

The Role of miRNA in Regulating the ZBP1-RIPK3-MLKL Pathway in Post-Influenza Inflammatory Responses

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Abstract. Excessive inflammatory response triggered by influenza virus infection is a critical factor contributing to severe disease and mortality. Recent studies have revealed that necroptosis mediated by the ZBP1-RIPK3-MLKL signaling pathway plays a significant role in pulmonary immunopathological injury. MicroRNAs (miRNAs), as crucial post-transcriptional regulators, are pivotal in maintaining inflammatory balance; however, their regulatory mechanisms on the ZBP1 pathway during post-influenza inflammation remain unclear. In this study, bioinformatic analysis predicted miRNAs targeting ZBP1, and the human microRNA-361-3p (hsa-miR-361-3p) was identified as a novel candidate regulatory molecule via a dual-luciferase reporter assay. In A549 cells, hsa-miR-361-3p was confirmed to act on the 3'-untranslated region (3'-UTR) of ZBP1, reducing ZBP1 protein expression and the phosphorylation levels of its downstream effectors RIPK3 and MLKL. Overexpression of hsa-miR-361-3p significantly suppressed the expression of the inflammatory cytokines CXCL10 and IL-8 induced by Poly I:C. This study reveals that hsa-miR-361-3p may negatively regulate necroptosis and associated inflammatory responses by targeting ZBP1, providing new insights into the mechanisms underlying influenza virus-associated inflammatory storm.**.

Keywords: Z-DNA binding protein 1 (ZBP1); microRNA (miRNA); hsa-miR-361-3p; Influenza virus; Bioinformatics**.

1. Introduction

Influenza remains a major global health challenge. Post-influenza infection, the bronchi and lungs can exhibit disease courses ranging from mild to severe inflammation. Severe inflammation is often accompanied by extensive tissue damage, constituting one of the primary pathological bases for patient mortality [1]. For instance, Influenza A virus (IAV) infection induces significant cell death in the upper and lower respiratory tracts, as well as in the lung parenchyma. In severe infections, excessive cell death exacerbates inflammation and disrupts the integrity of the epithelial barrier, potentially culminating in acute respiratory failure [2].

While scientists have made considerable progress in understanding inflammatory control in recent years, more effective strategies for the prevention and treatment of severe post-influenza inflammatory responses remain an imperative area of exploration.

Research has revealed that multi-organ damage following influenza is not solely a direct consequence of the pathogen; rather, the host's excessive immune response plays a critical role. This dysregulated reaction is termed a "cytokine storm" (CS) [3]. Its essence is a systemic hyperinflammatory response, triggered by the uncontrolled activation of the immune system leading to the overproduction of pro-inflammatory cytokines. This manifests as a rapid surge in circulating cytokine levels and, in severe cases, can precipitate multi-organ failure and death [4].

However, the body possesses intrinsic mechanisms to prevent the immune response from causing fatal self-harm, employing various pathways to regulate immunity to an appropriate level. MicroRNAs (miRNAs) are short non-coding single-stranded RNAs approximately 21-23 nucleotides in length. First discovered in the 1990s, their pivotal role in post-transcriptional gene regulation was recognized by the 2024 Nobel Prize in Physiology or Medicine (awarded to Victor Ambros and Gary Ruvkun) [5]. Mature miRNAs typically bind to partially complementary sequences within the 3' untranslated region (3'-UTR) of target messenger RNAs (mRNAs), leading to mRNA inhibition or degradation, thereby effecting post-transcriptional gene regulation. miRNAs have been demonstrated

to modulate the innate immune response to influenza virus infection, potentially promoting viral replication by suppressing the host's antiviral response, or influencing the intensity of the inflammatory response by regulating pro-inflammatory signaling pathways.

Studies show that miRNAs can precisely target key protein molecules within host immune pathways, "fine-tuning" or "suppressing" overactivated immune-inflammatory reactions to prevent damage from an inflammatory storm to host cells. For example, miR-146a targets TNF receptor-associated factor 6 (TRAF6) in human nasal epithelial cells, thereby modulating the Nuclear Factor Kappa B (NF- κ B) pathway. Beyond regulating the host, some miRNAs can directly "recognize and attack" influenza viral nucleic acids or proteins, blocking the viral replication cycle at its source [6].

In addition to miRNA-mediated regulation of inflammatory signaling, cell death can also exacerbate inflammation. Programmed cell death (PCD) is a deliberate suicide mechanism that eliminates unwanted cells from the body, such as tumor cells or self-reactive lymphocytes, to maintain homeostasis. During viral infection, infected cells may undergo PCD as part of the host cell defense mechanism, aiming to eliminate infected cells and limit viral spread. Accumulating evidence indicates that PCD is involved in suppressing viral replication and modulating inflammation and pathogenicity in influenza disease [7].

During IAV infection, Z-DNA binding protein 1 (ZBP1) is recognized as a key molecule inducing PCD. ZBP1 specifically detects the left-handed Z-conformation of double-stranded RNA (Z-RNA) within infected cells; this conformational change serves as a stress signal expressed by the cell. While primarily cytoplasmic, ZBP1 is recruited to the nucleus following IAV infection, where it senses Z-RNA species generated during active viral replication. Upon Z-RNA recognition within the nucleus, ZBP1 interacts with Receptor-Interacting Serine/Threonine-Protein Kinase 3 (RIPK3), promoting RIPK3 oligomerization and activation of Mixed Lineage Kinase Domain-Like Protein (MLKL). This protein complex is termed the necrosome [8].

Current research highlights the particular importance of the ZBP1 pathway in post-influenza inflammation. In recent years, growing evidence indicates that ZBP1-mediated excessive necroptosis following IAV infection is a central mechanism driving severe lung injury, inflammatory storms, and disease exacerbation. The aforementioned ZBP1-RIPK3-MLKL axis not only leads to massive death of alveolar epithelial cells and immune cells but also triggers the release of substantial amounts of Damage-Associated Molecular Patterns (DAMPs) and pro-inflammatory cytokines (e.g., Interleukin-1 β , Interleukin-6, Tumor Necrosis Factor- α). These factors activate macrophages and neutrophils, promote CS, and exacerbate pulmonary inflammation and acute respiratory distress syndrome (ARDS), representing a major cause of mortality in severe influenza patients [9-10]. Given the significant impact of ZBP1 in influenza severity, targeting the ZBP1-RIPK3-MLKL pathway has emerged as a promising new focus for antiviral and anti-inflammatory therapies.

2. Materials and Methods

2.1. Construction of Dual-Luciferase Reporter Plasmids

The psiCHECK-2 vector (Promega, C8021) served as the backbone vector. SnapGene v6.0 software was used to analyze its multiple cloning site, identifying XhoI (CTCGAG) and NotI (GCGGCCGC) restriction sites as the insertion region for the 3'-UTR, ensuring no internal restriction sites within the target fragment. The full-length 3'-UTR sequences of ZBP1 (NM_001160417.2), RIPK3 (NM_006871.4), and MLKL (NM_001142497.3) were selected. Sequence maps for the recombinant plasmids were designed and ordered from GenePharma (Shanghai).

2.2. Cell Culture

A549 cells were used. Cells recovered from cryopreservation were passaged twice prior to transfection. When cell proliferation reached 70% confluence, they were passaged. The medium was removed, and cells were washed twice with PBS. Then, 1 mL of 0.25% trypsin was added, and digestion was monitored under a microscope. Digestion was stopped by adding 2 mL of F12K

complete medium (Procell, PM150910) containing 10% Fetal Bovine Serum and 1% Penicillin-Streptomycin-Glutamine. The cell suspension was transferred to a centrifuge tube and centrifuged at 1500 rpm for 5 minutes. After discarding the supernatant, the cell pellet was resuspended in medium and counted. Cells were diluted to a density of 1×10^5 cells/mL, seeded into 6-well plates (2 mL per well), and cultured overnight to allow attachment. Transfection was performed when cells reached 70-90% confluence.

2.3. Dual-Luciferase Assay

Following the cloning of the ZBP1, RIPK3, and MLKL 3'-UTRs into the plasmids, the recombinant reporter plasmids were transfected into A549 cells. Transfection was performed using Lipofectamine 3000 reagent (Invitrogen, L3000001). Transfection Mixture A (125 μ L Opti-MEM + 5 μ L Lipofectamine 3000) and Mixture B (125 μ L Opti-MEM + 2.5 μ g recombinant plasmid + 5 μ L P3000 Reagent) were prepared. While mixtures were incubated for 15 minutes, fresh complete medium (0.75 mL) was added to the wells of a 12-well plate. After 15 minutes, the mixtures were combined and added to the respective wells of the 12-well plate, mixed gently, and placed in the incubator. After 6 hours, the medium was replaced with complete medium. At 24 hours post-transfection, cells were treated according to the following groups: ① Carbonyl cyanide m-chlorophenyl hydrazone (CCCP) group [10 μ M, Sigma, C2759, dissolved in Dimethyl Sulfoxide (DMSO)], ② Polyinosinic-polycytidylic acid (Poly I:C) group (1 μ g/mL, TargetMol, 24939-03-5, dissolved in sterile PBS), ③ Combination group (CCCP + Poly I:C), ④ & ⑤ Two solvent control groups (PBS or DMSO, respectively). At 48 hours post-treatment, the lysis reagent was prepared fresh according to the manufacturer's instructions. Carefully, 100 μ L of cell lysate supernatant was transferred to a black 96-well plate, and Renilla Luciferase activity was immediately measured using a multifunctional microplate reader. Firefly Luciferase activity was used as an internal control, and the Renilla/Firefly (R/F) luciferase ratio was calculated.

2.4. Bioinformatic Prediction and Candidate Screening of miRNAs

Three bioinformatic databases—TargetScan (www.targetscan.org/vert_80/), miRDB (mirdb.org), and miRNA Tissue Atlas (ccb-compute2.cs.uni-saarland.de/mirnatissueatlas_2025)—were used to predict miRNAs targeting the 3'-UTRs of ZBP1 and RIPK3 genes. Data intersecting all three databases were compiled using Excel, grouped, and processed. miRNAs with literature validation available in miRTarBase (awi.cuhk.edu.cn/~miRTarBase/miRTarBase_2025/php/index.php) were selected as positive controls.

2.5. Co-transfection of Dual-Luciferase Reporter Plasmids with miRNA Mimics/Inhibitors

Following the method described in section 2.3, co-transfection of ZBP1 or RIPK3 3'-UTR plasmids with miRNA mimics or inhibitors (see Table 1) was performed. All miRNA mimics/inhibitors were purchased from GenePharma (Shanghai). The R/F ratio for each group was detected using the multifunctional microplate reader.

Tab. 1 miRNA Mimic/Inhibitor Sequences

Primer Name	Sequence (5' to 3')
Negative Control of Mimic	UUCUCCGAACGUGUCACGUTT ACGUGACACGUUCGGAGAATT
hsa-miR-339-5p Mimic	UCCUGUCCUCCAGGAGCUCACG UGAGCUCCUGGAGGACAGGGAUU
hsa-miR-654-3p Mimic	UAUGUCUGCUGACCAUCACCUU GGUGAUGGUCAGCAGACAUAUU
hsa-miR-361-3p Mimic	UCCCCCAGGUGUGAUUCUGAUUU AUCAGAAUCACACCUGGGGGAUU
hsa-miR-98-5p Mimic	UGAGGUAGUAAGUUGUAUUGUU CAAUACAACUUACUACCUCAUU
hsa-miR-99b-5p Mimic	CACCCGUAGAACCGACCUUGCG CAAGGUCGGUUCUACGGGUGUU
hsa-miR-29a-3p Mimic	UAGCACCAUCUGAAAUCGGUUA ACCGAUUUCAGAUGGUGCUAUU
hsa-miR-325-3p Mimic	UUUAUUGAGGACCUCCUAUCAA GAUAGGAGGUCCUCAUAAAUU
hsa-miR-4443 Mimic	UUGGAGGCGUGGGUUUU AACCCACGCCUCCAAUU
hsa-miR-18a-3p Mimic	ACUGCCCUAAGUGCUCUUCUGG AGAAGGAGCACUUAGGGCAGUUU
hsa-miR-223-3p Mimic	UGUCAGUUUGUCAAAUACCCCA GGGUAUUUGACAAACUGACAUU
Inhibitor Negative Control	CAGUACUUUUGUGUAGUACAA
hsa-miR-339-5p Inhibitor	CGUGAGCUCCUGGAGGACAGGGA
hsa-miR-654-3p Inhibitor	AAGGUGAUGGUCAGCAGACAUA
hsa-miR-361-3p Inhibitor	AAAUCAGAAUCACACCUGGGGGA
hsa-miR-98-5p Inhibitor	AACAAUACAACUUACUACCUCA
hsa-miR-99b Inhibitor	CGCAAGGUCGGUUCUACGGGUG
hsa-miR-29a-3p Inhibitor	UAACCGAUUUCAGAUGGUGCUA
hsa-miR-325-3p Inhibitor	UUGAUAGGAGGUCCUCAUAAA
hsa-miR-4443 Inhibitor	AAAACCCACGCCUCCAA
hsa-miR-18a-3p Inhibitor	CCAGAAGGAGCACUUAGGGCAGU
hsa-miR-223-3p Inhibitor	UGGGGUAUUUGACAAACUGACA

2.6. Western Blot Analysis

Cells were divided into four experimental groups: control group, Poly I:C (1 µg/mL) treatment group, hsa-miR-361-3p mimic group, and Poly I:C (1 µg/mL) + hsa-miR-361-3p mimic group. Transfection was performed as described in section 2.3. Treatment was administered when cells reached 70-90% confluence, with three replicates per group. At 24 hours post-transfection, cells were treated with Poly I:C or corresponding controls. Samples were collected 48 hours post-treatment. After collection, cells were lysed on ice using RIPA Lysis Buffer (EpiZyme, PC101) supplemented with phosphatase and protease inhibitors. The lysates were centrifuged at 12,000 rpm for 10 minutes at 4°C to obtain the protein supernatant. Protein concentration was determined using the bicinchoninic

acid (BCA) assay. After normalizing concentrations, 5× loading buffer (Solarbio, P0100) was added, and samples were heated in a boiling water bath for 10 minutes. Proteins were separated by electrophoresis on 12.5% SDS-PAGE gels (constant voltage: 80V for 15 min; 120V for 60 min) and subsequently transferred onto PVDF membranes (constant current: 150 mA for 90 min). After blocking at room temperature, membranes were incubated with primary antibodies (1:1000 dilution) at 4°C overnight. The following day, membranes were washed and incubated with HRP-conjugated secondary antibodies (1:10000 dilution). Finally, protein bands were visualized using ECL chemiluminescent substrate (EpiZyme, SQ201). The primary antibodies used were: anti-ZBP1 (Proteintech, 13285-1-AP), anti-p-RIPK3 (Ser227) (CST, 93654), and anti-p-MLKL (Ser358) (CST, 91689).

2.7. Real-Time Quantitative PCR (RT-qPCR)

Using the same grouping strategy as in section 2.6, with three replicates per group, total RNA was extracted from collected cell samples 48 hours post-transfection and treatment. RNA was reverse transcribed into complementary DNA (cDNA), which was subsequently used as the template for real-time quantitative PCR (RT-qPCR). The expression levels of target genes and internal reference genes were detected, and relative expression levels were calculated using the $2^{-\Delta\Delta C_t}$ method. Primer sequences, designed based on the NCBI database and literature searches, are listed in Table 2.

Tab. 2 Primer Sequences Used in Experiments of Table 1

Primer Name	Sequence (5' → 3')
GAPDH-F	ATTCCACCCATGGCAAATTCC
GAPDH-R	GACTCCACGACGTACTCAGC
CXCL10-F	AAGTGGCATTCAAGGAGTACCT
CXCL10-R	ACACGTGGACAAAATTGGCT
IFNB1-F	GACAGGATGAACTTTGACATCCC
IFNB1-R	CTCAACAATAGTCTCATTCAGCC
IL8-F	GCTGGCCGTGGCTCTCTTGG
IL8-R	GACAGAGCTCTCTTCCATCAG

The culture medium was removed, and the cells were washed with an appropriate amount of pre-cooled PBS. RNA lysis buffer was added to the culture plate according to the volume of 1 mL per well of a 6-well plate. The plate was gently swirled to ensure the cells at the bottom were fully covered by the lysis buffer. Cells were then lysed by repeated pipetting, and the lysate was transferred to a centrifuge tube, followed by incubation at room temperature for 5 minutes.

For every 1 mL of RNA lysis buffer used, 200 µL of chloroform was added. The tube was then vigorously vortexed for 15 seconds and allowed to stand at room temperature for 5 minutes. Subsequently, it was centrifuged at $12,000 \times g$ for 15 minutes at 4°C. The upper colorless aqueous phase was carefully transferred to a new centrifuge tube.

To the collected aqueous phase, 500 µL of isopropanol (Hengxing Reagent, 1010) was added. The tube was inverted several times to mix thoroughly and then incubated at room temperature or 4°C for 10 minutes to precipitate the RNA. The sample was centrifuged at $12,000 \times g$ for 10 minutes at 4°C, after which the supernatant was discarded.

The RNA pellet was washed by adding 1 mL of 75% ethanol (Hengxing Reagent, Analytical Grade AR500) and inverting the tube several times. The sample was centrifuged again at $12,000 \times g$ for 5 minutes at 4°C, and the supernatant was discarded. A brief high-speed centrifugation ($5,000 \times g$ for 3-5 seconds) was performed to collect any residual liquid, which was then carefully removed using an RNase-free micropipette tip.

The tube containing the RNA pellet was left uncovered for 5 minutes to allow any residual ethanol to evaporate. The RNA was then thoroughly dissolved in 20-50 µL of RNase-free water.

The reverse transcription reaction mixture was prepared according to the system outlined in Table 3, using the NovoScript Reverse Transcription Kit (E047-01B).

Tab. 3 RNA Reverse Transcription Reaction System of Table 1

Reagent	Volume
2×NovoScript®Plus1st Strand cDNA Synthesis SuperMix	10µL
Template RNA	Total RNA (1µg)
gDNA Purge	1µL
RNase-Free Water	Up to 20µL

The mixture was gently vortexed and centrifuged. It was then incubated at 50°C for 15 minutes, followed by incubation at 85°C for 5 seconds to terminate the reaction. The resulting cDNA was stored at -20°C.

The reaction mixture for mRNA quantitative real-time PCR was prepared according to Table 4, using the ChamQ Universal SYBR qPCR Master Mix (Vazyme, Q711-03).

Tab. 4 mRNA Quantitative Real-Time PCR Reaction System

Reagent	Volume
2×ChamQ Universal SYBR qPCR Master Mix	10µL
Forward Primer	0.4µM (Final Concentration)
Reverse Primer	0.4µM (Final Concentration)
Template	1µL
RNase-Free Water	Up to 20µL

The mixture was gently vortexed and centrifuged. It was then incubated at 50°C for 15 minutes, followed by heating at 85°C for 5 seconds to terminate the reaction. The resulting cDNA was stored at -20°C.

The reaction mixture for mRNA fluorescence quantification was prepared according to Table 4, using the ChamQ Universal SYBR qPCR Master Mix (Vazyme, Q711-03).

Tab. 5 mRNA Quantitative Real-Time PCR Reaction System

Reagent	Volume
2×ChamQ Universal SYBR qPCR Master Mix	10µL
Forward Primer	0.4µM (Final Concentration)
Reverse Primer	0.4µM (Final Concentration)
Template	1µL
RNase-Free Water	Up to 20µL

The mixture was gently vortexed and centrifuged. It was then incubated at 50°C for 15 minutes, followed by 85°C for 5 seconds to terminate the reaction. The resulting cDNA was stored at -20°C.

The mRNA quantitative PCR reaction mixture was prepared according to Table 4, using the ChamQ Universal SYBR qPCR Master Mix (Vazyme, Q711-03). The PCR protocol was as follows: initial pre-denaturation at 95°C for 1 minute; followed by 40 cycles of amplification, each consisting of denaturation at 95°C for 20 seconds, annealing at 60°C for 20 seconds, and extension at 72°C for 30 seconds. A dissociation curve was generated after the amplification cycles. The results were calculated using the $2^{-\Delta\Delta C_t}$ method and subsequently analyzed. The real-time quantitative PCR instrument used was the DLAB Accurate 96.

3. Results

3.1. Changes in Luciferase Activity of ZBP1, RIPK3, and MLKL 3'-UTR Reporter Plasmids under Poly I:C and CCCP Stimulation

To identify genes regulated during IAV infection, the 3'-UTR sequences of the human ZBP1, RIPK3, and MLKL genes were cloned downstream of the Renilla luciferase gene in the psiCHECK-2 vector using a double-enzyme digestion and ligation method. Sequencing verification and BLAST alignment confirmed 100% identity with the original 3'-UTR sequences of the respective genes.

Using these reporter plasmids, we found that Poly I:C treatment significantly reduced the reporter luciferase activity of ZBP1, RIPK3, and MLKL ($P < 0.05$). Under Poly I:C stimulation, the R/F (Renilla/Firefly luciferase) ratios for the ZBP1, RIPK3, and MLKL reporter plasmids decreased to 40% ($P < 0.0001$), 57% ($P = 0.0014$), and 58% ($P = 0.0017$) of the control group levels, respectively. In contrast, under CCCP stimulation, the R/F ratios for all three reporter plasmids showed an increase compared to the DMSO control group, which was inconsistent with the expected results (this will be discussed in detail later).

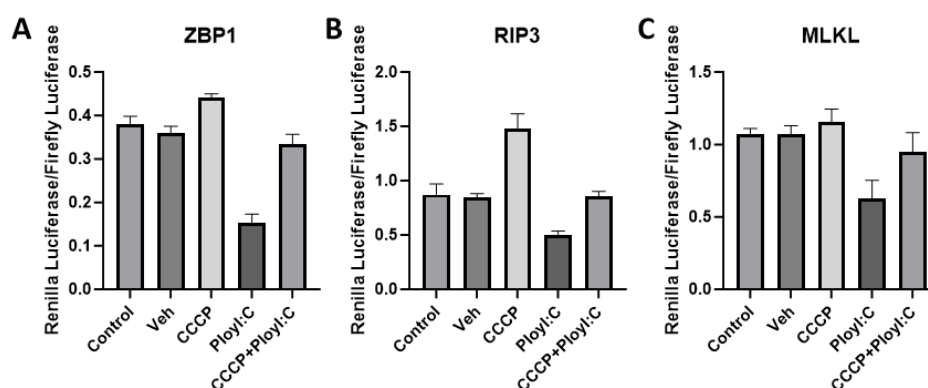


Fig. 1 A549 cells were transfected with ZBP1, RIPK3, or MLKL 3'-UTR reporter plasmids. Renilla luciferase activity was measured under solvent control (PBS or DMSO) and stimulation (Poly I:C or CCCP) conditions, normalized to Firefly luciferase activity. Data are presented as mean $\pm$ SD ($n = 3$) and were analyzed by one-way ANOVA with Tukey's multiple comparisons test. The Control group refers to the PBS solvent control, while the Veh group refers to the DMSO solvent control.

3.2. Bioinformatic Prediction Results

We performed bioinformatic screening using miRDB and TargetScan. All predicted miRNAs potentially targeting ZBP1 and RIPK3 from both databases were extracted, and their intersections were obtained to infer miRNAs with potential regulatory roles. Results from TargetScan for ZBP1 indicated a lack of support from conserved data, with only poorly conserved information available. From these results, miRNAs were prioritized based on the following criteria: a Weighted Context++ Score ≤ -0.15 , a Percentile ≥ 85 , and a Seed type of either 7mer-m8 (indicating perfect pairing of 7 nucleotides with pairing at the 8th position) or 8mer (indicating perfect consecutive pairing of 8 nucleotides). For miRDB retrievals of ZBP1 and RIPK3, results were sorted by Target Score, where a higher score indicates greater prediction reliability.

The intersection of results from miRDB, TargetScan, and miRTarBase revealed no common miRNAs across all three databases. Therefore, the intersection between miRDB and TargetScan was selected, indicating that the predicted miRNAs possess relatively strong novelty. Subsequently, these intersecting miRNAs were queried in the Tissue Atlas database, and miRNAs with higher average expression values were selected. For ZBP1, these included hsa-miR-339-5p, hsa-miR-654-3p, and hsa-miR-361-3p. For RIPK3, they included hsa-miR-4443 and hsa-miR-18a-3p.

Consulting miRTarBase for miRNAs with literature and database support for regulating the target genes, and based on strong experimental validation evidence including reporter gene assays, Western

blot, and qPCR data, hsa-miR-98-5p and hsa-miR-93-5p were further confirmed to have clear interactions with ZBP1 and were used as positive controls. Literature searches identified hsa-miR-99b-5p [11], hsa-miR-325-3p [12], and hsa-miR-223-3p [13] as additional positive controls for ZBP1 and RIPK3, respectively.

3.3. Validation of Bioinformatically Predicted Candidate miRNAs by Dual-Luciferase Assay

We selected the ZBP1 and RIPK3 3'-UTR reporter plasmids and conducted dual-luciferase assays by co-transfecting them with the predicted miRNA mimics and inhibitors targeting the respective genes.

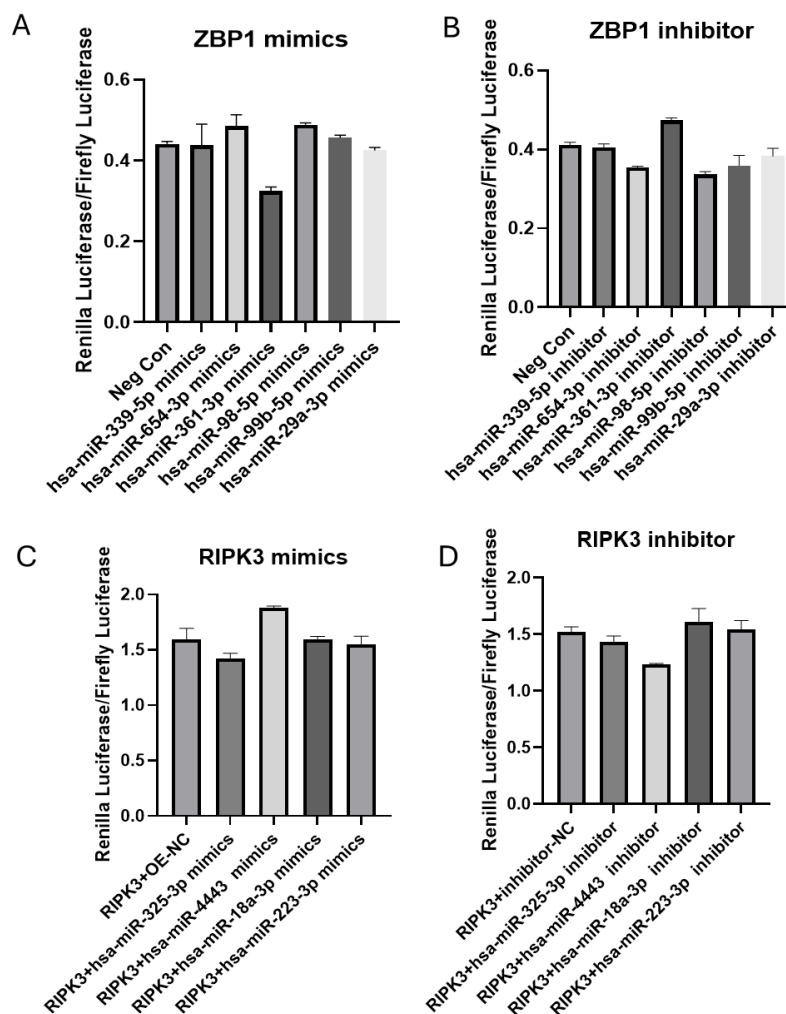


Fig. 2 A549 cells were transfected with ZBP1 (A, B) or RIPK3 (C, D) 3'-UTR reporter plasmids. Panels A and B (from left to right) show cells co-transfected with the ZBP1 reporter plasmid and mimics (A) or inhibitors (B) of hsa-miR-339-5p, hsa-miR-654-3p, hsa-miR-361-3p, hsa-miR-98-5p, hsa-miR-99b-5p, or hsa-miR-29a-3p. Panels C and D (from left to right) show cells co-transfected with the RIPK3 reporter plasmid and mimics (C) or inhibitors (D) of hsa-miR-325-3p, hsa-miR-4443, hsa-miR-18a-3p, or hsa-miR-223-3p.

Renilla luciferase and Firefly luciferase activities were measured separately. Firefly luciferase activity was used for normalization. Data are presented as mean $\pm$ SD ($n = 3$) and were analyzed by one-way ANOVA with Tukey's multiple comparisons test.

Using these reporter plasmids in dual-luciferase assays, we observed the regulatory effects of miRNAs on ZBP1 and RIPK3. For the ZBP1 3'-UTR reporter plasmid, transfection with the hsa-miR-361-3p inhibitor and mimic resulted in R/F ratios of 115.0% ($P=0.0003$) and 73.5% ($P=0.0001$) of the control group, respectively. For the RIPK3 3'-UTR reporter plasmid, transfection with the hsa-

miR-4443 inhibitor and mimic resulted in R/F ratios of 81.0% ($P=0.0017$) and 117.8% ($P=0.0006$) of the control group, respectively. Among the various candidate miRNAs, although hsa-miR-4443 regulation of RIPK3 also showed statistical significance, its effect direction was inconsistent with our research hypothesis. In contrast, the negative regulatory effect of hsa-miR-361-3p on ZBP1 was consistent with expectations. Therefore, we focused subsequent experiments on validating the function of hsa-miR-361-3p.

3.4. hsa-miR-361-3p Directly Suppresses ZBP1 Protein Expression

To validate the regulatory role of hsa-miR-361-3p at the protein level, we performed Western blot analysis. As shown in Figure 3A, Poly I:C stimulation increased ZBP1 protein expression. Transfection with the hsa-miR-361-3p mimic significantly reduced ZBP1 protein levels. In the combination treatment group, the hsa-miR-361-3p mimic effectively suppressed the Poly I:C-induced upregulation of ZBP1 protein. These results provide key evidence that hsa-miR-361-3p directly targets and negatively regulates ZBP1 protein expression.

Furthermore, we examined the activation status of the downstream ZBP1 signaling pathway, namely the phosphorylation levels of RIPK3 and MLKL (p-RIPK3 and p-MLKL), which are core markers of necroptosis activation. As shown in Figures 3C and 3D, Poly I:C stimulation effectively activated this pathway, demonstrated by significantly increased levels of p-RIPK3 and p-MLKL. Transfection with the hsa-miR-361-3p mimic suppressed the phosphorylation levels of these two key molecules. In the combination treatment group, the hsa-miR-361-3p mimic partially reversed the Poly I:C-induced phosphorylation of RIPK3 and MLKL.

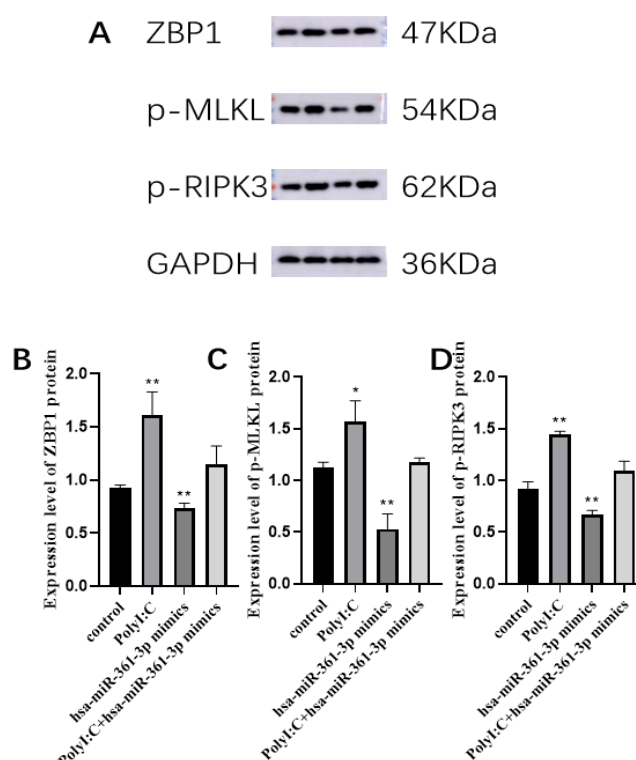


Fig. 3 Effect of hsa-miR-361-3p on ZBP1 protein expression and downstream pathway molecules.

A549 cells were subjected to different treatments: control group, Poly I:C treatment group, hsa-miR-361-3p mimic treatment group, and Poly I:C + hsa-miR-361-3p mimic co-treatment group. Target proteins were detected by Western blot: ZBP1 (B), p-MLKL (C), and p-RIPK3 (D). GAPDH served as the loading control. Statistical significance compared to the control group is indicated as * for $P \leq 0.05$ and ** for $P \leq 0.01$.

3.5. hsa-miR-361-3p Negatively Regulates the ZBP1-RIPK3-MLKL Pathway and Inflammatory Response

To investigate the potential role of hsa-miR-361-3p in the ZBP1-RIPK3-MLKL-mediated necroptosis signaling pathway, we transfected A549 cells with the hsa-miR-361-3p mimic and stimulated them with the viral double-stranded RNA analog Poly I: C. Gene expression levels were analyzed by quantitative PCR, with detailed results shown in Figure 4.

We first examined the mRNA expression of key inflammatory factors. As shown in Figure 4, Poly I:C stimulation significantly upregulated the expression of the pro-inflammatory chemokines CXCL10 and IL-8. Transfection with the hsa-miR-361-3p mimic alone significantly suppressed the basal expression levels of both cytokines. In the co-treatment group, the hsa-miR-361-3p mimic partially attenuated the Poly I:C-induced upregulation of CXCL10 and IL-8 expression.

Regarding IFNB1 expression, we observed a particular phenomenon (Figure 4C). Contrary to initial expectations, at the specific time point assessed in this experiment, Poly I:C stimulation resulted in a trend towards downregulation of IFNB1 mRNA levels. Conversely, transfection with the hsa-miR-361-3p mimic alone elevated the basal IFNB1 expression level. In the co-treatment group, IFNB1 expression was partially restored compared to the Poly I:C treatment alone. The potential reasons underlying these observations will be discussed later.

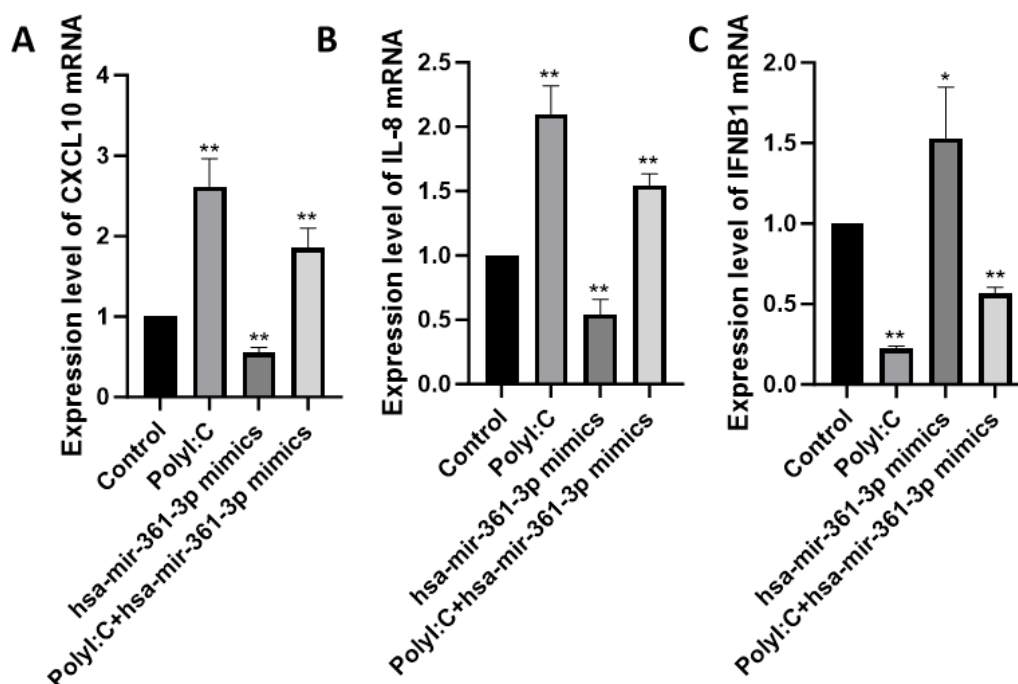


Fig. 4 miR-361-3p modulates Poly I:C–induced inflammatory cytokine expression in vitro

4. Discussion

This study initially constructed 3'-UTR reporter plasmids for the ZBP1, RIPK3, and MLKL genes concurrently, establishing a foundational experimental platform for subsequent targeted regulation research. In the screening for Poly I:C stimulation response, the ZBP1 signal demonstrated the most significant reduction, whereas the signal changes for RIPK3 and MLKL were relatively minor and did not align with the anticipated regulatory trend.

From a mechanistic perspective, these findings are consistent with the functional roles of these proteins within the signaling pathway: ZBP1 functions as an upstream sensor for viral infection, and its expression regulation directly responds to viral mimic stimulation, thus exhibiting the strongest regulatory effect. In contrast, RIPK3 and MLKL act as downstream signaling molecules potentially subject to more complex regulatory networks involving upstream and downstream factors (e.g.,

protein phosphorylation, ubiquitination). This complexity may obscure miRNA-mediated targeting effects at the 3'-UTR level, resulting in comparatively weaker responses.

It is important to note that the negative or non-significant results for RIPK3 and MLKL also hold scientific value. They not only exclude these two genes as core targets responsive to Poly I:C in this experimental system but also provide exclusionary evidence supporting the subsequent focus on ZBP1. This helps prevent distraction by non-core targets and suggests that the regulation of downstream molecules may involve mechanisms independent of miRNAs.

In this study, we initially hypothesized that CCCP, similar to Poly I:C, would decrease ZBP1 3'-UTR reporter activity. Contrary to expectations, the results indicated increased activity in the CCCP treatment group. We speculate this phenomenon might be related to global expression changes in miRNAs that regulate ZBP1. Specifically, under Poly I:C stimulation, these miRNAs might be upregulated, leading to stronger negative regulation of ZBP1 and consequently reduced reporter signal. Under CCCP treatment conditions, the expression levels of these miRNAs might instead be downregulated, thereby attenuating the inhibition on ZBP1 and enhancing its signal.

Existing reviews have reported that cellular miRNA expression profiles can undergo significant alterations under stress or damage conditions, with a common trend being global miRNA downregulation [14-15]. This pattern indirectly supports our observation: CCCP might induce miRNA downregulation through mitochondrial damage and mitochondrial DNA release, consequently relieving the inhibition on ZBP1 [16].

To identify miRNAs regulating ZBP1, this study employed a combined screening strategy utilizing three databases—TargetScan, miRDB, and miRTarBase—to enhance prediction reliability and mitigate biases inherent to individual databases. TargetScan emphasizes the conservation of miRNA-target gene interactions and seed region matching, facilitating the identification of evolutionarily more functionally significant targeting relationships. miRDB utilizes a machine learning model capable of covering non-conserved binding sites, thereby complementing potential omissions from conservation analysis. miRTarBase integrates published experimental validation data, providing preliminary experimental support for the predictions. By implementing stringent screening parameters (Weighted Context++ Score ≤ -0.15 , Percentile ≥ 85 , and binding types of 7mer-m8 or 8mer), we ultimately identified hsa-miR-361-3p as a candidate miRNA. Dual-luciferase reporter gene assays confirmed its direct binding to the ZBP1 3'-UTR region.

Validation in the A549 cell model demonstrated that hsa-miR-361-3p not only significantly suppressed ZBP1 protein expression but also, via ZBP1 downregulation, further reduced the phosphorylation levels of downstream RIPK3 and MLKL. This ultimately led to attenuated expression of pathway-associated downstream inflammatory factors (CXCL10, IL-8), forming a regulatory cascade: "hsa-miR-361-3p $\rightarrow$ ZBP1 $\rightarrow$ RIPK3/MLKL $\rightarrow$ Inflammatory Factors".

A particular observation was made regarding IFNB1: its expression did not show the expected increase upon Poly I:C stimulation but rather decreased. Literature examining type I interferon expression in mouse mesenteric lymph nodes and thymus reported three consecutive expression waves in mesenteric lymph nodes, peaking at one hour. Our sample collection occurred after 72 hours, coinciding with the declining phase [17]. Considering this literature, our results align with the kinetic profile of IFNB1 expression—an early rapid increase following viral stimulation to initiate antiviral responses, followed by decreased expression during later stages due to negative feedback regulation. The detection time point in this experiment likely captured this declining phase.

From a biological perspective, this regulatory cascade suggests that hsa-miR-361-3p may function as a negative regulator of ZBP1. By inhibiting ZBP1 and its downstream pathway, it potentially mitigates inflammatory factor release, possibly exerting a protective role against influenza virus infection-induced inflammatory storms. Due to time constraints, this study did not complete site-directed mutagenesis experiments for the predicted binding site within the ZBP1 3'-UTR. However, such experiments are crucial for verifying the specificity of miRNA targeting: if the inhibitory effect of hsa-miR-361-3p on ZBP1 is abolished following mutation of its predicted binding site in the ZBP1 3'-UTR, it would provide further site-specific confirmation of their direct interaction, ruling out

indirect regulatory effects. Therefore, future studies should include this experiment to strengthen the evidence chain for the hsa-miR-361-3p–ZBP1 regulatory axis and enhance the robustness of the conclusions. Additionally, this research was conducted solely using the A549 in vitro cell model, lacking in vivo validation in animal models. This limits insights into the regulatory role of hsa-miR-361-3p on the ZBP1 pathway and inflammatory responses within a whole-organism physiological context, and the in vivo applicability of the findings remains to be confirmed. The study also measured outcomes at only a single time point, failing to capture the complete expression kinetics of IFNB1, inflammatory factors, and pathway proteins, thus limiting a comprehensive understanding of the dynamic changes these molecules undergo during different stimulation phases. Furthermore, miRNA-mediated regulation of target genes often involves cooperative effects of multiple molecules. This study focused exclusively on hsa-miR-361-3p and did not explore other miRNAs that might coregulate ZBP1, resulting in an incomplete depiction of the miRNA regulatory network governing ZBP1.

5. Prospects and Summary

Future work could consider establishing an influenza virus mouse infection model to observe the effects of overexpressing or knocking down hsa-miR-361-3p on lung inflammatory storms and ARDS in mice, thereby validating the in vivo regulatory function of hsa-miR-361-3p. Multi-timepoint dynamic detection experiments could be conducted to profile the expression change curves of IFNB1, ZBP1, RIPK3/MLKL, and inflammatory factors (e.g., CXCL10, IL-8) following Poly I:C stimulation, clarifying the temporal sequence of regulation among these molecules. Based on the negative regulatory function of hsa-miR-361-3p, further research could investigate the interventional effects of its mimic or inhibitor on influenza-associated inflammatory storms, assessing its potential as a clinical target for anti-inflammatory therapy.

The core innovation of this study lies in the first discovery and validation of the novel hsa-miR-361-3p–ZBP1 regulatory axis. On one hand, through combined multi-database screening and experimental validation, it clearly defines the direct targeting relationship of hsa-miR-361-3p on ZBP1, supplementing the understanding of ZBP1 expression regulation mechanisms. On the other hand, it reveals the downstream impact of this axis on the ZBP1–RIPK3–MLKL inflammatory pathway, providing a new molecular target and research direction for understanding the regulation of influenza-associated inflammatory storms.

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