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 immunofluorescence. 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

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the pathogenesis of infectious diseases.[10] miRNA alterations have been reported after various viral infections, including hepatitis B, enterovirus 71, coxsackievirus 16, and influenza virus.[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.[14] MiR-155 expression has been shown to be elevated following influenza A viral infection using both *in vivo* and *in vitro* microarrays.[15,16] In addition, miR-155 has been shown to be remarkedly upregulated during vesicular stomatitis viral infection, and it promoted type I interferon signaling,[17] which is potentially pathogenic in influenza infection according to a former study.[18] In autoimmune diseases, miR-155 has been well characterized as a promotor of inflammation.[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 Madin-Darby canine 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 with 5% CO_2.

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-κB 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.[20] The viruses were propagated and titrated as described previously.[9] HPEMC cells were seeded onto 6-cm Petri dishes at 5×10^6 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 HPMEC cells were cultured at 37°C with 5% CO_2 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 Primescript 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 $2^{-\Delta\Delta CT}$ 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.[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

with 4% paraformaldehyde for 30 min, permeabilized with 0.1% Triton X-100 for 10 min, and washed with phosphate-buffered saline three times. Cell non-specific antigens were blocked using 5% bovine serum albumin (Sigma-Aldrich) in phosphate-buffered saline for 30 min at 37°C. Coverslips were then incubated with mouse anti-NP monoclonal antibodies at a dilution of 1:1000 at 4°C overnight. After washing, the coverslips were incubated with fluorescein isothiocyanate-conjugated anti-mouse antibody (Bioworld) and stained with 1 μg/mL of 4′,6-diamidino-2-phenylindole (Beyotime, Jiangsu, China) for 5 min. The coverslips were observed using an Olympus confocal fluorescence microscope (Olympus, Tokyo, Japan).

Transfection

MiR-155 mimic, miR-155 inhibitor, the corresponding negative controls (NCs), S1PR1-specific small interfering RNA (siRNA), and nonspecific siRNA control were synthesized by RiboBio. The HPMECs were plated in six-well plates (3×10^5 cells/well) for 24 h before transfection using the Hiperfect transfection reagent (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. The cells were exposed to H1N1 virus 48 h after transfection.

Luciferase assays

The sequence in the 3′-untranslated region (UTR) of the human *S1PR1* gene targeted by miR-155 was predicted using TargetScan (www.targetscan.org). The 3′-UTR region and mutant 3′-UTR (AGCATTA to TCGTAAT) of S1PR1 were cloned into the pmiR-RB-REPORT™ (RiboBio) vector to generate the luciferase reporter gene plasmid, pmiR-S1PR1-WT, and pmiR-S1PR1-MT. The HPMECs were seeded into 24-well plates and co-transfected with 500 ng of the wild-type (WT) or mutated plasmid together with 30 nmol/L of the miR-155 mimic or NC. A total of 48 h after transfection, the luciferase activity was determined using the Dual-luciferase Reporter Assay System following the manufacturer's instructions (Promega, Madison, WI, USA).

Statistical analysis

All cellular experiments were repeated at least three times. Student *t* test was performed to analyze the difference 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.[20] 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 followed mimic 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*.

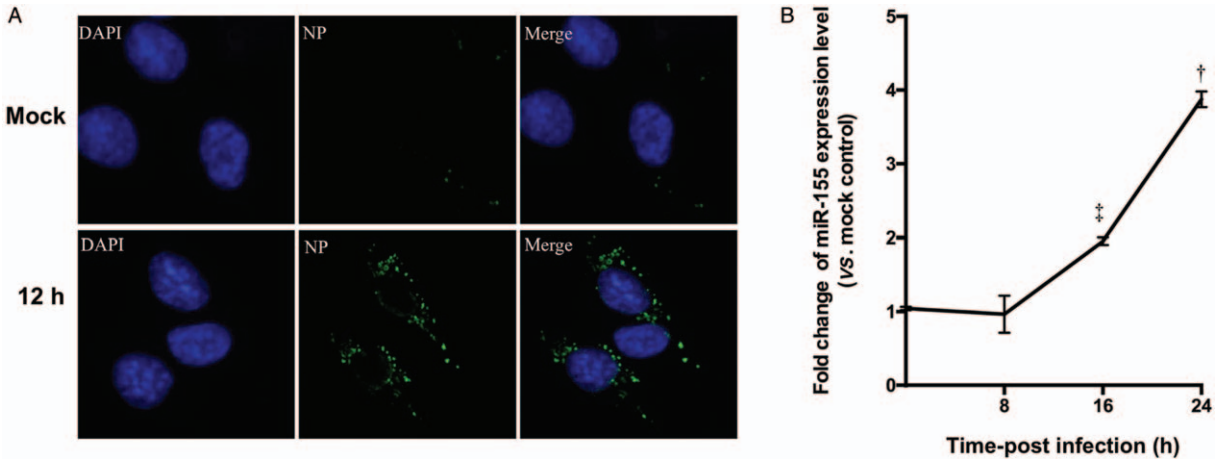

Figure 1: H1N1 infection increases the expression of miR-155 in HPMECs. (A) Immunofluorescence staining for influenza A virus NP in H1N1-infected HPMECs. HPMECs grown on coverslips were fixed and stained for NP (green) or DAPI (blue) at 12 h p.i. Original magnification, ×1000. (B) The levels of miR-155 in virus-infected or vehicle-treated HPMECs were measured using real-time polymerase chain reaction at the indicated time points. The experiments were repeated at least three times, and the data are presented as mean ± standard error. All data were analyzed by analysis of variance with a *post hoc* Tukey multiple comparison post-test. MiR-155 expression levels of the infected groups were normalized by the mock control groups. $^{\dagger}P < 0.01$; $^{\ddagger}P < 0.001$, compared with the miR-155 expression level at 0 h p.i. DAPI: 4′,6-diamidino-2-phenylindole; HPMECs: Human pulmonary microvascular endothelial cells; NP: Influenza nucleoprotein; p.i.: Post-infection.

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-1β; $P = 0.162$ in IL-6; $P = 0.044$ in IL-8; $P = 0.051$ in CCL2; $P = 0.088$ in CCL5; $P = 0.093$ in TNF-α; $P = 0.225$ in IFN-β) [Figure 3D].

Down-regulation of miR-155 inhibits H1N1-driven NF-κB activation

Previous studies have shown that NF-κB activation is involved in the proinflammatory response to influenza.[9,22] It has been found that the over-expression/activation of S1PR1 inhibited NF-κB 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-κB activation, while treatment with miR-155 inhibitor led to a decrease in pohsopho-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-κB 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-

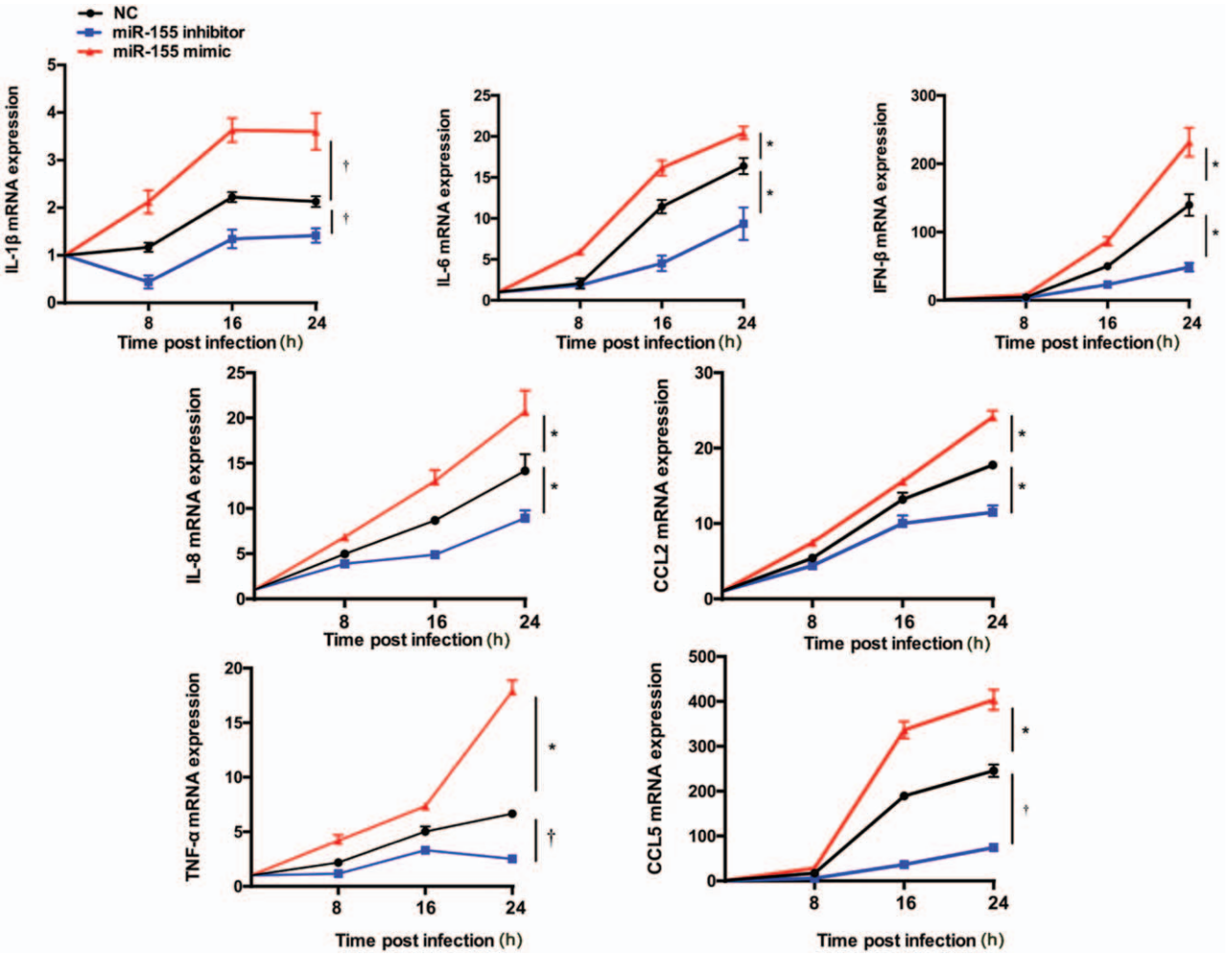

Figure 2: MiR-155 regulates cytokine production in H1N1-infected HPMECs. Cultured HPMECs were transfected with miR-155 mimic, inhibitor, or NC. After 48 h of transfection, the cells were infected with H1N1 at an MOI of 1 for 1 h. Total RNA was isolated at indicated time points and cDNA synthesized for analysis by real-time polymerase chain reaction. The expression levels of the proinflammatory cytokines were detected using real-time polymerase chain reaction. The experiments were repeated three times, and the data are presented as mean ± standard error. Data were analyzed by analysis of variance ($^{*}P < 0.05$; $^{\dagger}P < 0.01$; compared with the cytokine levels in virus-treated NC group cells). CCL: CC motif chemokine ligand; HPMECs: Human pulmonary microvascular endothelial cells; IFN-β: Interferon-β; IL: Interleukin; MOI: Multiplicity of infection; NC: Negative control; p.i.: Post-infection; TNF-α: Tumor necrosis factor-α.

155 in lung inflammation.[23-25] 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.[15] miR-155 knockout mice have been found to recover from influenza infection faster than wide type mice.[26] Similar to this, another study found that knocking out of miR-155 could protect mice from influenza and *Staphylococcus aureus* superinfection.[24] 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.[27,28] 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-induce ARDS *in vivo*.

SIPR1 was further identified in this study as a direct functional target of miR-155 in HPMECs. S1PR1, also known as endothelial differentiation gene 1, is one of the five receptor subtypes of sphingosine-1-phosphate. S1PR1 is widely expressed on vascular endothelial cells and plays an important role in the vascular barrier and the immune system.[29,30] Lately, S1PR1 has received great attention because of its regulatory function on endothelial activation.[31,32] Increasing evidence has indicated that endothelial cell activation is the key to the pathogenesis of influenza-induced ARDS.[32] 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.

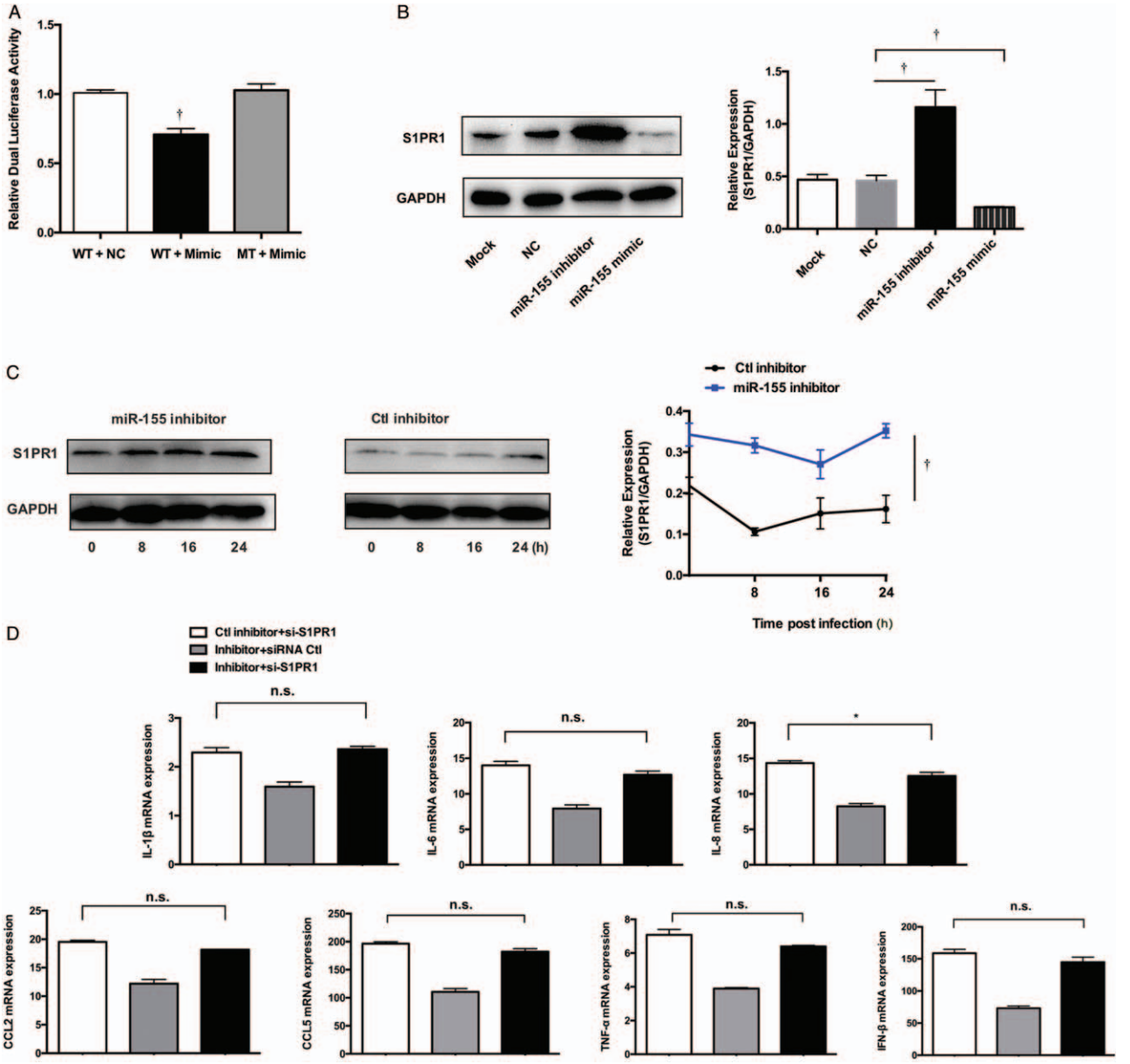

Figure 3: MiR-155 regulates the expression of S1PR1 in H1N1-infected HPMECs. (A) A luciferase reporter assay of the HPMECs transfected with WT or a mutant 3′-untranslated region vector together with miR-155 mimic or negative control. (B) HPMECs were transfected with the indicated miRNAs, and the S1PR1 protein was measured 48 h later ($^{\dagger}P < 0.01$, compared with the protein level of S1PR1 in NC group HPMECs). (C) The S1PR1 protein was detected in the HPMECS transfected with the miR-155 inhibitor or control inhibitor following H1N1 infection during different periods (Ctl inhibitor *vs.* miR-155 inhibitor, $P = 0.003$). (D) The HPMECs were co-transfected with miR-155 inhibitor and S1PR1-specific siRNA or negative control. After 48 h, the cells were infected with H1N1 at an MOI of one for 1 h, and then incubated for 24 h. The total RNA was prepared, and the mRNA levels of the selected cytokines were analyzed by real-time reverse transcription polymerase chain reaction. The experiments were repeated three times. All data were analyzed by analysis of variance with a *post hoc* Tukey multiple comparison post-test and were presented as mean ± standard error ($^{\dagger}P < 0.01$). CCL: CC motif chemokine ligand; Ctl: Control; HPMECs: Human pulmonary microvascular endothelial cells; IFN-β: Interferon-β; IL: Interleukin; MOI: Multiplicity of infection; MT: Mutant type; n.s.: Not statistically significant; NC: Negative control; S1PR1: Sphingosine1-phosphate receptor 1; siRNA: Small interfering RNA; TNF-α: Tumor necrosis factor-α; WT: Wild type.

These inflammatory cascade reactions eventually lead to ARDS.[7] 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

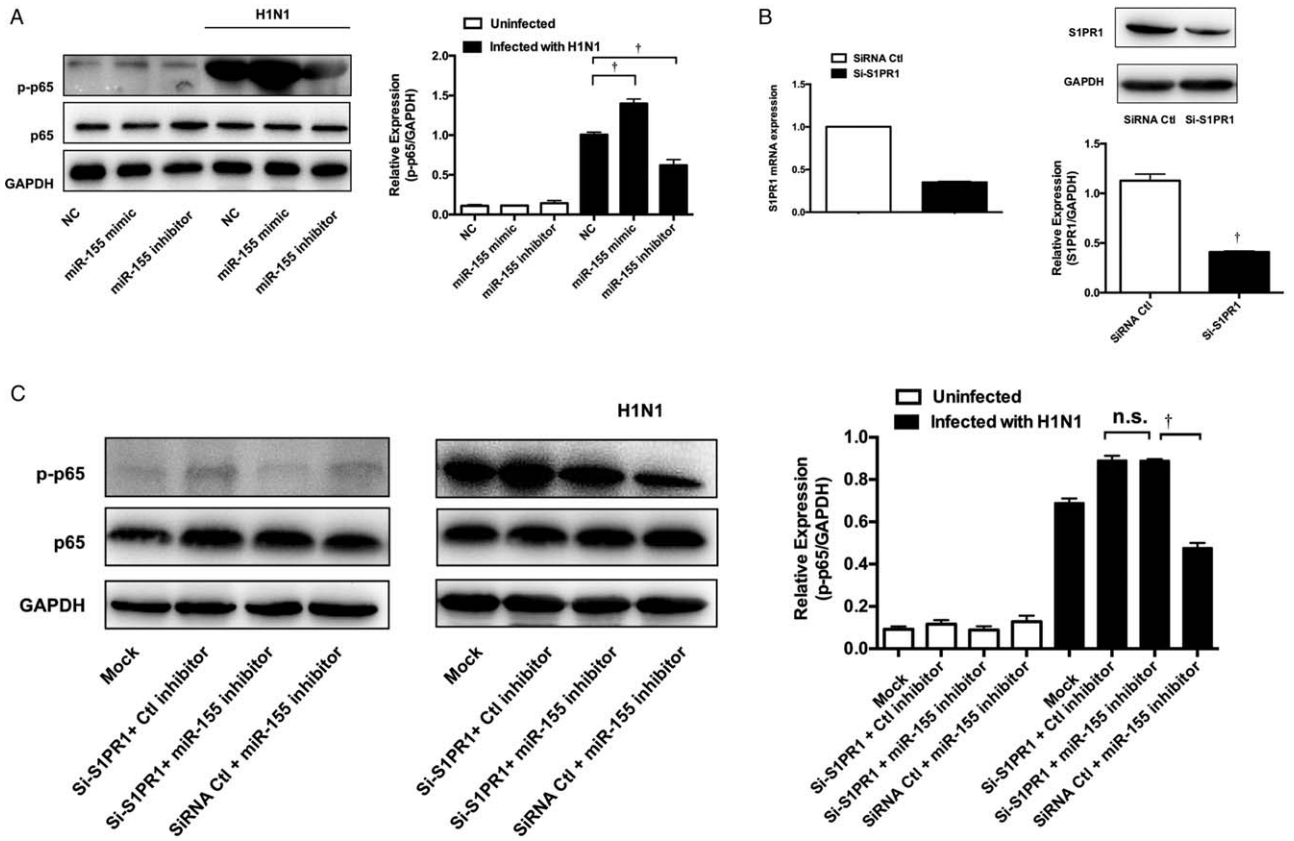

Figure 4: MiR-155 activates NF-κB signaling in H1N1-infected HPMECs via S1PR1. HPMECs were transfected with different substances. After 48 h, the HPMECs were infected with H1N1 or a vehicle for 1 h and then incubated for 24 h. A Western blot analysis was performed to analyze the phosphorylation of p65. (A) Over-expression of miR-155 promoted the activation of NF-κB in the H1N1-infected HPMECs, whereas inhibition of miR-155 had the opposite effect. In the uninfected HPMECs, the changes in the miR-155 expression did not affect the activation of NF-κB. (B) Both the mRNA and protein levels of S1PR1 decreased significantly after transfection of si-S1PR1 ($^{\dagger}P < 0.001$ *vs.* siRNA control group). (C) The expression of p-p65 was measured after co-transfection and H1N1 stimulation. The experiments were repeated three times. All data were analyzed by analysis of variance with a *post hoc* Tukey multiple comparison post-test and were presented as mean ± standard error ($^{\dagger}P < 0.01$). Ctl: Control; HPMECs: Human pulmonary microvascular endothelial cells; n.s.: Not statistically significant; NC: Negative control; NF-κB: Nuclear factor-kappa B; S1PR1: Sphingosine1-phosphate receptor 1; siRNA: Small interfering RNA.

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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