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Induction of the cellular miR-29c by influenza virus inhibits the innate immune response through protection of A20 mRNA

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ABSTRACT

Influenza A viruses (IAVs) are negative-sense, single-stranded, segmented RNA viruses, which primarily targets respiratory epithelial cells and produces clinical outcomes ranging from mild upper respiratory infection to severe pneumonia. MicroRNAs (miRNAs) represent a family of small noncoding RNAs controlling translation and transcription of many genes. The human miR-29 family of miRNAs has three mature members, miR-29a, miR-29b, and miR-29c. Recent studies have revealed that miR-29 is involved in regulation of the innate and adaptive immune responses. However, the function of miR-29 in the immune response to IAV infection remains to be further explored. Our previous study has shown that miR-29 family members are up-regulated during IAV infection, especially miR-29c. Here we report that miR-29c is involved in inhibition of IAV-induced innate immune responses. We found that posttranscriptional regulation was involved in IAV-induced A20 expression in A549 cells. Consistent with a previous report, miR-29c functionally protected A20 transcripts in A549 cells. Overexpression of miR-29c with miR-29c mimic enhanced IAV-induced A20 protein expression and conversely that miR-29c inhibitor significantly blocked IAV-induced A20 protein expression in A549 cells. Furthermore, functional results showed that IAV-induced miR-29c expression correlated with decreased NF-κB activity and expression of several antiviral and proinflammatory cytokines via up-regulation of A20. Together, the findings indicate a new role of miR-29c in IAV infection and suggest its induction may contribute to counteract the innate immune response.

1. Introduction

Influenza viruses are enveloped negative-stranded RNA viruses that cause approximately 500,000 deaths worldwide per year and many animal species [1]. IAV replicates throughout the respiratory tract, where the viral antigen is found predominantly in the epithelial cells. The clinical responses range from mild disease to fatal viral pneumonia.

The innate immune response provides the first line of defense against viruses and other pathogens. In the pulmonary environment, lung epithelial cells are the primary cellular targets for IAV. IAV infection leads to the exposure in the host cell of single-stranded genomic RNA and double stranded RNA as an intermediate of viral replication, which are recognized by endosomal toll-like receptor (TLR) 3 and 7 [2], by cytoplasmic RNA helicase retinoic acid inducible gene I (RIG-I) [3] and drives the activation of antiviral programs during viral infection. This antiviral response is mediated in part by the production of both antiviral and proinflammatory cytokines regulated by interferon regulatory factor (IRF) 3/7 and NF-κB signaling pathway, respectively [4]. As many other viruses, IAV infection activates IRF and NF-κB pathway in respiratory epithelial cells, resulting in the production of antiviral mediators and inflammatory cytokines, such as IFNs [5], TNFα [6], IL-1β [7], IL-6 [8] and IL-8 [9]. IAV, not surprisingly, has evolved strategies to counteract the cellular innate immune response, which may benefit the regulation of the delicate balance between the infection process and host defense mechanisms [10]. One of the strategies involves the IAV multifunctional NS1 protein that suppresses virus-induced activation of the transcription factors IRF 3, AP1 and NF-κB which impairs host antiviral responses [10–13]. In addition, IAV PB1-F2 protein with a serine at position 66 can inhibit type I IFN production by binding and inactivating the mitochondrial antiviral signaling protein (MAVS) [14].

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The deubiquitinating enzyme A20 (also known as TNFAIP3) is essential for the maintenance of immune homeostasis. It has become increasingly clear that A20 plays an important role in terminating the antiviral immune response by inhibiting NF-κB and IRF pathways [15–18]. A20 expression is induced in the lung from mice and bronchial epithelial cells, and overexpression of A20 attenuates NF-κB promoter activation in bronchial epithelial cells upon IAV infection [19]. *In vivo*, myeloid cell specific A20 gene knockout mice ($A20^{myel\text{-}KO}$) are protected against lethal IAV infection by an enhanced innate immune response, which demonstrates that boosting the innate immune response in myeloid cells is beneficial for the host survival [20].

MicroRNAs (miRNAs) are crucial regulators of gene expression, and exert their activity through the modulation of target mRNA stability or translation efficiency. Recently, regulation of the innate immune response has also been associated with changes in the expression of selected miRNAs, such as miR-146 [21], miR-155 [22,23] and miR-125b [23]. Furthermore, bidirectional interplays between the A20 and miRNAs have been recently illustrated [24–26]. MiR-125 a/b mediated activation of NF-κB enhances diffuse large B-cell lymphoma (DLBCL) aggressiveness by targeting of A20 [24]. Positive regulation of NF-κB signaling by miR-19b involves the coordinated suppression of negative regulators of NF-κB signaling, including A20, Rnf11, Fbxl11 and Zbtb16 [25]. In contrast to miR-125 and miR-19 that function by suppressing the activity of the 3′-UTR, expression of miR-29 increases A20 abundance and inhibits RIP1-mediated activation of NF-κB. To stabilize A20 mRNA, miR-29 is capable of acting as an RNA decoy to prevent HuR (human antigen R) from binding to the A20 3′-UTR and recruiting the RNA degradation complex RISC (RNA-induced silencing complex), thus protecting A20 mRNA [26].

Given the above mentioned information, this study was aimed to determine the as yet unraveled a novel role of miR-29c on IAV-induced innate immune response in A549 human pulmonary type II-like epithelial cells. We herein report that IAV-induced expression of miR-29c increases A20 abundance and promotes A20-mediated inhibition of NF-κB, as part of the mechanisms contributing to regulation of the innate immune responses.

2. Materials and methods

2.1. Virus and cell culture

Influenza virus A/Jingfang/01/1986(H1N1) was grown in 11-day-old embryonated eggs. A549, a human pulmonary epithelial cell line (ATCC CCL-185) was cultured in RPMI 1640 (Invitrogen) medium with 2 mM glutamine, 10 U/ml penicillin, 10 μg/ml streptomycin, and 10% fetal bovine serum at 37 °C in a CO_2 incubator. Serum-starved monolayers of A549 cells were infected with at a 50% tissue culture infective dose ($TCID_{50}$) of 100/cell. Actinomycin D (ActD) was purchased from Thermo Fisher Scientific.

2.2. Reverse transcription and real-time PCR

For quantitative analysis of mRNA expression, total RNA was isolated from cells with TRIzol reagent (Invitrogen). cDNA was synthesized using a PrimeScript™ 1st Strand cDNA Synthesis Kit (Takara). Comparative real-time PCR was performed using the SYBR Green PCR Master Mix (Takara) on the ABI 7500 Real-Time PCR System (Applied Biosystems). The level of the housekeeping gene GAPDH mRNA was measured as control. Primer sequences used in real-time PCR were as follows: IFN-β forward 5′-TGGGAGG CTTGAATACTGCCTCAA-3′ and reverse 5′-TCCTTGGCCTTCAGGTAAT GCAGA-3′; TNF-α forward 5′-AGCCCATGTTGTAGCAAACC-3′ and reverse 5′-TGAGGTACAGGCCCTCTGAT-3′; IL-1β forward 5′-GGACA AGCTGAGGAAGATGC-3′ and reverse 5′-TCGTTATCCCATGTGTCG AA-3′; IL-6 forward 5′-TACCCCCAGGAGAAGATTCC-3′ and reverse 5′-TTTTCTGCCAGTGCCTCTTT-3′; IL-8 forward 5′-AAGAAACCACCGG AAGGAAC-3′ and reverse 5′-ACTCCTTGGCAAAACTGCAC-3′; A20 forward 5′-ACCCCATTGTTCTCGGCTAT-3′ and reverse 5′-CGGTCTCT GTTAACAAGTGGAA-3′; GAPDH forward 5′-CAATGACCCCTTCATTG ACC-3′ and reverse 5′-TTGATTTTGGAGGGATCTCG-3′. All reactions were run in triplicate. The cycle threshold (Ct) value was converted to relative abundance using the $2^{-\Delta\Delta Ct}$ method for further analysis.

2.3. Transient transfection

Briefly, plate the appropriate dilution of cells to provide a confluency of 50–70% in 24 h before transfection. Cells were transiently transfected with plasmids or miRNAs with X-tremeGENE Transfection Reagent (Roche) according to the manufacturer's instructions.

2.4. Overexpression of miRNA mimic and inhibitor

To manipulate cellular function of miR-29c in A549 cells, we transfected cells with 50 nM of miRNA mimic hsa-miR-29c (Ambion) to increase miR-29c function or 50 nM of miR-29c inhibitor (Ambion) to inhibit miR-29c expression.

2.5. Luciferase reporter constructs

Complementary DNA oligonucleotides containing the putative miR-29 target site within 3′-UTR of human A20 were synthesized (sense: 5′-aaacTAGCGGCCGCTAGTGTCATCA**TGGTGCTA**TCCTCTGt-3′; antisense: 5′-ctagaCAGAGGA**TAGCACCA**TGATGACACTAGCGGCC GCTAgttt-3′) and cloned into the multiple cloning sites of the pmirGLO vector (Promega). Another pmirGLO luciferase construct containing mutant 3′-UTR (TGGTGCTA to CGATACGA) was also generated as a control. The cloning was confirmed by restriction digestion and sequencing.

2.6. Luciferase activity assay

For 3′-UTR luciferase reporter assay, we transfected HEK293 cells with each reporter construct, as well as miR-29c oligonucleotides (Ambion), followed by assessment of luciferase activity 24 h after transfection using the Dual-Luciferase Reporter Assay (Promega). pRL-CMV plasmid (Promega) expressing renilla luciferase was cotransfected to determine the transfection efficiency. Luciferase activity was measured using the microplate reader (Tecan). The firefly luciferase activity was normalized to that of renilla luciferase, and the relative luciferase activity is the comparison among various samples.

For NF-κB promoter luciferase reporter assays, NF-κB-TK-Luc plasmid was transfected into A549 cells. After treatment, luciferase activity was measured as described above. Each transfection was performed in triplicate.

2.7. Western blot analysis

Whole cell lysates were obtained from cells with mammalian protein extraction reagent (Beyotime) containing 1× protease inhibitor cocktail (Roche). Equal amounts of the proteins were electrophoresed in SDS–PAGE gel (10–12%), followed by transfer to PVDF membranes (Millipore). The western blots were then probed with antibodies recognizing the A20 (1:200; eBioscience), β-actin (1:2000; Cell Signaling), phosphor-NF-κB p65 (Ser536) and NF-κB p65 (1:1000, Cell Signaling). The secondary antibodies were from Santa Cruz Biotechnology (1:2000). Enhanced chemiluminescence (ECL) or SuperSignal West Femto chemiluminescence

kits (Pierce) were used for detection. Western blots were further quantified using standard densitometric analysis (NIH ImageJ software).

2.8. Statistical analysis

Data were expressed as mean ± standard deviation (SD) from at least three separate experiments. Significance of differences between groups was determined by Student's t test. Values of $P < 0.05$ were taken as statistically significant.

3. Results

3.1. A20 reporter activity and abundance is regulated by miR-29c

We cotransfected HEK293 cells with an A20 3′-UTR luciferase reporter and RNA oligonucleotides. The addition of miR-29c mimic significantly increased the 3′-UTR activity of A20, and cotreatment with an antagomir to miR-29c inhibited this increase (Fig. 1A). In addition, mutating the miR-29 binding sites in the A20 3′-UTR also increased the activity of the A20 3′-UTR reporter (Fig. 1B). These findings are consistent with a recent report showing that the miR-29, as a decoy, prevents HuR from binding to the A20 3′-UTR, but that this mechanism is indirect and independent of the seed sequence of miR-29. As shown in Fig. 1C, A549 cells transfected with miR-29c mimic showed increased A20 protein expression and conversely cells transfected with miR-29c-specific inhibitor displayed reduced A20 protein expression. These results suggested that miR-29c was involved in regulation of A20 gene expression in A549 cells.

3.2. IAV infection increases A20 expression through up-regulation of miR-29c

Previously, we have identified that miR-29 family members are up-regulated during IAV infection, especially miR-29c [27]. In this study, we first assessed A20 expression both at mRNA and protein levels in IAV-infected A549 cell. When A549 cells were infected with IAV, a time-dependent increase of A20 at both mRNA (Fig. 2A) and protein levels (Fig. 2B) was detected. To clarify whether posttranscriptional regulation was involved in IAV-induced A20 expression, we evaluated the expression of A20 mRNA in A549 cells after IAV infection in the presence of a transcription inhibitor, ActD. The result showed that IAV infection increased A20 mRNA stability when compared with controls (Fig. 2C), suggesting that posttranscriptional mechanisms could be involved in the up-regulation of A20 in A549 cells following IAV infection. Next, we transfected A549 cells with inhibitor or mimic of miR-29c and then infected those cells with IAV followed by western blot for A20 protein. As shown in Fig. 2D, miR-29c mimic significantly increased IAV-induced A20 protein expression and conversely that miR-29c inhibitor significantly blocked IAV-induced A20 protein expression in A549 cells. These data suggested that miR-29c-mediated post-transcriptional regulation was involved in IAV-induced A20 expression A549 cells.

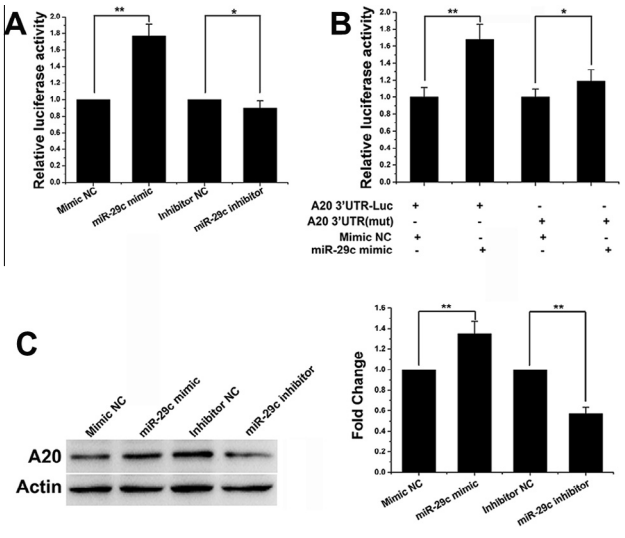

Fig. 1. miR-29c regulates A20 reporter activity and abundance. (A) A20 3′-UTR luciferase reporter activity in HEK293 cells cotransfected with the indicated miRNAs or control, normalized to that in nontransfected cells and those transfected with the reporter alone. (B) A20 3′-UTR luciferase reporter assays in HEK293 cells cotransfected with a wild-type (WT) UTR reporter construct or a reporter containing mutated miR-29 binding site [A20 3′-UTR (mut)] and the indicated miRNAs. (C) Western blot in A549 cells transfected with the indicated miRNAs. Bars are represented from three independent experiments, and data are means ± SD; $^{*}P < 0.05$ and $^{**}P < 0.01$.

3.3. miR-29c-mediated enhancement of A20 expression negatively regulates IAV-induced NF-κB activation in A549 cells

Because A20 as a negative regulator in NF-κB signaling pathways is commonly described [28], we try to assess whether miR-29c-mediated enhancement of A20 abundance affect the activation of NF-κB in IAV-infected A549 cells. There was a significant increase in the levels of the phosphorylation of p65-NF-κB subunit from IAV-infected A549 cells, which is indicator of NF-κB signaling activation (Fig. 3A). As expected, the activity of NF-κB reporter induced by IAV was inhibited by up-regulation of miR-29c and promoted by down-regulation of miR-29c (Fig. 3B). Subsequently, western blot results showed that miR-29c mimic significantly blocked IAV-induced the phosphorylation of p65 and miR-29c inhibitor significantly attenuated this effect (Fig. 3C). To further confirm the role of miR-29c and A20 in influenza virus-induced activation of NF-κB pathways, A549 cells were cotransfected with A20 expression plasmid and miR-29c inhibitor following IAV infection. As shown in Fig. 4D, overexpression of A20 abrogated miR-29c inhibitor-induced the phosphorylation of p65 in the presence of IAV. These findings suggested that miR-29c was involved in regulation of NF-κB activation induced by IAV infection at least in part through A20.

3.4. miR-29c-mediated enhancement of A20 expression prevents IAV-induced antiviral and proinflammatory gene expression in A549 cells

Previous studies have demonstrated that A20 negatively regulates innate antiviral pathways and inflammation [15–19]. To verify the role of miR-29c in innate immune response in IAV-infected A549 cells, we examined the effect of miR-29c on the mRNA expression of antiviral and proinflammatory cytokines, including TNF-α, IFN-β, IL-1β, IL-6, and IL-8, in IAV-infected A549 cells. As expected, overexpression of miR-29c significantly blocked the mRNA expression of these cytokines in A549 cells in response to IAV infection, and conversely, these cytokines expression was enhanced in A549 cells transfected with miR-29c inhibitor (Fig. 4A). Similarly, overexpression of A20 abrogated miR-29c inhibitor-enhanced the mRNA expression of antiviral and pro-inflammatory cytokines in the presence of IAV (Fig. 4B). These data suggested that miR-29c was involved in regulation of expression of antiviral and pro-inflammatory cytokines induced by IAV infection at least partially through A20.

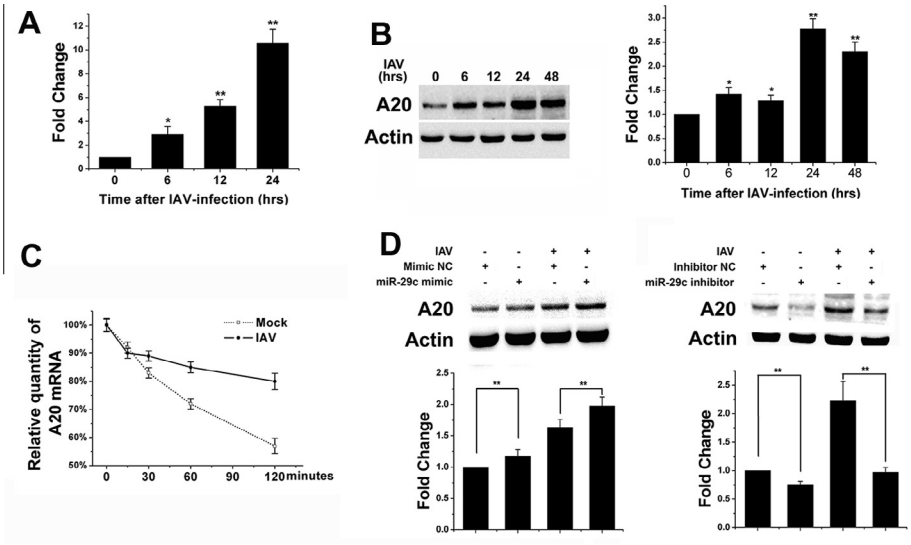

Fig. 2. IAV infection increases A20 expression through upregulation of miR-29c. Up-regulation of A20 mRNA (A) and protein (B) expression in A549 cells were analyzed at times as indicated after 100TCID50 IAV infection by real-time PCR or western blot, respectively. (C) At 24 h post-infection, A549 cells were exposed to culture medium with ActD (10 μg/ml) following by real-time PCR for A20 at the indicated time points. (D) A549 cells were transfected with the indicated miRNAs mimic or inhibitor for 24 h and then infected with 100TCID50 IAV for 24 h followed by western blot for A20. Bars are represented from three independent experiments, and data are means ± SD; $^{*}P < 0.05$ and $^{**}P < 0.01$.

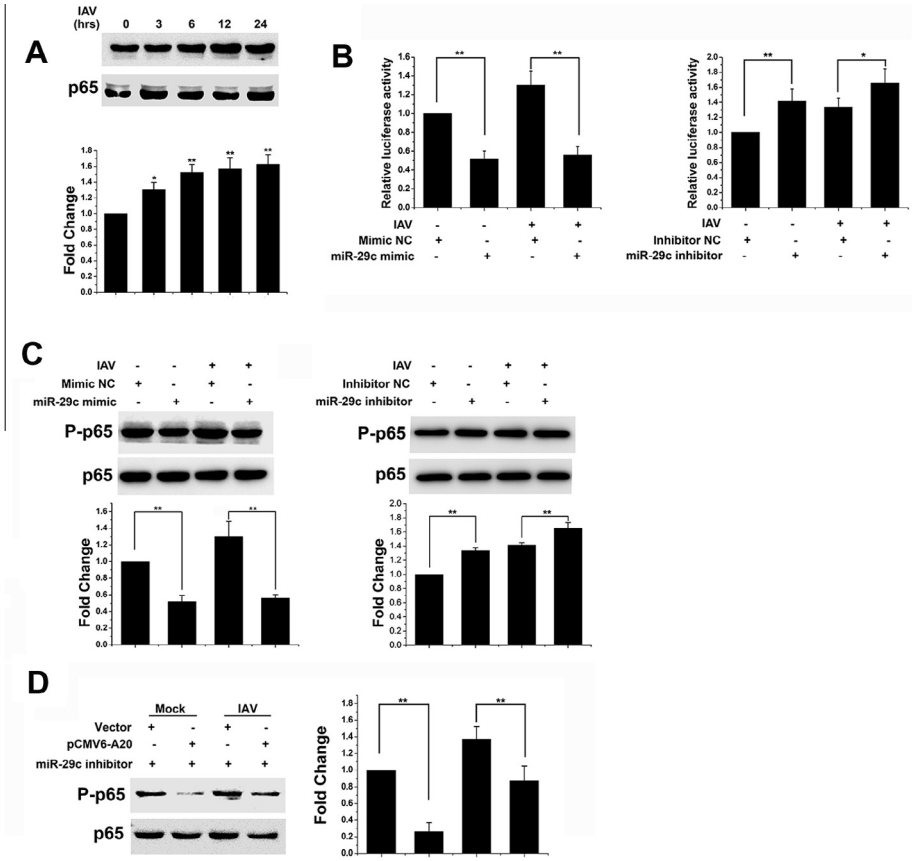

Fig. 3. miR-29c-mediated enhancement of A20 expression negatively regulates IAV-induced NF-κB activation in A549 cells. (A) Western blot analysis for the phosphorylation of p65 in A549 cells after 100TCID50 IAV infection at the indicated time points. (B) NF-κB reporter assays in A549 cells transfected with the indicated miRNAs or control for 24 h following IAV infection for 6 h. (C) A549 cells were transfected with the indicated miRNAs mimic or inhibitor for 24 h and then infected with 100TCID50 IAV for 12 h followed by western blot for phosphorylation of p65. (D) Western blot for phosphorylation of p65 in A549 cells cotransfected with the indicated miRNAs inhibitor and A20 plasmid following IAV infection for 12 h. Bars are represented from three independent experiments, and data are means ± SD; $^{*}P < 0.05$ and $^{**}P < 0.01$.

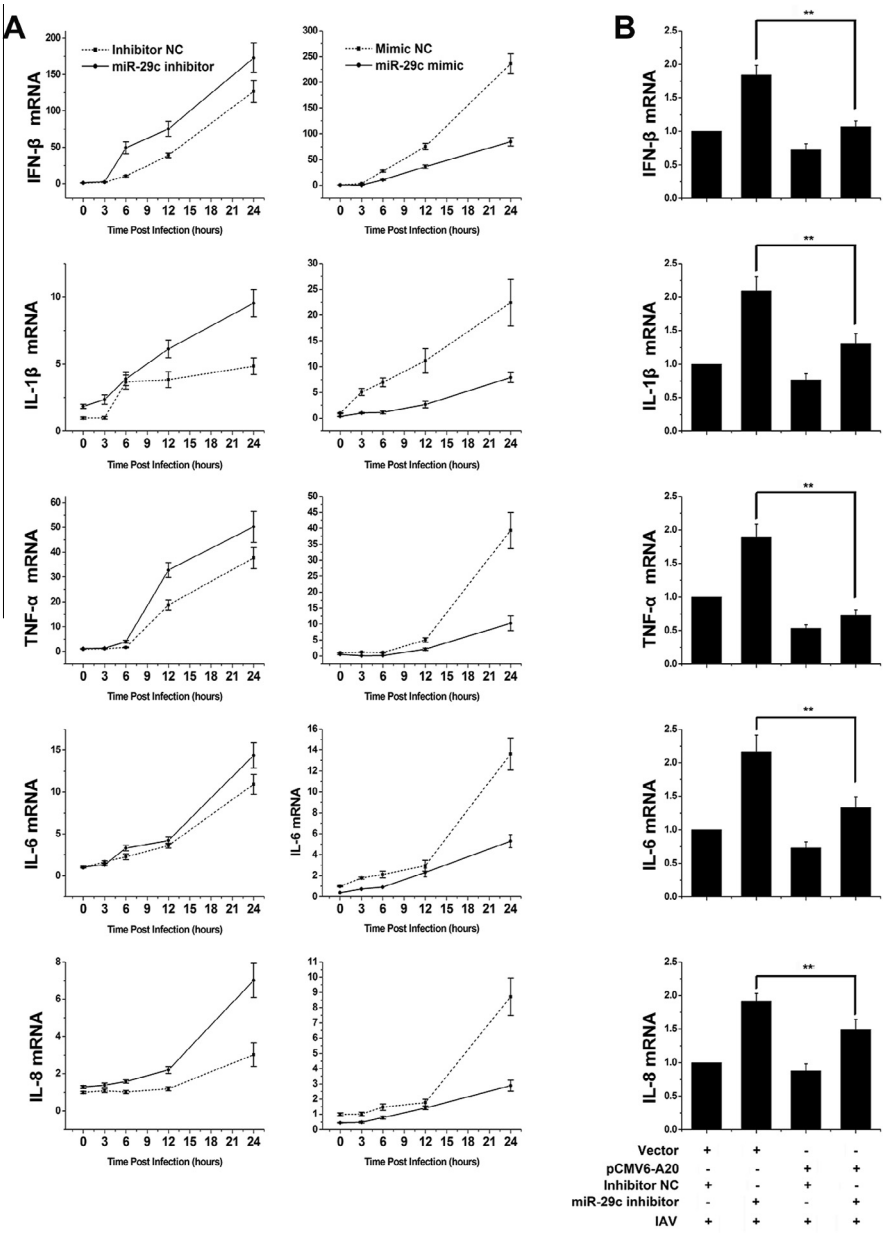

Fig. 4. miR-29c-mediated enhancement of A20 expression prevents IAV-induced antiviral and proinflammatory gene expression in A549 cells. (A) Real-time PCR analysis for the mRNA expression of cytokines (TNF-α, IFN-β, IL-6, IL-1β and IL-8) in A549 cells transfected with the indicated miRNAs or control following 100TCID50 IAV infection at the indicated time points. (B) Real-time PCR analysis for the mRNA expression of cytokines in A549 cells with miR-29c inhibitor and A20 following IAV infection for 24 h. Bars are represented from three independent experiments, and data are means ± SD; $^{*}P < 0.05$ and $^{**}P < 0.01$.

4. Discussion

A20, as a key regulator of inflammatory and antiviral signaling pathways, is essential for maintaining immune homeostasis [29]. A20 inhibited coxsackie virus B3-induced NF-κB signaling by restricting TNF receptor associated factor 6 (TRAF6) ubiquitylation [30]. Sendai virus-induced activation of ISRE (interferon-stimulated response element) and IFN-β promoter was suppressed by A20 as a negative feedback regulator for TLR3 signaling and cellular antiviral response [15]. Measles virus phosphoprotein suppressed NF-κB and AP-1 activation in monocytic cells by activating the transcription of A20, which was involved in immunological silencing and served as mechanism for dissemination of the virus throughout the body via blood and lymphatic fluids [31]. Recently, it was shown that IAV also induce the expression of A20 in the lung from mice and bronchial epithelial cells during infection [19]; however, the precise molecular mechanism of up-regulation of A20 caused by IAV infection has not been fully elucidated.

It is important to note that viruses are known to manipulate host signaling machinery to regulate virus replication and host response, which is not an all-or-nothing event, but instead, a fine-tuning of gene expression. miRNAs represent an elegant layer for both fine-tuning and dramatically altering the activity and output of cell signaling. Multiple families of miRNAs target immune transcripts to fine-tune gene expression and turn on negative feedback loops [21–23]. miR-146 was shown to be upregulated during influenza infection similarly to other viruses, and affected viral production, being involved in the antiviral response [32]. YWHAZ levels were inhibited by miR-451 specifically induced by influenza infection. Reduced YWHAZ levels relieved repression of ZFP36,

resulting in negative regulation of proinflammatory cytokine expression by DCs [33].

Within the immune response, miR-29 has been identified to be of elevated importance, due to functions in regulating key immunological pathways. For instance, miR-29 downregulated IL-23 by targeting IL-12p40 directly and IL-23p19 indirectly, likely via reduction of ATF2 [34]; miR-29 suppressed immune responses to intracellular pathogens by targeting IFN-γ [35]; More recently, Balkhi and coworkers reported an interesting study in which the expression of miR-29 acted as a decoy to inhibit HuR from binding to the 3′-UTR of A20, thereby maintaining A20 mRNA stability and NF-κB repression [26]. In earlier work, we have shown that miR-29 family members are up-regulated during IAV infection, especially miR-29c. We also observed up-regulation of A20 induced by IAV infection in A549 cells in this study. Therefore, we sought to investigate the contribution of miR-29c expression via up-regulation of A20 in A549 cells-infected with IAV. First, we confirmed that miR-29c protected A20 transcripts by luciferase reporter assay and western blot in A549 cells, and mRNA degradation of A20 was delayed in A549 cells infected with IAV. Second, transfection with miR-29c mimic enhanced IAV-induced A20 protein expression and conversely that miR-29c inhibitor significantly blocked IAV-induced A20 protein expression in A549 cells. Third, functional results showed that IAV-induced miR-29c expression associated with NF-κB activity and significantly inhibited expression of several antiviral and proinflammatory cytokines. Therefore, we suppose a novel mechanism by which miR-29c-mediated A20 expression contributes to IAV infection against the innate immune response.

Conflict of interest

The authors declare no competing financial interests.

Acknowledgments

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