Porcine reproductive and respiratory syndrome virus and bacterial endotoxin act in synergy to amplify the inflammatory response of infected macrophages

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1. Introduction

Porcine reproductive and respiratory syndrome (PRRS) was first discovered in the late 1980s in the United States, and has subsequently spread worldwide. It is characterized by severe respiratory disease in young pigs and reproductive failure in sows (Albina, 1997). It has become endemic in countries with high levels of pig rearing and causes devastating economic loss each year (Y.J. Zhou et al., 2009; Albina, 1997). PRRS virus replicates preferentially in cells of the macrophage lineage, cells which play a major role in the inflammatory and immune responses (Chen et al., 2010; Duan et al., 1997; Mengeling et al., 1996).

The inflammatory cytokines TNF-α and IL-1β are produced by macrophages during virus infection and if their circulating levels become excessive, high fever, depression, anorexia and even death of the host can occur (Schroder and Tschopp, 2010; van Reeth and Nauwynck, 2000).

PRRSV is considered to be one of the most important etiological agents in multifactorial respiratory disease of swine, and can predispose pigs to infection by bacterial such as Steptococcus suis, Haemophilus parasuis, Myco-...
plasma hyopneumoniae, Actinobacillus pleuropneumoniae,
and Salmonella spp (Fraile et al., 2009; Thanawongnuwech et al., 2004; Solano et al., 1997; Benfield et al., 1992). Pigs infected with both PRRSV and S. suis developed more severe clinical disease and lesions than when infected by S. suis alone (Thanawongnuwech et al., 2000) and so the synergistic effects of infection by PRRSV and a secondary bacterial infection in the induction of multifactorial respiratory diseases is already recognized (Van Gucht et al., 2004). It has been shown that European strains of PRRSV can sensitize the lungs for the production of pro-inflammatory cytokines and respiratory signs upon exposure to bacterial lipopolysaccharides (LPS) (van Gucht et al., 2003).

Since May 2006 there have been outbreaks of a severe form of PRRS in China characterized by prolonged high fever, red discoloration of the body, and blue ears and associated, irrespective of age, with a high mortality. Several studies have confirmed that the causative agent of these outbreaks was a highly pathogenic strain of PRRSV, characterized by a genomic marker of a 30 amino acid deletion in the Nsp2-coding region, and that secondary bacterial infection may also contribute to the generation of this severe form of PRRS (Zhou et al., 2008; Li et al., 2007; Tian et al., 2007). Understanding the processes by which a virus causes disease is a prerequisite in the search for better therapy. The aims of the present study were to investigate the potential for inflammatory cytokine production by macrophages infected with PRRSV and exposed to LPS to obtain a further understanding of the origin of the symptoms in this disease.

2. Materials and methods

2.1. Virus strains

Two PRRSV strains of differing levels of virulence, HN07-1 and BJ-4, were used for the study. HN07-1 strain was isolated during an atypical PRRSV outbreak from an intensive rearing pig farm in Henan province of China in 2007 and was propagated on Marc-145 cells, maintained in Dulbecco modified Eagle medium (DMEM) (Invitrogen) supplemented with 10% fetal bovine serum (FBS) (Sigma) at 37 °C under 5% CO2. The BJ-4 strain was a typical North American (VR2332)-like PRRSV isolated in 1996 in China and its complete genomic sequence has been determined and deposited in GenBank (accession no. AF331831).

2.2. Characterization of HN07-1 pathogenicity

To determine the pathogenicity of HN07-1, laboratory infection experiments were performed using 6-week old healthy crossbred piglets. All animals were confirmed to be initially free of PRRSV and porcine circovirus type 2 (PCV2) infections by the use of commercial enzyme-linked immunosorbent assay (ELISA) kits for the detection of antibodies and by reverse transcription-PCR (RT-PCR) or PCR for viral nucleic acid detection. The animals were also shown to be negative for classical swine fever virus, pseudorabies virus, swine influenza virus and porcine parvovirus infections by serological methods or by PCR. The pigs were divided into three groups of 5 animals, two infection groups (HN07-1 and BJ-4) and a control group, raised separately in different isolation rooms with individual ventilation. The animals were challenged by nasal and injection routes with 2.0 ml and 1.0 ml of viral culture respectively. Viral culture contained 105 tissue culture infective doses (TCID50) per ml, from the fifth passage on Marc-145 cells. All pigs in the control group were similarly inoculated with 2 ml of DMEM. All pigs were clinically examined and their rectal temperatures measured daily until 21 days postinfection. The date and time of death of each animal was recorded.

2.3. ORF5 and Nsp2 genes sequencing of HN07-1

The ORF5 and Nsp2 genes of HN07-1 were amplified from viral isolates by RT-PCR, cloned and sequenced. Viral RNA was extracted from the viral cultures using TRIzol reagents (TAKARA), and cDNA was subsequently synthesized using an oligo-dt primer (TAKARA). The cDNA was used for PCR with the primers F5.1 (5'-CTGAGACCATGAGGTGGGCAAC-3') and F5.2 (5'-TCAAAAGGTGCAAGGCTCC-3') for the ORF5 gene and with F1.1 (5'-CGGCCGTGAAAGACAGCAA-3') and F1.2 (5'-GCCGAGTAAACCCGCAAAG-3') for the Nsp2 gene. The resulting PCR products were isolated, cloned and sequenced.

2.4. Preparation of porcine alveolar macrophages (PAMs)

The PAMs were collected from 4-week-old piglets free of PRRSV through lung lavage as previously described (Zhang et al., 2006). PAMs were dispensed at 2 × 105 cells/well into 24-well plates and maintained in RPMI-1640 (Sigma) supplemented with 10% fetal bovine serum (FBS, Life Technologies) containing an antibiotic–antimycotic mixture composed of 100 mg/ml streptomycin, 100 IU/ml penicillin and 25 mg/ml amphotericin B (Sigma) at 37 °C in a humidified atmosphere containing 5% CO2. The FBS was free of LPS, as specified by the manufacturer.

2.5. Induction of TNF-α and IL-1β secretion in PAMs

To evaluate the appropriate dose of LPS (purified from Escherichia coli; 011:B4, Sigma) concentrations from 1 to 1000 ng/ml were used to treat cells for 1, 4, 6, 10, 12 and 24 h. The culture fluid levels of IL-1β and TNF-α were measured by ELISA, and the cellular expression level of mRNAs for IL-1β and TNF-α were assayed by Real-time PCR.

2.6. Synergistic effect of PRRSV and LPS induction of TNF-α and IL-1β secretion

To determine the effects of combined viral infection and exposure to LPS six groups of confluent PAM cells (n = 3 in each group) were taken, of which two groups of cells provided the controls, one receiving PBS alone (denoted as the PBS group) and the second group 100 ng/ml LPS (the LPS group). A further two groups of cells were infected with 105 TCID50 per well of PRRSV HN07-1 strain and 24 h after infection one group was treated with 100 ng/ml LPS. Two other groups of cells were infected with the BJ-4 strain and similarly treated. After exposure to LPS for 4 h cell cultures were harvested and IL-1β and TNF-α mRNA and secreted levels were measured.
2.7. Quantitative reverse-transcriptase polymerase chain reaction assay

Messenger RNAs from macrophages inoculated with virus and/or LPS were prepared for quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) analyses of TNF-α, IL-1β, and hypoxanthine phosphoribosyltransferase (HPRT) as previously described (Qiao et al., 2009, 2006). The oligonucleotide sequences of the upstream and downstream primers for these mRNA analyses were 5’-CACCAGCTCTTCTGCCTAC-3’ and 5’-AGGGGGTTATCTGAGGTGAG-3’ for TNF-α, 5’-CCCCAAGGTACCCGAAAGG-3’ and 5’-TCTGCTTGAGGTTCTGATG-3’ for IL-1β, and 5’-GCCGA GGATTGGGAAAAGG-3’ and 5’-GCACACAGAGGGTACGATG-3’ for HPRT. All real-time PCR reactions were performed on a Bio-Rad I-Cycler Real Time PCR Instrument (Bio-Rad) using SYBR green system (TAKARA). The thermal cycling conditions included an initial denaturation step at 95 °C for 30 s, then 40 cycles at 94 °C for 5 s, and 60 °C (HPRT and IL-1β) or 62 °C (TNF-α) for 20 s.

2.8. Cytokine ELISA

The amounts of the IL-1β and TNF-α proteins in the culture supernatants were quantified following the standard protocols of the enzyme-linked immunosorbent assay kits purchased from Biosource.

2.9. Flow cytometric analysis

The comparison of surface CD14 expression was performed by flow cytometry on CD14 expression. Cells were fixed with 2% formaldehyde in PBS and incubated with anti-pCD14 IgG2b monoclonal antibody (Serotec) or isotype-specific IgG2b control. After incubation for 1 h, the cells were washed and incubated with FITC-conjugated goat anti-mouse IgG secondary antibody at 4 °C, pelleted and washed twice with cold PBS and kept on ice. Fluorescence intensity was measured using a BD FACSCalibur flow cytometer counting 10,000 cells per sample.

2.10. Statistical analysis

Data were subjected to one-way analysis of variance (one-way ANOVA). If the P value from the ANOVA was less than or equal to 0.05, pairwise comparisons of the different treatment groups were performed by a least-significant difference test at a rejection level of a P value < 0.05.

3. Results

3.1. HN07-1 isolate exhibited high virulence for pigs

On day two post-inoculation, all of the pigs inoculated with strain HN07-1 showed an increased body temperature (>40 °C) (Fig. 1A), and had marked clinical symptoms including depression, anorexia, rubefaction of skin and ears, respiratory distress, shivering and diarrhea. Two of five animals died, at days 8 and 10, the remainder surviving to the end of the experimental period (Fig. 1B). All of the animals infected with strain BJ-4 and controls survived the experimental period and did not show any clinical symptoms. This experiment demonstrated that strain HN07-1 is a highly virulent PRRSV isolate that can produce fatal disease.

The ORF5 sequence of HN07-1 shared 89% nucleotide identity with the North American PRRSV prototype VR-2332, and 99% identity with the highly virulent PRRSV isolates JXA1 and HuN4 recently described in China (data not shown) (Tian et al., 2007). Amino acid alignment revealed that HN07-1 had the same unique deletion of 30 discontinuous amino acids in its Nsp2 coding region as JXA1, HuN4, in contrast to the sequences of VR-2332 and the earlier Chinese isolate BJ-4 (data not shown) (Tian et al., 2007).

3.2. LPS induces TNF-α and IL-1β secretion in PAMs

We first evaluated the ability of PAMs to release IL-1β and TNF-α after LPS stimulation. Secretion of both IL-1β and TNF-α showed dose dependency over a range of LPS concentrations between 1 and 100 ng/ml (Fig. 2A). The release of IL-1β and TNF-α begins after 4 h of incubation and reaches a maximum at 12 h (Fig. 2B). The time-course of IL-1β and TNF-α induction by LPS was confirmed at gene level by real time PCR (data not shown).

3.3. Synergistic effect of PRRSV and LPS on IL-1β and TNF-α production

PAM cells infected with either of the PRRSV isolates showed an increased secretion of IL-1β and TNF-α over the 28 h period following addition of virus (Table 1). HN07-1 was a stronger inducer of IL-1β secretion than BJ-4 (p = 0.046) and of TNF-α, although the difference was not significant. Secretion by uninfected control cells over this time period was below the reliable detection limits. The addition of LPS to cultures for 4 h led to an increased secretion of both cytokines. Secreted IL-1β levels were greatly increased in the infected cells over the controls.

Table 1

<table>
<thead>
<tr>
<th>Group</th>
<th>IL-1β (pg/ml) mean ± sem</th>
<th>TNF-α (pg/ml) mean ± sem</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No addition</td>
<td>Plus LPS</td>
</tr>
<tr>
<td>Controls</td>
<td>28.2 ± 8.9</td>
<td>116.8 ± 29.5</td>
</tr>
<tr>
<td>HN07-1</td>
<td>164 ± 42.215</td>
<td>831.1 ± 103.625</td>
</tr>
<tr>
<td>BJ-4</td>
<td>46 ± 10.125</td>
<td>286.1 ± 107.83</td>
</tr>
</tbody>
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Table 1: Secretion of pro-inflammatory cytokines IL-1β and TNF-α. Macrophages were infected with PRRSV strains at 10^6 TCID₅₀ in the presence or absence of LPS (100 ng/ml). Supernatants were assayed for IL-1β and TNF-α by ELISA. Data are shown as mean ± SE of three independent experiments. Controls are uninfected PAMs. Differences of values 1, 2, 3, 4, 5 and 6 significant at p < 0.005; values 5, p < 0.05; values 7 p = 0.096, NS.
(p < 0.01) and were greater than the expected sum. However, although the secretion of TNF-α was again greater in cells infected with HN07-1 (p < 0.01) and greater than the expected sum, this was not seen in those infected with BJ-4 (Table 1). These differences in the responses seen in cells infected with HN07-1 versus BJ-4 were significant (p < 0.01). The induction of IL-1β and TNF-α under these conditions was confirmed by real-time PCR (data not shown).

3.4. Upregulation of CD14 expression in PRRSV-infected PAMs

In order to gain insight into the possible mechanism behind the observed synergy between viral infection and the LPS induction of the proinflammatory cytokines, particularly of IL-1β, the level of CD14 on PRRSV-infected and non-infected cells was evaluated. Incubation of PAMs for 24 h following the addition of PRRSV HN07-1 strain resulted in a significant increase in CD14 expression, as assessed by mean fluorescence intensity (MFI), compared with cells from controls. The pre-infection of PAMs with PRRSV BJ-4 strain caused an increase of CD14 expression intermediate to that of the control and the HN07-1 infected cells (Fig. 3).

4. Discussion

Since 2006, atypical PRRS (called porcine high fever in China) has been endemic in China. Studies have confirmed
that the causative agent of this clinical outbreak was a highly pathogenic PRRSV strain, with a genomic marker consisting of a 30-aa deletion in Nsp2 (Tian et al., 2007; L. Zhou et al., 2009; Zhou et al., 2008). First, we determined by an animal inoculation trial, that our PRRSV isolate HN07-1 was a highly pathogenic virus. Sequence determination further showed that the isolate had the same genomic characteristics as in previously described isolates (L. Zhou et al., 2009; Zhou et al., 2008; Tian et al., 2007). This confirmed that highly virulent PRRSV is the etiological agent of atypical PRRS in China.

In order to undertake an investigation into the mechanisms by which the highly pathogenic PRRSV strain causes high fever we set up an in vitro model to investigate the production of pro-inflammatory cytokines produced by lung macrophages in response to exposure to PRRSV with or without LPS. LPS was chosen because it is the main endotoxin of Gram-negative bacteria. LPS is released at high concentrations in the lungs during pulmonary infection with Gram-negative bacteria (Kolle et al., 1996; Pugin et al., 1992). Previous in vivo studies had shown that the PRRSV-LPS combination is a simple and
Fig. 3. Representative histograms show a difference in CD14 expression levels. Surface protein expression comparisons were performed by flow cytometric assessment of HN07-1 PRRSV strain and BJ-4 strain infected (red histograms; MFI of M1-gated cells in bold) and control PAMs (light histograms; MFI of M1-gated cells not bold) using anti-porcine CD14 monoclonal antibody. Negative control represents PAMs incubated with isotype-specific control monoclonal antibody after infection with HN07-1 PRRSV (red histograms; MFI in bold). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)
reproducible experimental model for multifactorial respiratory disease in pigs (Van Gucht et al., 2004; van Gucht et al., 2003).

The proinflammatory cytokines, IL-1β and TNF-α, are important mediators in several infectious and inflammatory lung diseases (Murtaugh et al., 1996). They are among the first cytokines to be produced by the alveolar macrophages during infection. Increased levels of these cytokines in the circulation are responsible for the signs of acute systemic inflammation, including fever, depression and anorexia, signs which have been associated with cytokine production in the lungs (Thanawongnuwech et al., 2004; Huang et al., 1999). We found that the LPS induction of IL-1β and TNF-α secretion in PAMs was concentration dependent and showed a latency of about 4 h (Fig. 1). LPS is widely used for the stimulation of macrophages. The data for proinflammatory cytokines response of PAMs to LPS titration presented here and other related reports will provide useful information for porcine macrophages activation (Baarsch et al., 1991; Lin et al., 1994; Sacco et al., 1996).

The effect of pre-inoculation of cultured PAMs with PRRSV and virus on their pro-inflammatory cytokine release was examined. PRRSV HN07-1 significantly enhanced IL-1β (p = 0.024) and TNF-α (p = 0.043) production in naïve cells whilst, there was no significant difference between PRRSV BJ-4 strain and mock-infected group in induction the pro-inflammatory cytokines (P = 0.735 and 0.659 for IL-1β and TNF-α, respectively). The subsequent addition of LPS to both HN07-1 or BJ-4 infected cells further increased IL-1β and TNF-α secretion (Table 1). The level of IL-1β and TNF-α protein were 1–4 times higher in PRRSV-LPS inoculated groups than in the singly PRRSV or LPS-inoculated controls. These results are in good agreement with those of previous in vivo experiments (Van Gucht et al., 2004). The combination of HN07-1 strain and LPS exhibits significantly (P < 0.01) greater secretion of IL-1β and TNF-α than that of BJ-4 strain with or without LPS. In HN07-1 treated cells a synergistic effect was seen on both IL-1β and TNF-α secretion, although the effect was about twice as great on IL-1β secretion as that seen on TNF-α. With BJ-4 treated cells a synergistic effect on TNF-α was not seen. The greater synergistic effect of HN07-1 strain on the LPS elicited secretion of IL-1β and TNF-α by PAMs may explain the “high fever” associated with this highly pathogenic PRRSV variant and confirms that secondary bacterial infections may well exacerbate disease symptoms.

To understand why PRRSV may sensitize PAM to LPS, CD14 expression on infected PAMs was examined by flow cytometry. Flow cytometry demonstrated up-regulation of CD14, the primary LPS receptor (Wright et al., 1990) on PRRSV-infected PAMs. An earlier study had also shown an increased number of high expressing CD14 lung macrophages in PRRSV infected animals (Van Gucht et al., 2005) and the authors concluded that the increased numbers of high CD14 macrophages were the result of infiltration of the tissue by activated blood monocytes. However our results were obtained in vitro, indicating that the indigenous PAMs when infected with PRRSV can up-regulate CD14 expression. The expression of CD14 is much higher on PRRSV HN07-1 infected group than that of BJ-4 infected group, suggesting a correlation between pro-inflammatory cytokine secretion and the PRRSV-induced PAM activation. Nevertheless, recognition of LPS seems to involve a complex protein–protein interactions that eventually leads to cellular activation. The crystal structure shows that Toll-like receptor 4 and myeloid differentiation factor 2 (MD-2) acts in concert with LPS-CD14 complexes to initiate the signaling process (Park et al., 2009). Further work is required to investigation the detailed mechanisms regarding PRRSV sensitizing PAM to LPS.

5. Conclusion

In conclusion, we set up an in vitro model to investigate the production of pro-inflammatory cytokines produced by macrophages in response to inoculation with PRRSV and the further addition of LPS. A highly pathogenic isolate PRRSV HN07-1 was a stronger inducer of IL-1β production compared to classical PRRSV strain BJ-4. What is more, the combination of HN07-1 strain and LPS exhibits significant synergistic effects on the secretion of IL-1β and TNF-α than that of BJ-4 strain and LPS. Part of the explanation for this synergistic effect on the further response of the cells to LPS may be explained by an increase in the PAM’s CD14 expression and which was greater following exposure to HN07-1. These results further confirm a possible synergism between PRRSV and secondary bacterial infection in the induction of a high pro-inflammatory cytokine secretion. The above plus the observation that HN07-1 strain can itself elicit a strong IL-1β secretion, could explain the occurrence of the characteristically high fever associated with this highly pathogenic form of PRRSV.

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References


