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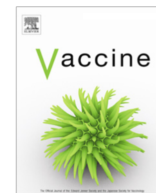

H9N2 avian influenza virus enhances the immune responses of BMDCs by down-regulating miR29c

Jian Lin [a], Jing Xia [a], Ya T. Chen [a], Ke Y. Zhang [a], Yan Zeng [a], Qian Yang [b,*]

[a] College of Life Science, Nanjing Agricultural University, Weigang 1, Nanjing, Jiangsu 210095, PR China
[b] College of Veterinary Medicine, Nanjing Agricultural University, Weigang 1, Nanjing, Jiangsu 210095, PR China

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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 AIVstimulation. 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-a. 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.

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1. 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

* Corresponding author.
E-mail addresses: linjian@njau.edu.cn (J. Lin), 2014816155@njau.edu.cn (J. Xia), 2013816153@njau.edu.cn (Y.T. Chen), keyunzhang@njau.edu.cn (K.Y. Zhang), zengyan@njau.edu.cn (Y. Zeng), zxbyq@njau.edu.cn (Q. Yang).

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[18]. The purpose of our study was to investigate how miRNAs regulate the immune response of DCs to H9N2 AIV.

2. Materials and methods

2.1. 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 (Nan Jing, 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** ($TCID_{50}$) 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.

2.2. 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.

2.3. 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 Fig. 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% CO_2, and used for luciferase reporter assays. Cells were transfected using Lipofectame2000 (Invitrogen).

2.4. 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 supplement 1. 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 $2{-}\Delta\Delta Ct$ method [21].

2.5. 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 μg/ml, positive control) for 48 h. 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).

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′ GCGAAGCTTACAATTAAAACCCCCCAC 3′	
miR339 Sense	5′ GGGATCCCAAGGTGGTGAGAGCAC 3′	524 bp
miR339 Anti-sense	5′ GCGAAGCTTGATTAGGACCCCAGAGG 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′	

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′ TTTGTTCGTTCGGCTCGCGT 3′
mmu-miR-29c-3p	MIMAT0000536	5′ GGCGTAGCACCATTTGAAATCG 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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