Virus Research 265 (2019) 20–29

Contents lists available at ScienceDirect

Virus Research

journal homepage: www.elsevier.com/locate/virusres

Porcine reproductive and respiratory syndrome virus infection of bone marrow: Lesions and pathogenesis

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ARTICLE INFO

Keywords:
PRRSV
Pathogenesis
Bone marrow
Apoptosis
Immunity

ABSTRACT

Red bone marrow is physiologically unique in that it is both the major hematopoietic organ and a primary lymphoid organ. Porcine reproductive and respiratory syndrome virus (PRRSV) affects normal bone marrow functions. The cumulative effect of PRRSV infection is acute bone marrow failure, i.e., hypoplasia characterized by the absence of normal myeloid and erythroid precursors and increased red bone marrow M:E ratios. The measurable clinical consequence of PRRSV infection on normal red bone marrow functions is a reduction in the number of cells emigrating to the peripheral blood resulting in leucopenia, anemia, and thrombocytopenia. These observations may be explained by the fact that bone marrow-derived mononuclear cells, i.e., imDCs, mDCs, monocytes, macrophages, and myeloid precursor cells are susceptible to PRRSV. Apoptosis in bone marrow-derived cells occurs both as a direct consequence of infection and indirectly via a bystander effect. Immunologically, PRRSV-susceptible mononuclear cells are the first line of defense against microbial infection and responsible for antigen recognition, processing, and presentation to T and B cells; a critical step in the initiation and development of an effective adaptive immune. Thus, impairment of normal immune function renders the host less able to resist and/or eliminate secondary infectious agents and partially explains the synergy between PRRSV and bacterial and viral co-infections.

1. Introduction

Porcine reproductive and respiratory syndrome viruses (PRRSV) are small, enveloped, positive single-stranded RNA viruses in the genus *Porartevirus*, family *Arteriviridae*, order *Nidovirales* (Adams et al., 2017). Previous genetic analyses established the existence of PRRSV as two distinct genotypes, PRRSV type 1 (European genotype, type strain Lelystad) and PRRSV type 2 (North American genotype, strain VR-2332) and showed that they shared ˜60% nucleotide identity at the genomic level (Nelsen et al., 1999). More recently, the International Committee on Taxonomy of Viruses has re-classified the two genotypes as two distinct virus species, i.e., PRRSV-1and PRRSV-2 (https://talk.ictvonline.org/taxonomy; 2016) (Adams et al., 2017), on the basis of pairwise sequence comparison (PASC) and the judgment that an appropriate species cut-off would be 71–77% identity (Kuhn et al., 2016). Although no PRRSV-2 subtypes have been identified, four subtypes of PRRSV-1 are recognized, i.e., Pan-European subtype 1 and Eastern European subtypes 2, 3, and 4 (Stadejek et al., 2017). Both PRRSV species are subject to frequent mutation and viral recombination events. Likewise, as a result of the infidelity of the viral RNA-dependent RNA polymerase, both species are considered to exist as quasispecies, i.e., viral populations comprised of close-related genetic variants (Domingo and Holland, 1997).

Since the earliest reports of the virus, it has been recognized that clinical signs produced by PRRSV infection are highly variable, ranging from inapparent to severe (Anon, 1991). Thus, PRRSV does not produce pathognomonic clinical signs; rather, clinical signs reflect viral virulence, the age and immune status of pigs, and the presence of concurrent infections (Galina et al., 1994; Zhou and Yang, 2010; Zimmerman et al., 1997, 2012). Overall, the clinical signs most commonly associated with PRRSV infection include anorexia, fever, lethargy, and respiratory disease. In breeding herds, clinical signs may include infertility, abortion, premature farrowing, increased numbers of stillborn, weak, and/or nonviable pigs, and acute diarrhea in neonatal

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https://doi.org/10.1016/j.virusres.2019.02.019
Received 11 February 2019; Received in revised form 26 February 2019; Accepted 28 February 2019
Available online 01 March 2019

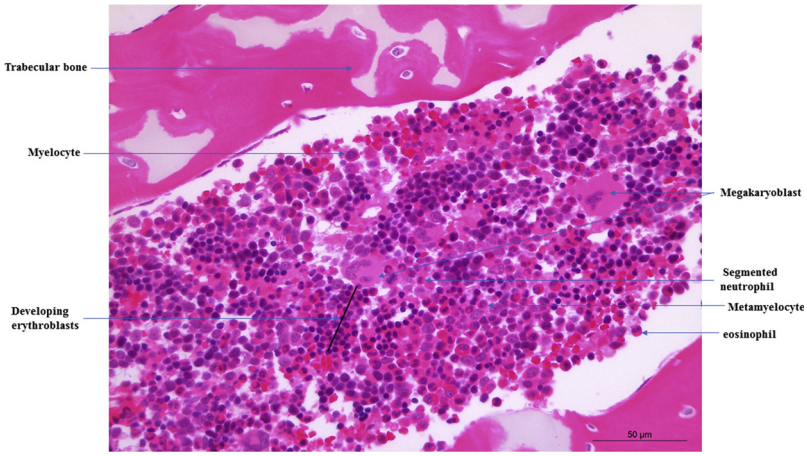

Fig. 1. Hisotological section of bone marrow from a neonatal Landrace × Yorkshire × crossbred pig. Photomicrograph provided by Dr. Xijun He.

piglets (Zimmerman et al., 2012).

Changes in hematological parameters often associated with PRRSV infection include significant reductions in blood leukocyte, erythrocyte, and platelet counts (Halbur et al., 2002; Stukelj et al., 2013). Christianson et al. (1993) observed leukopenia in PRRSV-inoculated sows early in the course of the infection, with temporal reductions in specific B lymphocyte and monocyte populations. Other investigators have made similar observations for PRRSV-infected pigs of various ages (Ladinig et al., 2014; Nielsen and Botner, 1997; Nielsen et al., 2003; Shi et al., 2008; Shimizu et al., 1996; Stukelj et al., 2013). In a study of field-infected 28-, 120- and 160-day-old pigs, Stukelj et al. (2013) reported thrombocytopenia in all infected age groups, albeit erythrocyte, hematocrit and hemoglobin values were within normal ranges (Stukelj et al., 2013). On the other hand, in a study including four PRRSV-2 isolates (VR-2385, ISU-984, ISU-22, and VR-2431), Halbur et al. (2002) found that infection led to reductions in hemoglobin, hematocrit, and platelet values. Non-regenerative anemia was observed in 5-week-old pigs, with statistically significant differences in the degree of anemia among the four virus isolates. Further analyses showed that the virus strain that produced more severe clinical signs and more extensive lung lesions also induced a more severe anemia and a higher myeloid:erythroid (M:E) ratio in red bone marrow.

PRRSV infection induces a cell-mediated immune response characterized by changes in T cell subpopulations, i.e., a decrease in $CD4^{pos}$ T cells and an increase in $CD8^{pos}$ and CD4/CD8 double-positive T cells, from 3 to 14 days post infection (DPI) (Nielsen and Botner, 1997; Feng et al., 2002; He et al., 2012; Shimizu et al., 1996; Wang et al., 2016b). Likewise, the virus dysregulates innate immune responses and cytokine expression, e.g., downregulates type I IFN, IL-1β, IL-6, and TNF-α expression (Amarilla et al., 2015; Calzada-Nova et al., 2011; Han and Yoo, 2014; Wang et al., 2007). PRRSV antibody can be detected from 7 to 14 DPI by various antibody-binding assays (Pejsak et al., 2015). Neutralizing antibodies are generally not detectable until 3 to 4 weeks post infection, do not reach high titers, and typically possess a limited ability to cross-neutralize non-homologous viruses (Molina et al., 2008; Martinez-Lobo et al., 2011; Robinson et al., 2015, 2018). Indeed, the role of the humoral immune response, and the precise contribution of neutralizing antibodies in particular, to the elimination of the infection from the host remains unresolved (Loving et al., 2015).

The mammalian immune system is functionally compartmentalized into primary lymphoid organs (bone marrow and thymus) and secondary lymphoid tissues (lymph nodes, spleen, tonsils, and mucosa- and skin-associated lymphoid tissues) (Mak et al., 2014). In general, primary lymphoid organs develop before secondary organs in the fetus and are the nidus for antigen-independent B and T lymphocytes differentiation from stem cells. For their part, secondary lymphoid tissues are the location for antigen-specific lymphocyte proliferation and differentiation, as well as the environment in which lymphocytes respond to pathogens and foreign antigens (Osmond, 1985). Halbur et al. (2002) postulated that the anemia observed in PRRSV-infected pigs was a direct or indirect effect of the virus on erythroid precursor cells in the red bone marrow. Likewise, PRRSV is known to induce apoptosis in immune cells located in bone marrow and thymus tissues, and cell apoptosis happened in both virus- infected and bystander cells (Amarilla et al., 2016, 2017; Feng et al., 2002; He et al., 2012; Novakovic et al., 2017; Wang et al., 2016a,2016b). Thus, PRRSV affects the primary organs involved in normal hematological and immune function, i.e., bone marrow and thymus. Herein we review the current information on the pathogenesis of this process.

2. Bone marrow

Bone marrow is found in the central cavities of axial and long bones and is of two types: red bone marrow (also known as myeloid tissue) and yellow bone marrow (Tavassoli and Yoffey, 1983). Red bone marrow is primarily found in the flat bones (hip bone, breast bone, skull, ribs, vertebrae, and shoulder blades) and in the cancellous material at the proximal ends of the long bones (femur and humerus), whereas yellow marrow is found in the middle portion of long bones (Tavassoli and Yoffey, 1983; Travlos, 2006). In newborn mammals, the marrow cavities of all bones contain functionally active red bone marrow, with yellow bone marrow appearing from the early postnatal period onwards (Gurevitch et al., 2007; Waitches et al., 1994).

Red bone marrow is unique in that it is both the major hematopoietic organ and a primary lymphoid organ. Histologically, red marrow consists of a hematopoietic component and a vascular component (Fig. 1, Kopp et al., 2005; Zhao et al., 2012). The hematopoietic component, located close to the endosteum and around blood vessels, produces myeloid stem cells and lymphoid stem cells. The vascular component consists of non-hematopoietic progenitor cells with the potential to differentiate into various tissues of mesenchymal origin (Kopp et al., 2005; Zhao et al., 2012).

Red bone marrow plays a major role in hematopoiesis and immunity, particularly humoral immunity (Zhao et al., 2012). During hematopoiesis, red bone marrow-derived stem cells differentiate into mature cells or precursors of cells that migrate out of the bone marrow and mature elsewhere, i.e., T cell precursors translocate to the thymus to develop and mature into fully functional T cells, or are induced to die via apoptosis (Pearse, 2006; Wilkins, 1992; Zhao et al., 2012). In addition to erythrocytes and platelets, all cells of the immune system are initially derived from red bone marrow, including natural killer cells, granulocytes, immature thymocytes, B cells, and antigen-specific, antibody producing, long-lived plasma cells (Travlos, 2006). In contrast, yellow bone marrow's primary function is to store fats. In locations

where yellow bone marrow is mixed with red bone marrow, it may be converted to red marrow under specific circumstances, e.g. acute anemia (Bigelow and Tavassoli, 1984; Maniatis et al., 1971; Tavassoli et al., 1974).

The temporal development of fetal bone marrow varies among species, but the basic skeletal structure of the fetal pig appears at ~18 days of gestation (DG) and ossification of bones by ~28 DG. Fetal pigs do not have yellow bone marrow, but red bone marrow begins detectable hematopoietic activity ~45 DG (Sinkora and Butler, 2009; Sinkora et al., 2003; Tlaskalova-Hogenova et al., 1994; Trebichavsky et al., 1996). The cellular components of innate immunity, e.g. polymorphonuclear leukocytes, macrophages, and dendritic cells appear concurrently with hematopoiesis (Sinkora and Butler, 2009; Sinkora et al., 2002), with the production of bone marrow-drived B lineage cells predominant after ~55 DG (Sinkora et al., 2002).

3. Effect of PRRSV infection on red bone marrow

The presence of the PRRSV-1 and PRRSV-2 in bone marrow has been clearly established. Wang et al. (2016a) detected PRRSV-2 in femoral bone marrow from ~5-week-old piglets inoculated with a highly pathogenic isolate (HuN4) using TaqMan™ fluorescent quantitative reverse transcription PCR (RT-PCR), immunohistochemistry (IHC), and an immunofluorescence assay (IFA) based on monoclonal antibody (mAb) SR30 F against the N protein. PRRS viral RNA and N protein were detected in the bone marrow of HuN4-infected piglets from 3 to 10 DPI. Amarilla et al. (2017) detected PRRSV-1 in femoral bone marrow from 5-week-old piglets inoculated with one of 3 PRRSV-1 isolates (LV, 215-06, and SU1-bel) using quantitative RT-PCR (RT-qPCR). PRRSV RNA was detected in the bone marrow of all LV-infected pigs (n = 14) through 35 DPI.

Both PRRSV-1 and PRRSV-2 isolates can produce lesions in red bone marrow. Feng et al. (2001) reported bone marrow hypoplasia characterized by the absence of normal myeloid and erythroid precursors in 12-day-old piglets infected *in utero* with PRRSV-2 (Fig. 2). Halbur et al. (2002) observed increased red bone marrow M:E ratios relative to uninoculated control animals between 3 and up to 21 DPI in 5-week-old pigs inoculated with PRRSV-2 and, likewise, reported that PRRSV-2 induced non-regenerative anemia in 5-week-old pigs, with differences in the degree of anemia among the four virus isolates evaluated in the experiment (VR-2385, ISU-984, ISU-22, and VR-2431). They observed that the most highly pneumovirulent isolates (VR2385, ISU-984 and ISU-22) induced more severe anemia than the least virulent isolate (VR2431). Amarilla et al. (2017) reported that PRRSV-1 strains also induced moderate, sustained hypoplasia of erythroid cells and increased bone marrow M:E ratios in 5-week-old pigs infected with three different virulent PRRSV-1 isolate (LV, 215-06, and SU1-bel). The percentage of affected hematopoietic tissue and stroma differed significantly among isolates at specific time-points, but this effect was independent of viral load and virulence (Fig. 3).

4. PRRSV-susceptible bone marrow-derived cells

Initially, Xiao et al (2004) recovered mononuclear cells from the femoral bone marrow of pigs inoculated with PRRSV-2 using lymphocyte separation medium. Among macrophages adherent to glass coverslips, ~5% stained positively for PRRSV, i.e., reacted with FITC-conjugated anti-N protein mAb SDOW17; however, marrow cell samples were negative for PRRSV nucleic acid by RT-PCR. To explain these results, the authors postulated that the anti-N mAb had reacted with residual viral protein in cells that had either survived PRRSV infection, but had successfully degraded viral RNA, or cells that had phagocytosed viral proteins from dead cells. Building on these original observations, *in vitro* culture of bone marrow-derived immature dendritic cells (imDCs), mature dendritic cells (mDCs), monocytes, and macrophages allowed for detailed studies of their response to PRRSV and achieved clarity regarding the question of their susceptibility.

4.1. Dendritic cells

Immature dendritic cells may be recovered by culturing bone marrow hematopoietic cells in medium supplemented with granulocyte-macrophage colony-stimulating factor either alone or in combination with interleukin-4. Cells can be used as imDCs or they can be stimulated to become mDCs by exposure to medium supplemented with lipopolysaccharide for 24–48 h (Carrasco et al., 2001; Chang et al.,

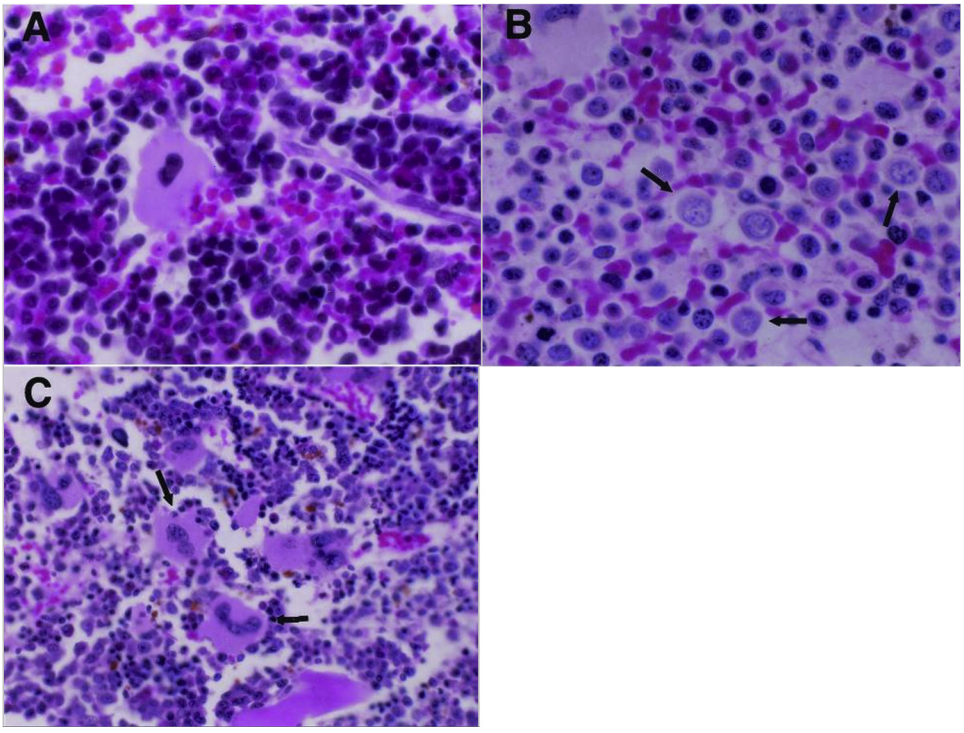

Fig. 2. Effect of PRRSV-2 on piglet bone marrow (hematoxylin and eosin stain). Bone marrow from a 12-day-old negative control piglet showing normal bone marrow histology. Magnification, ×600; (B) Bone marrow from a 12-day-old PRRSV-2-infected piglet showing reduced cellularity and increased number of mononuclear cells (arrows). Magnification, ×600; (C) Bone marrow from a 12-day-old PRRSV-infected piglet showing increased numbers of megakaryocytes (arrows). Magnification, ×396 (C).
(From Feng et al., 2001 with the permission of the publisher - 4534390246323)

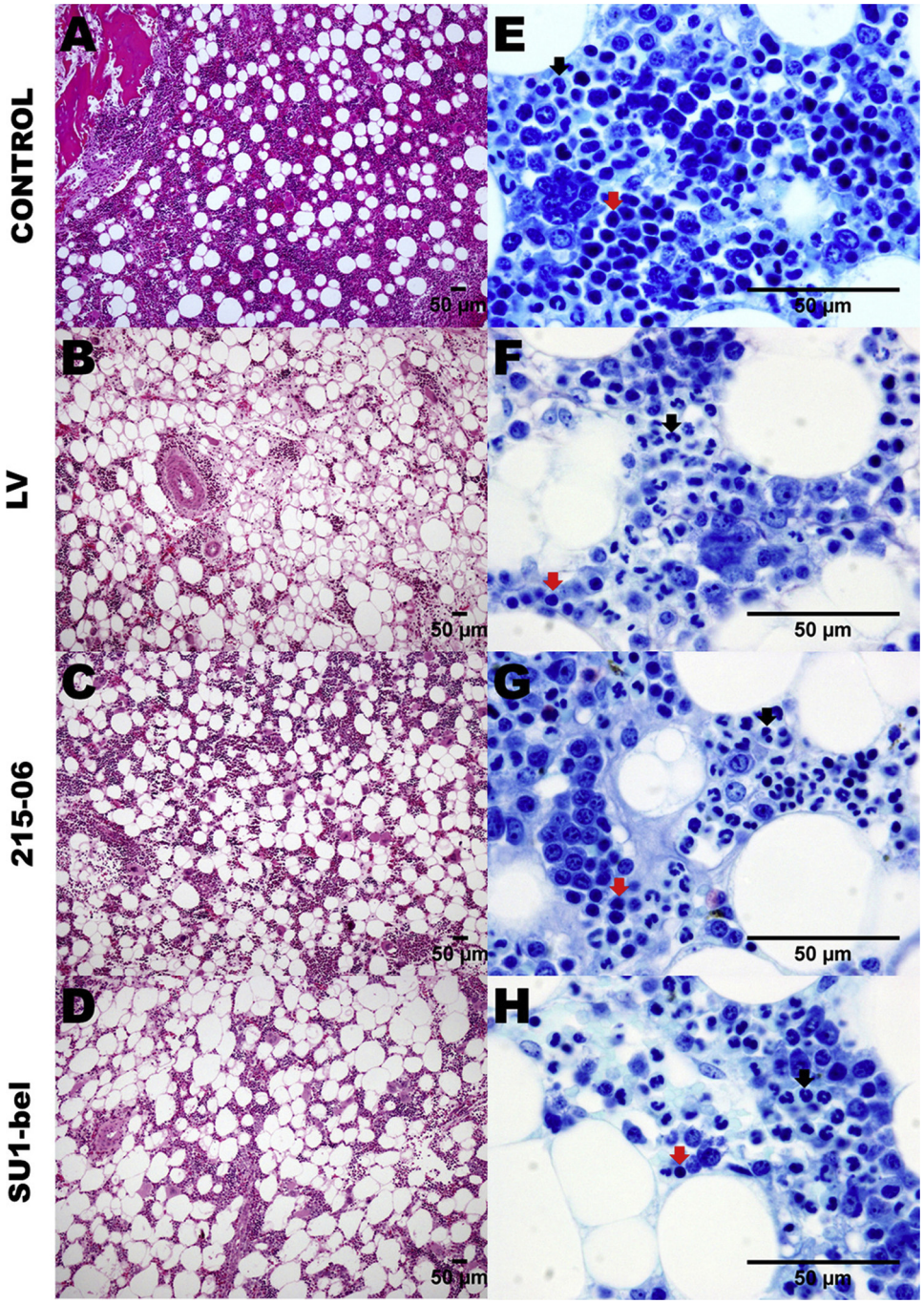

Fig. 3. Effect of PRRSV-1 on piglet bone marrow. (A–D) Hematoxylin and eosin stained bone marrow sections illustrating hypoplasia of erythroid cells and increased M:E ratio in piglets infected with PRRSV-1 isolates Lelystad (LV), moderately virulent 215-06, and highly virulent SU1-bel at 3 days post inoculation versus controls. (E–H) Giemsa stained bone marrow sections with erythroid and myeloid cells indicated by small red arrow or black arrows, respectively.
(From Amarilla et al., 2017 with the permission of the publisher - 4534381287060)

2008; Li et al., 2018).

Chang et al. (2008) compared the pattern of PRRSV replication in humeral and femoral imDCs versus pulmonary alveolar macrophages (PAMs) using a Taiwanese PRRSV-2 field isolate (HF6-2). Viral replication was confirmed and quantified by flow cytometry using anti-N protein mAb SDOW17 and R-phycoerythrin (PE)-conjugated anti-mouse IgG1 secondary antibody to identify PRRSV-infected cells. In cells exposed to PRRSV at a multiplicity of infection (MOI) of 0.1, N protein was detected as early as 6 h post infection (HPI) in both cell types, with peak infection at 36 HPI (88.7%) and 48 HPI (59.3%) in PAMs and imDCs, respectively. Similarly, virus titers in cell supernatants increased from $1 \times 10^{4.27}$ and $1 \times 10^{2.57}$ $TCID_{50}$ at 6 HPI to $1 \times 10^{6.1}$ and $1 \times 10^{7.3}$ $TCID_{50}$ per ml at 24 and 36 HPI for imDCs and PAMs, respectively.

Li et al. (2018) compared PRRSV-1 replication in imDCs, mDCs, and PAMs recovered from the same pigs. Following exposure to PRRSV at a MOI of 0.1, viral titers of isolates 3249, 3262, and 3267 were significantly higher in imDCs at 12 HPI ($1 \times 10^{5.7}$, $1 \times 10^{4.9}$, and $1 \times 10^{5.8}$ $TCID_{50}$/ml) and 24 HPI ($1 \times 10^{6.7}$, $1 \times 10^{6.1}$, and $1 \times 10^{6.8}$ $TCID_{50}$/ml) than in mDCs at 12 HPI ($1 \times 10^{4.3}$, $1 \times 10^{4.0}$, and $1 \times 10^{4.8}$ $TCID_{50}$/ml) and 24 HPI ($1 \times 10^{6.0}$, $1 \times 10^{5.3}$, and $1 \times 10^{6.3}$ $TCID_{50}$/ml) (Kruskal-Wallis test, $p < 0.05$). Maximum titers were observed in

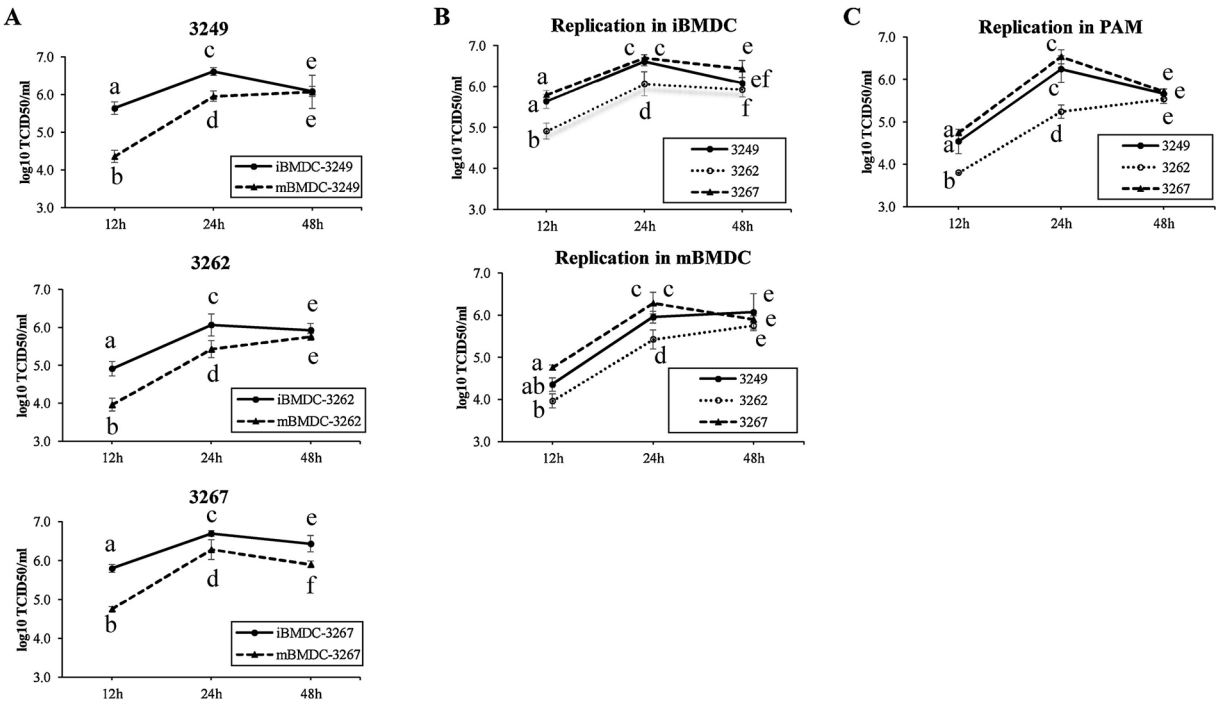

Fig. 4. Concentration of PRRSV-1 isolates 3249, 3262, and 3267 over time in supernatant from immature and mature bone marrow-derived dendritic cell (iBMDC, mBMDC) cultures as determined by titration on porcine alveolar macrophages (PAM). Error bars indicate standard deviations; different letters indicate significant differences in virus concentration ($p < 0.05$).
(From Li et al., 2018 with the permission of the publisher - 4534390779155).

imDCs at 24 HPI. In mDCs, isolate 3267 and isolates 3249 and 3262 reached peak titers at 24 and 48 HPI ($1 \times 10^{6.1}$ and $1 \times 10^{5.8}$ $TCID_{50}$/ml) in mDCs, respectively. In PAMs, viral titers of isolates 3249, 3262, and 3267 at 12 and 24 HPI were $1 \times 10^{4.6}$, $1 \times 10^{3.8}$, and $1 \times 10^{4.8}$ $TCID_{50}$/ml and $1 \times 10^{6.3}$, $1 \times 10^{5.2}$, and $1 \times 10^{6.5}$ $TCID_{50}$/ml, respectively, with peak titers observed at 24 HPI. Among the three isolates evaluated in the study, isolate 3262 replicated to the lowest titers in imDCs, mDCs, and PAMs at both at 12 and 24 HPI (Kruskal-Wallis test, $p < 0.05$) (Fig. 4).

Cumulatively, the results reported by Chang et al. (2008) and Li et al. (2018) demonstrated that imDCs and mDCs were permissive to both PRRSV-1 and -2. Further, although the data is sparse, the $TCID_{50}$ estimates suggested that PRRSV replicated more efficiently in imDCs and PAMs than mDCs.

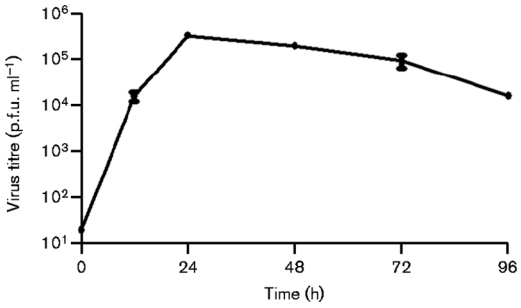

Fig. 5. Concentration of PRRSV-2 isolate NVSL 97–7895 over time in bone marrow-derived macrophage culture as determined by titration on MARC-145 cells. Error bars account for standard deviation.
(From Chaudhuri et al., 2016 with the permission of the publisher - 4534400475259).

4.2. Macrophages

Macrophages may be recovered from bone marrow by culturing bone marrow hematopoietic cells in medium supplemented with macrophage colony stimulating factor (M-CSF) for 6 to 10 days to stimulate the differentiation of porcine monocytic precursors to macrophages. Chaudhuri et al. (2016) reported that glass-adherent cell populations expressed the CD45 leukocyte marker and had cytoplasmic and nuclear morphologies characteristic of cultured macrophages.

Chaudhuri et al. (2016) analyzed the pattern of PRRSV replication in porcine mid-thoracic costal rib macrophages using PRRSV-2 (NVSL 97-7895). Viral replication was quantified by plaque assay titration of supernatants on MARC145 cells. In cells exposed to PRRSV at a MOI of 0.01, maximum virus concentration was observed at 24 HPI (1×10^5 plaque-forming units per ml) at 24 HPI, with similar titers observed for 72–96 h thereafter (Fig. 5).

4.3. $CD163^{neg}$ and $CD163^{pos}$ monocytes

$CD163^{neg}$ and $CD163^{pos}$ monocytes can be recovered from bone marrow hematopoietic cells using cell sorters. For example, Fernandez-Caballero et al. (2018b) described a protocol for the concentration of cells by centrifugation over a discontinuous 53% Percoll gradient; staining with anti-CD172a mAb, anti-CD163 mAb, R-phycoerythrin (PE)-conjugated anti-mouse IgG2b, and allophycocyanin (APC)-conjugated goat anti-mouse IgG1 antibodies; and simultaneous sorting of SSC^{low}, $CD172a^{high}$, $CD163^{neg}$, and $CD163^{pos}$ cells using a cell sorter (FACSAria™ III, BDbiosciences, San Jose, CA). Mature $CD163^{neg}$ and $CD163^{pos}$ monocytes were generated by culturing $CD163^{neg}$ and $CD163^{pos}$ sorted monocytes in medium supplemented with M-CSF for 3 days (Fernandez-Caballero et al., 2018a,b).

Fernandez-Caballero et al. (2018a) compared the pattern of PRRSV-1 replication in humeral, tibial, and femoral bone marrow $CD163^{pos}$ monocytes, $CD163^{neg}$ monocytes, and PAMs from the same pigs using PRRSV-1 isolate 5710. Cells were exposed to PRRSV at a MOI of 0.01 and viral replication confirmed by PRRSV RT-qPCR. Cycle threshold (Ct) values were re-expressed as $TCID_{50}$/ml using a standard curve created from ten-fold serial dilutions of a known concentration

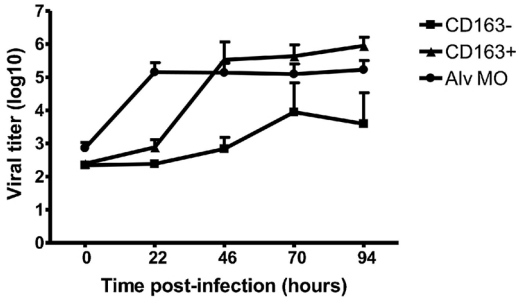

Fig. 6. Concentration of PRRSV-1 isolate 5710 over time in bone marrow $CD163^{neg}$ monocytes, $CD163^{pos}$ monocytes, and alveolar macrophages as determined by PRRSV RT-qPCR. Significant differences in virus concentration were observed between alveolar macrophages and $CD163^{neg}$ or $CD163^{pos}$ bone marrow monocytes at 22 h ($p = 0.0079$ and $p = 0.0159$, respectively), and between alveolar macrophages and $CD163^{pos}$ monocytes or $CD163^{neg}$ monocytes at 46 h ($p = 0.0079$ and $p = 0.0159$, respectively).
(From Fernandez-Caballero et al., 2018a with the permission of the publisher - 4534400878768).

($TCID_{50}$/ml) of PRRSV. At 22 HPI, virus titers in PAMs ($1 \times 10^{5.1}$ $TCID_{50}$/ml) were significantly higher than titers in $CD163^{pos}$ monocytes ($1 \times 10^{3.0}$ $TCID_{50}$/ml) (Mann-Whitney rank test, $p = 0.0159$) and $CD163^{neg}$ monocytes ($1 \times 10^{2.4}$ $TCID_{50}$/ml) (Mann-Whitney rank test, $p = 0.0079$). At 46 HPI, no significant difference in virus concentration was detected between PAMs ($1 \times 10^{5.1}$ $TCID_{50}$/ml) and $CD163^{pos}$ monocytes, but virus concentrations in both cell types were significantly higher than in $CD163^{neg}$ monocytes (Mann-Whitney rank test, $p = 0.0079$, and 0.0159, respectively). Relative to PAMs and $CD163^{pos}$ monocytes, virus replication in $CD163^{-}$ monocytes was lower and slower, with peak virus replication ($1 \times 10^{3.94}$ $TCID_{50}$/ml) at 70 HPI (Fig. 6).

4.4. Myeloid cells

A standardized approach to differentiating among leukocytes using mAb reactivity to swine leukocyte differentiation antigens was established in three International Swine Cluster of Differentiation Workshops (Haverson et al., 2001; Lunney et al., 1994; Saalmuller et al., 1998). In cases where two or more mAbs were reactive, "swine workshop cluster" (SWC) numbers were assigned. SWC3 was established as a myeloid monocytic-specific antigen and the porcine homolog of CD172a (SIRPα), a signal regulatory membrane glycoprotein (Alvarez et al., 2000; Blecha et al., 1994). SWC8 was found to be expressed on granulocytes, B lymphocytes, epithelial cells, fibroblasts, and $CD8^{pos}$ T lymphocytes, although the functional identify of SWC8 has not yet been established (Dawson and Lunney, 2018; Haverson et al., 1998; Saalmuller et al., 1998). Using this classification system, bone marrow myeloid progenitors have been shown to be $SWC3^{low/neg}SWC8^{neg}$, monocytic cells are $SWC3^{high}SWC8^{neg}$, granulocytic lineage cells are $SWC3^{pos}SWC8^{pos}$, and B cells are $SWC3^{neg}SWC8^{pos}$ (Summerfield et al., 2003; Summerfield and McCullough, 1997).

Two experiments showed that PRRSV-susceptible cells in bone marrow are $SWC3^{pos}SWC8^{neg}$ reactors. Wang et al. (2016a) collected femoral bone marrow myeloid cells from 5-week-old pigs inoculated with PRRSV-2 isolate HuN4 10 days prior. In brief, cells were incubated with primary mouse anti-porcine SWC8 antibody overnight and then APC-goat anti-mouse antibody for one hour. Thereafter, cells were incubated with SR30 F anti-PRRSV N protein mAb and R-phycoerythrin (PE)-conjugated-anti-porcine SWC3 for one hour. Visualization of the reactions using confocal microscopy showed that PRRSV-infected cells were $SWC3^{pos}SWC8^{neg}$ reactors (Fig. 7). Zheng et al. (2018) recovered femoral bone marrow hematopoietic cells from 3-week-old piglets and inoculated them *in vitro* with a green fluorescing PRRSV isolate (HuN4-EGFP). Construction of HuN4-EGFP is fully described elsewhere (Liu et al., 2018; Tang et al., 2016). At 72 h post inoculation, cells were treated for SWC3 and SWC8 reactivity using a procedure similar to that described by Wang et al. (2016a) and then analyzed by flow cytometry. As shown in Fig. 8, PRRSV-infected cells included both $SWC3^{high}SWC8^{neg}$ and $SWC3^{low}SWC8^{neg}$ cells. That is, the results demonstrated that both myeloid precursor cells ($SWC3^{low}SWC8^{neg}$) and monocytic cells ($SWC3^{high}SWC8^{neg}$) are susceptible to PRRSV.

5. PRRSV-induced apoptosis in red bone marrow

Research on PRRSV-induced apoptosis relies on the detection of targets associated with specific stages of the process. For example, early stage apoptosis involves the translocation of phosphatidylserine to the outer plasma membrane (Fadok et al., 1992). This signal can be detected using assays that use annexin V to detect phosphatidylserine on the cell surface (van Engeland et al., 1998). DNA fragmentation, a key feature of late-stage apoptosis, can be detected using "terminal dUTP nick end-labeling" (TUNEL) (Collins et al., 1997). These procedures have been used both *in vitro* and *in vivo* to provide insight on PRRSV-2-induced apoptosis in bone marrow-derived cells, as described below.

Using an *in vitro* approach, Chang et al. (2008) used annexin V to detect phosphatidylserine on the cell surface, propidium iodide to determine cell viability, and flow cytometry to evaluate the reactions. Thus, early apoptotic cells were (annexin V^{pos}, propidium $iodide^{neg}$) and late apoptotic or necrotic cells were (annexin V^{pos}, propidium $iodide^{pos}$). With these procedures Chang et al. (2008) was able to determine that the proportion of apoptotic imDCs was significantly higher in cells exposed to PRRSV isolate HF6-2 (MOI of 0.1) than in unexposed cells at both 24 HPI (38.2% vs 16.9%, Student's *t*-test $p < 0.05$) and 48 HPI (52.6% vs 21.5%, Student's *t*-test $p < 0.05$). Thereafter, Chang et al. (2008) separated imDCs from PRRSV-exposed wells (48 HPI) into annexin V^{pos} and annexin V^{neg} fractions by magnetic sorting and evaluated cell PRRSV infection status using mAb SDOW17. In the annexin V^{pos} fraction, 47% of the cells were positive for PRRSV versus. The absence of PRRSV infection in 53% of apoptotic cells provided evidence that the virus induced apoptosis in bystander cells.

In vivo studies of bone marrow cells have also provided evidence of virus-induced apoptosis in pigs infected with PRRSV. Wang et al. (2016a) collected femoral bone marrow myeloid cells from 5-week-old pigs inoculated with PRRSV-2 isolate HuN4 and found that the proportion of early apoptotic cells (annexin V^{pos}, propidium $iodide^{neg}$) in the HuN4-infected group was significantly higher than the control group at 3 DPI (25.9% vs 19.3%, Student's *t*-test $p < 0.05$), 7 DPI (40.8% vs 16.7, Student's *t*-test $p < 0.05$), and 10 DPI (42.1% vs 11.6%, Student's *t*-test $p < 0.05$) respectively. The total proportion of apoptotic cells, i.e., all annexin V^{pos} reactors, in the HuN4-infected group was also significantly higher than the control group at 7 DPI (51.1% vs 29.0%, Student's *t*-test $p < 0.05$), and 10 DPI (61.3% vs 22.0%, Student's *t*-test $p < 0.05$), respectively. Furthermore, an evaluation of femoral bone marrow cells recovered on glass slides at 3 and 7 DPI found that most apoptotic ($TUNEL^{pos}$) cells colocalized with $PRRSV^{pos}$ cells at 3 DPI, but apoptosis was approximately equivalent among $PRRSV^{pos}$ cells and $PRRSV^{neg}$ cells at 7 DPI (Wang et al., 2016a).

Cumulatively, these data provided by Chang et al. (2008) and Wang et al. (2016a) support the conclusion that PRRSV can induce apoptosis in bone marrow-derived cells as a direct consequence of infection and indirectly via a bystander effect.

6. Implications and conclusions

Early investigators noted changes in hematological parameters associated with PRRSV infection, e.g., significant reductions in blood leukocyte, erythrocyte, and platelet counts. Christianson et al. (1993) observed leukopenia in PRRSV-inoculated sows early in the course of the infection, with temporal reductions in specific B lymphocyte and monocyte populations. Other investigators have made similar

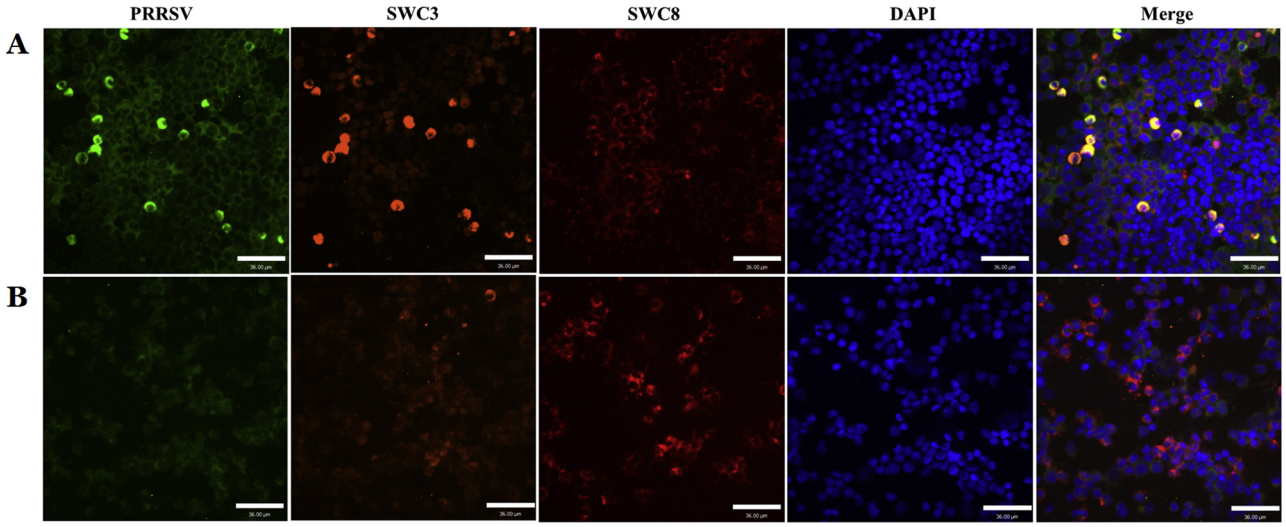

Fig. 7. Detection of virus-infected cells in bone marrow. Co-localization of PRRSV-infected cells and relevant cell subpopulations in (A) a PRRSV-2 (HuN4)-infected piglet and (B) negative control piglet. Triple labeling of PRRSV-infected cells shows co-localization of PRRSV N protein (green) and PRRSV-susceptible cell subpopulations, as indicated by SWC3 (orange) and SWC8 (red) markers. DAPI-stained nuclei appear blue.
(From Wang et al., 2016a with the permission of the publisher - 4534410107943).

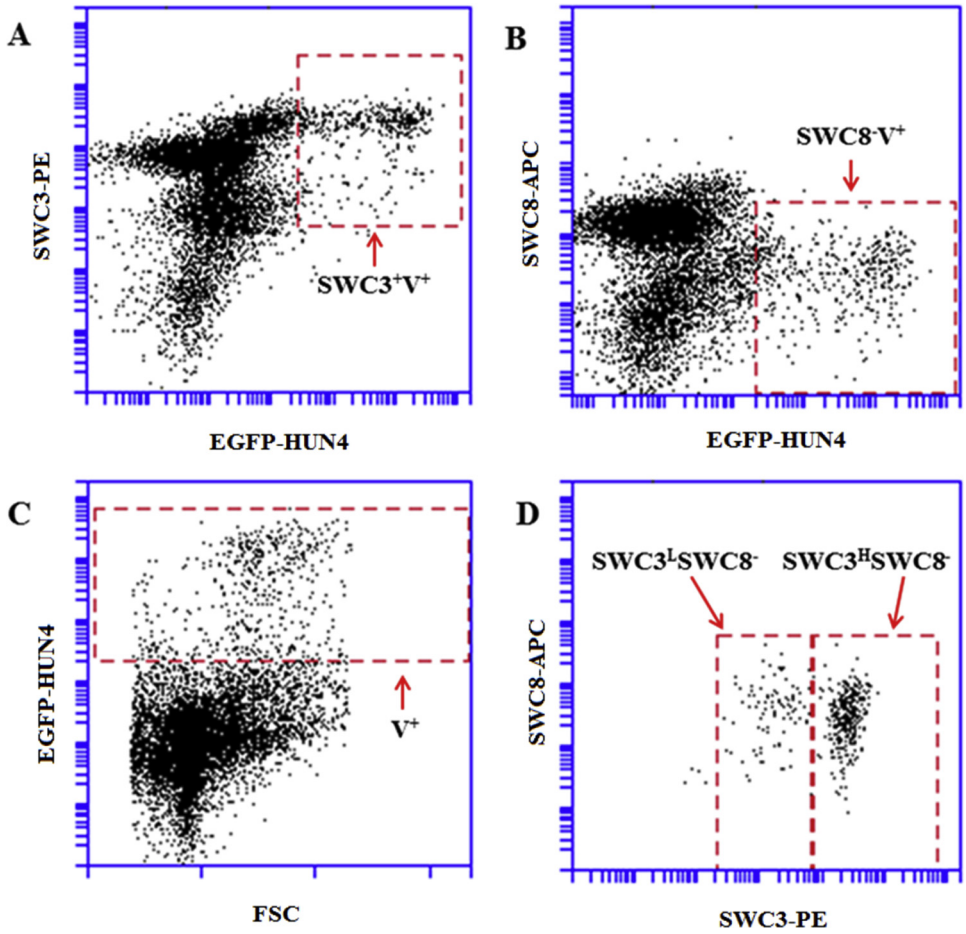

Fig. 8. Identification of bone marrow cell type infected with HP-PRRSV EGFP-HuN4. The cells expressed were (A) $SWC3^{pos}V^{pos}$, (B) $SWC8^{neg}V^{pos}$, (C) all virus-infected cells and (D) the myeloid precursors ($SWC3^{low}SWC8^{neg}$) and monocytes ($SWC3^{high}SWC8^{neg}$).
(From Zheng et al., 2018).

observations for PRRSV-infected pigs of various ages (Ladinig et al., 2014; Nielsen and Botner, 1997; Nielsen et al., 2003; Shi et al., 2008; Shimizu et al., 1996; Stukelj et al., 2013). As the primary site of erythrocyte, granulocyte, monocyte, lymphocyte and platelet production (Travlos, 2006), small changes in the functionality of red bone marrow may produce significant changes in the cellular constituents of peripheral blood and tissues. In the case of PRRSV, infection produces acute bone marrow failure, i.e., hypoplasia characterized by the

absence of normal myeloid and erythroid precursors and increased red bone marrow M:E ratios (Figs. 2, 3, 7 and 8; Amarilla et al., 2017; Feng et al., 2001; Halbur et al., 2002; Wang et al., 2016a; Zheng et al., 2018). Apoptosis results both as a direct consequence of cell infection and as a bystander effect (Chang et al., 2008; Wang et al., 2016a). Thus, the direct consequence of PRRSV infection on red bone marrow is a reduction in progenitor cells, which ultimately results in changes in both hematological and immunological parameters, including a decrease in the number of erythrocytes, and platelets and the number of total leukocytes and lymphocytes in peripheral blood (Christianson et al., 1993; Christopher-Hennings et al., 1997; Halbur et al., 2002; Nielsen and Botner, 1997; Stukelj et al., 2013).

Beginning with the earliest reports of "the new pig disease", an increase in morbidity and mortality due to secondary bacterial infections, e.g., *Pasteurella multocida*, *Haemophilus parasuis*, *Streptococcus suis*, and others, was noted in herds undergoing PRRSV outbreaks (de Jong et al., 1991; Done and Paton, 1995; Van Alstine, 1991). These field observations were reproduced under experimental conditions by Galina et al. (1994), who found that PRRSV-2 (ATCC VR-2332) coinfection exacerbated the clinical signs and lesions produced by *Streptococcus suis* infection in young pigs. Likewise, Wills et al. (2000) found that concurrent PRRSV (ATCC VR-2402) and *Salmonella choleraesuis* infections were far more severe that either pathogen alone. Similar observations were made for viral coinfections, e.g., Narita and Ishii (2004) found that PRRSV infection enhanced the severity of brain lesions caused pseudorabies virus and Allan et al. (2007) reported that PRRSV in combination with PCV2 was associated with more severe histopathology and a higher PCV2 antigenic load versus PCV2-only infected pigs. Subsequent research suggested that PRRSV infectious could profoundly modulate immune responses. Suradhat et al. (2006) investigated the effect of PRRSV on classical swine fever virus (CSFV) modified live vaccine efficacy by inoculating 17-day-old pigs with PRRSV-2 (isolate 01NP1) one week before CSFV vaccination, followed by challenge with CSFV (strain Bangkok 1950) 3 weeks post-vaccination. The response of the PRRSV-infected vaccinated pigs to CSFV challenge resembled those of the non-vaccinated pigs. That is, CSFV vaccination during the acute phase of PRRSV infection resulted in vaccination failure.

The research data have unequivocally demonstrated that PRRSV infection compromises the immune system and promotes synergy with bacterial and viral co-infections. In bone marrow and throughout the body, PRRSV-susceptible mononuclear cells, i.e., imDCs, mDCs, monocytes, and macrophages play a variety of important roles in the many aspects of tissue remodeling, development, immunity, and immunopathology (Gomez-Laguna et al., 1995; Hume, 2008; Rodriguez-Gomez et al., 2013). These cells are the first line of defense against microbial infection and responsible for antigen recognition, processing, and presentation to T and B cells; a critical step in the initiation and development of an effective adaptive immune response (Notkins et al., 1970). In addition, DCs prime naive T lymphocytes and play an important role in the control of T cell activation and regulation, as well as in B cell responses and NK cell activation (Carrasco et al., 2001; Summerfield and McCullough, 2009). Macrophages clear the interstitial environment of extraneous cellular materials, remove cellular debris generated during tissue remodeling and rapidly clear cells that have undergone apoptosis (Gordon, 1998; Mosser and Edwards, 2008). Logically, impairment of the normal functions of these cells must render the host less able to resist and/or eliminate infectious agents and partially explain the synergy between PRRSV and bacterial/ viral co-infections observed in the clinical setting.

In conclusion, PRRSV has adversely affected the health and well-being of swine populations worldwide since its appearance in the late 1980s. To a large extent, the research has described and defined the complex relationship that exists between PRRSV and the host, including the negative impact of PRRSV infection on bone marrow reviewed herein. The effect of PRRSV on the bone marrow is clear, but many questions remain, including the basis for differences in molecular and cellular interactions among isolates within the same species that vary in virulence or, indeed, between the two PRRSV species. Thus, the task of unraveling the complex molecular interactions between PRRSV and the pig remains in the future. Regardless, on-going research based on advanced techniques and improved reagents will reveal the secrets of "mystery swine disease" one by one to ultimately achieve control of PRRSV.

Conflict of interests

The authors declare that they have no conflict of interests.

Acknowledgements

This work was supported by grants from China Scholarship Council [grant number No. 201703250019], Heilongjiang Province Natural Science Foundation [grant number C2016068], and State Key Laboratory of Veterinary Biotechnology Foundation [grant number SKLVBP2018002].

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