Gene-edited pigs are protected from porcine reproductive and respiratory syndrome virus

To the Editor:

Porcine reproductive and respiratory syndrome (PRRS) is the most economically important disease of swine in North America, Europe and Asia, costing producers in North America more than $600 million annually[1]. The disease syndrome was first recognized in the United States in 1987 and described in 1989 (ref. 2). The causative agent, porcine reproductive and respiratory syndrome virus (PRRSV), was subsequently isolated and characterized in Europe in 1991 (ref. 3). Vaccines have been unable to control the disease. It has been suggested that CD163 is the receptor for entry of PRRSV into cells[4]. Thus, we hypothesized that pigs with defective CD163 would be immune to PRRSV. Previously we used CRISPR-Cas9 to generate pigs lacking functional *CD163* (ref. 5). Here we demonstrate that these animals are resistant to the PRRSV isolate NVSL 97-7895, a well-characterized, relatively virulent viral isolate that is commonly used in experimental PRRSV infection trials. After infection, they showed no clinical signs (fever or respiratory signs), lung pathology, viremia or antibody response and remained healthy for the 35 d after infection measured in this study. Because *CD163* was edited using CRISPR-Cas9, the pigs challenged in this study do not contain any transgenes[5].

PRRSV is a member of the mammalian arterivirus group, which also includes murine lactate dehydrogenase-elevating virus, simian hemorrhagic fever virus and equine arteritis virus. The arteriviruses share important pathogenesis properties, including macrophage tropism and the capacity to cause both severe disease and persistent infection. In young pigs, infection with PRRSV results in respiratory disease, including cough and fever and reduced growth performance. In pregnant sows, PRRSV infection can result in reproductive failure, as well as persistently infected and low birth weight piglets.The virus is associated with polymicrobial disease syndromes, including porcine respiratory disease syndrome and porcine circovirus–associated disease, and can establish a lifelong subclinical infection[6]. In 2006, a more severe form of the disease, called highly pathogenic PRRS, decimated pig populations throughout China[7]. Although genetic selection for natural resistance is an option, success to date has been limited, possibly due to the genetic diversity of the virus[8].

It had been proposed that PRRSV infects alveolar macrophages using the surface protein SIGLEC1 (CD169) as the primary viral receptor[4]. In this proposed model, after binding to CD169 and being taken up into the cell by receptor-mediated endocytosis, the virus is uncoated by CD163 in the endosome, and the viral genome is released into the cytoplasm. However, when *SIGLEC1*$^{-/-}$ pigs (which were generated by homologous recombination and somatic cell nuclear transfer) were infected with PRRSV and compared with infected wild-type pigs, no difference in virus replication was found[9]. To test the role of CD163 in infection, we previously created 45 live-born piglets with insertions ranging from 1 bp to 2 kb, deletions from 11 bp to 1.7 kb, as well as a partial domain swap in *CD163* using CRISPR-Cas9 technology[5].

One founder male and one founder female, both of whom had mutations in exon 7 of *CD163*, were bred to produce offspring (**Supplementary Methods**). The founder male (67-1) possessed an 11-bp deletion in exon 7 on one allele. The other allele had a 2-bp addition in exon 7 and a 377-bp deletion in the preceding intron and was predicted to be a null animal (*CD163*$^{-/-}$).

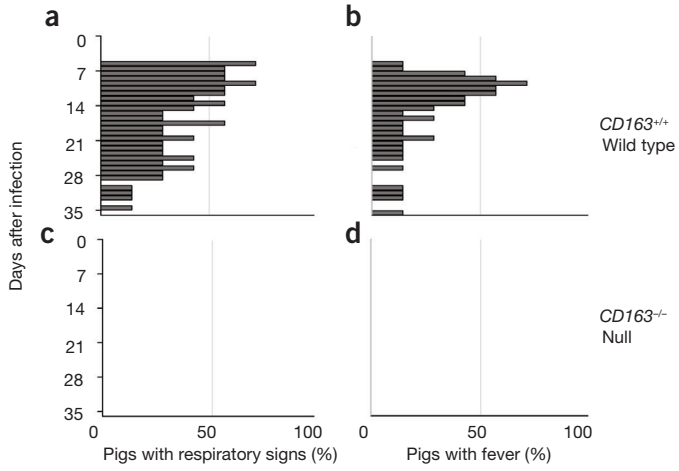

Figure 1 Clinical signs during acute PRRSV infection. (**a**–**d**) Results shown are compiled daily assessments for the presence of respiratory signs and fever for *CD163*$^{+/+}$ ($n = 7$) and *CD163*$^{-/-}$ ($n = 3$) pigs. The percentage of pigs with respiratory signs (**a**,**c**). The percentage of pigs with a fever (**b**,**d**). Fever was considered positive if it was ≥104 °F (normal body temperature, 101.6–103.6 °F). Respiratory scores ranged from 0: normal, to 1: mild dyspnea and/or tachypnea when stressed (when handled), 2: mild dyspnea and/or tachypnea when at rest, 3: moderate dyspnea and/or tachypnea when stressed (when handled), 4: moderate dyspnea and/or tachypnea when at rest, 5: severe dyspnea and/or tachypnea when stressed (when handled), 6: severe dyspnea and/or tachypnea when at rest. The percentage of piglets that had a fever or any sign of respiratory stress (a score of ≥1) at the various days of the challenge are shown. Note that the CD163$^{-/-}$ piglets displayed no signs of either respiratory stress or fever.

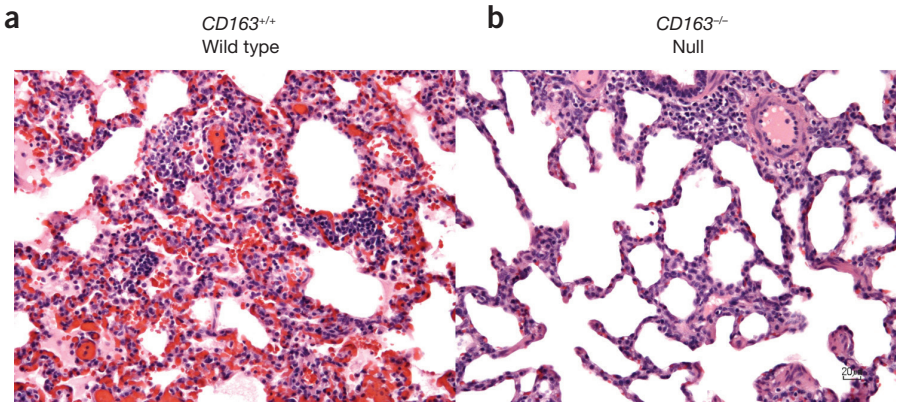

Figure 2 Lung histopathology during acute PRRSV infection. Each photo is from a single representative pig. The pathologist evaluated tissue from every pig. (**a**,**b**) Representative photomicrographs of hematoxylin & eosin–stained tissues from wild-type and knockout pigs at day 35. Edema and infiltration of mononuclear cells in the wild-type pig (**a**), and normal lung architecture in the *CD163*$^{-/-}$ pig (**b**).

The female (65-1) had a 7-bp addition in exon 7 in one allele and an uncharacterized edit in the other allele. When 65-1 was 372 days of age, she was mated to 67-1 who was 264 days of age. This mating resulted in an unremarkable gestation of 115 days and delivery of eight piglets: one stillborn, three piglets that carried two null alleles (*CD163*$^{-/-}$) and four that carried the uncharacterized allele. Because the functionality of the uncharacterized allele could not be predicted, piglets that carried the uncharacterized allele are not further described.

At 3 weeks of age the piglets were weaned. The three *CD163*$^{-/-}$ piglets together with eight wild-type piglets matched by age and breed (Large White by Landrace cross) were randomly assigned ear tags and transported to Kansas State University. Randomly assigned ear tags ensured that the researchers at Kansas State University who were responsible for the PRRSV challenge would be blinded to the litter and genotype. At the challenge facility, the piglets were maintained as a single group and the PRRSV challenge was conducted as previously described (ref. 9). All experiments were initiated after approval of institutional animal use and biosafety committees. After acclimation, the pigs were challenged with a PRRSV isolate, NVSL 97-7895 (ref. 10), propagated on MARC-145 cells[11]. Pigs were challenged with approximately 10^5 $TCID_{50}$ (i.e. approximately 100,000 infectious viral particles) of virus. One-half of the inoculum was delivered intramuscularly and the remainder delivered intranasally. Maintaining the piglets in a single group allowed continuous exposure of virus from infected pen mates. One *CD163*$^{+/+}$ piglet developed severe diarrhea, poor body condition and muscle wasting despite supplemental feedings, and was humanely euthanized by intravenous injection of sodium pentobarbital on day 1 of the study. Blood samples were collected periodically for 35 days after infection and the remaining piglets were humanely euthanized on day 35. Day 35 was chosen as this is sufficiently long to observe the peak of viremia and subsequent decay, and to demonstrate that those pigs that did not develop viremia initially did not develop viremia as a result of exposure to other pigs. Pigs were necropsied and tissues fixed in 10% buffered formalin, embedded in paraffin and processed for histopathology. PRRSV-associated clinical signs including respiratory distress, inappetence, lethargy and fever were recorded using a scoring system (**Fig. 1**). All seven of the wild-type *CD163*$^{+/+}$ pigs showed typical signs of PRRSV infection, which peaked between days 5 and 14 and persisted for the remainder of the study (**Fig. 1**). The percentage of febrile pigs peaked on day 10. In contrast, *CD163*$^{-/-}$ piglets ($n = 3$) showed no evidence of clinical signs over the entire study period. The respiratory signs seen during acute PRRSV infection are accompanied by substantial histopathological changes in the lung. Infected wild-type pigs ($n = 7$) showed histopathology consistent with PRRS, including interstitial edema with the infiltration of mononuclear cells (**Fig. 2** and **Supplementary Table 1**). The mononuclear infiltrate consisted of lymphocytes and plasma cells together with lesser numbers of macrophages. In contrast there was no evidence of pulmonary changes in the *CD163*$^{-/-}$ pigs.

Peak clinical signs correlated with the levels of PRRSV in the blood. Viral nucleic acid was measured by isolation of total RNA from serum followed by amplification of PRRSV RNA by using a commercial reverse transcriptase real-time PRRSV PCR test as previously described (ref. 9). A standard curve was generated using quantification standards supplied in the RT-PCR kit and results were reported as the number of templates per 25 µl PCR reaction. PRRSV viremia followed a typical course in the wild-type *CD163*$^{+/+}$ pigs (**Fig. 3a**). Viremia was apparent at day 4, reached a peak at day 11, and declined until the end of the study. In contrast, viral RNA was not detected in the *CD163*$^{-/-}$ pigs at any time point during the study period. The antibody response was measured weekly to day 28 (**Fig. 3b**) and plateaued on day 14 in the *CD163*$^{+/+}$ pigs, whereas there was no response in the *CD163*$^{-/-}$ piglets. Taken together, these data show that wild-type pigs support PRRSV replication resulting in clinical signs consistent with PRRS. In contrast, the knockout pigs experienced no viremia and no clinical signs, despite being inoculated

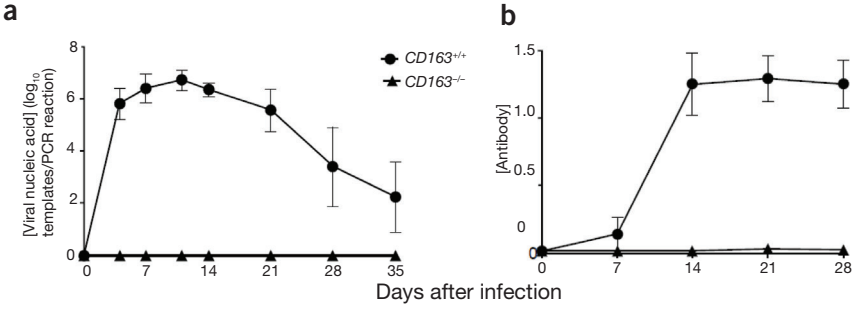

Figure 3 PRRSV-specific nucleic acid and antibody. (**a**,**b**) Mean and s.d. of PRRSV nucleic concentrations (**a**) and antibody (**b**) in serum from *CD163*$^{+/+}$ ($n = 7$) and *CD163*$^{-/-}$ ($n = 3$) pigs (one replication) are shown. Sample to positive ratio = the median fluorescent intensity (MFI) of the sample divided by the MFI of the positive control.

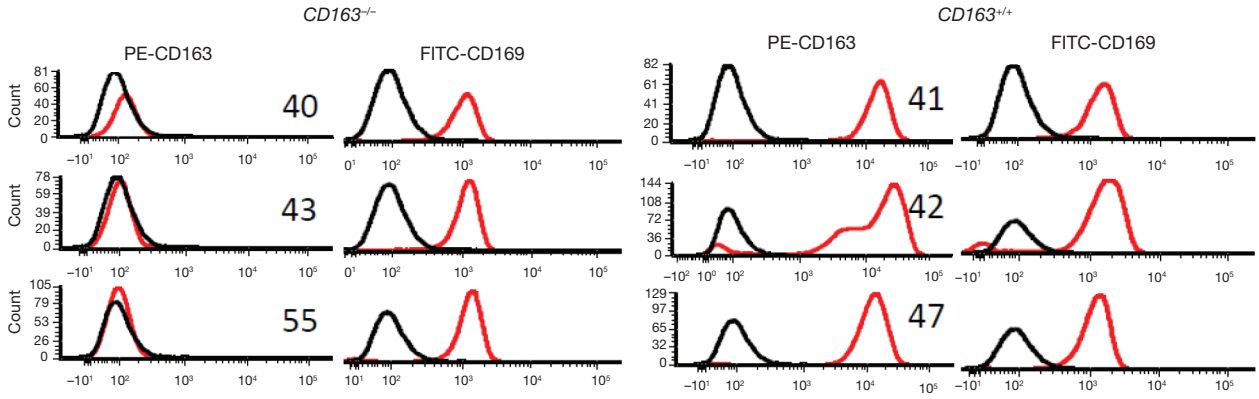

Figure 4 Surface expression of CD163 and CD169 on alveolar macrophages. The panels show representative histograms for CD163$^{+/+}$ and CD163$^{-/-}$ pigs. (FITC, fluorescein isothiocyanate; PE, PE-Cy7). The y axis shows the number of cells and the x axis shows fluorescence intensity. The black lines show the background fluorescence and the red lines show fluorescence of labeled antibody. Data are shown for individual animals. Three representative samples from the CD163$^{+/+}$ piglets are shown.

with PRRSV and continually exposed to infected pen mates.

At the end of the study, porcine alveolar macrophages were collected by lung lavage, subjected to DNA sequencing to confirm their genotype, and stained for surface expression of SIGLEC1 (CD169, clone 3B11/11) and CD163 (clone 2A10/11), as described previously[9]. Relatively high levels of CD163 expression were detected in *CD163*$^{+/+}$ wild-type animals (**Fig. 4**). In contrast, *CD163*$^{-/-}$ pigs showed only background levels of anti-CD163 staining, thus confirming the knockout phenotype (**Fig. 4**). Expression levels for CD169 were similar for both wild-type and *CD163* knockout pigs. Levels for other macrophage surface markers, including CD14, MHC II and CD172, were similar for both genotypes (data not shown).

CD163, a member of the scavenger receptor cysteine-rich (SRCR) superfamily, consists of an intracellular domain and nine extracellular SRCR domains. In humans, endocytosis of CD163-mediated hemoglobin-heme uptake by SRCR3 protects cells from oxidative stress[12–14]. CD163 also serves as a receptor for tumor necrosis factor–like weak inducer of apoptosis (TWEAK: SRCR1-4 & 6-9), several pathogens (African swine fever virus; bacteria: SRCR2), and erythroblast binding (SRCR2). Although subtle differences in phenotype may exist, no gross phenotypic abnormalities have been observed with our *CD163*-modified pigs. Additionally, *CD163* knockout in mice results in "no apparent phenotype change… and offspring were viable and fertile"[15–17]. Nevertheless, as *in vitro* studies implicated domain 5 from exon 7 as responsible for virus binding[18], it may be advantageous to delete or modify only domain 5. Pigs with various mutations in domain 5 (these include predicted partial domain swaps, deletion of 41, 43 or 44 amino acids within domain 5, and deletion of domains 5 and 6) of CD163 have been produced[5] and future challenge experiments will determine the *in vivo* sensitivity of these edits to various PRRSV isolates that represent both European and North American genotypes. For agricultural applications, CD163 modifications will need to be exhaustively characterized for subtle phenotypes related to growth performance, feed efficiency and susceptibility to additional pathogens. The data presented here include only three genetically related animals and infection with a single PRRSV isolate. Clearly, evaluation of additional pig *CD163* genotypes and viral isolates is warranted.

Although vaccination programs have been successful in the prevention or amelioration of many other swine pathogens, PRRSV has proven to be more of a challenge. Genetic selection programs have also failed to identify animals that are resistant to PRRSV challenge[8]. Our finding that CD163 is required for infection with one PRRSV isolate paves the way for vaccine development and genetic selection programs. Use of such genome-edited animals in agriculture could substantially reduce PRRS-related economic losses.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper (http://dx.doi.org/10.1038/nbt.3434).

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The authors declare competing financial interests: details are available in the online version of the paper (*http://dx.doi.org/10.1038/nbt.3434*).

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