Exosomal miR-181a-5p Protects against MG Infection by Targeting PPM1B and Activating the TLR2-Mediated MyD88/NF-κB Pathway

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Research

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Abstract

Background

*Mycoplasma gallisepticum* (MG) is one of the most important pathogens that causes chronic respiratory disease (CRD) in chickens. Exosomes secreted from cells have been well documented to deliver miRNAs to recipient cells to modulate cellular function. The purpose of this current study was to explore the functions of exosomal miR-181a-5p in MG infection and the underlying mechanisms.

Results

Here, we found that miR-181a-5p expression in *vivo* and in *vitro* was significantly upregulated after MG infection. Exosomes enriched in MG-infected chicken type-II pneumocytes (CP-II) could selectively load miR-181a-5p and transfer it into recipient DF-1 cells. PPM1B was further identified as the target gene of miR-181a-5p. Overexpression of miR-181a-5p and/or knockdown of PPM1B activated the TLR2-mediated MyD88/NF-κB signaling pathways, whereas inhibition of miR-181a-5p and the overexpression of PPM1B led to the opposite results. In addition, depressing miR-181a-5p significantly reduced the expression of tumor necrosis factors alpha (TNF-α) and interleukin-1β (IL-1β) by MG-induced. Upregulated miR-181a-5p promoted cell proliferation and cell cycle progression and inhibited apoptosis to resist MG infection. Moreover, overexpression of miR-181a-5p significantly depressed pMAG1.2 expression by directly inhibiting PPM1B.

Conclusions

Taken together, we conclude that the newly identified primary CP-II cells exosomal miR-181a-5p activates the TLR2-mediated MyD88/NF-κB pathway by directly targeting PPM1B to promote pro-inflammatory cytokines expression for defending against MG infection in recipient DF-1 cells.

1. Introduction

*Mycoplasma gallisepticum* (MG) is the primary etiologic agent of chronic respiratory disease (CRD) in poultry and is known to invade, survive and multiply inside a variety of non-phagocytic cells such as chicken red blood cells, HeLa cells, and chicken fibroblasts [1–3]. MG has been proven to colonize its host mainly via the mucosal surfaces of the respiratory tract, causing air sacculates within a few days, and disseminates throughout the body [4]. Once avian is infected with MG, it is difficult to eradicate the effects on cells adhesion, metastasis, proliferation and differentiation in whole body as its long incubation period and strong infectivity [5]. Therefore, CRD induced by MG causes millions of economic losses to the poultry industry every year. MicroRNAs (miRNAs), as 19–22 nucleotide non-coding RNAs, involve in multiple important biological processes by binding partially complementary sequences of its target gene mRNA 3'-untanslated region (3'-UTR) and repressing target gene functions. Many studies have demonstrated that miRNAs play important roles in resisting kinds of disease in birds, like Marek's disease virus, avian influenza virus, bursal disease virus and others virus infection [6–8]. Our previous studies have also highlighted the critical role of miRNAs during MG-HS (*Mycoplasma gallisepticum*-Huangshi) infection in lung tissue of chicken embryo, including miR-19a, miR-130b-3p, miR-451 ect [9–13].
miR-181a-5p belongs to the miR-181 family, and its nucleic acid sequence is highly conserved among species [14]. Several studies suggest that miR-181a differentially expressed in a variety of poultry diseases, such as avian influenza [15,16], and Marek's disease [17,18], which indicates miRNA-181a may play an important role among various pathogenic microorganism infection. More interesting, our previous miRNAs deep sequencing showed miR-181a-5p was increased in the lungs of MG-infected chicken embryo [19], indicating its potential significant role in MG infection. Mg$^{2+}$/Mn$^{2+}$-dependent protein phosphatase 1B (PPM1B or PP2C) is a member of the Ser/Thr protein phosphatase (PP2C) family [20,21]. PPM1B regulates various cellular functions by dephosphorylating several substrates proteins, including TAK1 (also known as MAP3K7), PPARγ, p53, and IKKβ (also known as IKBKB), there by terminating IKKβ-mediated NF-κB activation [22,23]. Ectopic expression of PPM1B significantly suppressed proliferation and tumorigenicity in bladder cancer cells in vitro and in vivo [24]. These studies indicate that PPM1B plays a potential role in regulating various signaling pathways and cellular functions by affecting many downstream targets.

Exosomes are the small membranous vesicles (30–150 nm) released by various of cells, including B lymphocytes, endothelial cells, epithelial cells, and body fluids. Increasing number of evidences indicate that exosomes play an important role in mediating cell-cell communication. Cell-derived exosomes cargo contains a variety of miRNAs, proteins, mRNAs and DNA, which can work locally or be stably transferred to recipient cells [25,26]. Among them, miRNA is the most investigated and has been shown to be involved in many physiological and pathological processes [27,28]. These findings prompted us to investigate whether miR-181a-5p could be loaded and transferred by exosomes and its role in immune responses to MG both in vivo and in vitro.

In this study, we found that miR-181a-5p expression was significantly upregulated in MG-HS-infected chicken embryonic lungs and DF-1 cells. Exosomal miR-181a-5p derived from CP-II cells could be absorbed by DF-1 cells. And then the correlation between miR-181a-5p expression and MG infection progression was analyzed. The results demonstrated that miR-181a-5p played an important role in cell proliferation, cell cycle progression, and regulation of apoptosis in MG infection by targeting PPM1B. Furthermore, up-regulated miR-181a-5p could promote inflammatory response by down-regulating the expression of PPM1B via activation of the TLR2-mediated MyD88/NF-κB pathway to resist to MG infection.

2. Results

2.1. miR-181a-5p Expression in Vivo and in Vitro Is Significantly Elevated after MG Infection

Our previous miRNAs deep sequencing showed miR-181a-5p expression was increased in the lungs of MG-infected chicken embryos [19]. To verify this result, we evaluated miR-181a-5p expression variation after MG infection in both chicken embryos and DF-1 cells. Chicken embryos were infected with MG on the 9th day of incubation (the eggs were hatched for a total of 21 days). On days 6 to 8 after infection (equivalent to days 15–17 of egg hatching), compared with non-infected groups, miR-181a-5p levels were increased by greater than 2-fold in MG-infected chicken embryos ($p < 0.01$; Figure 1A). Furthermore, the average expression level of miR-181a-5p in MG-infected DF-1 cells increased more than 5-fold compared to uninfected cells (Figure 1B). These results indicates that miR-181a-5p was significantly increased in MG-infected chicken embryos and DF-1 cells and may have an important potential role in MG infection.
2.2. Exosomes Derived from CP-II Cells Load and Transfer miR-181a-5p into DF-1 Cells

To investigate whether miR-181a-5p could mediate cell-cell communication through exosomes in MG infection, we first collected the conditioned medium of CP-II cells at 48 hours in vitro culture. Differential centrifugation was adopted to separate the exosomes from the CP-II cells medium. Exosomes were identified as rounded particles with approximately 80–100 nm size and a double-layer membrane by transmission electron Microscopy (Figure 2A). CD63 and CD9, the surface markers of exosome, were confirmed by western blot (Figure 2B). These results indicate that exosomes secreted by CP-II cells isolated successfully.

We examined the expression levels of miR-181a-5p in MG-infected CP-II cells and its derived exosomes. Compared with normal cells, the expression level of miR-181a-5p in infected CP-II cells was significantly increased (Figure 2C). Similarly, miR-181a-5p level was significant higher in exosomes isolated from MG-infected CP-II cells relative to control group (Figure 2D).

To further determine whether exosomes with miR-181a-5p transfers from CP-II cells into the recipient DF-1 cells. Firstly, the primary CP-II cells were transfected with 500 ng miR-181a-5p mimics plate labeled with Cy3 red fluorescence (Cy3-miR-181a-5p) for 48h at 75 cm² flask, and then cell culture medium was used to extract exosomes. Secondly, the extracted exosomes were labeled by PKH67 (a green fluorescence) and incubated at DF-1 cells for 48h at 37 °C. Both Cy3 fluorescence and PKH67 lipid dye were observed in the incubated DF-1 cells (Figure 2E). The above results indicate that exosomes derived from CP-II cells can package miR-181a-5p and transferred it into DF-1 cells.

2.3. miR-181a-5p Directly Targets PPM1B in Recipient DF-1 Cells

As above, we have identified that miR-181-5p could be absorbed by recipient DF-1 cells. To investigate the potential regulatory mechanism of miR-181a-5p in MG-HS infection, a combination of three algorithms, including Targetscan, miRDB, and miRTarBase were used. As a result, PPM1B was identified as a target gene of miR-181a-5p by all programs. In addition, Targetscan showed that the sequence of target sites in the 3'-UTR of PPM1B is highly conserved across species (Figure 3A). RNAhybrid revealed that the minimum free energy (MFE) of the RNA duplex was -22.6 kcal/mol (Figure 3B), suggesting a stable combination between miR-181a-5p and PPM1B. The expression of PPM1B in chicken embryos and DF-1 cells was then examined. On days 6 to 8 after infection (equivalent to days 15–17 of egg hatching), compared with normal solid tissues, PPM1B levels were significantly decreased in MG-infected chicken embryos (Figure 3C). Furthermore, the expression level of PPM1B in MG-infected DF-1 cells was decreased more than 1.5-fold compared to uninfected cells (Figure 3D). Western blot also showed that PPM1B was down-regulated after MG infection. This result shows contrary expression pattern compared with miR-181a-5p and indicates that PPM1B may be negatively related to miR-181a-5p expression after MG infection both in vivo and in vitro.

In order to verify whether PPM1B was the target gene of miR-181a-5p, the luciferase reporter gene plasmid containing the potential binding site for miR-181a-5p in 3'-UTR was constructed (Figure 4A). The wild-type or mutant 3'-UTR luciferase constructs was transfected into DF-1 cells and treated with miR-181a-5p mimics or mimic NC. We found that transfection of the miR-181a-5p mimics decreased the luciferase activity of Luc-PPM1B 3'-UTR and transfection of miR-181a-5p inhibitor increased the luciferase activity. However, there was
no effect on the mutant 3'-UTR type (Figure 4B). Collectively, miR-181a-5p could directly bind to the nucleotide sequence in the 3'-UTR of *PPM1B*.

To further investigate whether *PPM1B* was regulated by miR-181a-5p, miR-181a-5p mimics and mimics-NC or inhibitor and inhibitor-NC were transiently transfected into DF-1 cells for 48 h. when miR-181a-5p expression was increased (Figure 5A), PPM1B expression was significantly down-regulated at both mRNA and protein levels (Figure 5B and 5C). On the contrary, when miR-181a-5p expression was decreased (Figure 5D), the expression of PPM1B was significantly up-regulated at both mRNA and protein levels (Figure 5E and 5F).

Taken together, all of the above results indicates that PPM1B is a direct target of miR-181a-5p and its expression is negatively regulated by miR-181a-5p.

2.4. NF-κB Signaling Pathway Activated by miR-181a-5p Overexpression

Bioinformatics prediction using Gene Ontology (GO), the Kyoto Gene and Genomic Encyclopedia (KEGG) database indicated that miR-181a-5p was involved in the NF-κB pathway. miR-181a-5p mimics or inhibitor was transiently transfected into DF-1 cells for 48 h, and qPCR evaluated the expression level of MAP3K7, IKBKB and NF-κB at the mRNA level. After transfection of miR-181a-5p mimics, the expression of MAP3K7, IKBKB, NF-κB were significantly up-regulated comparing to the NC group (Figure 6A). In contrast, the expression of MAP3K7, IKBKB and NF-κB were significantly down-regulated after transfection with miR-181a-5p inhibitor (Figure 6B). MAPK and IKBKB proteins further play a regulatory role through phosphorylation [29]. And we found that overexpression of miR-181a-5p induced phosphorylation of MAP3K7 and IKBKB proteins comparing to the NC group (Figure 6C). In contrast, p-MAP3K7 and p-IKBKB were high manifest upon knockdown of miR-181a-5p by western blot (Figure 6D). To further investigate whether NF-κB was activated by miR-181a-5p overexpression, the NF-κB p65 expression was analyzed by an immunofluorescence. When DF-1 cells were transfected with the miR-181a-5p mimics, p65 (red) protein was observed to enter the nucleus from the cytosol, whereas the other groups had no clear change (Figure 6E). Taken together, our results shows that up-regulation of miR-181a-5p significantly promotes phosphorylation of MAP3K7 and IKBKB to activate the NF-κB signaling pathways.

2.5. miR-181a-5p Activates NF-κB Signaling Pathway by Directly Inhibiting PPM1B

To further investigate whether miR-181a-5p actually activates the NF-κB signaling pathway via PPM1B, we constructed over-expression plasmid of PPM1B or siRNA directed against PPM1B, the efficiencies of which were examined by qPCR. Firstly, co-transfection of overexpressed PPM1B vector and miR-181a-5p-silenced into DF-1 cells for 48 hours, qPCR analysis demonstrated an 70% up-regulation of PPM1B mRNA level in the presence of overexpressing PPM1B vector compared with cells transfected with pcDNA3.1 vector. Interestingly, when co-transfection miR-181a-5p-Inh, PPM1B expression was further significantly increased (Figure 7A). However, overexpression of PPM1B inhibited the expression of MAP3K7, IKBKB and NF-κB, and when co-transfection with miR-181a-5p-Inh, their expressions were significantly lower (Figure 7A). The western blot assay showed the similar results at phosphorylation of MAP3K7 and IKBKB proteins (Figure 7B).

In contrast, co-transfection of PPM1B siRNA and miR-181a-5p mimics enhanced down-regulation of PPM1B expression by PPM1B siRNA alone and increased MAP3K7, IKBKB and NF-κB expression as compared with
cells transfected with PPM1B siRNA alone (Figure 7C and D). These results indicates that miR-181a-5p mimics and PPM1B siRNA has synergistic effects on MAP3K7, IKKB and NF-κB expression, and overexpression of miR-181a-5p could promote the activation of NF-κB pathway by directly inhibiting PPM1B.

2.6. miR-181a-5p Significantly Increases TLR-2, MYD88, TNF-α and IL-1β Expression during MG Infection

The effects of miR-181a-5p on TLR2 and cellular inflammatory factors during MG infection were further investigated. We transiently transfected the miR-181a-5p mimics or miR-181a-5p-inhibitor and NC into DF-1 cells co-cultured with MG or not at transfection 24h. At 36 h post-infection, the expressions of TLRs and cellular inflammatory factors were detected, including TLR2, MYD88, TNF-α and IL-1β. qPCR analysis indicated that the expressions of TLR2, MYD88, TNF-α, IL-1β were significantly up-regulated along with increased miR-181a-5p expression, at both groups co-cultured with MG or not (Figure 8A). In contrast, the four factors expressions were significantly down-regulated along with decreased miR-181a-5p expression (Figure 8B). While TLR2, MYD88, TNF-α and IL-1β expressions were significantly up-regulated after MG infection, no matter in increased or decreased miR-181a-5p expression (Figure 8). These results indicates that miR-181a-5p significantly increases the expression of TLR-2, MYD88, TNF-α and IL-1β during MG infection.

2.7. miR-181a-5p Promotes Cell Proliferation and Inhibites Cell Apoptosis to Resist MG Infection

The NF-κB signaling pathway has been widely demonstrated to regulate various cellular processes, such as involvement in the regulation of cell proliferation and apoptosis. Therefore, we further investigated the effects of miR-181a-5p on cell proliferation, cell cycle and apoptosis.

DF-1 cells were infected with MG or not, after 6h of transfection with miR-181a-5p mimics, inhibitor or NC, for 24, 48 and 72h to detect cell proliferation. Detailed grouping as shown in Figure 9. Cell viability was measured by Cell Counting Kit-8 (CCK8) assay. The results showed that MG infection significantly decreased cell viability at infection 48 and 72h. Upregulated miR-181a-5p could significantly enhance cell viability compared with NC groups (Figure 9A). On the contrary, depressed miR-181a-5p could significantly reduce cell viability at MG infection 48 and 72h (Figure 9B).

In order to validate the protective effects of miR-181a-5p against MG infection in DF-1 cells, cell cycle and cell apoptosis were further checked through flow cytometry. Detailed grouping as shown in Figure 10, 11. We found that MG infection significantly inhibited cell mitosis by inducing G1 cell cycle arrested in DF-1 cells. Overexpression of miR-181a-5p could significantly reduce the percentage of cells in the G1 phase, while the percentage of cells in the S and G2 phases was significantly increased, which is opposite to depressed miR-181a-5p (Figure 10).

Apoptosis experiments showed that MG infection significantly stimulated apoptosis of DF-1 cells (Figure 11). Apoptotic cell amount was significantly increased after MG infection. More importantly, at MG-infected DF-1 cell, overexpression of miR-181a-5p could reduce the apoptotic DF-1 cells amount (Q2 and Q3) compared to control groups, while the opposite was observed when miR-181a-5p was significantly decreased.

Taken together, above data suggestes that miR-181a-5p could promote cell proliferation and inhibit cell apoptosis to resist MG infection.
2.8. miR-181a-5p Depresses pMAG1.2 Expression by Directly Inhibiting PPM1B

Finally, to verify whether miR-181a-5p resisted MG infection by directly targeting PPM1B. pMAG1.2 expression variation was tested by co-transformation trials and qPCR. pMAG1.2 is a major adhesion protein gene of MG, which is required for MG infection. PPM1B siRNA or/and miR-181a-5p mimics was/were (co-)transfected into DF-1 cells for 24 hours, which were then infected with the 8 μL of MG strain. We found that overexpression of miR-181a-5p could significantly down-regulate pMAG1.2 expression. Knockdown of PPM1B alone also extremely depressed the expression of pMAG1.2 and further significantly decreased pMAG1.2 expression, compared with overexpression of miR-181a-5p alone. While knockdown PPM1B along with overexpression of miR-181a-5p showed the same inhibitory effect on pMAG1.2 expression, as knockdown PPM1B alone (Figure 12), which indicated that PPM1B played key role in depressing pMAG1.2 expression during MG infection and miR-181a-5p depressed pMAG1.2 expression by directly inhibiting PPM1B.

3. Discussion

MG is the main pathogen of chronic respiratory diseases in chicken, it can adhere to respiratory tract mucous membrane, and cause inflammation and the damage of respiratory tract tissue. MG-HS strain is a virulent strain with markedly pathogenicity. The adhesin protein pMGA1.2, a crucial adhesin on the surface of MG-HS strain, is responsible for MG-HS to propagate in animal tissues through binding to apolipoprotein A–I (ApoA–I) on host [30]. Many researches have shown that miRNAs play an important role in the pathogenesis of various diseases. And in recent years, a raising number of studies indicates exosomal miRNAs exert important regulatory effects on recipient cells, suggesting that the exosomal transfer of miRNAs could be a novel mechanism for intercellular communication [27,31].

Our previous study had indicated that miR-181a-5p expression was significantly up-regulated in the lungs of MG-infected chicken embryos [19]. Therefore, we firstly verified its expression verification in vivo and in vitro. The upregulated miR-181a-5p result implied its potential role in MG infection. Recently, the function of miR-181a-5p in immune system was also unveiled in many researches. The down-regulated expression of miR-181a-5p in non-small cell lung cancer is directly related to the low survival rate of patients, suggesting that miR-181a may be a diagnostic biomarker for non-small cell lung cancer [32]. miR-181a-5p expression was up-regulated in the lung tissues of LPS-challenged mice to regulate LPS-induced apoptosis in A549 cells and in vivo by targeting Bcl-2 [33]. Meanwhile, up-regulated serum miR-181a is found to be associated with the early pathogenic process of chronic obstructive pulmonary disease in asymptomatic heavy smokers [34]. miR-181a-5p is also reported to inhibit non-small cell lung cancer A549 cells proliferation and migration by targeting KRAS [35]. All these studies suggest and consolidate that miR-181a-5p plays important regulatory role in diseases and host infection.

MG infection is systemic and throughout whole body. Once MG enter the body, almost all tissues and organs, especially lung tissues will be infected. Exosomes are well documented to be enriched with miRNAs and able to deliver miRNA from host cells to target cells, and can further regulate the function of recipient cells throughout the body. Previous study shows that miR-181-5p of exosome suppresses hepatic stellate cells activation and induces autophagy activation through direct targeting Bcl-2 and STAT3 [36]. Exosomal miR-124 derived from M2 microglia can be delivered to neurons and attenuates ischemic brain injury, consequently
promotes neuronal survival [37]. In this study, we found that Cy3 fluorescence could be detected in DF-1 cells incubated with exosomes derived from CP-II cells which before were transfected with Cy3-labeled miR-181a-5p. This result uncovered that exosomes derived from MG-infected CP-II cells could load and delivery miR-181a-5p into recipient DF-1 Cells.

miRNAs play their regulatory role in kinds of biological process is through the regulation by repressing the translation of downstream target mRNAs into protein. PPM1B is a member of the metal-dependent serine/threonine protein phosphatase (PPM) family with important regulatory functions in cellular signaling pathways [38]. Further, we identified PPM1B was the target gene of miR-181a-5p. One common functional feature of PPM family members is their involvement in the cellular stress response. According to the report, PP2Cβ negatively regulates the TAK1 pathways by dephosphorylating and inactivating TAK1 [22]. TAK1 is a key component required for cytokine-induced IKK activation [39]. And IKKβ knock-out mice confirms that IKKβ is the dominant kinase in regulating NF-κB activity [40]. PP2Cβ negatively regulates the NF-κB pathway post-TNFα treatment by dephosphorylating IKKβ and thus reducing its kinase activity [23]. In our study, we found the phosphorylated MAP3K7 and IKBKB were significantly induced through PPM1B directly depressed by miR-181a-5p, which indicated that PPM1B played an important role in MG infection. NF-κB is a general nuclear transcription factor consisting of two glutelin subunits (p65 and p50). More interesting, we found p65 subunit protein was expressed from the cytoplasm to the nucleus when overexpressed miR-181a-5p in MG infection, indicating the NF-κB pathway being activated. Therefore, we further investigated the underlying mechanism of NF-κB activation. And we found that MG highly increased the expression of TLR-2, MYD88, TNF-α and IL-1β in DF-1 cells. Overexpression or knockdown of miR-181a-5p resulted in an up-regulation or down-regulation in the expression of TLR-2, MYD88 and pro-inflammatory cytokines (IL-1β and TNF-α). NF-κB acts at the center of the inflammatory response and controls the gene expression of numerous inflammation-associated substances, including IL-1β, IL-6, IL-8, and TNF-α, which induce a cascade of inflammatory responses and related lung damage [41–44]. Several researches reported that MG activates IL-1β production through the NF-κB pathway via TLR2 and MyD88. And up-regulate inflammatory genes in chicken tracheal epithelial cells via TLR-2 ligation through an NF-κB Dependent Pathway [45,46]. Our previous research also indicated that MG infection stimulated the IL2/IL6-mediated inflammatory responses through TLR6-MyD88-NF-κB pathway [47].

The NF-κB pathway involves various cellular processes, such as involvement in the regulation of cell proliferation and apoptosis. Last, we found that miR-181a-5p promoted MG-infected DF-1 cell proliferation, accelerated cell cycle transition, and inhibited apoptosis through down-regulation of PPM1B. Other studies also suggested that upregulated miR-181a promoted cell proliferation and G1/S transition, and suppressed apoptosis in gastric cancer cell lines [48]. Overexpression of miR-181a promoted proliferation and G1/S transition apoptosis in ccRCC 786-O and 769-P cells, and inhibited their apoptosis [49]. In addition, miR-181a is also reported to enhanced G1/S transition and cell proliferation in pediatric acute myeloid leukemia by regulating EGR1 expression [50]. And miR-181a is identified to increase cell proliferation and inhibits apoptosis through activating the AKT pathway in keloid fibroblasts. Moreover, PPM1B often negatively regulates cell reaction pathways, such as regulating stress response, cell cycle, cell proliferation and apoptosis [24,51].

Correctly, we highly believe miR-181a-5p directly targets PPM1B and further activate NF-κB pathway to against MG infection in DF-1 cells.
In summary, we found that miR-181a-5p expression was up-regulated after MG infection in the lungs of chicken embryos and DF-1 cells. miR-181a-5p could be packaged in exosome derived from MG-infected CP-II cells and transferred into recipient DF-1 cells to further resist MG infection through its target gene PPM1B, activating TLR2-mediated MyD88/NF-κB pathway and depressing pMAG1.2 expression (Figure 13). Therefore our study suggests the TLR2-mediated MyD88/NF-κB pathway may be the therapeutic target for protecting against MG-induced CRD diseases.

4. Materials And Methods

4.1. MG Culture

The MG virulent strain used in this study was MG-HS strain isolated from henneries in Hubei, China [52] and was donated by the State Key Laboratory of Agricultural Microbiology, College of Veterinary Medicine, Huazhong Agricultural University (Wuhan, China). MG-HS was cultured at 37 °C in modified FM-4 medium supplemented with 12% (v/v) porcine serum and 10% yeast extract until the mid-log phase. The concentration of MG-HS was determined by the acid-mediated shift of phenol red dye from red to orange as previously described. The number of viable Mycoplasmas in a suspension was then determined by a color-changing unit (CCU) assay.

4.2. Infection Experiments

One hundred embryos of White Leghorn specific-pathogen-free (SPF) chickens were incubated on the ninth day and the all were injected with 300 µL of MG-HS at 10 CCU/mL. Other 100 chicken embryos were injected with the same dosage of the diluent to serve as controls. The viability of the chicken embryos was examined by eye under a candling machine. The dead embryos were eliminated. The mortality rates of the chicken embryos of the infection and control groups were 12.3% and 7%, respectively. Whole-lung tissue samples from six infected live chicken embryos and six controls were collected on days 6, 10, and 11 post-infection and stored in RNA xer (BioTeke Co., Ltd., Beijing, China).

4.3. Cell Culture and Treatment

The chicken embryonic fibroblast cell line (DF-1) was obtained and authenticated from American Type Culture Collection (Rockville, MD, USA). Cells were maintained in Dulbecco's modified eagle medium (DMEM, Invitrogen, Carlsbad, CA, USA), supplemented with 10% fetal bovine serum (FBS, Invitrogen, USA) and 1% penicillin-streptavidinglutamine (PSG, Invitrogen, USA). Cells were incubated at 39 °C in a 5% CO₂ humidified atmosphere. For transient transfection, DF-1 cells were plated evenly in 6-, 24- or 96-well plates and grown to 60% confluency without antibiotics and subsequently transfected with RNAs and/or plasmids using Lipofectamine 3000 (Invitrogen, USA). After 48 h, the cells in different groups were collected for further use. For MG-HS infection experiments, at 24 h post-transfection, cells were infected with MG-HS at the log phase (1 × 1010 CCU/ml) for the times mentioned in the figure legends.

4.4. microRNA Target Prediction and Sequences
Putative miR-181a-5p target genes were identified using a miRNA database (http://www.mirbase.org/) and target prediction tools: miRDB (http://www.mirdb.org/miRDB/), PicTar (http://pictar.mdc-berlin.de/), and TargetScan (http://www.targetscan.org/). The conservation of the target gene was analyzed by TargetScan (http://www.targetscan.org/). The duplex and mfe between miR-181a-5p and the 3'-UTR of the potential targets were analyzed by RNAhybrid (https://bibiserv.cebitec.uni-bielefeld.de/rnahybrid/). AmiGO (http://amigo.geneontology.org) was used to analyze the functions of the target genes of gga-miR-181a-5p in Gallus gallus. The sequences of all of the primers used in this study are shown in Table 1. All RNA oligonucleotides were designed and synthesized by GenePharm (Shanghai, China) and are shown in Table 2.

Table 1. Sequences of DNA primers.
| Name                      | Primer Sequence (5'-3')                              | Accession No.          |
|---------------------------|-------------------------------------------------------|------------------------|
| **Primers for CDS Cloning** |                                                       |                        |
| PPM1B-CDS-F               | CGCGGATCCATTGCATAAACATGGGTGCAATT                      | NM_001031052.1         |
| PPM1B-CDS-R               | GGAATTCCTACCACGGATCTTCTAGATCTCCA                     |                        |
| **Primers for 3′-UTR Cloning** |                                                       |                        |
| PPM1B 3′UTR-F             | TTCCTCGAGATGGGCATGGTACAGAGTGG                       | NM_001031052.1         |
| PPM1B 3′UTR-R             | AATGCGGCCGCTGCTATGAGAGACGTGTGG                       |                        |
| Mut-PPM1B 3′UTR-F         | GGAGGGGAAACAATACGTGTATCCATCTGATACATGATTACATGCTGTG   | NM_001031052.1         |
| Mut-PPM1B 3′UTR-R         | ATCATGTACAGATGGGACGTATTGGTTTCCCCCTCCAATAGCTTTTACT    |                        |
| **Primers for RT-qPCR**   |                                                       |                        |
| GAPDH-F                   | GAGGGTAGTGAAGGCTGCTG                                 | NM-204305              |
| GAPDH-R                   | CACAACACGGTTGCTGTATC                                 |                        |
| PPM1B-F                   | AATCTGGGATGTAATGAGCAACG                             | NM_001031052.1         |
| PPM1B-R                   | TCCAAGTGCTTATCCAACCTCTGC                            |                        |
| MAP3K7-F                  | GAGATCGAAGTGGAAGAGGT                                | XM_015284677.2         |
| MAP3K7-R                  | TGGGTTTAGACAGGCTCCAT                                |                        |
| IKBKB-F                   | CTGCCCGTGATGTTTCTGA                                 | NM_001031397.1         |
| IKBKB-R                   | TGAGAAGCAGAGGCAATATCAGAC                           |                        |
| NF-κB-F                   | GCCAGGGTCCATCGTGT                                  | NM-205129              |
| NF-κB-R                   | CGTGCCTGTCGCTCTTC                                   |                        |
| TLR-2-F                   | CGCAAGCTTATGTTCAACCAAG                              | NM_001161650.1         |
| TLR-2-R                   | CGCCTCGAGCTATGACTTCAAGG                            |                        |
| MYD88-F                   | TCAGTTTGTCAGGAGATG                                 | NM-001030962           |
| MYD88-R                   | GGTGTAATGACCGCAAGATA                                |                        |
| TNF-α-F                   | GGACAGCCTATGCCAACAAG                                | XM-015294124           |
| TNF-α-R                   | ACACGACAGCCAAGTCAG                                  |                        |
### Table 2. Sequences of RNA oligonucleotides.

| Name                          | Sequences (5′–3′)                                    |
|-------------------------------|-----------------------------------------------------|
| gga-miR-181a-5p mimics        | AACAUUCAACGCUGUCGGUGAGU                              |
|                               | UCACCGACAGCGUUGAAUGUUUU                             |
| gga-miR-181a-5p NC            | UUCUCCGAACGUGUCACGUTT                               |
|                               | ACGUGACACGUGUUCGGAGAATT                             |
| gga-miR-181a-5p inhibitor     | ACUCACCGACAGCGUUGAAUGUUU                            |
| gga-miR-181a-5p inhibitor-NC  | CAGUACUUUUGUGUAGUACAA                               |
| si-gga-PPM1B                  | GCAGGATCTCGTGTTGCAA                                 |

#### 4.5. Primary CP-II cells culture

CP-II cells were isolated according to our established method [53]. Briefly, it was prepared from lung tissue of 15 day SPF chicken embryos. The lung tissue was cut, washed three times with Hank's solution; then, 0.25% trypsin was added, each embryo was added in an amount of 1 ml, digested in a water bath at 37 °C for 10 minutes, centrifuged at 800 rpm/min for 10 minutes, and the supernatant was discarded; then, 0.1% IV collagenase was added, each embryo was added in an amount of 1 ml, digested in a water bath at 37 °C for 15 min, centrifuged at 800 rpm/min for 10 minutes, the supernatant was discarded, and a moderate volume of
10% FBS in DMEM complete medium was gently pipetted. The cells were mixed and filtered through a 75 μm mesh to a sterile plate for adherence. The unattached suspension was centrifuged at 1200 rpm/min for 5 min, resuspended and centrifuged, and repeated 3 times. After resuspending the cells in DMEM complete medium supplemented with 20% FBS, the cells were filtered through a 38 μm sieve into a culture flask, and the cells were cultured in a humidified incubator at 37 °C, 5% CO₂ for 18 hours, and then the culture solution was changed. At this time, the adherent cells are chicken embryo type II epithelial cells.

4.6. Exosome isolation, identification and labeling

Exosomes were purified from the cell culture supernatant of CP-II cells. Prior to culture medium collection, CP-II cells were washed twice with PBS, and the medium was switched to exosome-free medium (ultracentrifugation at 100,000X g for 16 h at 4 ℃) upon MG stimulation. The cells were then cultured for 48 h. The supernatant was collected and went through sequential ultracentrifugation at 2000 X g for 30 min, 10,000X g for 30 min, and 100,000 X g for 70 min at 4 ℃. The exosomes were washed once with PBS at 100,000X g for 70 min and suspended for further characterization.

A transmission electron microscope (TEM, Thermo Scientific, Waltham, MA) was used to identify the form of the exosomes. Nanoparticle tracking analysis (NTA, Brookhaven, New York) was used to measure exosome diameter and particle number. The protein content was measured using BCA protein assay (Thermo Scientific, Waltham, MA), and exosome markers CD9 and CD63 were detected by western blot analysis.

Fluorescence labeling of exosomes was performed according to the protocol previously described [54]. The PKH26 kit was used according to the instruction manual (Sigma-Aldrich, San Louis, MO). The labeled exosomes were washed at 100,000 g for 1 h, and the exosome pellet was diluted in PBS and used for the uptake experiment.

4.7. Dual-Luciferase Reporter Assay

The psi−CHECK™-2 dual-luciferase reporter vector (Promega, Madison, WI, USA) harboring the wild-type and mutant PPM1B 3’-UTR, which were inserted into the Xho I and Not I restriction sites 3’ to the end of the Renilla gene, were used to check the effect of miR-181a-5p on Renilla activity. The psi−CHECK™-2 mutant PPM1B′-UTR construct was generated by inducing a point mutation using the overlap extension PCR method. DF-1 cells were seeded on 24-well plates at a density of 3 × 10⁵ cells per well and cultured until the cells reached approximately 60% confluence. Cells were then transfected with 200 ng of the luciferase reporter plasmid and 10 pmol of miR-181a-5p, miR-181a-5p-NC, miR-181a-5p-Inh or miR-181a-5p-Inh-NC using Lipofectamine 3000 (Invitrogen, USA). At 48 h post-transfection, the cells were collected, the Firefly and Renilla luciferase activities were determined using EnSpire & Multimode Reader (PerkinElmer, Inc., Waltham, USA) according to the manufacturer's protocol (Promega, USA). Three independent repeats were performed for all above transfection experiments.

4.8. RNA Extraction and Quantitative Real-Time PCR (qPCR)

Total RNA was extracted from the cultured cells and frozen lung tissue specimens of chicken embryos using the TRIzol reagent (Invitrogen). According to the manufacturer’s instructions, 1 μg RNA from each sample was
used to synthesize cDNA using the Prime Script™ RT reagent kit with gDNA Eraser (TaKaRa, Tokyo, Japan). The real-time PCR was performed with TransStart Top Green qPCR SuperMix (TRANSGEN, Beijing, China) on a CFX96 or CFX384 Touch™ instrument (Bio-Rad, Hercules, CA, USA). The relative mRNA levels were calculated using the Bio-Rad, CR SuperMix (TRANSGEN, Beijing, China). The expression levels of miR-181a-5p and PPM1B were measured by qPCR. 5S-RNA and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA were used as internal controls, respectively. The expression of 5S-RNA and GAPDH are stable in the course of MG infection. The experiment was repeated three times, The primers are listed in Table 1.

4.9. Western blot analysis

Total proteins were extracted using RIPA lysis buffer, separated using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and transferred onto polyvinylidene difluoride membrane (Roche Life Sciences, Switzerland). Membranes were blocked with 5% skimmed milk and incubated with the appropriate antibodies. The PPM1B antibody was obtained from Proteintech, and phosphorylated MAP3K7 and phosphorylated IKBKB antibodies acquired from Cell Signaling Technology (Danvers, MA, China). Primary antibodies were applied to the membrane, followed by horseradish-peroxidase-conjugated secondary antibodies. The antigen-antibody complex on the membrane was detected with enhanced chemiluminescence reagent (Thermo Scientific, Waltham, MA, China).

4.10. Cell Proliferation, Cell Cycle and Cell Apoptosis

Cell proliferation was determined using the Cell Counting Kit-8 according to the manufacturer's protocol (CCK-8, DOJINDO, Shanghai, China). DF-1 cells were plated at a density of 8 × 10³ cells/well in a flat-bottomed, 96-well cell culture plate and allowed to grow for 4 h at 39 °C 37°C with 5% CO2. The DF-1 cells were then transfected with miR-181a-5p, miR-181a-5p-NC, miR-181a-5p-Inh, or miR-181a-5p-Inh-NC. At 24 h post-transfection, the cells then were infected with 8 µL of MG-HS strain at 10¹⁰ CCU/mL. At 24 h, 48 h, and 72 h post-transfection, 10 µL of the CCK-8 solution was added to each well of the plate, which was then incubated at 37 °C for 4 h. The infected-MG cells (denoted as miR-free MG + ) and the uninfected-MG cells (blank) were cultured in a sterile incubator to avoid MG contamination. The optical density at 450 nm of each well plate was measured using a microplate reader (Bio-Rad, Hercules, CA, USA). The cell cycle and cell apoptosis assay were performed in 6-well plates. The DF-1 cells were transfected with the indicated RNA oligonucleotides. At 24 h post-transfection, the cells were infected with 130 µL of MG-HS strain at 10¹⁰ CCU/mL. At 24 h post-infection, the cell cycle was analyzed with a flow cytometer, using the cell cycle detection kit (KeyGEN, Nanjing, China). Similarly, miR-free-MG+ and blank MG MGrly, miR-free-MG+ and blank MGankMGlow cytometer, using the cell cycle detection kit (t-transfection, the cells were infected with 130 µL of MG-HS strain at 10¹⁰ CCU/ch

4.11. Statistical Analysis

SPSS software (SPSS 20.0, IBM, Armonk, NY, USA) was used for statistical analyses. All results are presented as the mean values the c. Statistical significance was determined by using one-way ANOVA or Student’s t-test, and p-values of < 0.01 or < 0.05 were considered the statistically significant difference between groups.

5. Conclusions
We conclude that the newly identified primary CP-II cells exosomal miR-181a-5p activates the TLR2-mediated MyD88/NF-κB pathway by directly targeting PPM1B to promote pro-inflammatory cytokines expression for defending against MG infection in recipient DF-1 cells. These findings provide potential novel targets for prevention or treatment of MG infection in chicken.

**Abbreviations**

| Abbreviation | Description |
|--------------|-------------|
| MG           | *Mycoplasma gallisepticum* |
| MG-HS        | *Mycoplasma gallisepticum*-Huangshi |
| 3'-UTR       | 3'-untranslated region |
| PPM1B        | Mg$^2+$/Mn$^2+$-dependent protein phosphatase 1B |
| MFE          | minimum free energy |
| DF-1         | chicken embryonic fibroblast cells |
| CP-II        | chicken type II pneumocytes |
| MAP3K7 (TAK1)| mitogen-activated protein kinase kinase kinase 7 |
| IKBKB (IKKβ)| Inhibitor Of Nuclear Factor Kappa B Kinase Subunit Beta |
| NF-κB        | nuclear factor-κB |
| TLR2         | Toll Like Receptor 2 |
| MyD88        | Myeloid Differentiation Primary Response 88 |
| TNF-α        | tumor necrosis factor-α |
| IL-1β        | interleukin-1β |
| GAPDH        | glyceraldehyde-3-phosphate dehydrogenase |
| pMGA1.2      | Protein Mycoplasma Gallisepticum AdheA-In 1.2 |

**Declarations**

**Acknowledgments:** Not applicable.

**Author Contributions:** Y.F. performed experiments, wrote the manuscript and analyzed the data. Y.Z. and H.P. helped to write, revise the manuscript. M.Z provided advices for the study. X.P. conceived and designed the study and helped to revise the discussion.

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Figures

Figure 1

miR-181a-5p expression is up-regulated after MG infection in the lungs of chicken embryos (A) and DF-1 cells (B). The total RNA was extracted, and miR-181a-5p expression was assessed by qPCR using 5s-rRNA as the internal quantitative control gene. Three independent experiments, each with three replicates, were performed. Student t test. The plotted data points show the means ± SDs, and the asterisks indicate statistically significant differences (** means p < 0.01).
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Figure 2

Exosomes-miR-181a-5p by CP-II cell-derived is absorbed by DF-1. (A) a representative transmission electron microscopy (TEM) images of exosomes. Scale bar 200 nm. (B) The positive markers of exosomes, CD63 and CD9, were detected in CP-II cells by Western blot. (C) miR-181a-5p levels in MG-infected CP-II cell (M- CP-II) or normal CP-II cell (B- CP-II). 5S was used as the internal control. (D) miR-181a-5p levels in MG-infected CP-II cell-derived exosomes (M-EXO) or normal CP-II cell-derived exosomes (B-EXO). 5S was used as the internal control.
The panels show presence of Cy3 fluorescence and PKH67 lipid dye in DF-1 cell after adding PKH67-labeled exosomes derived from CP-II cells for 24 h. Scale bar represent 20 µm.

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The panels show presence of Cy3 fluorescence and PKH67 lipid dye in DF-1 cell after adding PKH67-labeled exosomes derived from CP-II cells for 24 h. Scale bar represent 20 µm.

Figure 3

The expression of PPM1B is decreased in MG infection. (A) Sequence alignment of PPM1B 3′-UTR from different species. The conserved target sequences are highlighted. (B) The secondary structure of the RNA duplex of gga-miR-181a-5p and the PPM1B 3′-UTR target site (red: PPM1B sequence; green: miR-181a-5p). PPM1B mRNA expression was down-regulated after MG infection in the lungs of chicken embryos in 6, 7 and 8 days (C) and in DF-1 cells (D). PPM1B protein expression showed the same trend (D). Three independent experiments, each with three replicates, were performed. Student t test. The plotted data points show the means ± SDs, and the asterisks indicate statistically significant differences (** means p < 0.01, * means p < 0.05).
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miR-181a-5p could directly bind to the nucleotide sequence in the 3′-UTR of PPM1B. (A) psiCHECK-2 dual-luciferase reporter vector containing the 3′-UTR (wild-type or mutant) of PPM1B. (B) DF-1 cells were co-transfected with Luc-PPM1B 3′-UTR (wild-type or mutant) and the indicated RNA oligonucleotides. At 48 h post-transfection, the cells were assayed for both firefly and renilla luciferase activity through a dual-luciferase glow assay. Three independent experiments, each with three replicates, were performed. The data are expressed as the means ± SDs. One-way ANOVA was used to analyze the significant differences (** means p < 0.01).
transfected with Luc-PPM1B 3′-UTR (wild-type or mutant) and the indicated RNA oligonucleotides. At 48 h post-transfection, the cells were assayed for both firefly and renilla luciferase activity through a dual-luciferase glow assay. Three independent experiments, each with three replicates, were performed. The data are expressed as the means ± SDs. One-way ANOVA was used to analyze the significant differences (** means p < 0.01).

Figure 5
miR-181a-5p negatively regulates PPM1B in DF-1 cells. The expression of miR-181a-5p (A) and mRNA (B) and protein (C) expression of PPM1B after treatment with miR-181a-5p mimic (n = 3, **, p < 0.01 vs. control group); The expression of miR-181a-5p (D) and mRNA(E) and protein(F) expression of PPM1B after treatment with miR-181a-5p inhibitor (n = 3, **, p < 0.01 vs. control group); GAPDH was used as the internal control (Different Capital letters represent p < 0.01; Different small letters represent p < 0.05 ).
Figure 5

miR-181a-5p negatively regulates PPM1B in DF-1 cells. The expression of miR-181a-5p (A) and mRNA (B) and protein (C) expression of PPM1B after treatment with miR-181a-5p mimic (n = 3, **, p < 0.01 vs. control group); The expression of miR-181a-5p (D) and mRNA(E) and protein(F) expression of PPM1B after treatment with miR-181a-5p inhibitor (n = 3, **, p < 0.01 vs. control group); GAPDH was used as the internal control (Different Capital letters represent p < 0.01; Different small letters represent p < 0.05 ).
miR-181a-5p activates the NF-κB signalling pathway. (A) mRNA expression of MAP3K7, IKBKB and NF-κB after treatment with miR-181a-5p mimic. (B) Protein expression of MAP3K7, IKBKB and NF-κB after treatment with miR-181a-5p mimic. (C) mRNA expression of MAP3K7, IKBKB and NF-κB after treatment with miR-181a-5p inhibitor. (D) Protein expression of MAP3K7, IKBKB and NF-κB after treatment with miR-181a-5p inhibitor. GAPDH was used as the internal control; (E) Translocation of the p65 subunit from the cytoplasm into the nucleus was evaluated by immunofluorescence. Blue spots represent cell nuclei, and red spots represent p-p65 staining. (Different Capital letters represent p < 0.01; Different small letters represent p < 0.05 ).
miR-181a-5p activates the NF-κB signalling pathway. (A) mRNA expression of MAP3K7, IKBKB and NF-κB after treatment with miR-181a-5p mimic. (B) Protein expression of MAP3K7, IKBKB and NF-κB after treatment with miR-181a-5p mimic. (C) mRNA expression of MAP3K7, IKBKB and NF-κB after treatment with miR-181a-5p inhibitor (D) Protein expression of MAP3K7, IKBKB and NF-κB after treatment with miR-181a-5p inhibitor. GAPDH was used as the internal control; (E) Translocation of the p65 subunit from the cytoplasm into the nucleus was evaluated by immunofluorescence. Blue spots represent cell nuclei, and red spots represent p-p65 staining. (Different Capital letters represent p < 0.01; Different small letters represent p < 0.05).
Figure 7

miR-181a-5p activates the NF-κB signaling pathway by directly inhibiting the expression of PPM1B (A,C) mRNA expression of PPM1B, MAP3K7, IKBKB and NF-κB after co-transfection of overexpression PPM1B vector and miR-181a-5p-inhibitor or co-transformation of PPM1B siRNA and miR-181a-5p mimics; (B,D) Protein expression of MAP3K7/IKBKB/NF-κB after co-transfection of overexpression PPM1B vector and miR-181a-5p-inhibitor or co-transformation of PPM1B siRNA and miR-181a-5p mimics; (different letters represent p < 0.01).
miR-181a-5p activates the NF-κB signaling pathway by directly inhibiting the expression of PPM1B (A,C) mRNA expression of PPM1B, MAP3K7, IKBKB and NF-κB after co-transfection of overexpression PPM1B vector and miR-181a-5p-inhibitor or co-transformation of PPM1B siRNA and miR-181a-5p mimics; (B,D) Protein expression of MAP3K7/IKBKB/NF-κB after co-transfection of overexpression PPM1B vector and miR-181a-5p-inhibitor or co-transformation of PPM1B siRNA and miR-181a-5p mimics; (different letters represent p < 0.01).
miR-181a-5p significantly increases the expression of TLR-2, MYD88, TNF and IL-1β during MG infection. DF-1 cells were transfected with miR-181a-5p, miR-181a-5p-NC (A), and miR-181a-5p-Inh or miR-181a-5p-Inh-NC (B), and were incubated for 24 h and then either left uninfected or infected with 130 µL of MG for 36 h. The mRNA expression of TLR2, MYD88, TNF-α, and IL-1β were analyzed by qPCR at 36 h post-infection. Three independent experiments, each with three replicates, were performed. The data are presented as the means ± SDs. One-way ANOVA was used to analyze significant differences (Different lowercase letters represent p < 0.01).
miR-181a-5p significantly increases the expression of TLR-2, MYD88, TNF and IL-1β during MG infection. DF-1 cells were transfected with miR-181a-5p, miR-181a-5p-NC (A), and miR-181a-5p-Inh or miR-181a-5p-Inh-NC (B), and were incubated for 24 h and then either left uninfected or infected with 130 µL of MG for 36 h. The mRNA expression of TLR2, MYD88, TNF-α, and IL-1β were analyzed by qPCR at 36 h post-infection. Three independent experiments, each with three replicates, were performed. The data are presented as the means ± SDs. One-way ANOVA was used to analyze significant differences (Different lowercase letters represent $p < 0.01$).
Figure 9

miR-181a-5p promotes DF-1 cell viability in MG infection. DF-1 cells were infected with MG or not, after 6h of transfection with miR-181a-5p mimics and NC (A), or inhibitor and NC (B), for 24, 48 and 72h to detect cell proliferation. Cell viability was assessed by CCK-8 assay to value cell proliferation. All values are represented as the mean ± SD of three independent experiments in triplicate. The asterisks represented statistically significant differences (* p < 0.05, ** p < 0.01).
**Figure 10**

miR-181a-5p promotes cell cycle progression in MG infection. DF-1 cells were infected with MG or not, after 6h of transfection with miR-181a-5p mimics and NC, or inhibitor and NC, for 24h to detect cell cycle by flow cytometry. All values are represented as the mean ± SD of three independent experiments in triplicate. The asterisks represented statistically significant differences (*Different lowercase letters represent p < 0.01).
Figure 10

miR-181a-5p promotes cell cycle progression in MG infection. DF-1 cells were infected with MG or not, after 6h of transfection with miR-181a-5p mimics and NC, or inhibitor and NC, for 24h to detect cell cycle by flow cytometry. All values are represented as the mean ± SD of three independent experiments in triplicate. The asterisks represented statistically significant differences (*Different lowercase letters represent p < 0.01).
Figure 11

miR-181a-5p inhibites cell apoptosis in MG infection. DF-1 cells were infected with MG or not, after 6h of transfection with miR-181a-5p mimics and NC, or inhibitor and NC, for 24h to detect cell apoptosis by flow cytometry. All values are represented as the mean ± SD of three independent experiments in triplicate. One-way ANOVA was used to analyze significant differences (Different lowercase letters represent p < 0.01).
Figure 11

miR-181a-5p inhibits cell apoptosis in MG infection. DF-1 cells were infected with MG or not, after 6h of transfection with miR-181a-5p mimics and NC, or inhibitor and NC, for 24h to detect cell apoptosis by flow cytometry. All values are represented as the mean ± SD of three independent experiments in triplicate. One-way ANOVA was used to analyze significant differences (Different lowercase letters represent p < 0.01).
miR-181a-5p depresses pMAG1.2 expression by directly inhibiting PPM1B. DF-1 cells were co-transfected with PPM1B siRNA or/and miR-181a-5p mimics for 24 h. The cells were then infected with 8 μL of MG strain. The expression of pMGA1.2 was then detected (Different letters represent p < 0.01).
miR-181a-5p depresses pMAG1.2 expression by directly inhibiting PPM1B. DF-1 cells were co-transfected with PPM1B siRNA or/and miR-181a-5p mimics for 24 h. The cells were then infected with 8 μL of MG strain. The expression of pMGA1.2 was then detected (Different letters represent p < 0.01).
Figure 13
