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# Porcine reproductive and respiratory syndrome virus infection triggers HMGB1 release to promote inflammatory cytokine production

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ABSTRACT

The high mobility group box 1 (HMGB1) protein is an endogenous damage-associated molecular pattern (DAMP) molecule involved in the pathogenesis of various infectious agents. Based on meta-analysis of all publicly available microarray datasets, HMGB1 has recently been proposed as the most significant immune modulator during the porcine response to porcine reproductive and respiratory syndrome virus (PRRSV) infection. However, the function of HMGB1 in PRRSV pathogenesis is unclear. In this study, we found that PRRSV infection triggers the translocation of HMGB1 from the nucleus to the extracellular milieu in MARC-145 cells and porcine alveolar macrophages. Although HMGB1 has no effect on PRRSV replication, HMGB1 promotes PRRSV-induced NF-κB activation and subsequent expression of inflammatory cytokines through receptors RAGE, TLR2 and TLR4. Our findings show that HMGB1 release, triggered by PRRSV infection, enhances the efficiency of virus-induced inflammatory responses, thereby providing new insights into the pathogenesis of PRRSV infection.



## Introduction

Porcine reproductive and respiratory syndrome (PRRS) is an economically important infectious disease characterized by severe reproductive failure in sows, and respiratory distress in piglets and growing pigs (Lunney et al., 2010; Meng, 2012). The causative agent, PRRS virus (PRRSV), is a single-stranded positive-sense RNA virus classified within the *Arteriviridae* family (Nelsen et al., 1999; Snijder et al., 2013). Since its emergence in the late 1980s, PRRS has been a threat to the global swine industry (Neumann et al., 2005). Infection with PRRSV causes high fever, severe interstitial pneumonia and mild lymphocytic encephalitis (Guo et al., 2013; Liu et al., 2010), suggesting that the inflammatory response plays an important role during infection and pathogenesis. Furthermore, a large number of studies have reported that PRRSV infection activates the inflammation-related transcription factor NF-κB, and induces production of pro-inflammatory cytokines (IL-6, IL-8 and TNF-α) (Lee and Kleiboeker, 2005; Luo et al., 2011a; Mateu and Diaz, 2008; Yoo et al., 2010). However, the underlying mechanisms of these responses have yet to be elucidated. More recently, Badaoui et al. (2013) used a new meta-analysis approach to assess the immune response in pigs to PRRSV infection. This meta-analysis involved the collection of all publicly available microarray datasets (six datasets comprising 31,353 probes in 278 chips). They identified that the most significant immune modulator during the porcine response to PRRSV infection was high mobility group box 1 (HMGB1). Thus, they proposed that HMGB1 might be a possible target of PRRSV in the manipulation of the host immune response.

HMGB1, formerly known as HMG-1 or amphoterin, is a chromatin protein ubiquitously expressed by cells. It is highly conserved and participates in the regulation of chromatin structure; it is also involved in various aspects of DNA replication, transcription and repair (Huang et al., 2010; Lotze and Tracey, 2005). HMGB1 can be either passively released from necrotic or damaged cells, or can be secreted by activated innate immune cells (Mazarati et al., 2011; Wang et al., 1999). In addition to its nuclear role, HMGB1 is also a damage-associated molecular pattern (DAMP) molecule that plays a role during endotoxin shock, vascular injury, lethal systemic and local pulmonary inflammation (Bianchi, 2007; Bianchi and Manfredi, 2007; Higgins et al., 2013; Hirata et al., 2013; Inoue et al., 2013; Lotze and Tracey, 2005; Tang et al., 2010). Extracellular HMGB1 acts on several immune cells to trigger inflammatory responses. Known receptors for HMGB1 include toll-like receptor (TLR) 2, TLR4, TLR9, and the receptor for advanced glycation end products (RAGE) (Park et al., 2006; Tian et al., 2007). HMGB1-

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mediated TLR2, TLR4, TLR9 and RAGE signaling pathways are involved in the activation of NF-κB, which contributes to the production of pro-inflammatory molecules by macrophages (Chen et al., 2012; He et al., 2012; Ivanov et al., 2007; Palumbo et al., 2007; Park et al., 2004; Tadie et al., 2012).

Considering that HMGB1 is associated with a variety of inflammatory diseases and PRRSV infection induces severe interstitial pneumonia, the present study was designed to determine any relationship between HMGB1 and PRRSV-induced inflammatory cytokines.

## Results

### *PRRSV infection triggers HMGB1 release from MARC-145 cells and PAMs*

To determine whether PRRSV infection triggers the release of HMGB1 from infected cells, MARC-145 cells were either mock-infected or infected with PRRSV WUH3. At 30 h post-infection (hpi), cells were examined using immunofluorescence. A large amount of nuclear-to-cytoplasmic translocation of HMGB1 was observed in PRRSV-infected cells (Fig. 1A), while HMGB1 was infrequently seen in the cytoplasm of mock-infected cells. To further explore whether PRRSV infection could induce the release of HMGB1 from the intracellular cytoplasm to extracellular, ELISAs were conducted to detect the secretion of HMGB1 in cell culture supernatants. As shown in Fig.1B, the amount of HMGB1 in supernatants of PRRSV-infected cell cultures is increased compared with that for mock-infected cells at 24 hpi. This increase was greater than 2-fold at 36 and 48 hpi. When MARC-145 cells were infected with PRRSV at different multiplicity of infections (MOI)s (0.001, 0.01, 0.1 and 1), we observed greater levels of HMGB1 secretion at higher MOIs (Fig. 1C), suggesting that HMGB1 release induced by PRRSV was in a dose-dependent manner. Furthermore, the release of HMGB1 by PRRSV was likely replication-dependent because UV-irradiated PRRSV did not significantly induce HMGB1 release.

As showed that MARC-145 cells release HMGB1 upon PRRSV infection, we investigated whether similar HMGB1 translocation takes place during PRRSV infection in PAMs, the target cells of PRRSV infection *in vivo*. As expected, HMGB1 was observed in the cytoplasm (Fig. 1D) and increasing amount of HMGB1 could be detected in the supernatant with the progression of infection (Fig. 1E) in PRRSV-infected PAMs. Also, PRRSV induced HMGB1 release in a dose-dependent manner in PAMs (Fig. 1F). Collectively, these results unequivocally confirmed that PRRSV infection triggers the release of HMGB1 from the nuclei to extracellular environment in both MARC-145 cells and PAMs.

### *HMGB1 does not significantly affect PRRSV proliferation*

Previous studies suggested that HMGB1 is involved in the replication of many viruses, such as Hepatitis C virus, influenza virus, and HIV (Jung et al., 2011; Moisy et al., 2012; Nowak et al., 2006). To investigate whether HMGB1 affects PRRSV replication, three different approaches were performed. Firstly, HMGB1 was knocked down by siRNA. The designed HMGB1-specific siRNA could significantly reduce the endogenous expression of HMGB1 in MARC-145 cells (Fig. 2A) and had no detectable cellular toxicity at the doses tested (data not shown). And then, MARC-145 cells were transfected with HMGB1-specific siRNAs (50 nM) or negative control (NC) (50 nM) prior to PRRSV infection (MOI = 0.1). The infected cells were collected at 12, 24, 36 and 48 hpi for plaque assay to determine virus titer. As shown in Fig. 2B, siRNA-mediated HMGB1 knockdown has no significant influence on PRRSV replication. In accord with the plaque assay results, expression of PRRSV Nsp2 was not significantly altered in cells transfected with either HMGB1-specific siRNAs or control siRNAs (Fig. 2C).

To further confirm the conclusion from siRNA-mediated knockdown assay, we also used the anti-HMGB1 neutralizing antibody and purified HMGB1 protein to evaluate the role of HMGB1 on PRRSV replication. MARC-145 cells were pretreated with an anti-HMGB1 neutralizing antibody or recombinant HMGB1 protein at the indicated concentrations for 18 h prior to PRRSV infection. The infected cells were cultured in the presence of anti-HMGB1 blocking antibody or HMGB1 protein and collected at different time points for plaque assay and Western blot analysis. The results showed that anti-HMGB1 neutralizing antibody did not affect PRRSV replication (Fig. 2D) and PRRSV Nsp2 expression (Fig. 2E). Also, purified HMGB1 protein had no detectable effect on PRRSV replication (Fig. 2F) and PRRSV Nsp2 expression (Fig. 2G). Moreover, there were no significant changes in PRRSV replication at any time point for the isotype control IgG and control GFP groups. Taken together, all results from three different assays demonstrated that HMGB1 might have no effect on PRRSV replication in MARC-145 cells.

### *HMGB1 is involved in PRRSV-induced NF-κB activation and the inflammatory response*

As a powerful DAMP, HMGB1 has pro-inflammatory and immunomodulatory action (Bianchi and Manfredi, 2007; Scaffidi et al., 2002), and previous studies have showed that PRRSV infection activates NF-κB pathway (Fu et al., 2012; Lee and Kleiboeker, 2005; Luo et al., 2011a). To examine whether HMGB1 participates in PRRSV-induced NF-κB activation, MARC-145 cells were co-transfected with HMGB1-specific siRNAs, pNF-κB-Luc and pRL-TK prior to infection with PRRSV. Knockdown of HMGB1 inhibited PRRSV-induced NF-κB transcriptional activity compared with that for the negative controls (Fig. 3A). Since NF-κB activation requires NF-κB subunit p65 phosphorylation (Hayden and Ghosh, 2004), we further examined the phosphorylation level of p65 subunit. As expected, HMGB1-specific siRNA significantly inhibited PRRSV-induced p65 phosphorylation, while total p65 level remained unchanged (Fig. 3B).

To further validate the effects of HMGB1 on PRRSV-induced NF-κB activation, pNF-κB-Luc and pRL-TK were co-transfected into MARC-145 cells before infection with PRRSV. Infected cells were treated with purified HMGB1 protein and collected at 36 hpi. Luciferase assay results showed that treatment with the purified HMGB1 protein enhanced PRRSV-induced NF-κB promoter activation (Fig. 3C) and p65 phosphorylation (Fig. 3D) to some degree. However no influence could be observed in cells treated with the control GFP protein.

NF-κB is an important transcription factor for pro-inflammatory cytokines production. Because HMGB1 promotes PRRSV-induced NF-κB promoter activation, it should enhance PRRSV-induced inflammatory cytokine expression. To test this hypothesis, PRRSV-infected MARC-145 cells were transfected with HMGB1-specific siRNAs or treated with purified HMGB1 protein. Cells were collected at 36 hpi for detecting cytokine message expression by real-time RT-PCR. Consistent with previous reports (Bi et al., 2014; Flores-Mendoza et al., 2008; Han et al., 2014; Luo et al., 2011a; Mateu and Diaz, 2008; Wang et al., 2011; Zhang et al., 2013a), PRRSV infection was able to induce IL-6, IL-8, RANTES and TNF-α expression in MARC-145 cells. However, knockdown of HMGB1 significantly reduced the production of IL-6, IL-8, RANTES and TNF-α (Fig. 4A). As expected, treatment with the recombinant HMGB1 resulted in an increase in IL-6, IL-8, RANTES and TNF-α mRNA expression levels (Fig. 4B). We also assessed the effects of HMGB1 on PRRSV-induced cytokine expression in PAMs and

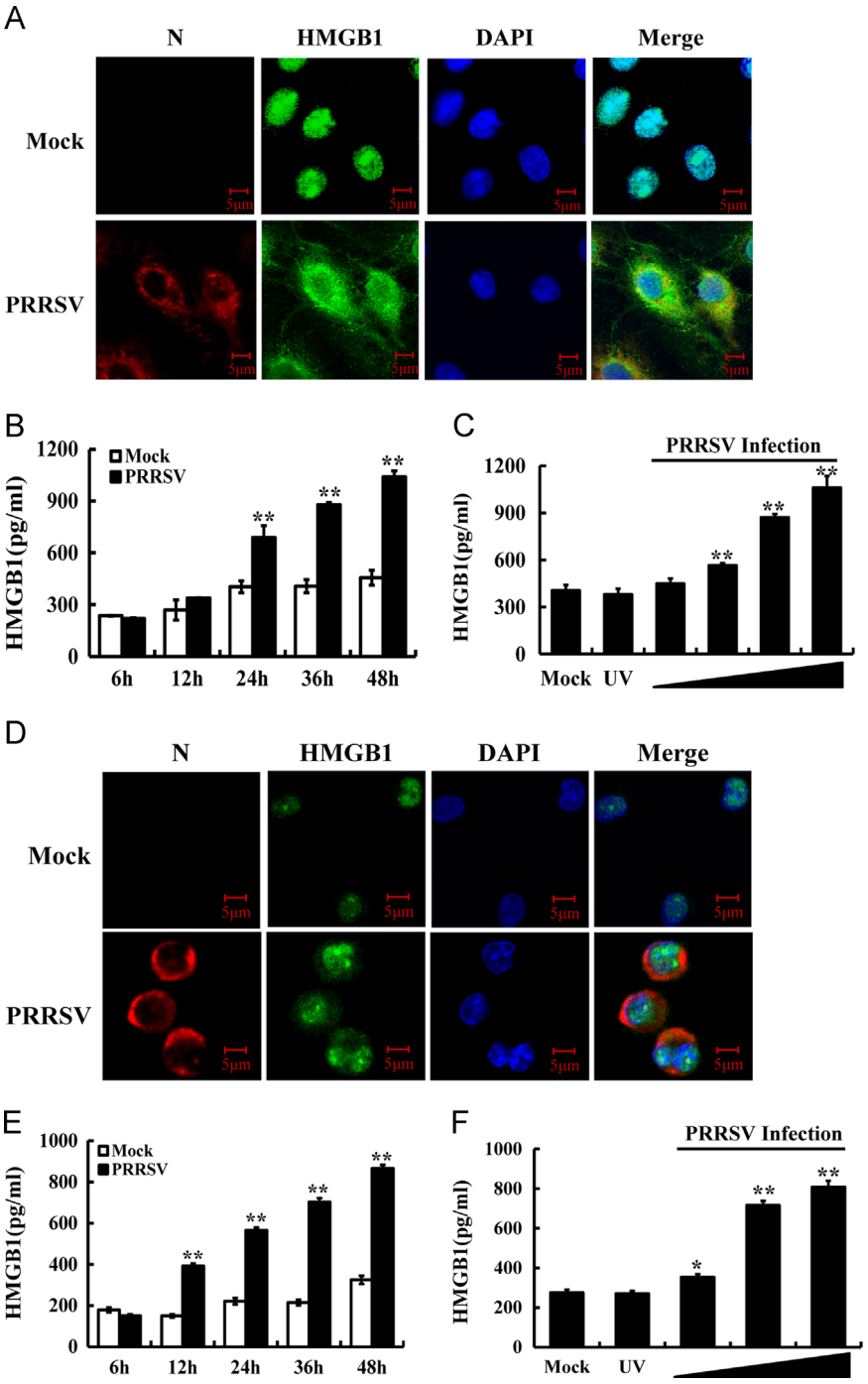


**Fig. 1.** Release of HMGB1 protein by PRRSV-infected cells. MARC-145 cells (A) and PAMs (D) are either mock-infected or infected with PRRSV at a multiplicity of infection (MOI) of 0.1. Cells are fixed and incubated with antibodies against the PRRSV N protein (red) and HMGB1 (green). The nuclei of cells are stained with DAPI (blue). The scale bar indicates 5 μm. MARC-145 cells (B) and PAMs (E) are mock-infected or infected with PRRSV at an MOI of 0.1. Culture supernatants are collected at the indicated time points and subjected to ELISA to analyze HMGB1 release. MARC-145 cells (C) and PAMs (F) are infected with PRRSV at different MOIs (0.001, 0.01, 0.1, 1.0 MOI in Marc-145 cells; 0.01, 0.1, 1.0 MOI in PAMs) or UV-inactivated PRRSV. Culture supernatants are collected to determine levels of HMGB1 protein using ELISA. The values shown in (B–F) represent the mean ± SD from three independent experiments performed in triplicate. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with mock-infected cells.

similar results could be obtained (Fig. 4C and D), further demonstrating that HMGB1 promotes expression of inflammatory cytokines during PRRSV infection.

*TLR2, TLR4 and RAGE are involved in PRRSV-induced NF-κB activation and expression of inflammatory cytokines*

TLR2, TLR4, TLR9 and RAGE are the known receptors for HMGB1-mediated NF-κB activation and subsequent inflammatory cytokine production (He et al., 2012; Hirata et al., 2013; Park et al., 2006). To dissect the signal pathways involved in HMGB1-promoted inflammatory effect during PRRSV infection, specific siRNA targeting RAGE, TLR2, TLR3, TLR4 and TLR9 were synthesized. The siRNA targeting TLR3 was included because TLR3 is not the receptor of HMGB1; thus, we can use this siRNA as an additional control in subsequent experiments. Transfection of MARC-145 cells with specific siRNAs significantly reduced the endogenous expression of corresponding target receptors compared with that by negative control siRNAs (data

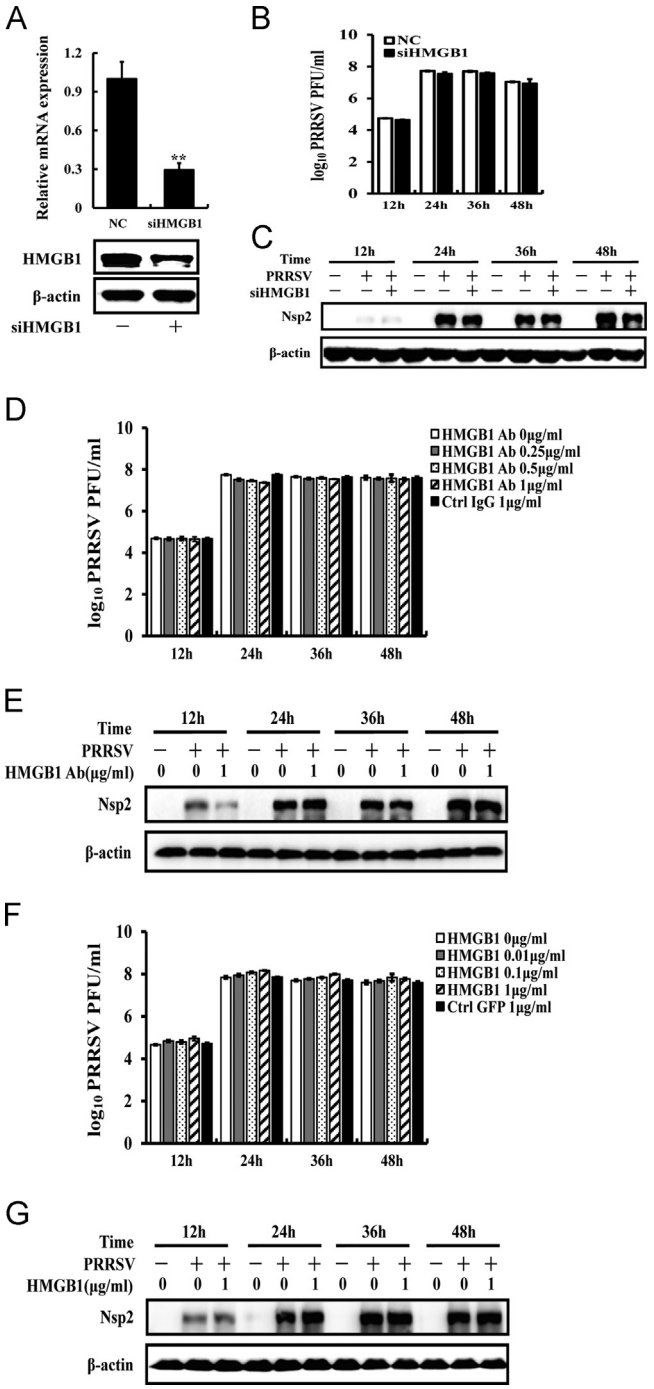


**Fig. 2.** HMGB1 does not affect PRRSV proliferation. (A) MARC-145 cells are transfected with 50 nM/well of HMGB1 siRNAs or negative control (NC) for 48 h, and then HMGB1 expression is determined by real-time RT-PCR and western blotting. HMGB1 mRNA level is first normalized to that of β-actin in the same sample and then compared with that of cells transfected with NC. ($^{**}P < 0.01$). (B) MARC-145 cells are transfected with siRNAs targeting HMGB1 for 24 h, and then infected with PRRSV (MOI=0.1). Plaque assays are conducted at 12, 24, 36 and 48 hpi. (C) MARC-145 cells are treated as in (B). At 12, 24, 36 and 48 hpi, cells are collected for western blotting analysis using a specific antibody against the PRRSV Nsp2 protein, with β-actin used as a loading control. (D, E) MARC-145 cells are pretreated with either control IgG or an antibody against HMGB1 at the indicated concentrations for 12 h. This is followed by infection with PRRSV (MOI=0.1). Infected cells are cultured in the presence of a HMGB1 antibody and collected at different time points for plaque assay (D) and western blot analysis (E). (F, G) MARC-145 cells are pretreated with either control GFP or purified HMGB1 protein at the indicated concentrations for 12 h, followed by PRRSV infection (MOI=0.1). Infected cells are cultured in the presence of HMGB1 and collected at different time points for plaque assay (F) and western blot analysis (G). All plaque results are the mean ± SD from three independent experiments performed in triplicate.

not shown). The siRNAs targeting RAGE or TLRs (TLR2, 3, 4, and 9) were co-transfected with pNF-κB-Luc and pRL-TK in MARC-145 cells before PRRSV infection. As shown in Fig. 5A, specific siRNAs targeting RAGE, TLR2 and TLR4 significantly inhibit NF-κB transcriptional activity, however this is not the case for TLR9. As expected, no influence could be observed in cells transfected with TLR3-specific siRNAs. Further studies showed that specific siRNAs targeting RAGE, TLR2 and TLR4 also inhibited IL-6, IL-8, RANTES and TNF-α mRNA expression in PRRSV-infected MARC-145 cells (Fig. 5B). Collectively, these findings suggested that HMGB1 may act as a mediator for NF-κB activation and inflammatory cytokine production through RAGE, TLR2 and TLR4 receptors in PRRSV-infected MARC-145 cells.

## Discussion

Although HMGB1 has recently been proposed as the most significant immune modulator during the porcine response to PRRSV infection, there is no experimental evidence to confirm this, only a meta-analysis (Badaoui et al., 2013). In our present study, we found that PRRSV infection triggers the translocation and secretion of HMGB1 in MARC-145 cells and PAMs. The released HMGB1 mediates PRRSV-induced NF-κB activation and subsequent inflammatory responses and may have no effect on PRRSV replication. HMGB1 appears to function as a pro-inflammatory mediator through TLR2, TLR4 and RAGE receptors during PRRSV infection.

HMGB1 is a non-histone chromosomal nuclear protein. Because there is no signal sequence at its N-terminus, HMGB1 cannot be released *via* the classical endoplasmic reticulum–Golgi secretory pathway (Wang et al., 2006). However, exogenous stimulation, including pathogen infection, can lead to cytoplasmic translocation of HMGB1 and its subsequent release into the extracellular milieu. The cytoplasmic translocation and release of HMGB1 in virus-infected cells have been reported previously following infection with West Nile virus, Dengue virus, HIV, hepatitis C virus (HCV) and herpes simplex virus type 2 (Barqasho et al., 2010; Chen et al., 2008; Chu and Ng, 2003; Gaillard et al., 2008; Jung et al., 2011; Kamau et al., 2009). In our present study, we found that following infection with PRRSV, the cytoplasmic translocation and release of HMGB1 were observed in MARC-145 cells and PAMs. It should be noted that secreted HMGB1 was only detected at 24 hpi and later in MARC-145 cells, and at 12 hpi and later in PAMs. Previous studies have revealed that HMGB1 shuttles from the nucleus to the cytosol, where it accumulates in intracellular vesicles prior to secretion. This process can take up to 8 h to complete (Andersson and Tracey, 2011; Gardella et al., 2002; Wang et al., 1999), suggesting that HMGB1 is a late cytokine. Although the molecular mechanisms regarding the translocation and release of HMGB1 are not fully understood, previous work has shown that HMGB1 proteins are translocated from the nucleus to the cytoplasm, or even secreted into the extracellular milieu under conditions in which intracellular reactive oxygen species (ROS) levels are elevated (Tang et al., 2010). It has been demonstrated that PRRSV infection significantly increases ROS production (Lee and Kleiboeker, 2007). Thus, the involvement of ROS is a likely mechanism for PRRSV-induced HMGB1 release.

The role of HMGB1 in the extracellular milieu has been intensively investigated during the last decade. To date, studies addressing the question of whether HMGB1 exerts salutary or detrimental effects on viral replication have generated conflicting results (Troseid et al., 2011). Some studies showed that HMGB1 increases HIV-1 replication and persistence (Saidi et al., 2008; Thierry et al., 2007), whereas other studies have reported that extracellular HMGB1 inhibits HIV-1 replication (Cassetta et al., 2009; Naghavi et al., 2003). In addition, HCV infection induces HMGB1 translocation from the nucleus to the cytoplasm and

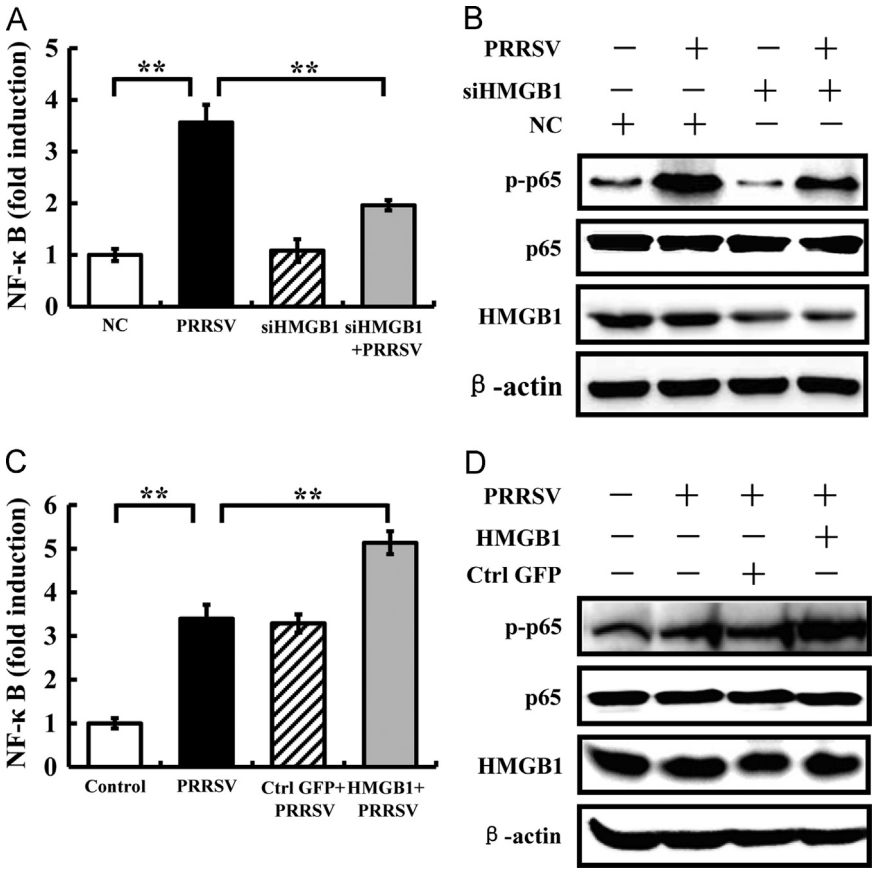


**Fig. 3.** HMGB1 enhances NF-κB activation during PRRSV infection. (A) MARC-145 cells are co-transfected with HMGB1 siRNA, pNF-κB-Luc and pRL-TK for 24 h, followed by PRRSV infection (MOI=0.1). At 36 hpi cells are lysed and analyzed using a NF-κB luciferase reporter system. Values are presented as the mean ± SD from three independent experiments performed in triplicate. ($^{*}P<0.05$ and $^{**}P<0.01$). (B) MARC-145 cells are transfected with HMGB1 siRNA or NC for 24 h, and then cells are infected with or without PRRSV (MOI=0.1). At 36 hpi, phosphorylated p65 protein levels are analyzed western blotting with β-actin used as a loading control. HMGB1 protein level is also analyzed by Western blot to detect silencing effect. (C) MARC-145 cells are co-transfected with pNF-κB-Luc and pRL-TK and then infected with PRRSV (MOI=0.1). At 30 hpi, infected cells are treated with purified HMGB1 (0.2 μg/ml) or control GFP for 6 h. Cells are lysed and analyzed with a NF-κB luciferase reporter system. Values are presented as the mean ± SD from three independent experiments performed in triplicate. ($^{*}P<0.05$ and $^{**}P<0.01$). (D) MARC-145 cells are mock-infected or infected with PRRSV (MOI=0.1). At 30 hpi, infected cells are treated with purified HMGB1 (0.2 μg/ml) or control GFP for 6 h. Cells are collected for western blotting analysis using an antibody specific for p65, with β-actin used as a loading control. HMGB1 protein level is detected by Western blot with HMGB1-specific antibody.

secreted HMGB1 from infected cells can block the infection of neighboring cells by HCV (Jung et al., 2011); however HMGB1 promotes influenza virus growth by enhancing viral polymerase activity (Moisy et al., 2012). In our current study, we used three different techniques to investigate the role of HMGB1 in PRRSV replication: knockdown of endogenous HMGB1 with specific siRNAs; blocking extracellular HMGB1 with an HMGB1 neutralizing antibody; and the extracellular addition of purified recombinant HMGB1 protein. Results from the three approaches clearly showed that HMGB1 did not significantly affect PRRSV proliferation under our experimental conditions. Previous studies revealed that HMGB1 can bind to the influenza virus nucleoprotein and promote viral replication (Moisy et al., 2012). We attempted to explore the possible interactions between HMGB1 and PRRSV-encoded proteins using a yeast two-hybrid screen; however no interactions were observed (data not shown).

Although HMGB1 is not associated with PRRSV replication, it enhances PRRSV-induced NF-κB activation and subsequent expression of inflammatory cytokines. PRRSV infection induces excessive production of inflammatory cytokines *in vivo*, which could be associated with PRRSV pathogenesis (Mateu and Diaz, 2008; Zhang et al., 2013b). HMGB1 is a well-known DAMP molecule and its accumulation can amplify cytokine cascades, stimulate inflammation, and mediate an adverse inflammatory response (Bae, 2012; Czura et al., 2001; Kudo et al., 2013). In our study, knockdown of HMGB1 inhibited PRRSV-induced NF-κB promoter activity, phosphorylation levels of p65, and expression of inflammatory cytokines. In contrast, addition of recombinant HMGB1 enhanced these effects, suggesting that it played an important role in enhancing NF-κB activation and expression of inflammatory cytokines.

RAGE, TLR2, TLR4 and TLR9 are known to be HMGB1 receptors (Park et al., 2006; Tian et al., 2007), with the RAGE, TLR2 and TLR4 signaling pathways implicated in the production of various inflammatory molecules through activation of NF-κB following tissue damage and injuries associated with burns (He et al., 2012; Palumbo et al., 2007; Park et al., 2006, 2004). In our work, knockdown of RAGE, TLR2, and TLR4 inhibited PRRSV-induced NF-κB activation and expression of inflammatory cytokines, indicating that RAGE, TLR2 and TLR4 receptors were involved in these processes. Previous studies have demonstrated that HMGB1 is an essential molecule of DNA-containing immune complexes that stimulates production of cytokines through a TLR9-MyD88 pathway (Ivanov et al., 2007; Tian et al., 2007). TLR9 is a sensor for CpG-DNA, not for RNA virus, possibly explaining why knockdown of TLR9 has no effect on the PRRSV-induced inflammatory response.

Although extracellular HMGB1 could act as a pro-inflammatory cytokine, we found that treatment with purified recombinant HMGB1 protein alone had no pro-inflammatory activity in MARC-145 cells and PAMs (data not shown); however, PRRSV infection coming with recombinant HMGB1 significantly potentiated NF-κB activation and IL-1β, IL-6, IL-8, RANTES, TNF-α production. Similar observations were reported in other studies

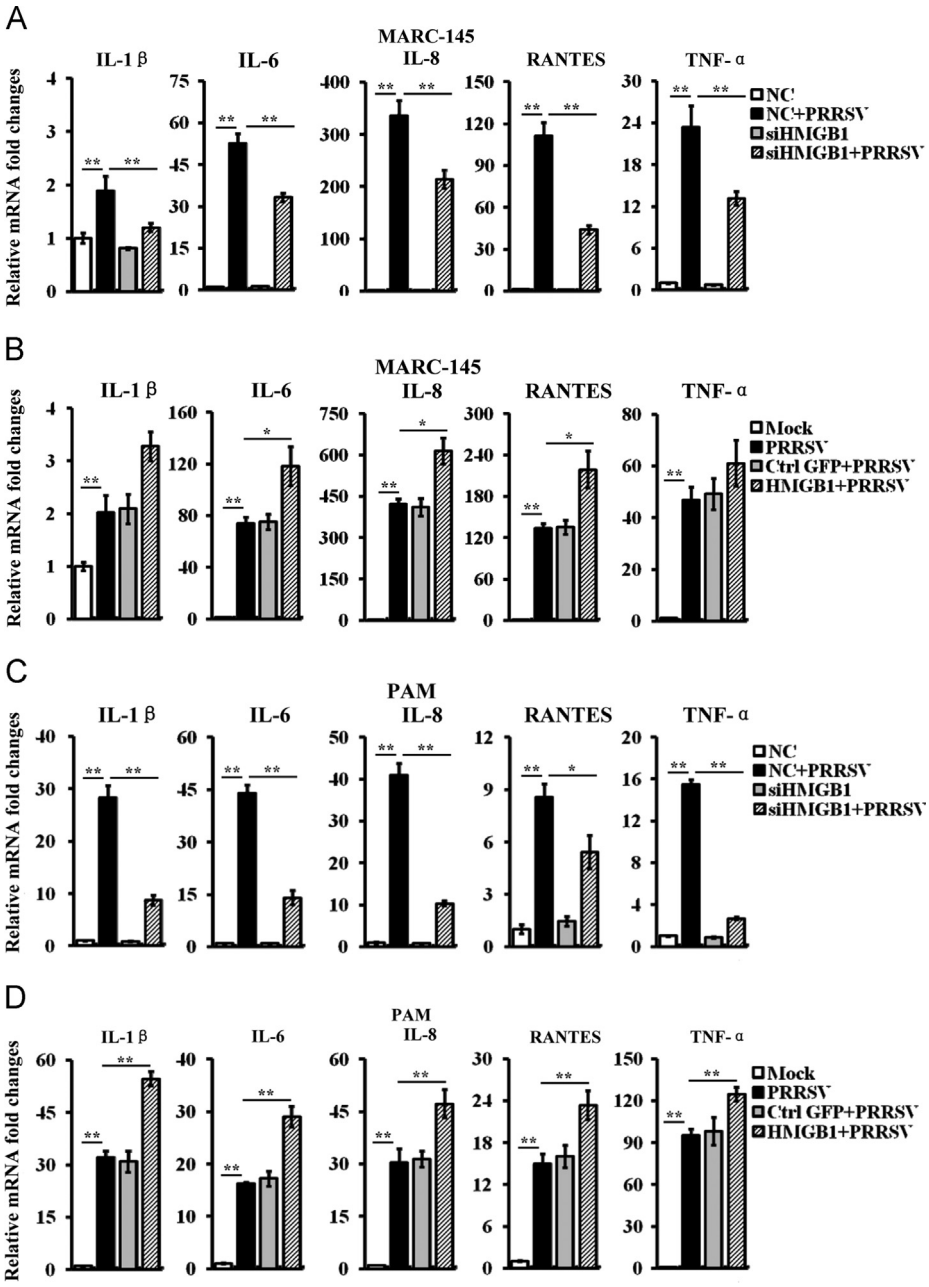


**Fig. 4.** HMGB1 mediates PRRSV-induced expression of cytokines. MARC-145 cells (A) and PAMs (C) are transfected with HMGB1-specific siRNAs or NC for 24 h, and then cells are infected with or without PRRSV (MOI=0.1). Cells are collected at 36 hpi for analysis of cytokine mRNA levels using real-time RT-PCR assays. MARC-145 cells (B) and PAMs (D) are infected with or without PRRSV (MOI=0.1). At 30 hpi, infected cells are treated with purified HMGB1 (0.2 μg/ml) or control GFP for 6 h. Cells are harvested for real-time RT-PCR determination of cytokine mRNA levels. Values for real-time RT-PCR results are presented as the mean ± SD from three independent experiments performed in triplicate. ($^{*}P < 0.05$ and $^{**}P < 0.01$).

indicating that purified recombinant HMGB1 has no pro-inflammatory activity and only acts as a chemoattractant and a mitogen (Bianchi, 2009; Dong et al., 2013). Indeed, extracellular HMGB1 can function by itself and/or forms inflammatory complexes with other molecules, including LPS, IL-1 and nucleosomes (Bianchi, 2009; Cassetta et al., 2009; Chen et al., 2009; He et al., 2013; Hreggvidsdottir et al., 2009; Qin et al., 2009; Tian et al., 2007). Thus, we speculate that HMGB1 itself does not perform pro-inflammatory activity, but may interact with other cytokines induced by PRRSV, and then interacts with TLR2, TLR4 and RAGE receptors to promote pro-inflammatory production.

In conclusion, our findings demonstrated that PRRSV infection appears to trigger the translocation and secretion of HMGB1, which in turn function through TLR2, TLR4 and RAGE receptors to mediate NF-κB activation and subsequent production of inflammatory cytokines. Our results provide new insight into understanding how PRRSV infections induce excessive inflammatory responses. Further investigations into the role of HMGB1 in PRRSV pathogenesis will hopefully provide potential therapeutic targets against PRRSV.

## Materials and methods

### *Cells, virus and reagents*

MARC-145 cells, a monkey kidney cell line highly permissive for PRRSV infection, were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented with 10%

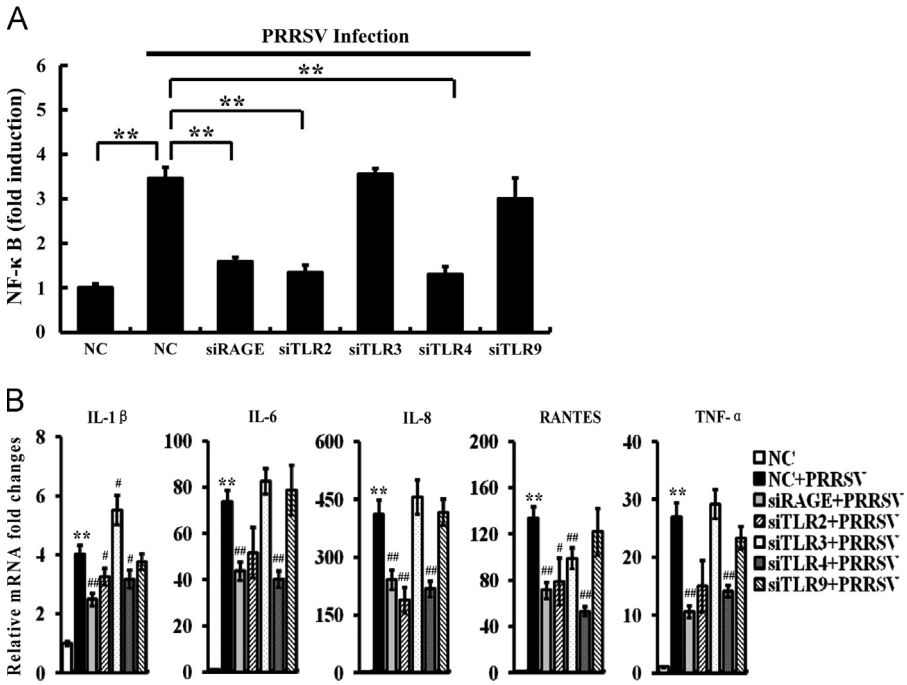


**Fig. 5.** HMGB1 receptors are involved in PRRSV-induced NF-κB activation and expression of inflammatory cytokines. (A) We co-transfected siRNAs targeting RAGE or TLRs (TLR2, 3, 4, 9) with pNF-κB-Luc and pRL-TK into MARC-145 cells. At 24 h post-transfection, cells are then infected with PRRSV (MOI = 0.1). At 36 hpi, cells are lysed and analyzed using an NF-κB luciferase reporter system. Values are presented as the mean ± SD from three independent experiments performed in triplicate. ($^{*}P < 0.05$ and $^{**}P < 0.01$). (B) MARC-145 cells are transfected with specific siRNAs targeting RAGE or TLRs (TLR2, 3, 4, 9) for 24 h, and then infected with PRRSV (MOI = 0.1). At 36 hpi, cytokine mRNA levels are analyzed by real-time RT-PCR. Values are presented as the mean ± SD from three independent experiments performed in triplicate. $^{**}P < 0.01$ (for positive PRRSV group compared to negative control); $^{\#}P < 0.05$; $^{\#\#}P < 0.01$ (for positive PRRSV compared to specific siRNAs group).

**Table 1**
The sequences of siRNAs used in this study.

| Gene name | siRNA sequence (sense 5′–3′) | siRNA sequence (anti-sense 5′–3′) |
|---|---|---|
| HMGB1-MARC | CUGCUAAGCUGAAGGAGAATT' | UUCUCCUUCAGCUUAGCAGTT |
| HMGB1-PAM | CTGCTAAGCTGAAGGAGAATT | TTCTCCTTCAGCTTAGCAGTT |
| RAGE | GCCGGAAAUUAUAGAUUCUTT | AGAAUCUAUAAUUUCCGGCTT |
| TLR2 | CGGAGAGACUUUGCUUACUTT | AGUAAGCAAAGUCUCUCCGTT |
| TLR3 | CAACUUUCUUGGGACUAAATT | UUUAGUCCCAAGAAAGUUGTT |
| TLR4 | GUGCAGGCAUAAUCUUCAUTT | AUGAAGAUUAUGCCUGCACTT |
| TLR9 | GCCUCAACCUCAAGUGGAATT | UUCCACUUGAGGUUGAGGCTT' |

heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin in a humidified incubator with 37 °C/5% $CO_2$. Porcine alveolar macrophages (PAMs) were isolated from a 5-week-old PRRSV-negative herd by bronchoalveolar lavage after necropsy. The isolated PAMs were also detected for common swine pathogens, including pseudorabies virus (PRV), porcine circovirus type 2 (PCV-2), swine influenza virus, porcine parvovirus (PPV), and Japanese encephalitis virus (JEV), by RT-PCR. Only RT-PCR negative PAMs were used for our study. Isolated PAMs were maintained in RPMI-1640 medium (Invitrogen) with 10% heated-inactivated FBS, an antibiotic–antifungal mix (100 U/ml penicillin, 100 μg/ml streptomycin; Invitrogen) at 37 °C/5% $CO_2$. PRRSV strain WUH3 (GenBank Accession No. HM853673), isolated from the brains of pigs suffering from "high fever" syndrome in China at the end of 2006, and identified as a highly pathogenic PRRSV (HP-PRRSV), was used throughout our study (Li et al., 2009). The anti-HMGB1 neutralizing antibody and isotype control IgG were purchased from Biolegend (Canada). Antibodies against NF-κB p65 and phosphor-NF-κB p65 were purchased from Cell Signaling Technology (USA). Secondary antibodies for immunostaining were purchased from Invitrogen and Sigma-Aldrich. The purified recombinant HMGB1 protein and control protein GFP were purchased from Abcam (Hong Kong).

*Plasmids, siRNA and cell transfection*

The luciferase reporter plasmid pNF-κB-Luc was purchased from Stratagene. We purchased small interfering RNAs (siRNAs) targeting HMGB1, TLR2, TLR3, TLR4, TLR9, and RAGE from GenePharma (Shanghai, China), and the sequences are listed in Table 1. Plasmids and siRNAs were transfected into cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol.

*Detection of cytokines using real-time RT-PCR*

MARC-145 cells or PAMs in 24-well plates were transfected with specific siRNAs (50 nM/well) and then infected with PRRSV at a multiplicity of infection (MOI) of 0.1. At 36 h post-infection (hpi), total RNA was extracted from cells using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. In alternative experiments, MARC-145 cells or PAMs were infected with PRRSV (MOI = 0.1); at 30 hpi cells were treated with recombinant HMGB1 or control GFP. Total RNA was extracted from cells at 36 hpi. Once RNA was extracted, we reverse transcribed 1 μg of each sample into cDNA using a Transcriptor First Strand cDNA Synthesis Kit (Roche). We then used 1 μL of the resulting cDNA in SYBR green qPCR assays. The abundance of individual mRNA transcripts in

each sample was assayed three times. Gene expression was measured as described previously (Wang et al., 2012). Primer sequences are listed in Table S1.

*Luciferase reporter assays*

MARC-145 cells cultured in 24-well plates were co-transfected with 100 ng/well of pNF-κB-Luc, 50 ng/well of pRL-TK (as an internal control to normalize the transfection efficiency, Promega), and specific siRNA or negative control (50 nM/well), followed by PRRSV infection (MOI=0.1). In alternative experiments, sub-confluent MARC-145 cells were co-transfected with pNF-κB-Luc (100 ng/well) and pRL-TK (50 ng/well) prior to PRRSV infection (MOI=0.1); cells were then treated with recombinant HMGB1 or control GFP at 30 hpi. At 36 hpi, cells were lysed to determine firefly luciferase and Renilla luciferase activities using a Dual-luciferase reporter assay system (Promega) as per the manufacturer's suggestions. We have presented our results as relative firefly luciferase activity normalized to Renilla luciferase activity from three independent experiments.

*Western blot analysis*

Briefly, MARC-145 cells cultured in 60-mm dishes were transfected with HMGB1-specific siRNA prior to PRRSV infection (MOI=0.1) or treated with purified HMGB1 protein at 30 h after PRRSV infection. At 36 hpi, the cells were harvested by adding lysis buffer, and protein concentrations were measured in whole cellular extracts. Equal amounts of samples were then subjected to SDS-PAGE and analyzed for expression of PRRSV nonstructural protein 2 (Nsp2) (Wang et al., 2013), HMGB1, p65 or p-p65 protein by western blotting using specific antibodies, respectively. Expression of β-actin was detected with an anti-β-actin mouse monoclonal antibody (MAb) (Beyotime, China) to demonstrate equal protein sample loading.

*PRRSV titration*

Plaque assays were conducted as described previously (Luo et al., 2011b). Briefly, MARC-145 cultures that were 95% confluent in 6-well culture plates were infected for 1 h with 10-fold serial dilutions (1 mL) of PRRSV-containing samples. After three washes with phosphate-buffered saline (PBS; pH 7.4), cells were overlaid with 1.8% (w/v) Bacto agar mixed with 2XDMEM at a 1:1 ratio supplemented with 0.05 mg/mL neutral red. Plaques were counted at 4 dpi, and average plaque number and standard deviations were calculated from three independent experiments. We expressed virus titer as plaque-forming units (PFU)/mL.

*Indirect immunofluorescence assays*

To examine the subcellular localization of HMGB1, MARC-145 cells or PAMs were infected with PRRSV (MOI=0.1). At 30 hpi, cells were fixed with 4% paraformaldehyde for 20 min at room temperature before permeabilization with 0.1% Triton X-100. Cells were then incubated with a mouse monoclonal antibody against the PRRSV N protein (SDOW17; Rural Technologies, USA) and a rabbit antibody against HMGB1 (Novus Biologicals, USA). Following incubation with the primary antibodies, cells were incubated with Alexa Flour 488-conjugated anti-rabbit and Alexa Fluor 555-conjugated anti-mouse antibodies (Invitrogen). Cells were counterstained with 1 μg/mL 4′,6′-diamidino-2-phenylindole (DAPI; Beyotime) for 15 min and visualized using an LSM 510 Meta confocal laser-scanning microscope (Carl Zeiss, Germany).

*Measurement of secreted HMGB1 protein*

Secreted HMGB1 in cell supernatants was quantified using a commercial sandwich enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer's protocol (USCN, China).

## Statistical analysis

Data are presented as the mean ± SD for at least three independent experiments. Student's *t*-test was used to analyze differences between two experimental groups. A *P*-value less than 0.05 was considered significant and a *P*-value less than 0.01 was considered highly significant.

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## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.virol.2014.07.046.

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