Macrophages derived from gene-edited pigs pose resistance to multiple isolates of Porcine Reproductive and Respiratory Syndrome virus

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Abstract

Porcine Reproductive and Respiratory Syndrome Virus (PRSSV) is one of the most economically important diseases in the global swine industry, costing producers an estimated $660 million annually. PRRSV is genetically diverse with a low replication fidelity, due to it being an RNA virus, resulting in multitudes of isolates being produced. This virus has a tropism for cells of the monocyte/macrophage lineage. Cluster of Differentiation 163 (CD163) is considered the primary PRRSV receptor located on porcine alveolar macrophages (PAMs). CRISPR/Cas9 technology was utilized to knock out CD163 via a frameshift mutation, resulting in pigs of the CD163 Null genotype. Also, a domain of porcine CD163 was deleted and replaced with the insertion of a CD163 homolog of human-like domain and neomycin cassette to serve as a genetic marker. This swap resulted in pigs that possessed a CD163L1 domain 8 mimic of porcine homolog human CD163-like (hCD163L-1) of SRCR domain 8. Previous work has demonstrated that CD163 Null pigs were resistant to one genotype 2 PRRSV isolate. An *in vivo* study was performed to observe whether hCD163L-1 pigs were also resistant to infection. Various diagnostic tests were performed to determine the presence or absence of PRRSV viremia levels in serum, CD163 receptor surface expression levels on PAMs, IgG antibody levels and haptoglobin (Hp) levels in serum. hCD163L-1 pigs did not support genotype 1 PRRSV replication, but were susceptible to genotype 2 PRRSV infections. In addition, *in vitro* infection experiments were performed on PAMs and macrophages derived from peripheral blood mononuclear cells (PBMCs) to determine resistance to multiple isolates. hCD163L-1 macrophages showed reduced infection with genotype 2 and no infection with genotype 1 PRRSV during *in vitro* infections. Null PAMs and PBMCs derived macrophages did not support infection towards any isolate of either PRRSV genotype.

Table of Contents

List of Figures

List of Tables

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Chapter 1 - Literature Review of the PRRS Virus, the Macrophage Receptor CD163, and Genetic Editing via the CRISPR/Cas9 System

Introduction

Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) has plagued the global swine industry since the early 1980s. It was first termed Mystery Swine Disease, as it caused a variety of clinical signs in pigs. Veterinarians were unable to determine the etiologic agent in the United States (Keffaber, 1989), and the syndrome later appeared in Europe. However, the first isolate was characterized in 1991 (Benfield *et al.,* 1992). The etiological agent was identified and sequenced in Europe in 1991 and called Lelystad virus (Wensvoort *et al.,* 1991). Clinical signs ranged from reproductive failure in breeding age females, reduced weight gain and pneumonia in post weaning pigs, and increased mortality among all age groups (Hill, 1993). Transmission occurs via contact (Willis *et al*., 1997), boar semen (Yaeger *et al.,* 1993), and as well as through fomites such as boots and coveralls (Otake *et al.,* 2002). Annual productivity losses associated with PRRSV in the U.S. were estimated to be $660 million in 2011 with 45% of the cost coming from losses in the breeding herd (Holtkamp *et al.* 2011; Neumann *et al.,* 2005).

Many to combat PRRSV have focused on vaccine development and selective mating of potential PRRSV tolerance in pigs. Both modified-live virus (MLV) and killed vaccines have been evaluated for efficacy and protection against PRRSV. MLV vaccines have been shown to provide heterologous protection against respiratory disease associated with PRRSV over killed autogenous vaccines (Roof *et al*., 1999). More recently, a MLV vaccine was shown to reduce viremia levels, PRRSV-induced lesions, and decrease nasal shedding of virus from challenged pigs (Park *et al*., 2014). However, most vaccines on the market are not completely effective at eliminating virus replication, as different responses of vaccinated animals have been observed.

Certain genotypes of pigs from various genetic lines have been observed to show a production of PRRSV-neutralizing antibodies (NA) against isolates as well as better growth and performance in mass infection studies (Rowland et *al.*, 2012). Nevertheless, genetic progress of these breeding lines with regard to tolerance via artificial selection can prove to be a timely process.

Genetic modification of animals through laboratory manipulation has been utilized for over 20 years via gene targeting with the predominant method being the use of zinc finger nucleases (ZFNs) and transcriptor activator like endonucleases (TALENS) (Capecchi, 1989). Unfortunately, these processes are time consuming have mediocre gene targeting efficiency. It was not until recently that a new and more efficient genetic editing system termed clustered regularly interspersed short palindromic repeats (CRISPR) paired with CRISPR associated protein 9 (Cas9) became available for genetic use, and has proved beneficial through quick and accurate genome editing (Jinek *et al.*, 2012).

Pigs are one of the many animal species that have undergone specific genetic manipulation through the CRISPR/Cas9 system, including the alteration of the PRRSV receptor, CD163. Genetic alteration of CD163 in macrophages of pigs has been proven successful, however many genotypes were produced (Whitworth *et al.*, 2013). A previous study demonstrated that one of the many genotypes of genetically edited pigs exhibited resistance to infection of a single PRRSV isolate (Whitworth *et al.*, 2015), however evaluation of disease resistance to the other produced genotypes as well as other isolates of PRRSV remained unknown.

The Arteriviridae Family

Arteriviridae is a family of enveloped positive-stranded RNA viruses which include Arterivirus, the only genus in the family. *Arteriviridae,* in addition to *Coronaviridae, Toroviridae, and Roniviridae,* comprise the order, *Nidovirales*. These virus families have characteristics that are distinct to each, such as *Coronaviridae, Toroviridae,* and *Roniviridae* comprising some of the largest known RNA viral genomes (23 to 26kb), while *Arterividae* RNA genomes are smaller (13-16kb). However, due to the organization and expression of their genomes, there was ample evidence to group these families under the same order. That evidence includes a nested subgenomic mRNA for expression of the 3' proximal ORFS, which encodes for the most conserved structural proteins and was the basis for the order (den Boon *et al.,* 1995; Gorbalenya *et al.,* 2006).

The Arterivirus Genus

Arteriviruses have a tropism for monocytes and macrophages of mammalian lineage and can cause persistent or subclinical infections in addition to respiratory disease in an acute phase (Snijder *et al*., 2013). Three other viruses that compose the Arterivirus genus in addition to PRRSV are equine arteritis virus (EAV), lactate dehydrogenase elevating virus (LDV), and simian hemorrhagic fever virus (SHFV) (Plagemann *et al*., 1992). Natural hosts of arteriviruses range from pigs (PRRSV), horses and donkeys (EAV), mice (LDV), and numerous genera of Asian and African monkeys (SHFV). PRRSV is categorized into two distinct genotypes, genotype 1 (termed European strain) and genotype 2 (termed North American strain), consisting of approximately 60% nucleotide identity thus allocating a wide variation of genetic diversity (Forsberg *et al*., 2002, Chen *et al*., 2011).

PRRS Virus

Economic Impact

The United States has been plagued with PRRSV in hog barns for well over 30 years. The reproductive losses associated with PRRSV infection are substantial. In 2013, it was recorded that breeding herds had reduced reproductive efficiency by 1.44 pigs weaned per sow per year. The total annual cost of productivity losses measured up to $302.06 million, equivalent to the loss of $52.19 per breeding female or $2.36 per weaned pig for the entire U.S. inventory. In 2005, the total annual losses were around $493.57 million in the growing pig population, and a loss of $66.75 million in breeding herd However just four years ago, PRRSV cost the industry $4.67 for every pig that was marketed leading to an annual cost of $663.91 million or $1.8 million per day. (Holtkamp *et al.*, 2013; Neumann *et al.*, 2005)..

Viral Organization

The PRRSV genome is comprised of positive-stranded polyadenylated RNA of about 15 kilobases in size which varies based on the isolate of the virus (Conzelmann *et al.,* 1992). The genomic RNA contains a 3'polyadenylated tail which follows the 5' cap. The untranslated region (UTR) of the 5' end has demonstrated a critical role in subgenomic RNA synthesis as a component in translational mechanisms (van den Born *et al*., 2005). The 3' poly A tail has been considered important for initiation of RNA-dependent RNA polymerase and replication (Godeny *et al*., 1993). Due to its positive stranded properties, PRRSV does not require RNA polymerase in its virion and is capable of being directly translated by the host during protein synthesis., thus not requiring RNA polymerase in the virion (Meulenberg *et al*., 1993). Ten open reading frames (ORFs) have been identified: ORFs 1a, 1b, 2a, 2b, 3, 4, 5a, 5b, 6, and 7 (Figure 1.1) (Firth *et al.,*

2011; Johnson *et al.,* 2011). Multiple ORFs perform a variety of functions and encompass the PRRSV virion (Figure 1.2)

ORF 1a and ORF 1b encode for the RNA-dependent RNA polymerase and genes coding for two large polyproteins (pp1a and pp1ab) that cleave into 14 nonstructural protein (Nsp) coding regions by a cascade for four virally encoded proteins (Fang and Snijder, 2010; Ziebuhr *et al*., 2010). The Nsps inhabit 75% of the genome and are required for virus replication. Nomenclature for these Nsp's range from Nsp 1 to Nsp 12. pp1a is thought to produce Nsp1α, Nsp 1β, and Nsp 2 to Nsp 8. Nsp1 and Nsp 7 are further broken down into Nsp 1α and Nsp 1β, and Nsp 7α and Nsp 7β, respectively. Papain-like proteases (PLPs) encoded by Nsp1α and Nsp 1β, Nsp 2, and a chromotrypsin-like serine protease encoded by Nsp 4, perform the entire polyprotein processing (den Boon *et al*., 1995; Han *et al*., 2009; Tian *et al*., 2009). Nsp 9 through 12, products of pp1ab, are associated with virus replication and transcription and are translated when the virus enters the cell. They are then processed by proteinases into at least 12 mature Nsps and intermediate precursors. These precursors and mature Nsps are potentially accountable for RNA synthesis via virus-induced double membrane vesicles (den Boon *et al*., 1995; Snijder and Meulenberg, 1998; van Dinten *et al*., 1997).

ORFs 2 through 7 encode for viral structural proteins (Allende *et al*., 1999). ORF 2a encodes GP_{2a}, a minor glycosylated envelope viral protein that is essential for virus infectivity and attachment (Wissink *et al*., 2004). Minor non-glycosylated viral envelope protein E is a product of ORF 2b. Reverse genetics utilizing EAV infectious clones have shown the potential function of protein E is required to produce infectious virions, however the exact function is still unknown (Snijder *et al*., 1999). ORF 3 encodes the minor glycosylated envelope viral protein GP_3. GP_3 is considered to be the most variable PRRSV proteins and is highly antigenic. It is also

considered to partake in viral neutralization (Cancel-Tirado *et al*., 2004; Gonin *et al*., 1998). GP_4 is the product of ORF 4. GP_4 is another minor glycosylated envelope viral protein in addition to GP_3. ORF 4 has been found to be crucial for virus replication via studies involving transfections of PRRSV permissive cells with ORF 4 deleted infectious cDNA clones (Welch *et al*., 2004). The ORF 5 gene encodes GP_5 glycoprotein and is one of the most variable regions in the PRRSV genome (Andreyev *et al*., 1997). GP_5, being one of the most important structural proteins, is a major glycosylated envelope viral protein and contains epitopes involved in protection and virus neutralization (Ansari *et al*.,2006). *In vivo*, most of the neutralizing antibodies are directed to GP_5 (Gonin *et al*., 1999) However, studies have found evidence that glycosylation of GP_5 has a role in escaping or decreasing the neutralizing antibody response by N-glycan shielding (Johnson *et al*., 2003; Wei *et al*., 2003). Additionally, GP5 appears to be involved with antibody dependent enhancement, allowing increased infection of macrophages via the fc receptor on PRRSV antibodies (Cancel-Tirado *et al*., 2004). Inducing apoptosis of the macrophage is another function of GP_5. During PRRSV infection, specific apoptotic enzymes are activated resulting in death of the cell and promoting the release of more PRRSV (Gagnon *et al*., 2003; Suarez *et al*., 1996). ORF 6 encodes the M membrane envelope protein and is one of the most conserved structural proteins. The M protein likely plays a role in virus assembly and budding (Meulenberg *et al*., 1993). The nucleocapsid, or N, protein is encoded by ORF 7 and is highly immunogenic in pigs (Meulenberg *et al*., 1995) The N protein encapsulates the genome and is the sole component of the capsid, comprising of approximately 20% to 40% of total viral proteins. The C-terminus of the protein plays an essential role in conformational epitopes (Meulenberg *et al.*, 1998). The N protein is the most commonly used protein in diagnosis via detection of the virus by N-specific antibodies due to its highly conserved immunogenic

properties, and being the target of the early immunological response in infected pigs (Loemba *et al*., 1996; Meulenberg *et al*., 1998). Part of the N protein localizes itself in the nucleus and nucleolus of the cell which may play a role in viral pathogenesis such as cell gene expression (Rowland *et al*., 1999). N protein has also demonstrated protein binding that is independent of other binding proteins via the formation of an N protein/ importin complex, which was discovered by an *in vitro* pull-down assay (Rowland and Yoo, 2003).

Genotypes

PRRSV falls into two distinct genotypes with multiple isolates in each, termed the European strain (genotype 1) and North American strain (genotype 2). The study of polyclonal antisera indicated a difference was present between the two genotypes (Wensvoort *et al*., 1992). Comparison studies have also been performed by sequencing genomes from isolates of each genotype. At the genome level, both isolates constitute 60% nucleotide identity. However, major differences have been detected at the protein level. Variations include at level of the 5' and 3' non-coding regions, and in the non-structural coding regions of Nsp and capsid protein 4 (CP4) located in ORFs 1a and 1b. Nsp 2 has appeared to possess only 32% amino acid identity and included 120 extra residues, while CP4 shared only 42% amino acid identity between the two genotypes (Allende *et al*., 1999).

Transmission

PRRSV has historically been known to be a highly infectious pathogen, needing less than 1 X10 ^{1} tissue culture infectious dose ($TCID_{50}$) to infect pigs via the intramuscular route (Yoon *et al.,* 1999). Transmission of PRRSV is primarily through close contact between diseased and naïve animals. Nose-to-nose contact with urine and feces are the main routes of transmission (Rossow *et al.,* 1994; Wills *et al.,* 1997). The minimum infectious dose (MID) varies with the

route of viral exposure. For exposure to PRRSV genotype 2 isolate VR-2332 via oral and intranasal routes, the infectious dose 50 (ID_{50}) was $10^{5.3}$ and tissue culture infectious dose 50 ($TCID_{50}$) was 10^4, respectively (Hermann *et al.,* 2005). There are also reports of virus being isolated from semen six days post inoculation and may persist up to 92 days in infected boars. Transmission of the virus via insemination has also been detected. PRRSV can replicate in male testicular germ cells, induce death via apoptosis, and alter spermatogenesis (Christopher-Hennings *et al.,* 1995; Reicks *et al.,* 2006; Sur *et al.,* 1997; Yaeger *et al.,* 1993). However, higher infectious doses (2×10^5) are needed for transmission via semen as compared to infection via the intranasal route (Nelson *et al.,* 2002). PRRSV can also infect fetuses via replication in the endometrium of the female and then crossing the maternal-fetal interface, ultimately infecting the fetuses. However, not all fetuses may be directly infected with virus. Due to the compartmentalizable nature of the placenta with each fetus receiving individual blood and nutrient supply and are at different distances from the uterine body, each fetus of which may attempt to induce an antiviral response (Karniychuk and Nauwynck, 2013; Rowland, 2010). Additionally, normal pig behavior plays a role in PRRSV transmission. Tail and vulva bites, scratches, and abrasions can all result from pigs fighting. Aggressive behaviors, especially in sows, have shown to promote PRRSV transmission to other animals in the specific pen the infected animal is housed in (Bierk *et al.,* 2001). Differences in infectivity have also been observed in various isolates, such as aerosol exposure to a genotype 2 isolate, MN-184. The recorded ID_{50} was less than 2, indicating MN-184 is far more infectious than VR-2332 (Cutler *et al.,* 2011). Fomites have served as vectors of PRRSV transmission including but not limited to: boots, tools, coveralls, and contaminated needles (Otake *et al.,* 2002a). Mechanical transmission of PRRSV from infected pigs to naïve pigs via the species of mosquito, *Aedes vexans* has also

been reported. However, it has not been verified whether mosquitoes could serve as biological vectors for the virus (Otake *et al.,* 2002b). Houseflies, particularly the species *Musca domestica* Linnaeus, can harbor PRRSV in their intestinal tract for up to 12 hours after feeding on infected animals (Otake *et al.,* 2003). Aerosol transmission of PRRSV has also been measured in controlled conditions as a means of pathogen quantification (Hermann *et al.,* 2009).

Clinical Signs

Clinical signs vary betweein the developmental stages of pigs. The hallmarks for signs of infection lie with reproductive and other various systemic signs. Particularly for breeding age gilts, sows, and boars, reproductive problems are the most obvious and may last 1-4 months post infection. These signs include: reduced conception rates, weak-born piglets that lead to higher pre-weaning mortality, mummified fetuses, abortions, stillborns, and premature farrowings. This age range can also experience lethargy, anorexia, malaise, respiratory distress, and potential vomition. Infected boars have produced semen of poor quality, low sperm motility, and a reduced number of spermatozoa with normal acrosomes. Infected suckling piglets are known for "thumping", which is associated with dyspnea, and being febrile. Postweaning pigs may have chronic infections leading to, rough hair coats, anorexia, lethargy, cutaneous hyperemia, and an 85% reduction in average daily gain which leads to uneven size within groups. Secondary opportunistic bacterial infections have also been observed in PRRSV infected pigs which include: *Streptococcus suis, Mycoplasma hyopneumoniae, Escherichia coli, Haemophilus parasuis,* and *Salmonella* Choleraesuis (Dee, 2016; Prieto *et al.,* 1996; Zimmerman *et al.,* 2012).

Pathogenicity

Interactions between PRRSV and its pig host are comprised of a variety of viral pathogenesis mechanisms. Detection of a vigorous anti-PRRSV antibody response can be as early as 7 to 9-day post infection (DPI). However, this production of early response antibodies has been found to promote the infection rather than work in neutralization, thus increasing viremia levels (Lopez *et al.*, 2007; Yoon *et al.*, 1996). Due to the mediocre cell mediated immune response, a persistence of infection results due to suppressed T lymphocyte recognition of infected macrophages (Xiao *et al.,* 2004). Subacute viremia covers approximately 28 days with mechanisms of respiratory distress and the releasing of cytokines to induce inflammation. A failure within the protective immune effectors allow the acute replication of the virus in the animal due to the late response of PRRSV neutralizing antibodies and PRRSV specific gamma interferon-producing cells (Meier *et al.,* 2003). Following peak viremia around day 7, the presence of virus tends to fade slightly with the periodical reappearance of viremia around day 28 (Boddicker *et al.,* 2011). Virus replication has a tendency to decrease from 9 DPI in the lungs potentially due to the appearance of anti-PRRSV antibodies, lack of susceptibility of penetrated monocytes, and the shortage of macrophages available for infection (Labarque *et al.,* 2000). PRRSV has been found to persist for up to 150 days post infection with persistent shedding during the asymptomatic period. Eventually the virus replication levels decrease to minimal and undetectable levels and are cleared from the body, however the mechanism responsible for this is not clear (Horter *et al.,* 2002, Rowland *et al.,* 2003b). The genetic stability of both non-coding regions (NCRs) in the PRRSV nucleocapsid as well as genes coding for the M protein and glycoproteins have been found to possess mutations which may account for the persistence of the virus (Allende *et al.,* 2000).

Pathological Lesions

Due to the nature of PRRSV, there are multiple lesions that occur prior to and upon necropsy, indicating that the results of infection are multisystemic. Gross lesions at necropsy include lymphadenopathy, encephalitis, myocarditis, vasculitis, pneumonia. Lymph node lesions are variable but may consist of germinal center hypertrophy and hyperplasia, lymphocyte necrosis, and multifocal cystic spaces. Light microscopic lesions can be found in the lymph nodes, blood vessels, lung, and heart. The greatest number of lesions under light microscopy can be found in the alveolar cells in the cranial lung lobes, with interstitial pneumonia being the primary lesion. Alveoli appear to be the most affected with lysed macrophages, syncytial alveolar cells, and karyorrhectic debris (Rossow *et al.,* 1995). However, there are no gross nor microscopic pathognomonic lesions. Aborted fetuses and stillborns have little value for diagnosis. For pigs in all age groups, systemic hypertrophic lymph nodes and interstitial pneumonia can be observed upon necropsy (Lager and Halbur, 1996; Stevenson *et al.,* 1993). Additionally, genotype 2 PRRSV has been observed to be more pneumovirulent than genotype 1 based on respiratory clinical signs and lesions both on the gross and microscopic level (Martinez-Lobo *et al.,* 2011.)

Detection of PRRSV

Diagnostic methods for PRRSV include real time reverse transcription-polymerase chain reaction (RT-PCR), enzyme linked immunosorbent assays (ELISA), microbead assay (MBA), indirect fluorescent antibody test (IFA), serum neutralization (SN), direct fluorescent antibody test (DFA), immunohistochemistry (IHC), and virus isolation. For PRRSV detection in live animals, a serum sample is the preferred retrieval method. Real time RT-PCR has been found to produce rapid, highly sensitive results with detection of low levels of PRRSV in serum. Real time RT-PCR can also detect the strain of PRRSV that is present. However, ELISAs have been

the gold standard for detection due to high sensitivity and specificity. Serum is utilized in an ELISA and antibodies can be detected 9-13 DPI. ELISAs are less costly than real time RT-PCR, however serum samples must be taken later to get a detectable amount of PRRSV antibodies. Also, the strain of PRRSV cannot be detected by ELISA. (Collins *et al.,* 1996; Lurchachaiwong *et al.,* 2007; Rovira *et al.,* 2007). More recently, microbead assays were developed as a means of measuring lower antibody levels and have the capability for simultaneous detection of antibodies for more than one disease that may be present in the sample serum (Lin *et al.,* 2011.) A historical diagnostic method of PRRSV is through IFA, which can detect PRRSV antibodies restricted to PRRSV infected cells. SN branched from IFA and could detect antibodies earlier with higher titers, and can differentiate between PRRSV isolates (Yoon *et al.,* 1994). However, both methods are used less today, due to in part more rapid results of real time RT-PCR. IHC is a standard tissue diagnostic method for deceased animals which has capabilities in detecting PRRSV antigen, particularly in the lung (Yaeger, 2002).

Prevention and Control

Prevention of the introduction of PRRSV is the utmost important strategy for farms that are free of the virus. Biosecurity is key when attempting to prevent an outbreak. Current protocols in use include: personnel entry via the Danish entry system or shower-in systems, proper facility upkeep with rodent and insect control, quarantine areas for incoming animals, sanitation and drying of equipment and trucks, and the use of filtration systems for barn air movement (Dee and Deen, 2006; Zimmerman *et al.,* 2012). Immunizations to defend against PRRSV have been in the market for many years, particularly modified live virus vaccines. Field virus inoculations have also been practiced inducing PRRSV in a region of farms with goals of immunizing all present animals. Unfortunately, vaccination has been unable to prevent a virus

outbreak. However, there have been reports of vaccinated animals that were infected with PRRSV showing reduced nasal shedding, decreased viremia levels, and less extensive lesions (Corzo *et al.,* 2010; Park *et al.,* 2014). Unfortunately, one or more of the above protocols is breached and the farm may become PRRSV positive. Many control measures can be utilized. Total herd depopulation/repopulation, partial depopulation, and herd closure have been shown to successfully eliminate PRRSV from a facility (Torremorell and Christianson, 2002). Additionally, unidirectional pig flow has shown promise in controlling disease spread. Ensuring diseased animals do not come back in contact with their previous locations is critical for management of a PRRSV outbreak (Dee and Philips, 1998).

The Peripheral Blood Mononuclear Cell (PBMC) and Macrophage

Peripheral blood mononuclear cells (PBMCs) are immune cells circulating in the blood with a round nucleus. Monocytes, and lymphocytes comprise the PBMC category. Isolation of PBMCs depends on the density of the PBMCs and the other components in whole blood. Successful isolation includes the use of a density gradient medium such as Ficoll™ (Riedhammer *et al*., 2014). Porcine 2A10 antigen expressed on tissue macrophages has been found to possess sequence homology with human CD163. However, most blood monocytes are negative 2A10. Receptor expression on porcine macrophages can be measured via the application of 2A10 mab (Bullido *et al*., Sanchez *et al*., 1999). If measuring particular receptor expression to evaluate its function, a means of cell stimulation is necessary. The use of colony stimulating factors are common to differentiate monocytes into macrophages. Colony stimulating factors (CSF) are essential for the survival of blood monocytes during *in vitro* cell culture. Granulocyte-monocyte cell stimulating factor (GM-CSF), is a useful tool for maturing blood monocytes to obtain differentiated macrophages that resemble alveolar macrophages (Akagawa,

2002; Metcalf, 1986). Under specific culture conditions, GM-CSF can be used to differentiate blood derived monocytes into mature macrophages, thus upregulating the expression of 2A10 and increasing the opportunity to measure CD163 expression with 2A10 mab.

Macrophages are derivatives of bone marrow cells and are a hallmark component of the innate as well as adaptive immune responses due to their phagocytic properties with engulfing large particulate matter. Macrophages ingest bacteria, viruses, fungi, immune complexes, and cellular debris. Additionally, they aid in the process of restoring homeostasis and are also involved in disease (Aderem and Underhill, 1999; Dale *et al*., 2008). Healing and tissue repair has also been discovered as a physiological component of macrophages via the release of angiogenic propagators and nitric oxide (NO) (Stallmeyer *et al*., 1999).

Macrophages have the capacity to differentiate and mature in response to various stimuli. Adapted from T lymphocyte nomenclature, macrophages can be classified as M1 and M2 based on the type of activation that was evoked (Mills *et al*., 2000). M1 macrophages are considered to be classically activated, as they perform phagocytosis and promote inflammation. M1 macrophages are stimulated via gamma interferon combined with lipopolysaccharide (LPS) or tumor necrosis factor (TNF). M1 macrophages participate in inflammatory immune responses. Alternatively, macrophages may be of the M2 phenotype, and are broken down into three subclasses. M2a macrophages are stimulated by IL-4 and IL-13. M2b macrophages are stimulated by immune complexes coupled with toll-like receptor. M2c macrophages are stimulated by secretion of IL-10. M2 macrophages are involved in immunoregulation, Th2 activation, and tissue remodeling (Mantovani *et al*., 2004; Murray *et al*., 2014; Sang *et al*., 2015; Singleton *et al*., 2016.)

The PRRSV receptor: CD163

Structure

Cluster of Differentiation 163 (CD163) is a 130 kDa long protein plays many roles in various biological processes in mammals. Expression of CD163 is restricted to cells specifically of the macrophage/monocyte lineage (Van den Huevel *et al.*, 1999). CD163 is a member of the scavenger receptor cysteine rich (SRCR) superfamily specifically Class B consisting of nine SRCR domains, 17 exons, 16 introns, two Proline Serine Threonine (PST) domains, and a cytoplasmic tail (Law *et al.*, 1993).

Physiological Properties

CD163 was first identified as a macrophage membrane receptor for the endocytosis of haptoglobin-hemoglobin (HpHb) complexes and acts as a scavenger for free haptoglobin as a preventative measure of oxidative toxicity (Kristiansen *et al.,* 2001). Particularly, SRCR domain 3 is the main determinant in coupling and interaction with endocytosis of HpHb complexes (Madesn en *et al.,* 2004). Additionally, metabolites are released following HpHb degradation. They include bilirubin, CO, and free iron and possess strong anti-inflammatory as well as anti-oxidative effects (Figure 1.2). The result of the release of these metabolites is a negative feedback loop that reduces inflammation (Soares and Bach, 2009). Macrophages are a crucial component to clearing infection, but can generate tissue damage if left unchecked. As a response to inflammation, CD163 on macrophages produces an anti-inflammatory effect via the secretion of IL-10, an anti-inflammatory cytokine, and creates a positive feedback loop. The result is the upregulation of IL-10 expression to induce an anti-inflammatory effect to the inciting stimulus, thus reducing the macrophage immune response (Figure 1.2) (Buechler *et al.,* 2000; Philippidis *et al.,* 2004). However, pro-inflammatory cytokines such as IL-1β, IL-6, GM-CSF, and TNF-α,

are also produced through the cross-linking of CD163 with bacteria or antibodies. An increased inflammatory response will promote more macrophage activity, thus creating risk of tissue damage (Fabriek *et al.,* 2009, Polfliet *et al.,* 2006,).

Soluble CD163

In addition to membrane-bound CD163, a soluble form of CD163 (sCD163) is present in serum and other fluids (Moller *et al*., 2002). HbHp complexes have been found to also bind to sCD163, but at a much lower affinity when compared to membrane CD163 (Møller *et al*., 2010). sCD163 has been found to be a result of shedding as a part of normal physiology and by a number of inflammatory mechanisms. These include the induction of toll-like receptor (TLR) 4 activation from exposure to LPS, thrombin, oxidative stress, and crosslinking of the Fcγ on antibodies (Chung *et al*., 2011; Hintz *et al*., 2002; Møller *et al*., 2002; Sulahian *et al*., 2004; Timmermann and Högger, 2005). The actual function of sCD163 is still unknown. However, it can serve as a useful biomarker of macrophage activation in response to inflammation with a variety of diseases (Møller *et al*., 2012).

Virus-Receptor Interaction

Porcine CD163 plays a role as a key mediator for infection of PRRSV (Calvert *et al.,* 2007). Of the 17 exons, exon 7 codes for SRCR domain 5, and serves as the critical facilitator for PRRSV infection and is therefore a potential target for genetic manipulation. (Van Gorp *et al.,* 2010). The PRRSV major envelope protein, GP5, was thought to bind to the alveolar macrophage receptor sialoadhesin (Sn/SIGLEC/CD169) prior to internalization of PRRSV (Van Breedham *et al.*2010). Pigs that were genetically edited to completely lack the once intact sialoadhesin receptor on alveolar macrophages revealed it is not required for PRRSV infection (Prather *et al.,* 2013). However, a heterotrimer (consisting of GP2, GP3and GP4 of the PRRSV

virion) interacts with SRCR domain 5 of CD163 on the extracellular membrane of macrophages resulting in propagation of virus uncoating and replication leading to eventual infection (Das *et al.,* 2010, Van Gorp *et al.,* 2010).

The CRISPR/Cas9 System

The CRISPR/Cas9 system has recently become an efficient genetic editing tool and was awarded the 2015 science breakthrough technology of the year due to incredibly precise and efficient genome editing. First discovered in 1987, CRISPRs were identified in *Escherichia coli* with a sequence element series consisting of 29-nucleotide repeats separated by 32-nucleotide 'spacer' sequences (Ishino *et al.,* 1987). However, this discovery was of interest in genetics in the 2000s when they were identified in many prokaryotes with multiple CRISPR-associated (Cas) proteins (Jansen *et al.,* 2002, Mojica *et al.,* 2000).

CRISPR/Cas9: A Genetic Engineering Breakthrough

Prior to CRISPR/Cas9 usage in genetic manipulation strategies, the primary route of genetic modification was through gene targeting with homologous recombination (Capecchi, 1989). However, this method is rather inefficient and was eventually replaced with customized zinc finger nucleases (ZFNs) and meganucleases which work by cleaving specific DNA target sequences *in vivo* and creating double stranded breaks at pre-selected sites. The breaks are then repaired via homologous recombination or non-homologous end joining (NHEJ) (Bibikova *et al.,* 2003; Cathomen and Joung, 2008). ZFNs had the spotlight of genetic engineering for many years until transcription activator-like effector nucleases (TALENs) showed signs of significant

improvements of traditional genetic modifications with increased applicability of targeting the desired edited sequence through larger amino acid modules compared to ZFNs (Boch *et al.,* 2009; Christian *et al.,* 2010).

Until a few years ago, the use of ZFNs or TALENS were considered the only method of genetic alteration. The CRISPR/Cas9 system has been found to be an efficient alternative for inducing targeted genetic editing. The editing mechanism is achieved through short segments of foreign DNA, also known as "spacers", which are incorporated with the CRISPR genomic loci and are then transcribed and processed into short CRISPR RNAs (crRNAs). Pathogenic DNA is then silenced by Cas proteins due to the crRNAs annealing to trans-activating crRNAs (tracrRNAs) producing sequence-specific cleavage (Figure 1.3). It has been found that the Cas9 protein requires a baseline sequence within the crRNA and a conserved dinucleotide-containing protospacer adjacent motif (PAM) sequence upstream of the crRNA binding region in order to perform target recognition and double stranded breaks (Jinek *et al.,* 2012). By re-designing the crRNA, the CRISPR/Cas9 system can be re-targeted to cleave practically any DNA sequence.

Designing of Genetically Edited Animals

Prior to genome editing of eukaryotes, prokaryotes were the first subjects of use for application of CRISPR technology for bacterial strain typing, particularly for *Mycobacterium tuberculosis* (Groenen *et al.,* 1993). In 2002, many CRISPR associated (Cas) proteins were found to be invariably adjacent to a CRISPR locus indicating a functional relationship as stated previously (Jansesn *et al.,* 2002). It was not until 2005 that CRISPRs were described to protect prokaryotes from invading foreign DNA, potentially from plasmids or viruses through a mechanism that is analogous to eukaryotic RNA interference mechanisms (Makarova *et al.*2006). The type II CRISPR/Cas9 adaptive immune system has been shown to facilitate RNA-

guided site-specific cleavage of DNA, which is now the tool in current use for CRISPR/Cas 9 system genetic targeting (Deveau *et al.,* 2010; Horvath and Barrangou, 2010; Makarova *et al.,* 2011; Bhaya *et al.,* 2011). A successful attempt at reconstituting a CRISPR system derived from *Streptococcus pyogenes* in a mammalian cell line, facilitating efficient genome editing occurred in 2013 with a later study confirming high efficiency RNA-genome targeting in several human and mice cell lines (Cong *et al.,* 2013; Mali *et al.,* 2013). CRISPR/Cas9 technology has exponentially skyrocketed in the genome editing realm and many animal species have now been subject to such manipulation ranging in species from: mice (Cong *et al.,* 2013), pigs (Whitworth *et al.,* 2013) cattle (Tan *et al.,* 2013), mosquitoes (Gantz *et al.,* 2015), and even dogs (Arnott *et al.,* 2015).

Genetically Edited Pigs Are Protected from PRRSV

In Whitworth, *et al.* 2013, the CRISPR/Cas9 system was utilized to produce genetically edited pigs from *in vitro* derived oocytes and embryos (Figure 1.5). After manipulation of somatic cells through homologous recombination (HR) or non-homologous end joining (NHEJ) by CRISPR/Cas9, the cells were used to produce genetically edited pigs through somatic cell nuclear transfer (SCNT). The edited embryos or oocytes were then surgically implanted into surrogate gilts via embryo transfer (ET) and an array of genotypes were produced. Challenge studies performed by Whitworth *et al.* 2015 revealed that pigs lacking the CD163 receptor, due to manipulation by the CRISPR/Cas9 system, were resistant to a single strain of PRRSV, a genotype 2 isolate NVSL 97-7895.

Proceeding with an additional *in vivo* study, more gene edited pigs were challenged against a genotype 1 strain of PRRSV, SD 13-15, and again with the genotype 2 strain, NVSL 97-7895. In addition to the Null pigs being challenged, hCD163L-1 pigs which have a swapped

domain of SRCR domain 5 with a human-like CD163 SRCR domain 8, were challenged as well. Blood was collected intravenously on days 0, 4, 7, 11, and 14 post infection. On day 15, pigs were euthanized and lung lavages were performed to collect PAMS.

Lung tissue was also collected at necropsy and aliquoted in 10% formalin, embedded in paraffin, and processed for histopathology. A board-certified pathologist performed histopathology. Scores ranged 0-4 with 0 for no microscopic lesions to 4 for severe interstitial pneumonia that includes ~75 to 100% of lung tissue. WT and hCD163L-1 lung scores ranged from 1 to 4 ranging from mild to severe interstitial pneumonia while the Null pigs presented scores of 0 with normal lung architecture. (Figure 1.6)

Viremia levels in serum determined by RT-PCR are shown in figures 1.7 A and B as averages per genotype in units of Log10 templates per reaction. The minimum threshold of determining infected sera was set at 500 relative fluorescence units (RFU). Figure 1.7 A represents viremia levels of genotypes of pigs challenged with PRRSV genotype 1 isolate SD 13-15. The WT group showed a typical PRRSV viremia curve with peak viremia approximately 7-11 DPI. The Null and hCD163L-1 group did not show signs of virus replication. Figure 1.7 B shows viremia levels of genotypes of pigs challenged with PRRSV genotype 2 isolate NVSL 97-7895. WT and hCD163L-1 pig groups revealed values of virus replication with a typical PRRSV viremia curve for both groups. The Null group did not show any signs of virus replication or viremia (Wells *et al*., 2017).

IgG antibody levels were determined via a multiplex assay in figure 1.8, and are represented in units of net Median Fluorescent Intensity (MFI) based on parameter from the bead sets that were used. For genotype 1 PRRSV, only WT pigs were observed to have a recognizable

MFI. For genotype 2 PRRSV infections, WT and hCD163L-1 pigs both had an observable MFI. MFI levels of Null pigs were unobservable for both PRRSV genotypes. (Wells *et al*., 2017).

CD163 performs a physiological function by scavenging HpHb complexes, as a means of prevention of oxidative toxicity (Kristiansen *et al.,* 2001). Questions were raised regarding the Hp levels of CD163 gene edited pigs, and the animal's homeostatic status. A double antibody sandwich ELISA was performed to measure Hp levels in the serum of PRRSV negative WT, hCD163L-1, and Null pigs. Elevated Hp levels were found in the gene edited pigs, while WT were shown to have less circulating levels of Hp (Figure 1.9) (Wells *et al*., 2017). This may be in part due to the receptor being rendered nonfunctional in Null pigs and less expressive in the hCD163L-1 pigs. However, when observed on the hoof, both edited genotypes of pigs appeared to be bright, alert, and responsive, just like their WT cohorts.

In Wells et al., 2017, it was concluded that pigs with a replacement of porcine CD163 SRCR domain 5 with a CD163-like homolog, also termed hCD163L-1 pigs, were resistant to genotype 1 PRRSV but not genotype 2 PRRSV. Additionally, we found the Null pigs to also be resistant to the genotype 1 PRRSV. These results led to the use of *in vitro* challenge studies to evaluate PRRSV resistance in a larger quantity.

Purpose

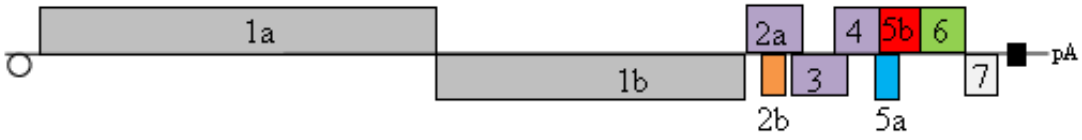
The purpose of this study was to evaluate the potential resistance that macrophages from gene edited pigs may have to multiple isolates of genotype 1 and 2 PRRSV. However, alternative methods for macrophage collection besides animal sacrifice has also warranted investigation. The low replication fidelity of PRRSV leaves it to be an international virus that has yet to be prevented. Control efforts have been in place for a number of years leading to some success for eventual eradication of PRRSV from individual hog sites, but eradication on a larger scale has

not been achieved. Unfortunately, a preventative cure has yet to established. The verification of gene edited pigs posing resistance to a breadth of PRRSV isolates may hold the key to propagating these animals into the worldwide hog commodity market.

Tables and Figures

Figure 1.1: Representation of the PRRSV genome

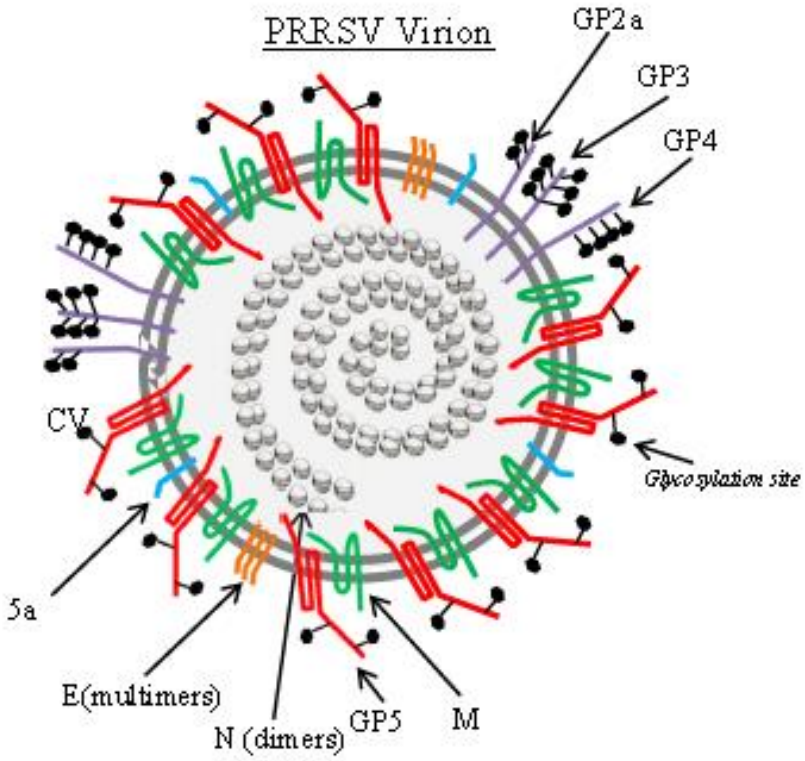
The PRRSV genome consists of 10 open reading frames 10 (ORFs). ORFs 1a and 1b produce polyproteins to generate nonstructural proteins. ORFs 2a and 2b as well as 3 through 7 produce viral structural glycoproteins.



Courtesy of Benjamin Trible

Figure 1.2: Schematic representation of the PRRSV virion

The PRRSV virion is consists of an envelope, N protein, M protein, as well as major glycoprotein 5 and minor glycoproteins 2a, 3, and 4. Das *et al.*2010 and Van Gorp *et al.*2010 has shown that GP2a, and GP4 interact with domain 5 of CD163 for attachment and internalization.



Courtesy of Benjamin Trible

Figure 1.3: Diagram of macrophage receptor porcine CD163

(A) Diagram showing CD163 protein SRCR (ovals) and PST (squares) domains and corresponding gene exons. (B) Peptide sequence comparison of porcine CD163 SRCR 5 with the HCD163L1 SRCR 8 homolog. The figure is based on GenBank accession numbers AJ311711 (pig CD163) and GQ397482 (hCD163L-1).

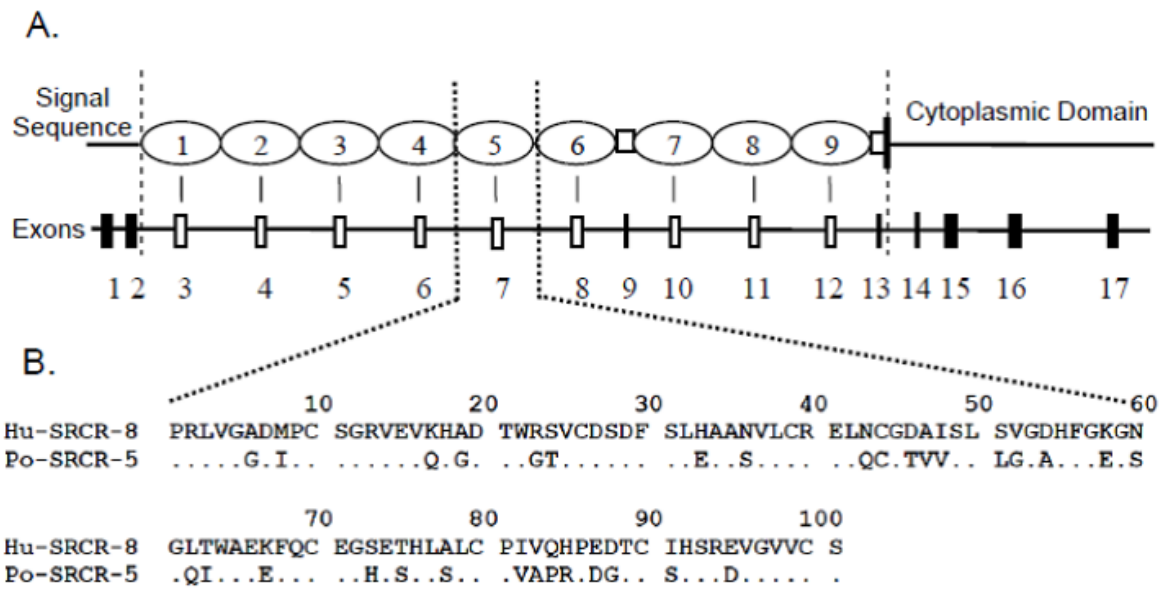


Figure 1.4: Regulation of inflammation via CD163 SRCR 3 breaking down Hp-Hb complexes

After breaking down Hp-Hb complexes, the macrophage incorporates the products in the lysosome where they are metabolized and excreted as metabolites of either free iron, carbon monoxide, or bilirubin which in turn reduces inflammation.

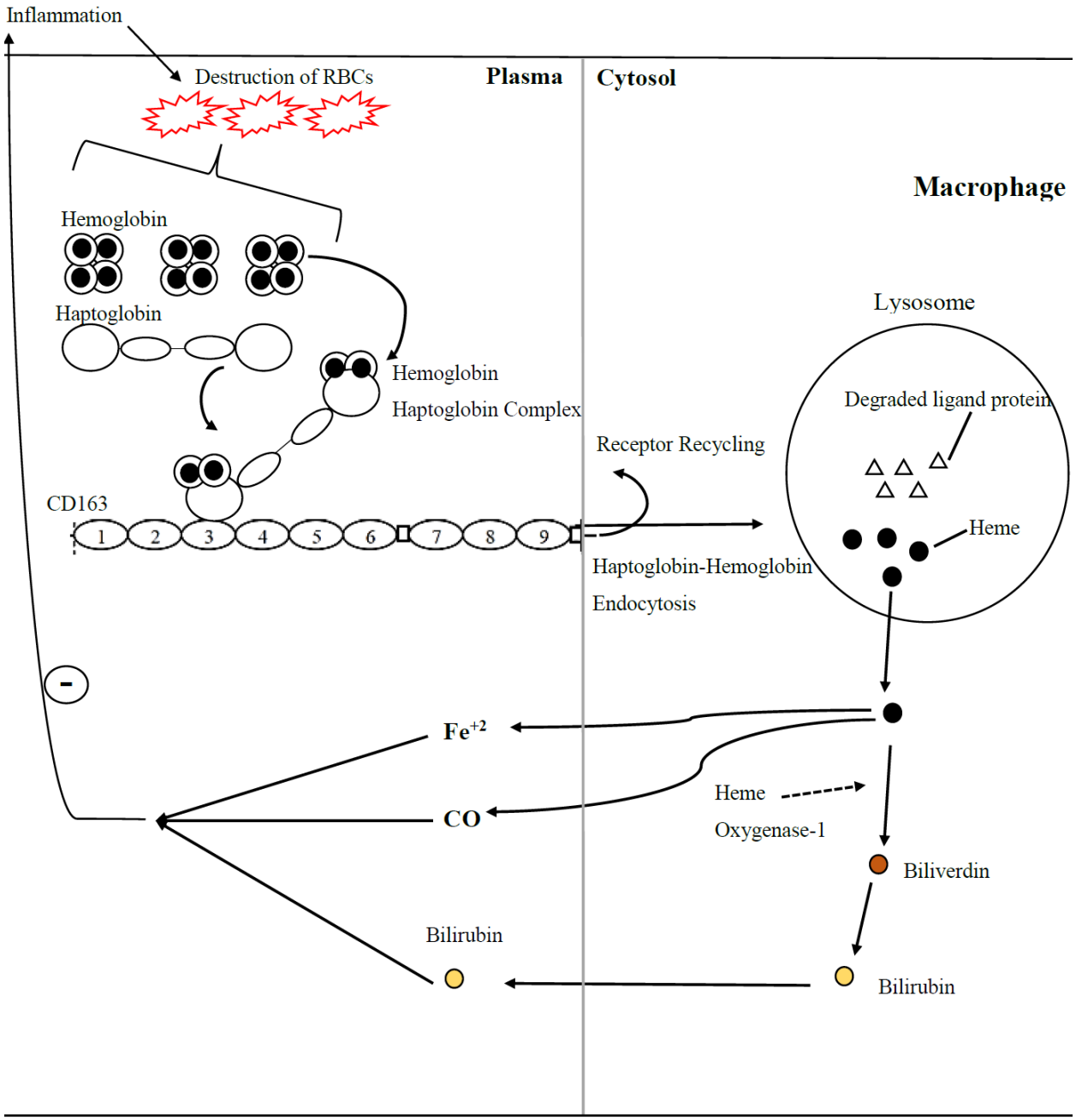


Figure 1.5:Various genetic constructs of pigs by CRISPR/Cas9 system.
From Wells et *al.* 2017. Various genetic constructs of WT, and CD163 edited pigs.

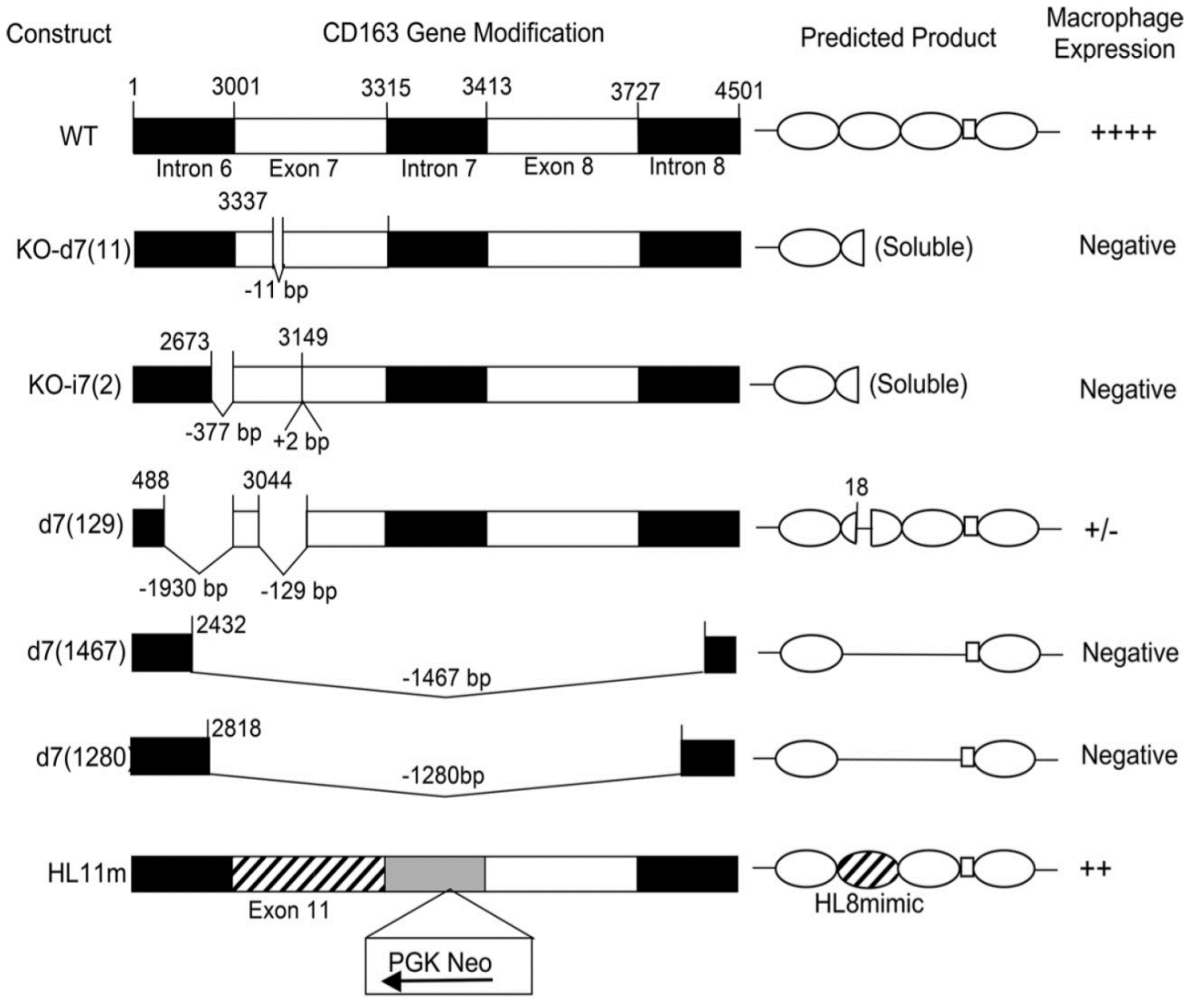


Figure 1.6: Lung scores of PRRSV infected pigs

Post mortem lung scores are below. WT and hCD163L-1 lung scores ranged from 1 to 4 ranging from mild to severe interstitial pneumonia while the Null pigs presented scores of less than 1 with normal lung architecture. hCD163L-1 pigs infected with a type 2 isolate were observed to have a higher lung score than those infected with a type 1 isolate.

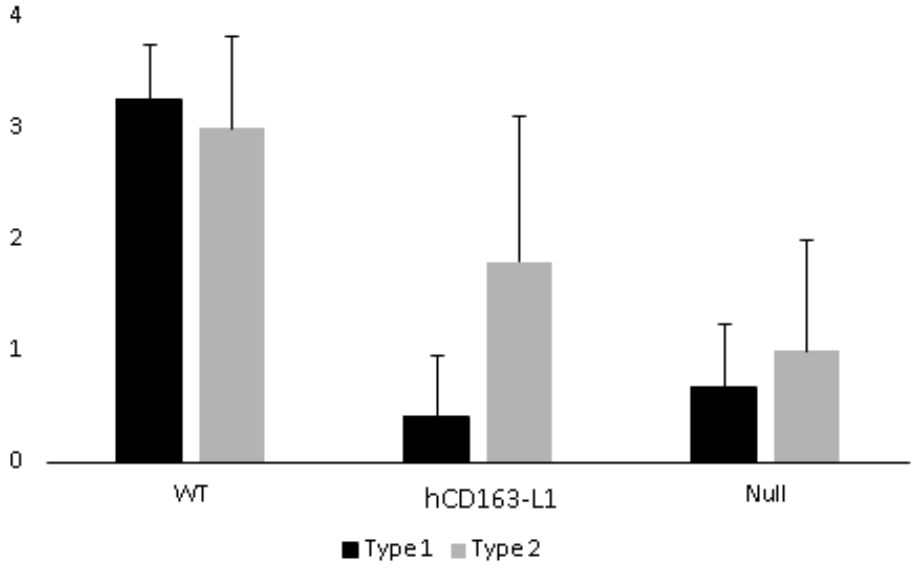


Figure 1.7: Average viremia levels in serum of PRRSV infected pigs by real time RT-PCR at log_{10} templates/ reaction.

Genotypes challenged include CD163 hCD163L-1, Null, and WT. A) Viremia levels of Type 1 (SD 13-15) infected pigs. Only WT pigs showed noticeable levels of viremia. B) Viremia levels of Type 2 (NVSL) infected pigs. Only pigs of the Null genotype showed no detectable levels of viremia.

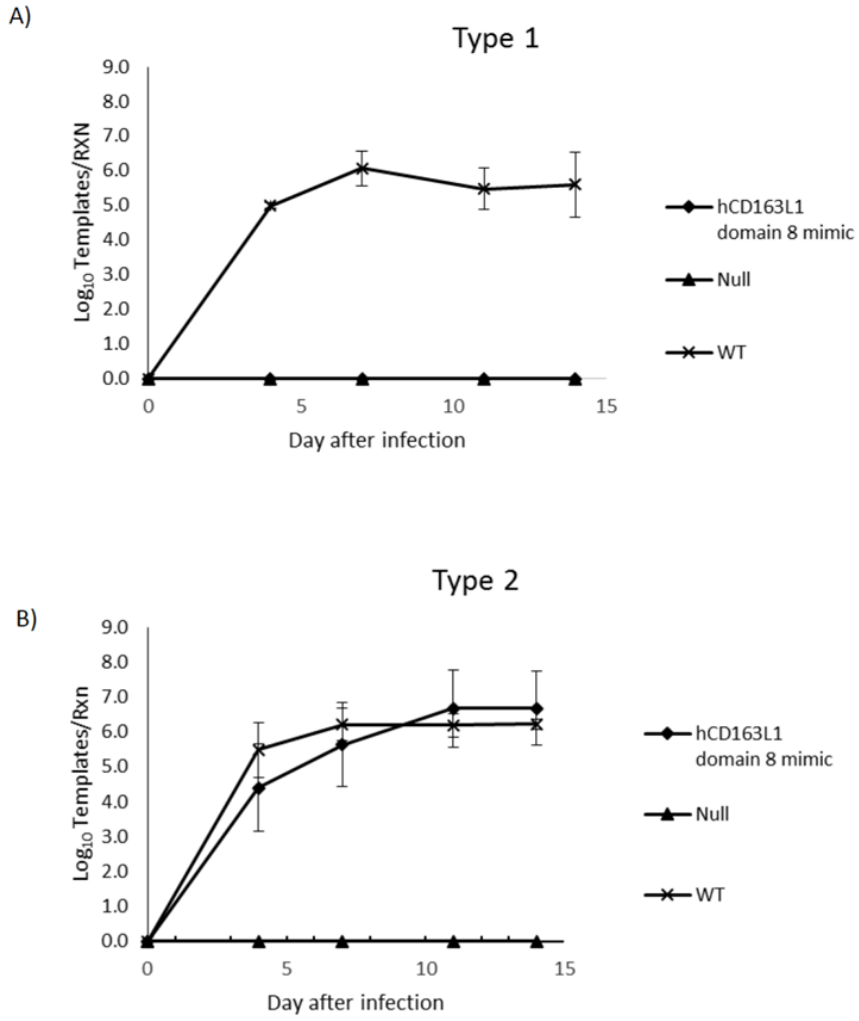


Figure 1.8: Median fluorescence intensity level of CD163 antibody produced in serum of PRRSV infected pigs.

Genotypes challenged include hCD163L-1, Null and wildtype (non-edited) pigs. The Y-axis indicates median fluorescence intensity of the parameter of microbeads in regard to hCD163L-1 to antibody levels, specifically IgG. The X-axis indicates DPI. A) Antibody levels of Type 1 (SD 13-15) infected pigs. B) Viremia levels of Type 2 (NVSL) infected pigs.

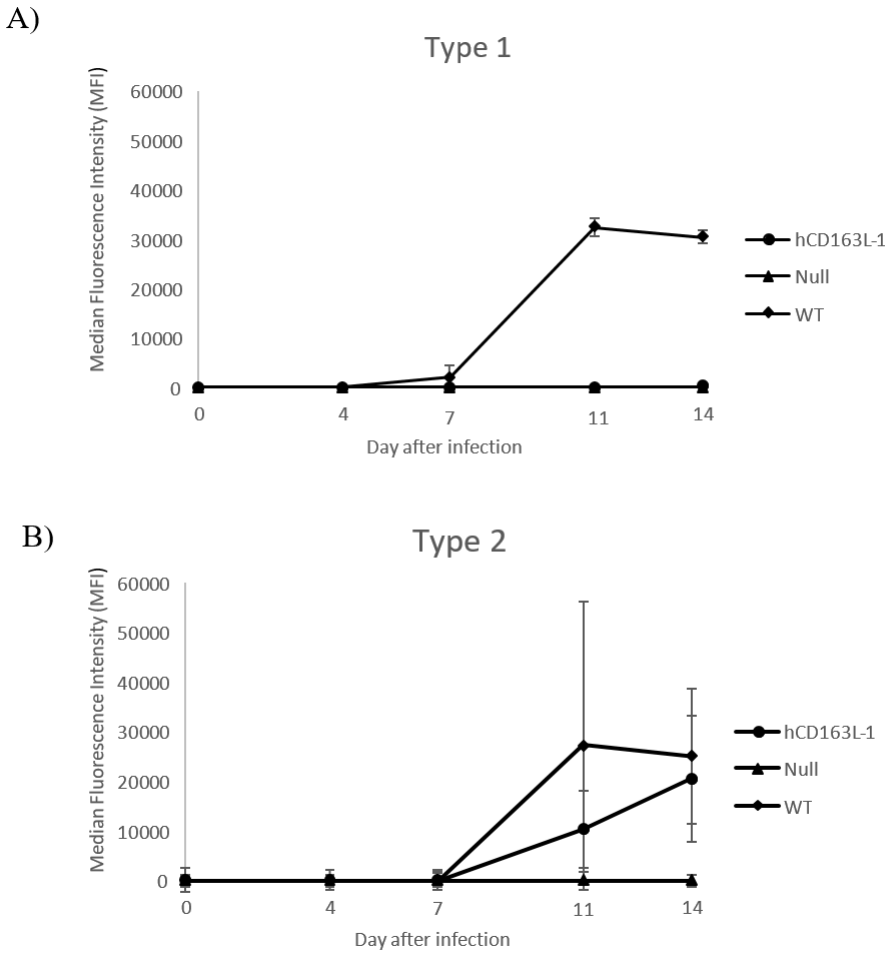
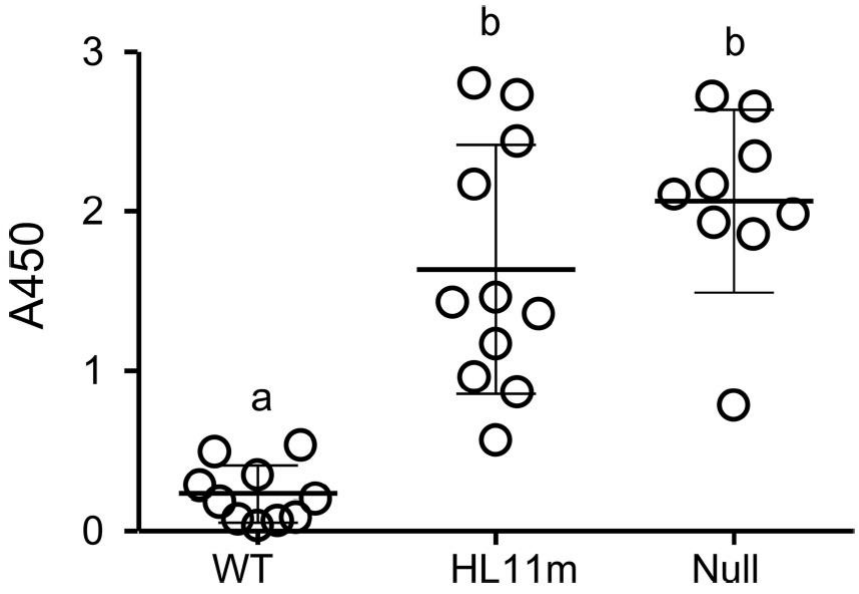


Figure 1.9: Haptoglobin levels in serum of WT, hCD163L-1, and Null pigs

Circulating haptoglobin levels for HL11m (hCD163L-1), and Null pigs were higher than WT pigs, potentially due to lower or no expression levels of CD163, a scavenger receptor that recycles haptoglobin



Chapter 2 - Materials and Methods

PAMs Collection

PAMs were collected 15 DPI at necropsy. Three collection tubes each containing 50 mL of phosphate buffered saline (PBS) were used to perform lung lavages to collect the PAMs and then temporarily stored on ice until further aliquoted. Lung tissue from each pig was also collected and stored in 200 mL sample cups with 150 mL formalin for histopathology. Lungs were removed from euthanized pigs and lavaged by pouring 100 ml of cold PBS into the trachea. The trachea was clamped with hemostats and the lungs gently massaged for a minimum of 30 seconds. The alveolar contents were poured into 50 ml centrifuge tubes and stored on ice. Macrophages and other cells were removed by centrifugation at 1200 x g for 10 minutes at 4°C. The resulting pellet was resuspended in cold sterile PBS and was washed once. The cell pellet was resuspended in freezing media comprised of 50% RPMI 1640, 50% FBS, and 10% DMSO (Thermofisher Scientific, Lenexa, KS, USA), and 45% FBS (Sigma Aldrich, St. Louis, MO, USA) then stored in liquid nitrogen until use.

PBMC Isolation

Peripheral blood mononuclear cell (PBMC) isolation was performed using 50 ml SepMate50 PBMC isolation tubes (Stem Cell Technologies, Vancouver, British Columbia, Canada). 3.5 ml Ficoll-Paque Premium™ density gradient medium (GE Healthcare Life Sciences, Pittsburgh, PA, USA) was added to empty 50 ml SepMate50 tubes. 15 ml blood samples were diluted with 15 ml PBS with 2% FBS. Diluted blood samples were pipetted down the sides of the of the SepMate50 tubes. SeptMate50 tubes with blood samples were centrifuged at 1200 x g for 20 minutes at room temperature. The top layer of mononuclear cells (MNCs) was

poured off into a new 50 ml conical tube and was washed with PBS with 2% FBS. MNCs were centrifuged at 300 x g for 8 minutes at room temperature and repeated once. MNCs were then resuspended in freezing media with 45% FBS, 45% DMEM, and 10% DMSO and stored at -80°C in 1ml aliquots in 1.5 ml Nalgene System 100 Cryogenic Tubes (Thermofisher Scientific, Lenexa, KS, USA).

Isolates

PRRSV isolates (Figure 2.2) grown from Marc-145 cell lines were titrated from 10^1 to 10^6 infectious units/ml. 100 µl of PRRSV isolate was added to 900 µl of RPMI culture media creating a 1:10 dilution. 100 µl of diluted PRRSV isolates were then added in triplicate to the to appropriate wells of naïve PAMs. Plates were incubated overnight at 37°C with 5% CO2. Isolates were chosen based on their genotype, different nucleotide and peptide sequences, date of initial isolation, and level of virulence ranging from low virulence to atypical (highly virulent).

Flow Cytometry of PAMs

For antibody staining, approximately $1X10^6$ PAMs were placed into 12 mm x 75 mm polystyrene flow cytometry (FACS) tubes and incubated for 15 minutes at room temperature in 1ml of PBS with 10% normal mouse serum. Cells were pelleted by centrifugation and re-suspended in 100 µl PBS and 1% BSA along with 5 µl of FITC-conjugated mouse anti-porcine CD169 mAb and 5ul of PE-conjugated mouse anti-porcine CD163 mAb (Abd Serotec/Bio-Rad companies, Hercules, CA, USA) (Table 2.1). After a 30-minute incubation, the cells were washed twice with PBS containing 1% BSA (Fraction V; Hyclone) and immediately analyzed on a BD LSR Fortessa flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) with FCS

Express 5 software (De Novo Software, Los Angeles, CA, USA). A minimum of 80,000 macrophages were analyzed for each sample.

PAMs *In Vitro* Infection (End-Point Dilution)

Frozen cells were thawed on ice, counted using a Scepter™ 2.0 handheld automated cell counter (EMD Millipore, Temecula, CA, USA) and adjusted to a concentration of $5x10^5$ cells/ml of media (RPMI 1640 with 10% FBS, PenStrep, and Fungizone: RPMI-FBS) (Gibco, Thermofisher Scientific, Lenexa, KS, USA). PAMs (100 ul/well) were plated onto 96 well plates and incubated overnight at 37°C in 5% CO2. The cells were gently washed and infected by adding serial 1:10 dilutions of PRRSV, in triplicate to rows in the 96 well plate. After an overnight incubation at 37°C in 5% CO2, cells were washed and then fixed for 10 min with 80% acetone. After drying, 50uL per well of the PRRSV specific SDOW-17 mAb (Rural Technologies Inc., Brookings, SD, USA), diluted 1:1000 in 1% fish gelatin (Sigma Aldrich, St. Louis, MO, USA) was added to each well. After a 30-minute incubation at 37°C, the antibody was removed and cells washed with PBS. Cells were stained with Alexafluor 488 mAb (Thermofisher Scientific, Lenexa, KS, USA) and diluted 1:200 in 1% fish gelatin. Cells were incubated for 30 minutes away from light at 37°C, then washed with sterile PBS, and viewed under a fluorescence microscope.

PAMs *In Vitro* Infection (Percent Infection)

Frozen cells were thawed on ice, counted using a Scepter™ 2.0 handheld automated cell counter (EMD Millipore, Temecula, CA, USA) and adjusted to a concentration of $5x10^5$ cells/ml of media (RPMI 1640 with 10% FBS, PenStrep, and Fungizone: RPMI-FBS) (Gibco, Thermofisher Scientific, Lenexa, KS, USA). PAMs (500 ul/well) were plated onto 48 well plates and incubated overnight at 37°C 5% CO2. The cells were washed and infected with 1:100 diluted

PRRSV of both Type 1 and Type 2 genotypes in singlicate to rows in the 48 well plate. After incubation, overnight at 37°C in 5% CO2, cells were washed then fixed for 10 min with 70% acetone. After drying, 250uL per well of the PRRSV specific SDOW-17 mAb (Rural Technologies Inc., Brookings, SD, USA), diluted 1:1000 in 1% fish gelatin (Sigma Aldrich, St. Louis, MO, USA) was added to each well. After a 30 minute incubation at 37°C, the antibody was removed and cells washed with PBS. Cells were then stained with 250µl/well Alexafluor 488 mAb (Thermofisher Scientific, Lenexa, KS, USA) diluted 1:200 in 1% fish gelatin. Cells were incubated for 30 minutes away from light at 37°C, then washed with sterile PBS. Cells were viewed under a fluorescence microscope and percent infections were recorded in counts of three windows/well counting 60-80 cells per window.

Cultured Macrophages Derived from PBMCs *In Vitro* Infection

Aliquoted PBMCs of uninfected WT, hCD163L-1 and Null pigs in freezing media with 50% FBS, 50% DMEM, and 10% DMSO were thawed from liquid nitrogen, washed and resuspended in 5ml of culture media RPMI 1640 with penstrep, 7%FBS, fungizone, and gentimicin and plated on a Costar® 6 Well Clear TC-Treated cell culture plate (Corning Inc., Corning, NY, USA) with a minimum cell concentration of $3X10^6$ cells/well. 24 hours later culture media was replaced with fresh media containing 20ng/ml of 1:100 diluted granulocyte/monocyte colony stimulating factor (GM-CSF) (Life Technologies, Carlsbad, CA, USA). Blood monocytes were cultured for approximately for three days with fresh media with GM-CSF being added on day one after initial plating. On the fourth day, fresh media was added along with 250 µl per well Type 2 isolate VR-2332 in a 1:20 dilution and incubated for 24 hours. After incubation, cells were washed with sterile PBS and fixed with 70% acetone for 15 minutes

and then washed with PBS Tween and PBS prior to application of 1ml per well of 1:2000 diluted PRRSV tagged mAb SDOW and 1:200 diluted fAb Alexafluor 594. Following fixation and staining, plates were evaluated on a fluorescence microscope to determine percent infection.

Freezing of Cultured Macrophages from PBMCs

PBMCs were isolated via the protocol above and then cultured in media (RPMI 1640 with 10% FBS, PenStrep, and Fungizone: RPMI-FBS) (Gibco, Thermofisher Scientific, Lenexa, KS, USA) for six days at 37°C, being stimulated with GM-CSF (Life Technologies, Carlsbad, CA, USA) every three days. On the sixth day, cells were washed with PBS and treated with 5 mls of Versene solution (Gibco, Thermofisher Scientific, Lenexa, KS, USA) and incubated at 37°C for 20 minutes. Cells were then removed with cell scrapers that consisted of a 25cm handle and 1.8cm blade (BD Biosciences, Franklin Lakes, NJ, USA), and then aliquoted into 15 ml conical tubes. The samples were then centrifuged at 800Xg for 10 minutes. The supernatant was decanted and the cells were resuspended in freezing media (50% C80 EZ (Cryocrate, Columbia MO, USA)), 50% DMEM, 10% DMSO), and aliquoted into 1 ml Nunc™ Cryotube™ vials (Thermofisher Scientific, Lenexa, KS, USA). Samples were frozen at -80°C in a Nalgene™ Cryo 1°C Freezing container (Thermofisher Scientific, Lenexa, KS, USA) for 24 hours. After the freezing period, samples were thawed and suspended in culture media and centrifuged at 800Xg for 10 minutes. Samples were then resuspended in culture media and plated on a Costar® 24 Well Clear TC-Treated cell culture plate (Corning Inc., Corning, NY, USA) at 1ml per well. 24 hours later, cells were washed with sterile PBS and fixed with 70% acetone for 15 minutes and then washed with PBS Tween and PBS prior to application of 1ml per well of 1:500 diluted Invitrogen™ CD163/M130 mab (Thermofisher Scientific, Lenexa, KS, USA) and 1:200 diluted

fAb Alexafluor 594. Following fixation and staining, plates were evaluated on a fluorescent microscope to determine percent fluorescence.

Tables and Figures

Table 2.1: Conjugated antibody types for flow cytometry

Antibody	Isotype	Concentration	Flurochrome
CD163	Mouse Anti-pig IgG1	.1mg/ml	PE
CD169	Mouse Anti-pig IgG1	.1mg/ml	FITC

Table 2.2:Viruses used for *In Vitro* PAM Infections

Virus	Type	Year Isolated	GenBank Acc#
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*	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

*NA, Not available

Chapter 3 - Results

CD163 Surface expression levels on PAMs

Surface expression levels of CD163 and CD169 were evaluated using flow cytometry. PAMs were prepared with PE and FITC conjugated antibodies for CD163 and CD169 respectively and ran through the flow machine. The machine was programmed to detect macrophages and monocytes in each sample that exhibit CD163 and CD169 surface expression. Dot plots were created in cooperation with the running samples to detect forward scatter (FSC) for cell granularity and side scatter (SSC) for cell size. A population of macrophages and monocytes was selected from the FSC/SSC dot plot and evaluated on another dot plot for CD163 expression on the y-axis and CD169 expression on the x-axis. Quadrants were divided up as Q1 (CD163 +/CD169 -), Q2 (CD163 +/CD169 +), Q3 (CD163 -/ CD169 -), and Q4 (CD163 -/CD169 +). WT macrophages showed an average of 80% of selected cells expressing both CD163 and CD169 in Q2. hCD163L-1 macrophages showed an average of 40% in Q2 and 50% in Q4. Null pigs showed an average of over 90% of selected cells negative for CD163 yet still expressing CD169 in Q4. PAMs from the CD163 Null pigs did not show evidence of CD163 surface expression, but expression levels of CD169 were normal. For the hCD163L-1 pigs, both CD163+ and CD169+ were detected on PAMs (Figure 3.1). However, expression of CD163 was reduced compared to the WT PAMs. Surface expression levels for CD163 on the CD169-positive cells ranged from no detectable CD163 expression to cells possessing moderate levels of CD163 expression. The results for the hCD163L-1 pigs suggest there is presence and location of a PGK-Neo marker which may have influenced the reduced expression of CD163.

In Vitro PRRSV Infection of PAMs (End-Point Dilution)

Infection rates for PAMs were assessed with the 50% tissue culture infectious dose ($TCID_{50}$)/ml and was calculated using the Reed-Muench method (Reed and Muench, 1938). Figure 3.2 compares all three genotypes challenged against both type 1 and type 2 PRRSV isolates. hCD163L-1 PAMs measure one half fold less $TCID_{50}$ values than WT PAMs. Null PAMs failed to support any PRRSV infection. Figure 3.3 also shows hCD163L-1 (HL11) PAMs measuring one half fold less $TCID_{50}$ values compared to WT PAMs when infected with only type 2 isolates.

In Vitro PRRSV Infection of PAMs (Percent Infection)

Infection rates for PAMs were assessed by calculating percent infection in three windows per well consisting of 60-80 cells per window at 40X magnification. In concurrence with $TCID_{50}$ measurements in the previous result, Figure 3.3 A reveals hCD163L-1 is one half fold less in percent infection compared to WT PAMs when infected with Type 2 isolates. Figure 3.3 B shows hCD163L-1 domain 8 mimic PAMs are resistant to Type 1 isolates.

In Vitro PRRSV Infection of Cultured Macrophages Derived from PBMCs

After three days of culture and stimulation by GM-CSF, mature macrophages were derived from PBMCs that were isolated from whole blood. Percent infections were counted in 3 windows/ well consisting of 40-60 cells per window. Due to decreased concentration of cultured PBMCs compared to PAMs there were less available cells to count in each window. Following the PBMC infections, in Figure 3.4 approximately 91% of WT macrophages were infected, 39% of hCD163L-1 macrophages were infected, and Null cells did not sustain any infection. No infection of Null cells is a likely result of the cultured macrophages lacking the expression of CD163 and the hCD163L1 domain 8 mimic (HL11m) cultured macrophages having an altered

CD163 receptor due to a domain swap and and potentially from the insertion of a PGK neomycin cassette. Figures 3.5 A, B, and C, represent images of PBMC derived macrophages under microscopic fluorescence. These images support figure 3.4 regarding pig genotype and PRRSV infectivity.

In Vitro Fluorescence of Frozen Macrophages Derived from PBMCs

Macrophages were cultured from PBMCs in whole blood and were frozen at -80° for 24 hours. Cells were then thawed, fixed with 70% acetone, and stained with CD163/M130 mab instead of being infected with PRRSV. Cells were then stained with a fluorescent antibody for CD163 positive cell detection. Figure 3.6 A exhibits the percentage of cells that expressed fluorescence in accordance with individual pig genotype. Figure 3.6 B shows the average percent fluorescence of cultured macrophages. Macrophages of the hCD163L-1 genotype exhibit approximately a half fold less fluorescence than WT macrophages, indicating an altered CD163 receptor. Null macrophages did not express any fluorescence.

Successful culture and infection of MNCs has exhibited results similar to *in vitro* infections of PAMs derived from sacrificed animals. Fixation and staining of the macrophages with a mab and fab led to the result of fluorescent cells under a fluorescent microscope, indicating CD163 was present on WT cells. Macrophages of the gene edited pigs showed little or no fluorescence, which portrayed similar results to *in vitro* PRRSV infection of cultured macrophages that were not stored, and *in vitro* infection of PAMs. However, further studies need to be conducted to account for the loss of cell numbers potentially due to freezing and thawing.

Tables and Figures

Figure 3.1:Receptor Expression Levels of CD163 and CD169 on PAMs
Receptor expression levels were observed via flow cytometry with FITC, (fluorescein isothiocyanate) and PE (PE-Cy7). A) The majority of WT PAMS fell into quadrant 2, indicating that both CD163 and CD169 was being expressed. The majority of hCD163L-1 PAMS fell into quadrants 2 and 4, indicating that both CD163 and CD169 was being expressed, but not at very high levels. The majority of Null PAMS fell into quadrant 4, indicating that CD163 is not expressed, but CD169 was being expressed. B and C) Levels of fluorescence for CD163 or CD169 on a linear scale. The black line indicates background fluorescence, while the red line represents the level of fluorescence indicated for the labeled antibody CD163. The Null PAMS did not show any observable level of fluorescence for CD163. There was an observable fluorescence for CD169 in Null PAMs.

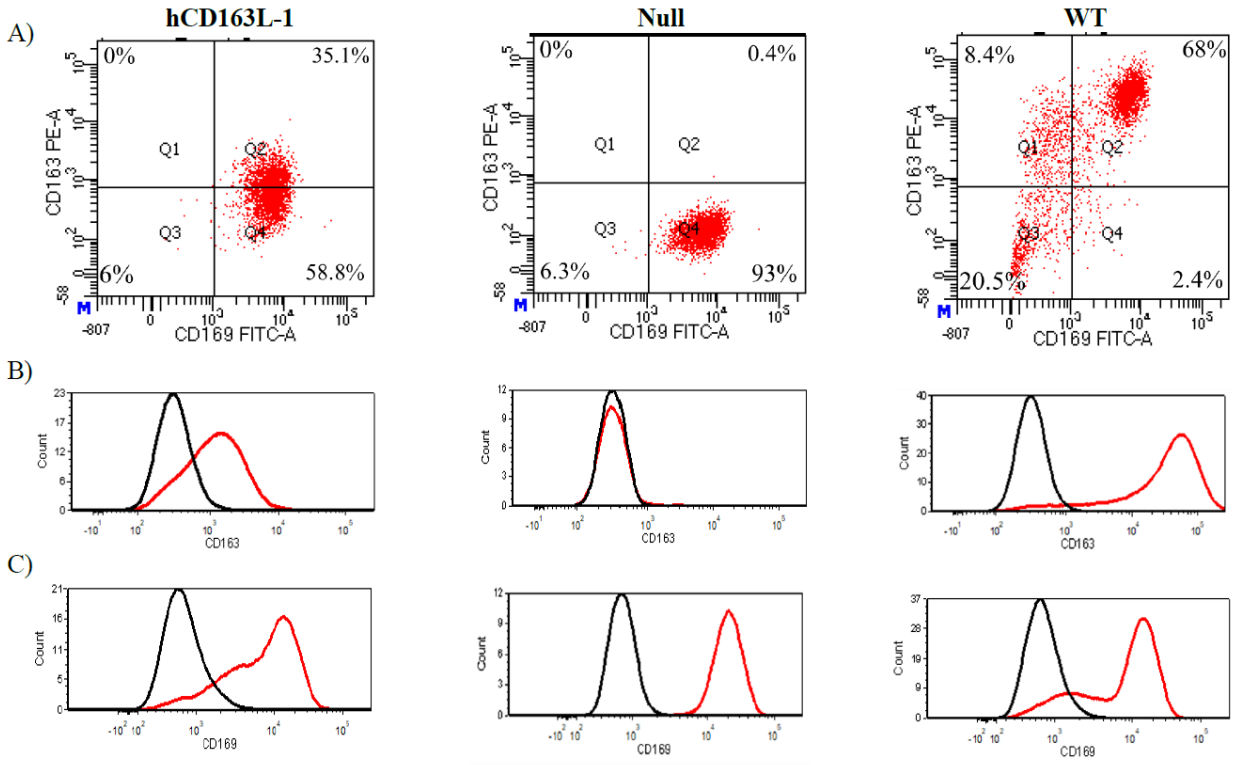


Figure 3.2:In Vitro infection of PAMs measured in units of $TCID_{50}$
Comparing WT, hCD163L-1, and Null pig PAM infections in units of $TCID_{50}$. A and B) hCD163L-1 pigs showed one half fold less infectivity compared to WT pigs when compared across seven various PRRSV isolates. C) Null PAMs failed to support virus infection.

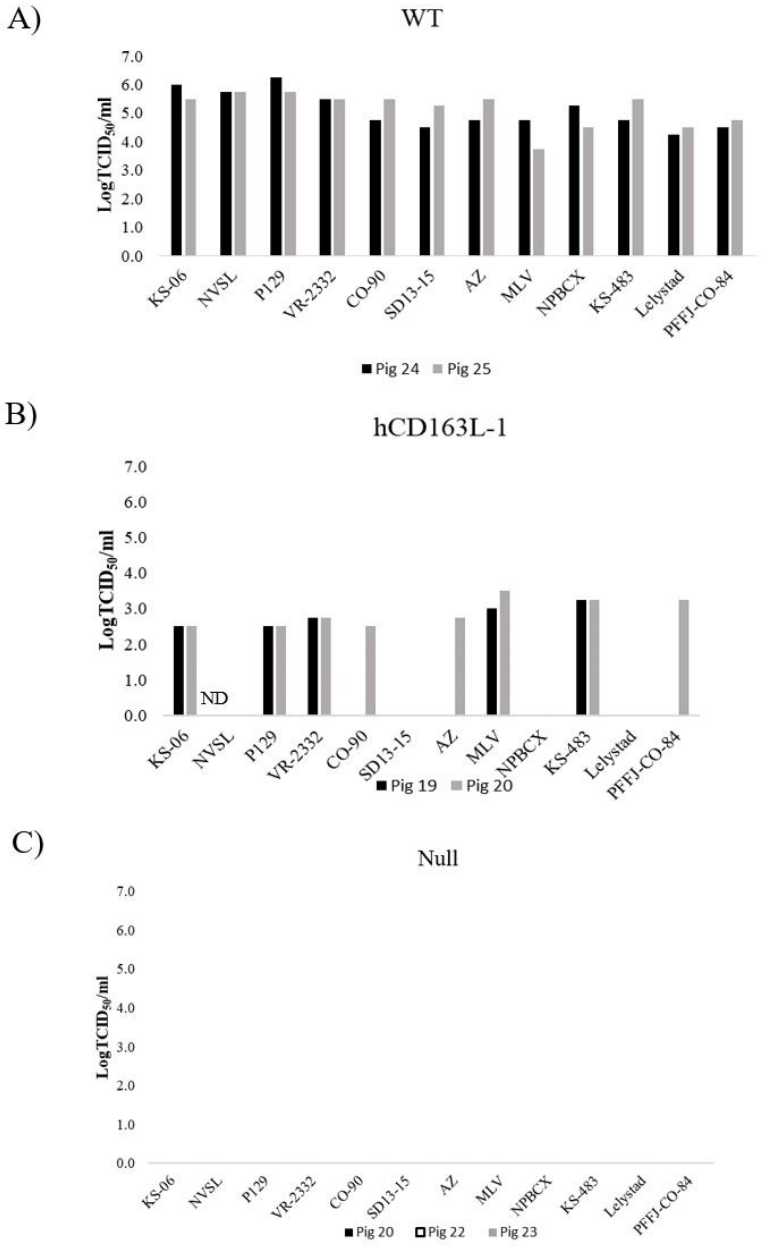


Figure 3.3:In Vitro infection of PAMs measured in units of $TCID_{50}$

From Wells et *al.* 2017. Comparing HL11 (hCD163L-1) pigs to WT pig in units of $TCID_{50}$. HL11 pigs showed one half fold less infectivity compared to WT pigs when compared across seven various PRRSV isolates.

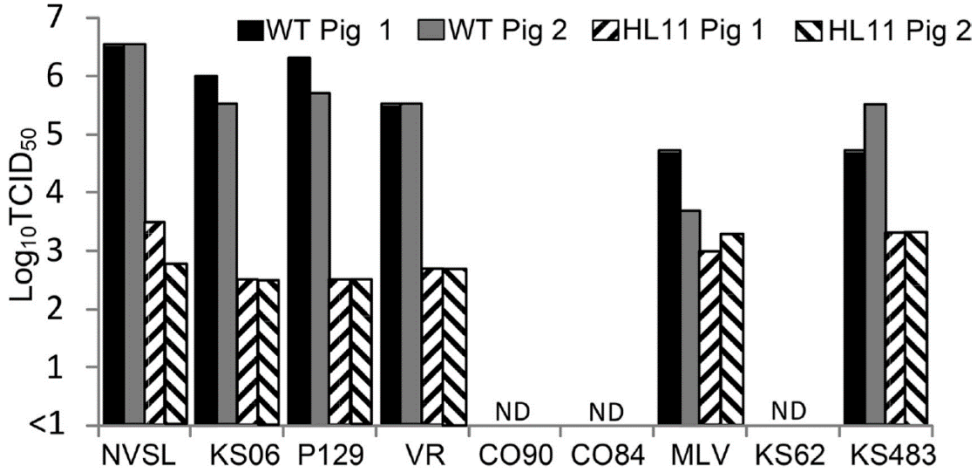
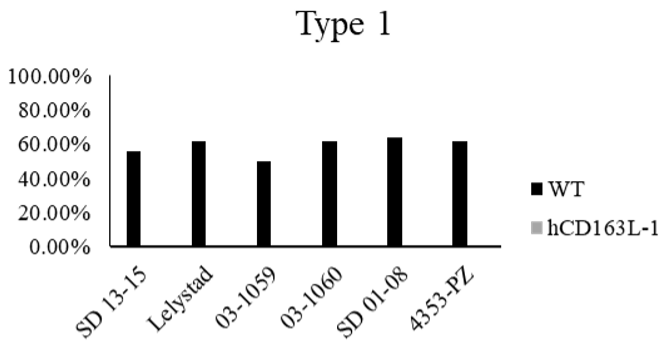


Figure 3.4: *In Vitro* infection of PAMs measured in percent infection
Comparing hCD163L-1 pigs to WT pig in percentage of PRRSV infection. A) There was no infectivity of hCD163L-1 pigs with genotype 1 PRRSV isolates. B) hCD163L-1 pigs showed one half fold less infectivity compared to WT pigs when compared across nine various genotype 2 PRRSV isolates.

A)



B)

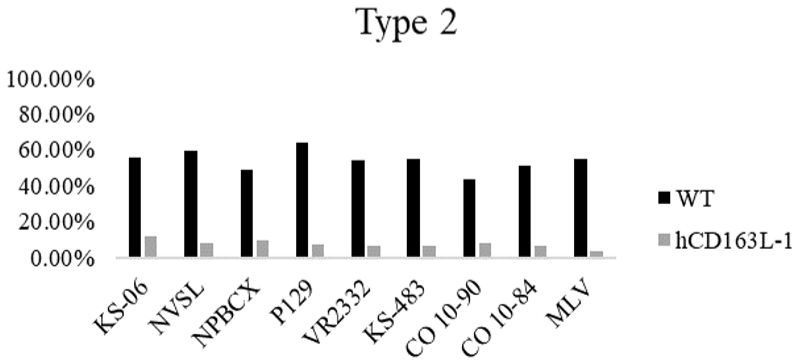


Figure 3.5:*In vitro* infection of PMBC derived macrophages

WT macrophages demonstrated almost full virus replication, while cells of the hCD163L-1 genotype displayed slightly less than one third of WT percent infection. Pigs of the Null genotype did not support virus replication.

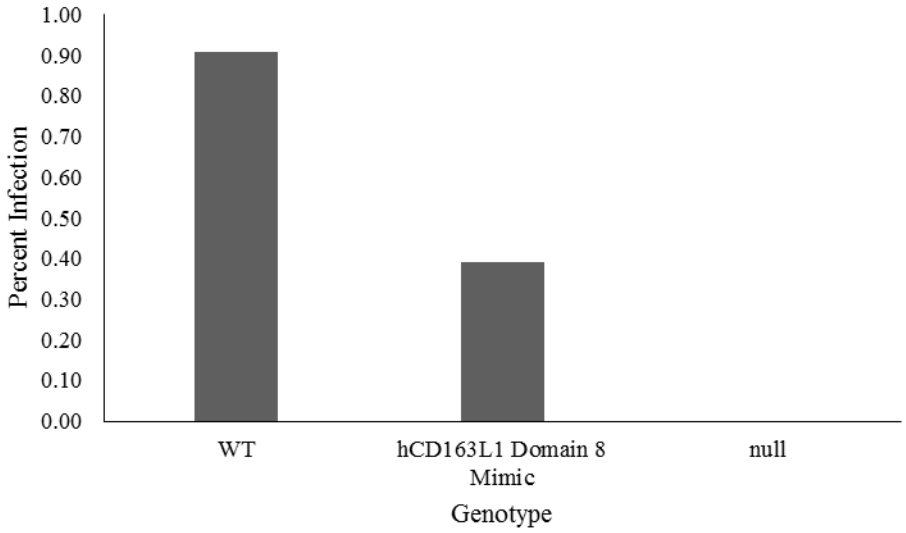


Figure 3.6:Images of PBMC derived macrophages infected with VR-2332 and stained with Alexafluor 594.

A) WT PBMCs expressing almost full fluorescence. B) hCD163L-1 PBMCs expressing markedly less fluorescence, but is still somewhat observable. C) PBMCs of the Null genotype showing no fluorescence.

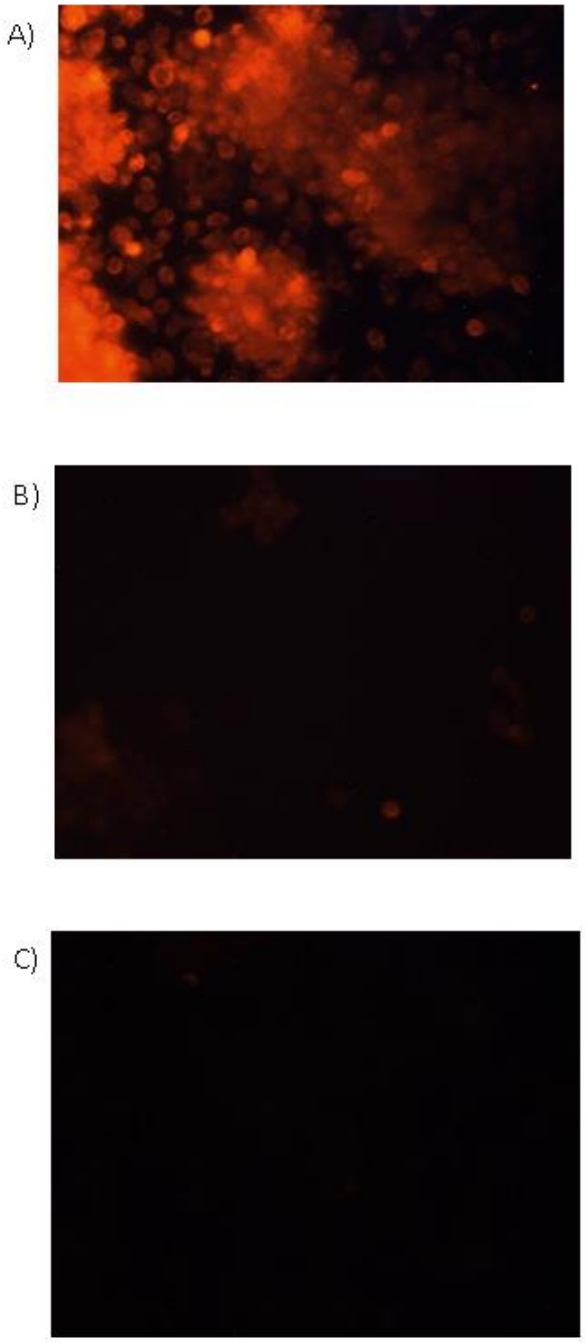
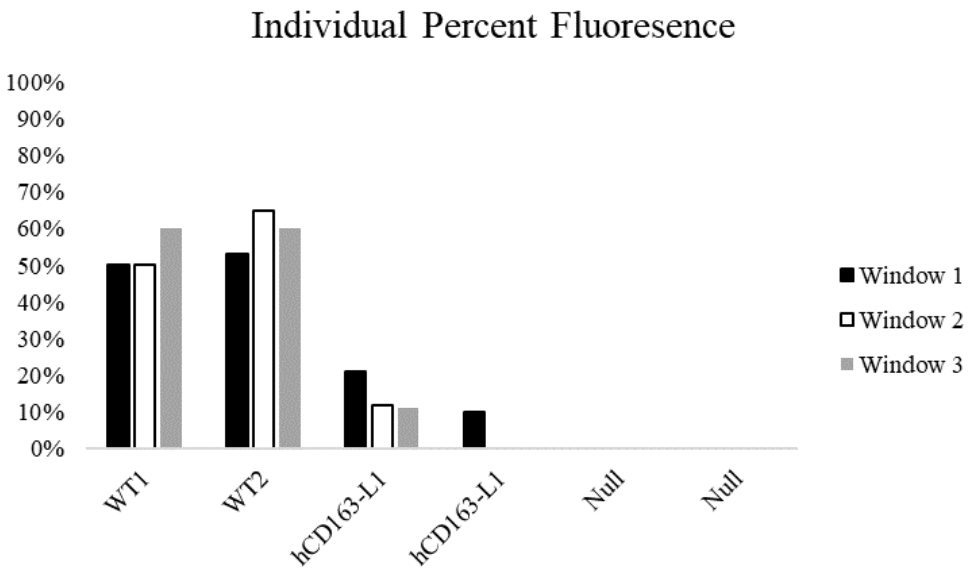


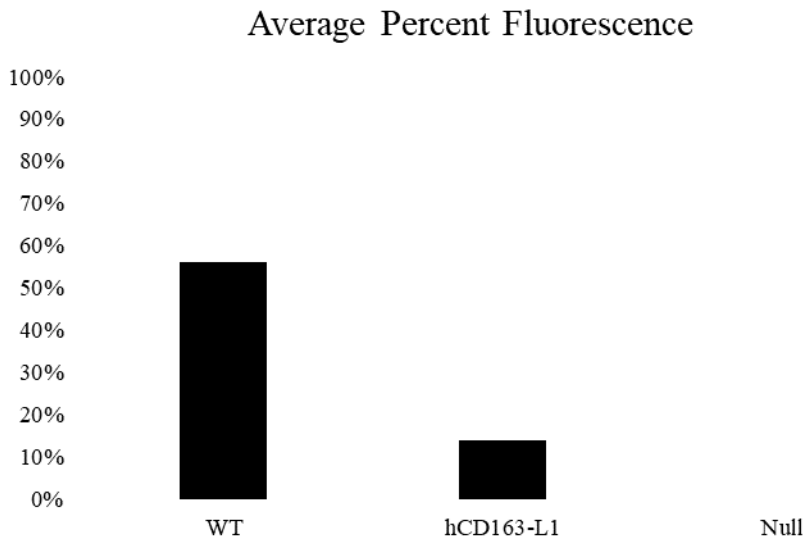
Figure 3.7:Figures of frozen cultured macrophages from PBMCs

Cultured macrophages from PBMCs that were frozen as a means of potential storage/transportability. Windows indicate the number of cells that were counted in the specific well. A) Individual percent fluorescence with windows of allotted pig genotypes. B) Average percent fluorescence of stained macrophages.

A)



B)



Chapter 4 - Discussions and Conclusion

The objective of this study was to verify the disease resistance capabilities of macrophages derived from genetically edited pigs when exposed to not only one isolate of PRRSV, but multiple isolates. Since CD163 was found to serve as the main receptor for PRRSV incorporation into the macrophage, many studies have been undergone as an effort to better understand the receptor and ways to manipulate it. The manipulation of CD163 was performed and successful by utilizing the CRISPR/Cas9 gene editing system (Calvert et *al.* 2007; Whitworth et *al.* 2013)

Previously, an *in vivo* challenge was performed on WT pigs and pigs that were genetically edited with CRISPR/Cas9 technology. Null gene-edited pigs were found to be resistant to both genotype 1 and 2 PRRSV isolates. Additionally, it was found that hCD163L-1 pigs were completely resistant to genotype 1 (European) but not to genotype 2 (North American) PRRSV (Wells et *al.*, 2017). The results from this study warranted further investigation of the resistance capabilities of Null and hCD163L-1 genotypes.

The discovery of newfound resistance led to *in vitro* challenges of PAMs to multiple isolates of PRRSV, either from genotype 1 or 2 lineage. Results from the *in vitro* challenges indicated that the Null pig genotype is completely resistant to all PRRSV isolates (both genotype 1 and 2), while the hCD163L-1 genotype is completely resistant to multiple genotype 1 PRRSV isolates, and reduced infection with genotype 2 isolates. (Wells et *al.*, 2017). However, *in vitro* experiments showing reduced infection of hCD163L-1 PAMs did not reflect the results from the *in vivo* PRRSV challenge. hCD163L-1 pigs infected with the genotype 2 virus had a mean viremia like the WT pigs, indicating the reduced of permissiveness of PAMs did not translate

back to the pig. There are limitations of deducing *in vitro* model results back to the live animal, and these results have proven correct of these limitations.

Extraction of PAMS from pigs requires a terminal procedure consisting of lung lavages. Upon necropsy, care must be taken to avoid PAM contamination from other bodily fluids or tissues. The isolation of PBMCs from whole blood can serve as an alternative option of using macrophages for *in vitro* experiments. Eventual differentiation of macrophages in culture from isolated PBMCs do not require the sacrifice of an animal.

The demonstration of CD163 gene edited pigs, showing no support of infection from multiple isolates of PRRSV from either genotype, can infer their potential resistance to a vast number of more isolates (Wells et *al*., 2017). Cell culture models can prove a valuable resource for evaluating the pathogenesis of PRRSV and the expression of CD163. Additionally, *in vitro* experiments have the capability of producing results in a more cost effective and timely fashion. Procedures for further *in vitro* investigations regarding the gene edited pig can expand our understanding of not only the CD163 receptor, but potentially other genes that have the capability of being edited by CRISPR/Cas9 technology. PRRSV resistant pigs emphasizes the outstanding opportunity to create a preventative cure for one of the most significant diseases affecting the pork industry throughout the globe.

Chapter 5 - References

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