic spread, the development of new therapeutics to ameliorate influenza-induced ARDS is urgently needed. In this study, it was demonstrated that the influenza A virus promoted the increased expression of miR-155 in HPMECs. An increase of miR-155 led to the suppression of endothelial S1PR1 and up-regulation of proinflammatory cytokines and chemokines. The data further suggested that miR-155 might promote cytokine production by targeting S1PR1. We had previously reported that S1PR1 was involved in cytokine production and NF-\( \kappa \)B activation in influenza-infected HPMECs.\(^{[9,22]}\) Here, we further illustrated the role of miR-155/S1PR1 axis on endothelial cells in influenza viral infection. These findings allow us to better understand the pathogenesis of ARDS caused by influenza and thus provide a target for early intervention to prevent the cytokine storm.

MiR-155 is a multifunctional microRNA transcribed by a non-coding gene B-cell integration cluster. The proinflammatory effects of miR-155 have been elucidated in asthma, idiopathic pulmonary fibrosis, and cigarette-induced inflammation, suggesting a vital role of miR-
However, few studies have examined the role of miR-155 in viral infection. Microarray analysis revealed that miR-155 was induced in influenza-infected mice. In addition, a higher expression of miR-155 was associated with the severity of infection. Knocking out miR-155 knockout mice have been found to recover from influenza infection faster than wild-type mice. Similar to this, another study found that knocking out of miR-155 could protect mice from influenza and Staphylococcus aureus superinfection. These findings suggest a crucial role of miR-155 in the pathogenesis of influenza infection, which was confirmed in this study. In agreement with earlier work, it was found that miR-155 expression was induced by influenza infection in HPMECs, and the down-regulation of miR-155 could suppress proinflammatory cytokine production. This indicates that miR-155 can positively regulate inflammation induced by influenza infection in pulmonary endothelium. Conversely, miR-155 expression in the liver, serum, and peripheral blood mononuclear cells of patients with chronic hepatitis B virus infection was decreased compared with healthy people. These findings suggest a nuanced miR-155 response in viral infection. Further investigations are warranted to fully understand the functional role of miR-155 in the pathogenesis of influenza-induced ARDS in vivo.

SIPR1 was further identified in this study as a direct functional target of miR-155 in HPMECs. SIPR1, also known as endothelial differentiation gene 1, is one of the five receptor subtypes of sphingosine-1-phosphate. SIPR1 is widely expressed on vascular endothelial cells and plays an important role in the vascular barrier and the immune system. Lately, SIPR1 has received great attention because of its regulatory function on endothelial activation. Increasing evidence has indicated that endothelial cell activation is the key to the pathogenesis of influenza-induced ARDS. In severe cases of influenza infection, activated endothelial cells secret many cytokines and chemokines, causing leukocyte recruitment and the destruction of the alveolar epithelial-endothelial barrier.
These inflammatory cascade reactions eventually lead to ARDS. Hence, finding a suitable target to intervene to avoid excessive activation of endothelial cells is essential. The results presented in this study revealed new molecular targets that intersect with influenza-mediated lung inflammation, and this might shed new light on targeted therapeutic strategies for influenza-induced ARDS.

The present study had several limitations. The experimental model was based on monocultures of pulmonary endothelial cells. Such a system cannot account for the complex cellular microenvironment of the lung. Furthermore, it is not clear if endothelial cells are the only target and effectors of H1N1-driven inflammation. Finally, cell culture data may not mirror in vivo findings. However, these new data provide support for further research.

In conclusion, this study illustrated that miR-155 might serve as a positive proinflammatory regulator in the inflammatory response induced by H1N1 infection in HPMECs. Inhibition of miR-155 significantly attenuated proinflammatory cytokine and chemokine production by...
promoting the expression of S1PR1. These results might provide a candidate therapeutic target for the clinical treatment of severe influenza A viral infection.

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Conflicts of interest

None.

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