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Pigs lacking the scavenger receptor cysteine-rich domain 5 of CD163 are resistant to PRRSV-1 infection.

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Abstract

Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) has a narrow host cell tropism, limited to cells of the monocyte/macrophage lineage. CD163 protein is expressed at high levels on the surface of specific macrophage types and a soluble form is circulating in blood. CD163 has been described as a fusion receptor for PRRSV, with the scavenger receptor cysteine-rich domain 5 (SRCR5) region having been shown to be the interaction site for the virus.

As reported earlier, we have generated pigs in which Exon 7 of the CD163 gene has been deleted using CRISPR/Cas9 editing in pig zygotes. These pigs express CD163 protein lacking SRCR5 (ΔSRCR5 CD163) and show no adverse effects when maintained under standard husbandry conditions. The ΔSRCR5 CD163 was not only detected on the surface of macrophage subsets, but the secreted, soluble protein can also detected in the serum of the edited pigs, as shown here by porcine soluble CD163-specific ELISA. Previous results showed that primary macrophage cells from ΔSRCR5 CD163 animals are resistant to PRRSV-1, subtypes 1, 2, and 3, as well as PRRSV-2 infection in vitro. Here, ΔSRCR5 pigs were challenged with a highly virulent PRRSV-1, subtype 2 strain. In contrast to the wildtype control group, ΔSRCR5 pigs showed no signs of infection and no viremia or antibody response indicative of a productive infection. Histopathological analysis of lung and lymph node tissue showed no presence of virus replicating cells in either tissue. This shows that ΔSRCR5 pigs are fully resistant to infection by the virus.
Porcine Reproductive and Respiratory Syndrome virus (PRRSV) is the etiological agent of PRRS, causing late-term abortions, stillbirths, and respiratory disease in pigs, incurring major economic losses to the world-wide pig industry. The virus is highly mutagenic and can be divided into two species, PRRSV-1 and PRRSV-2, each containing several subtypes. Current control strategies mainly involve biosecurity measures, depopulation, and vaccination. Vaccines are at best only partially protective against infection with heterologous subtypes and sublineages and modified-live vaccines have frequently been reported to revert to virulence. Here we demonstrate that a genetic control approach results in complete resistance to PRRSV infection \textit{in vivo}. CD163 is edited such as to remove the viral interaction domain while maintaining protein expression and biological function, averting any potential adverse effect associated with protein knock-out. This research demonstrates a genetic control approach with potential benefits in animal welfare as well as to the pork industry.
Introduction

Porcine reproductive and respiratory syndrome (PRRS) is arguably the most economically important infectious diseases affecting pigs worldwide. The causative agent of PRRS is PRRS virus (PRRSV), a member of the *Arteriviridae* family and the order *Nidovirales*. Infected pigs of all ages may present with symptoms involving inappetence, fever, lethargy, and respiratory distress. However, the most devastating effects of PRRSV infection are observed in young piglets and pregnant sows. In pregnant sows full abortions or death and mummification of fetuses *in utero* are observed and live-born piglets from an antenatal infection are often weak and display severe respiratory symptoms (1-3). Piglets infected with PRRSV in early life can show diarrhea and more commonly severe respiratory distress due to active PRRSV replication in pulmonary macrophages and subsequent damage in lung tissues (4). Due to reduction or loss of pregnancies, death in young piglets, and decreased growth rates in all PRRSV infected pigs it is estimated that the economic impact of PRRSV to pork producers in the United States alone is more than $650m annually (5, 6).

There are two different species of PRRSV with distinct geographic distributions, PRRSV-1 is found primarily in Europe and Asia, overlapping the range of PRRSV-2, which is found in Asia and the Americas. PRRSV-1 can be further divided into at least three subtypes, currently based on ORF7 sequences and geographical distribution, with subtype 1 being pan-European whilst subtypes 2 and 3 are currently limited to Eastern Europe (7).

PRRSV has a very narrow host cell range, infecting only specific subsets of porcine macrophages (8-10). Entry of PRRSV into macrophages has been shown to occur via pH-dependent, receptor mediated endocytosis (11, 12). Various attachment factors and receptors have been indicated to be involved in the PRRSV entry process (reviewed in (13)). However,
only the scavenger receptor CD163, also known as a hemoglobin/haptoglobin scavenger receptor or p155, has been confirmed to be an essential fusion receptor in vitro and in vivo (14-16). CD163 is expressed on specific subtypes of macrophages. The extracellular portion of CD163 forms a pearl-on-a-string structure of nine scavenger receptor cysteine-rich (SRCR) domains and is anchored by a single transmembrane segment and a short cytoplasmic domain (17). A proteolytically cleaved, soluble form of the protein ectodomain is found in the bloodstream, and is involved in the inflammation and ischemic repair response (18, 19). Transmembrane anchoring and interaction with the SRCR domain 5 (SRCR5) of CD163 were found to be essential for successful infection with PRRSV (20, 21). CD163 has a variety of biological functions, including mediating systemic inflammation and the removal of hemoglobin from blood plasma (reviewed in (21, 22)). Overexpression of CD163 renders non-susceptible cells permissive to PRRSV infection (20), and it was found that CD163 does not mediate internalization but is crucial for fusion (16).

Recent in vivo challenge experiments of pigs in which both copies of the CD163 gene had been knocked-out using gene editing technology confirmed that CD163 is required for infection by PRRSV-2 and highly pathogenic PRRSV-2 (HP-PRRSV) (14, 23). Gene editing technology has also been used to generate pigs in which the CD163 SRCR5 encoding sequence has been replaced with a sequence encoding human CD163L1 domain SRCR8 (24), and in effect replicating in vivo the earlier in vitro domain swapping experiment of Van Gorp and colleagues (25). This attempt to maintain CD163 function rendered pigs and macrophages resistant to PRRSV-1 but not PRRSV-2 infection (24), making this strategy ineffective in combating both PRRSV species. CD163 has important biological functions and the complete knockout could have a negative physiological impact on the animal, particularly with respect to inflammation.
response and/or infection by other pathogens. Interestingly, whereas all the other eight SRCR domains have been shown to be involved in different biological functions, no specific role has been associated with SRCR5, other than in PRRSV infection (21). Therefore, we generated pigs lacking SRCR5 by deletion of exon 7 of $CD163$ using CRISPR/Cas9 editing and showed that macrophages from these pigs were resistant to both PRRSV-1 and PRRSV-2 infection in vitro (15). The aim of the experiments described here was to determine whether our in vitro results would translate directly in vivo by conducting a PRRSV challenge of pigs with a CD163 SRCR5 deletion. Further, we aimed to characterize the biological function of the modified CD163 protein ($\Delta$SRCR5 CD163) both as a soluble and a cell-bound protein.
Results

Genome editing in zygotes for ΔSRCR5 CD163 pigs and breeding genotypically uniform F2 pigs

Founder generation F0 animals carrying a deletion of exon 7 in the CD163 gene, which encodes SRCR5 of the protein, were generated by CRISPR/Cas9 gene editing as previously described (ΔSRCR5 pigs, (15)). Briefly, zygotes were microinjected with a combination of Cas9mRNA and guide RNAs targeting sites flanking exon 7, resulting in double-strand breaks (DSBs) and deletion of the exon (Figure 1A). Cross-breeding of heterozygous founder animals and outbreeding with wildtype pigs yielded a first generation composed of both heterozygous and biallelic edited animals (F1 generation, (15)). We selected for breeding heterozygous F1 animals displaying a “clean” deletion between the DSBs, i.e. no ‘on-target’ sequence changes beyond the desired deletion region. To generate a cohort with comparable genetic background half sibling heterozygous animals and wildtype animals were bred to yield homozygous ΔSRCR5 animals (Figure 1A) and wildtype half and full sibling animals.

As previously described, ΔSRCR5 animals express the ΔSRCR5 CD163 mRNA and protein at equivalent levels to CD163 transcripts and protein in wildtype siblings. Furthermore, the native-structure ΔSRCR5 CD163 is recognized on the surface of pulmonary alveolar macrophages (PAMs) by a commercial antibody (15). We further analyzed whether a template-based protein structure analysis by RaptorX predicted folding of each subdomains compatible with a functional ΔSRCR5 CD163 protein (26). As demonstrated in figure 1B, all subdomains in both the full-length and ΔSRCR5 CD163 are predicted to adopt the globular structure and retain the pearl-on-a-string configuration of the native CD163 protein. This supports our findings indicating proper folding and expression of the ΔSRCR5 protein.
Previously, we have shown that PAMs and *in vitro* differentiated peripheral blood monocytes are resistant to infection with both PRRSV-1 and PRRSV-2. Now, we aimed to assess the resistance of these pigs to PRRSV-1 infection *in vivo*. We selected four homozygous ΔSRCR5 F2 animals and four wildtype controls that were co-housed from weaning (Figure 1C). Blood samples were taken from all eight pigs and a full blood count conducted by the diagnostics laboratory at the Royal (Dick) School of Veterinary Sciences, University of Edinburgh. The blood counts of all animals were within reference values indicating good general health and the absence of infection or inflammation. Furthermore, the hemoglobin levels of all animals were within reference values, indicating normal function of the hemoglobin/haptoglobin scavenging activity of CD163 (Table 1).

At 6 weeks of age a serum sample was collected from all animals prior to movement to a specified pathogen-free (SPF) unit. The cohort were co-housed for the duration of the experiment and allowed to settle for one week prior to initiation of the challenge. On day 0 of the challenge with PRRSV-1 a second serum sample was taken and soluble CD163 (sCD163) serum levels were assessed using a commercially available enzyme-linked immunosorbent assay (ELISA) recognizing soluble porcine CD163. Serum CD163 levels were found to be 433.2 ± 69.57 ng/ml in wildtype pigs and 463.5 ± 68.99 ng/ml in ΔSRCR5 pigs (Figure 2). These results are not significantly different from each other (P=0.7512) and are comparable to sCD163 levels in humans (for example (27)).

ΔSRCR5 pigs are resistant to PRRSV-1 infection

At 7-8 weeks of age the pigs were inoculated intranasally with the PRRSV-1, subtype 2 strain BOR-57 (28). Eastern European strains are often associated with higher pathogenicity than other PRRSV-1. For BOR-57, we previously observed mild respiratory symptoms, elevated
core temperature, extensive lung pathology, and high viral loads in serum. For this study, the strain was selected due to the high viremia and shedding levels expected to occur under study conditions. The experiment was conducted for a period of 14 days following inoculation of each pig on days 0 and 1 with 5E6 TCID₅₀, as determined by assessment on PAMs, of the virus isolate used. Rectal temperature, respiration, nasal discharge, coughing, and demeanor were recorded every day and serum samples were collected on days 0 (prior to challenge), 3, 7, 10, and 14 (prior to euthanasia). Weights were recorded on day 0, 7, and 14 (prior to euthanasia). People assessing the pigs clinically, conducting the challenge, and analyzing the pathology were blinded to the genotype status of the animals.

The rectal temperatures were significantly elevated on days 6-9 of the challenge in the wildtype animals, whereas no fever was observed in the ∆SRCR5 animals (Figure 3A). The average daily weight gain of the ∆SRCR5 pigs was higher compared to their wildtype counterparts over the entire challenge period and significantly higher over days 7-14 (p=0.0465, Figure 3B). One wildtype pig showed decreased demeanor on days 7-8, no respiratory symptoms or other abnormalities in behavior were observed in any of the other animals during the course of the study, as expected in a PRRSV-1 infection at this age. Viral RNA was isolated from serum and virus levels quantified using a DNA fragment template standard and viral RNA extracted from virus stocks of known infectivity. Whereas the wildtype pigs showed a high viremia no viral RNA was detected in the serum of ∆SRCR5 pigs (Figure 3C). The presence of antibodies against PRRSV was assessed using a commercial ELISA able to detect antibodies against all PRRSV-1 subtypes and PRRSV-2. PRRSV antibodies were detected in wildtype pigs from day 7 and present at significant levels (as per the manufacturers
indicated positive threshold >0.4 s/p ratio) on days 10 and 14 (Figure 3D), but were not
detected in samples from the ΔSRCR5 pigs collected during the course of the experiment.

During necropsy lungs were assessed initially and detailed photographs taken of the dorsal
and ventral side. Lungs were scored for the presence of lesions. An established scoring system,
based on the approximate contribution of each lung section to the complete lung volume was
employed (29). On average, 61% of the lung surface of control pigs was found to be mottled-
tan with areas of consolidations compared to 0.25% of lung surfaces in ΔSRCR5 pigs (Figure 3
E & G). Samples of the lungs were fixed in formalin, embedded in paraffin, cut into sections,
and stained for further analysis. To assess general lung histology samples were stained with
hematoxylin and eosin. Sections from each pig were assessed for the presence of interstitial
pneumonia on a scale of 0-6 (0, normal; 1, mild multifocal; 2, mild diffuse; 3, moderate
multifocal; 4, moderate diffuse; 5, severe multifocal; 6, severe diffuse). Microscopic lung
lesions characterized by multifocal to diffuse interstitial pneumonia with type 2 pneumocytes,
hypertrophy, and hyperplasia were only observed in wildtype animals (averaged 4) and were
not present in lung sections of ΔSRCR5 pigs (Figure 3 E & F, top). The presence of PRRSV
antigens was assessed by immunohistochemistry on lung sections and lymph node sections
using a mixture of two different antibodies against the PRRSV-N protein as described before
(30). PRRSV antigens were detected in 3 out of 4 lung sections and 1 out of 4 lymph node
sections of wildtype animals, but no PRRSV antigens were detected in sections from ΔSRCR5
pigs (Figure 3 E & F, bottom).

Overall, no signs of infection were detected in ΔSRCR5 animals despite the high initial
inoculation volume and persistent exposure to infected wildtype animals that actively shed
virus (the wildtype and edited pigs were co-housed throughout the experiment). This is a clear
demonstration that ΔSRCR5 animals are resistant to PRRSV-1 infection, confirming our previous in vitro results (15).

ΔSRCR5 pigs show no cytokine response to PRRSV-1 infection and generally normal cytokine levels

CD163 is involved in the cytokine response to infection and immune stimuli, as well as hemoglobin-haptoglobin (Hb-Hp) uptake. Hb-Hp stimulation has been shown to lead to increased levels of IL-6 and IL-10 as well as IL-1 and TNFα downregulation (31, 32). SRCR domains 1-4 and 6-9 contain binding sites for TNF-like weak inducer of apoptosis (TWEAK), an anti-inflammatory cytokine that negatively regulates CD163 expression (33, 34).

Stimulation with inflammatory mediators can induce secretion of soluble CD163 and TNFα in an ADAM17-mediated manner (35). In order to assess these biological functions in the ΔSRCR5 pigs we assayed expression levels of key cytokines. Quantitative antibody arrays were used to assess the expression levels of 20 cytokines in serum collected from pigs on day 0 (prior to challenge), 3, 7, 10, and 14 of the challenge. Overall, baseline cytokine levels as determined on day 0, considered a baseline were similar between ΔSRCR5 and wildtype pigs.

However, the monokine induced by gamma interferon (MIG, also known as CXCL9) was found to show consistently higher levels in wildtype pigs until day 10, after which no significant difference was detected. MIG is a T-cell chemoattractant to inflammation sites and involved in repair of tissue damage. In wildtype animals MIG was strongly upregulated on days 7 and 10 of the challenge (36) (Figure 4H). The chemokine ligand 3-like 1 (CCL3L1, an isoform of MIP-1α), involved in the inflammation response via CCR5 signaling and downregulated by IL-10 (37), was found to be higher in wildtype compared to ΔSRCR5 animals (Figure 4J). As IL-10 levels were found to be comparable in both genotype groups IL-10-mediated downregulation
is unlikely to be the cause of low CCL3L1 levels. In wildtype animals CCL3L1 was elevated in the serum on days 10 and 14, whereas no significant IL-10 elevation was found to occur over the period of the challenge (Figure 40).

We observed sequential cytokine responses to PRRSV-1 infection in wildtype animals, with an early increase in interferon α (IFNα), interleukin-17A (IL-17A), and the IL 1 receptor antagonist (IL-1ra) (Figure 4A, B, and C), followed by an increase in ILs 4, 6, and 8 (IL-4, IL-6, and IL-8, respectively) at the high point of viremia, from 7 days post inoculation (dpi) (Figure 4 D, E, and F). Increased levels of MIG, and the macrophage inflammatory protein 1β (MIP-1β, also known as CCL4) were only observed transiently at 10 dpi (Figure 4 G and H). In the last period of the challenge, with moderate but reducing levels of viremia, elevations in CCL3L1, granulocyte macrophage colony stimulating factor (GM-CSF), ILs 12 and 1β (IL-12 and IL-1β) were detected (Figure 4 I, J, K, L, and M). All of these cytokine responses were restricted to wildtype animals, with no cytokine response recorded in ΔSRCR5 pigs. IL-10, transforming growth factor β1 (TGFβ1), and IFN γ (IFNγ) levels showed no significant difference between wildtype and ΔSRCR5 pigs at any of the time points but were found to change significantly over time in the wildtype animals (calculated using a two-way ANOVA & Sidak's multiple comparison test) (Figure 4 N, O, P). IL 18 (IL-18) levels decreased significantly over time in wildtype animals but were not significantly different from those of ΔSRCR5 pigs at each time point (Figure 4 Q). Platelet endothelial cell adhesion molecule (PECAM1) was significantly elevated on day 3 of the challenge and decreased on day 10 compared to levels of ΔSRCR5 pigs (Figure 4 R). No significant difference in levels of IL 1α (IL-1α) and IL 13 (IL-13) was found between ΔSRCR5 and wildtype pigs or over time (Figure 4 S and T).
Discussion

The results of this study show that ΔSRCR5 pigs are healthy under standard husbandry conditions and maintain biological function of the CD163 protein, whilst being resistant to PRRSV infection. So far, we have bred three generations of edited animals with over 10 litters and didn’t observe any abnormalities in breeding.

ΔSRCR5 pigs were generated, as previously described (15), by using two sgRNAs flanking exon 7 of CD163 CRISPR/Cas9. Here we have shown that heterozygous and homozygous animals can be bred naturally and yield normal litter size offspring. Our previous data showed that primary PAMs and peripheral blood monocyte (PBMC)-derived macrophages from ΔSRCR5 pigs are fully resistant to PRRSV-1, subtype 1, 2, and 3 infection as well as to both typical and atypical PRRSV-2 (15). To confirm that these results translated to the in vivo model we showed here that ΔSRCR5 pigs are completely resistant to infection with a highly virulent PRRSV-1, subtype 2 strain. The edited animals displayed no clinical or pathological signs of infection, no viral replication was observed, and no cytokine response (indicative of low level virus replication) was observed. This confirms that our previous in vitro results directly translate to the in vivo situation.

The ΔSRCR5 CD163 protein was previously detected on the surface of CD163-expressing macrophages using a native-confirmation antibody (15). Analysis of the ΔSRCR5 CD163 amino acid sequence in silico using RaptorX predicts that post-translational folding will yield a protein that closely mimics the structure of full length CD163. The expression of ΔSRCR5 CD163 in animals has several advantages over previously described PRRSV-resistant CD163 knockout animals generated by the random introduction of a premature stop codon in exon 3 or exon 7 of the CD163 gene (14, 23). Free hemoglobin, often released following hemolytic
events, can cause serious toxicity to a system (reviewed in (38)). CD163 is a direct mediator of the Hb-Hp complex uptake into macrophages, which sequesters and degrades this potentially toxic compound (39). We have previously shown that PBMC-derived macrophages from ΔSRCR5 animals are still capable of CD163-mediated Hb-Hp uptake, as demonstrated by HO-1 upregulation and uptake of fluorescently labelled Hb<sub>AF488</sub>-Hp complex (15). All ΔSRCR5 animals show normal hemoglobin levels in their blood, confirming the proper clearance of Hb-Hp complexes. Yang et al. recently re-made pigs with a premature stop codon in exon 7 of CD163, resulting in a functional CD163 knock-out, as previously reported (40). Surprisingly, this new paper claims that PBMC-derived macrophages from CD163 knock-out animals are able to uptake Hb<sub>AF488</sub>-Hp complexes <i>in vitro</i> (23), a result that directly contradicts the findings by Schaer et al., Nielsen et al. and others (39, 41) in human macrophages, Etzerodt et al. in cells with murine CD163 (42), Boretti et al. in canine macrophages, all of which highlight the essential requirement for CD163 to be present for Hb-Hp uptake in macrophages. CD163 both in its cell-bound as well as its secreted form has been shown to have multiple other functions in addition to Hb-Hp scavenging (reviewed in (18)). One important aspect is the regulatory function of soluble CD163 following inflammation and ischemic repair, which was found to result in enhanced regeneration activity in CD163 knock-out mice resulting in abnormal peripheral blood vessel development and systemic rather than local regeneration after injury (43). Soluble CD163 is also able to bind <i>Staphylococcus aureus</i>, which promotes recognition, phagocytosis, and killing of this important livestock and human pathogen (44). Soluble CD163 is not the result of alternative splicing but results from proteolytic cleavage, likely by the metalloprotease ADAM17 in the juxtamembrane area following SRCR domain 9 of the protein (45). Proper folding of CD163 and accessibility of this area are essential for the
secretion of soluble CD163. Here we showed the presence of soluble CD163 in the serum of ΔSRCR5 pigs at comparable levels to wildtype animals.

CD163 knock-out mice have been reported to be significantly more susceptible to intra-abdominal sepsis, which is linked to haptoglobin-HMGB1 signaling and cytokine response (46). It was also found that CD163 plays a role in asthmatic human patients and CD163 knock-out mice were found to have increased airway eosinophils and mucus cell metaplasia linked to CCL24 chemokine signaling upon dust mite challenge (47). We analyzed the function of ΔSRCR5 CD163 in signaling and cytokine response by measuring the base line cytokine levels of ΔSRCR5 pigs compared to their wildtype counterpart. We also monitored the cytokine levels during the course of the in vivo PRRSV challenge to identify any changes that could result from low-level PRRSV replication. Whereas we found an orchestrated sequence of inflammation and immune response signaling in the PRRSV infected wildtype animals no cytokine response was observed in the ΔSRCR5 pigs. Of the panel of 20 cytokines analyzed only one cytokine, CCL3L1, was significantly different between the two groups of animals over the entire course of the challenge. The inflammation response protein CCL3L1 is involved in inflammation response and is downregulated by IL-10 but no significant differences in IL-10 between the two genotype groups could be found. We have no explanation for the higher CCL3L1 level in ΔSRCR5 pigs but the limited timeframe and animal number of this study warrant further investigation of this cytokine in the future. Another cytokine, MIG, showed higher levels in wildtype animals up to day 10 but no significant difference was observed on day 14. It will be interesting to investigate this observation further, over a longer period and with larger numbers of animals.
The creation of ΔSRCR5 pigs holds tremendous opportunity for the pork industry worldwide to improve both animal welfare and productivity at the same time. PRRSV infection has immunomodulatory outcomes and plays an important role in polymicrobial disease, such as the porcine respiratory disease complex. As such PRRSV-resistant animals could benefit general health and decrease the need for antimicrobial use at the same time. However, for the implementation of the next generation breeding / genome editing techniques in animal production, both consumer acceptance and the legislative framework need to be in place.
Materials and Methods

All animal work was approved by The Roslin Institute's and the University of Edinburgh's Protocols and Ethics Committees as well as the ethics group at Moredun Scientific Ltd. The experiments were carried out under the authority of U.K. Home Office Project Licenses PPL60/4518, PPL60/4482, and PPL70/8827 under the regulations of the Animal (Scientific Procedures) Act 1986. Humane endpoints were clearly defined.

Cells and viruses

Primary pulmonary alveolar macrophages (PAMs) for the propagation of PRRSV-1, subtype 2 strain BOR-57 (isolated from a sample from a Belarussian pig in 2009 by T. Stadejek et al.) were harvested from wild type research animals aged 6–9 weeks as previously described (48). Briefly, animals were euthanized according to a schedule I method. Lungs were removed and transferred on ice to a sterile environment. Lung lavage with warm PBS and gentle massage was used to recover PAMs. Cells were collected by centrifugation for 10 min at 400 x g. When necessary red blood cells were removed using red cell lysis buffer (10 mM KHCO₃, 155 mM NH₄Cl, 0.1 mM EDTA, pH 8.0) for 5 min before washing again with PBS. Cells were collected by centrifugation as before and frozen in 90% FBS (HI, GE Healthcare), 10% DMSO (Sigma). Cells were frozen gradually at 1°C/min in a -80°C freezer before being transferred to -150°C.

PAM cells were cultivated in RPMI-1640, Glutamax (Invitrogen), 10% FBS (HI, Gibco), 100IU/ml penicillin and 100μg/ml streptomycin (Invitrogen) (RPMI +/+).

Breeding and genotyping of animals

Unrelated founder animals generated by zygote injection of Cas9 mRNA and sgRNAs SL26 and SL28 as described (15) were bred to each other or to wildtype animals to generate
heterozygous F1 offspring. F1 animals with a double-strand break and re-ligation without insertions or deletions at the cutting sites of SL26 and SL28 were selected for breeding the F2 generation. Animals were genotyped as described previously (15), briefly, genomic DNA was extracted from ear biopsies using the DNeasy Blood and Tissue Kit (Qiagen). The region spanning intron 6 to exon 8 was amplified using primers oSL46 (ACCTTGATGATTGCGCTCTT) and oSL47 (TGTCCCAGTGAGAGTTGCAG), generating a 904 bp product from the intact allele and a 454 bp product if complete deletion of exon 7 had occurred. The PCR products were analyzed by separation on a 1% agarose gel and by Sanger sequencing.

**Animal challenge with BOR-57**

Four days prior to transfer of the animals to the specific pathogen-free unit (SPFU) blood and serum samples were taken from all animals by jugular venepuncture and the blood samples subjected to whole blood count analyses. Sera were screened using the IDEXX PRRSV X3 ELISA test to confirm that none of the animals had previously been exposed to PRRSV. Animals were acclimated in the SPFU for 1 week prior to challenge. Infectivity of BOR-57 stocks was assessed using a TCID$_{50}$ assay on PAMs immediately after production, prior to challenge, and on both challenge dates after administration. BOR-57 inoculum was tested for the absence of mycoplasma and other major viral pathogens. Animals were inoculated intranasally in the left nostril with 5 ml of 1E6 TCID$_{50}$/ml BOR-57 in RPMI +/- . Body weights of the animals were measured on day 0 prior to challenge, day 7 and day 14 prior to euthanasia. Serum samples were collected on day 0 prior to challenge, and days 3, 7, 10, and 14 prior to euthanasia by jugular venepuncture into vacutainer tubes. After clotting samples were centrifuged at 2000 x g for 10 min at 4°C and samples aliquoted and frozen at -80°C for further analysis. Clinical observations were recorded daily making note of
the rectal temperature, demeanor, nasal discharge, coughing and respiration. Feeding and water consumption and general health were observed and recorded daily. Humane endpoints were defined prior to challenge. No animal reached the criteria for premature termination during the challenge.

**Necropsy, Histopathology, & Immunohistochemistry**

On day 14 of the challenge animals were euthanized according to a schedule I method. During necropsy the lungs were removed, initially assessed and detailed photographs taken from the dorsal and ventral side for detailed scoring of macroscopic lung lesions. An established scoring system, based on the approximate contribution of each lung section to the complete lung volume was employed as previously described (29). Briefly, each lung lobe is assigned a number to reflect the approximate volume percentage of the entire lung represented. The affected area of each lobe is scored relative to the assigned volume percentage. Lung, mediastinal lymph node, and PAM samples were collected and frozen and lung and lymph node samples were fixed in 10% neutral-buffered formalin. Formalin-fixed sections were embedded in paraffin and routinely processed for histological examination with hematoxylin & eosin staining. Lung sections were scored for the presence and severity of interstitial pneumonia ranging from 0 (normal) to 6 (severe diffuse) as previously described (29).

Immunohistochemical analysis of lung and lymph nodes for the detection of PRRSV antigen was performed as previously described (30) using a mixture of two monoclonal antibodies, SDOW-17, 1/5,000, and SR-30, 1/1,500, (both RTI) as primary antibodies. Sections were counterstained with hematoxylin.

**Assessment of PRRSV virus and anti-PRRSV antibody levels in serum**
Viral RNA was extracted from the sera collected on day 0, 3, 7, 10, and 14 using the QIAmp Viral RNA Mini Kit (Qiagen) according to the manufacturer's instructions. Viral RNA levels were assessed by RT-qPCR using the GoTaq 1-Step RT-qPCR system (Promega) analyzed on a LightCycler II 480 (Roche). Viral RNA extracted from PAM cell culture supernatant with known multiplicity of infection (MOI) and a custom synthesized DNA fragment (Invitrogen) with known concentration (GAGAGCGGCCGCTAATACGACTCACTATAGTCAGCTGTGTCAGCTGGGAAAAATGATGAAATCCAGCGCCAGCAACCCAGGGAGGACAGCCCAAACAAAAAGGAAAAAGCTGAGAAGCCTCATTTTCCCTTTGGCTGCTGAAGATGACGTTCGGCACCATCTCACCGCAACTGAGCGTTCCCTCTGTCTGCAATCGACTCCAGACAGCCTTCAATCAGGGTGCGAGAAGCTCGTCTGTGCTCAATTCCAGCTTATGCGAGAAGGACAGCGTTTTCCAG) were used as standards. The primers used were BOR57_FWD: GAAATCCCAGCGCCAGCAAC and BOR57_REV: TTCCCACTGGGTGAAAGCGA.

**Assessment of soluble CD163 serum levels**

Serum samples collected a week prior and on day 0 of the challenge were analyzed for the presence and level of soluble CD163. A sandwich ELISA was performed using the porcine CD163 ELISA kit (Elabscience) according to the manufacturer's instructions and using serial dilutions of serum.

**Analysis of serum cytokine levels using cytokine arrays**

Serum samples collected on days 0 (prior to challenge), 3, 7, 10, and 14 of the challenge were analysed for the levels of 20 different cytokines. Cytokine arrays were performed using the Porcine Cytokine Antibody Array A (ab197479) and Array B (ab197480; both Abcam) according to the manufacturer's instructions.
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References


pigs lacking CD163 SRCR5 domain are fully resistant to both PRRSV genotypes while maintaining biological function. PLoS pathogens 13:e1006206.


32. Ugozsi P, Barlage S, Dada A, Schmitz G. 2006. Regulation of surface CD163 expression and cellular effects of receptor mediated hemoglobin-haptoglobin uptake on human monocytes and


Figure Legends

Figure 1: Generation of ΔSRCR5 pigs and experimental set-up. A) Genome editing to generate ΔSRCR5 pigs. Genome-edited founder animals were generated by zygote injection of CRISPR/Cas9 editing reagents using Cas9 mRNA and two guide RNAs, sgSL26 and sgSL28, in combination to generate a deletion of exon 7 in CD163. Animals were breed to generate F1 and F2 generations, focusing on one genotype showing clean religation at the cutting sites of both guide RNAs. Homozygous F2 animals carry this genotype in both alleles (bottom). B) Structure prediction and expression of ΔSRCR5 in pulmonary alveolar macrophages of F2 animals. Protein structure prediction using RaptorX points towards an intact protein product upon deletion of SRCR5. C) Experimental design of challenge study. 4 homozygous (green) and 4 wildtype (orange) siblings from heterozygous/heterozygous mating of the F1 generation animals were co-housed from weaning. Genotypes were confirmed by PCR amplification across exon 7 (see figure 1A) and by Sanger sequencing. Piglets were co-housed after weaning and, after acclimation to the specific pathogen-free unit for 1 week and throughout the 14-day challenge experiment that was initiated by inoculating each pig intranasally with 5E6 TCID50 of the PRRSV-1, subtype 2 strain BOR-57 at day 0 & day 1 of the challenge. The piglets were 7-8 weeks of age at the start of the acclimation period. D) Piglets 1 day before the start of the challenge.

Figure 2: Serum levels of soluble C163. Serum samples collected 2 week prior to and on day 0 of the challenge were assessed for the level of sCD163 using a commercial ELISA. n=2*2*3, displaying min/max and 90 percentile. Statistical analysis using an unpaired t-test showed no significant difference.
Figure 3: ΔSRCR5 pigs show no clinical signs or pathology of a PRRSV-1 infection. A) Rectal temperature of ΔSRCR5 (green) and wildtype piglets (orange) during the challenge with BOR-57. Rectal temperatures were measured daily during feeding. Error bars represent SEM, n=4. B) Average daily weight gain based on weight measurements at day 0, 7, and 14 of the challenge. A&B; Statistical analysis was performed using a two-way ANOVA & Sidak’s multiple comparison test. C) Viremia during the challenge with BOR-57. Serum samples were collected at day 0, 3, 7, 10, and 14 from the jugular vein using vacuum tubes, viral RNA isolated and quantified using RT-qPCR with primers specific to ORF5 of BOR-57. D) Antibody response to PRRSV-1 during the challenge. Serum samples were analyzed for the presence of PRRSV antibodies using the IDEXX PRRSV X3 ELISA test. <0.40=negative; ≥0.4=positive. Each data point / line represents a single animal; 4 animals per genotype group. E) Lung and Lymph node pathology, histopathology and immunohistochemistry scores. Lung pathology was assessed in a blind fashion and a subjective score for severity of gross lung lesions using an established scoring system was applied (scale 0-100). Lung histopathology sections were scored for the presence and severity of interstitial pneumonia ranging from 0 to 6 (0, normal; 1, mild multifocal; 2, mild diffuse; 3, moderate multifocal; 4, moderate diffuse; 5, severe multifocal; 6, severe diffuse). Immunohistochemistry staining against PRRSV-N of lung and lymph node sections was scored ranging from 0-3 (0, no signal; 1, low numbers of positive cells; 2, moderate numbers of positive cells; 3, abundant). Numbers represent average n=4±SEM. F) Lung histology and immunohistochemistry. Top: formalin-fixed, paraffin-embedded, haemotoxylin and eosin stained lung sections from the necropsy on day 14 post challenge. Left: ΔSRCR5, right: wildtype piglets. The scale bar represents 100 µm. Bottom:
formalin-fixed, paraffin-embedded immunohistochemical stain against PRRSV antigen (brown) and hematoxylin counterstain. Left: ΔSRCR5, right: wildtype piglets. The scale bar represents 50 µm. G) Lung pathology. Lungs from pigs at necropsy 14 days post challenge; left, lungs from two ΔSRCR5 pigs and right, lungs from two wildtype pigs.

**Figure 4: Cytokine response to BOR-57 PRRSV infection.** Cytokine levels in serum samples collected prior to challenge on day 0, and challenge days 3, 7, 10, and 14 were measured using cytokine antibody arrays. **A) Interferon α (IFNα), B) Interleukin 17A (IL-17A), C) Interleukin 1 receptor antagonist (IL-1ra), D) Interleukin 4 (IL-4), E) Interleukin 6 (IL-6), F) Interleukin 8 (IL-8), G) Monokine induced by gamma interferon (MIG/CXCL9), H) Macrophage inflammatory protein-1β (MIP-1β/CCL4), I) Chemokine ligand 3-like 1 (CCL3L1), J) Granulocyte macrophage colony-stimulating factor (GM-CSF), K) Tumor necrosis factor alpha (TNFα), L) Interleukin 12 (IL-12), M) Interleukin 1 beta (IL-1β), N) Interleukin 10 (IL-10), O) Transforming growth factor beta 1 (TGFβ1), P) Interferon gamma (IFNγ), Q) Interleukin 18 (IL-18), R) Platelet endothelial cell adhesion molecule (PECAM-1/CD31), S) Interleukin 1 alpha (IL-1α), T) Interleukin 13 (IL-13). Error bars represent SEM, n=2*4. Statistical analysis was performed using a two-way ANOVA & Sidak's multiple comparison test. p-values indicated are: *p≤0.5; ** p≤0.01; ***p≤0.001; ****p≤0.0001.

**Table 1:** Whole blood count results of ΔSRCR5 (animals 4-7) & wildtype piglets at week 6 (animals 8-11)
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