Replacement of Porcine CD163 Scavenger Receptor Cysteine-Rich Domain 5 with a CD163-Like Homolog Confers Resistance of Pigs to Genotype 1 but Not Genotype 2 Porcine Reproductive and Respiratory Syndrome Virus

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ABSTRACT CD163 knockout (KO) pigs are resistant to infection with genotype 2 (type 2) porcine reproductive and respiratory syndrome virus (PRRSV). Furthermore, the substitution of CD163 scavenger receptor cysteine-rich (SRCR) domain 5 with a homolog of human CD163-like (hCD163L1) SRCR 8 domain confers resistance of transfected HEK cells to type 1 PRRSV. As a means to understand the role of domain 5 in PRRSV infection with both type 1 and type 2 viruses, pigs were genetically modified (GM) to possess one of the following genotypes: complete knockout (KO) of CD163, deletions within SRCR domain 5, or replacement (domain swap) of SRCR domain 5 with a synthesized exon encoding a homolog of hCD163L1 SRCR domain 8. Immunophenotyping of porcine alveolar macrophages (PAMs) showed that pigs with the KO or SRCR domain 5 deletion did not express CD163. When placed in culture, PAMs from pigs with the CD163 KO phenotype were completely resistant to a panel consisting of six type 1 and nine type 2 isolates. PAMs from pigs that possessed the hCD163L1 domain 8 homolog expressed CD163 and supported the replication of all type 2 isolates, but no type 1 viruses. Infection of CD163-modified pigs with representative type 1 and type 2 viruses confirmed the *in vitro* results. The results confirm that CD163 is the likely receptor for all PRRS viruses. Even though type 1 and type 2 viruses are considered phenotypically similar at several levels, there is a distinct difference between the viral genotypes in the recognition of CD163.

IMPORTANCE Genetic modification of the *CD163* gene creates the opportunity to develop production animals that are resistant to PRRS, the costliest viral disease to ever face the swine industry. The results create further opportunities to develop refinements in the modification of *CD163* with the goal of making pigs refractory to infection while retaining important CD163 functions.

KEYWORDS CD163, genetic modification, porcine reproductive and respiratory syndrome virus

In 1989, Keffaber (1) was the first to describe a mystery swine disease in the United States associated with a seemingly diverse set of clinical signs, including respiratory distress in young pigs, late-term abortions in gilts and sows, and reduced growth in

Received 29 July 2016 Accepted 2 November 2016

Accepted manuscript posted online 9 November 2016

Citation Wells KD, Bardot R, Whitworth KM, Tribble BR, Fang Y, Mileham A, Kerrigan MA, Samuel MS, Prather RS, Rowland RRR. 2017. Replacement of porcine CD163 scavenger receptor cysteine-rich domain 5 with a CD163-like homolog confers resistance of pigs to genotype 1 but not genotype 2 porcine reproductive and respiratory syndrome virus. *J Virol* 91:e01521-16. <https://doi.org/10.1128/JVI.01521-16>.

Editor Stanley Perlman, University of Iowa

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FIG 1 Diagram of porcine CD163 protein and gene. (A) CD163 protein SRCR (ovals) and PST (squares) domains (top), along with the corresponding gene exons (bottom). (B) Peptide sequence comparison of porcine CD163 SRCR 5 with the human CD163L1 SRCR 8 homolog peptide sequence. The figure is based on GenBank accession numbers AJ311716 (pig CD163) and GQ397482 (hCD163L1).

finishing pigs. Soon afterward, a similar syndrome appeared in Europe. The etiological agent, porcine reproductive and respiratory syndrome virus (PRRSV), was identified and sequenced in Europe in 1991 and called Lelystad virus (2), followed in 1999 by the complete sequence of a North American isolate, VR2332 (3–5). Molecular comparisons between North American and European viruses place all PRRSV isolates into one of two genotypes, type 2 or type 1, respectively. Even though the two genotypes possess only about 70% identity at the nucleotide level (5), they share a tropism for CD163-positive cells, establish long-term infections, and produce similar clinical signs.

CD163 is a 130-kDa type 1 membrane protein composed of nine scavenger receptor cysteine-rich (SRCR) domains (6). As illustrated in Fig. 1A, porcine *CD163* contains 17 exons that code for a peptide signal sequence followed by nine SCRC domains; two linker domains, also referred to as proline serine threonine (PST) domains (located after SRCR 6 and SRCR 9); a cytoplasmic domain; and a short cytoplasmic tail. Surface expression of CD163 is restricted to cells of the monocyte-macrophage lineage. The protein was first identified in human tissues based on its ability to bind hemoglobin-haptoglobin (HbHp) complexes (7). HbHp scavenging is a major function of CD163 and is located in SRCR 3 (8). Metabolites released by macrophages following HbHp degradation include bilirubin, CO, and free iron. Therefore, one important anti-inflammatory function of CD163 is the prevention of oxidative toxicity that results from free hemoglobin (7, 9).

CD163, as a receptor for PRRSV, was first described by Calvert et al. (10). Transfection of PRRSV-nonpermissive cell lines with CD163 cDNAs from a variety of species, including simian, human, canine, and mouse, can make cells permissive for PRRSV infection with type 2 genotype viruses. Subsequently, Van Gorp et al. (22) mapped the individual SRCR domains involved in determining the permissiveness of a type 1 genotype virus. The approach was to transfect HEK cells with cDNAs possessing various SRCR domain deletions and/or substitutions with domain homologs from human CD163-like 1 protein (hCD163L1). Cells transfected with CD163 cDNA possessing a swap between SRCR 5 and the hCD163L1 homolog, SRCR 8, are not permissive, identifying SRCR 5 as the domain involved in PRRSV infection by type 1 viruses. In addition to CD163, a second receptor protein, CD169 (also known as sialoadhesin or SIGLEC1), was previously identified as being involved in the initial interaction with the GP5-matrix (M) heterodimer, the major protein on the surface of the virion (11). In this model, the subsequent interaction between CD163 and the GP2-GP3-GP4 heterotrimer in an endosomal compartment mediates uncoating and the release of the viral genome into the cytoplasm (12, 13). However, our previous work showed that genetically modified (GM) pigs that lacked SIGLEC1 supported virus replication to levels comparable to those

FIG 2 Summary of *CD163* gene modifications used in the study. The genomic region covers the sequence between intron 6 and intron 8 of the porcine *CD163* gene. The numbers begin at exon 1 of the *CD163* gene. The introns and exons are not drawn to scale. The predicted protein product is illustrated on the right of each genomic structure. The relative macrophage expression, as measured by the level of 2A10 MAb staining of PAMs, is shown on the far right. Black regions, introns; white regions, exons; hatched region, *hCD163L1* exon 11 mimic constructed by making 38-bp substitutions in exon 7 of porcine *CD163*, resulting in 31-amino-acid substitutions in the HL8 mimic; gray area, synthesized intron with the *PGK Neo* construct used for selection.

of wild-type pigs after infection with a type 2 virus (14). These results supported previous *in vitro* studies showing that PRRSV-resistant cell lines lacking surface CD169 and CD163 supported virus replication after transfection with a CD163 plasmid (15).

Recently, we showed that GM pigs lacking CD163 expression on macrophages failed to support infection with a type 2 virus (16). The purpose of the current study was to determine if CD163 knockout (KO) pigs were resistant to type 1 PRRSV genotype viruses and, second, to determine if the replacement of SRCR 5 with an *hCD163L1* peptide sequence would be sufficient to confer on pigs resistance to both type 1 and type 2 viruses. GM pigs were constructed that either possessed deletions in SRCR 5 or had CD163 SRCR 5 swapped with a synthesized exon that expressed the SRCR 8 domain from *hCD163L1*. Pigs with deletions in SRCR 5 were negative for staining with the anti-CD163 monoclonal antibody (MAb) 2A10 and were refractory to PRRSV infection, whereas the replacement of SRCR 5 with *hCD163L1* SRCR 8 conferred resistance of macrophages and pigs to infection with type I viruses but retained the ability to be infected with type 2 viruses.

RESULTS

Phenotypic properties of porcine alveolar macrophages (PAMs) from CD163 gene-modified pigs. The forward and side scatter properties of cells in the lung lavage material were used to gate on the mononuclear subpopulation of cells. Representative CD169 and CD163 staining results for the different gene modifications are summarized in Fig. 2 and presented in Fig. 3. In the representative example shown in Fig. 3A, greater than 91% of PAMs from the wild-type (WT) pigs were positive for both CD169 (CD169⁺) and CD163 (CD163⁺). The results for 12 WT pigs used in this study showed a mean of 85% $\pm$ 8% double-positive cells. As shown in Fig. 3B, PAMs from the *CD163* KO pigs showed no evidence of CD163 and retained normal surface levels of CD169. Although it was predicted that the CD163 polypeptides derived from the *d7(1467)* and *d7(1280)*

FIG 3 Surface expression of CD163 and CD169 on PAMs from wild-type and CD163-modified pigs. Shown are representative results for the expression of *CD163* gene constructs diagrammed in Fig. 2.

deletion genotypes should produce modified CD163 polypeptides anchored to the PAM surface, immunostaining results showed no surface expression of CD163 (Fig. 3D). Since MAb 2A10, used for detection of CD163, recognizes similar linear epitopes located in SRCR domains 3, 7, and 8, the absence of antibody recognition was likely not the result of the deletion of an immunoreactive epitope in SRSR domain 5 or 6 (17). The *d7(129)* genotype was predicted to possess a 43-amino-acid deletion in SRCR 5 (Fig. 2). In the example presented in Fig. 3C, only 2.4% of the cells were located in the double-positive quadrant. The analysis of PAMs from nine *d7(129)* pigs used in this study showed percentages of double-positive cells ranging from 0% to 3.6% (mean = 0.9%). Surface expression of CD169 remained similar to that of WT PAMs. For the purpose of this study, pigs possessing the KO, *d7(1467)*, *d7(1280)*, and *d7(129)* genotypes were all categorized as possessing a CD163-null phenotype.

All PAMs from pigs with the *HL11m* CD163 modification expressed both CD163 and CD169 (Fig. 3E); however, the surface expression of CD163 was markedly reduced compared to that of the WT PAMs. The levels of CD163 fell on a continuum of expression, ranging from no detectable CD163 to cells possessing moderate levels of CD163. In the example shown in Fig. 3E, approximately 60% of the cells could be placed in the double-positive quadrant while the remainder stained for only CD169. The

FIG 4 Serum Hp levels in wild-type and CD163-modified pigs. The mean and standard deviation for each group were as follows: WT, 0.23 ± 0.18, n = 10; HL11m, 1.63 ± 0.8, n = 11; and CD163-null pigs, 2.06 ± 0.57, n = 9. The CD163-null group was composed of genotypes that did not express CD163. Hp measurements were made on a single ELISA plate. Groups with the same letter (a or b) were not significantly different (*P* > 0.05, Kruskal-Wallis one-way ANOVA with Dunnett's posttest).

analysis of PAMs from a total of 24 *HL11m* pigs showed that 38% ± 12% of PAMs were single positive for CD169 compared to 54% ± 14% of cells that were double positive.

Circulating haptoglobin levels in WT and CD163-modified pigs. As a scavenging molecule, CD163 is responsible for removing HbHp complexes from the blood (6–8). Therefore, the level of Hp in serum provides a convenient method for determining the overall functional state of CD163-expressing macrophages. Hp levels in sera from WT, *HL11m*, and CD163-null pigs were measured at 3 to 4 weeks of age, just prior to infection with PRRSV. The results, presented in Fig. 4, showed that sera from WT pigs had the smallest amounts of Hb (mean *A*₄₅₀ = 23 ± 0.18; n = 10). The mean *A*₄₅₀ value for WT pigs was significantly different from those for the *HL11m* and CD163-null pigs (*P* < 0.05). Although the mean *A*₄₅₀ value was lower for the *HL11m* group than for the CD163-null group (*A*₄₅₀ = 1.6 ± 0.8 versus 2.1 ± 0.6), the difference was not statistically significant. Since the interaction between HbHp and CD163 occurs through SRCR 3 (8), increased circulating Hp in the *HL11m* pigs was not a consequence of a reduced affinity of CD163 for HbHp but likely the result of reduced numbers of CD163⁺ macrophages, along with reduced expression on the remaining CD163-positive macrophages (Fig. 3E).

Infection of PAMs with type 1 and type 2 viruses. The permissiveness of the CD163-modified pigs for PRRSV was initially evaluated by infecting PAMs *in vitro* with a panel of six type 1 and nine type 2 PRRSV isolates (Table 1 lists the viruses). The viruses represent different genotypes and have differences in nucleotide and peptide

TABLE 1 PRRSV isolates used in the study

Virus	Genotype	Yr isolated	GenBank accession no.
NVSL 97-7895	2	1997	AY545985
KS06-72109	2	2006	KM252867
P129	2	1995	AF494042
VR2332	2	1992	AY150564
CO90	2	2010	KM035799
AZ25	2	2010	KM035800
MLV-ResPRRS	2	NA ^a	AF066183
KS62-06274	2	2006	KM035798
KS483 (SD23983)	2	1992	JX258843
CO84	2	2010	KM035802
SD13-15	1	2013	NA
Lelystad	1	1991	M96262
03-1059	1	2003	NA
03-1060	1	2003	NA
SD01-08	1	2001	DQ489311
4353PZ	1	2003	NA

^aNA, not available (not sequenced).

TABLE 2 Infection of PAMs from wild-type and *CD163* gene-modified pigs with different PRRSV isolates^a

Virus	% infection for genotype/phenotype		
	WT	<i>HL11 m</i>	Null ^b
Type 1			
13-15	56 ± 9	0	0
Lelystad	62 ± 15	0	0
03-1059	50 ± 18	0	0
03-1060	61 ± 12	0	0
01-08	64 ± 20	0	0
4353-PZ	62 ± 15	0	0
Type 2			
NVSL 97	59 ± 15	8 ± 08	0
KS-06	56 ± 20	12 ± 09	0
P129	64 ± 11	8 ± 06	0
VR2332	54 ± 05	6 ± 03	0
CO 10-90	43 ± 18	8 ± 08	0
CO 10-84	51 ± 22	7 ± 04	0
MLV-ResP	55 ± 12	3 ± 01	0
KS62	49 ± 03	10 ± 11	0
KS483	55 ± 23	6 ± 03	0

^aThe viruses are listed in Table 1. Three pigs were used for each genotype. The results are shown as means ± standard deviations of the percentages of PAMs infected.

^bThe CD163-null PAMs were from pigs expressing the *d7(129)* allele (see Fig. 2 and 3 for CD163 gene constructs and CD163 expression on PAMs, respectively).

sequences, pathogenesis, and numbers of years since isolation. The data presented in Table 2 show the results of experiments using PAMs from three pigs for each *CD163* genotype group. As expected, the WT PAMs were infected by all the viruses. In contrast, the CD163-null phenotype PAMs were negative for infection by all the viruses. These data support our earlier work describing the nonpermissiveness of CD163 KO pigs after infection with a type 2 virus (16). A marked difference was observed in the response of PAMs from the *HL11m* pigs. None of the type 1 viruses were able to infect the *HL11m* PAMs, whereas all the viruses in the type 2 panel infected the *HL11m* PAMs, albeit at much lower percentages than the WT PAMs.

Besides a reduction in the percentage of macrophages positive for infection, the effect of the *HL11m* domain swap was studied by comparing the permissiveness of PAMs from WT and *HL11m* pigs for infection with a panel of type 2 viruses. The results shown in Fig. 5 are for two WT and two *HL11m* pigs. The log₁₀ 50% tissue culture infectious dose (TCID₅₀) titration endpoints for PAMs from the *HL11m* pigs were 1 to 3 log units lower than those for WT PAMs infected with the same virus. The only exception was infection with a modified-live-virus vaccine strain. Taken together, the results suggest that PAMs from *HL11m* pigs possess a reduced susceptibility or permissiveness for infection with type 2 viruses.

FIG 5 Relative permissiveness of wild-type and *HL11m* PAMs for infection with type 2 PRRSV isolates. The log₁₀ TCID₅₀ values were calculated based on the infection of PAM cultures with the indicated type 2 viruses, listed in Tables 1 and 2. Infection results are shown for two pigs from each genotype. *HL11*, *HL11m*; ND, not determined.

FIG 6 Infection of CD163-modified pigs with type 1 and type 2 PRRSV isolates. (A) Wild-type (circles), *HL11m* (squares), and CD163-null (triangles) pigs were infected with a contemporary type 1 isolate, SD-1305 (left), or the type 2 isolate, NVSL 97-7895 (right). The open squares in the right graph represent a single *HL11m* pig, 101, that showed lower viremia levels than the other three *HL11m* pigs. The null-phenotype pigs carried the *d(1567)* allele (Fig. 2). Pigs infected with type 1 virus: WT, $n = 4$; *HL11m*, $n = 5$; and null, $n = 3$. Pigs infected with type 2 virus: WT, $n = 4$; *HL11m*, $n = 4$; and null, $n = 3$. (B) Anti-PRRSV antibody levels from the same pigs at 14 days after infection. The error bars indicate standard deviations.

Infection of CD163-modified pigs with type 1 and type 2 viruses. WT, *HL11m*, and CD163-null pigs were infected with representative type 1 (SD13-15) and type 2 (NVSL 97-7895) viruses. Pigs inoculated with the same virus were comingled in one pen, which allowed the continuous exposure of CD163-modified pigs to virus shed from WT pen mates. As shown in Fig. 6A and B, the CD163-null pigs infected with either the type 1 or type 2 virus were negative for viremia at all time points and did not seroconvert. As expected, the WT pigs were productively infected, with mean viremia levels approaching 10^6 templates/reaction at 7 days after infection for both viruses. By 14 days, all the WT pigs had seroconverted (Fig. 6B). Consistent with the PAM infection results (Table 2), all five *HL11m* pigs infected with the type 1 virus showed no evidence of viremia or PRRSV antibody. All the *HL11m* pigs infected with the type 2 isolate, NVSL, supported infection and seroconverted (Fig. 6B). The *HL11m* PAM infection results showing reduced infection by type 2 viruses (Table 2 and Fig. 5) did not translate to pigs. The mean viremia for three of the four *HL11m* pigs was similar to that for the WT pigs. However, for one *HL11m* pig, 101 (Fig. 6A, open squares), viremia was greatly reduced compared to the other pigs in the *HL11m* genotype group. An explanation for the 3- to 4-log-unit reduction in viremia for pig 101 was not clear but suggested that some *HL11m* pigs may be less permissive for PRRSV, an observation supporting the *in vitro* PAM infection results (Table 2). Since all the pigs were comingled with the WT pigs, the lower viremia in pig 101 was not the result of receiving a smaller amount of virus. Flow cytometry of macrophages showed that CD163 expression for pig 101 was comparable to that for the other *HL11m* pigs (data not shown). There was also no difference in the nucleotide sequence of the *HL11m* exon. Therefore, additional virus infection trials were conducted to identify other outliers. Pigs were infected with two type 2 viruses, NVSL 97-7895 and KS06-72109, and followed for 35 days after infection. The data shown in Fig. 7 are presented as the area under the viremia curve (AUC) for individual pigs. The mean AUC value for the seven WT pigs infected with NVSL was 168 ± 8 , versus 165 ± 15 for the seven *HL11m* pigs. For KS06, the mean AUC values for the six WT and six *HL11m* pigs were 156 ± 9 and 163 ± 13 , respectively. For both viruses, there was no statistically significant difference between the WT and *HL11m* pigs

FIG 7 Virus loads for wild-type and CD163-modified pigs infected with type 2 viruses. The virus load was determined by calculating the AUC for each pig over a 35-day infection period. The AUC calculation was performed using $\log_{10}$ PCR viremia measurements taken at 0, 4, 7, 10, 14, 21, 28, and 35 days after infection. The horizontal lines show means and standard deviations. HL11, *HL11m* genotype pigs; null, CD163-null genotype.

($P > 0.05$). Furthermore, none of the pigs could be identified as outliers. Taken together, the results showed that the *HL11m* pigs failed to support infection with type 1 PRRSV but retained permissiveness for infection with type 2 viruses. Even though there was a reduction in the PRRSV permissiveness of *HL11m* PAMs infected *in vitro* with type 2 isolates, the difference was not apparent in pigs.

DISCUSSION

CD163 is a macrophage surface protein important for scavenging excess Hb from the blood and is involved in modulating inflammation in response to tissue damage (18). In previous work, we demonstrated that GM pigs that lack CD163 expression on macrophages fail to support the replication of a single type 2 PRRSV isolate (16). In the present study, we demonstrated that macrophages from KO pigs were resistant to infection with a large panel of type 1 and type 2 isolates (Table 2), suggesting that all PRRSV isolates, regardless of genotype or clade, require CD163. The resistance of the CD163-null phenotype macrophages to type 1 and type 2 viruses was confirmed *in vivo* (Fig. 6 and 7). Based on these results, we can rule out the contribution of other PRRSV receptors previously described in the literature (19). For example, Shanmukhappa et al. (20) showed that nonpermissive BHK cells transfected with a CD151 plasmid acquired the ability to support PRRSV replication, and incubation with a polyclonal anti-CD151 antibody reduced the infection of MARC-145 cells. In addition, a simian cell line, SJPL, originally developed for use in propagating swine influenza viruses, was shown by Provost et al. (21) to support PRRSV replication. Important properties of the SJPL cell line include the presence of CD151 and the absence of CD163. The absence of PRRSV infection in macrophages and pigs possessing a CD163-null phenotype indicates that CD151 as a sole receptor for PRRSV is not biologically relevant.

The viral proteins GP2a and GP4, which form part of the GP2a-GP3-GP4 heterotrimer complex on the virion surface, are coprecipitated with CD163 in pulldown assays from cells transfected with GP2 and GP4 plasmids (13). It is interesting to speculate that the stable interaction between GP2/GP4 and CD163 occurs through SRCR 5. Additional viral glycoproteins, such as GP3 and GP5, may further stabilize the virus-receptor complex or may interact with a coreceptor. The requirement for SRCR 5 was investigated in this study by infecting macrophages and pigs possessing the *HL11m* allele, which recreated the CD163L1 SRCR 8 domain swap described by Van Gorp et al. (22) by making 31-amino-acid substitutions in porcine SRCR 5. The *HL11m* pigs expressed CD163 on PAMs, albeit at reduced levels compared to WT PAMs (compare Fig. 3A and E). Reduced CD163 expression was likely due to the presence of a neomycin cassette, which was located between the exon 11 mimic and the following intron. *HL11m* pigs were not permissive for infection with a type 1 virus, confirming the importance of SRCR 5. However, *HL11m* macrophages and *HL11m* pigs did support infection with type 2 viruses. Based on virus titration and percent infection results, the PAMs from the *HL11m* pigs showed an overall decrease in permissiveness for virus compared to the WT macrophages (Table 2 and Fig. 2). Decreased permissiveness may be due to reduced

levels of CD163 on the *HL11m* macrophages combined with a reduced affinity of the virus for the modified CD163 protein. The lower affinity can be explained by the difference in peptide sequences between human SRCR 8 and porcine SRCR 5 (Fig. 1B). However, the reduced permissiveness of PAMs in culture did not translate into reduced viremia in pigs. The mean viremia for the *HL11m* pigs was not significantly different from that for WT pigs (Fig. 7). In addition to PAMs, other cell populations, such as intravascular, septal, and lymphoid tissue macrophages, also contribute to viremia (23, 24). The contributions of these and other CD163-positive cell populations to maintaining the overall virus load in *HL11m* pigs deserve further study.

Even though CD163 plasmids possessing deletions of SRCR domains are stably expressed in HEK cells transfected with cDNAs (22), the deletion of exons 7 and 8 in *d7(1467)* and *d7(1280)* pigs resulted in a lack of detectable surface expression of CD163, as determined using the 2A10 MAb (Fig. 3). Since the linear epitope recognized by MAb 2A10 is replicated in SRCR domains 3, 7, and 8 (17, 22), the absence of staining was likely not the result of removing all the 2A10 epitopes in the mutated proteins. While a small amount of CD163 expression was detected on PAMs from some of the *d7(129)* pigs (Fig. 3C), the quantity of expressed protein was likely not sufficient to support PRRSV infection in PAMs or in pigs. Alternatively, the absence of infection may be the result of the deletion of 43 amino acids from the N-terminal region of SRCR 5. The absence of CD163 expression on the surfaces of macrophages from pigs possessing deletions of exon 7 and 8 is not fully understood but is likely the result of the inability of the nuclear transcription products to be properly processed into mRNA and/or the result of degradation of the mutated protein.

In 2003, CD163 was first described as a receptor for African swine fever virus (ASFV) (25). This conclusion was based on the observations that infected macrophages possess a mature CD163-positive phenotype and that anti-CD163 antibodies, such as 2A10, block ASFV infection of macrophages *in vitro*. It remains to be determined if CD163 KO pigs are resistant to ASFV infection.

Cell culture models incorporating modifications to the PRRSV receptor have provided valuable insight into the mechanisms of PRRSV entry, replication, and pathogenesis. One unique aspect of this study was the conduct of parallel *in vivo* experiments in receptor-modified pigs. This research has important impacts on the feasibility of developing preventative cures for one of the most serious diseases ever to face the global swine industry.

MATERIALS AND METHODS

Ethics statement. Experiments involving animals and viruses were performed in accordance with the Federation of Animal Science Societies Guide for the Care and Use of Agricultural Animals in Research and Teaching and the USDA 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.

Genomic modifications of the porcine CD163 gene. Pigs possessing deletions in *CD163* were prepared using the CRISPR/Cas9 technology as previously described by Whitworth et al. (26), and the deletions are diagrammed in Fig. 2. The *CD163* gene knockout constructs, *KO-d7(11)* and *KO-i7(2)*, possessed a deletion of 11 bp and an insertion of 2 bp in exon 7, respectively. These edits were predicted to result in frameshifts that introduced early stop codons. If expressed, the partial SRCR 5 would produce a soluble polypeptide lacking a cell membrane anchor. Three other constructs possessed deletions in exon 7; one was a 129-bp deletion in exon 7 of *d7(129)* and two were complete deletions of exons 7 and 8 in *d7(1467)* and *d7(1280)*. For these deletion constructs, the remaining *CD163* exons remained intact. The last construct, *HL11m*, was produced using a homologous-recombination targeting event that replaced exon 7 of *CD163* with a synthesized exon that encoded a mimic or homolog of SRCR 8 in the human CD163-like 1 protein (hCD163L1 domain 8 is encoded by hCD163L1 exon 11). The CD163L1 SRCR 8 peptide sequence was created by making 38-nucleotide substitutions that produced 31-amino-acid changes to porcine exon 7, as shown in Fig. 1B. A neomycin cassette was included in the synthesized exon to enable screening for the modification.

Viruses. The viruses used in this study are listed in Table 1. The isolates were propagated and titrated on MARC-145 cells (27). For titration, each virus was serially diluted 1:10 in minimal essential medium (MEM) supplemented with 7% fetal bovine serum (FBS), Pen-Strep (80 units/ml and 80 µg/ml), 3 µg/ml amphotericin B (Fungizone), and 25 mM HEPES. Diluted samples were added in quadruplicate to confluent MARC-145 cells in a 96-well plate to a final volume of 200 µl per well and incubated for 4 days at 37°C in 5% CO₂. The titration

endpoint was identified as the last wells in each dilution series with cytopathic effect (CPE). The TCID₅₀ per milliliter was calculated using the method of Reed and Muench (28).

Infection of alveolar macrophages. The preparation and infection of macrophages were performed as previously described (29, 30). Lungs were removed from euthanized pigs and lavaged by pouring 100 ml of cold phosphate-buffered saline (PBS) into the trachea. The tracheas were clamped, and the lungs were gently massaged. The alveolar contents were poured into 50-ml centrifuge tubes and stored on ice. The PAMs were sedimented by centrifugation at $1,200 \times g$ for 10 min at 4°C. The pellets were resuspended and washed once in cold sterile PBS. The cell pellets were resuspended in freezing medium (45% RPMI 1640, 45% FBS, 10% dimethyl sulfoxide [DMSO]) and stored in liquid nitrogen until use. The frozen cells were thawed on ice, counted, and adjusted to 5×10^5 cells/ml in medium (RPMI 1640 supplemented with 10% FBS, PenStrep, and amphotericin B [RPMI-FBS]). Approximately 10^3 PAMs per well were added to 96-well plates and incubated overnight at 37°C in 5% CO₂. The cells were gently washed to remove nonadherent cells. Serial 1:10 dilutions of virus in medium were added to triplicate wells. After incubation overnight, the cells were washed with PBS and fixed for 10 min with 80% acetone. For detection of infected macrophages, the wells were stained with PRRSV N-protein-specific MAb (SDOW-17; Rural Technologies Inc.) diluted 1:1,000 in PBS with 1% fish gelatin (PBS-FG) (Sigma-Aldrich). After a 30-min incubation at 37°C, the cells were washed with PBS and stained with Alexa Fluor 488-labeled anti-mouse IgG (Thermo Fisher Scientific) diluted 1:200 in PBS-FG. The plates were incubated for 30 min in the dark at 37°C, washed with PBS, and viewed under a fluorescence microscope. The TCID₅₀ per milliliter was calculated according to the method of Reed and Muench (28).

Measurement of CD169 and CD163 surface expression on PAMs. Staining for surface expression of CD169 and CD163 was performed as described previously (14). Approximately 1×10^6 PAMs were placed in 12-mm by 75-mm polystyrene flow cytometry (fluorescence-activated cell sorter [FACS]) tubes and incubated for 15 min at room temperature in 1 ml of PBS with 10% normal mouse serum to block Fc receptors. The cells were pelleted by centrifugation and resuspended in 5 μ l of fluorescein isothiocyanate (FITC)-conjugated mouse anti-porcine CD169 MAb (clone 3B11/11; AbD Serotec) and 5 μ l of phycoerythrin (PE)-conjugated mouse anti-porcine CD163 MAb (clone 2A10/11; AbD Serotec). After a 30-min incubation, the cells were washed twice with PBS containing 1% bovine serum albumin (BSA) (Fraction V; HyClone) and immediately analyzed on a BD LSR Fortessa flow cytometer (BD Biosciences) with FCS Express 5 software (De Novo Software). A minimum of 10,000 cells were analyzed for each sample.

Measurement of PRRS viremia. RNA was isolated from 50 μ l of serum using Ambion's MagMax 96 viral isolation kit (Applied Biosystems) according to the manufacturer's instructions. PRRSV RNA was quantified using EZ-PRRSV MPX 4.0 real-time reverse transcription (RT)-PCR target-specific reagents (Tetracore) according to the manufacturer's instructions. Each plate contained Tetracore quantification standards and control sets designed for use with the RT-PCR reagents. PCR was carried out on a CFX96 Touch real-time PCR detection system (Bio-Rad) in a 96-well format using the recommended cycling parameters. The PCR assay results were reported as log₁₀ PRRSV RNA copy number per 50- μ l reaction volume, which approximates the number of copies per milliliter of serum. The AUC for viremia over time was calculated using GraphPad Prism version 6.00 for Windows.

Measurement of PRRSV antibody. The microsphere fluorescent immunoassay (FMIA) for the detection of antibodies against the PRRSV nucleocapsid (N) protein was performed as described by Stephenson et al. (31). Recombinant PRRSV N protein was coupled to carboxylated Luminex MagPlex polystyrene microsphere beads according to the manufacturer's directions. For FMIA, approximately 2,500 antigen-coated beads, suspended in 50 μ l PBS with 10% goat serum (PBS-GS), were placed in each well of a 96-well polystyrene round-bottom plate. The sera were diluted 1:400 in PBS-GS, and 50 μ l was added to each well. The plate was incubated in the dark for 30 min at room temperature with gentle shaking. The plate was placed on a magnet, and the beads were washed three times with PBS-GS. For the detection of IgG, 50 μ l of biotin-SP-conjugated affinity-purified goat anti-swine secondary antibody (IgG; Jackson ImmunoResearch) was diluted to 2 μ g/ml in PBS-GS, and 100 μ l was added to each well. The plate was incubated at room temperature for 30 min and washed three times, followed by the addition of 50 μ l of streptavidin-conjugated phycoerythrin (2 μ g/ml in PBS-GS) (SAPE). After 30 min, the microspheres were washed, resuspended in 100 μ l of PBS-GS, and analyzed using a MagPix instrument (Luminex) and Luminex xPONENT 4.2 software. The mean fluorescence intensity (MFI) was calculated on a minimum of 100 microsphere beads.

Measurement of Hp. The amount of Hp in serum was measured using a porcine-specific Hp enzyme-linked immunosorbent assay (ELISA) kit (Genway Biotech Inc.) according to the manufacturer's instructions. Serum samples were diluted 1:10,000 in diluent solution and pipetted in duplicate on a precoated anti-pig Hp 96-well ELISA plate, incubated at room temperature for 15 min, and then washed three times. Anti-Hp-horseradish peroxidase (HRP) conjugate was added to each well and incubated in the dark at room temperature for 15 min. The plate was washed, and 100 μ l of chromogen substrate solution was added to each well. After incubating in the dark for 10 min, 100 μ l of stop solution was added to each well. The plate was read at 450 nm on a Fluostar Omega filter-based microplate reader (BMG Labtech).

ACKNOWLEDGMENTS

The work was supported by Genus PLC, Food for the 21st Century at the University of Missouri, and Kansas NBAF Transition funds.

We acknowledge the many people who made this set of experiments possible, including Mykel Anderson's help with genotyping the CD163 pigs.

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