Title: An intact sialoadhesin (Sn/SIGLEC1/CD169) is not required for attachment/internalization of the porcine reproductive and respiratory syndrome virus (PRRSV)

Running Title: SIGLEC1 knockout pigs are not resistant to PRRSV.

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Surface expression of SIGLEC1, also known as sialoadhesin (SN) or CD169, is considered a primary determinant for the permissiveness of porcine alveolar macrophages (PAMs) for infection by porcine reproductive and respiratory syndrome virus (PRRSV). In vitro the attachment and internalization of PRRSV is dependent on the interaction between sialic acid on the virion surface and the sialic acid binding domain of the SIGLEC1 gene. To test the role of SIGLEC1 in PRRSV infection, a SIGLEC1 gene knockout pig was created by removing part of exon 1 and all of exons 2 and 3 of the SIGLEC1 gene. The resulting knockout ablated SIGLEC1 expression on the surface of alveolar macrophages, but had no effect on the expression of CD163, a co-receptor for PRRSV. After infection, PRRSV viremia in SIGLEC1 \(-/-\) pigs followed the same course as \(-/+\) and \(+/+\) litter mates. The absence of SIGLEC1 had no measurable effect on other aspects of PRRSV infection, including clinical disease course and histopathology. The results demonstrate that the expression of the SIGLEC1 gene is not required for infection of pigs with PRRSV and the absence of SIGLEC1 does not contribute to the pathogenesis of acute disease.
Introduction

One viral disease that continues to have the greatest economic impact on swine production is porcine reproductive and respiratory syndrome (PRRS). It was first reported in the United States in 1987 (14) and in Europe in 1990 (38). Molecular analysis of the prototype PRRS viruses (PRRSV) VR-2332 and Lelystad (U.S. and European isolates, respectively) suggests that divergently evolved strains emerged on the two continents almost simultaneously, perhaps due to similar changes in swine management practices (22, 24). Since its initial emergence, PRRSV has spread worldwide, including the circulation of European genotype viruses in U.S. swine herds (28). PRRSV infection of pigs results in respiratory disease, reproductive failure, and participates in polymicrobial disease syndromes, such as porcine respiratory disease complex and porcine circovirus associated disease (4). The most consistent pathological lesion associated with acute PRRS in nursery age pigs is interstitial pneumonia ((27) and references therein). The acute phase of PRRSV infection is typically characterized by viremia and clinical disease, after which many pigs fully recover, although many continue to carry subclinical viral loads. Persistently infected pigs shed the virus, either intermittently or continuously, and may infect naïve pigs (1, 2, 5, 6, 29, 40).

A receptor for PRRSV has been identified, purified, sequenced (36, 39), and named SIGLEC1, CD169 or sialoadhesin. SIGLEC1 is a transmembrane protein belonging to a family of sialic acid binding immunoglobulin-like lectins. It was first described as a sheep erythrocyte binding receptor on macrophages of hematopoietic and lymphoid tissues (8). SIGLEC proteins consist of an N-terminal V-set domain containing the sialic acid binding site followed by a variable number of C2-set domains, a transmembrane domain and cytoplasmic tail. In contrast to other SIGLEC proteins, SIGLEC1 does not have a tyrosine-based motif in the cytoplasmic tail.
SIGLEC1, which is expressed on macrophages, functions in cell-to-cell interactions through the binding of sialic acid ligands on erythrocytes, neutrophils, monocytes, NK cells, B cells, and some cytotoxic T cells. The SIGLEC1-sialic acid interaction participates in several aspects of adaptive immunity, such as antigen processing and presentation to T cells, and activation of B cells and CD8 T cells (reviewed in (21, 25)).

An intact N-terminal domain on SIGLEC1 has been suggested to be both necessary and sufficient for PRRSV binding and internalization by cultured macrophages (3, 10). Transfection of SIGLEC1 negative cells, such as PK-15, with SIGLEC1 is sufficient to mediate virus internalization. Incubation of PRRSV-permissive cells with anti-SIGLEC1 mAb is blocks PRRSV binding and internalization (36). On the virus side, removing the sialic acid from the surface of the virion or pre-incubation of virus with sialic acid-specific lectins blocks infection (8, 9, 33). A model illustrating the role of SIGLEC1 in PRRSV infection is presented in Figure 1. The interaction between SIGLEC1 and sialic acid on viral proteins, such as GP5/M heterodimer, promotes the attachment and internalization of the virion in a clathrin-coated pit (9). Subsequent entry of PRRSV occurs by receptor-mediated endocytosis (23). A second receptor, CD163, located within the endosome compartment, participates in the uncoating of the virion. The interaction between CD163 and the virion occurs via binding of the GP2,3,4 heterotrimer, a minor group of viral proteins. In combination with a decrease in pH, the virus fuses with the endosome envelope and the viral genome is released into the cytoplasm (32).

To test the hypothesis that SIGLEC1 is required for PRRSV infection, we infected transgenic pigs that possessed a knockout of the SIGLEC1 gene.

**Material and Methods**

Unless otherwise stated all chemicals are from Sigma, St. Louis.
Targeted disruption of porcine SIGLEC1 gene. The use of animals and virus were approved by university animal care and institutional biosafety committees at the University of Missouri and/or Kansas State University. Homologous recombination was incorporated to remove the protein coding exons 2 and 3 from SIGLEC1, and introduce premature stop codons to eliminate expression of the remaining coding sequence (see Figure 2). Porcine SIGLEC1 cDNA (GenBank accession no. NM214346) encodes a 210-kDa protein from an mRNA transcript of 5,193 bases (36). Genomic sequence from the region around SIGLEC1 (GenBank accession no. CU467609) was used to prepare oligonucleotide primers to amplify genomic fragments by high-fidelity PCR [AccuTaq (Invitrogen)] for generation of a targeting construct. Based on comparisons with the mouse and human genomic sequences, porcine SIGLEC1 is predicted to possess 21 exons. In addition, exon 2 is conserved between pig, mouse, and human. Peptide sequence alignments reveal that the six amino acids in the exon 2 coding region in mouse SIGLEC1, known to be involved with sialic acid binding, are conserved in pig SIGLEC1. One fragment, the ‘upper arm’, represented part of the first coding exon and 3,304 bp upstream from the start codon. The second fragment, or ‘lower arm’ was 4,753 bp in length and represented most of the intron downstream of the third coding exon and extended into the 6th intron (including the 4th, 5th and 6th coding exons). Between the lower and upper arms was a neo cassette, inserted in the opposite direction and placed under the control of a phosphoglycerol kinase (PGK) promoter.

Male and female fetal fibroblast primary cell lines, from day 35 of gestation, were isolated from large commercial white pigs (Landrace). The cells were cultured and grown for 48 hours to 80% confluence in Dulbecco’s Modified Eagles Medium (DMEM), containing 5 mM glutamine, sodium bicarbonate (3.7 g/L), penicillin-streptomycin and 1 g/L d-glucose was further
supplemented with 15% fetal bovine serum (FBS, Hyclone), 10 µg/mL gentamicin, and 2.5 ng/mL basic fibroblast growth factor. Media was removed and replaced four hours prior to transfection. Fibroblast cells were washed with 10 mL of phosphate buffered saline (PBS) and lifted off the 75 cm² flask with 1 mL of 0.05% trypsin-EDTA (Invitrogen). The cells were resuspended in DMEM and collected by centrifugation at 600Xg for 10 min then washed with Opti-MEM (Invitrogen) and centrifuged again at 600xg for 10 min. Cytosalts (75% cytosalts [120 mM KCl, 0.15 mM CaCl₂, 10 mM K₂HPO₄; pH 7.6, 5 mM MgCl₂] and 25% Opti-Mem (Invitrogen) were used to resuspend the pellet (34). The cells were counted with a hemocytometer and adjusted to 1 x 10⁶ cells per mL. Electroporation of cells was performed with 0.75-10 µg of double or single stranded targeting DNA (achieved by heat denaturation) in 200 µl of transfection media containing 1x10⁶ cells/mL. The cells were electroporated in a BTX ECM2001 electro cell manipulator by using three 1 msec pulses of 250V the medium to resuspend the pellet (see above). The electroporated cells were diluted in DMEM/FBS/bFGF at 10,000 cells per 13 cm plate and cultured overnight without selective pressure. The following day, medium was replaced with culture medium containing G418 (Geneticin, 0.6 mg/ml). After 10 days of selection, G418-resistant colonies were isolated and transferred to 24-well plates for expansion. PCR was used to determine if targeting of SIGLEC1 was successful. PCR primers “f” and “b”, and “a” and “e” (Figure 2B) were used to determine the successful targeting of both arms. Primers “f” and “e” annealed outside the region of each targeting arm. PCR primers “c” and “d” were used to determine the insertion of an intact Neo gene.

Somatic cell nuclear transfer. Pig oocytes were purchased from ART Inc. (Madison, WI) and matured according to the supplier’s instructions. After 42-44 h of in vitro maturation, the oocytes were stripped of cumulus cells by gentle vortexing in 0.5 mg/mL hyaluronidase. Oocytes
with good morphology and a visible polar body (metaphase II) were selected and kept in the micromanipulation medium at 38.5°C until nuclear transfer.

Using an inverted microscope, a cumulus-free oocyte was held with a holding micropipette in drops of micromanipulation medium supplemented with 7.5 μg/mL cytochalasin B and covered with mineral oil. The zona pellucida was penetrated with a fine glass injecting micropipette near the first polar body, and the first polar body and adjacent cytoplasm, containing the metaphase-II chromosomes, was aspirated into the pipette. The pipette was withdrawn and the contents discarded. A single, round, and bright donor cell with a smooth surface was selected and transferred into the perivitelline space adjacent to the oocyte membrane (16, 17). The nuclear transfer complex (oocyte + fibroblast) was fused in fusion medium with a low calcium concentration (0.3 M mannitol, 0.1 mM CaCl₂, 2H₂O, 0.1 mM MgCl₂, 6H₂O and 0.5 mM HEPES). The fused oocytes were then activated by treating with 200 μM thimerosal for 10 min in the dark, followed by rinsing and treatment with 8 mM dithiothreitol (DTT) for 30 min; the oocytes were rinsed again to remove the remaining DTT (18, 19). Following fusion/activation, the oocytes were washed three times with Porcine Zygote Culture Medium 3 supplemented with 4 mg/mL of BSA (12), and cultured at 38.5°C in a humidified atmosphere of 5% O₂, 90% N₂ and 5% CO₂ for 30 min. Those complexes that had successfully fused were cultured for 15-21 hours until surgical embryo transfer.

Embryo transfer. The surrogate gilts were synchronized by administering 18-20 mg Regu-mate (Intervet, Millsboro, DE) mixed into the feed for 14 days according to a scheme dependent on the stage of the estrous cycle. After the last Regu-mate treatment (105 hours), an IM injection of 1000 units of hCG was given to induce estrus. Surrogate pigs on the day of (day 0), or the first day after standing estrus were used (17). The surrogates were aseptically prepared and a caudal
ventral incision was made to expose the reproductive tract. Embryos were transferred into one oviduct through the ovarian fimbria. Pigs were checked for pregnancy by abdominal ultrasound examination around day 30, and then checked once a week through gestation until parturition at 114 days of gestation.

**PCR and Southern blot confirmation in transgenic piglets.** For PCR and Southern blots, genomic DNA was isolated from tail tissue. Briefly, the tissues were digested overnight at 55°C with 0.1 mg/mL of proteinase K (Sigma, St Louis, MO) in 100 mM NaCl, 10 mM Tris, pH 8.0, 25 mM EDTA, pH 8.0, 0.5% SDS. The material was extracted sequentially with neutralized phenol and chloroform and the DNA was precipitated with ethanol (11). Detection of both wild-type and targeted **SIGLEC1** alleles was performed by using PCR with primers that annealed to DNA flanking the targeted region of **SIGLEC1** (to amplify TK lower arm region (black arrows in Figure 2): a-forward 5′-agaggccactgtgtagcgc, e-reverse 5′-caggtaccaggaaaaacgggt; to amplify upper arm Neo region (blue arrows in Figure 2): f-forward 5′-ggaacaggctgagccaataa, b-reverse 5′-ggttctaagtactgtggtttcc; to amplify exon 1 and Neo gene (red arrows in Figure 2): c-forward 5′-gcattcctaggcacagc, d-reverse 5′-ctccttgccatgtccag). The incorporation of ‘c’ and ‘d’ primers (red arrows in Figure 2) were designed to yield ~2,400 bp and ~2,900 bp for the wild-type and targeted alleles, respectively.

For Southern blots, the genomic DNA was digested at 37°C with *ScaI* and *MfeI* (New England Biolabs). Sites for *MfeI* reside in the genomic regions upstream of the translation start site and in intron 6. A *ScaI* site is present in the Neo cassette. Digested DNA was separated on an agarose gel, transferred to a nylon membrane (Immobilon NY+, EMD Millipore) by capillary action and immobilized by UV-crosslinking (11). A genomic fragment consisting of intron 4 and portions of exons 4 and 5 was amplified by PCR (oligonucleotides: 2789 F gatctggtcaccctcagct;
3368 R  gcgcttccttaggtgtattg) and labeled with Digoxigenin according the manufacturer’s protocols (Roche). Hybridization, washes and signal detection were performed as per manufacturer’s recommendations (Roche). Predicted sizes for wild-type and targeted SIGLEC1 genes was 7,892 bp and 7,204 bp, respectively.

**Infection of pigs.** A single litter of 11 pigs, at three weeks of age, were brought into the challenge facility. The litter was derived from the mating of heterozygous parents. Three genotypes were represented: homozygous +/+ (4 pigs); heterozygous +/- (3 pigs); and homozygous -/- (4 pigs). The pigs were housed in the same room and allowed to freely intermingle. After acclimation for one week, the pigs were challenged with a low-passage PRRSV isolate, KS-06, a North American isolate obtained during a PRRS outbreak in 2006. Virus was propagated by three rounds of amplification on MARC-145 cells (15). The virus retained the ability to grow on primary cultures of porcine alveolar macrophage cells (PAMs) and reproduced PRRS clinical signs following experimental infection. Pigs were challenged with approximately 10^5 TCID₅₀ of virus, diluted in 3 mL of culture medium. One-half of the innoculum was delivered intramuscularly and the remaining delivered intranasally. Blood samples were collected at days 0, 4, 7, 14, 21, 28, and at termination, day 35. Blood was allowed to clot and serum stored at -80°C until use. At termination of the study, pigs were necropsied and tissues fixed in 10% buffered formalin, embedded in paraffin and processed for histopathology by the Kansas State Veterinary Diagnostic Laboratory. Paraffin-embedded thin sections were mounted on slides, deparaffinized, and stained with hematoxylin and eosin (H&E). Histopathology was assessed by a board certified pathologist.

**Measurement of PRRSV viremia.** Two approaches were used to measure the amount of PRRSV in serum. Virus titration was performed by adding serial 1:10 dilutions of sera to
confluent MARC-145 cells in a 96 well plate. Sera were diluted in EMEM supplemented with 8% FBS, penicillin, streptomycin, and Fungizone. After four days’ incubation at 37°C, the wells were examined under a microscope for the presence of cytopathic effect (CPE). The last well showing CPE was scored as the titration endpoint. For the measurement of viral nucleic acid, total RNA from serum was isolated using Life Technologies’ MagMAX™-96 Viral RNA Isolation Kit according to the manufacturer’s recommendations. RT-PCR was performed using the EZ-PRRSV MPX 4.0 Kit from Tetracore according to the manufacturer’s instructions and reactions were performed on a CFX-96 Real-Time PCR System (BioRad) in a 96-well format. Each 25 uL rxn contained RNA from 5.8 uL of serum. A standard curve was constructed by preparing serial dilutions of an RNA control, supplied in the RT-PCR kit (Tetracore). RT-PCR results were reported as the number of templates per PCR reaction.

**SIGLEC1 (CD169) and CD163 surface staining of PAM cells.** PAMs were collected by lung lavage. Briefly, excised lungs were filled with approximately 100 mL of cold PBS. After a single wash, the pellet was resuspended in approximately 5 mL of cold PBS and stored on ice. Approximately 10^7 PAM cells were incubated in 5 mL of 20 µg/mL anti-porcine CD169 (clone 3B11/11; AbD Serotec) or anti-porcine CD163 (Clone: 2A10/11, AbD Serotec) diluted in PBS with 5% FBS and 0.1% sodium azide (PBS-FBS) for 30 minutes on ice. Cells were centrifuged, washed and re-suspended in 1/100 FITC-conjugated goat-anti mouse IgG (Life Technologies) diluted in staining buffer and incubated for 30 minutes on ice. At least 10^4 cells were analyzed using a FACSCalibur flow cytometer and Cell Quest software (Becton Dickinson).

**Measurement of PRRSV-specific Ig and IgM.** Recombinant PRRSV N protein, expressed in bacteria (31), was conjugated to magnetic Luminex beads using a kit from Luminex Corp. N protein-coupled beads were diluted in PBS with 10% goat serum (PBS-GS) to a working
concentration of 2500 beads in 50 µL and placed wells on a 96-well polystyrene round bottom plate. Fifty microliters of a 1:400 dilution of serum in PBS-GS was added to duplicate wells and incubated for 30 min at room temp with gentle shaking. The plate was washed three times PBS-GS followed by the addition of 50 µL of biotin-SP-conjugated affinity purified goat anti-swine secondary antibody (IgG, Jackson ImmunoResearch) or biotin-labeled affinity purified goat anti-swine IgM (KPL) diluted to 2 µg/mL in PBS-GS. After an 30 min incubation, the plates were washed 3 times followed by the addition of 50 µL of streptavidin-conjugated phycoerythrin (2 µg/ml in PBS-GS; Moss, Inc.). After 30 min, the plates were resuspended in 100 µL of PBS-GS and analyzed using the MAGPIX with the Luminex® xPONENT 4.2 software. The results were reported as mean fluorescence intensity (MFI).

RESULTS

Creation of SIGLEC1 knockout pigs. The knockout strategy, diagrammed in Figure 2, focused on creating drastic alterations of SIGLEC1 such that exons two and three were eliminated and no functional protein was expected to be obtained from the mutated gene. In addition, further disruption of the gene was accomplished by replacing part of exon 1 and all of exons 2 and 3 with a neomycin selectable cassette, oriented in the opposite direction (20). Thirty-four transfections were conducted using a variety of plasmid preparations (0.75-10 µg/µL; both single and double stranded constructs; and both medium and large sized constructs). Also included were male and female cells representing 5 different porcine fetal cell lines. Over 2,000 colonies were screened for the presence of the targeted insertion of the neo cassette. The PCR primers pair, “f” and “b” and “a” and “e” (Figure 2B and C) were used to check for the successful targeting of the upper and lower arms of the construct. Two colonies tested positive for the presence of the correct insertion: one male and one female (data not shown).
Cells from the male clone, 4-18, were used for somatic cell nuclear transfer, and the transfer of 666 embryos into surrogates. The transfer of cloned embryos into two surrogates produced a total of 8 piglets. One surrogate delivered 6 normal male piglets on day 115 of gestation. A C-section was performed on the second surrogate on day 117 of gestation resulting in two normal male piglets. Three embryo transfers were also conducted with the female cells (658 embryos), but none established a pregnancy. Figure 3 shows the results for PCR reactions performed on genomic DNA extracted from the eight male piglet clones (F0) generated from the 4-18 targeted fetal fibroblast line. To detect both alleles, PCR was performed with primers “c” and “d” (see Figure 2C). The predicted PCR products were ~2,400 bp for the wild-type allele and ~2,900 bp for the targeted allele. The results for the PCR using the “c” and “d” primers are shown in Figure 3. All pigs tested positive for the presence of the the wild-type, 2,400 bp, and targeted, 2,900 bp, alleles (Figure 3B). Control PCR reactions, incorporating DNA from the cell line used for cloning, the targeted 4-18 fibroblast cell line, and non-targeted 4-18 cell line produced the predicted products (see Figure 3A). The presence of the targeted mutation was further confirmed by amplifying regions using primer pairs identified by the blue and black arrows (see Figure 2C), which were predicted to yield products of ~4,500 and ~5,000 bp, respectively. Results showed the presence of both products in the eight founder pigs (data not shown).

Five of the F0 males were used for mating to wild-type females that resulted in 67 F1 offspring (40 males and 27 females), 39 (58%) of which were SIGLEC1+/-. One of the F1 males was mated to one of the F1 females (litter 52) to yield a litter of 12 pigs; 11 of which remained viable until weaning. Identification of wild-type and targeted alleles in the offspring was made by Southern blotting of genomic DNA. The results in Figure 4 shows 4 SIGLEC1+/+, 3 SIGLEC1+/-, and 4 SIGLEC1-/- F2 animals. These 11 animals were infected with PRRSV.
Expression of CD169 (SIGLEC1) and CD163 on PAM cells. Cells for antibody staining were obtained from pigs at the end of the study. As shown in Figure 5, greater than 90% of PAMs from SIGLEC1+/+ and SIGLEC1+/− pigs were double-positive for CD169 and CD163. In contrast, all of the SIGLEC1−/− pigs were negative for surface expression of CD169, but remained positive for CD163. The results showed that the absence of CD169 expression on cells from all of the SIGLEC1−/− pigs. The absence of CD169 surface expression did not alter the expression the PRRSV co-receptor, CD163. To determine if infection had an effect on the level of CD169 surface expression, staining was also performed on pigs not infected with PRRSV. The results for CD169 and CD163 expression on PAM cells from the +/+, +/- and −/− pigs were similar to the infected pigs (data not shown).

Clinical outcome and histopathology following infection with PRRSV. The infected pigs were assessed daily for clinical signs, including overall body condition, respiratory signs, ambulation and general activity. There were no apparent clinical signs prior to virus infection. All pigs maintained a healthy body condition and remained active throughout the study period. The only exceptions were two pigs in the SIGLEC1−/− group, which developed lameness, a condition during PRRSV infection generally linked to a secondary bacterial infection. Swelling of the affected joint is the principal clinical sign of bacteria-induced lameness. In one pig, lameness first appeared on the 2nd day after infection, and was resolved by day 28. Lameness in the second pig appeared at 3 days after infection and was resolved 6 days later. In both cases, joint swelling was absent, suggesting an etiology not related to a secondary bacterial infection caused by PRRS. Respiratory distress is the principal clinical sign associated with acute PRRSV infection. Beginning at day 3 after infection, all pigs began to exhibit mild respiratory signs, including sneezing, coughing and increased breathing effort. These signs, typical of PRRS, were
resolved within one to two weeks after onset. In one SIGLEC1 ^+/+^ pig, more severe respiratory
signs, including dyspnea and coughing, re-appeared on day 28 after infection and continued until
the end of the study. A scoring system, between 0 and 4, was used to assess changes in the
lungs at a microhistological level: 0 = no significant lesion, 1 = mild interstitial pneumonia (<25%
of lung lobe involvement), 2 = moderate multifocal interstitial pneumonia (50-75% lung
involvement), 3 = moderate to severe, multifocal interstitial pneumonia (50-75% lung
involvement), and 4 = severe diffuse, interstitial pneumonia (> 75% of lung involvement). The
mean scores for the SIGLEC1 ^+/+^, (+/-) and (-/-) groups were 2.8 (n=4), 2.7 (n=3) and 2.8 (n=4),
respectively. There were no statistically significant differences between groups in the mean
scores related to histopathology. However, it should be noted that the group sizes were
relatively small. Taken together, the clinical signs and histopathology were consistent with acute
PRRSV infection, and there were no significant differences between groups of pigs.

**PRRSV viremia and antibody.** The course of PRRSV viremia over 36 days of infection was
measured by virus isolation and RT-PCR of viral nucleic acid. As shown in Figure 6A, the
results for virus isolation showed a peak in mean virus titer for all groups at between 7 and 14
days after infection. Virus titers declined until reaching undetectable levels for all pigs by 36
days after infection. Statistical analysis showed no significance differences between groups on
any day. Viremia, as measured by RT-PCR (Figure 6 B), showed a similar pattern of infection
for all groups. The principal difference relative to the virus isolation results, was the presence of
detectable levels of viral nucleic acid in serum at 36 days after infection. Again, there were no
statistically significant differences between groups on any of the days after infection.

PRRSV-specific IgM and IgG responses were evaluated by measuring antibodies against the
PRRSV N protein. Results for PRRSV-specific IgM are presented in Figure 6C. Consistent with
a primary immune response to PRRSV infection, N protein-specific IgM appeared by day 7, peaked at about day 11, then rapidly declined, reaching background levels by the end of the study. SIGLEC1\(^{+/-}\) and SIGLEC1\(^{+/+}\) showed higher peak IgM MFI levels compared to the (-/-) group. However, because of a large degree of variation between pigs within each group, the differences in IgM levels between groups were not statistically significant. The secondary anti-swine antibody used for the detection of IgG is specific for the light chain; therefore, it can be assumed that the anti-swine antibody detected total Ig, including IgG, IgM and circulating IgA. N-specific Ig was first detected at day 7 after infection, peaked at about day 21 and remained elevated for the remainder of the study. Mean Ig was elevated for SIGLEC1\(^{+/-}\) and SIGLEC1\(^{+/+}\) groups relative to the (-/-) group. Similar to IgM, there was large degree of variation between pigs in each group and differences between groups were not statistically significant.

**Discussion**

Previous models, developed based on the virus infection of cultured cells, identified the interaction between sialic acid on the surface of PRRSV and SIGLEC1 on the surface of macrophages as the initial interaction required for PRRSV attachment and internalization into permissive cells (see Figure 1). The subsequent interaction of CD163 with the virion GP2/GP3/GP4 heterotrimer mediates further internalization and uncoating. The current work describes the course of PRRS virus infection and pathogenesis in SIGLEC1 knockout pigs with the goal of creating pigs that are resistant to PRRSV. The knockout of SIGLEC1 was accomplished by the removal of exons 2 and 3 of the SIGLEC1 gene and demonstrated by the absence of SIGLEC1 expression on the surface PAMs from SIGLEC-/- pigs (Figure 5). Macrophages from SIGLEC1\(^{+/-}\) pigs retained the ability to express CD163, and at the same level...
as heterozygous and wild-type littermates. The absence of SIGLEC1 expression did not appear to significantly alter the development or maturation of PRRSV-permissive macrophages. Furthermore, the absence of SIGLEC1 did not significantly alter the level of the second PRRSV receptor.

Infection of \textit{SIGLEC1}\textsuperscript{-/-} pigs with PRRSV resulted in a productive infection as demonstrated by the presence of circulating virus nucleic acid and viable virus (Figure 6). The peak and duration of infection in \textit{SIGLEC1}\textsuperscript{-/-} pigs were no different from \textit{SIGLEC1} heterozygous (+/-) and wild-type (+/+ ) littermates. One explanation for the presence of virus replication in the absence of SIGLEC1 could be related to the different experimental systems incorporated to investigate PRRSV receptors. The current model supporting SIGLEC1 as an important virus receptor is based on PRRSV infection of cultured macrophages. Under these experimental conditions, the blocking of SIGLEC1 from cells or the removal of sialic acid from the virion is sufficient to block infection. Within the more complex environment of the pig, there may exist other cell populations that can support PRRSV replication. For instance, immunohistochemical staining of tissues from infected pigs reveals the presence of PRRSV antigen in non-macrophage cells, such as pneumocytes and epithelial germ cells of the testes (29, 30). However, the presence of a PRRSV receptor or productive virus infection of these cell types is not known. Another explanation for replication of PRRSV in \textit{SIGLEC1}\textsuperscript{-/-} pigs is the potential for leaky expression of the \textit{SIGLEC1} gene, which could provide a source for virus binding. However, the complete removal of exons 2 and 3, combined with the insertion of a Neo gene cassette, cloned in the opposite direction, as well as the addition of stop codons flanking the Neo cassette, suggested that a non-functional or truncated SIGLEC1 protein was produced. And finally, Welsh et al. (37) reported that transfection of PRRSV non-permissive, SIGLEC1 minus, PK-15 cells with a vector...
that expressed CD163 was sufficient for establishing PRRSV permissiveness. Since CD163 can
be released as a soluble form and taken back up into cells, it is possible that CD163 could bind
and internalize virus (as discussed in (35)). Together with the results presented in the current
work, the previous observations place into question the importance of SIGLEC1 as the primary
receptor for PRRSV.

Presumably, the entry of PRRSV into MARC-145 cells is independent of SIGLEC1 (13).
Therefore, a MARC-adapted virus would lose the requirement for SIGLEC1, perhaps through
increased affinities for heparin sulfate and/or other receptors on the MARC cell surface. In fact,
repeated passage of PRRSV on MARC-145 leads to increased virus yield on MARC-145 cells,
loss of the ability of the virus to replicate in macrophages and attenuation of clinical signs in
pigs. However, these phenotypic changes occur after approximately 100 serial passages in
MARC-145 cells (personal observation). The virus used in this study, KS06, was only passaged
a limited number of times in MARC-145 cells, retained the ability to replicate in macrophages
and produced clinical signs in pigs. Furthermore, VR-2333, the prototypic representative for
genotype II North American PRRSV isolates retains the requirement for SIGLEC1 (36), even
though it was originally isolated and passaged on a monkey kidney cell line, from which the
MARC-145 cell line was derived. Therefore, it is unlikely that the virus used in this study would
be sufficiently MARC-145 cell adapted to lose a requirement for SIGLEC1.

Conversely, the serial passage of PRRSV on macrophages should increase the tropism of the
virus for macrophages, increase the dependence of the virus on SIGLEC1 for binding and
internalization and produce enhanced clinical disease signs. There is no documented evidence
that demonstrates enhanced pathogenesis of PRRSV after propagation in macrophages.
SIGLEC1 participates in a variety of macrophage-associated immune functions. The immune response against several sialic acid-containing pathogens is initiated by the interaction of sialic acid with SIGLEC1 (reviewed in (21, 25)). Furthermore, SIGLEC1 modulates CTL and B cell function. In SIGLEC1 (-/-) mice, there are increased CD8⁺ cells in spleen and lymph nodes and a corresponding decrease in B220⁺ B cells. In addition the level of circulating IgM were reduced by 50%. De Baere et al. (7) reported that the engagement of SIGLEC1 by PRRSV significantly reduced the capacity of macrophages to phagocytize fluorescent beads. The degree of inhibition was dependent on the amount of virus present, and the effect of PRRSV on phagocytosis was blocked by the pre-incubation of cells with a SIGLEC1 antibody. (26).

Even though PRRSV replication was unaffected by the absence of SIGLEC1, there was the possibility that PRRSV-specific pathogenesis could be altered during infection of SIGLEC1⁻/⁻ pigs. SIGLEC1 knockout pigs showed lower levels of PRRSV N protein-specific IgM and IgG in serum; however, the differences were not statistically significant (see Figure 6 C and D). Furthermore, knocking out SIGLEC1 expression did not appear have a measurable effect on the overall clinical signs or disease progression. Even though the absence of SIGLEC1 expression on macrophages had no apparent effect on PRRSV infection or pathogenesis, the availability of a SIGLEC1 knockout pig creates the opportunity to study the role of SIGLEC1 in porcine immunology and response to other infectious diseases.

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Figure 1. Proposed steps for the attachment, internalization and uncoating of PRRSV. In step 1, virus permissive macrophages co-express SIGLEC1 and CD163. The initial binding step is through SIGLEC1 with sialic acid on GP5/M heterodimer in a clathrin coated pit (steps 2 and 3). The engagement of the GP2/3/4 heterotrimer combined with the acidification of the endosome results in uncoating and release of the viral genome (steps 4 and 5).

Figure 2. SIGLEC1 knockout strategy. (A) shows the organization of porcine SIGLEC1, which contains 21 exons and spans approximately 20 kb (GenBank accession no. CU467609). (B) illustrates the targeting construct used for homologous recombination. The primer sequences for PCR amplification and cloning are labeled (F) and (R). The ‘upper arm’ DNA fragment is ~3.5 kbp upstream of exon 1 and includes part of exon 1 (after the start codon). The sialic binding domain is located in exon 2. The ‘lower arm’ DNA fragment includes exons 4, 5, 6 and most of intron 6. Most of exon 1 and all of exons 2 and 3 were substituted with a neomycin (neo) cassette under the control of a PGK promoter. A thymidine kinase (TK) cassette was available immediately downstream of the lower arm but was not used for selection. Three in frame stop codons (sss) were introduced into the end of the upper and lower arms by including them in the antisense and sense PCR primers used to amplify the region. The bottom figure (C) shows the mutated SIGLEC1 gene after homologous recombination. The horizontal arrows show the location of PCR primers used for screening.

Figure 3. PCR screening of wild-type and targeted SIGLEC1⁻/⁻ alleles in transgenic founder pigs. PCR primers, “c” and “d” (Red arrows in Figure 2) were used to amplify genomic DNA.
from the eight founder pigs, derived from the male 4-18 clone. Panel A shows DNA from KW2 
cells (the initial cells used for transfection), the targeting plasmid, the targeted cells 4-18 (note 
the two bands, ~2,400 and ~2,900 bp), a non-targeted fibroblast and water blank as a negative 
PCR control. Arrow shows the location of a faint 2,900 bp band for the 4-18 clone. Panel B 
shows the results for eight F0 transgenic pigs. Note the presence of two bands (~2,400 and 2,900 
bp) for each piglet. A wild-type 4-18 clone, 11-1 and targeting plasmid show only a single band. 
Standard is shown on the right side of Panel B in kilobases (kbs).

Figure 4. Southern blot identification of knockout pigs in F2 litter #52. The upper arrow 
points to the location of the wild-type band (7,892 bp), while the lower arrow identifies the 
predicted location of the gene knockout (7,204 bp). Molecular size standards are shown (STD). 
In addition to the SIGLEC1⁻/⁻ (-/-) pigs, examples of wild-type (+/+), and heterozygous (+/-) pigs 
and are depicted.

Figure 5. Expression of SIGLEC1 (CD169) and CD163 on the surface of PAM cells. Fresh 
PAM cells were stained for CD169 (mAb 3B11/11) or CD163 (mAb 2A10/11). PAM cells 
stained with only FITC-conjugated goat-anti mouse IgG were included as a background control.

Figure 6. Viremia and antibody responses following infection with PRRSV. Pigs, at 2-3 
weeks of age, were challenged with North American PRRSV isolate, KS-06. Serum samples we 
analyzed for the presence of virus by virus isolation (panel A) and RT-PCR of viral nucleic acid
(panel B). PRRSV N protein-specific IgM (panel C) and IgG (panel D) were measured using fluorescence microsphere immunoassay (FMIA).