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The integrity of PRRSV nucleocapsid protein is necessary for up-regulation of optimal interleukin-10 through NF-κB and p38 MAPK pathways in porcine alveolar macrophages

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ABSTRACT

Porcine reproductive and respiratory syndrome (PRRS), a highly contagious disease, has been constantly causing huge economic losses all over the world. PRRS virus (PRRSV) infection results in immunosuppression and IL-10 up-regulation. The relationship between them is still in dispute. Previous studies demonstrated the protein of PRRSV nucleocapsid (N) protein is able to up-regulate IL-10, yet the underlying molecular mechanisms remain unknown. In this study, the expression kinetics of IL-10 up-regulation induced by PRRSV N protein were analyzed in immortalized porcine alveolar macrophages (PAMs). N protein induced IL-10 expression in a time- and dose-dependent manner. Inhibition experiments of signaling pathways suggested NF-κB and p38 MAPK pathways are both involved in N protein-induced IL-10 up-regulation. Besides, the integrity of N protein is essential for significant IL-10 up-regulation. This research is beneficial for further understanding of the interplay between PRRSV and host immune system.

1. Introduction

Porcine reproductive and respiratory syndrome (PRRS), a highly contagious disease, is characterized by reproductive failures, including late-term abortions, increased number of stillborn and mummified fetuses in sows, and respiratory problems in pigs of all ages [31]. Since its first epidemic in the United States in 1987, PRRS has been constantly causing huge economic losses all over the world [9,21,23]. So far, commercial vaccines are still not able to provide high efficacy and guaranteed safety.

PRRSV infection leads to immunosuppression which includes weak innate immune responses and poor induction of acquired immune responses, especially a delayed appearance of neutralizing antibodies [13,17,25]. As a matter of fact, host innate immune responses play a critical role in initiating resistance and developing subsequent adaptive immune responses. Although IFNα/β could significantly enhance host immune responses and suppress PRRSV replication, they are scarely secreted no matter in target cells or in infected lungs [14,24]. However, PRRSV infection could significantly up-regulate interleukin-10 (IL-10) both in vitro and in vivo [7,15,29,32,33]. IL-10 is an immuno-regulatory cytokine that plays an important role in protecting the host from infection associated immunopathology, autoimmunity and allergy [22].

Previous studies found PRRSV infection could activate NF-κB, PI3K and p38 MAPK signaling pathways [18,29,38]. NF-κB is a family of inducible transcription factors involving pathogen- or cytokine-induced immune and inflammatory responses as well as cell proliferation and survival [18]. PI3K signaling pathways are

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involved in many cellular processes, such as cell proliferation, differentiation, and survival [2]. A number of viruses, including RNA viruses, have been shown to utilize the PI3K pathways to enhance their replication and pathogenesis. Many viruses, especially epidemic immunosuppressive viruses can induce IL-10 expression through MAPK subfamilies signaling pathways in different cell types [8,10,11].

PRRSV contains 10 open reading frames (ORFs) that encode 14 non-structural proteins (NSPs) and 8 structural proteins. The virion contains three major structural proteins: a nucleocapsid protein N (ORF7), a non-glycosylated membrane protein M (ORF6), and a major envelope glycoprotein GP5 (ORF5) [6,28]. N protein is the most abundant virion component and the most immunogenic protein in PRRSV-infected pigs [1]. Antibodies first produced are against N protein and could last for up to twelve months, but they couldn't neutralize viruses [5]. To some extent, this special immunogenicity prevents PRRSV from effective host immune clearance. Up to now, N protein has been demonstrated to possess several regulative functions, such as participating in ribosome biogenesis, delaying cell cycle progression and modulating interferon-β production [30,39]. More important, N protein is proved to be able to up-regulate IL-10 in immortalized porcine alveolar macrophages (PAMs, ATCC 3D4/2) [35], but the regulatory manners are poorly understood. In this study, we investigated the underlying molecular mechanisms of IL-10 up-regulation by PRRSV nucleocapsid protein.

2. Material and methods

2.1. Viruses and cells

PRRSV ZCYZ strain, 2 deletions were identified: a novel 25-aa deletion at positions 476–500, and a 29-aa deletion at positions 533–561, originally isolated from Hybrid wild boars in Shandong Province, China [37], was preservated in Shandong Key Laboratory of Animal Disease Control and Breeding. MARC-145 cells were maintained in DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 units/ml penicillin G and 100 μg/ml streptomycin sulfate. Immortalized PAMs (porcine alveolar macrophages, ATCC 3D4/2) was cultured in RPMI-1640 medium (Invitrogen, Carlsbad, CA, USA) with 10% heat-inactivated FBS, 100 units/ml penicillin G and 100 μg/ml streptomycin sulfate in a humidified 37°C/5% CO_2 incubator.

2.2. Construction of plasmids for transfection experiments

A pair of specific primers was designed to clone the plasmid encoding the N protein from PRRSV ZCYZ strain (F: CGGAATTCATG*GATTACAAGGATGACGACGATAAG*CCAAATAACAACGG; R: TGCTCTAGATTATCATGCTGAGGGTGATGCTGTGG). In order to analyze the expression of N protein, a flag tag (italic part) was inserted into the front primer. The PCR products and pCI-neo vector were ligated after subjected to double enzyme digestion by EcoRI and XbaI. Five truncated N protein (tN1, tN2, tN3, tN4 and tN5) expression vectors were constructed referred to pCI-neo-N.

2.3. Transfection experiments

For transfection of PAMs, a porcine PAM cell line (ATCC 3D4/2) was split into a 12-well plate at 3–6 × 10^5 cells in a volume of 1 ml per well and incubated overnight to allow complete attachment on the plate. Cells were transfected with 1 μg pCI-neo-N, pCI-neo-tN1, pCI-neo-tN2, pCI-neo-tN3, pCI-neo-tN4, pCI-neo-tN5 using Lipofectamine 2000 (Invitrogen) and incubated for 24 h at 37 °C in a 5% CO_2 incubator according to the manufacturer's protocol. At the end of the incubation period, cells were harvested and washed once with PBSA. The positive-control group was PAMs cultured with LPS (10 μg/mL). The negative-control groups were cultured PAMs alone and PAMs transfected with pCI-neo.

2.4. Inhibition of signal transduction pathways

MAPK inhibitors (SP600125, SB202190 and U0126) and NF-κB inhibitor (BAY11-7082) were purchased from Sigma-Aldrich; PI3K inhibitor (LY294002) was purchased from Enzo life Sciences. They were all dissolved in DMSO prior to use. Inhibitors treatment was conducted 2 h after vector transfection.

2.5. Real-time PCR

Total cellular RNA was extracted from PAMs using an RNAprep pure cell kit (Qiagen, Valencia, CA, USA). RNA (≤1 μg) was reverse transcribed in a 20 μL reaction mixture. The cDNA product was amplified in a 20 μL reaction mixture containing SYBR Green Real-time PCR Master Mix (TaKaRa, Japan). Gene-specific primers designed for real-time RT-PCR are listed in Table 1. Each cDNA sample was performed in triplicate. PCR amplifications were performed using a Roche Light Cycler 480 Real-Time System (Roche, Basel, Switzerland). Thermal cycling conditions were 30 s at 95 °C and 40 cycles of 5 s at 95 °C, 20 s at 60 °C. Gene expression was measured as a relative quantity as described previously [34].

2.6. Immunofluorescent assay (IFA)

Transfected PAMs were washed three times with PBSA, fixed with 4% formaldehyde in PBSA for 10 min, and permeabilized with 0.1% NP-40 (Sigma- Aldrich) in PBSA for 15 min at room temperature. After blocking with 1% BSA in PBSA, the cells were incubated with anti-Flag M2 mAb (Sigma-Aldrich) for 1 h. Then the cells were incubated with Goat Anti-Mouse IgG(H + L) (Invitrogen) for another 1 h. The presence of the N protein was determined with a fluorescent microscope.

2.7. Western blot analysis

Proteins were separated by SDS-PAGE (12% acrylamide) and transferred to NC membrane (Pierce) at 100 mA for 90 min. After blocking with 5% BSA(BD) in PBSA, the membrane was incubated with anti-Flag M2 mAb diluted at 1:2000 for 1 h at room temperature, washed three times with PBST (0.05% Tween-20 (Sigma-Aldrich) in PBSA), following by incubation with 1: 8000 HRP-conjugated goat anti-mouse IgG(H + L) (Invitrogen) in 1% BSA in PBSA for 1 h. At the end of incubation period, the antibodies were visualized using ECL reagent (GE Healthcare) according to the manufacturer's instructions.

2.8. Statistical analysis

All experiments were performed with at least three independent replicates. Data were presented as mean ± standard deviation

Table 1
Primers used in real-time PCR.

Name	Sequence 5′–3′
β-actin forward	GCGGGACATCAAGGAGAAG
β-actin reverse	AGGAAGGAGGGCTGGAAGAG
IL-10 forward	TTCAAACGAAGGACCAGATG
IL-10 reverse	CACAGGGCAGAAATTGATGA
IL-12 p40 forward	GGGTGGGAACACAAGAGAT
IL-12 p40 reverse	GGCTAAACTTGCCTAGAGGT