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Genetically edited pigs lacking CD163 show no resistance following infection with the African swine fever virus isolate, Georgia 2007/1

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ABSTRACT

African swine fever is a highly contagious, often fatal disease of swine for which there is no vaccine or other curative treatment. The macrophage marker, CD163, is a putative receptor for African swine fever virus (ASFV). Pigs possessing a complete knockout of CD163 on macrophages were inoculated with Georgia 2007/1, a genotype 2 isolate. Knockout and wild type pen mates became infected and showed no differences in clinical signs, mortality, pathology or viremia. There was also no difference following *in vitro* infection of macrophages. The results do not rule out the possibility that other ASFV strains utilize CD163, but demonstrate that CD163 is not necessary for infection with the Georgia 2007/1 isolate. This work rules out a significant role for CD163 in ASFV infection and creates opportunities to focus on alternative receptors and entry mechanisms.

1. Introduction

African swine fever virus (ASFV) is a large enveloped double-stranded DNA virus and the single member of the family *Asfarviridae*. The ASFV genome ranges in size from 170 to 193+ kbp, and encodes 150–167 genes (Chapman et al., 2008; de Villiers et al., 2010). Currently, 23 ASFV genotypes have been identified based on partial sequencing of the gene encoding the major capsid protein p72 (Achenbach et al., 2016). ASFV is endemic throughout Sub-Saharan Africa and the island of Sardinia, but in 2007, the virus produced outbreaks in the Republic of Georgia, followed by entry of the virus into the Russian Federation and Eastern Europe (Costard et al., 2013; Rowlands et al., 2008). The Georgia 2007/1 isolate, a highly virulent genotype 2 virus, is of great concern to the global swine industry (Rowlands et al., 2008; Chapman et al., 2011).

ASFV is maintained in nature through the interactions between African wild pigs (*Phacochoerus aethiopicus, Potamochoerus sp.*) and soft ticks of the genus *Ornithodoros* (Costard et al., 2013). In these hosts the virus causes a non-clinical, persistent infection. However, infection of domestic or wild pigs of the species *Sus scrofa* results in a febrile illness and widespread systemic hemorrhage that typically results in death within days (Blome et al., 2013). Isolates can be divided into high, moderate and low virulence categories, each associated with a range of clinical and pathological presentations (Blome et al., 2013; Galindo-Cardiel et al., 2013). Highly virulent strains can cause acute and hyperacute infections that result in 100% mortality. Death as a result of hyperacute infection can occur within 2–4 days, often before the onset of clinical symptoms other than fever. Infections with low to moderate virulent viruses are associated with clinical symptoms, and death occurs between 12 and 20 days in 30–60% of pigs; but mild or unapparent clinical signs with no mortality may also be observed.

The principal targets for ASFV include cells of the monocyte/macrophage lineage, and cells of later maturation stages are preferentially infected. Early *in vitro* studies identified a receptor-mediated mechanism for ASFV entry using Vero cell adapted virus. Those studies employed metabolic inhibitor drugs to block specific components of endocytosis and found ASFV infection was reduced (Alcami et al., 1989a; Valdeira and Geraldes, 1985). Moreover, the binding of ASFV to the surface of Vero cells was saturable, indicating that there were specific receptors being competitively bound by the virus (Alcami et al., 1989b). These results were replicated in primary macrophages (Alcami et al., 1990; Galindo et al., 2015). More recent studies have shown that clathrin-mediated, dynamin dependent endocytosis is a primary pathway for ASFV entry (Galindo et al., 2015; Hernaez and Alonso, 2010; Cuesta-Geijo et al., 2012). Despite the broad body of literature in

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support of a receptor-mediated entry mechanism, little work has been done to identify receptors for ASFV.

The cell tropism of ASFV suggests that a macrophage-specific receptor is required for infection. CD163 is a surface marker primarily expressed on mature tissue macrophages (Pulford et al., 1992), and it was previously identified as a receptor for ASFV (Sanchez-Torres et al., 2003). This conclusion was based on the observation that infected macrophages possess a mature CD163-positive phenotype, combined with the capacity of anti-CD163 monoclonal antibodies to block infection of primary alveolar macrophages in culture (Sanchez-Torres et al., 2003). Furthermore, the location of epitopes on the N-terminal end of CD163 recognized by the virus-neutralizing monoclonal antibody, 2A10, identified scavenger receptor cysteine-rich (SRCR) domains 1–3 as the region on CD163 recognized by the virus (Van Gorp et al., 2010). However, non-permissive cells transfected with a CD163 plasmid remained refractory to infection (Lithgow et al., 2014). One conclusion from those studies was that CD163 may be necessary but not sufficient for infection, suggesting that other macrophage surface proteins, such as CD45 and MHC II, may participate in the infection process (Lithgow et al., 2014).

Another macrophage-tropic virus that utilizes CD163 for infection is porcine reproductive and respiratory syndrome virus (PRRSV). Recently, we demonstrated that genetically edited pigs lacking CD163 are non-permissive for PRRSV infection (Whitworth et al., 2016). The current study tests the hypothesis that CD163 plays an important role in ASFV infection by infecting the same line of CD163 knockout (KO) pigs along with wild type (WT) pen mates with the Georgia 2007/1 ASFV isolate, and evaluating outcomes in clinical disease, mortality and infection of macrophages.

2. Materials and methods

2.1. Ethical statement

Experiments involving animals and virus were performed in accordance with the Federation of Animal Science Societies Guide for the Care and Use of Agricultural Animals in Research and Teaching, the United States Department of Agriculture Animal Welfare Act and Animal Welfare Regulations, and were approved by the Kansas State University and University of Missouri Institutional Animal Care and Use Committees and Institutional Biosafety Committees. Animals were humanely euthanized by pentobarbital overdose following the American Veterinary Medical Association guidelines for the euthanasia of animals, and all efforts were made to minimize suffering.

2.2. Virus

The ASFV Georgia 2007/1 isolate (Rowlands et al., 2008; Chapman et al., 2011) was kindly provided by Linda Dixon at the Pirbright Institute, and obtained through the generosity of David Williams at the Commonwealth Scientific and Industrial Research Organization's Australian Animal Health Laboratory. Virus was propagated on primary cultures of porcine alveolar macrophages (PAMs) collected by lung lavage from 3 to 5 week old piglets. PAMs were washed with PBS, re-suspended in freezing medium containing 50% fetal bovine serum (FBS) plus 10% DMSO, and stored in liquid nitrogen until use. Prior to infection, PAMs were cultured for 2 days in RPMI-1640 culture medium (Life Technologies) supplemented with 10% FBS and 1x antibiotics-antimycotic (Gibco), in a 37 °C 5% CO_2 incubator. Red blood cells (RBCs) used for hemadsorption were isolated from heparinized whole pig blood and washed twice with 0.9% saline solution and maintained at 4 °C in Alsever's solution (Sigma-Aldrich). Virus in serum or tissue culture media was quantified by hemadsorption. Briefly, PAMs cultured on 96-well plates were incubated with serial dilutions of sample along with a 1% RBC suspension. Samples were titrated in duplicate and negative controls included uninfected PAMs incubated with RBCs. The extent of hemadsorption was determined at 5 days after infection and the $\log_{10}$ of 50% hemadsorption dose (HAD_{50}) calculated using the Spearman-Karber method (Hierholzer and Killington, 1996).

2.3. Gene-edited CD163 knockout pigs

The CD163 KO pigs were produced using the CRISPR/Cas9 system, an efficient method of introducing biallelic and homozygous mutations in pigs, as described previously (Whitworth et al., 2016, 2014). In brief, founder male and female pigs are produced by two CRISPR/Cas9 techniques. Both utilize the same guide RNA (gRNA) sequence and human optimized *Staphylococcus pyogenes* Cas9 endonuclease to target exon 7 of the CD163 gene. One technique involves transfection of swine-harvested fibroblasts with the gRNA and Cas9 expressed *via* the commercially available pX330 vector. The nucleus from the transfected fibroblast is transferred to commercially harvested oocytes *via* somatic cell nuclear transfer (SCNT) and the reconstructed oocyte is then *in vitro* fertilized (IVF). The IVF embryo is then transferred to a surrogate sow for gestation. Alternatively, unmodified embryos can undergo IVF and are then cultured until they become zygotes. The zygotes are directly injected with messenger RNAs coding for the gRNA and Cas9 sequences. The modified zygotes are then transferred on day 6 post-standing estrus into a surrogate sow. The resulting founder males and females created by either procedures, are bred to produce offspring that are either wild type (WT), full CD163 knockouts (CD163 $^{-/-}$), or partial knockouts where only one allele of the CD163 gene is modified. For this study, only full knockouts are described and are referred to as CD163 KO.

2.4. Animal infection and monitoring

Infection studies were performed at the biosafety level 3 (BSL3) and BSL3-Ag biocontainment facilities at the Kansas State University Biosecurity Research Institute. Five-week-old CD163 WT (n=10) and KO (n=6) Large White x Landrace pigs were housed in a single room and acclimated for four days prior to infection. Ear-tags were randomly assigned so that caretakers and researchers were blind as to the genetic identity of the individual pigs. Pigs were infected with 10^4 HAD_{50} of ASFV Georgia 2007/1 *via* intramuscular injection (IM) in the right hamstring muscle group. Clinical signs and rectal temperatures were monitored twice daily. Blood samples were collected on day 0 and 3 post-infection (PI) *via* jugular venipuncture. At the termination of the study, three pigs from each CD163 genotype were selected for necropsy. Following a gross examination, tissue samples collected from lungs, spleen, tonsils, and inguinal or mesenteric lymph nodes were placed in 10% PBS-buffered formalin. Slide-mounted hematoxylin and eosin (H & E) stained thin sections were evaluated by a board-certified pathologist in the Kansas State Veterinary Diagnostic Laboratory. The pathologist was blind as to the CD163 genotype of the pigs.

2.5. Infection of PAMs and measurement of CD169 and CD163 expression

Primary cultures of PAMs were collected by lung lavage from WT and CD163 KO pigs and stored as described above until further use. PAMs were cultured for 2 days, then inoculated in duplicate with ten-fold serial dilutions of the Georgia 2007/1 virus and a 1% RBC suspension. End-point virus titration was determined by hemadsorption as described above.

Aliquots of PAMs from the same pigs used in the infection experiments were subjected to surface staining for flow cytometry analysis. PAMs were washed with PBS and blocked with 5% mouse/5% rat serum in PBS for 15 min, then incubated for 30 min at 4 °C with phycoerythrin (PE)-CD163 and fluorescein isothiocyanate (FITC)-CD169 conjugated antibodies (AbD Serotec; MCA2311PE and

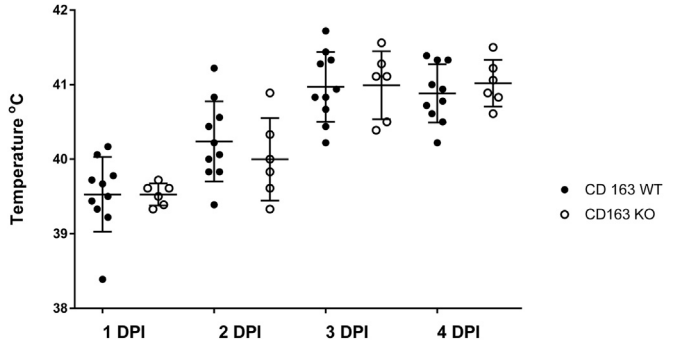

Fig. 1. Rectal temperatures of wild type (WT) and knockout (KO) pigs infected with Georgia 2007/1. Means with standard deviations are shown. A one-way ANOVA analysis showed no significant difference between temperatures of WT and KO pigs on any day ($p > 0.96$). The analysis did show a significant increase in mean temperatures from day 1–3 for both WT and KO groups ($p < 0.0001$).

MCA2316F). Labeled isotype controls were included in each experiment (AbD Serotec: MCA928PE, MCA928F). Cells were washed and fixed for 15 min at 4 °C with BD Cytofix solution (BD Biosciences) and analyzed on a BD LSR Fortessa flow cytometer (BD Biosciences) with BD FACSDiva 8.0.1 software (BD Biosciences). A total of 10,000 events were collected for analysis.

3. Results

3.1. Clinical signs

After infection, the overall attitude of all pigs quickly declined from bright, alert and responsive on day 0, to depressed by day 3. One WT pig displayed cyanosis of the left ear on day 3; however, no other outward clinical signs were apparent. As shown in Fig. 1, mean temperatures increased from 39.5 °C on day 1 and peaked at 41.0 °C on day 3 for both genotypes. There was no statistically significant difference between the two CD163 genotypes on any day post infection; however, there was significant difference between the means of baseline temperatures at 1 DPI and peak temperatures at 3 DPI ($p < 0.0001$). On days 2 and 3 after infection, all pigs were treated with the nonsteroidal anti-inflammatory drug (NSAID), flunixin meglumine (Banamine, 50 mg/mL; Merck Animal Health). The study was terminated on day 4 due to the appearance of pyrexia, anorexia, dehydration, and the failure of pigs to respond to NSAID treatment. Overall, there was no observable difference in clinical signs between the CD163 WT and KO pigs. Acute fever and unapparent clinical symptoms are consistent with hyperacute ASFV infection (Blome et al., 2013; Galindo-Cardiel et al., 2013).

3.2. Pathology

At termination of the study, three pigs from each CD163 genotype group were randomly selected for necropsy. Anatomic pathology results showed moderate to severe hemorrhagic congestion and necrosis of the lungs, spleen, and inguinal and mesenteric lymph nodes. The lungs appeared grossly edematous. At the microscopic level, acute edema was observed in five of the six pigs; one WT pig displayed mild hemorrhage with no edema. Multifocal lymphoid necrosis in the white and red pulp were present in the spleen of all pigs. Lymph node samples from five of the six pigs exhibited a multifocal necrosis, with one WT pig showing no significant microscopic lesions. Tonsils were mostly within normal limits, with only one WT pig exhibiting widely scattered multifocal necrosis. The results showed that CD163 KO and WT pigs exhibited signs consistent with hyperacute ASFV infection, typical of what has been reported in the literature for infection with the Georgia 2007/1 isolate (Blome et al., 2013; Galindo-Cardiel et al., 2013).

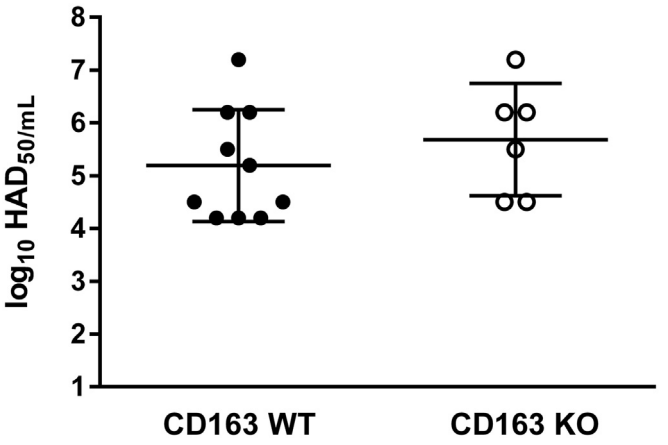

Fig. 2. Viremia of wild type (WT) and knockout (KO) pigs infected with Georgia 2007/1. Virus titers of day 3 post infection serum samples were determined by 50% hemadsorption (HAD_{50}) on alveolar macrophages. Closed and open circles represent individual pigs from each group with the means and standard deviations indicated. A paired *T*-test showed no significant difference between virus titers of WT and KO pigs ($p > 0.93$).

3.3. Viremia

Viremia was measured in sera collected from all pigs at 3 days after infection. Virus levels ranged from 1.6×10^4 to 1.6×10^7 HAD_{50}/mL in the group of WT pigs, and 5×10^4 to 1.6×10^7 HAD_{50}/mL for the CD163 KO pigs (see Fig. 2). There was no statistical difference in the levels of viremia between the WT and CD163 KO pigs.

3.4. Infection of PAMs

Previous work that identified a role for CD163 in ASFV infection was performed on primary macrophage cultures. Those studies demonstrated that PAMs (~76%) are more permissive for ASFV infection *in vitro* compared to blood monocytes or bone marrow precursor cells, and infection positively correlates with CD163 expression (Sanchez-Torres et al., 2003). In the current study, permissiveness of PAMs from WT and CD163 KO pigs was evaluated by comparing the titration endpoints after *in vitro* inoculation with the Georgia 2007/1 isolate. As shown in Fig. 3, similar end-point infectious titers were achieved for macrophages from one representative WT and three CD163 KO pigs. PAMs from these same pigs were assessed by flow cytometry for surface expression of the macrophage markers CD163 and CD169. Relatively high levels of surface CD163 was expressed on WT PAMs, while staining of CD163 KO PAMs was comparable to the negative isotype control, shown in Fig. 3. Both CD163 genotypes of PAMs expressed similar levels of another alveolar macrophage marker, CD169.

4. Discussion

The role of CD163 in ASFV infection is controversial. Previous work by Sanchez-Torres et al. (2003) strongly supported the requirement of CD163 as a receptor for ASFV on macrophages. However, subsequent work failed to recover permissiveness of CD163-negative cell lines after transfection with a CD163 plasmid (Lithgow et al., 2014). Nonetheless, those negative results do not rule out CD163 as a necessary co-receptor. CD163 alone may not be sufficient for infection but require the cooperation of additional macrophage-specific proteins. The results from the current study clearly demonstrate that CD163 is not essential for infection of PAMs with the Georgia isolate. While this study and previous work both assessed infection of PAMs, the strains of ASFV used differ. This work does not entirely rule out the receptor requirements of other ASFV isolates.

In the absence of CD163, it is plausible that other surface markers may be upregulated to compensate; however, we saw no evidence to

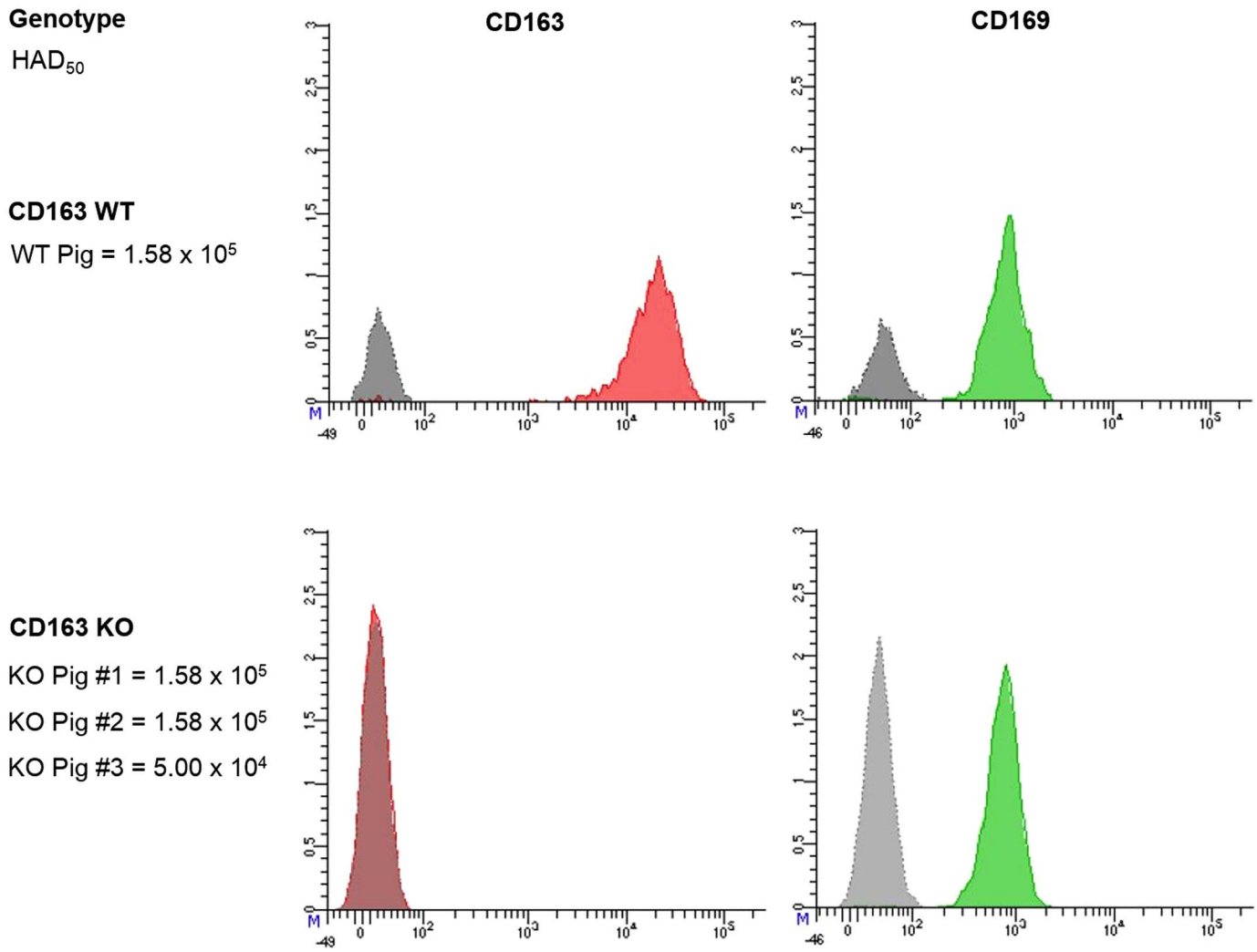

Fig. 3. Infection of wild type (WT) and CD163 knockout (KO) PAMs with ASFV Georgia 2007/1. Flow cytometry for CD163 and CD169 surface expression on PAMs of representative animals are shown. Percentage of labeled cells is expressed on the Y-axis and fluorescence intensity is indicated by the X-axis. Red and green peaks show cells labeled with CD163 and CD169 antibodies, respectively; grey peaks represent non-specific background fluorescence of the corresponding antibody isotype. HAD_{50} titrations of Georgia 2007/1 on WT and CD163 KO PAMs are shown on the right. ASFV=African swine fever virus; PAMs=pulmonary alveolar macrophages; HAD_{50}=50% hemadsorption dose. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

support this. Here we used the alveolar macrophage-specific surface protein, CD169, as a control. CD169 and CD163 are normally expressed at similar levels on the surface of porcine PAMs (Whitworth et al., 2016). Based on results from this study and previous characterization of PAMs from CD163 modified pigs by our lab (Whitworth et al., 2016), we found that surface expression of CD169 remains similar to WT PAMs in the absence of CD163. Other macrophage surface markers, including CD14, MHC II and CD172, were also found to be expressed at similar levels for both WT and CD163 KO genotypes (Whitworth et al., 2016). However, this does not exclude changes in expression of other macrophage surface proteins which were not evaluated.

While there is support for receptor-dependent entry mechanisms, such as clathrin-mediated endocytosis (CME) (Galindo et al., 2015; Hernaez and Alonso, 2010), there is also evidence that ASFV may exploit non-receptor mechanisms, such as macropinocytosis (Sanchez et al., 2012; Hernaez et al., 2016). Macropinocytosis is the non-selective, actin-dependent uptake of molecules (Kerr and Teasdale, 2009), and is utilized by several large DNA viruses, including vaccinia virus, herpes simplex virus 1, and adenovirus 3, and others (Mercer and Helenius, 2009). Recently, Hernaez et al. (2016) demonstrated that both pathways, CME and macropinocytosis, are utilized by ASFV for entry into permissive cells. That study incorporated fluorescence and transmission electron microscopy (TEM) to document the early events during infection of PAMs. Purified labeled virions were shown to co-localize with dextran, a macropinocytosis marker, as well as transferrin, a marker for CME. Sixty-eight percent of virions co-localized with dextran. Further support comes from TEM studies, which show virus particles in macropinocytic-like processes; a small percentage of virions were also found in dense-membrane invaginations typical of clathrin-coated pits. Chemical inhibitors of either CME (Chlorpromazine, pitstop 2 and dynasore) or macropinocytosis (EIPA, IPA-3 and cytochalasin D) inhibited ASFV entry into PAMs (Hernaez et al., 2016). Nonetheless, if ASFV utilizes macropinocytosis as a non-specific mechanism for entry, then other intracellular macrophage-specific interactions must be involved which define ASFV tropism. The mechanism of entry for ASFV in the KO PAMs is currently unknown and a topic for further study.

Even though the current study rules out the requirement of CD163 for ASFV infection, the absence of CD163 in the KO pigs could affect the host response to infection. For example, the expression of CD163 is linked to a subpopulation of macrophages described as anti-inflammatory. Both CD163 expression and the polarization of macrophages to an M2 phenotype are induced by interleukin (IL)-6, IL-10 and glucocorti-

coids (Hogger et al., 1998; Buechler et al., 2000; Gordon, 2003). M2 regulatory macrophages are upregulated in response to both innate and adaptive immune responses, and appear to play an immunosuppressive role by producing high levels of the anti-inflammatory cytokine IL-10 (Mosser and Edwards, 2008). CD163 is a component of a haptoglobin/hemoglobin scavenging complex which reduces inflammation that results from oxidative stress caused by excess circulating heme (Kristiansen et al., 2001). Furthermore, heme degradation products produced by CD163-positive macrophages act as potent anti-inflammatory compounds (Jeney et al., 2002; Soares and Bach, 2009). Upregulation of soluble CD163 is associated with inflammatory and hemorrhagic diseases in patients with virus infections (Buechler et al., 2013; Wang et al., 2014). One possibility is that the soluble form of CD163 may act like an anti-inflammatory cytokine to downregulate inflammation and T-cell activation as demonstrated *in vitro* (Frings et al., 2002). Thus, in the absence of CD163, it would be predicted that the presence of a hemorrhagic disease would enhance the inflammatory response in the CD163 KO pigs. However, there appeared to be no differences in the clinical outcome and histopathology between CD163 KO and wild type pigs.

5. Conclusion

Taken together, the results of this study indicate that CD163 is not critical for ASFV infection of PAMs or progression of the disease in pigs. Nonetheless, because ASFV replication is primarily restricted to macrophages, it is clear that other macrophage-associated proteins are involved in ASFV tropism and may represent important targets for genetic modification.

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Conflict of interest

The authors report no conflict of interest, but the University of Missouri has filed for patent protection of intellectual property associated with this manuscript.

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