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Mycoplasma hyopneumoniae Mhp597 is a cytotoxicity, inflammation and immunosuppression associated nuclease

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ABSTRACT

Nucleases are ubiquitously recognized as essential proteins in mycoplasmas because these organisms lack most capacities for *de novo* synthesis of nucleotides. Some of these proteins were proved to be important pathogenic factors during the infection of mycoplasms. In this study, the protein Mhp597 from *M. hyopneumoniae* was expressed and purified in *Escherichia coli*. Analysis of nuclease activity showed that recombinant Mhp597 (rMhp597) was a Ca_{2+} or Mg_{2+} dependent thermostable nuclease with very high activity and neutrophil extracellular traps (NETs) induced by *M. hyopneumoniae* were completely degraded by this nuclease. In addition, when PK15 cells were incubated with different concentrations of rMhp597, their viability was reduced and cell apoptosis was observed in a dose-dependent manner. To further investigate the host immune system response, we report that rMhp597 up-regulated the exression of inflammatory genes showing that TLR4/MyD88/NF-κB signal pathway was involved. On the other hand, rMhp597 down-regulated the expression of type I IFN (IFN-α/β) and promoted the multiplication of porcine reproductive and respiratory syndrome virus (PRRSV). Recombinant rMhp597$\delta^{315-377}$ lacking C-terminal 63 amino acids exhibited all biological functions mentioned above except for nuclease activity. In summary, Mhp597 is a dynamic secreted nuclease involved in cytotoxicity, inflammation and immunosuppression.

1. Introduction

Mycoplasmas are prokaryotic organisms between bacteria and viruses. And they are widespread in nature as conditional pathogens of several diseases in humans and animals (Kovalenko et al., 1969), such as *Mycoplasma pneumoniae*, *Mycoplasma gallisepticum*, *Mycoplasma hyopneumoniae,* among others. *Mycoplasma hyopneumoniae* is generally considered one of the most important pathogens of porcine respiratory disease, causing important economic losses every year in many countries (Razin et al., 1999). And both *M. hyopneumoniae* and PRRSV are regarded as significantly important pathogens associated with porcine respiratory disease complex and it is found that *M. hyopneumoniae* potentiates pneumoniae induced by PRRSV (Thacker et al., 1999). Regardless, the pathogenic mechanisms of *M. hyopneumoniae* have not been well described (Deutscher et al., 2010) and few methods are currently used to prevent and cure the disease (Marchioro et al., 2016).

In *M. hyopneumoniae*-infected hosts, the bacteria can be detected in the ciliated epithelium cells of the trachea, bronchi and bronchioles, leading to damage of cilia and epithelium cell death. Histological lesions are characterized by well-defined dark red to purple areas of cranioventral pulmonary consolidation and inflammatory exudate in the airway that usually contains accumulated neutrophils and macrophages (Maes et al., 2009). Like many other mycoplasmas, *M. hyopneumoniae* exert its pathogenetic role through some virulence factors, which can be proteins or toxins associated with colonization, persistence or cytotoxicity (Deutscher et al., 2010; Xu et al., 2015; Zhang et al., 2016). Although virulence factors associated with cytotoxicity and inflammation have not been completely elucidated, adhesion proteins have been well characterized (Whittlestone, 2012; Hsu and Minion, 1998; Deutscher et al., 2012) and some vaccines developed (Marchioro et al., 2016; Maes et al., 2017).

Several studies suggest that nucleases are important cytotoxic factors during the infection of mycoplasmas (Xu et al., 2015; Zhang et al., 2016). *Mycoplasma gallisepticum* MGA_0676 is a membrane-associated cytotoxic nuclease with a staphylococcal nuclease region essential for nuclear translocation and apoptosis induction in chicken cells (Xu et al.,

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2015). *Mycoplasma bovis* MBOV_RS02825 encodes a secretory nuclease associated with cytotoxicity (Zhang et al., 2016). Like many other Mollicutes, *M. hyopneumoniae* cannot synthesize *de novo* pyrimidine and purine bases due to its limited bio-synthetic capabilities and its small genome size (Schmidt et al., 2007). To grow and replicate, the pathogen must assimilate metabolic precursors from the host. Mhp379 of *M. hyopneumoniae* is a Ca_2+ dependent, sugar-nonspecific exonuclease exposed on the cell surface, and it comprises part of a conserved ABC transport operon in the cells and its nuclease activity may be associated with the conserved function of the ABC transport system in the import of nucleic acid precursors (Schmidt et al., 2007). Thus, nucleases may be indispensable constituents of mycoplasma for scavenging nucleotides from the extracellular environment.

There are reports indicating that nucleases released by pathogens are able to destroy the DNA matrix of neutrophil extracellular traps (NETs) (Yamamoto et al., 2016; Zhang et al., 2016). The destruction of NETs efficiently protect pathogens from being captured and killed by neutrophils (Yamamoto et al., 2016). In addition, many nucleases are confirmed to be lipoprotein and lipid-associated membrane proteins of *M. hyopneumoniae* and could induce inflammation in porcine peripheral blood mononuclear cells (Bai et al., 2014).

Mhp597 was previously described as a *M. hyopneumoniae* putative virulence factor (Ferreira and Castro, 2007) and MHP7448_0580, homologous to Mhp597, was found to be a secretory nuclease in *M. hyopneumoniae* (Paes et al., 2017), while no further studies had been made on this nuclease. In this way, the focus of this study was to characterize the enzymatic properties of *M. hyopneumoniae* Mhp597 nuclease and confirm its role in cytotoxicity and host pathogenicity.

2. Material and methods

2.1. Reagents and antibodies

The calf thymus DNA, lipopolysaccharides (LPS), Phorbol-12-myristate-13-acetate (PMA), SYTOX Green reagent and nucleus fluorescent dye 49,6-diamidino-2-phenylindole (DAPI) were purchased from Sigma (Sigma-Aldrich, Shanghai, China). Restriction enzymes *Nco*I and *Xho*I were acquired from the New England BioLabs Company (Ipswich, MA, USA). Detoxi-GelTM Endotoxin Removing Columns Kit was purchased from ThermoFisher Scientific (USA). ToxinSensorTM Chromogenic LAL Endotoxin Assay Kit was purchased from Kingsy Biotechnology (Nanjing, China). ANNEXIN V-FITC/PI Apoptosis Detection Kit was obtained from the BD Company (Franklin Lakes, NJ, USA). Alexa Fluor 555-conjugated phalloidin (red) was obtained from Invitrogen (USA). TLR4 inhibitor TAK-242, MyD88 inhibitor ST2825 and NF-κB inhibitor Bay11-7082 were purchased from MedChem Express (USA). The small interfering RNA (siRNA) constructs targeting TLR2, TLR3, TLR4, TLR8, TLR9, RIG-I, MyD88, TRIF and P65 were synthesized by GenePharma (China). HRP-conjugated goat-anti-rabbit antibody, mouse anti-His-tag monoclonal antibody, FITC-conjugated goat-anti-mouse antibody and HRP-conjugated goat-anti-mouse antibody were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Rabbit anti-PRRSV-Nsp4 protein antibody was purchased from GeneTex (USA). All of the chemical reagents used in the study were of analytical grade.

2.2. Bacterial strains and cell culture conditions

M. hyopneumoniae V11 strain (ATCC® 27714™, acquired from China Veterinary Culture Collection Center, Beijing, China) was grown in Friis medium with 20% porcine serum at 37 °C in 5% CO_2. Highly pathogenic PRRSV strain (JXwn06) (GeneBank accession, EF641008.1) was kindly donated by professor Xinna Ge, China Agricultural University, Beijing, China. *Escherichia coli* (*E. coli*) Simple-T1 (Invitrogen, USA) and *E. coli* BL21(DE3) (TransGen Biotech, Beijing, China) were propagated in Luria–Bertani (LB) broth. Neutrophils were isolated from the peripheral blood of two 6-week-old specific-pathogen-free (SPF) piglets (Beijing Centre of SPF Swine Breeding and Management, Beijing, China) by using Porcine Peripheral Blood Isolation Kits (TBD science, Tianjin, China). Neutrophils were maintained in RPMI-1640 medium with 5% heat-inactivated FBS (Gibco, Life Technologies). Porcine alveolar macrophage cells (PAMs) were isolated from the lung lavage of the SPF piglets, and multiplied in RPMI-1640 medium (100 U/ml penicillin and 100 μg/ml streptomycin) with 10% heat-inactivated fetal calf serum (FBS) as previously described (Patel et al., 2008). PK15 cell lines were maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, Life Technologies), supplemented with 10% FBS containing 100 U/ml penicillin and 100 μg/ml streptomycin, respectively.

2.3. Cloning, site-directed mutagenesis, expression and purification of rMhp597 and rMhp597δ$^{315-377}$

Chromosomal DNA of *M. hyopneumoniae* was isolated using TIANamp Kit (TIANGEN Biotech Co., Beijing, China). The intact open reading frame of the Mhp597 gene was cloned into SimpleT1 plasmid (TransGen Biotech, Beijing, China) and sequenced by Shanghai Majorbio Bio-Pharm Technology Co., Ltd. (Shanghai, China) to confirm if the DNA fragment produced by PCR was the target fragment. Signal peptide cleavage sites were analyzed using SignalP Server (http://www.cbs.dtu.dk/services/SignalP/). Strongly basic (+) amino acid rich region (C-terminal 63 amino acids) was analyzed by DNAStar software. The primers used in TGA mutation, amplifying the Mhp597 gene lacking the signal peptide sequence and without both signal peptide sequence and C-terminal 63 amino acids are listed in Table S1. The DNA product, TGA-corrected and with the signal peptide sequence removed, was cloned into SimpleT1 plasmid to obtain T1-Mhp597 and then recombined into PET28a plasmid to produce PET-Mhp597, followed by transformation into *E. coli* BL21(DE3) competent cells.

E. coli BL21(DE3) / PET-Mhp597 transformants were cultivated at 37 °C for 4 h with constant shaking, followed by induction using 1 mM IPTG for 5 h. Cells were collected and disrupted by sonication and then the supernatant was separated by centrifugation. Saturated ammonium sulfate was added into the supernatant to 40% saturation to remove irrelevant proteins. The supernatant was collected 20 min later, then re-suspended into saturated ammonium sulfate at 55% saturation. The precipitate was re-suspended and dialyzed in phosphate buffer saline (PBS, 10 mM, PH7.2), followed by purification using nickel affinity chromatography Ni-Affinity Chromatography (Qiagen, NY, USA). The expression and purification of rMhp597δ$^{315-377}$ was performed following previously described protocols (Xu et al., 2015). Endotoxins from bacteria were removed using the Detoxi-GelTM Endotoxin Removing Columns Kit, and the endotoxin residue in purified rMhp597 or rMhp597δ$^{315-377}$ was detected with the ToxinSensorTM Chromogenic LAL Endotoxin Assay Kit (Kingsy Biotechnology, Nanjing, China). Protein suspension was separated by SDS-PAGE electrophoresis and stained using a Silver Stain Kit (TransGen Biotech, Beijing, China).

2.4. Preparation of rabbit anti-rMhp597 polyclonal antibody

Two New Zealand Rabbits of clean grade were immunized with recombinant rMhp597 protein as described previously (Harlow and Lane, 1999). After three separate immunizations, the serum was collected and purified by ammonium sulfate graded precipitation.

2.5. Analysis of nuclease activity

Nuclease activity of rMhp597 was visualized by agarose gel electrophoresis and analyzed as described by Xu et al. (2015). Briefly, 0.1 μg of rMhp597 was incubated at 37 °C in 25 μl nuclease reaction buffer (25 mM Tris-HCl, pH 8.0, 1 mM $MgCl_2$, 1 mM $CaCl_2$) containing 1 μg of calf thymus DNA (double stranded DNA, dsDNA; Sigma, USA), M13 phage DNA (single stranded DNA, ssDNA; New England BioLabs), pCMV-HA plasmid DNA or total RNA from PK15 cells. Then, 10 μl

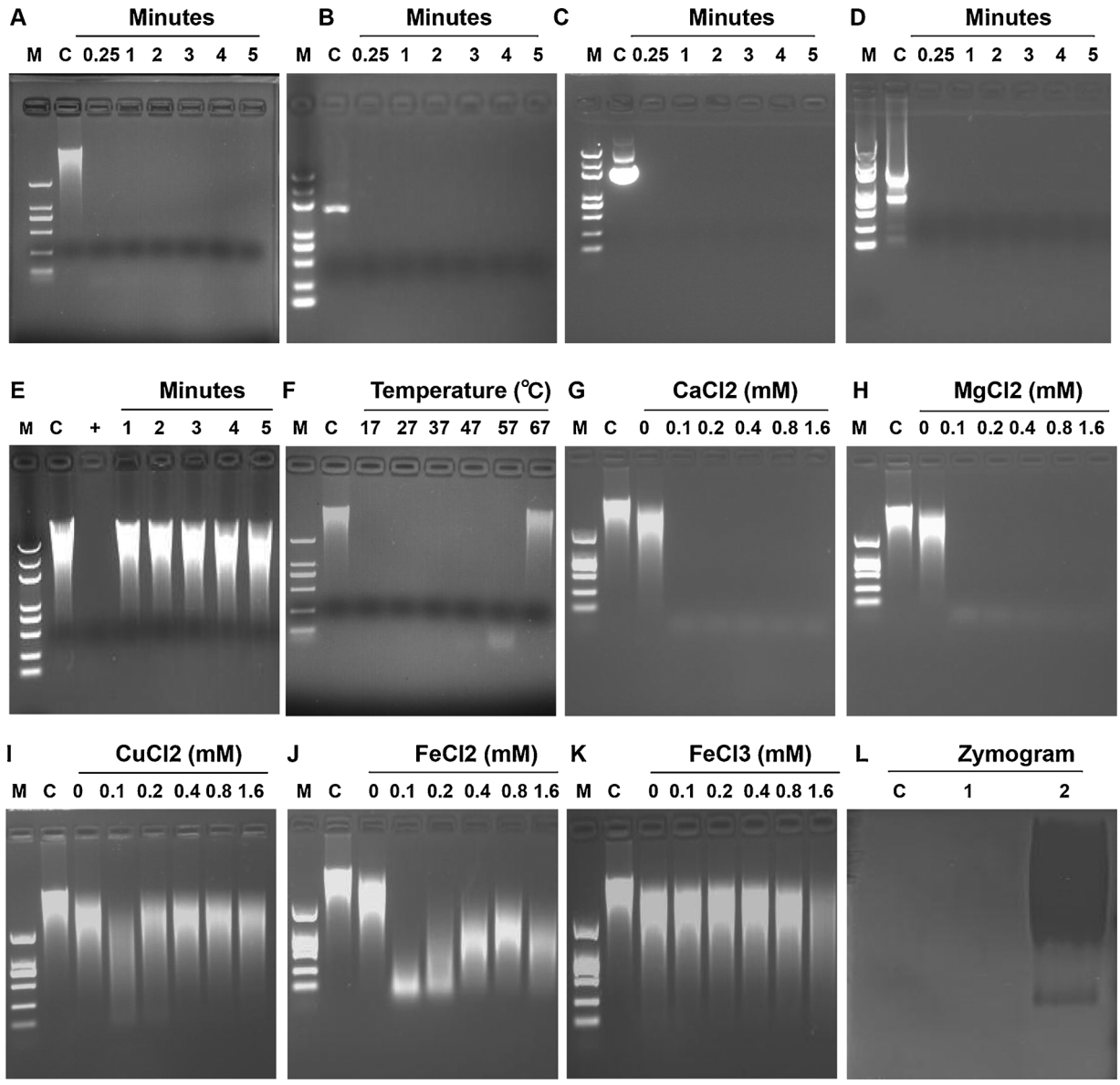

Fig. 1. Analysis of nuclease activity. 1 μg dsDNA (Calf thymus DNA) (**A**), ssDNA (M13 phage DNA) (**B**), plasmid DNA (**C**) or RNA (from PK15 cells) (**D**) treated with 0.1 μg of rMhp597 in 1 mM $CaCl_2$ and $MgCl_2$ reaction buffer. (**E**) dsDNA (Calf thymus DNA) treated with rMhp597$\delta^{315-377}$ at different time intervals in 1 mM $CaCl_2$ and $MgCl_2$ reaction buffer; +, rMhp597 was used as positive control. (**F**) rMhp597 nuclease activity detection at different temperatures ranging from 17 °C to 57 °C. The nuclease activity was examined in the presence of $CaCl_2$ (**G**), $MgCl_2$ (**H**), $CuCl_2$ (**I**), $FeCl_2$ (**J**), $FeCl_3$ (**K**). (**L**) Zymogram analysis of rMhp597. C, rMb-EF-Tu protein was used as control; 1, rMhp597$\delta^{315-377}$ protein; 2, rMhp597 protein.

aliquots were removed at different time intervals, and EDTA was added to arrest the reactions. Reaction products were subsequently visualized by 1% agarose gel electrophoresis. To investigate the effect of divalent or trivalent cations or salts, 0.1 μg of rMhp597 were pre-incubated with $CaCl_2$ (0.1–1.6 mM), $MgCl_2$ (0.1–1.6 mM), $CuCl_2$ (0.1–1.6 mM), $FeCl_2$(0.1–1.6 mM) or $FeCl_3$ (0.1–1.6 mM) for 30 min; then, 1 μg of calf thymus DNA was added followed by incubation for 5 min. To determine the optimal temperature for nuclease activity, both the nuclease reaction buffer (containing 1 μg of calf thymus DNA) and rMhp597 were preheated and assayed at a temperature range from 17 to 67 °C for 5 min, followed by the addition of 0.1 μg of rMhp597 with continuous incubation for 5 min. Reactions were visualized as described above as well as nuclease activity of rMhp597$\delta^{315-377}$.

2.6. Zymogram analysis

Nuclease detection was performed following Xu et al. (2015). The native-PAGE gels were performed as usual SDS-PAGE gels except that no SDS was added into the former. rMhp597 was electrophoresed on 10% native-PAGE gel with a discontinuous denaturing buffer and saturated with 160 μg/ml of herring DNA. Gels were washed three times with PBS and incubated for 30 min at 37 °C in nuclease reaction buffer. The gels were stained with GoldView dye and visualized under gel imaging system. Translation elongation factor (EF-Tu) of *M. bovis* was used as negative control.

2.7. Western blot analysis

The extracellular fraction of *M. hyopneumoniae* was extracted following Zhang et al. (2016). Briefly, 25 ml log-phase culture *M. hyopneumoniae* medium was centrifuged at 12,000 × g for 30 min to obtain supernatant, then the supernatant was concentrated to 1 ml by ultrafiltration. 15 μg of fraction from *M. hyopneumoniae* growth medium was subjected to SDS-PAGE gel, followed by wet transfer into a

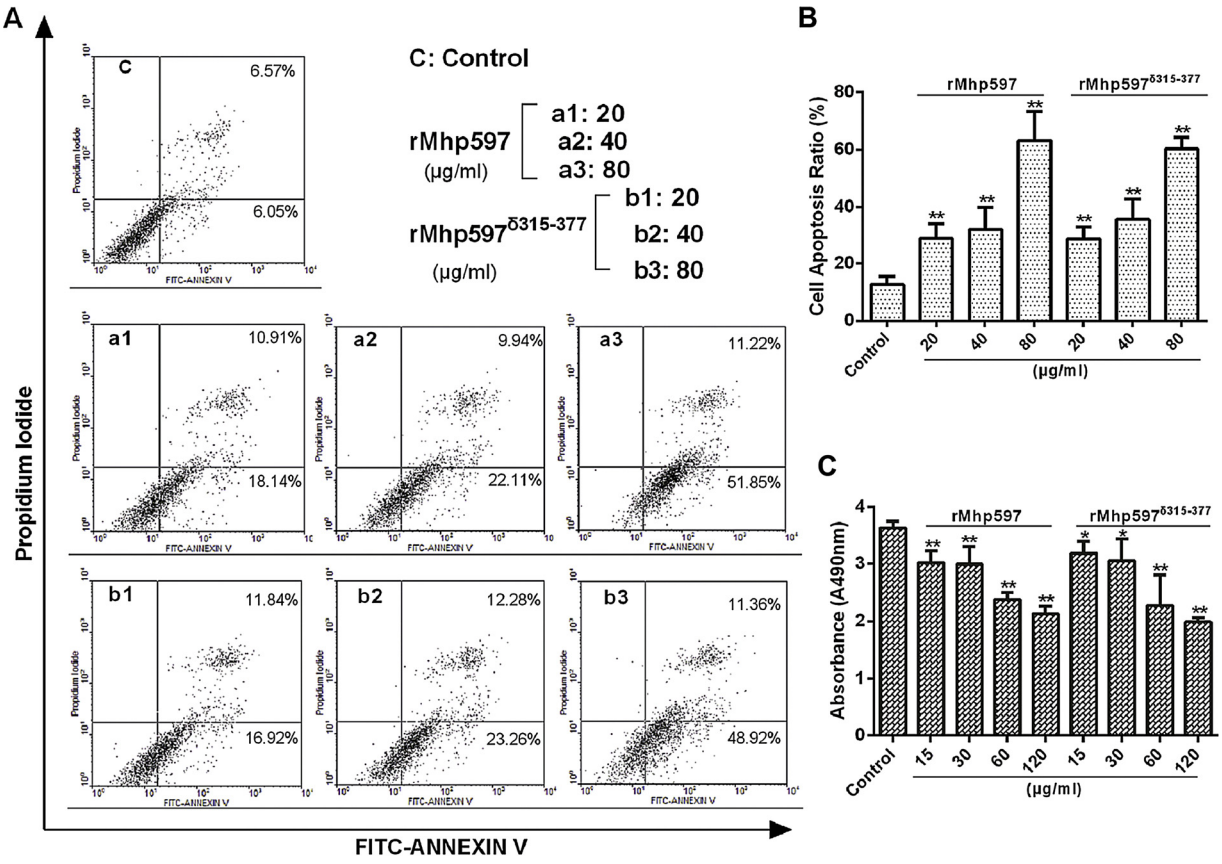

Fig. 2. Apoptosis and cytotoxicity of PK15 cells induced by rMhp597. (**A, B**) Flow cytometry analysis of PK15 cells apoptosis after treatment with different concentrations of rMhp597 or rMhp597$\delta^{315-377}$. (**C**) MTT assay to detect the cytotoxicity of rMhp597 or rMhp597$\delta^{315-377}$ on PK15 cells. The results are expressed as the mean ± S.E.M. $^{*}P < 0.05$, $^{**}P \leq 0.01$ vs. control group.

nitrocellulose membrane for 90 min. Membranes were blocked for 1 h at 37 °C in Tris-buffered saline (TBS), 5% non fat dry milk followed by 1 h incubation at 37 °C with the anti-rMhp597 polyclonal antibody at a concentration of 1:500 in 5% non fat dry milk. After 5 min rinses in TBST for 3 times, the immunoblots were incubated in 5% non fat dry milk with HRP-conjugated goat-anti-rabbit antibody at a concentration of 1:3000 for 45 min at 37 °C. Protein bands were detected using an ECL substrate kit (TransGen Biotech, Beijing, China).

2.8. Flow cytometry

Induced apoptosis in PK15 cells was measured by flow cytometry. Briefly, 5×10^5 PK15 cells suspension were plated in 12 well flat-bottomed plates and incubated for 12 h. Then, PK15 cells were incubated with rMhp597 or rMhp597$\delta^{315-377}$ at 20, 40, and 80 μg/ml concentrations for 24 h. The apoptosis level was detected using the ANNEXIN V-FITC/PI Apoptosis Detection Kit.

2.9. Methylthiazol tetrazolium (MTT) assay for viability of cells

1×10^4 PK15 cells were plated in 96-well flat-bottomed plates and incubated at 37 °C in 5% CO_2 overnight. PBS, rMhp597 or rMhp597$\delta^{315-377}$ at final concentrations of 15, 30, 60,120 μg/ml were added to each well and incubated for 24 h. A volume of 20 μl of 5 mg/ml MTT was added into each well and incubated for 4 h. The MTT solution was removed and 150 μl DMSO was added into each well, then the plates were shocked at low volatility for 10 min. The light absorption value at 490 nm was detected by Universal Microplate Spectrophotometer.

2.10. NET induction and degradation

NET induction was achieved following previous described protocols (Zhang et al., 2016). Briefly, 5×10^5 neutrophils per well were plated in 12-well flat-bottomed plates. Two hours later, the cells were stimulated by 10, 20 and 40 μg/ml PMA or 4×10^6, 8×10^6 and 1.6×10^7 ccu/ml of *M. hyopneumoniae* cells for 3 h at 37 °C and 5% CO_2 allowing NETsosis. Then, the cells were stained with SYTOX Green for 10 min and visualized under the fluorescence microscope.

To examine the NETs-degradation activity of rMhp597 and its natural pattern in the culture supernatant of *M. hyopneumoniae*, neutrophils were stimulated by *M. hyopneumoniae* at optimum conditions to achieve NET induction, and then 0.1 μg rMhp597 or rMhp597$\delta^{315-377}$, 2 μl culture supernatant of *M. hyopneumoniae* or 2 μl positive/negative antibody-treated culture supernatant of *M. hyopneumoniae* were added into each respective experimental well. Then cells were stained with SYTOX Green and visualized under fluorescence microscopy. Rabbit anti-rMhp597 polyclonal antibody was used as a positive control and rabbit anti-rMb-EF-Tu polyclonal antibody as a negative control.

2.11. Quantitative real-time RT-PCR (RT-qPCR)

A RT-qPCR assay was used to detect the expression of genes related to immune response pathways. Total RNA of treated cells was isolated using the RNAprep pure Cell/Bacteria Kit (TransGen Biotech, Beijing, China) according to the manufacturer's instructions. 1 μg RNA sample was used for cDNA synthesis by employing a TransScript First-Strand cDNA Synthesis SuperMix (TransGen Biotech, Beijing, China). Primers for inflammatory cytokines, including IL-1β, IL-8, and TNF-α (Cho and Chae, 2003) as well as primers for TLRs, including TLR2, TLR3, TLR4,

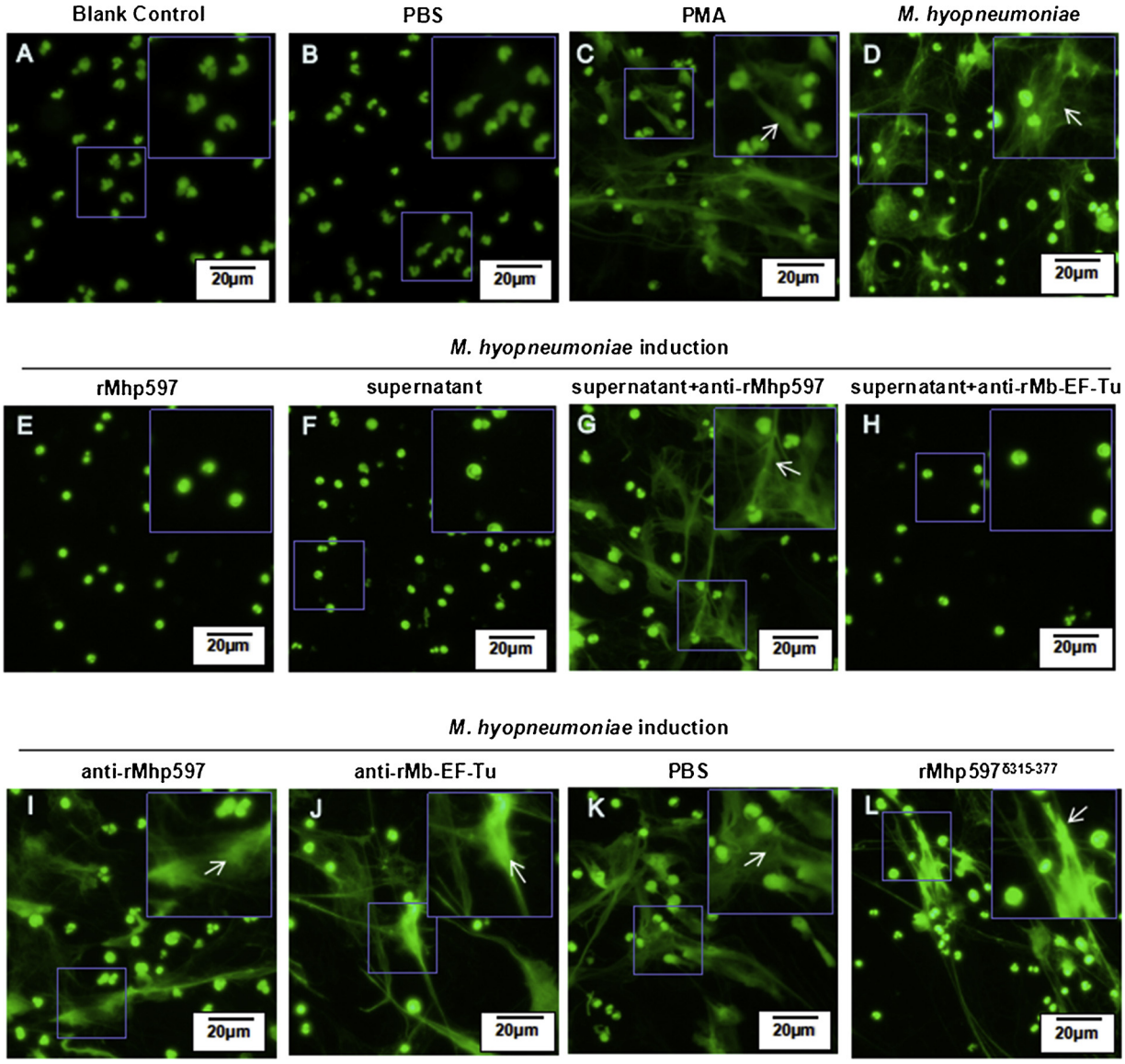

Fig. 3. NET induction and disintegration. Neutrophils without any treatment (**A**), exposed to PBS (**B**), PMA (**C**) or *M. hyopneumoniae* (**D**) after NET induction by *M. hyopneumoniae*, neutrophils were incubated with 0.1 μg of rMhp597 (**E**), 2 μl culture supernatant of *M. hyopneumoniae* (**F**), 2 μl rabbit anti-rMhp597 antibody treated culture supernatant of *M. hyopneumoniae* (**G**), 2 μl rabbit anti-rMb-EF-Tu antibody treated culture supernatant of *M. hyopneumoniae* (**H**), rabbit anti-rMhp597 antibody (**I**), rabbit anti-rMb-EF-Tu antibody (**J**), PBS (**K**) or 0.1 μg of rMhp597$\delta^{315-377}$ (**L**). Arrows indicate NETs released by the neutrophils.

TLR8, TLR9 (Duan et al., 2014), and RIG-I, MyD88, TRIF (Song et al., 2013), P65 (Santos et al., 2007), IFN-α (Loving et al., 2006), IFN-β (De et al., 2006), nucleocapsid protein of PRRSV (PRRSV-N) (Zhang et al., 2015) were manufactured as previously described, and they were listed in Table S2. The RT-qPCR step was carried out by incubating samples at 95 °C for 10 min, followed by 45 cycles at 95 °C for 15 s, 56 °C for 30 s and 72 °C for 30 s. The reaction was performed in a LightCycler 96 system and SYBR Green probe-base detection (TransGen Biotech, Beijing, China). Results were calculated by the $2^{-\triangle\triangle Ct}$ method and reported as a relative fold change to negative control.

2.12. Quantification of IL-1β, IL-8 and TNF-α by ELISA

The levels of IL-1β, IL-8 and TNF-α in the culture supernatants were quantified by ELISA using the Porcine IL-1β ELISA Kit, Porcine IL-8 ELISA Kit and Porcine TNF-α ELISA Kit (Cusabio Biotech Co., China). Concentration of samples was determined by comparing the O.D. samples to the standard curve and the intensity was measured at 550 nm using a spectrophotometer.

2.13. Small RNA interference

To silence the expression of genes, PAMs were treated with corresponding siRNAs. Sequences of siRNAs targeting TLR2, TLR3, TLR4, TLR8, TLR9, RIG-I, MyD88, TRIF (Cao et al., 2015) and P65 (Li et al., 2015) were previously described, and they were listed in Table S3. Briefly, PAM cells were seeded onto 12-well plates at a density of 1×10^6 cells/well in antibiotic-free normal growth medium containing 10% FBS. After culture at 37 °C in a 5% CO_2 incubator for 24 h, the cells were washed twice with plain RPMI-1640 medium, followed by the transfection of siRNAs using Lipofectamine RNAiMAX Reagent (Invitrogen, USA) following the manufacturer's instructions. Negative control siRNA of these siRNAs was included in the experiment.

2.14. Effects of rMhp597 on the expression of IFN-α/β and the multiplication of PRRSV in PAMs

PAMs at concentrations of 1×10^6 per well were seeded in 12-well flat-bottomed plates, after being cultured overnight. rMhp597 or

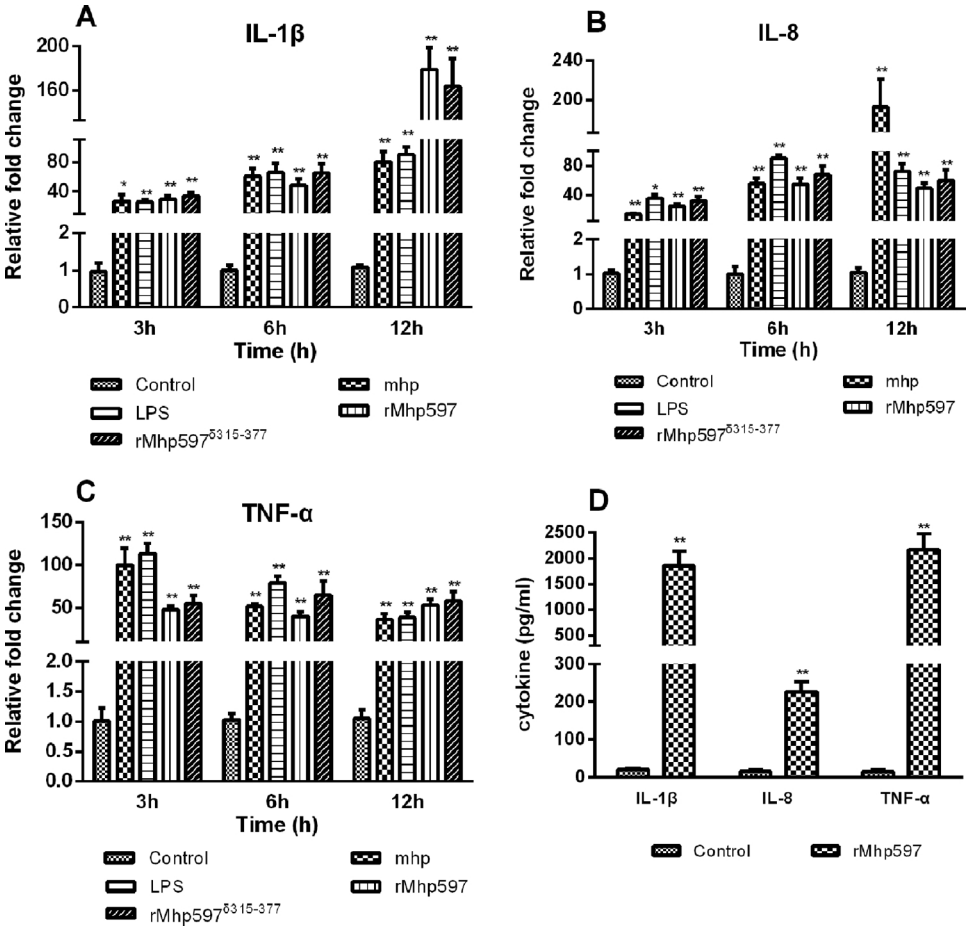

Fig. 4. Activation of inflammatory genes after the exposure to *M. hyopneumoniae*, LPS, rMhp597 and rMhp597δ$^{315-377}$. (**A, B, C**) Relative fold change to negative control of IL-1β, IL-8, and TNF-α respectively. (**D**) PAMs exposed to 2 μg/ml of rMhp597 for 12 h. Protein quantification of IL-1β, IL-8 and TNF-α was determined by ELISA. $^{*}P < 0.05$, $^{**}P < 0.01$, compared with the corresponding control group.

rMhp597δ$^{315-377}$ at final concentrations of 0.2, 0.4, 0.6, 0.8 and 1.6 μg/ml were added into each well, followed by incubation for 24 h, and then the expression levels of IFN-α/β of the cells were determined by RT-qPCR assay.

After PAMs were incubated with 1 μg/ml rMhp597 or rMhp597δ$^{315-377}$ for 24 h, 2×10^4 $TCID_{50}$ JXwn06 PRRSV was added into each well, followed by incubation for 24, 36 and 48 h, and then, the cells were lysed by RIPA buffer and the reproduction of PRRSV was quantified by RT-qPCR following previous described protocols.

Protein quantification was assessed by western blot technique where 30 μg of the cell lysate was used and Anti-PRRSV-Nsp4 rabbit polyclonal antibody at a concentration of 1:800 and HRP-conjugated goat-anti-rabbit antibody (Santa Cruz Biotechnology, USA) at a concentration of 1:3000 were used as primary and secondary antibody respectively.

2.15. Statistical analysis

All experiments were carried out in triplicates, and the given data are means ± S.E.M. Significant differences between different groups were assessed by Student's *t*-test using GraphPad software, and P values were represented as $^{*}P \leq 0.05$, $^{**}P \leq 0.01$.

3. Results

3.1. Expression and purification of rMhp597 and rMhp597δ$^{315-377}$

Sequence analysis of Mhp597 showed that there was a N-terminal signal sequence (aa 1–18) (Figs. S1A, S2A) and a strongly basic (+) amino acid rich region in the C-terminal ends of the sequence (Figs. S1B, S2A). The recombinant C-terminal His-tagged rMhp597 and rMhp597δ$^{315-377}$ protein were expressed in *E. coli.*and purified. Finally, very pure recombinant His-tagged rMhp597 (40 kDa) and rMhp597δ$^{315-377}$ (33 kDa) were harvested (Fig. S2B).

3.2. Analysis of rMhp597 nuclease activity

The nuclease activity of rMhp597 was assessed with different nucleic acid substrates and under different conditions. In the presence of $CaCl_2$ and $MgCl_2$ and at 37 °C, rMhp597 showed high nuclease activity even at 15 s of digestion with 1 μg of calf thymus DNA (Fig. 1A), M13 phage DNA (Fig. 1B), pCMV-HA plasmid DNA (Fig. 1C) and total RNA from PK15 cells (Fig. 1D). On the other hand, rMhp597δ$^{315-377}$ did not exhibit nuclease activity in digesting these nucleotides (Figs. 1E, S3A, S3B, S3C). Besides, rMhp597 showed high nuclease activity at a wide range of temperatures from 17 °C to 57 °C (Fig. 1F). Divalent or trivalent cations or salts requirement assays showed whether $CaCl_2$ (Fig. 1G) or $MgCl_2$ (Fig. 1H) was essential for rMhp597 to digest nucleic acid substrates but not for $CuCl_2$ (Fig. 1I), $FeCl_2$ (Fig. 1J) and $FeCl_3$ (Fig. 1K). The nuclease activity of rMhp597 was further confirmed by zimography analysis (Fig. 1L). The black stripe represented nucleic acid substrates were degraded.

3.3. Detection of Mhp597 in the culture supernatant of M. hyopneumoniae

In Fig. S4A, immunoblots showed that Mhp597 was detected in the culture supernatant of *M. hyopneumoniae* but not in the negative control supernatant, indicating that Mhp597 was a secreted protein of *M. hyopneumoniae,* agreeing with the data from Paes et al. (2017). To further confirm that Mhp597 was a secreted protein, the DNA-degradation ability of the supernatant of *M. hyopneumoniae* was examined. The results showed that a 2-μl supernatant of *M.*

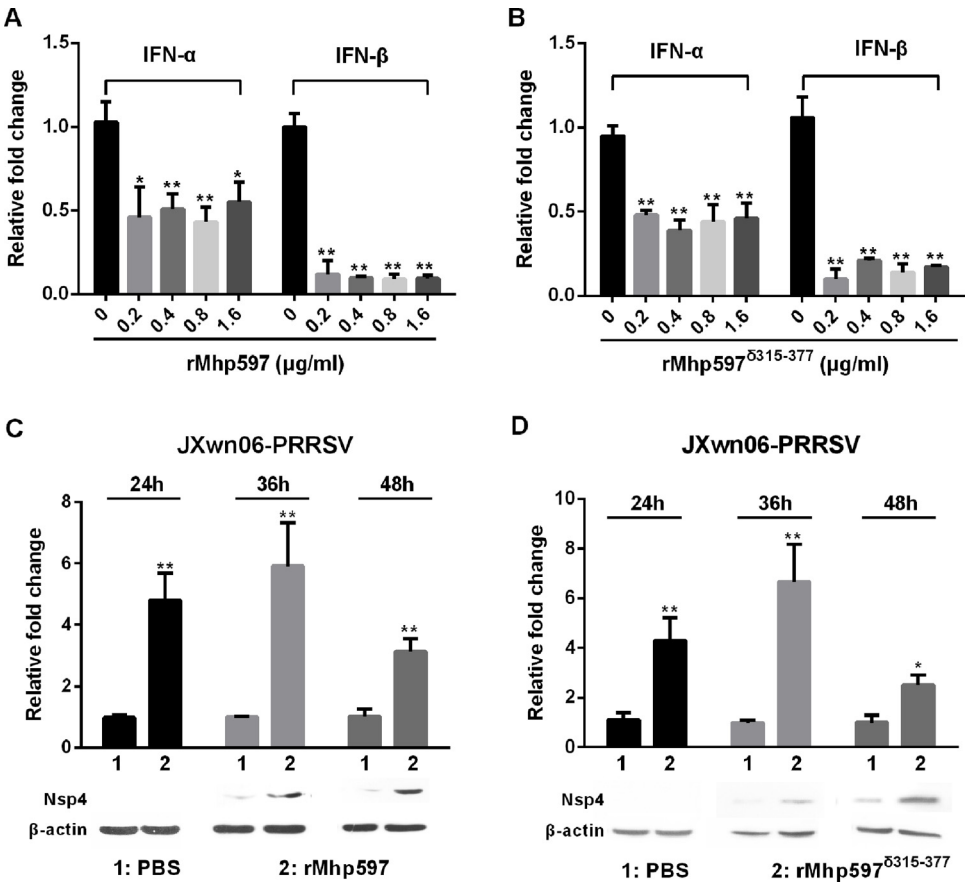

Fig. 5. rMhp597 down-regulates IFN-α/β and promotes the reproduction of PRRSV. (A, B) Effect of rMhp597 and rMhp597$\delta^{315-377}$ on the expression of IFN-α/β in PAMs. PAMs were incubated with rMhp597 or rMhp597$\delta^{315-377}$ at concentrations of 0.2, 0.4, 0.8, 1.6 μg/ml for 24 h, then the expression of IFN-α/β was measured by RT-qPCR assay. 0 μg/ml rMhp597 or rMhp597$\delta^{315-377}$ was used as control group. $^{*}P < 0.05$, $^{**}P < 0.01$, relative to the control group. (**C, D**) Effect of rMhp597 and rMhp597$\delta^{315-377}$on the reproduction of PRRSV in PAMs. After PAMs were incubated with 1 μg/ml rMhp597 or rMhp597$\delta^{315-377}$ for 24 h, 2×10^4 $TCID_{50}$ JXwn06 PRRSV was added into the wells, and then 24 h, 36 h and 48 h later, the multiplication of PRRSV was determined by RT-qPCR and western blot assay. $^{*}P < 0.05$, $^{**}P < 0.01$, relative to corresponding control group (PBS).

hyopneumoniae was able to digest 1 μg of the Mhp597-f1 DNA fragment, which was blocked by more than 2 μl of rabbit anti-rMhp597 antibody (Fig. S4B). In the western blot analysis, it seems that the polyclonal antiserum also bind to proteins present in the complete medium and not specific to Mhp597 of *M. hyopneumoniae*, which problem also present in Zhang et al (2016). We suspect that there may be some epitopes in these nucleases which are similar to other proteins in the complete medium. Besides, a large amount of concentrated supernatant (15 μg) was used in the western blot assay, and it may strengthen the non-specific binding.

3.4. rMhp597-induced apoptosis and cytotoxicity of PK15 cells

As shown in Fig. 2A and B, both rMhp597 and rMhp597$\delta^{315-377}$ induced PK15 cell apoptosis in a dose-dependent manner. The cytotoxicity of rMhp597 on PK15 cells was determined by MTT assay, and found that the viability of cells was extremely reduced by rMhp597 and rMhp597$\delta^{315-377}$ in a dose-dependent manner (Fig. 2C).

3.5. NET induction an disruption

The NETs-inducing ability of both PMA (a frequently-used NETs-inducing reagent)and *M. hyopneumoniae* was evaluated under different concentrations. We found that both PMA (Fig. 3C) and *M. hyopneumoniae* (Fig. 3D) induced the production of NETs with optimum concentrations of 20 μg/ml and 8×10^6 ccu/ml, respectively (Fig. S5). The NETs in the well were completely destroyed by both 0.1 μg of rMhp597 (Fig. 3E) and a 2-μl culture supernatant of *M. hyopneumoniae* (Fig. 3F).

We also demonstrated that the NETs-disruption ability in the supernatant of *M. hyopneumoniae* was blocked by rabbit anti-rMhp597 antibody (Fig. 3G), further confirming that Mhp597 was a secreted protein.

3.6. rMhp597 up-regulates the inflammatory genes of PAMs

To examine the possible association of Mhp597 with inflammation, genes involved in this pathway were assessed by RT-qPCR. PAMs were incubated with 1×10^7 ccu/ml *M. hyopneumoniae*, 2 μg/ml of LPS, 2 μg/ml rMhp597 or rMhp597$\delta^{315-377}$ for 3, 6 or 12 h respectively. *M. hyopneumoniae* and LPS were used as positive control. The total RNA of the cells was then isolated and analyzed through qPCR assay. As shown in Fig. 4, compared with the negative control group, the mRNA levels of IL-1β (Fig. 4A), IL-8 (Fig. 4B) and TNF-α (Fig. 4C) of each experimental group were in average about 100 fold change up-regulated. To further confirm the effect of rMhp597, cytokines secreted to the cell culture supernatant of each group were measured by ELISA. As shown in Fig. 4D, the quantities of IL-1β, IL-8 and TNF-α in the cell culture supernatant in the rMhp597-incubated group were about 80 times larger than those in the negative control group.

3.7. Silencing TLR4 expression inhibits rMhp597-induced inflammatory gene up-regulation

TLRs (Toll-like receptors) and RLRs (RIG-I-like receptors) are major receptors recognizing most types of various pathogen-associated molecular patterns. To identify the role of the TLR and RLR signaling pathways in rMhp597-induced inflammation, the expression of TLR2, TLR3, TLR4, TLR8, TLR9 and RIG-I were silenced by siRNAs. The interference efficiency of these siRNAs in PAMs was examined by RT-qPCR. As shown in Fig. S6A, compared with the negative control, the mRNA expression of TLR2, TLR3, TLR4, TLR8, TLR9 and RIG-I was in average 80% reduced by the corresponding siRNA. After the treatment with these siRNAs, 2 μg of rMhp597 was added into the cell culture, and incubated for 4 h and then the expression of these genes measured. As shown in Fig. S6B, C, D, compared with the negative control siRNA,

siTLR4 significantly suppressed the rMhp597-induced up-regulation of IL-β, IL-8 and TNF-α, while no significant differences were detected for other siRNA treatment groups, indicating TLR4 as the main receptor recognizing rMhp597.

3.8. Silencing MyD88 and P65 expression inhibits rMhp597-induced inflammatory gene up-regulation

MyD88 and TRIF are two main adaptor molecules of TLRs and they act as a bridge between TLRs and subsequent signaling pathways, such as NF-κB. P65 is a key molecule in NF-κB-associated signaling pathways and usually combines with IκB (inhibitor of NF-κB) in the cytoplasm under normal circumstances. To investigate the role of these molecules in rMhp597-induced inflammation, the expression of MyD88, TRIF and P65 in PAMs was silenced and the interference efficiency was evaluated by RT-qPCR. As shown in Fig. S7A, compared with the negative control, the expression of MyD88, TRIF and P65 was in average 80% reduced by siMyD88, siTRIF and siP65 respectively. After the supression of MyD88, TRIF and P65 in PAMs, cells were incubated with 2 μg/ml rMhp597 for 4 h, and the expression of IL-8, IL-1B and TNF-a were quantified. As shown in Fig. S7B, C, and D, compared with the negative control siRNA, the rMhp597-induced up-regulation of IL-β, IL-8 and TNF-α could be efficiently decreased by siMyD88 and siP65, but were not reduced by siTRIF, indicating that MyD88 and P65 rather than TRIF acted as essential molecules in the signaling associated with rMhp597-induced inflammation.

3.9. Inhibitors targeting TLR4, MyD88 and IKKα reverse rMhp597-induced inflammatory gene up-regulation

TAK-242 is a small-molecule specific inhibitor of TLR4 signaling. TAK-242 binds to the intracellular Cys747 residue of TLR4. ST2825 is a pharmacologic inhibitor targeting MyD88. Bay11-7082 is an irreversible inhibitor of IKKα and phosphorylation of cytokine-inducible P65. The molecular structures of the three are shown in Fig. S8A. To further confirm the role of TLR4, MyD88 and NF-κB in rMhp597-induced inflammation, PAMs were treated with 15 μM TAK-242, 0.1 mM ST2825 and 5 μM Bay11-7082, and incubated with 2 μg/ml rMhp597 for 4 h, and assessed for inflammatory gene expression.

As shown in Fig. S8B, C, D, all TAK-242, ST2825 and Bay11-7082 actively reverse the rMhp597-induced inflammatory gene up-regulation, providing more evidence that TLR4, MyD88 and NF-κB were indispensable molecules in the signaling.

3.10. Mhp597 down-regulates IFN-α/β and promotes the multiplication of PRRSV

Clinically, pigs co-infected with *M. hyopneumoniae* and PRRSV are very common in farms and IFN-α/β is a very important cytokine associated with innate immune response against this type of viral infection. In this way, to validate the assumption that rMhp597 is an immunosuppression protein, the expression of IFN-α/β in PAMs was determined. As shown in Fig. 5A, B, the expression of IFN-α in PAMs was about 0.5 fold suppressed by rMhp597 or rMhp597$\delta^{315-377}$, and at the same time, IFN-β was around 0.2 fold suppressed.

Given that the expression of IFN-α/β in PAMs was strongly suppressed by rMhp597, the multiplication levels of PRRSV in the cells was further explored. As shown in Fig. 5C, D, the expression of PRRSV-N in rMhp597 or rMhp597$\delta^{315-377}$-treated group was in average about 5 fold change up-regulated at 24 h and 36 h time intervals and about 2 fold change up-regulated at 48 h compared with the corresponding negative control group. Respectively, Nsp4 protein quantification of PRRSV in rMhp597 or rMhp597$\delta^{315\text{-}377}$ incubated group increases compared with the corresponding negative control group, when the cells were incubated with rMhp597 or rMhp597$\delta^{315-377}$ for 36 or 48 h. The results indicating that rMhp597 down-regulates IFN-α/β and promotes the multiplication of PRRSV.

4. Discussion

In comparison to other pathogenic bacteria, pathogenic mycoplasmas seem to lack typical virulence factors such as toxins, invasins and adhesins (Pilo et al., 2005; Maes et al., 2017). In this way, virulence genes in mycoplasma are described as any nonessential genes for in vitro conventional growth, which are essential for the optimal survival (colonization, persistence or pathology) inside the host (Browning et al., 2014).

Adhesins and lipoproteins, for instance, are generally seen as important virulence factors in mycoplasma (Debey and Ross, 1994; Masukagam et al., 2013; Maes et al., 2017; Browning et al., 2011). While several putative adhesion proteins (such as P97, P50 and P60) have been reported in *M. hyopneumoniae* (Whittlestone, 2012; Hsu and Minion, 1998; Deutscher et al., 2012) most lipoproteins have not been well studied and characterized.

Although many other mycoplasma nucleases have been studied to this day (Xu et al., 2015; Zhang et al., 2016; Schmidt et al., 2007), no other study reported the high activity we showed for Mhp597. Interestingly, the size of the mature protein in *M. hyopneumoniae* was about 5 KDa smaller than rMhp597, resulting from a proteolytic cleavage during the process of maturation, which was found to be similarly to the MbovNase of *M. bovis* (Zhang et al., 2016). Maybe due to the proteolytic cleavage, Mhp597 is extracellular and not membrane-anchored as expected because of its predicted transmembrane-domain, like its MHP7448_0580 homologue.

Sequence analysis showed that there was a strongly basic (+) amino acid rich region in the C-terminal ends. When the C-terminal 63 amino acid residues were removed, the nucleotide degradation activity of the protein disappeared, indicating that these residues were essential for Mhp597 nuclease activity.

However, both rMhp597 and rMhp597$\delta^{315-377}$ strongly adhered to PAMs and colocalized with the cytomembrane (Fig. S9A, B, C), providing significant information for future studies on the pathogenicity of this species. Bai and collaborators have reported that lipid-associated membrane proteins of *M. hyopneumoniae* could induce inflammation in porcine peripheral blood mononuclear cells (Bai et al., 2014), but which protein has this function has not been reported. In this study, we show for the first time the link between a lipoprotein of *M. hyopneumoniae,* Mhp597, and the occurrence and development of inflammation, as evidenced by the activation of IL-1β, IL-8 and TNF-α.

In addition to the biological functions of adhering to PAMs, inducing apoptosis and up-regulating inflammatory genes, Mhp597 was also able to disrupt NETs and suppress the expression level of type I IFN (IFN-α/β), two mechanisms of defense from the host.

NETs are a mechanism by which a host catches and kills invasive pathogens either by engulfment, secretion of antimicrobials or by elimination of the pathogen, minimizing damage of the host cell (Brinkmann et al., 2004). Here we found that *M. hyopneumoniae* exposure induced porcine neutrophils to release NETs, which in turn were digested by both rMhp597 and *M. hyopneumoniae* culture supernatant. Furthermore, we found that NETs-disrupting function of the supernatant could be blocked by anti-rMhp597 polyclonal antibodies, providing further evidence that Mhp597 is a secreted protein.

IFN-α/β is a very important cytokine in host cells for preventing virus infection. Many viruses have developed effective strategies to avoid the host innate immune system. For instance, some proteins encoded by viruses demonstrated their ability to suppress the expression of IFN or inhibit IFN-associated signaling pathways (García-Sastre et al., 2006; Randall et al., 2008). rMhp597 modulated the expression of IFN-α/β (Fig. 5A), and in turn promoted the multiplication of PRRSV in PAMs (Fig. 5C). These observations suggest that Mhp597 may be a very important factor associated with the mixed infection of *M. hyopneumoniae* and PRRSV.

It was interesting to note that although rMhp597$\delta^{315-377}$ had no nuclease activity, it could still adhere to PAMs, induce apoptosis and cytotoxicity of PK15 cells, over-express inflammatory genes, and down-regulate IFN-α/β, revealing that the nuclease activity of Mhp597 had nothing to do with its biological function associated with cytotoxicity, inflammation and immunosuppression. We were unable to generate a mutant with a deleted Mhp597 gene (with and without complementing plasmid). Hence, in this paper we provide supporting information that Mhp597 is a major extracellular nuclease with very high activity, implicated in cytotoxicity, inflammation and immunosuppression which is also probably an essential protein for *M. hyopneumoniae* survival. Considering these results in this study, we believed that Mhp597 is a cytotoxicity, inflammation and immunosuppression associated nuclease, and all of results ware summarized in Fig. S10.

At last, most of experimental data were generated using the recombinant protein, and further studies are needed to investigate that can all the properties associated in this work to the rMhp597 protein (except the nuclease activity demonstrated to be present in culture supernatant) really be expected in the natural extracellular form of the protein? And it would have been interesting to look for Mhp597-nuclease activity also in *M. hyopneumoniae* whole cell extracts to see if the two forms (extracellular and membranous) can co-exist.

Conflicts of interest

The authors declare that they have no conflicts of interest.

Author contributions

Conceived and designed the experiments: Wenxue Wu. Performed the experiments: Peng Li. Data analysis: Xia Li and Wenyan Zhou participated in the analysis. Wrote the manuscript: Peng Li, Yunke Zhang, Xuni Li and Fei Jiang. All authors read and approved of the final manuscript.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetmic.2019.05.011.

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