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Original Article

PRRSV regulates cytokine secretion from PAMs cultured *in vitro* via activation of MyD88-dependent TLRs signaling pathway

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Abstract: Porcine reproductive and respiratory syndrome (PRRS) is a highly contagious and lethal disease in the global swine industry. The major target cells of PRRS virus (PRRSV) are porcine alveolar macrophages (PAMs). With the aim to elucidate the pathogenic mechanism of PRRS and thus to help prevention and control of the disease, we investigated the functional changes of PAMs caused by PRRSV infection. PAMs from fifteen 35-day-old piglets that were free of PRRSV and porcine circovirus type 2 (PCV2) antibody and antigen, were cultured *in vitro* and divided into 4 groups, *i.e.*, control group, PRRSV group, myeloid differentiation 88 (MyD88) interference group (interference group), and MyD88 interference-PRRSV infection group (interference-PRRSV group). The PRRSV-infected cells was observed using confocal scanning laser microscope; the MyD88 gene of PAMs was knocked down by siRNA; the transcription of Toll-like receptors (TLRs) in PAMs was detected by fluorescent quantitative real-time PCR; the protein concentrations of IL-1β, IL-6, IL-10 and TNF-α in the culture supernatant were detected by ELISA; the protein concentrations of MyD88, NF-κB and phosphorylated IκB (p-IκB) in cytoplasm and NF-κB in nucleus were detected by western blot; and the NF-κB-DNA binding activity in nucleus was detected using EMSA. The results showed PRRSV infection led to the increase in the TLRs mRNA and protein concentrations of IL-1β, IL-6, IL-10 and TNF-α in PAMs and derived supernatants. After PRRSV infection, the protein concentrations of MyD88 and p-IκB in cytoplasm and NF-κB in nucleus were higher than those in the control group. In addition, the protein concentrations of MyD88 and p-IκB in cytoplasm and NF-κB in nucleus and the NF-κB-DNA binding activity in the former group were significantly lower than those in the latter one. In conclusion, PRRSV could up-regulate the protein expression of IL-1β, IL-6, IL-10 and TNF-α in PAMs through TLRs-MyD88-NF-κB signaling pathway.

Keywords: PRRSV, PAMs, cytokine, TLRs, cellular signaling pathway

Introduction

Porcine reproductive and respiratory syndrome (PRRS) is composed of porcine reproductive and respiratory syndrome virus (PRRSV) to reproductive disorders, respiratory disease characterized piglets appear infectious diseases. The disease was first reported in the late 1980s that has impacted the development of the global swine industry, now widely popular in the world. The disease is one of the animal epidemics diseases OIE statutory reporting. There are two kinds of PRRSV genotypes: The European type (genotype I) [1] the North American type (genotyp II) [2]. In our contrary, PRRSV was separation from aborted fetuses in 1997 [3], and confirmed that there is also a PRRSV in China. With the heredity and variation of the virus, China was broke out highly pathogenic porcine reproductive and respiratory syndrome characterized by high piglet mortality in 2006, make our swine industry suffered huge economic losses. The secretion of some cytokines such as TNF-α, IL-1, IL-6 and IL-10, etc. was regulated by PRRSV. This will finally caused immunosuppression and persistent infection [4].

Macrophages are important antigen-presenting cells, plays a key role in recognizing, presenting and processing of antigens. Distinguishes pathogen-associated molecular patterns (PAMPs), such as Toll like receptors (TLRs), to start early response to invading pathogens, regulate the expression of cytokines. TLRs signal path-

way including myeloid differentiation factor 88 (MyD88) dependent pathway and MyD88 independent pathway. TLRs except TLR3, after stimulation with an appropriate ligand, relay the signal via MyD88 dependent pathway, to regulation the secretion of inflammatory cytokines [5]. Macrophages are also the target cell of pigs' infection PRRSV. Many papers have rep-orted the effect of PRRSV on cytokines produced in macrophages, but the signaling pathways of these cytokines induced changes are still unknown. in this study, Porcine alveolar macrophages (PAMs) cultured in vitro as the object, the protein concentrations of IL-1β, IL-6, IL-10 and TNF-α in the culture supernatant were detected. The expression levels of TLRs in PAMs were detected, and its signal pathway was designed to investigate the regulation of cytokine secretion of PRRSV.

Materials and methods

Animals and virus

Animals: Fifteen, 35-day-old, healthy, crossbred piglets obtained from a pig farm that was negative for PCV2 and PRRSV infections. The pigs were confirmed to be free of PRRSV and porcine circovrius type 2 (PCV2) infections with commercial ELISA kits for PRRSV antibody (IDEXX, Westbrook, Maine, USA) and PCV2 antibody (INGEZIM CIRCOVIRUS IgG/IgM Kit, Ingenasa, Spain) and real-time RT-PCR was performed to verify they were free of PRRSV and PCV2.

Virus: The virus isolate PRRSV (GenBank No. HQ315835) was provided by the Key Laboratory of Animal Disease Diagnosis and Immunology at Nanjing Agricultural University. The PRRSV stock titers were 5×10^6 mL^{-1} TCID, as determined by cytopathic effect (CPE) in MARC-145 cells [6].

Experimental design

The PAMs were isolated from bronchoalveolar lavage by centrifugation as [7], the purity can reach more than 99%. The viability and numbers of PAMs determined by trypan blue dye exclusion. The cell concentration of PAMs was adjusted to 5×10^6/mL with RPMI-1640 medium containing 10% fetal bovine serum (GIBCO, Invitrogen Corporation, CA) and 1% Penicillin-Streptomycin Solution (GIBCO, Invitrogen Corporation, CA). Added to 25 cm^2 culture flasks (Corning Incorporated, USA) and incubated for 4~6 h at 37°C in a humidified compartment to allow cells to adhere to flasks. Cells were washed two times in phosphate-buffered saline (PBS) and randomly divided into four groups: control group, PRRSV group, myeloid differentiation 88 (MyD88) interference group (interference group), and MyD88 interference-PRRSV infection group (interference-PRRSV group). The MyD88 gene was silenced by using small interfering RNA method. The PRRSV group was inoculated with 200 μL of virus, the interference-PRRSV group was inoculated with equivalent virus and detected efficiency of interference. The control group and interference group was inoculated with 200 μL of RPMI-1640 medium. After incubation at 37°C in 5% CO_2, the adherent cells and supernatants of cultured PAMs were collected at 0 h, 6 h, 12 h, 24 h and 36 h, respectively. Prior to use, the cells were confirmed as negative for PCV2, PRRSV by PCR or RT-PCR.

The MyD88 gene was silenced by using small interfering RNA (siRNA) method

According to siRNA construction principle, synthesis of siRNA sequence and nonsense control siRNA sequence (Zimmer gene company, Shanghai), interference sense: 5'-AUGCCUGAGCAUUUUGAUGTT-3', antisense: 5'-CAU-CAAAAUGCUCAGGCAUTT-3'; non sense control sequence sense: 5'-CUGCCCCAGCGAUAUCCAGTT-3', antisense: 5'-CUGGAUAUCGCUGGGGCAGTT-3'. Each group siRNA (5 μmol/l) were added to 250 mL serum-free RPMI-1640 medium, and mix well; 4 mL LipofectamineTM RNAiMax reagent (Life technologies Inc. USA) was added to 250 mL serum-free RPMI-1640 medium and mix well; Mixed these two diluted solution gently and incubated 15 min at room temperature. Added to the culture medium of six-well culture plates (Corning Incorporated, USA) with glass covers lips and incubated at 37°C in 5% CO_2. Gently shaking the culture plate, make the final concentration of siRNA for 10 nmol/L.

Indirect immunofluorescence assay

Infected cells growing on glass covers lips were washed with PBS, fixed with cold ethanol for 20 min at 4°C. After fixation, PAMs were incubated for 1 h with pig anti-PRRSV polyclonal antiserum at 37°C and washed three times with PBST

(0.05%Tween-20 in PBS, pH 7.4), and further incubated with staphylococcal protein A (SPA) conjugated to FITC (Boshide, Wuhan, China) at 37°C for 1 h in the dark. After three washes with PBST, stained with 5 μg/ml DAPI (Sigma, Aldrich) for 5 min for visualization of nuclei. Finally, cells washed three times with PBST and examined by confocal laser scanning microscopy (Zeiss, Germany). Select 500 cells in the field of vision randomly, calculated the rate of virus infection.

Quantitative Real-time RT-PCR detection of TLRs in PAMs

Total RNA was extracted from PAMs using TRIzol reagent (Life technologies Inc, USA) according to the manufacturer's instructions. Reverse transcription was performed using M-MLV Reverse Transcriptase (Takara Bio Inc, JAPAN). The PCR primers were designed by Primer Premier 5.0 software and the primers GAPDH sequence sense: 5'-CTGCCCCTTCTGCTGATGC-3', antisense: 5'-TCCACGATGCCGAAGTTGTC-3'. The primers of TLR2, 3, 4, 6, 7, 8, 9 is same as [8]. Real-time PCR was performed in an ABI PRISM 7300 sequence detection system (Applied Biosystems, USA). The PCR reaction system was consisted of 0.6 μL of sense primer, 0.6 μL of anti-sense primer, 10 μL of SYBR® Green Realtime PCR Master Mix-plus (TOYOBO Bio Inc., JAPAN), 2 μL cDNA, and 6.8 μL RNase-free water. The levels of TLRs mRNA from cDNA in the samples were calculated using the following formula: $2^{-\Delta\Delta Ct} = 2^{\Delta Ct\ TLRs} / 2^{\Delta Ct\ GAPDH}$.

Cytokine measurement

The levels of IL-1β, IL-6, IL-10 and TNF-α in culture supernatants were measured by using porcine specific ELISA kits (R&D Systems, USA) according to the manufacture's instructions. A standard curve was generated using known concentrations. For each cytokine, concentration was calculated resulting from a regression formula according to the standard curve.

Western blotting to detect NF-κB/P65, p-IκB and MyD88

Protein was extracted from PAMs using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pirece, USA) according to the manufacture's instructions. Pierce BCA Protein Assay Kit for the determination of protein concentration. The quantities of NF-κB/p65 protein and p-IKB and MyD88 protein extracts were measured by Western blotting as DUAN [8] etc. have previously described. Protein samples were separated by 12% sodium dodecylsulfate polyacrylamide gel electrophoresis. Samples were transferred to nitrocellulose membranes (Takara Bio Inc. JAPAN). Then blocked for 2 h in 5% nonfat dry milk suspended in 0.1% Tween-20 Tris-buffered saline (TBST, pH 7.4). Membranes were incubated overnight at 4°C with monoclonal antibodies against NF-κB/p65 (Cell Signaling Technology, USA), p-IκB, MyD88 (Abcam, USA) (Histone H3 (Santa Cruz Biotechnology, USA), GAPDH and β-actin (Cell Signaling Technology, USA). After three washes with TBST, membranes were incubated with horseradish peroxidase-conjugated secondary antibody. Signals were performed in ImageQuant LAS 4000 biomolecular imager (Fuji Corp, JAPAN). The results analyzed with Quantity One software.

Electrophoretic mobility shift assay (EMSA)

EMSA was used to detect the DNA-binding activity of NF-KB as we have previously described [9].

Statistical analysis

Results are presented as mean ± standard error. Statistical analysis of experimental data was performed using SPSS v18.0 software. Results were analyzed for significance between groups with the One-way ANOVA. A P value <0.05 was considered significantly significant, and labeled with an asterisk (*) on each graph.

Results

PAMs infection and interference efficiency detection

PAMs infected with PRRSV for 6, 12, 24 and 36 h were examined by confocal laser scanning microscopy. No PRRSV antigens were found in the control group. After 6 h of incubation with PRRSV, the PRRSV group was seen in the cytoplasm of PRRSV in PAMs (green), and the number of infected PAMs increased with incubation time. After 36 h of incubation with PRRSV, the percentage of infected PAMs cells was 98% (**Figure 1A**). The results of MyD88 mRNA interference efficiency detected by Real-Time PCR as shown in **Figure 1B**, after 24 h interference,

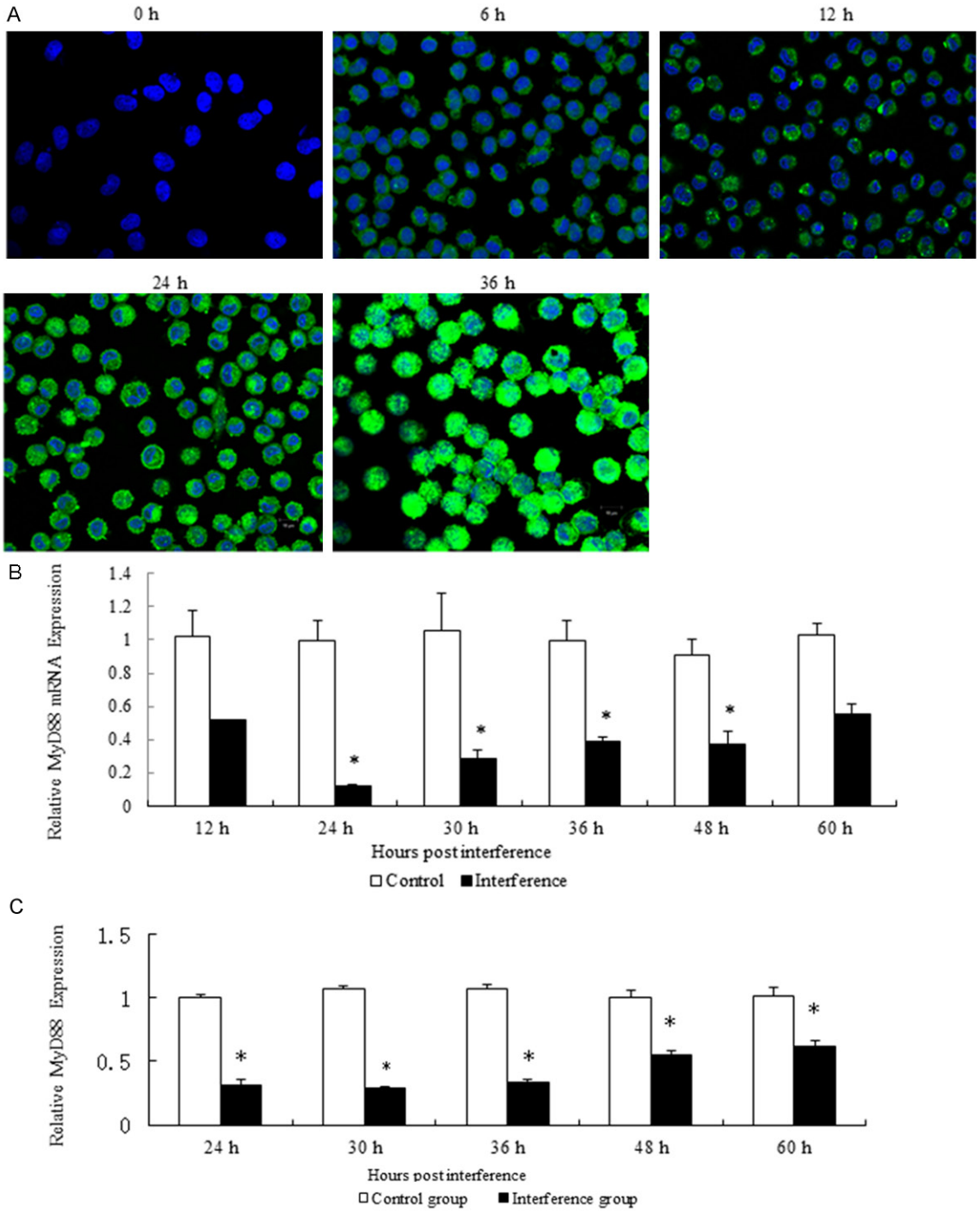


Figure 1. PAMs Infection and Interference Efficiency Detection. A. PRRSV specific staining in PAMs by indirect immunofluorescence. DAPI for nucleus. The picture epresents the nucleus-specific staining (DAPI, bule) and the PRRSV-specific staining (FITC, green). B: PRRSV infection rate in PAMs. Bar: 10 μm. B. Relative expression of MyD88 mRNA interfered with siRNA; (*) indicate a significant difference ($P<0.05$) in comparison with the corresponding control group. C. Relative expression of MyD88 protein interfered with siRNA.

85% of MyD88 gene was silent. The interference efficiency of MyD88 decreased with incubation time, but was still significantly lower than the control group. The levels of MyD88 protein were measured by Western blotting (**Figure 1C**). The levels of MyD88 protein in the interference

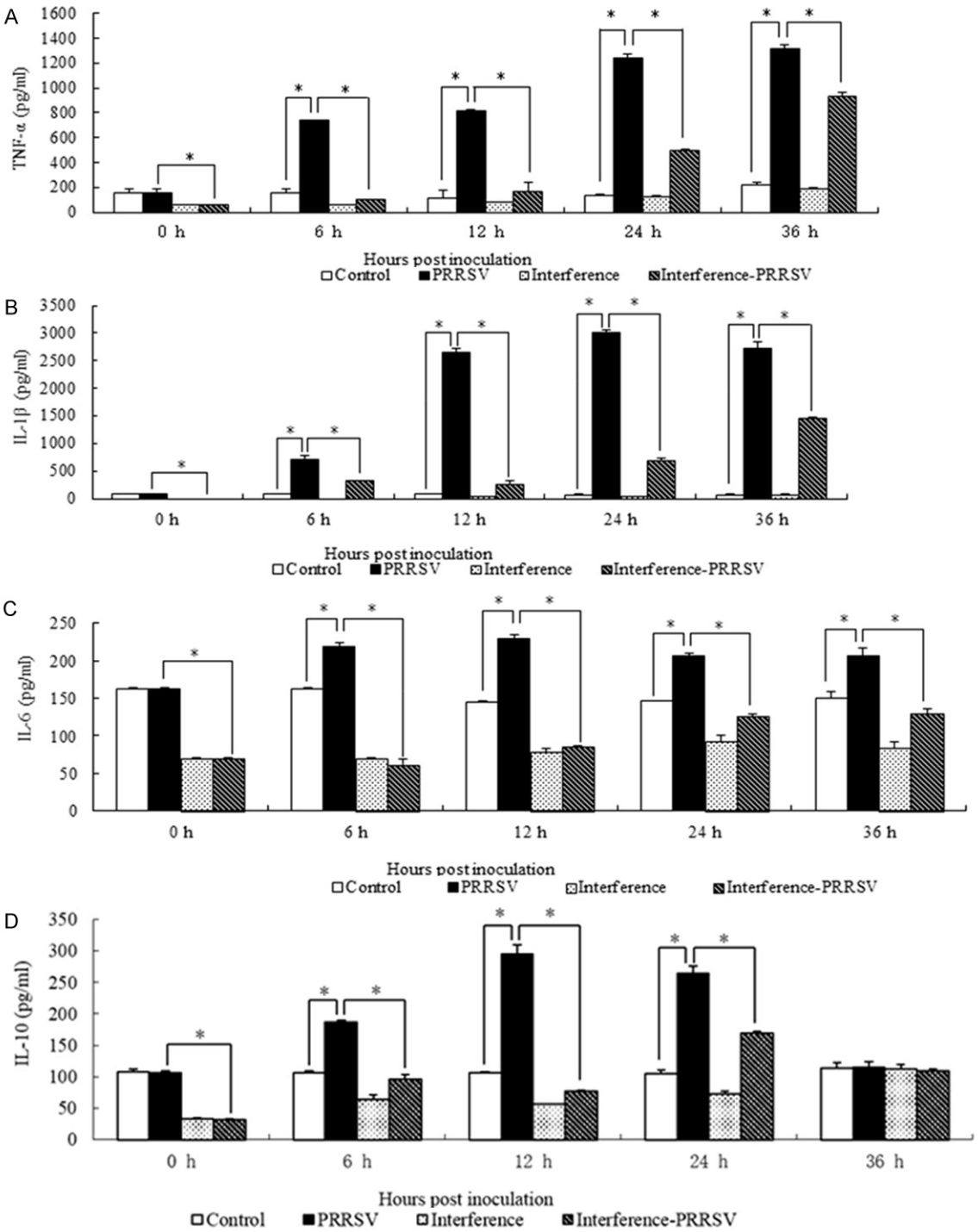


Figure 2. Effect of PCV2 on the Production of TNF-α, IL-1β, IL-6 and IL-10. Kinetics of TNF-α (A), IL-1β (B), IL-6 (C), IL-10 (D) concentration in supernatants of PAMs infected with PRRSV. The supernatant levels of porcine TNF-a (A), IL-1β (B), IL-6 (C), IL-10 (D) were measured with commercial ELISA kits. (*) indicate a significant difference ($P<0.05$) between groups. One-way ANOVA was used for the analysis.

group down to the lowest after 24 h (85%). The expression of MyD88 protein decreased with incubation time, but was still significantly lower than the control group (P<0.05).

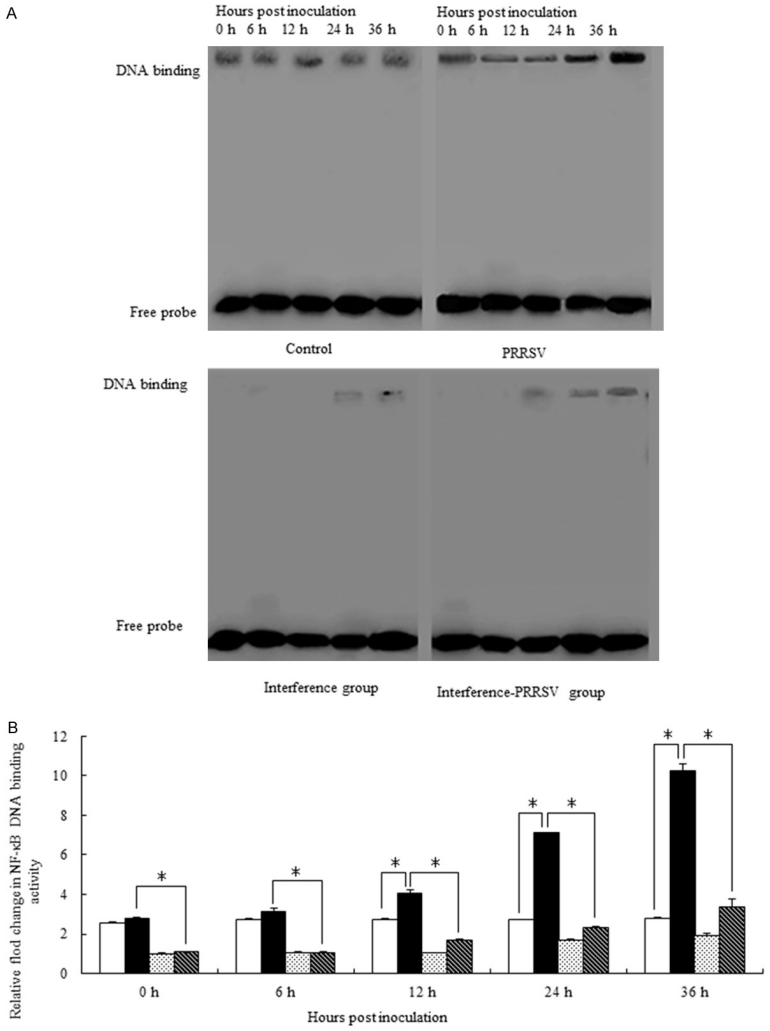


Figure 3. Effect of PRRSV on the DNA-binding Activity of NF-κB. An electrophoretic mobility shift assay (EMSA) was used to measure NF-κB DNA-binding activity after 6, 12, 24 and 36 h in controls group and PRRSV group, interference group and interference-PRRSV group. PRRSV increased NF-KB DNA-binding activity, and this effect was abrogated by interference of MyD88 gene. A. The picture of EMSA; B. The histogram of EMSA analysis. (*) indicate a significant difference (P<0.05) between groups. One-way ANOVA was used for the analysis.

Effect of PCV2 on the production of TNF-α, IL-1β, IL-6 and IL-10

The levels of TNF-α, IL-1β, IL-6 and IL-10 in the culture supernatant were determined by ELISA. The level of TNF-α (**Figure 2A**), IL-1β (**Figure 2B**) and IL-6 (**Figure 2C**) in the PRRSV group after 6, 12, 24, 36 h of incubation was higher than in the control group (P<0.05), The level of IL-10 (**Figure 2D**) in the PRRSV group after 6, 12, 24 h of incubation was higher than in the control group (P<0.05), but decreased rapidly after 36 h of incubation, and no significant differences between each groups. This suggesting that PRRSV infection led to increased TNF-α, IL-1β, IL-6 and IL-10 secretion significantly in PAMs. The levels of TNF-α, IL-1β, IL-6 and IL-10 in the interference-PRRSV group were higher than in the interference group, but lower than in the PRRSV group (P<0.05). These results demonstrate that effects of PRRSV on the levels of TNF-α, IL-1β, IL-6 and IL-10 were reversed by the interference of MyD88.

Effect of PRRSV on the DNA-binding activity of NF-κB

To determine whether NF-κB signaling was activated after PAMs were infected with PRRSV, EMSA was used to detect the ability of NF-κB to bind to DNA. Changes was shown in NF-κB DNA-binding activity in nuclear of PAMs infected with PRRSV. The binding activity of NF-κB in the PRRSV group after 12, 24 and 36 h of incubation was significant higher than in the control group (P<0.05; **Figure 3A** upper panel). The binding activity of NF-κB in the interference group was significant lower than in the control group (P<0.05). The binding activity of NF-κB in the interference-PRRSV group after 12, 24 and 36 h of incubation was significant lower than in the PRRSV group (P<0.05; **Figure 3A** lower panel), demonstrating that interference of MyD88 inhibited the binding activity of NF-κB caused by PRRSV. The analysis of the EMSA was shown in **Figure 3B**.

Nuclear translocation of NF-κB/p65 protein

Nuclear translocation of NFκB/p65 protein was detected by indirect immunofluorescence in PAMs incubated with PRRSV. Nuclear translocation of NF-κB/p65 protein can see in most PAMs

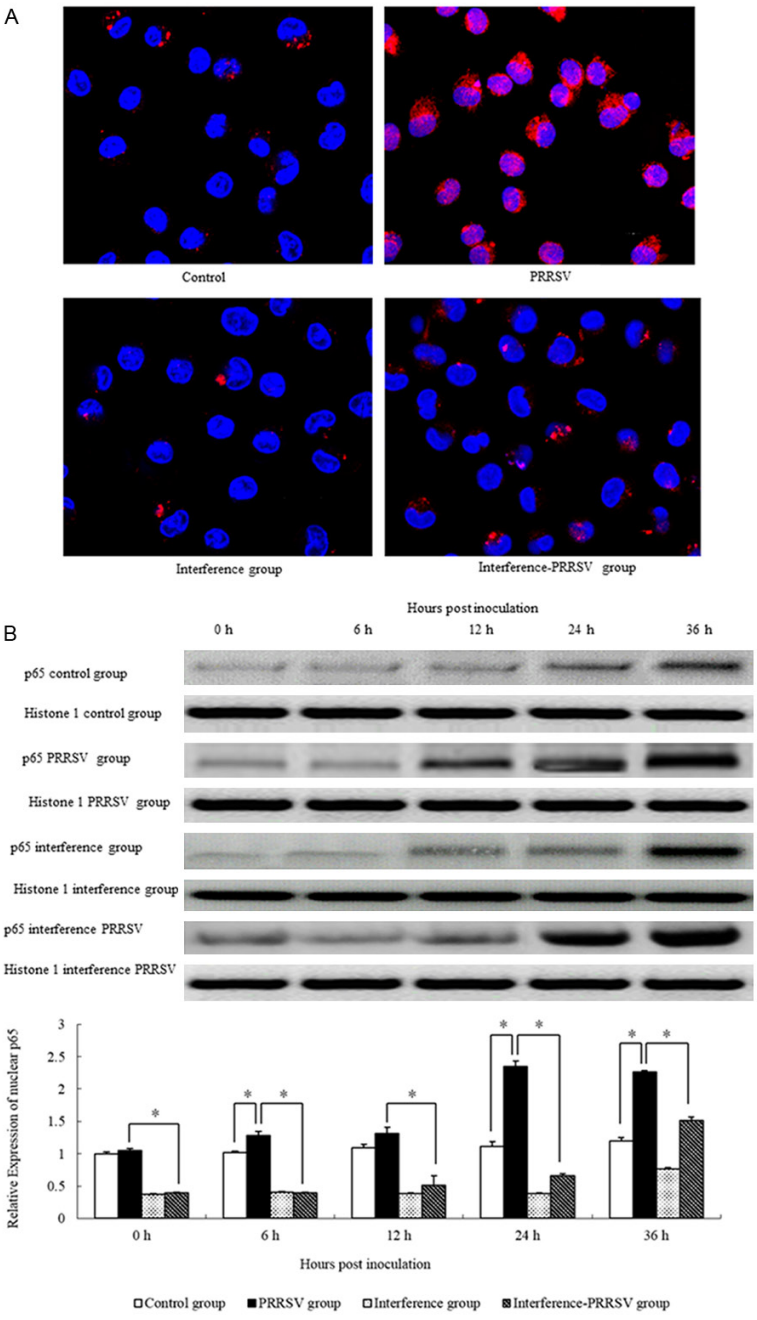


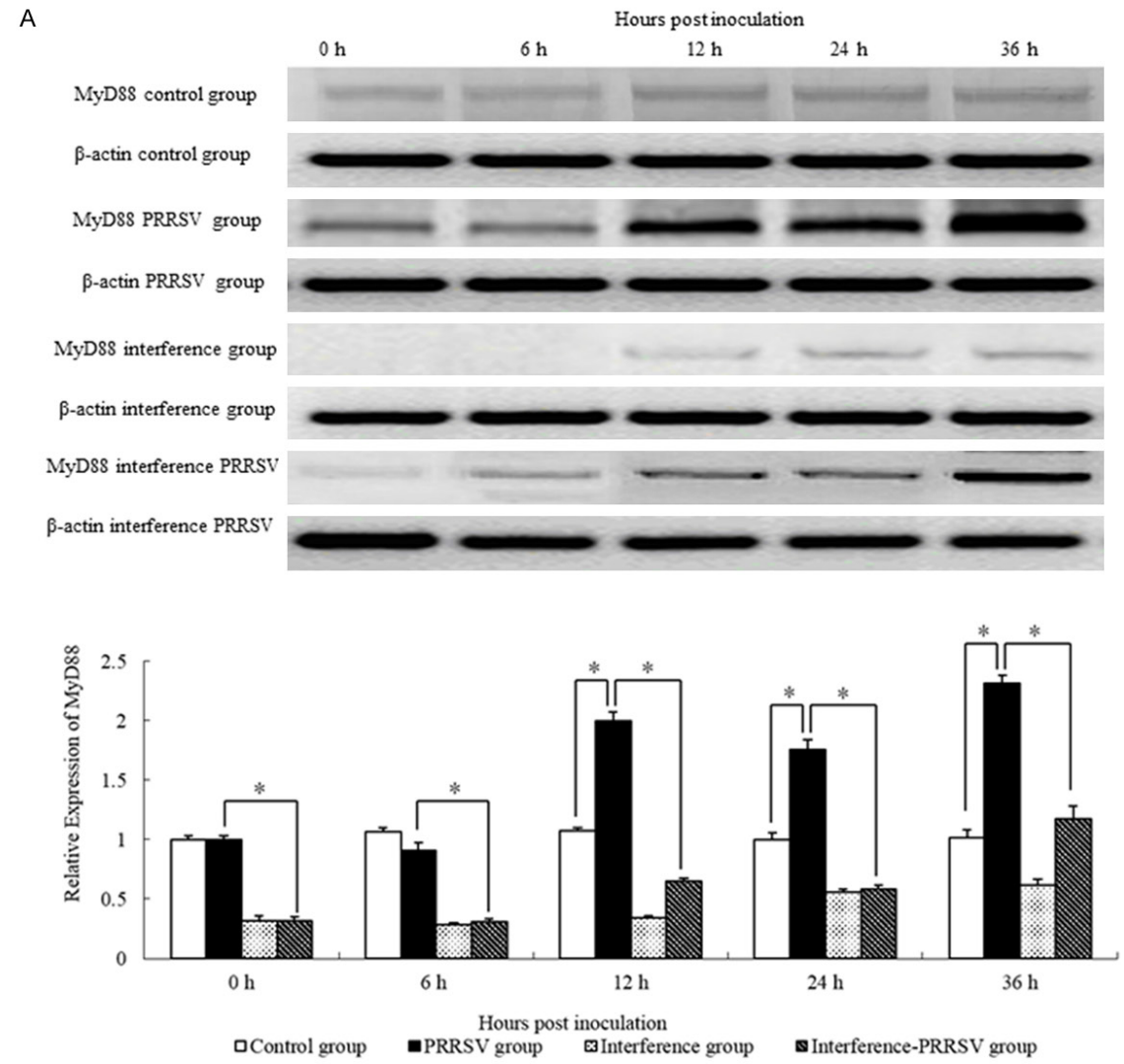
Figure 4. Nuclear translocation of NF-κB/p65 protein. A. NF-κB/p65 protein specific staining in PAMs by indirect immunofluorescence. DAPI for nucleus (blue). TRITC for NF-κB/p65 (red). Bar: 10 μm. B. Western blotting was used to measure expression of NF-κB/p65 protein in the nucleus at 6, 12, 24 and 36 h in each group of PAMs. Levels of p65 increased after PRRSV infection and this effect was abrogated by the silence of MyD88 gene. Expression of histone H3 was used as a positive control. (*) indicate a significant difference (P<0.05) between groups. One-way ANOVA was used for the analysis.

in the PRRSV group while less in the control group and interference group (**Figure 4A**).

To further investigate the nuclear translocation of NF-κB/p65 protein after PAMs were infected with PRRSV, levels of NF-κB/p65 protein was measured by Western blotting. The levels of p65 protein in the nucleus in the PRRSV group after 6, 24 and 36 h of incubation were significantly higher than in the control group (**Figure 4B**, $P<0.05$), indicating an increase in translocation of NF-κB/p65 from the cytoplasm to the nucleus. In the interference group and interference-PRRSV group after 6 h of incubation, the nuclear p65 protein levels were significantly lower than in the PRRSV group (**Figure 4B**, $P<0.05$), demonstrating that interference of MyD88 inhibits the nuclear translocation of NF-κB/p65.

Effect of PRRSV on MyD88, p-IκB, NF-κB/p65 in the cytoplasm

To further investigate whether NF-κB/p65 signaling was activated after PAMs were infected with PRRSV, levels of MyD88 (**Figure 5A**), p-IκB (**Figure 5B**), NF-κB/p65 (**Figure 5C**) protein in the cytoplasm was measured by Western blotting. The levels of MyD88, p-IκB protein in the PRRSV group after 12, 24 and 36 h were significantly higher than in the control group ($P<0.05$; **Figure 5A**, **5B**), description PRRSV can accelerate the process of phosphorylation of IκB. The levels of NF-κB/p65 protein in the cytoplasm in the PRRSV group after 12, 24 and 36 h were significantly lower than in the control group ($P<0.05$; **Figure 5C**). The levels of MyD88, p-IκB protein in the interference-PRRSV group after 6,


A
Hours post inoculation
0 h
6 h
12 h
24 h
36 h
MyD88 control group
β-actin control group
MyD88 PRRSV group
β-actin PRRSV group
MyD88 interference group
β-actin interference group
MyD88 interference PRRSV
β-actin interference PRRSV
Relative Expression of MyD88
2.5
2
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1
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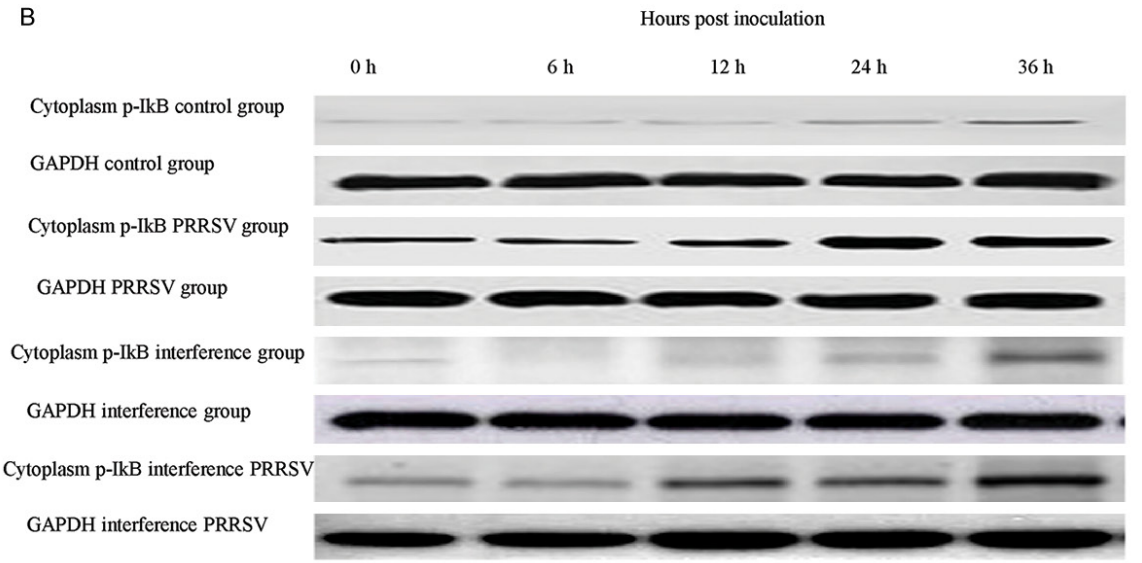
Hours post inoculation
Control group
PRRSV group
Interference group
Interference-PRRSV group


B
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Cytoplasm p-IkB control group
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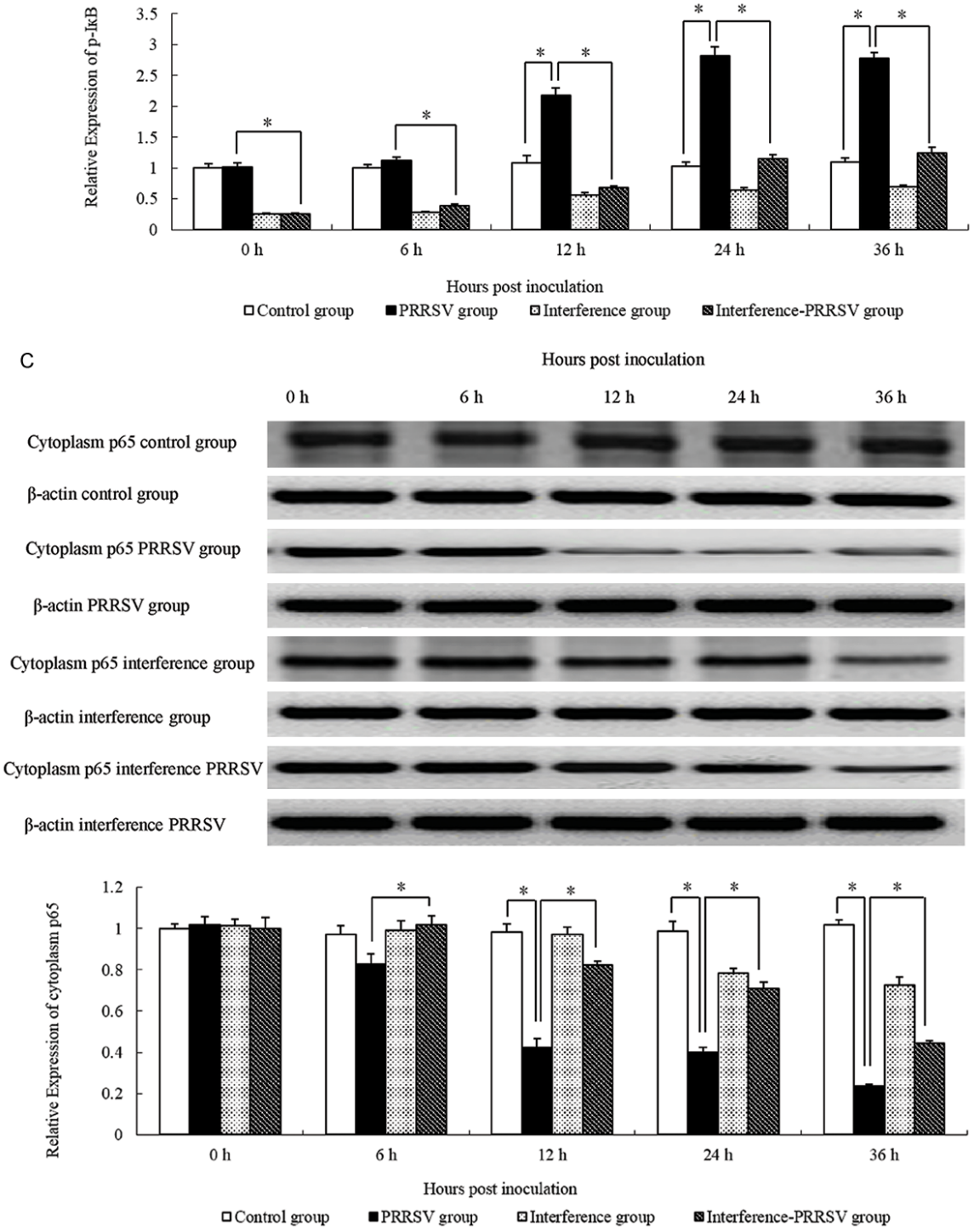
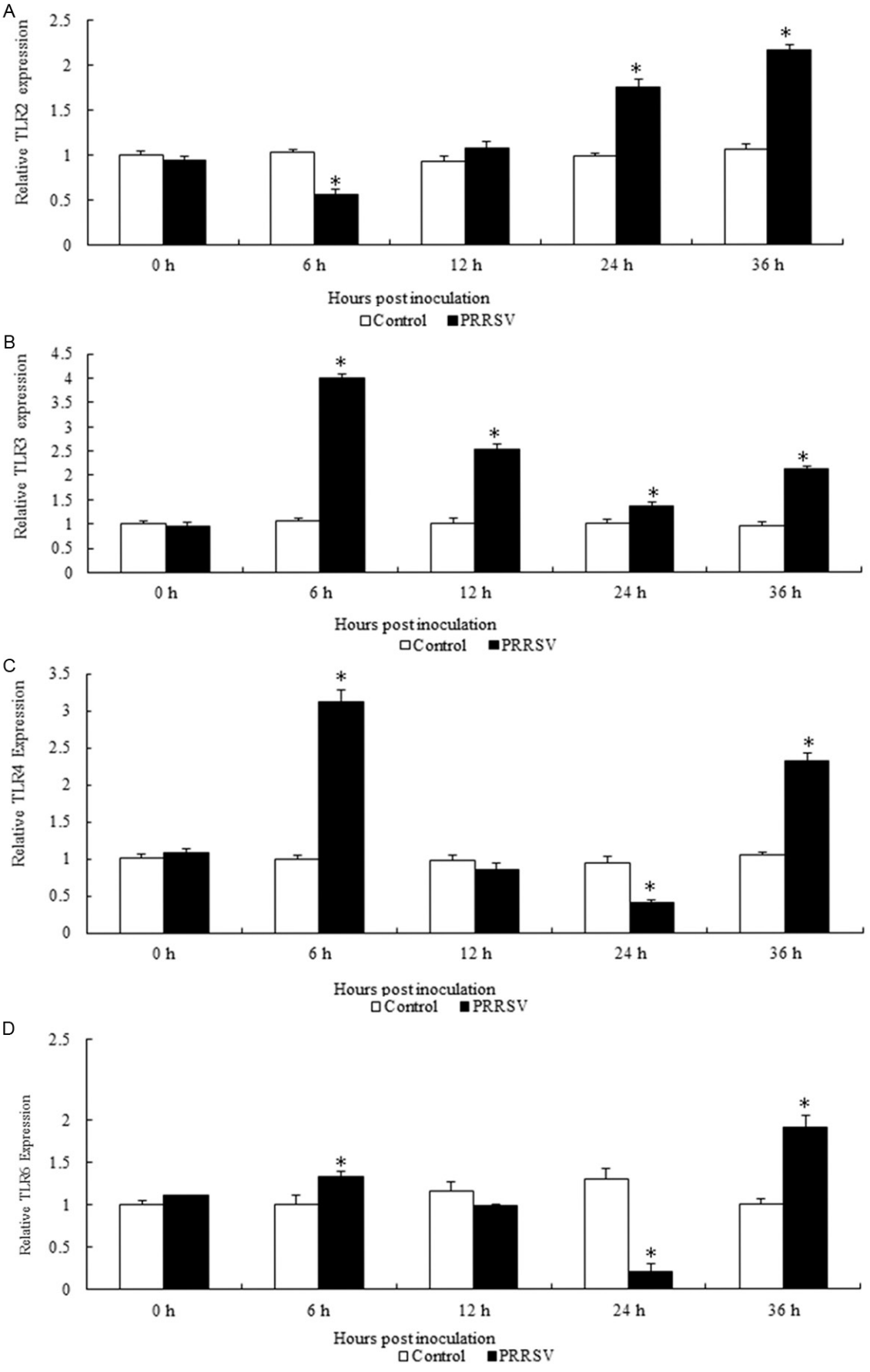


Figure 5. Effect of PRRSV on MyD88, p-IκB, NF-κB/p65 in the cytoplasm. A. Western blotting was used to measure expression of MyD88 protein in the cytoplasm at 6, 12, 24 and 36 h in each group of PAMs. Levels of MyD88 increased after PRRSV infection and this effect was abrogated by the silence of MyD88 gene. Expression of β-actin was used as a positive control. (*) indicate a significant difference (P<0.05) between groups. B. Western blotting was used to measure expression of p-IκB protein in the cytoplasm at 6, 12, 24 and 36 h in each group of PAMs. Levels of p-IκB increased after PRRSV infection and this effect was abrogated by the silence of MyD88 gene. Expression of GAPDH was used as a positive control. (*) indicate a significant difference (P<0.05) between groups. C. Western blotting was used to measure expression of NF-κB/p65 protein in the cytoplasm at 6, 12, 24 and 36 h in each group of PAMs. Levels of NF-κB/p65 decreased after PRRSV infection and this effect was abrogated by the silence of MyD88 gene. Expression of β-actin was used as a positive control. (*) indicate a significant difference (P<0.05) between groups. One-way ANOVA was used for the analysis.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Relative TLR2 expression
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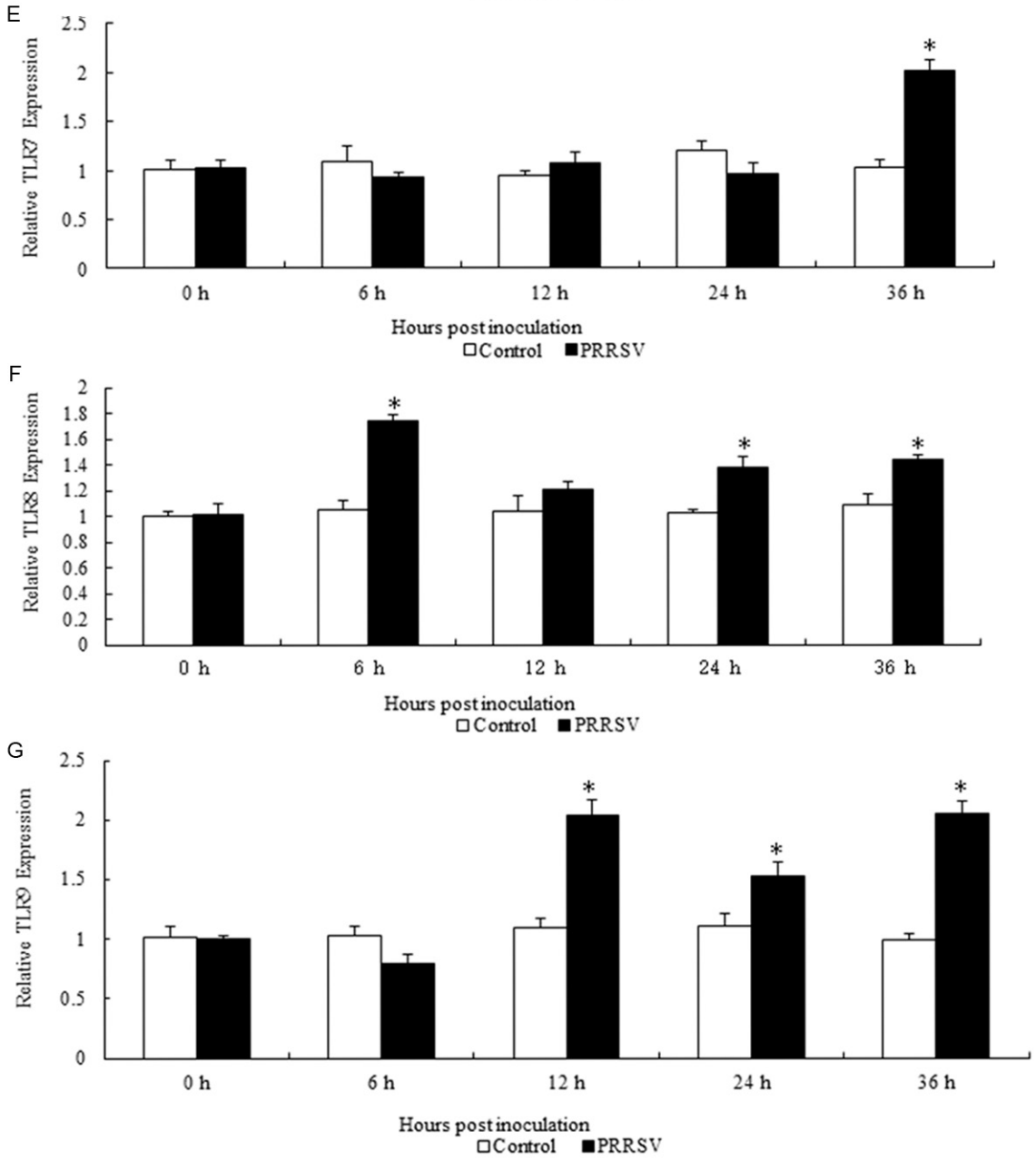

Figure 6. Effect of PRRSV on TLRs mRNA expression. Real-time RT-PCR was used to determined the mRNA expression of TLR2 (A), 3 (B), 4 (C), 6 (D), 7 (E), 8 (F) and 9 (G) in PAMs incubated with PRRSV and controls after 6, 12, 24 and 36 h. (*) indicate a significant difference ($P<0.05$) between groups. One-way ANOVA was used for the analysis.

12, 24 and 36 h were significantly lower than in the PRRSV group ($P<0.05$), demonstrating that interference of MyD88 inhibited the phosphorylation of IκB.

Effect of PRRSV on TLRs mRNA expression

To determine whether TLRs were activated after PAMs were inoculated with PRRSV, The expression levels of TLR2, 3, 4, 6, 7, 8 and 9 mRNA were examined individually by real-time RT-PCR in PAMs infected with PRRSV. The expression of TLR2 (**Figure 6A**) mRNA in the PRRSV group was significantly lower than in the control group at 6 h, higher at 24, 36 h ($P<0.05$). The expression of TLR3 (**Figure 6B**) mRNA in the PRRSV group was significantly higher than in the control group at 6, 12, 24, 36 h ($P<0.05$). The expression of TLR4 (**Figure 6C**) and TLR6

(**Figure 6D**) mRNA in the PRRSV group was significantly higher than in the control group at 6 and 36 h ($P<0.05$), lower at 24 h ($P<0.05$). The expression of TLR7 (**Figure 6E**) mRNA in the PRRSV group was significantly higher than in the control group at 36 h ($P<0.05$). The expression of TLR8 (**Figure 6F**) mRNA in the PRRSV group was significantly higher than in the control group at 6, 24, 36 h ($P<0.05$). The expression of TLR9 (**Figure 6G**) mRNA in the PRRSV group was significantly higher than in the control group at 12, 24, 36 h ($P<0.05$).

Discussion

After 6 h of PAMs were inoculated with PRRSV, the PRRSV antigen was seen in the cytoplasm of PAMs, and the number of infected PAMs increased with the incubation time, demonstrating that PAMs can be infected with PRRSV. Many studies show that PAMs is the major target cells of PRRSV, PRRSV can promote the apoptosis of cells after PAMs infection, in line with previous studies [10].

TNF-α is an important inflammatory cytokines secreted by activated macrophages and T cells, can activate T and B lymphocytes. Gomez and Chang found that the expression level of TNF-α protein significantly increased in PAMs infected with PRRSV. In this est, the virus of PRRSV-BB0907 is a highly pathogenic. Zhang [6, 11] also confirmed that this virus can significantly up-regulated the expression of TNF-a in serum and PAMs infection in piglets, This may be one of the reasons leading to the apoptosis of macrophages infected with PRRSV.

IL-1β is one of the main cytokines produced by alveolar macrophages immune response, with a variety of immune regulation function, including participation in the body's defense and promote systemic inflammatory reaction. The experiment shows that the expression level of IL-1β protein was up-regulated after 6 h infected with PRRSV, and continued until 36 h. This result is in line with Gomez previous studies, maybe a kind of embodiment of alveolar macrophages in response to viral infections after infected with PRRSV.

IL-6 is considered with cytokine in promoting the dual role of proinflammatory and antiinflammatory [12], is also a important early indicators of pig health, high level of IL-6 produced after seventh days in pigs infected with PRRSV. In the present study, the expression level of IL-6 protein was significantly higher than that in the control group after 6 h in PAMs infected with PRRSV. The increasing of IL-6 also showed the body begins to produce the immuneinflammatory reaction in the presence of PRRSV.

IL-10 is an important anti-inflammatory cytokine, can inhibit the synthesis and release of IFN-α, IL-12 and TNF-α. Subramaniam thought that IL-10 expression levels were correlated to PRRSV virulence. Some strain can up-regulated the expression level of IL-10, and some strains can down-regulate the expression level of IL-10. The PRRSV virulence we used in this study can enhanced production of IL-10 in PAMs culture supernatant. The expression level of IL-10 protein was significantly higher than that in the control group after 6 h. The Literature indicates that [13], some pathogens of the macrophage as a target cell, usually induced the expression level of IL-10 that can inhibit the body's cellular immune response and prevent clearance of the pathogen. Therefore, in this study, high expression of IL-10 caused by PRRSV is an important reason of immune inhibition and persistent viral infections.

Signaling pathway plays a central role in the process of secretion of various cytokines, TLRs are an important class of pattern recognition receptors (PRRs). They start innate immune response against the pathogen by distinguishing pathogen-associated molecular patterns (PAMPs). Lee [14] demonstrated PRRSV can activate NF-κB pathway, and NF-κB protein are downstream regulatory factors of TLRs signaling pathways. We fiest detected the ability of NF-κB to bind to DNA, found the binding activity of NF-κB in the PRRSV group after 12 h of incubation was significant higher than in the control group, and was increased with the incubation time. Meanwhile, indirect immunofluorescence and western blot results also showed nucleus NF-κB/p65 protein in PRRSV group was significantly higher than in the control group after 6 h, this is the same trend with the ability of NF-κB binding to DNA, indicating that PRRSV can promote NF-κB translocation into the nucleus and bind to DNA.

In resting cells, NF-κB homodimer (p65/p50) and its inhibitor (IκB) complex formed in the cytoplasm [15]. Once the cells are stimulated

by LPS or viruses, non-active NF-κB-IκB complexes incurs phosphorylated, IκB degradation, nuclear localization sequence of NF-κB revealed that NF-κB translocation into the nucleus and activates transcription of target genes [16]. Therefore, the content of the p-IκB change in the cytoplasm can reacted the activation status of NF-κB. The levels of p-IκB protein in the PRRSV group after 12 h was significantly higher than the same time in the control group, indicating that PRRSV can activate NF-κB degradation pathway through IκB phosphorylation. The results are in line with Lee [14] et al in MARC-145 cells studies. Virus infection associated TLRs mainly TLR2, 3, 4, 7, 8 and 9, TLR2 and TLR4 mainly recognize the lipid and protein composition of virus. In this study, TLR2 and TLR4 mRNA was complementary expression in PRRSV after 36 h infection. Namely 6 h after infection, the down-regulation of TLR2 expression and up-regulation of TLR4 expression; infection in 12 h and 24 h, increase the expression of TLR2 and decrease the expression of TLR4, and were both significantly higher than the control group after 36 h infection. Studies have found that, TLR2 and TLR4 can shuttled between the cell surface and Golgi. So there will be a increase expression of TLR2 and decrease expression of TLR4 in dynamic balance.

TLR3, TLR7, TLR8, TLR9 were located in the intracellular, mainly recognize the nucleic acid components of virus [17]. In this study, the expression of TLR3 was increased in PAMs infected with PRRSV, probably related a large number of dsRNA produced during PRRSV replication in cells [18]. According to reports, the expression of TLR3 and TLR7 are up-regulated in PRRSV infected brain tissue and peripheral nerve. Liu found in lymphoid tissue of infected pigs TLR2, 3, 4, 7, 8 increased and Zhang [6, 11] reported that TLR3, 8, 9 increased in PAMs infected with PRRSV, our study is in line with their results.

Interestingly, in this study, the most (85%) MyD88 gene was silenced by using small interfering RNA (siRNA) method. And then the PAMs infection of PRRSV, not only the expression of MyD88 protein was significantly decreased, and the downstream p-IκB, NF-κB/p65 protein in the nucleus and the ability of NF-κB to bind to DNA were lower than the control group. The trend of their changes are consistent. At the same time, the PAMs infected with PRRSV after MyD88 gene interference (interference-PRRSV group), the expression of TNF-α, IL-1β, IL-6 and IL-10 were significantly lower than the PRRSV group in culture supernatants, indicating that once MyD88 gene was knocked out, effect of PRRSV on activation of MyD88-NF-κB signal pathway in PAMs will decreased, the secretion via this way regulation of TNF-α, IL-1β, IL-6 and IL-10 also reduced. This fully confirmed, PRRSV regulation the secretion of TNF-α, IL-1β, IL-6 and IL-10, and these changes were regulated by the TLR-MyD88-NF-κB signal pathway.

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Disclosure of conflict of interest

None.

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