**H9N2 avian influenza virus enhances the immune responses of BMDCs by
down-regulating miR29c**

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ABSTRACT

Avian influenza virus (AIV) of the subtypes H9 and N2 is well recognised and caused outbreaks- due to its high genetic variability and high rate of recombination with other influenza virus subtypes. The pathogenicity of H9N2 AIV depends on the host immune response. Dendritic cells (DCs) are major antigen presenting cells that can significantly inhibit H9N2 AIV replication. MicroRNAs (miRNAs) influence the ability of DCs to present antigens, as well as the ability of AIVs to infect host cells and replicate. Here, we studied the molecular mechanism underlying the miRNA-mediated regulation of immune function of mouse DCs. We first screened for and verified the induction of miRNAs in DCs after H9N2 AIV stimulation. We also constructed miR29c, miR339 and miR222 over-expression vector and showed that only the induction of miR29c lead to a hugely increased expression of surface marker MHCII and CD40. Whilst the inhibition of miR29c, miR339 and miR222 in mouse DCs would repressed the expression of DCs surface markers. Moreover, we found that miR29c stimulation not only up-regulate MHCII and CD40, but also enhance the ability of DCs to activate lymphocytes and secrete cytokines IL-6 or TNF- α . Furthermore, we found that *Tarbp1* and *Rfx7* were targeted and repressed by miR29c. Finally, we revealed that the inhibition of miR29c marvelously accelerated virus replication. Together, our data shed new light on the roles and mechanisms of miR29c in regulating DC function and suggest new strategies for combating AIVs.

Keywords: H9N2 AIV, miR29c, dendritic cells, target gene, immune regulation

Introduction

The H9N2 subtype avian influenza virus (AIV), classified as a low pathogenic AIV, is still prevalent since its isolation in 1966 [1, 2]. It has high genetic variability and has shown both increases in virulence and ability to cross the host barrier, including transmission to swine and mammals, such as hamsters and ferrets [3-8]. The frequent recombination of H9N2 AIV plays a major role in reassorting new AIV strains that enables them to cross the interspecies barrier[4, 6]. Six segments of the H7N9 virus are from H9N2 origin. But again H9N2 is not a pandemic strain. Since the H7N9 and H10N8 AIV outbreaks in 2013 resulted from recombination between H9N2 and other influenza subtypes [4, 9], H9N2 AIV is a subject of intense research.

There is a constant struggle between viruses and the host immune system, and the pathogenicity of a virus is determined not only by its characteristics but also by the host immune response [10]. Dendritic cells (DCs), the professional and effective antigen-presenting cells *in vivo*, play an essential role in the innate immune response [11]. AIV infection affects the maturation, antigen presenting ability, and cytokine secretion of DCs [12]. Hundreds of host proteins have been characterised and a functional map of host-influenza interactions has been drawn for epithelial cells. The binding of pathogen-associated molecular patterns to receptors expressed by DCs may activate DCs [13], but it remains unclear how AIVs produce changes in DCs and how DCs respond to AIV infection.

MicroRNAs (miRNAs) have emerged as key regulators of diverse biological processes, including innate immune responses [14, 15]. miRNAs affect the development of DCs and

their ability to present antigens and secrete cytokines [16]. For example, the disruption of miR155 caused defective DC-mediated antigen presentation and reduced the numbers of germinal center B cells and Th2 T cell responses [17]. In addition, miR148 and miR152 can influence DC functions, including the capture of antigens via Toll-like receptors and the processing and presentation of antigens to T cells. Furthermore, AIV infection leads to the differential expression of cellular miRNAs in chickens and mice, and miR491 and miR654 inhibit the replication of H1N1 virus through binding to PB1 in MDCK cells [18]. The purpose of our study was to investigate how miRNAs regulate the immune response of DCs to H9N2 AIV.

MATERIALS AND METHODS

Virus and animals

Influenza A virus (A/duck/Nanjing/01/1999(H9N2)) was provided by the Institute of Animal Husbandry and Veterinary Medicine, Jiangsu Academy of Agricultural Science (Nanjing, China). Allantoic fluid was concentrated 10-fold (10^9 egg infectious doses 50 (EID₅₀) / 0.1 ml) and purified on a discontinuous sucrose density gradient as described [19]. The viral 50% -tissue culture infectious dose (TCID₅₀) of the purified H9N2 was calculated using the Reed and Muench method[20]. SPF C57BL/6 and BALB/c mice were obtained from Comparative Medical Center of Yang Zhou University.

Ethics Statement

This study was approved by the Ethical Committee of Animal Experiments of the College of Veterinary Medicine, Nanjing Agricultural University. All animal care and use

were conducted in strict accordance with the Animal Research Committee guidelines of the College of Veterinary Medicine, Nanjing Agricultural University.

Cell isolation and culture

Bone marrow-derived dendritic cells (BMDCs) were prepared from the femurs and tibias of sacrificed 4-6 wk wild-type male C57BL/6 mice and treated with red blood cells lysing buffer (Beyotime) [12]. Nonadherent granulocytes were removed by discarding the culture medium after 60 h of culture. At day 6, the non-adherent, relatively immature DCs (1×10^6 cells/ml) were harvested and centrifuged to remove debris and dead cells, then transferred into six-well plates, and cultured overnight in complete medium. transfect with different plasmid for subsequent assays. The transfection efficiency of DCs can be proved in supplementary figure 3, which a plasmid with RFP protein were transfect into DCs for 24 h. Transferred cells samples (1×10^6 cells) were washed twice with PBS and incubated at 4°C for 30 min with the following monoclonal antibodies (anti-mouse CD11c (N418), anti-mouse CD40(1C10), anti-mouse CD86(GL1), anti-mouse MHC (major histocompatibility complex) class II (M5/114.15.2) and anti-mouse CD80 antibody(16-10A1), respectively (eBioscience)). At last, cells were analyzed with Fluorescence Activated Cell Sorter (FACS) (BD, FACS Aria) after twice washing.

293T cells were cultured in Earle's modified Eagle's medium containing 10% fetal bovine serum (HyClone), 100 units/ml penicillin, and 100 g/ml streptomycin at 37°C under 5% CO₂, and used for luciferase reporter assays. Cells were transfected using Lipofectame2000 (Invitrogen).

The choice of miRNAs and quantitative PCR validation

Small RNAs were selected from our previous Microarray data [12]. The complete data set for each miRNA have been listed in supplement1. According to microarray result, 1 up-regulated and 8 down-regulated genes were selected for quantitative PCR (qPCR) verification. Small RNAs were purified using the miRNeasy mini kit (Qiagen) and reverse transcribed to cDNA by miScript Reverse Transcriptase. QuantiTect SYBR Green PCR master mix (Qiagen) was used to perform qPCR according to instructions. miRNAs expression was normalized to the internal control 5S rRNA. Primers for 9 selected miRNAs were listed in table 1. All assays were performed in triplicate. Relative expression levels were calculated using the $2^{-\Delta\Delta C_t}$ method [21].

Immune response of BMDCs stimulated by miRNAs

Plasmid construction and phenotypic detection: MiRNAs over-expression vector were constructed based on pSilencer4.1 vector (Invitrogen). Selected miRNAs (miR222, miR29c and miR339) were amplified and then cloned into pSilencer4.1(Invitrogen), whose primers were listed in table 2. The isolation of BMDCs was as previous description. Plasmids were transfected with lipofectamine 2000 reagent (Invitrogen). Immature BMDCs were plated into fresh medium (1×10^6 cells/ml) and transfected with constructed vector (miR222, miR29c and miR339), pSilencer4.1 (negative control) and LPS ($1 \mu\text{g/ml}$, positive control) for 48h. Then cells samples (1×10^6 cells, 1.5 ml tube) were collected, washed twice with PBS and incubated at 4°C for 30 min with the following monoclonal antibodies (anti-mouse CD11c, anti-mouse CD40, anti-mouse CD86, anti-mouse MHC class II and anti-mouse CD80 antibody or the respective isotype controls, respectively). After washing, cells were analyzed with Fluorescence Activated Cell Sorter (FACS) (BD, FACS Aria)

Allogeneic mixed leukocytes reaction (MLR) proliferation assays: The primary T-cell stimulatory capacity of BMDCs was examined in a MLR. Untreated and variously treated BMDCs (pSilencer4.1- stimulated, miR29c-stimulated and LPS-stimulated (1 µg /ml)) were used as the stimulator cells. Allogeneic lymphocytes were obtained from BALB/c as follows. Leukocytes were isolated from the spleens of 4 to 6 week-old allogeneic BALB/c mice with T cell isolation kit (Miltenyi, Bergisch Gladbach, Germany) and cultured in complete RPMI 1640 medium supplemented with 10% FCS in 96-well plates at 37°C for 48 h. Graded numbers of responder cells (1×10^5 cells/well) were added to 96-well round bottomed plates, giving responder: stimulator ratios of 1:1 or 5:1. Cell proliferation assays was conducted with the Cell Counting Kit-8 (CCK-8, Beyotime). Each well received 20 µL CCK-8 solution and was incubated for a further 2 h at 37°C before absorbance measurement at 450 nm. All experiments were conducted in triplicates. The Stimulation Index was calculated using the formula: $SI = (OD_{\text{sample}} - OD_{\text{stimulator cells only}}) / (OD_{\text{responder cells only}} - OD_{\text{blank control}})$ [22].

Cytokines analysis: BMDC culture supernatants were collected at 24 h after treatments (Groups were divided as MLR experiments). Concentrations of TNF-α, IL-6, IL-10 and IL-12p₇₀ in the supernatants were measured using the Quantikine Elisa kit (Boster) according to the manufacturer's instructions. The sensitivity of the assay was 2 pg/ml for TNF-α, and 4 pg/ml for IL-6, IL-10 and IL-12p₇₀.

qRT-PCR validation: BMDCs was cultured and collected at 24 h after treatments with miR29c over-expression vector. Then, qPCR was conducted to determine the alteration of Tarbp1, Rfx7 and Mxd1, targets of miR29c. Primers were listed in Supplementary table 1.

Meantime, small RNAs were collected and evaluated when miR29c, miR222 and miR339 were over-expressed or inhibited. QPCR was performed as previously described with QuantiTect SYBR Green PCR master mix (Qiagen) and 5S rRNA was used as the internal control to normalize miRNAs expression. All assays were performed in triplicate. Relative expression levels were calculated using the $2^{-\Delta\Delta C_t}$ method [21].

MiRNAs inhibiting experiment

To detect whether the phenotypic alteration of BMDCs induced by H9N2 AIV was mediated by miRNAs, miRNAs inhibitors were designed and purchased from Ribbio (Guangzhou, China). MiRNA inhibitor is a chemically modified single chain RNA, which can be easily obtained and used in miRNA function analysis. Each 100 nM miRNAs inhibitor (microOFF™ mmu-miR-29c-3p inhibitor (miR20000536-1-5), microOFF™ mmu-miR-222-5p inhibitor (miR20017061-1-5), microOFF™ mmu-miR-339-5p inhibitor (miR20000584-1-5) and microOFF™ inhibitor Negative Control #24 (miR02201-1-5)) was transfected into BMDCs for 2h, before inactivated H9N2 AIV was added. After another 24 h, BMDCs were collected for phenotypic detection with FACS.

MiRNAs target prediction and validation

Target genes of miR-29c were predicted by online database (miRanda and Targetscan) [23]. Only target genes identified by these two algorithms were considered. Besides, we matched predicted genes with our previous microarray data to narrow the target. The 3'-UTRs of selected target genes (MAX dimerization protein 1 (Mxd1), Radical S-adenosyl methionine domain containing 2 (Rsd2), Claudin 1 (Cldn1), TAR RNA binding protein 1

(Tarbp1), Chemokine (C-X-C motif) ligand 9 (CXCL9) and Regulatory factor X 7 (Rfx7)), harboring the predicted miR-29c binding sequences, were amplified and cloned into pMIR-Report luciferase vector (Ambion, TX), with primers listed in table 2. In the same time, the mutated vector was constructed by overlap PCR methods. The predicted genes target position and mutated position of miR29c was listed in Supplementary table 2. To determine whether miR-29c could repress the expression of target genes, 293T cells were cultured and transfected with pSilencer4.1-miR-29c and a pMIR-Report vector, along with pRL-TK to normalize transfection efficiencies. Luciferase assays were performed using the dual-luciferase reporter assay system kit (Promega), according to the manufacturer's protocol, on a Modulus single-tube multimode reader (Promega).

Western blot assay

Since DCs were the only bridge communicated the innate and acquired immunity, we tried to evaluate how miRNAs affect the TLR and NF- κ B signal pathways by western blot. BMDCs were transfect with miR-29c over expression vector for 24h. Then cells were collected and washed with PBS three times for the next experiments. Western blot detection was performed as our previous article [12]. Mouse P-I κ Ba (#5205, CST), P-p38 (#9216, CST), IRF-3 (ab63468, abcam), IRF-7 (ab70069, abcam), TRAF-3 (ab172240, abcam) and TRAF-6 (ab58369, abcam) were purchased from Abcam or Cell Signaling Technology and detected according to each manufacturer's protocol. Protein bands were visualized using the Super ECL Plus system. GAPDH was used as a loading control (Abcam).

Virus replication experiments

DCs were seeded onto 6-well plates (5×10^5 cells/well) and pretreated with miR-29c inhibition or over-expression plasmid for 24 h. Then, pretreated DCs were inoculated with H9N2 AIV for 1 h at 37 °C. We chose the concentrations ($TCID_{50} = 1$) of H9N2 strain interactions with mouse DCs. After that, DCs were washed five times with 1000 rpm/5 min to eliminate the non-absorbed virus and resuspended in fresh RPMI 1640 medium. After another 24 h, BMDCs were collected and total RNA was extracted with trizol reagents. RT-PCR was then performed according to instructions (TaKaRa). Virus PB1 and NP gene was choose as the target gene and the mouse GAPDH gene was used as the internal parameter (primers were list in Supplementary table 3) [24, 25]. Quantification of the target gene was determined by relative standard curves. PB1, NP and GAPDH plasmids were used to produce a double-standard curve. The mouse GAPDH quantity at each sampling time was taken as the standard to determine the target genes quantity.

Statistical analyses

Data were evaluated by unpaired two-tailed Student's t-test using GraphPad Prism 5, with p values<0.05 considered to be statistically significant. The significance of the data was also determined by one-way ANOVA, followed by Tukey's multiple comparison tests. FACS data were analysed by FlowJo software (FlowJo, China). All data are expressed as mean $\pm$ standard error of the mean.

RESULTS

The miRNAs expression following H9N2 AIV infection

Previously, we studied the influence of H9N2 AIV infection on global RNA expression in mice. Of total 349 conserved miRNAs, we found that 9 miRNAs were significantly up-regulated by viral infection, whilst 8 were down-regulated (Figure 1A and Supplementary table 4). Then, we selected 8 down-regulated miRNAs and 1 up-regulated miR222 (positive control) for further experiment. Quantitative PCR (qPCR) showed that miR181b1, miR339, miR375, miR29c and miR24-1 were significantly decreased, which was consistent with the microarray results (Figure 1B).

The immune function of miR29c in regulating mice BMDCs

Recent studies have shown that miRNAs regulate the immune responses of BMDCs, we tried to examine the functions of these miRNAs in BMDCs. There are three standards for evaluating the immune function of BMDCs, including phenotypic alterations, the ability to activate T lymphocytes and the ability to secrete cytokines [26]. We first constructed the miR339, miR29c and miR222 over-expression vector based on pSilencer4.1 and validated by digesting with restriction enzyme Hind III and BamHI (Supplementary figure 1). Then, we examined the phenotypic changes in BMDCs transfected with miR339, miR29c and miR222. Fluorescence-activated cell sorting (FACS) showed that miR222 significantly increased the mean fluorescence intensity (MFI) of MHCII-positive cells, compared with pSilencer4.1 group (Figure 2A and 2B). Whilst, only miR29c increased the MFI of CD40- and MHCII-positive cells, which had no significant effects on CD80- and CD86-positive cells. Moreover, miR29c stimulated DCs showed enhanced stimulation at a ratio of 1:1 or 1:5, and

they expressed higher levels of interleukin (IL)-6 and tumor necrosis factor (TNF)- α than did the pSilecer4.1-transfected controls ($P<0.05$), and had no effect in inducing IL-10 and IL-12 (Figure 2C and 2D). Besides, we performed qPCR to evaluate the expression of miRNAs when miR29c, miR222 and miR339 were over-expressed or inhibited. Results indicated that the expression of miR29c, miR222 and miR339 was all tremendously increased in miRNAs over-expression groups, as well as a significant decreased expression of miR29c and miR339 in their inhibited groups (Figure 2E). However, the expression of miR222 showed no difference when it was inhibited. In addition, we found that over expressed miR29c could significantly decrease the expression of its target genes Tarbp1 and Rfx7, compared with pSilencer4.1 group. However, the repression of miR29c greatly up-regulated the target Tarbp1 and Rfx7, compared with blank group (Figure 2F).

Inhibition of endogenous miRNA function blocked inactivated avian influenza virus-induced phenotypic alterations in BMDCs

Previous studies demonstrated that H9N2 AIV can influence the phenotype of BMDCs. Thus, we investigated whether the virus-induced surface marker up-regulation on DCs are mediated by miRNAs. To test this hypothesis, miRNA inhibitors were synthesized and added to DCs to repress endogenous miRNAs before inactivated AIV stimulation. In general, FACS revealed that the inhibition of endogenous miR339 and miR29c significantly repressed the expression of co-stimulatory molecules (CD80/CD86 and CD40) and MHCII, which was induced by inactivated AIV. Whilst, the inhibition of endogenous miR222 only increased the MFI of CD40 and MHCII, without changing the surface marker CD80 and CD86 (Figure 3A and 3B).

MiR29c target prediction and validation

Numerous studies have shown that miRNAs exert their functions by inhibiting the expression of target genes. To study the mechanism of action of miR29c, we used Miranda and Targetscan to predict miR29c target genes, considered only the overlap of both algorithms and further matched the prediction based on our previous microarray data. In the end, we selected *Mxd1*, *Rsad2*, *Cldn1*, *Tarbp1*, *Rfx7* and *Cxcl9* for further testing. Luciferase reporter assays showed that *Mxd1*, *Tarbp1*, and *Rfx7* significantly decreased luciferase activity ($P<0.05$), whilst *Rsad2*, *Cldn1* and *Cxcl9* non-significantly reduced luciferase activity (Figure 3C, Supplementary table 2 and Supplementary figure 2).

Effects on signalling pathways stimulated by miR29c

We previously demonstrated that H9N2 AIV activates interferon (IFN) regulatory factor (IRF)-7 and TNF receptor-associated factor (TRAF)-6. Thus, we continued to explore the signalling pathways that may be activated by miR29c. On the one hand, we found that the protein expression levels of p-IkBa and p-P38 in the miR29c over-expression group were higher than that in the pSilencer4.1 group (Figure 4B and 4C). On the other hand, over expressed miR29c greatly reduced the levels of TRAF-3/6 and IRF-3/7, compared with the pSilencer4.1 group (Figure 4A and C).

Effects of miR29c inhibition or over-expression on virus replication

Since miR29c, decreased by H9N2 AIV stimulation, significantly affected the immune function of DCs, we further evaluated that whether miR29c could influence the replication of H9N2 AIV. We first created standard curves for PB1, NP and GAPDH (Figure 4D) and then conducted absolute qPCR to detect the replication of H9N2 AIV. Our results show that

279 the inhibition of miR29c significantly accelerated viral replication from 3 to 24 h, whilst the
280 over-expression of miR29c had no effect on the replication of virus (Figure 4D).

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DISCUSSION

The interactions between cellular factors, such as miRNAs, and H9N2 AIV are important for AIV infection. In this report, we focused on 9 miRNAs whose expressions were altered by AIV in BMDCs. DCs represent a central element in the generation and maintenance of immune responses [16]. Our study suggests that the immune function of DCs, including phenotypic alteration and T lymphocyte activation, was greatly stimulated by miR29c. Members of the miR29 family can be activated by IFN signaling, suggesting a role for these miRNAs in the immune system and host-pathogen interactions, especially in response to viral infections [27]. For example, the influenza virus induced miR29c, which inhibited the innate immune response through the protection of A20 mRNA [28]. Since the level of miR29c is correlated with immune function, we evaluated the potential role of miR29c in regulating DC immune responses. Our research suggests that increased miR29c enhanced the DC immune responses by increasing the expression of CD40 and MHCII, whilst the inhibition of endogenous miR29c had the opposite effect. The surface markers are characteristics of fully mature DCs and represent different functionalities [29]. CD40 is a member of the TNF receptor family, which promotes the development of T cell immune responses by binding to CD40L [30]. Thus, miR29c may enhance DC-T cell interactions by up-regulating DC surface maturation molecules. Over-expression of miR29c also up-regulated the ability of DCs to activate lymphocytes and secrete inflammatory cytokines IL-6 and TNF- α . DCs promote Th1 responses via IL-12 [31], while our data suggest that miR29c could slightly increase the expression of IL-12 (difference was no significance).

Activation of the innate immune system by IFN- α is crucial for host defence against virus infection [32]. The transcription of IFN- α is controlled primarily by members of the IFN regulatory factor family (specifically, IRF-3 and IRF-7) [33, 34]. Viral infection resulted in the activation of IRF-3 and high-level secretion of IFN- α on non-plasmacytoid DCs [35, 36]. Our results show that IRF-3 and IRF-7 were down-regulated by miR29c, suggesting that miR29c inhibits IFN- α production. Besides, we also found that TRAF-6 and TRAF-3 were both decreased in miR29c over-expression group, indicating that the production of TNF- α might be inhibited by miR29c over-expression.

Most of the genes targeted by miR29c are involved in immune and inflammatory responses. In addition, *Mxd1*, *Rsad2*, *Cldn1*, *Tarbp1*, *Rfx7* and *Cxcl9* were especially distinctive in the gene interaction network based on the identified genes. Our results show that miR29c significantly repressed the expression of *Tarbp1* and *Rfx7*. *TARBP1*, a double-stranded RNA-binding protein, was always highly expressed in disease situation[37]. Whilst, regulatory factor X (*Rfx*) transcription factors are likely involved in the pathogenesis of serious human diseases[38]. These two target genes may represent a mechanism for miR29c to regulate the immune function of DCs. In summary, our results suggest that cellular miRNAs are important factors in regulating mouse DC function to defend against viral infection and they provide a deeper understanding of the mechanisms underlying DC's immune function. Here, we demonstrated a previously unidentified role for miR29c in the regulation of mice immune responses of DCs. We suggest that miR29c mediates the H9N2 AIV-induced immune responses of DCs by targeting *Tarbp1* or *Rfx7* (Figure 4E). Thus,

324 miR29c may be a new immune reinforcing agent that can be used to activate DCs for defence
325 against H9N2 AIV.

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DECLARATIONS

Ethics approval and consent to participate

This study was approved by the Ethics Committee for Animal Experiments of the College of Life Science, Nanjing Agricultural University. All animal care and use were conducted in strict accordance with the Animal Research Committee Guidelines of the College of Life Science, Nanjing Agricultural University.

AUTHORS' CONTRIBUTIONS

Jian Lin design and carried out the molecular genetic studies, bio-information analyses, data analyses and manuscript drafting. Jing Xia and Ya T Chen developed chicken dendritic cells and performed qRT-PCR analyses. Keyun Zhang and Yan Zeng anticipated in the interpretations of the data and participated in its design and coordination experiment in the lab. Qian Yang is the principal investigator of the project, who conceived the study, participated in its design and helped to draft the manuscript. All authors read and approved the final manuscript.

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FIGURE LEGEND

Figure 1. qPCR analysis of select miRNAs following H9N2 AIV stimulation.

A: The heat map of different expressed miRNAs selected from microarray data (1: blank DCs; 3:H9N2 AIV treated DCs; 5: Inactivated H9N2 AIV treated DCs)

B: The expression levels of miRNAs stimulated by H9N2 AIV ($*P < 0.01$, the significance of the data was determined by one-way ANOVA with Duncan test.

Figure 2. The immune function of BMDCs stimulated by miR29c.

A: Flow cytometric analysis of the phenotypic alterations (CD40, CD80/86, and MHCII) in DCs stimulated by miR29c, miR339 and miR222 . (Isotype: IgG2a for CD40, IgG1 for CD80 and CD86, IgG2b for MHCII; Blank: DCs without any treatment; Lipo: DCs added the same lipofectame as transfection groups; miR29c, miR339 and miR222 over-expression plasmids transfected DCs; pSilencer group: plasmid pSilencer4.1 transfected DCs; LPS: Positive control, 1 μ g/ml LPS).

B: The MFI of CD40, CD80/86, and MHCII. ($*P < 0.05$ or $**P < 0.01$ the significance of the data was determined by one-way ANOVA with Tukey's multiple comparison test).

C: miR29c-stimulated BMDCs stimulated the proliferation of naive T cells in MLR. The stimulator cells were BMDCs stimulated with or without miR29c, pSilencer, or LPS at 37 °C for 24 h. Significant differences between the treated and pSilencer4.1 groups are expressed as $*P < 0.05$ or $**P < 0.01$, determined by one-way ANOVA with Tukey's multiple comparison test.

D: Cytokine release from BMDCs stimulated by miR29c was measured by ELISA. Data for IL-6, IL-10, IL-12, and TNF- α are shown as means $\pm$ SD of three samples. Significant differences between the treated and pSilencer4.1 groups are expressed as $*P<0.05$ or $**P<0.01$, determined by one-way ANOVA with Tukey's multiple comparison test.

E: Results of the qPCR analysis of miRNAs when miR29c, miR222 and miR339 were over-expressed or inhibited. Significant differences between the treated and pSilencer4.1 groups are expressed as $*P<0.05$, $**P<0.01$ or $***P<0.001$, determined by one-way ANOVA with Tukey's multiple comparison test.

F: Results of the qPCR analysis of select target genes (*Mxd1*, *Tarbp1* and *Rfx7*) following stimulation by miR29c. Significant differences between the treated and pSilencer4.1 groups are expressed as $*P<0.05$, $**P<0.01$ or $***P<0.001$, determined by one-way ANOVA with Tukey's multiple comparison test.

Figure 3. Results of miRNA inhibitor treatment and dual luciferase reporter assays for miR29c and its target genes.

A: The expressions of CD40, CD80/86, and MHCII on BMDCs when miR29c, miR339 and miR222 were inhibited (100 μ g/ml inhibitor for each miRNA). (Isotype: IgG2a for CD40, IgG1 for CD80 and CD86, IgG2b for MHCII; Blank: DCs without any treatment; Ncortrol+ inactivated H9N2 AIV: DCs first treated with microOFF™ inhibitor Negative Control #24 for 2h, then added inactivated H9N2 AIV; In-miR29c/miR222/miR339+ inactivated H9N2 AIV: DCs first treated with microOFF™ mmu-miR-29c-3p/ miR-222/ miR-339 inhibitor for 2h, then added inactivated H9N2 AIV.)

B: The MFI data for CD40, CD80/86, and MHCII. (* $P < 0.05$ or ** $P < 0.01$ the significance of the data was determined by one-way ANOVA with Tukey's multiple comparison test).

C: The map position of gene Mxd1 and its mutated site targeted to miR29c, and the dual luciferase assay results for miR-29c and its target genes (* $P < 0.05$, ** $P < 0.01$ or *** $P < 0.001$, the significance of the data was determined by one-way ANOVA with Tukey's multiple comparison test).

Figure 4. Regulatory protein expression on BMDCs stimulated by miR29c as determined by Western blotting.

A and B: Western blot results for p-IKKa, p-P38, IRF-3, IRF-7, TRAF-3, and TRAF-6 in cells stimulated by miR29c (lane 1: Blank dendritic cells; lane 2: pSilencer4.1-stimulated cells; lane 3: miR29c-stimulated cells).

C: The expression levels of p-IKKa, p-P38, IRF-3, IRF-7, TRAF-3, and TRAF-6 in cells stimulated by miR29c (the data shown are the means $\pm$ standard error of the mean of double wells from three independent experiments. * $P < 0.05$, ** $P < 0.01$ or *** $P < 0.001$, the significance of the data was determined by one-way ANOVA with Tukey's multiple comparison test).

D: The detection of viral replication in DCs when miR29c was inhibited or over-expressed. (First picture: Standard curve for the PB1 and NP gene from H9N2 AIV with different dilution (10^n , $n=0,-1,-2,-3,-4,-5,-6,-7$)).

Second picture: Standard curve for the mouse GAPDH gene with different dilution (10^n , $n=0,-1,-2,-3,-4,-5,-6,-7$)).

523 Third picture: Replication of H9N2 AIV following pre-treatment of DCs with miR29c from 3

524 h. to 24 h.)

525 E: The summary map of the research strategy

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TABLES

Table 1: qRT-PCR primers used for detecting miRNAs alteration

MiRNA	Mirbasenumber	Sense primer
mmu-miR-222-5p	MIMAT0017061	5' GGGTCAGTAGCCAGTGTAGATCCT 3'
mmu-miR-375-3p	MIMAT0000739	5' TTTGTTCGTTTCGGCTCGCGT 3'
mmu-miR-29c-3p	MIMAT0000536	5' GGCCTAGCACCATTGAAATCG 3'
mmu-miR-146b-5p	MIMAT0003475	5' GGGGTGAGAACTGAATTCCATAGGCT 3'
mmu-miR-687	MIMAT0003466	5' GGGCTATCCTGGAATGCAGCAATGA 3'
mmu-miR-24-1-3p	MIMAT0000219	5' GGTGGCTCAGTTCAGCAGGAAC 3'
mmu-miR-339-5p	MIMAT0000584	5' TCCCTGTCCTCCAGGAGCTCAC 3'
mmu-miR-181b-5p	MIMAT0000673	5' AACATTCATTGCTGTCGGTGGGT 3'
mmu-miR-679-3p	MIMAT0017248	5' GGAGCAAGGTCCTCCTCACAGTAG 3'

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Table 2: Primers used in amplified miRNAs and their target genes

Gene		Sequence	Products
MiRNAs			
miR222	Sense	5' GCGGGATCCAAAGGTAGTTAAGGTTTAT 3'	503 bp
miR222	Anti-sense	5' GCGAAGCTTTGTGTGTGCCTATGTGT 3'	
miR29c	Sense	5' GGGATCCGGACTGCTTCATTCACATC 3'	514 bp
miR29c	Anti-sense	5' GCGAAGCTTACAATTA AAAACCCCCCAC 3'	
miR339	Sense	5' GGGATCCCAAGGTGGTGAGAGCAC 3'	524 bp
miR339	Anti-sense	5' GCGAAGCTTGATTAGGACCC CAGAGG 3'	
Target genes			
Mxd1	Sense	5' ATAGAGCTCGGTGAGGAAGACGCAGGTA3'	557 bp
Mxd1	Anti-sense	5' GCAAGCTTAGAAGGGCCACAGGAAAC 3'	
Rsad2	Sense	5' ATAGAGCTCCTATCTCCTGCGACAGCT 3'	766 bp
Rsad2	Anti-sense	5' GCAAGCTTCTCCTCTGAGAACCGAAC 3'	
Cldn1	Sense	5' ATAGAGCTCGACTGTTGATGATGGTTA 3'	436 bp
Cldn1	Anti-sense	5' GCAAGCTTTGAGTTCATAAGGAAGTCT 3'	
Tarbp1	Sense	5' ATAGAGCTCAGCACCACCACCTCATTC 3'	511 bp
Tarbp1	Anti-sense	5' GCAAGCTTGCCTCCAGGACAAGTTCA 3'	
Rfx7	Sense	5' ATAGAGCTCGATGGGTTGGAAGGAGTT 3'	698 bp
Rfx7	Anti-sense	5' GCAAGCTTATCTGAGGGTAGCGGTGT 3'	
Cxcl9	Sense	5' ATAGAGCTCACACTGAAGAACGGAGAT 3'	487 bp
Cxcl9	Anti-sense	5' GCAAGCTTTATGAAGGAAAGGGACAC 3'	

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Table 1 qRT-PCR primers used for detecting miRNAs alteration

MiRNA	Mibase number	Sence primer
mmu-miR-222-5p	MIMAT0017061	5'GGGTCAGTAGCCAGTGTAGATCCT3'
mmu-miR-375-3p	MIMAT0000739	5'TTTGTTCGTTTCGGCTCGCGT3'
mmu-miR-29c-3p	MIMAT0000536	5'GGCGTAGCACCATTTGAAATCG3'
mmu-miR-146b-5p	MIMAT0003475	5'GGGGTGAGAACTGAATTCCATAGGCT3'
mmu-miR-687	MIMAT0003466	5'GGGCTATCCTGGAATGCAGCAATGA3'
mmu-miR-24-1-3p	MIMAT0000219	5'GGTGGCTCAGTTCAGCAGGAAC3'
mmu-miR-339-5p	MIMAT0000584	5'TCCCTGTCCTCCAGGAGCTCAC3'
mmu-miR-181b-5p	MIMAT0000673	5'AACATTCATTGCTGTCGGTGGGT3'
mmu-miR-679-3p	MIMAT0017248	5'GGAGCAAGGTCCTCCTCACAGTAG3'

Table 2 Primers used in amplified miRNAs and their target genes

Gene		Sequence	Products
MiRNAs			
miR222	Sence	5' GCGGGATCCAAAGGTAGTTAAGGTTTAT3'	503 bp
miR222		5' GCGAAGCTTTGTGTGTGCCTATGTGT3'	
Anti-sence			
miR29c	Sence	5' GGGATCCGGACTGCTTCATTCACATC3'	514 bp
miR29c		5' GCGAAGCTTACAATTAAAACCCCCCAC3'	
Anti-sence			
miR339	Sence	5' GGGATCCCAAGGTGGTGAGAGCAC3'	524 bp
miR339		5' GCGAAGCTTGATTAGGACCCCAGAGG3'	
Anti-sence			
Target genes			
Mxd1	Sence	5' ATAGAGCTCGGTGAGGAAGACGCAGGTA3'	557 bp
Mxd1	Anti-sence	5' GCAAGCTTAGAAGGGCCACAGGAAAC3'	
Rsad2	Sence	5' ATAGAGCTCCTATCTCCTGCGACAGCT3'	766 bp
Rsad2	Anti-sence	5' GCAAGCTTCTCCTCTGAGAACCGAAC3'	
Cldn1	Sence	5' ATAGAGCTCGACTGTTGATGATGGTTA3'	436 bp
Cldn1	Anti-sence	5' GCAAGCTTTGAGTTCATAAGGAAGTCT3'	
Tarbp1	Sence	5' ATAGAGCTCAGCACCACCACCTCATTC3'	511 bp
Tarbp1	Anti-sence	5' GCAAGCTTGCCTCCAGGACAAGTTCA3'	
Rfx7	Sence	5' ATAGAGCTCGATGGGTTGGAAGGAGTT3'	698 bp
Rfx7	Anti-sence	5' GCAAGCTTATCTGAGGGTAGCGGTGT3'	
Cxcl9	Sence	5' ATAGAGCTCACACTGAAGAACGGAGAT3'	487 bp
Cxcl9	Anti-sence	5' GCAAGCTTTATGAAGGAAAGGGACAC3'	

Figure.1

Figure-1

Figure.2

Figure-2

Figure.3

Figure-3

Figure.4

Figure-4