

## Development and application of a porcine specific ELISA for the quantification of soluble CD163

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### ABSTRACT

The cellular marker CD163 is a type 1 transmembrane scavenger protein found either on the surface of antigen-presenting cells or in a soluble form (sCD163), released in response to inflammation. Despite an obligatory role in porcine reproductive and respiratory virus (PRRSV) infection, information on sCD163 as a biomarker of disease outcome in swine remains limited. In the present study, we developed a sandwich ELISA using an anti-bovine CD163 antibody, LND68A, in conjunction with the porcine specific 2A10/11 antibody. The ELISA demonstrated that CD163 shedding from porcine alveolar macrophages increased following in vitro exposure to lipopolysaccharide or PRRSV-2 strain NVSL 97–7895. Evaluation of serum sCD163 in healthy feeder pigs identified a significant age effect with concentration rising after birth to a peak at day 19 ( $P < 0.05$ ) followed by a sharp decline to a minimal level of detection at 9 weeks of age ( $P < 0.05$ ). Healthy sows showed substantial variation but no significant change in average concentration between early and late lactation. The serum concentration of sCD163 from pigs with homozygous gene edits disrupting translation of the CD163 protein was below the threshold of detection. However, when reformatted as a competitive ELISA the assay identified an interfering substance consistent with the release of a truncated form of the CD163 protein in sera from gene edited animals. With sCD163 shown to be both dynamic and responsive, the described ELISA represents a novel tool for investigation of this molecule as a potential biomarker of disease response in the pig.

### 1. Introduction

CD163 is a cysteine-rich cell surface receptor found primarily on monocytes, macrophages (Buechler et al., 2000) and specific subsets of dendritic cells (Maniecki et al., 2006). Under normal circumstances, this scavenger type receptor plays a vital role in the clearance of otherwise toxic hemoglobin released during the natural turnover of red blood cells or following injury (Kristiansen et al., 2001). The protein has also been shown to function as an innate bacterial immune sensor, capable of inducing pro-inflammatory cytokine production following the binding of either gram positive or negative bacteria (Fabriek et al., 2009). Unfortunately, the accessibility of CD163 on the cell surface combined with its apparent capacity for internalization of bound ligands has been co-opted by pathogens such as HIV to increase the efficiency of viral replication within target cells (Tuluc et al., 2014).

This interaction between viruses and CD163 is of particular importance in swine, where it has been extensively studied in relation to pathogens such as African swine fever virus (ASFV) and porcine reproductive and respiratory syndrome virus (PRRSV). Both viruses have a devastating impact on the swine industry with ASFV causing a lethal hemorrhagic fever and PRRSV causing a combination of reproductive failure, increased pre-weaning mortality, respiratory disease, immune suppression and reduced growth performance (Lunney et al., 2016). In the case of ASFV, viral replication is a corollary of cellular CD163 abundance (Sanchez-Torres et al., 2003), but the virus has not been shown to be critically dependent on CD163 to initiate infection (Popescu et al., 2017). In contrast, PRRSV is not only directly dependent on the cellular abundance of CD163 (Wang et al., 2018), but strictly requires the protein to initiate a productive infection (Van Gorp et al., 2008). This strict requirement for CD163 during PRRSV infection

**Abbreviations:** sCD163, soluble CD163 protein; PRRSV, porcine reproductive and respiratory virus; ASFV, African swine fever virus; PAMs, porcine alveolar macrophages; NH, non-hemolyzed; LH, low level hemolysis; HH, high degree of hemolysis; Het, heterozygous for genetic modification of CD163 gene; Homo, Homozygous for genetic modification of CD163 gene; Tr, edited version of CD163 gene resulting premature protein truncation; Fl, full length, unedited version of CD163 gene; TBS, tris-buffered saline

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combined with the overwhelming impact of PRRSV on the swine industry has resulted in a number of independent efforts to target this protein using gene editing (Burkard et al., 2017; Prather et al., 2017; Wells et al., 2017; Whitworth et al., 2016; Yang et al., 2018). Although these edits have resulted in either partial (Wells et al., 2017), or complete (Burkard et al., 2017; Prather et al., 2017; Yang et al., 2018) resistance to viral infection, both the side effects of these edits and the potential for introduction of these animals into the human food chain remain an open question.

In addition to the well described functionality of CD163 when anchored in the cell membrane, the extracellular region of the molecule is proteolytically cleaved from its membrane anchor by metalloproteases such as ADAM17 (Etzerodt et al., 2010; Guo et al., 2014). This mechanism of cleavage, which has been shown to be activated in response to inflammatory signals, results in the release of the extracellular region as a soluble form (sCD163). Although the complete functional role of sCD163 has not been determined, it is widely considered to be a marker of macrophage activation and has been extensively studied as a biomarker for a variety of human conditions ranging from insulin resistance (Parkner et al., 2012) to cancer progression (Andersen et al., 2014). In swine, a CD163 ELISA based on a non-commercialized monoclonal antibody (Pérez et al., 2008) has been used to demonstrate increased activity of this shedding mechanism following challenge with either ASFV (Cabezón et al., 2017) or *Haemophilus parasuis* (Costa-Hurtado et al., 2013). More recently, commercial porcine CD163 ELISAs have become available (Burkard et al., 2017), however, their utility for large scale testing is limited by their high per sample cost. As a result, information on the potential utility of serum sCD163 as a biomarker for industry relevant traits remains limited, owing in large part to the lack of a low cost, sensitive and specific assay capable of quantifying the porcine protein in large populations.

Herein, we describe the development and validation of an ELISA protocol for quantification of porcine sCD163 based on a commercially available pair of monoclonal antibodies shown to efficiently recognize the porcine protein. We then employed this assay to evaluate: 1) in vitro shedding of sCD163 by porcine alveolar macrophages (PAMs) in response to lipopolysaccharide and PRRSV-2 infection, 2) the natural variability in the abundance of sCD163 in postnatal and lactating animals, and 3) the level of sCD163 in serum from animals following targeted gene editing of CD163.

## 2. Methods

### 2.1. Porcine alveolar macrophages collection

PAMs were collected post mortem by bronchoalveolar lavage of 4–8 week old pigs. Cells were isolated from lavage fluid by centrifugation at  $500 \times g$  for 15 min, followed by purification on a Ficol (GE Healthcare) density gradient for 45 min at  $1000 \times g$ . The resulting buffy coats were collected, washed three times and then quantified with a hemocytometer. To evaluate induced in vitro shedding of CD163,  $1 \times 10^6$  PAMs from 8 week old pigs ( $N = 13$ ) were cultured in triplicate with either LPS (from *E. coli* O127:B8 at 1  $\mu\text{g}/\text{ml}$  = LPS low, 5  $\mu\text{g}/\text{ml}$  = LPS high) or PRRSV-2 strain NVSL 97–7895 (MOI 0.025 = PRRSV low, MOI 0.25 = PRRSV high) and the supernatant collected for quantification at 24 and 48 h of culture. For the purpose of generating PAM cell lysates, aliquots of cells were pelleted at 500 g, the supernatant completely removed, and the pellets lysed at  $5 \times 10^7$  cells per ml in RIPA buffer containing a protease inhibitor cocktail (Roche).

### 2.2. Porcine serum collections

To determine the impact of sample hemolysis on the measurement of serum sCD163, triplicate blood samples were collected by jugular puncture from eight-week old pigs into plain vacutainers, clotted at room temperature for a minimum of 30 min, and the serum separated

by centrifugation at  $1000 \times g$ . Non-hemolyzed (NH) serum was separated from one tube and the remaining serum either continuously vortexed at high speed for 3 min to induce low level hemolysis (LH) or partially frozen at  $-20^\circ\text{C}$  for 30 min to induce a high degree of hemolysis (HH). To evaluate the impact of repeated freeze-thaw cycles, serum from additional 8-week-old pigs ( $N = 5$ ), was freshly aliquoted and subjected to as many as 10 sequential cycles of rapid freezing at  $-80^\circ\text{C}$  and slow thawing at room temperature. To determine the level and natural variation of sCD163 in feeder pigs, archived sera collected from neonates at birth ( $N = 17$ ), 19 days, 9 weeks and 15 weeks of age were used. Archived sera from sows ( $N = 9$ ) collected twice during lactation at days 5 and 19 relative to farrowing were used. Finally, sCD163 levels were assessed in a set of serum samples from pigs either Heterozygous (Het,  $N = 7$ ) or Homozygous (Homo,  $N = 13$ ) for Crispr/Cas9 edits to the CD163 gene known to cause premature protein truncation (Tr) (Prather et al., 2017; Whitworth et al., 2016), or their wild type (WT,  $N = 14$ ) full length (Fl) counterparts (generously provided by Dr. R. Rowland, Kansas State University). The collection of blood samples from live pigs was approved by the University of Saskatchewan Animal Research Ethics Board (protocols 20070046, 20060020, 20150035) in accordance with guidelines provided by the Canadian Council for Animal Care.

### 2.3. Western blot analysis

Seventy  $\mu\text{g}$  of total protein from porcine alveolar macrophages (PAMs) was separated on an 8% acrylamide gel under non-reducing or reducing conditions, with 0.2  $\mu\text{g}$  of recombinant, full length and eukaryotic expressed, human CD163 (R&D Systems, 1607-CD-050) used as a control. Proteins were electroblotted to nitrocellulose membranes (BioRad Laboratories) using a semi-dry system (Pierce) onto a nitrocellulose membrane (BioRad). Blots were blocked in 1% (w/v) bovine serum albumin (BSA) in tris-buffered saline (TBS), separated into strips and incubated overnight at  $4^\circ\text{C}$  with 1  $\mu\text{g}/\text{ml}$  of either mouse anti-CD163 monoclonal antibodies derived from clones 2A10/11, ED-Hu, ED2 (BioRad Antibodies), 215930 (R&D systems), LND68A (WSU Monoclonal Antibody Center) or a 1:20 dilution of cell culture supernatant from 2H12BM (Dr. Ezquerro, INIA). Blots were washed in TBS with 0.5% tween and incubated in 1 mg/ml goat anti-mouse Alexa-647 (ThermoFisher Scientific) for 3 h at room temperature followed by imaging on a Typhoon Trio (GE Life Sciences).

### 2.4. Phylogenetic analysis

Full length CD163 protein sequences from all species from which tested antibodies were either generated against, or known to cross react with, were obtained from the RefSeq protein database. In species where multiple isoforms were known to exist, the primary full length isoform was selected for analysis. Multiple sequence alignment was carried out with Clustal Omega (Sievers et al., 2011) and a phylogenetic tree constructed using the neighbor-joining method (Saitou and Nei, 1987).

### 2.5. Sandwich ELISA development

Flat bottom Immulon IV extra high binding plates (Thermo Scientific) were coated overnight at  $4^\circ\text{C}$  with 1  $\mu\text{g}/\text{ml}$  mouse anti-CD163 specific monoclonal (Clone LND68A, WSU Monoclonal Antibody Center) in 0.05 M carbonate/bicarbonate buffer pH9.6. All washing steps were carried out with TBS + 0.05% v/v Tween 20 and blocking was achieved with 0.1% BSA in wash buffer for 30 min at room temperature. Binding of sCD163 from samples was carried out at room temperature for 2.5 h. A biologically relevant standard curve was generated from a pool of PAM lysates derived from six animals, serially diluted to produce a five-point standard curve ranging from  $0.2 \times 10^5$  to  $3.3 \times 10^5$  cells/ml equivalent. Interplate controls in the form of two unique pools of porcine serum were included on each plate. Mouse anti-

CD163-specific monoclonal (clone 2A10/11, BioRad Antibodies) was biotinylated with EZlink Sulfo-NHS-biotin (ThermoFisher Scientific), buffer exchanged on a 10 kDa Vivaspin 2 column (GE Healthcare) and used as a detection antibody at 0.5 µg/ml for 2.5 h at room temperature. Colorimetric detection was achieved through the binding of Streptavidin-HRP (R&D Systems) followed by the addition of TMB substrate (Bethyl). The enzymatic activity was stopped with 0.18 M H<sub>2</sub>SO<sub>4</sub> and absorbance was assessed at 450 nm using a microplate reader (Molecular Devices) with quantity relative to PAM standard curve calculated using the associated software. Intra-assay variation was calculated as the average percent variation between replicates for all samples. Inter-assay variation was determined using a pair of inter-plate controls included with every assay run.

## 2.6. Competitive binding ELISA Development

ELISA plates were coated with mouse anti-CD163-specific monoclonal (Clone LND68A) and blocked as described above. A pooled PAM cell lysate was diluted in sample buffer to make a working stock with a concentration of CD163 equivalent (eq) to  $1.25 \times 10^5$  PAM cells/ml. Serum samples from WT, Het, and Homo gene edited pigs were then diluted 1:10 in this working stock before applying to the ELISA plate. The remainder of the assay was carried out as described above and the apparent sCD163 concentration assessed relative to the signal obtained from a non-competitive control (sample buffer diluted into working stock in place of sera).

## 2.7. Statistics

Statistical analysis was performed in R (Team, 2018) using a mixed model from nlme package (Pinheiro et al.) with animal as a random variable to account for repeated measures where appropriate. Pairwise comparisons were conducted using the emmeans (Lenth, 2018) package with Tukey's correction for multiple testing. The estimated model means (lsmeans) and standard error were graphed using the ggplot2 (Wickham, 2016) package, with observed statistical differences ( $P < 0.05$ ) marked by unique superscripts.

## 3. Results

### 3.1. Antibody selection and ELISA development

Although a wide array of monoclonal antibodies raised against CD163 from various species are presently available, information on their potential cross reactivity and/or specificity with the porcine protein is often lacking. In an effort to identify an appropriate matched antibody pair for use in the porcine CD163 ELISA, we tested six different monoclonal antibodies by western blot analysis (Fig. 1A). Varying degrees of reactivity with the porcine protein by antibodies derived from four clones including 2A10/11, LND68A, ED-Hu1 and 2H12BM were observed, while ED2 and 215930 were unable to recognize the porcine protein at a detectable level. As expected, the recombinant human protein was recognized by both antibodies developed against the human protein, ED-Hu1 and 215930, but was also recognized by 2H12BM and LND68A. Antibody from clones 2A10/11, LND68A and ED-HU1 were also evaluated for reactivity with PAM cell lysate and recombinant human CD163 separated under reducing conditions, with weak detection for both samples only with ED-HU1 antibody (Fig. 1B). Two distinct proteins with similar molecular weights were consistently detected in PAM cell lysate by antibodies showing reactivity with the porcine sequence under non-reducing conditions, and no other proteins species indicative of non-specific binding were found. Phylogenetic comparison of the protein sequences for CD163, from species for which at least one of the six antibodies have been shown to interact, revealed a close relationship between the porcine, human and bovine sequences (Fig. 1C) consistent with the results of the

western blot analysis. Based on these results, LND68A and 2A10/11 were chosen to act as the capture and detection antibodies respectively, with their working concentrations for use in a sandwich ELISA established by checkerboard titration (data not shown).

The lack of reactivity between 2A10/11 antibody and the commercially available recombinant human CD163 protein (Fig. 1A) necessitated the development of an alternative standard. We initially evaluated a series of individual cell number normalized PAM cell lysates that revealed considerable natural variation between animals (Fig. 2A). To reduce the impact of this variation, a pool of PAM cell lysates from seven animals was generated and used for all subsequent experiments. Intra-assay variation, defined as the average percent variation between replicates for all samples was 2.7%. Inter-assay variation, as determined using two inter-plate controls (moderate low and moderate high pooled serum) run on each plate was found to be 12.1% across 10 plates. Although the inter-assay variation was found to be moderately high it remains below the level that would preclude the detection of biologically relevant differences in sCD163.

To determine the impact of sample quality on the quantitative accuracy of the ELISA for sCD163 in porcine serum, we first evaluated the impact of pre-separation hemolysis (Fig. 2B). Although a minor but significant increase in LH samples, as measured by sample OD<sub>450</sub> relative to NH, could be generated by aggressive mixing of blood samples, the observed numerical decrease in the apparent sCD163 concentration was not significant. In contrast, the HH samples, with severe hemolysis produced by partial freezing prior to serum separation resulted in a marked increase in raw sample OD accompanied by a significant decrease in apparent sCD163 concentration. Finally, we evaluated the impact of repeated freeze thaw cycles on the quantification of sCD163 in serum and found no significant change in quantity across 10 freeze-thaw cycles (Fig. 2C).

### 3.2. In vitro shedding of CD163 from PAMs

To demonstrate the utility of the porcine CD163 ELISA, we evaluated the concentration of this molecule in the cell culture supernatant from PAMs at 24 and 48 h after isolation (Fig. 3A). Unstimulated PAM cells released sCD163 into culture media, with the concentration rising in cell culture supernatant between 24 and 48 h. With the addition of a proinflammatory stimulant in the form of LPS at 1 µg/ml, we observed a numerical but non-significant increase in CD163 levels in culture supernatant relative to the unstimulated control, whereas inclusion of LPS at 5 µg/ml resulted in a significant increase at both 24 and 48 h relative to the respective control. To determine if shedding increased during PRRSV infection, PAMs were inoculated with virus strain NVSL 97-7895 at two MOIs (MOI 0.025 = PRRSV low, MOI 0.25 = PRRSV high). At 24 h, a significant increase in sCD163 was observed for both low and high PRRSV infection whereas at 48 h, only the high level PRRSV infection was significantly different relative to the time matched control. Collectively, the data show shedding in response to LPS and PRRSV is both dose and time dependent.

### 3.3. Natural variation in sows and postnatal pig

To characterize the degree of natural variation in serum sCD163 levels between animals and during key stages of development, the assay was applied to archived serial sera from normal healthy pigs. First, serum samples from a cohort of pigs at 4 time points between birth and 15 weeks of age were analyzed (Fig. 3B). In piglets, levels of sCD163 were  $2.9 \times 10^5$  PAM eq at birth and significantly increased thereafter to reach peak levels at 19 days of age before dropping to a minimal level 9 and 15 weeks after birth. To evaluate the role of the sow in this temporal pattern, we next assessed serially collected sera from sows at 5 and 19 days of lactation but found no significant change during this period (Fig. 3C). While these two sets of serum samples were the product of separate collections, the observed concentration of serum

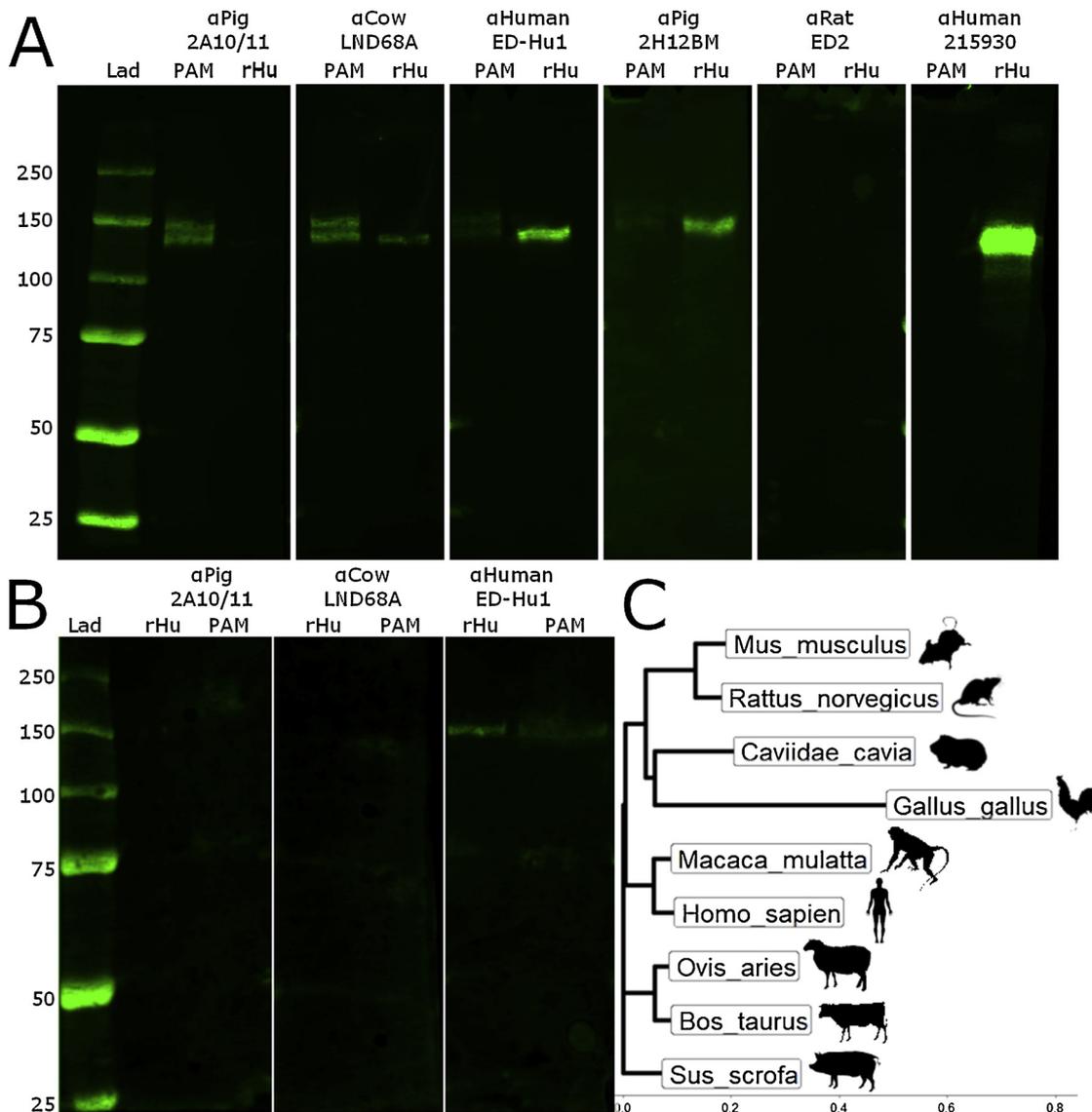


Fig. 1. Evaluation of antibody cross reactivity under: A) non-reducing and B) reducing conditions in western blot analysis of CD163 in porcine alveolar macrophage (PAM) lysate and of recombinant human (rHu) CD163. Monoclonal antibodies raised against porcine (2 A10/11 & 2H12BM), bovine (LND68 A), human (ED-Hu1 & 215,930) and rat (ED2) CD163 proteins were utilized. C) Phylogenetic comparison of target proteins for species known to be recognized by at least one antibody used in the study.

sCD163 in lactating sows was more consistent with the range observed in neonatal pigs compared to 9 or 15 week old pigs. The lack of a significant change between 5 and 19 days of lactation in sows suggests the observed increase in suckling pigs was not associated with maternal factors.

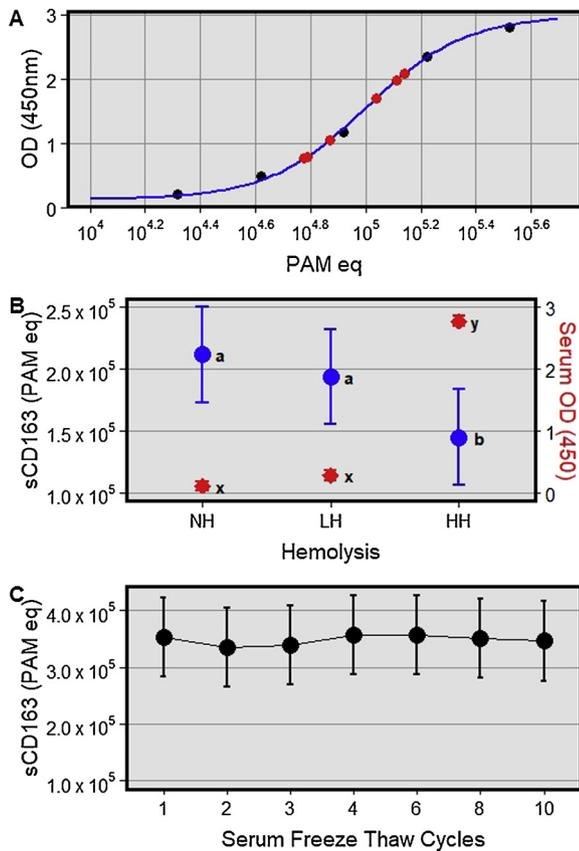
### 3.4. sCD163 levels in gene edited pigs

To further validate the ELISA protocol, sera from pigs either Homo or Het for Crispr/Cas9 edits to exon 7 of the CD163 gene, along with matched WT controls was obtained. When tested using a standard sandwich ELISA protocol (Fig. 4A), all samples obtained from animals with homozygous edits for sCD163 were below the threshold of detection regardless of sample dilution. The levels among heterozygous animals were detectable but significantly reduced relative to the wild-type control. Perhaps most interesting, the sCD163 concentration in the serum of heterozygous animals was found to be far lower than expected when compared to the wild type controls suggesting some form of interference in the assay. Based on the understanding that the genetic edits performed on these animals resulted in premature truncation of

the protein during translation, the ELISA protocol was altered to create a competitive binding assay to reassess these serum samples (Fig. 4B). As expected, the combination of wild type serum and PAM cell lysate showed significantly higher CD163 levels relative to the lysate alone. In contrast, the addition of serum from either the heterozygous or homozygous animals significantly decreased the observed concentrations. Based on these results, and the format of the ELISA assay, we conclude that the truncated protein produced by the gene edited pigs is present in sera and competes for binding sites on the LND68A coated plates but is not recognized by the 2A10/11 antibody used for detection.

## 4. Discussion

The primary goal of this research was to develop a porcine specific ELISA for the quantification of CD163 in sera and other experimental samples utilizing commercially available antibodies. To this end, we evaluated a variety of existing monoclonal antibodies and demonstrated that the anti-bovine CD163 antibody derived from hybridoma clone LND68A (Elnaggar et al., 2016) recognized the porcine protein



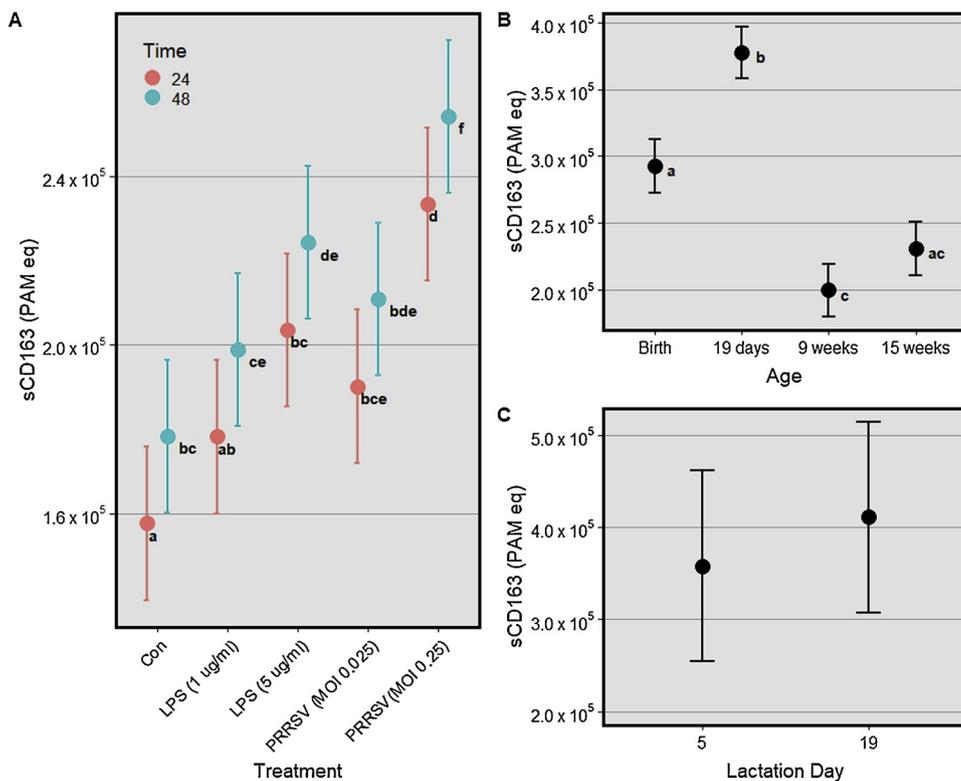
**Fig. 2.** A) Representative five-point standard curve generated from pooled PAM lysate (black) with individual cell lysates from  $N = 6$  animals (red) demonstrating inter-animal variation. B) Impact of sample hemolysis on sCD163 measurement (blue) in relation to serum sample OD at 450 nm (red). Replicate serum samples from animals ( $N = 8$ ) either immediately separated (NH; no hemolysis) or pre-treated to induce Low (LH) or High (HH) levels of hemolysis via prolonged agitation or sample freezing, respectively. C) Impact of repeated freeze-thaw cycles of serum samples on sCD163 measurement from animals ( $N = 5$ ). CD163 quantities are presented in the form of  $\text{lsmeans} \pm \text{SE}$  of PAM cell equivalents (PAM eq) with statistical differences ( $P < 0.05$ ) noted with unique subscripts. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

with an affinity comparable to that of the widely used anti-porcine 2A10/11. The cross reaction of the LND68A antibody is perhaps unsurprising given both the high degree of sequence similarity observed in our phylogenetic analysis and the known cross reaction of 2A10/11 with the bovine sequence (Hussen et al., 2013). The anti-human ED-Hu1 and anti-porcine 2H12BM were also both shown to recognize the porcine protein; however, neither showed particularly high affinity by western blot analysis. Other monoclonal antibodies have previously been generated against (Pérez et al., 2008) or shown to cross react with the porcine sequence (Saalmuller et al., 2005), but 2A10/11 and LND68A were chosen for development of the ELISA due to their commercial availability along with apparent high affinity and specificity. Interestingly, all 4 antibodies shown in this study to cross react with the porcine protein detected two distinct protein species of similar molecular weight under non-reducing conditions. A smaller paralog of CD163, CD163 like 1, has been identified in the pig, but is known to be unrecognized by 2A10/11 (Van Gorp et al., 2010), suggesting the observed dual banding pattern in the result of transcript variants from porcine CD163 gene. One alternative variant of porcine CD163 lacking SRCR domains 1 through 5 has previously been described in an artificially immortalized PAM cell line (Wang et al., 2018), though such a large deletion from the protein would result in a molecular weight roughly a third of what was observed. On the other hand, transcript

variants resulting in more similar isoforms differing only in their C-terminal regions, have been sequenced in both mice (NM\_001170395 and NM\_053094) and humans (NM\_004244 and NM\_203416). While only a single mRNA has actually been sequenced in the pig, the current genome annotation includes a prediction of similar transcripts (XM\_021091120, XM\_021091121, XM\_021091122 & XM\_021091123), missing either portions of the C or N terminal regions, all of which could conceivably result in the second band observed. More importantly, the dual reactivity of both antibodies used in our assay means it does not distinguish between these apparent isoforms, but rather measures total CD163 in samples.

As with many analytical techniques, the quantitative accuracy of immune assays can be compromised by sample quality or handling (Boadella and Gortázar, 2011). Prior to application we attempted to determine the limitation of our assay with respect to two of the most common factors known to influencing serum sample quality: hemolysis and repeated freeze-thaw cycles. In some cases, assay interference due to sample hemolysis has been determined through the direct addition of hemoglobin to samples (Simoni et al., 1995); however, this does not account for potential interference from the plethora of other cellular compounds and components released during lysis of erythrocytes. An alternative approach is to add an erythrocyte lysate to samples, which has been used to demonstrate both positive and negative interference in an assay specific manner (Ji and Meng, 2011). Similar to this approach, hemolysis in our experiments was generated in replicate samples by either prolonged agitation or pre-separation freezing in an effort to mimic the typical issues encountered during on-farm sample collection. Severe hemolysis induced by freeze/thaw was found to significantly reduce the measurable level of sCD163 in samples demonstrating a negative impact of sample hemolysis on the accuracy of the assay. Although the hemoglobin released in this process is a well-established ligand of the CD163 receptor, the interaction between the two is dependent on the presence of haptoglobin (Kristiansen et al., 2001). While the specific interfering substance remains unknown, all subsequent assays were conducted on samples with minimal evidence of hemolysis. Freeze-thaw cycles have also been shown to significantly alter the results obtained from some immunoassay procedures (Sgoutas and Tuten, 1992); however, the degree of impact has been shown to be assay dependent (Lee et al., 2015). In the case of our CD163 ELISA, we found no significant impact of freeze-thaw cycles, which is consistent with the human sCD163 ELISA (Wang et al., 2015). While the antibodies used in these two assays likely target different epitopes, the results suggest that the CD163 protein is reasonably stable under normal conditions.

PRRS remains one of the most significant challenges facing the global swine industry, and while significant natural variation in host response to infection is known to exist (Reiner, 2016), there are currently no known biomarkers of disease resistance on which to base genetic selection. The ability of the PRRSV to efficiently infect and replicate in host cells has been shown to be dependent on the level of full length CD163 expressed the surface of target cells (Wang et al., 2018; Zhu et al., 2014). Combined with the knowledge that the level of sCD163 in serum is inversely correlated with the amount of CD163 found on expressing cells (Davis and Zarev, 2005), there is potential for an indirect link between the concentration of sCD163 and the response to infection of an individual animal. Furthermore, a fusion protein formed from the extracellular region of CD163 and porcine FC has been shown to bind PRRSV and limit replication when expressed in vitro (Chen et al., 2014) and in vivo (Xia et al., 2018), opening the possibility for sCD163 to directly inhibit viral replication. In the present research, we not only observed substantial variation in serum sCD163 among healthy animals but also demonstrated increased in vivo shedding of CD163 by PAM following infection with PRRSV. Based on the combination of these results, further investigation into the relationship between response to PRRSV infection and serum sCD163 levels, either prior to or during challenge, is warranted. Serum sCD163 levels were stable within a lactation in sows; however, levels varied significantly in



**Fig. 3.** Application of the porcine CD163 ELISA to: A) cell free culture media obtained after 24 and 48 h of porcine alveolar macrophage (PAM) culture. Cells were derived from 8 week old pigs (N = 13) and cultured with either media alone (Con), LPS at low (1 ug/ml) and high (5 ug/ml) concentrations or inoculated with PRRSV at low (0.025) or high (0.25) MOI. B) sCD163 in serum serially collected from feeder pigs (N = 17) at birth, 19 days, 9 weeks and 15 weeks of age. C) sCD163 in serum from sows (N = 9) collected at either day 5 or 19 of lactation. CD163 quantities are presented in the form of lsmeans ± SE of PAM cell equivalents (PAM eq) with statistical differences ( $P < 0.05$ ) noted with unique subscripts.

young feeder pigs from birth to 15 weeks of age, which is potentially the result of neonatal immune activation brought about by exposure to the extra-uterine environment. Regardless of the underlying cause, this result would suggest that the rate of CD163 shedding may not be stable across all stages of the pig production cycle. It will therefore be necessary to establish both the temporal variation across the production cycle and the stability of an individual phenotype over time.

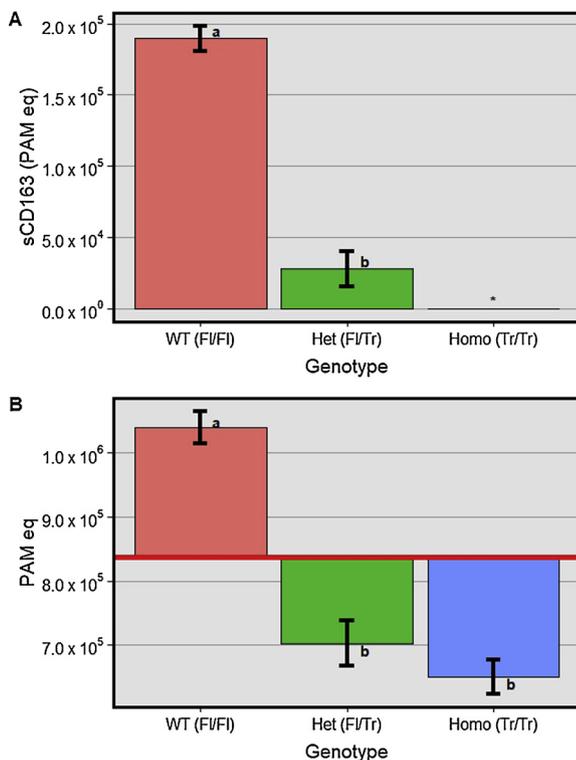
Owing to its obligatory role of CD163 in PRRSV infection a number of research groups have targeted genes for editing in an effort to create transgenic pigs immune to the PRRS virus (Burkard et al., 2017; Prather et al., 2017; Yang et al., 2018). The particular gene-edited pigs used in this study were the product of Crispr/Cas9 targeting of exon7 encoding the SRCR domain 5 (Whitworth et al., 2016), thought to be the sole binding site to interact with the PRRSV (Van Gorp et al., 2010). This approach resulted in a variety of indels all of which altered the reading frame and result in premature truncation of the CD163 protein during translation (Whitworth et al., 2016). Regardless of which of these specific gene edits was used, the resulting protein was predicted to include only the signal peptide and the first 4 SRCR domains. Interestingly, near full length versions of porcine CD163 lacking only the C-terminal transmembrane and intracellular regions have previously been shown to accumulate in the cytoplasm (Das et al., 2010). The results of our initial assessment of sera from these gene edited animals is consistent with this previous finding. However, when the protocol was modified to act as a competitive binding assay, the results revealed an inhibition consistent with a truncated form of the protein in sera recognized by the capture but not the detection antibody. Based on recognition of truncated or domain swapped clones of the porcine CD163 protein, the 2A10/11 antisera was proposed to bind an epitope located in domains 1–3 (Van Gorp et al., 2010). However, the sensitivity of this particular antibody to reducing conditions suggests a structural epitope that may be sensitive to protein misfolding in altered versions of the protein. Thus, the lack of recognition by 2A10/11 of the edited form of CD163 assayed in this experiment could presumably be conferred by either premature truncation prior to the target epitope or disruption of epitope secondary structure. In contrast, the apparent ability of the

LND68A capture antibody to maintain recognition of the edited porcine CD163 limits the location of its epitope to the four N-terminal domains encoded by exons prior to the Crispr/Cas9 editing target. Should these particular CD163 knock out pigs be brought to market, the differential recognition of these antibodies employed in a competitive binding format may represent a simple and cost-effective method for distinguishing between wild type and genetically edited swine.

The CD163 gene edited animals used in this study are widely referred to in the literature as knockouts. While this descriptor clearly meets the definition, in terms of rendering the gene inoperative in its functional role at the cell surface, the presumptive truncated form we observed in sera may still possess some functionality. For instance, the interaction between CD163 and hemoglobin-haptoglobin complexes is known to depend on electrostatic interaction with SRCR domains 2 and 3 (Nielsen et al., 2013), both of which would remain in the truncated form. Serum sCD163 has also been shown to sequester the proinflammatory cytokine TWEAK, reducing its biological activity by acting as a decoy receptor (Akahori et al., 2015). More recently an association between the level of serum sCD163 and fatty acid metabolism in adipose tissue has been identified (Rittig et al., 2017), and while no causative link has been identified it suggests the possibility for additional functionality as a signaling molecule. In spite of widespread study, the complete physiology of sCD163 is not yet well described but the presence of a truncated form of the protein in circulation as reported herein, combined with the recent availability of other CD163 gene edited lines (Burkard et al., 2017; Yang et al., 2018) makes the pig an intriguing model for further study of the non-cell associated functionality of this protein.

### 5. Conclusions

In this report we demonstrate functional method for quantifying CD163 based on commercially available monoclonal antibodies. Using this protocol, we have shown that the shedding of CD163 is responsive to both inflammatory stimuli and PRRSV infection, which combined with its obligatory role during PRRSV infection, makes it a high-value



**Fig. 4.** Evaluation of sCD163 levels in serum taken from pigs with either wild type (WT, N = 14) full length (F1) CD163 gene, or Heterozygous (Het, N = 7) and Homozygous (Homo, N = 13) for Crispr/Cas9 editing of the CD163 gene resulting in premature truncation (Tr) of the protein. Serum was measured by using: A) sandwich ELISA protocol or B) a competitive binding ELISA against a PAM cell lysate with results presented relative to the lysate alone (solid red line). CD163 quantities are presented in the form of lsmeans  $\pm$  SE of PAM cell equivalents (PAM eq) with statistical differences ( $P < 0.05$ ) noted with unique subscripts, \* indicates values were below the threshold of detection. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

target for use as a biomarker of susceptibility to this economically devastating disease. However, due to the temporal dynamics we observe among healthy feeder pigs, a more in-depth analysis of the natural variation in serum sCD163 across the pig producing system will be required before its value as a biomarker can be effectively determined.

#### Conflict of interest

The authors declare no conflict of interest.

#### Author's contributions

JAP, DJM and JCH planned and organized development of the ELISA and designed experiments. JAP performed assays/analysis and drafted the manuscript, all authors read and revised the final manuscript.

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