Short Communication

Highly pathogenic porcine reproductive and respiratory syndrome virus infection and induction of apoptosis in bone marrow cells of infected piglets

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Highly pathogenic porcine reproductive and respiratory syndrome virus (HP-PRRSV) has been shown to have a wide range of tissue tropism, and can directly and indirectly induce cellular apoptosis. However, the impact of HP-PRRSV infection on the bone marrow (BM) of piglets remains unclear. In this study, we investigated the BM as a novel site of infection by the HP-PRRSV strain in piglets. HP-PRRSV infected SWC3+SWC8/C0 cells in the BM and induced BM cells to undergo apoptosis. The number of apoptotic cells highlights the striking effects of HP-PRRSV on the central immune organs (BM and thymus) that may enhance the susceptibility of pigs to secondary infections and lead to high mortality. This study is, to the best of our knowledge, the first to report the impact of HP-PRRSV on the BM and implicate the depletion of BM cells during HP-PRRSV infection in the development of immunosuppression in this disease.

Porcine reproductive and respiratory syndrome (PRRS), caused by the PRRS virus (PRRSV), is one of the most economically important viral diseases in the pig industry worldwide. After the catastrophic porcine reproductive failures observed in the United States and Europe in the late 1980s and early 1990s, a pandemic of the disease affected North America, Europe and Asia in subsequent years (Kim et al., 2007). In 1996, the first PRRSV strain (CH-1a) was isolated in China (Guo et al., 2011), where the disease has since caused significant losses in the pig production industry. In 2006, the highly pathogenic PRRSV (HP-PRRSV) emerged in China, and has since become an intractable problem for the development of the pig industry (Li et al., 2007; Tian et al., 2007; Tong et al., 2007).

PRRSV subverts early innate immunity, which leads to host immunodeficiency or immunosuppression (Vu et al., 2011). Piglets infected with the PRRSV SD 23983 strain in utero, as well as weaned piglets infected with the HP-PRRSV HuN4 strain, have been reported to exhibit lesions as well as atrophy and cell apoptosis in the thymus, which is a primary lymphoid organ (Feng et al., 2001; He et al., 2012; Wang et al., 2014). As another important primary lymphoid organ, bone marrow (BM) plays a critical role in immune responses (Kvisgaard et al., 2013; Liu et al., 2013) and is able to supplant secondary lymphoid tissues as a site of primary immune responses or as a cache for excess T-cell precursors (Silva-Campa et al., 2009). Previous studies demonstrated that viral antigens were undetectable in both piglets infected in utero and those infected after birth with the ATCC VR-2332 strain, while lesions were observed in the BM of infected piglets (Feng et al., 2002).

Our previous studies showed that the HP-PRRSV HuN4 strain caused severe thymic atrophy in infected piglets after birth (He et al., 2012; Wang et al., 2011). Thus, to fully characterize the tissue damage caused by the HuN4 strain, a representative HP-PRRSV, further investigations of infected piglets were deemed to be necessary, particularly in the BM compartment. In this study, we identified the BM as a novel site for infection by HuN4 in these piglets. The

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subpopulations of BM cells infected by the virus, as well as those undergoing apoptosis, were analyzed.

In this study, 18 PRRSV-negative weaned piglets (aged 4–5 weeks) were randomly divided into two groups (9 piglets per group) and housed separately in isolated rooms. The piglets in one group were inoculated intranasally with HP-PRRSV HuN4 \(10^{5.5}\) TCID\(_{50}\) in 3 ml Dulbecco’s modified Eagle’s medium (DMEM) (GenBank accession no. EF635006) (Tian et al., 2009), while those in the other group were sham-inoculated with 3 ml DMEM. Three piglets from each group were euthanized humanely at 3, 7 and 10 days post-inoculation (DPI). All animal experiments were conducted in accordance with the guidelines of the Ethical and Animal Welfare Committee of the Harbin Veterinary Research Institute, Chinese Academy of Agricultural Sciences.

Following euthanization of the piglets at the designated DPI, BM cells were harvested from the femurs according to a method described elsewhere (Chang et al., 2008). Briefly, BM cells were collected by flushing the bone from both ends with 40 ml PBS containing 2 % FBS. The BM cells were then filtered through a 70 µm cell strainer, and erythrocytes were lysed with RBC lysis buffer (ACK lysis buffer containing 1 % NH\(_4\)Cl; Biolegend) for 30 min at 4 °C, followed by three washes with PBS at 250 g for 10 min at 4 °C. The collected cell samples were processed for subsequent virus detection and apoptosis analysis.

Viral load in BM cells was quantified by TaqMan fluorescent quantitative reverse transcription PCR (RT-PCR) using our previously described method (Liu et al., 2010). Briefly, \(1 \times 10^7\) BM cells (erythrocytes lysed) from each sample were resuspended in 200 µl PBS, and total RNA was extracted using the TRIzol total RNA extraction kit (Sangon Biotech Shanghai) according to the manufacturer’s instructions. Reverse transcription was performed using 10.5 µl total RNA as the template, 4 µl 5× reverse transcription buffer, 2 µl dNTP mixture (10 mmol l\(^{-1}\)), 1 µl 9-mer random primer (50 pmol l\(^{-1}\)), 2 µl avian myeloblastosis virus (5 U µl\(^{-1}\)) and 0.5 µl RNase inhibitor (40 U µl\(^{-1}\)). The reagents were mixed gently, placed in a water bath at 42 °C for 1 h, and incubated on ice for 2 min prior to analysis by fluorescence quantitative PCR. Two pairs of primers were used for the quantitative PCR analysis. The first pair, PNF (5¢-AAAACCAGTCCAGGGCAAG-3¢) and PNR (5¢-CGGATCACGCACGAT ATG-3¢), was designed for the

**Fig. 1.** Detection of HP-PRRSV and identification of virus-infected cells in the BM of HuN4-infected piglets. Identification of viral expression was carried out by IHC and confocal microscopy. (a, b) IHC analysis of viral expression in BM cells of one piglet from the HuN4-infected group at 10 DPI (a) and BM from one negative control piglet at 10 DPI (b) (magnification \( \times 400 \)). (c, d) Co-localization of PRRSV-infected cells and the relevant cell subpopulation in HuN4-infected piglets (c) and one negative control piglet (d); triple labelling of PRRSV-infected cells showed the localization of PRRSV N protein (green) and the relevant cell subpopulations by the SWC3 (orange) and SWC8 (red) markers, DAPI-stained nuclei appeared blue.
amplification of a conserved region of the PRRSV ORF 7 sequence (250 bp) for a standard curve. The second pair, designated PNPF (5'-CCCTAGTGGAGGCGAATTTG-3') and PNPR (5'-TCCAGGCCCTGATTG AA-3'), was designed for the amplification of the truncated region of ORF 7 (60 bp) for detection of genomic copies. The NP probe (FAM-TCTGTCGATTCAGA-MGB) was labelled at the 5' end with 6-carboxylfluorescein (FAM) dye as a reporter. The PCR system (25 µl) comprised 2 µl cDNA template, 1 µl (20 pmol l⁻¹) specific primers, 1 µl (10 mM) dNTPs, 2.5 U Taq DNA polymerase and 5 µl 5× PCR buffer (all from TaKaRa). The final volume was obtained by the addition of RNase-free water. The PCR conditions were as follows: pre-denaturing at 95°C for 15 min; and 45 cycles of denaturing at 94°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 45 s. In the HuN4 group, the genomic copies in BM cells were 10⁶.5 copies per 10⁶ cells at 3 DPI and peaked with 10⁶.5 copies per 10⁶ cells at 7 DPI. The peak genomic copies persisted until 10 DPI with 10⁷.1 copies per 10⁶ cells. No virus was detected in the control group.

Since viral RNA was detectable in BM, the viral nuclear capsid protein antigen was detected by immunohistochemistry (IHC) analysis to further confirm the presence of the virus in BM from piglets in the HuN4 group. BM samples were collected from femoral bones during necropsy. The samples were fixed in 10% neutral buffered formalin, embedded in paraffin and cut into 4 µm sections. The PRRSV N protein was detected using the mAb SR30A (RTI) for the specific detection of the PRRSV viral capsid antigen. The reverse transcription PCR (RT-PCR) was performed using oligonucleotides designed for the amplification of the truncated region of ORF 7 (1036 bp). The primers are 5'-CTGTCGATCCAGA-3' and 5'-CCCTAGTGGAGGCGAATTTG-3'. The PCR conditions were as follows: pre-denaturing at 95°C for 15 min; and 45 cycles of denaturing at 94°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 45 s. The final volume was obtained by the addition of RNase-free water. The PCR conditions were as follows: pre-denaturing at 95°C for 15 min; and 45 cycles of denaturing at 94°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 45 s. The HuN4 group, the genomic copies in BM cells were 10⁶.5 copies per 10⁶ cells at 3 DPI and peaked with 10⁶.5 copies per 10⁶ cells at 7 DPI. The peak genomic copies persisted until 10 DPI with 10⁷.1 copies per 10⁶ cells. No virus was detected in the control group.

The cells were placed on glass slides and incubated with the primary mouse anti-porcine SWC8 antibody overnight. After washing the sections three times with TBS for 5 min, the APC-goat anti-mouse antibody was added. The slides were incubated in the dark at room temperature for 1 h and then washed with TBS three times for 5 min. Thereafter, the slides were incubated with SR30F anti-PRRSV N protein and PE-anti-porcine SWC3 in the dark at room temperature for 1 h. After washing, the slides were stained with DAPI (Sigma) for 2 min and then washed again as described above. BM cells targeted by HP-PRRSV were identified by confocal microscopy. The localization of the PRRSV N protein (green) and the relevant cell subpopulations expressing the SWC3 (orange) and SWC8 (red) markers were determined by triple labelling of PRRSV-infected cells. The yellow areas of co-localized orange and green signals showed that the SWC3-SWC8 monocytic lineage cells were infected by HP-PRRSV (Fig. 1c); no virus-positive cells were observed in the control group (Fig. 1d).

Apoptosis and necrosis of BM cells were evaluated by flow cytometric analysis as described previously (Wang et al., 2015) using the Annexin V: FITC apoptosis detection kit I (BD Biosciences). Briefly, cell samples (1 × 10⁶ BM cells) were washed twice with cold PBS and then re-suspended in 1× binding buffer at a concentration of 1 × 10⁶ cells ml⁻¹. One hundred microlitres of the solution (1 × 10⁶ cells) was transferred to a 5 ml culture tube, and then 5 µl FITC Annexin V and 5 µl propidium iodide were added. After vortexing gently, the samples were incubated for 15 min at room temperature (25°C) in the dark. The samples were analyzed by flow cytometry after adding 400 µl of 1× binding buffer. Statistical analyses were performed using GraphPad PRISM software (version 5.02 for Windows; GraphPad Software) for ANOVA (Student’s t-test). The results showed that a higher level of early and total apoptosis appeared in the BM cells of the HuN4 group from 3 DPI and remained high until 10 DPI (Fig. 2a, c), while significantly higher

![Fig. 2](image-url)  
**Fig. 2.** Kinetics of apoptosis development in BM cells at different DPI. Percentages of early (a), late (b) and total (c) apoptotic cells in BM cells (±SD) (*P < 0.05).
levels of late apoptosis were observed in the BM cells of the HuN4 group at 10 DPI (Fig. 2b).

To detect the subpopulations of apoptotic cells and infected cells, the following antibodies were used: SR30F (1:100; RTI) anti-PRRSV N protein and TMR red–cell death. The cells were placed on glass slides, incubated with SR30F in the dark at room temperature for 1 h and washed three times with TBS. Detection of apoptotic cells was performed by the TUNEL technique using an In Situ Cell Death detection kit (Roche) according to the manufacturer’s instructions (Rodriguez-Ropon et al., 2003). After incubation, the cells were washed three times with TBS, and cell nuclei were then stained with DAPI (Sigma). The PRRSV+ cells (green) and TUNEL+ cells (red) were observed under a laser scanning confocal microscope. Most of the PRRSV+ BM cells co-localized with the TUNEL+ cells at 3 DPI, indicating that most of the virus-infected BM cells underwent apoptosis at 3 DPI (Fig. 3a). However, in addition to the virus-positive cells, a large number of apoptotic cells were virus-negative at 7 DPI (Fig. 3b). Only a few apoptotic cells and no virus-positive BM cells were observed in the control piglets (Fig. 3c).

In this study, we demonstrated the presence of both viral RNA and viral proteins in the BM of HuN4-infected piglets (Fig. 1). Furthermore, the virus in the BM appeared to have induced apoptosis in a large number of cells at this site (Figs 2 and 3). This study is, to the best of our knowledge, the first to show HP-PRRSV infection in the BM of piglets and confirmed it as a novel site affected by this highly pathogenic virus. The HP-PRRSV HuN4 strain was thought to target a wide range of cells and tissues following detection of a high viral load (Garcia-Nicolas et al., 2014; Li et al., 2012; Zhou et al., 2009). In this study, the HuN4 strain showed BM cell tropism to BM cells of piglets. These results differed from those of previous reports that did not detect the virus in the BM compartment after inoculation of piglets with the Lelystad PRRSV isolate, ATCC VR-2332 or other classical strains (Feng et al., 2001; Rossow et al., 1994; Xiao et al., 2004). The presence of HP-PRRSV in the BM of infected piglets indicated a new characterization of the virus infection, which may be related to the virulence of this highly pathogenic virus.

Apoptosis plays an essential role in the development and maintenance of homeostasis in multicellular organisms. PRRSV has been shown to induce apoptosis in lung lavage cells and lung tissue, as well as lymphoid tissues (thymus and secondary lymph nodes) in vivo. Furthermore, PRRSV was shown to induce apoptosis in Marc-145 cells in vitro, with the majority of apoptotic cells consisting of bystander cells (Feng et al., 2002; He et al., 2012; Labarque et al., 2003; Sur et al., 1998). In our study, HuN4 also induced apoptosis in BM cells, including infected cells and a large number of bystander cells (Fig. 3). The BM hematopoietic system is crucial for the regulation of cellular homeostasis in the peripheral blood (Alvarez et al., 2000; Sinkora et al., 2002; Summerfield & McCullough, 1997) and is the major source of leukocytes for the immune system throughout post-natal

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**Fig. 3.** Co-localization of PRRSV-infected cells and apoptotic cells. (a, b) Co-localization of PRRSV-infected cells and apoptotic cells was detected in HuN4-infected piglets at 3 DPI (a) and 7 DPI (b). (c) BM cells from one piglet of the negative control group at 7 DPI. PRRSV was stained with SR30F mAb against PRRSV N protein. Apoptotic cells were detected using an In Situ Cell Death detection kit (red), and the nuclei were stained with DAPI (blue).
life (Summerfield & McCullough, 1997; Terada et al., 2002). It is also particularly important for short-lived granulocytes, as well as lymphoid precursor cells. In our study, the apoptotic cells in BM may reduce premature migration of lymphocyte precursors out of the BM as a result of the initiation of inflammation, which transiently facilitates the death of extra-medullary cells in the thymus and lymph nodes. The deletion of a large proportion of the BM cells would reduce the number of lymphocytes emigrating to the peripheral blood, thereby impacting the immune status of virus-infected piglets during the early stage of infection.

In conclusion, the research presented here provides what is believed to be the first identification of the BM as a novel tissue site targeted by HP-PRRSV infection. The depletion of BM cells may be a major factor leading to immunosuppression during virus infection. These findings contribute to our understanding of the effect of HP-PRRSV on host immunity and provide evidence to guide further studies on the mechanism of viral pathogenesis.

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