Journal of General Virology (2016), **97**, 2719–2731 DOI 10.1099/jgv.0.000586

Immunogenicity of a recombinant parapoxvirus expressing the spike protein of *Porcine epidemic diarrhea virus*

Kyle S. Hain,[1]† Lok R. Joshi,[1]† Faten Okda,[1,2]† Julie Nelson,[1] Aaron Singrey,[1] Steven Lawson,[1] Mathias Martins,[3] Angela Pillatzki,[1] Gerald F. Kutish,[4] Eric A. Nelson,[1] Eduardo F. Flores[3] and Diego G. Diel[1]

Correspondence
Diego G. Diel
diego.diel@sdstate.edu

[1]Animal Disease Research and Diagnostic Laboratory, Department of Veterinary and Biomedical Sciences, South Dakota State University, Brookings, SD 57007, USA

[2]National Research Center, Giza, Egypt

[3]Department of Preventive Veterinary Medicine, Federal University of Santa Maria, Santa Maria, RS, Brazil

[4]Department of Pathobiology, University of Connecticut, Storrs, CT, USA

Received 28 June 2016
Accepted 22 August 2016

The parapoxvirus Orf virus (ORFV), has long been recognized for its immunomodulatory properties in permissive and non-permissive animal species. Here, a new recombinant ORFV expressing the full-length spike (S) protein of *Porcine epidemic diarrhea virus* (PEDV) was generated and its immunogenicity and protective efficacy were evaluated in pigs. The PEDV S was inserted into the *ORFV121* gene locus, an immunomodulatory gene that inhibits activation of the NF-κB signalling pathway and contributes to ORFV virulence in the natural host. The recombinant ORFV-PEDV-S virus efficiently and stably expressed the PEDV S protein in cell culture *in vitro*. Three intramuscular (IM) immunizations with the recombinant ORFV-PEDV-S in 3-week-old pigs elicited robust serum IgG, IgA and neutralizing antibody responses against PEDV. Additionally, IM immunization with the recombinant ORFV-PEDV-S virus protected pigs from clinical signs of porcine epidemic diarrhoea (PED) and reduced virus shedding in faeces upon challenge infection. These results demonstrate the suitability of *ORFV121* gene locus as an insertion site for heterologous gene expression and delivery by ORFV-based viral vectors. Additionally, the results provide evidence of the potential of ORFV as a vaccine delivery vector for enteric viral diseases of swine. This study may have important implications for future development of ORFV-vectored vaccines for swine.

INTRODUCTION

Orf virus (ORFV) is the type member of the genus *Parapoxvirus* of the family *Poxviridae* (Mercer *et al.*, 2007). ORFV is a highly epitheliotropic virus, and keratinocytes and epithelial cells in the oral mucosa are the most important – if not the only – cell type(s) to support ORFV replication in natural hosts (sheep and goats) (Jenkinson *et al.*, 1990). The ORFV genome consists of a dsDNA molecule of approximately 138 kbp in length and contains 131 putative genes (Delhon *et al.*, 2004). ORFV has been long known for its immunomodulatory properties (Weber *et al.*, 2007; Weber *et al.*, 2013). Many genes with immunomodulatory functions have been identified in the ORFV genome, including a homologue of interleukin 10 (IL-10) (Fleming *et al.*, 1997), a chemokine binding protein (CBP) (Seet *et al.*, 2003), a secreted inhibitor of granulocyte-monocyte colony-stimulating factor (GM-CSF) and IL-2 (Deane *et al.*, 2000), a vascular endothelial growth factor (VEGF) (Wise *et al.*, 1999), an interferon (IFN)-resistance gene (McInnes *et al.*, 1998) and more recently, three inhibitors of the NF-κB signalling pathway (*ORFV002, ORFV024* and *ORFV121*) (Diel *et al.*, 2010, 2011a, b). Among these, the IL-10 homologue, the VEGF gene and the NF-κB inhibitor *ORFV121* have been shown to contribute to ORFV virulence in the natural host (Diel *et al.*, 2011b; Fleming *et al.*, 2007; Meyer *et al.*, 1999).

ORFV has been historically used as a preventive or therapeutic agent in veterinary medicine (Weber *et al.*, 2007). The potential of ORFV as a recombinant vaccine delivery vector has been explored, and recombinant ORFV vectors

†These authors contributed equally to this work.

based on the highly attenuated ORFV strain D1701 have been shown to induce protective immunity against several viral diseases in permissive and non-permissive animal species (Amann *et al.*, 2013; Dory *et al.*, 2006; Fischer *et al.*, 2003; Henkel *et al.*, 2005; Marsland *et al.*, 2003; Rohde *et al.*, 2011, 2013; van Rooij *et al.*, 2010; Voigt *et al.*, 2007). Notably, ORFV D1701-based recombinants expressing the pseudorabies virus (PRV) glycoproteins gC and gD induced protective immunity against PRV infection in pigs (Dory *et al.*, 2006), while a recombinant ORFV expressing the classical swine fever virus (CSFV) E2 glycoprotein protected swine against intranasal challenge with a virulent CSFV strain (Voigt *et al.*, 2007). These studies demonstrate the efficacy of ORFV-based vectors in eliciting protective immune responses in swine. In the present study, the potential of ORFV as a vaccine delivery vector for enteric viral diseases of swine was investigated. The porcine epidemic diarrhea virus spike (PEDV S) glycoprotein was used as a model antigen to evaluate the immunogenicity and protective efficacy ORFV-based vectors in pigs.

Porcine epidemic diarrhea virus (PEDV), a member of the genus *Alphacoronavirus* of the family *Coronaviridae*, causes severe enteric disease (porcine epidemic diarrhoea; PED) in pigs (Debouck *et al.*, 1981; Pensaert & de Bouck, 1978). PEDV infects pigs of all ages, producing high mortality rates (50–100 %) in suckling piglets and weight loss due to diarrhoea in older animals (Pensaert & de Bouck, 1978). The virus replicates primarily in enterocytes of the small intestines leading to villous atrophy and malabsorptive diarrhoea followed by electrolyte imbalance, metabolic acidosis and death (Madson *et al.*, 2014). Characteristic clinical signs of PED include watery diarrhoea, vomiting, anorexia and dehydration (Lee, 2015). In older pigs, the disease is usually milder resulting in low mortality rates (Lee, 2015).

The PEDV genome is a large (~28 kb) positive-sense RNA molecule that contains six ORFs, encoding the replicase proteins (pp1a and pp1ab), four structural proteins [spike (S), envelope (E), membrane (M) and nucleoprotein (N)] and one accessory protein (ORF3) (Lee, 2015; Song & Park, 2012). Among the structural proteins, the S protein is the major envelope glycoprotein responsible for virus attachment and entry (Cruz *et al.*, 2008; Sun *et al.*, 2007, 2008). Given its critical function in attachment and entry, the S glycoprotein is the main target for neutralizing antibodies (NAs) (Chang *et al.*, 2002; Cruz *et al.*, 2008; Paudel *et al.*, 2014; Suo *et al.*, 2012), with several neutralizing epitopes being mapped to this glycoprotein (Chang *et al.*, 2002; Cruz *et al.*, 2006, 2008; Sun *et al.*, 2007, 2008).

Here a novel recombinant ORFV expressing the full-length PEDV S protein (ORFV-PEDV-S) was generated, and its immunogenicity and protective efficacy were evaluated in pigs. The PEDV S coding sequences were inserted into the *ORFV121* gene locus, a recently characterized immunomodulatory gene of ORFV that contributes to the virus virulence in the natural host (Diel *et al.*, 2011b). Results from immunization studies in pigs show that intramuscular (IM) immunization with the ORFV-PEDV-S elicited S-specific IgG, IgA and NA responses. Notably, animals immunized with the ORFV-PEDV-S via the IM route were protected from clinical signs of PED, and presented reduced virus shedding in faeces after oral challenge with a virulent PEDV strain.

RESULTS

Generation and characterization of the ORFV-PEDV-S recombinant virus

The full-length spike protein of PEDV was inserted into the *ORFV121* (Diel *et al.*, 2011b) gene locus of the ORFV genome by homologous recombination. *ORFV121* (Diel *et al.*, 2011b), was deleted from the ORFV genome and replaced by a DNA fragment encoding the full-length PEDV S protein and the reporter gene encoding the GFP under the control of individual early/late *VV7.5* poxviral promoters (Fig. 1a). The recombinant virus was selected and purified by limiting dilution followed by plaque assays based on expression of the GFP (Fig. 1c). Deleted *ORFV121* gene sequences were not detected in the purified recombinant virus (Fig. 1b). In contrast, PEDV S sequences were detected in the recombinant virus but not in the wild-type ORFV genome (Fig. 1b). Complete genome sequence of the ORFV-PEDV-S recombinant virus confirmed the insertion of the full-length coding sequences of PEDV S (data not shown).

Replication properties of the ORFV-PEDV-S recombinant virus were assessed *in vitro*. No differences in replication kinetics and viral yields were observed when multiple-step or one-step growth curves of ORFV-PEDV-S were compared to those of the wild-type OV-IA82 virus in primary ovine foetal turbinate (OFTu) cell cultures (data not shown). Replication kinetics of ORFV-PEDV-S were also assessed in porcine kidney cells (PK-15). Notably, replication of ORFV-PEDV-S in PK-15 cells was markedly impaired when compared to its replication in natural host OFTu cells (Fig. 2a, b). Similar replication kinetics were observed in swine testicle cells (ST, data not shown). These results indicate a marked growth defect for ORFV-PEDV-S in swine cells, demonstrating only minimal virus replication in cells of porcine origin.

Recombinant ORFV-PEDV-S expresses PEDV S *in vitro*

Expression of PEDV S by ORFV-PEDV-S recombinant virus was assessed by immunofluorescence (IFA) and Western blot assays. Expression of PEDV S by the recombinant ORFV-PEDV-S was assessed during virus infection in OFTu cells by using an S-specific mAb in an indirect IFA assay. High levels of PEDV S were detected in ORFV-PEDV-S-infected cells (Fig. 1c). Similar results were observedin PK-15 cells (data not shown). High levels of PEDV S

(~150 kDa) were detected in ORFV-PEDV-S-infected cells by Western blot assays (Fig. 1d).

Intracellular or surface expression of PEDV S by the recombinant ORFV-PEDV-S was assessed by IFA assays. As shown in Fig. 3a, PEDV S expression was detected in permeabilized (Triton X-100) and in non-permeabilized cells, indicating abundant expression/localization of PEDV S on the surface and intracellular compartment of ORFV-PEDV-S-infected cells.

The stability of PEDV S gene inserted into the *ORFV121* locus of the ORFV-PEDV-S genome was assessed by IFA and confirmed by PCR and DNA sequencing following serial passages of the recombinant virus *in vitro*. Expression of PEDV S was consistently detected in ORFV-PEDV-S-infected cells after 1, 5 or 10 passages of the recombinant virus in OFTu cell cultures (Fig. 3b). Additionally, the full-length PEDV S was amplified from the recombinant virus on passages 1, 5 and 10 and sequencing of the p.10 full-length S revealed 100 % identity with the S sequences inserted in the recombinant virus (data not shown). Together, these results demonstrate the stability of PEDV S inserted into the *ORFV121* locus.

Intramuscular immunization with ORFV-PEDV-S induces serum IgG and IgA antibody responses against PEDV in 3-week-old pigs

The immunogenicity of ORFV-PEDV-S recombinant virus was evaluated in pigs following transcutaneous (TC) or IM immunizations. Three-week-old pigs were immunized with ORFV-PEDV-S via the TC or IM routes on day 0 and received booster immunizations on days 21 and 45 post-

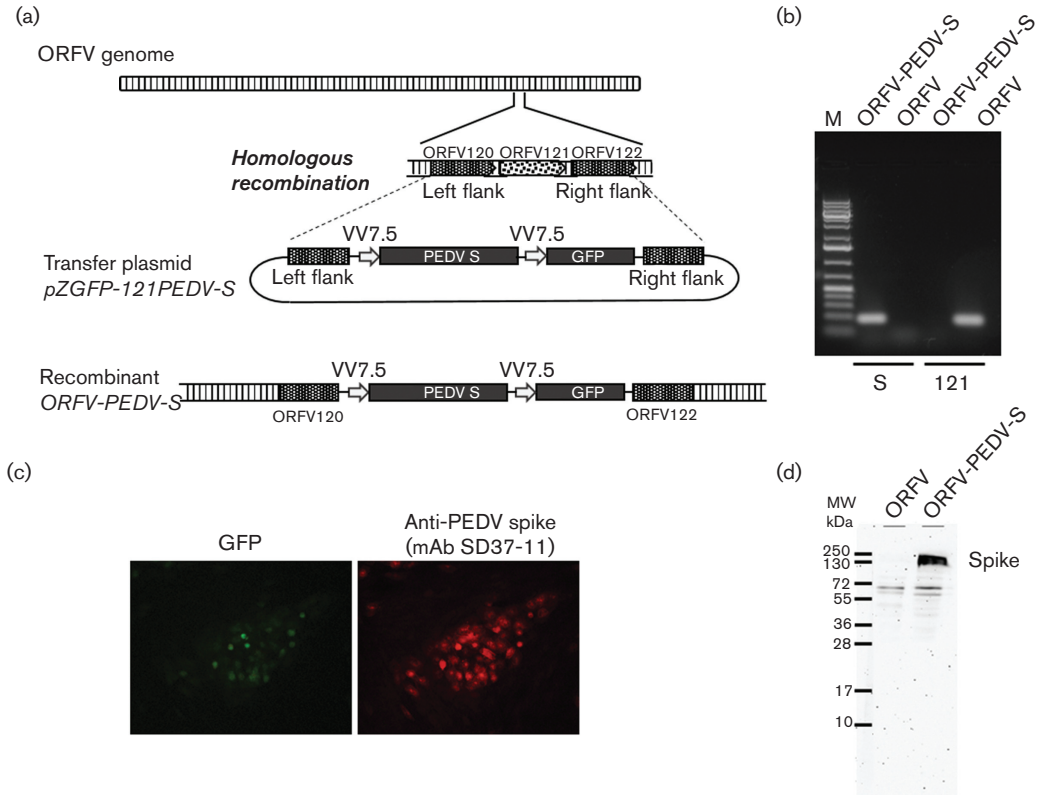

Fig. 1. Generation of recombinant ORFV-PEDV-S virus. (a) Schematic representation of the ORFV genome depicting *ORFV121* insertion site and flanking regions (*ORFV120* and *ORFV122*) used to generate the recombinant ORFV-PEDV-S virus. pZGFP-121PEDV-S transfer plasmid containing the full-length PEDV S gene plus the GFP selection reporter under the control of individual early/late VV7.5 poxviral promoters. Recombinant ORFV-PEDV-S genome depicting insertion of the PEDV S and the GFP reporter gene into the *ORFV121* gene locus. (b) Agarose gel (1 %) demonstrating PCR amplification of an internal region of the S gene (~150 bp) from the genome of the recombinant ORFV-PEDV-S gene and absence of *ORFV121* gene sequences in the recombinant ORFV-PEDV-S virus. Wild-type ORFV DNA was used as a negative and positive control on the PCR amplifications with S- and ORFV121-specific primers, respectively. (c) IFA demonstrating expression of PEDV S in cell cultures infected with the recombinant ORFV-PEDV-S virus. Left panel shows expression of the reporter GFP protein by the recombinant virus. Right panel shows expression of PEDV S detected with a mouse mAb against PEDV S. (d) Western blot demonstrating expression of the full-length PEDV S (~150 kDa) by the recombinant ORFV-PEDV-S virus in cell culture *in vitro*. Cell lysates from cells infected with wild-type ORFV were used as negative controls. Blot was developed with an anti-His tag mAb.

primary immunization (Table 1). Serological responses elicited against PEDV were monitored by indirect S IgG- and IgA-isotype ELISAs. All animals immunized via the IM route with the recombinant ORFV-PEDV-S virus developed IgG and IgA antibody responses against PEDV S (Group 3; Fig. 4a, b), whereas no antibody responses were detected in the animals immunized via the TC route (Group 2) or in animals from control groups (Group 1 and Group 4), immunized with ORFV-GFP vectors (Group 1 and 4; Fig. 4a, b). Antibodies were first detected on animals from Group 3 (IM) on day 28 post-immunization (p.i.), following the booster immunization on day 21 (Fig. 4a, b). Notably, while a second booster immunization elicited anamnestic antibody responses in animals immunized by the IM route, no serological responses were detected in animals immunized by the TC route (Fig. 4a, b). Similar results were observed when serological responses were monitored using a whole virus (PEDV) indirect ELISA (data not shown). These results demonstrate that IM immunization with ORFV-PEDV-S elicits robust antibody (IgG and IgA) responses in immunized pigs.

Intramuscular immunization with ORFV-PEDV-S induces neutralizing antibody responses against PEDV in pigs

The ability of ORFV-PEDV-S to induce NA responses against PEDV was assessed using a fluorescent focus neutralization (FFN) assay (Okda *et al.*, 2015). Similar to the serological responses detected with the IgG and IgA isotype ELISAs, virus neutralization assays revealed that IM immunization with ORFV-PEDV-S elicited NA responses against PEDV in all immunized animals (Fig. 4c). No NA responses were detected in animals immunized via the TC route or in control sham-immunized animals (Groups 1 and 4; Fig. 4c).

Intramuscular immunization with ORFV-PEDV-S protects pigs from clinical PED

To evaluate the protective efficacy of the recombinant ORFV-PEDV-S, pigs from Groups 2, 3 and 4 (Table 1) were challenged orally with the virulent PEDV strain CO13 on day 60 p.i. (2×10^5 $TCID_{50}$ $animal^{-1}$). Clinical signs of PED and virus shedding in faeces were monitored after challenge infection. Notably, characteristic clinical signs of PEDV infection were observed in 2/4 (50 %) animals from the sham-immunized group (Group 4) and in 3/4 (75 %) animals from the TC immunized group (Group 2), while no animals immunized via the IM route (0/4) (Group 3) developed clinical signs of PED. Daily average clinical scores were recorded for each group, and are presented in Fig. 5a. Control sham-immunized animals (Group 4) and TC immunized animals (Group 2) presented significantly higher clinical scores when compared to sham-immunized non-challenged animals (Group 1) or to ORFV-PEDV-S immunized (IM) PEDV challenged animals (Group 3) (Fig. 5).

Virus shedding in faeces was evaluated after challenge infection using real-time reverse transcription PCR (RT-qPCR). Rectal swabs collected from all animals on days 0, 3, 5, 7, 9, 11 and 14 post-challenge (p.c.) were tested by PEDV RT-qPCR and the duration of virus shedding as well as PEDV genome copy numbers were compared between treatment groups. All challenged animals (Groups 2, 3 and 4) shed PEDV in faeces, while no virus shedding was detected in non-challenged animals (Group 1) (Table 2). Notably, both duration and magnitude of PEDV shedding were markedly decreased in animals from Group 3, immunized via the IM route with the ORFV-PEDV-S virus (Table 2; Fig. 5b). While 3/4 animals in Group 2 (75 %) and 4/4 animals in

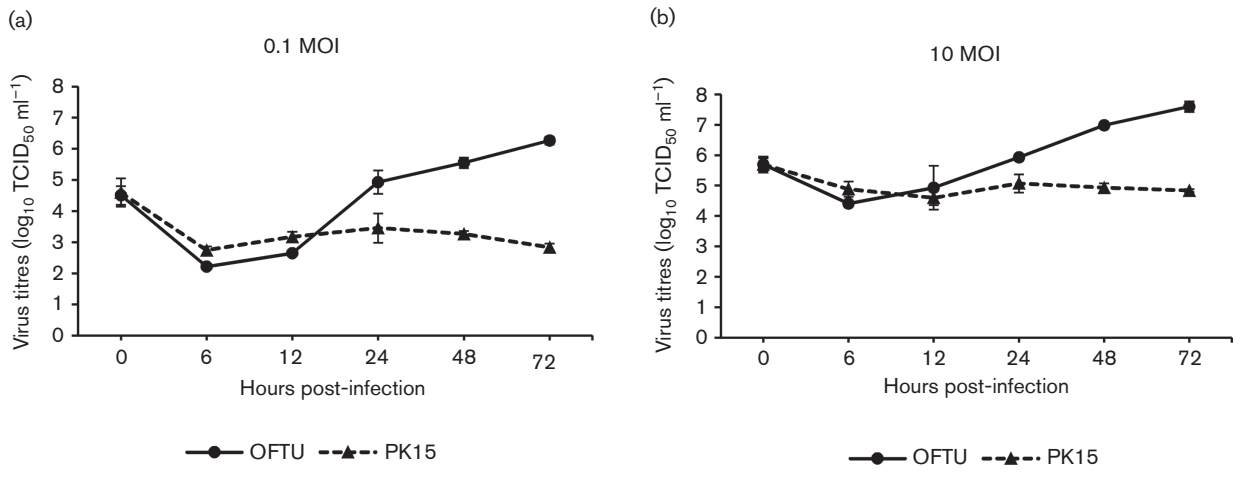

Fig. 2. Replication properties of the recombinant ORFV-PEDV-S. (a) Multistep growth curve of the recombinant ORFV-PEDV-S in primary OFTu and porcine kidney (PK15) cells. (b) Single-step growth curve of the recombinant ORFV-PEDV-S in primary OFTu and porcine kidney (PK15) cells. Cells were collected at indicated time points and virus titres determined by the Spearman and Karber's method and expressed as log_{10} tissue culture infections dose 50 ($TCID_{50}$) per millilitre. Error bars represent SEM calculated based on the results of three independent experiments.

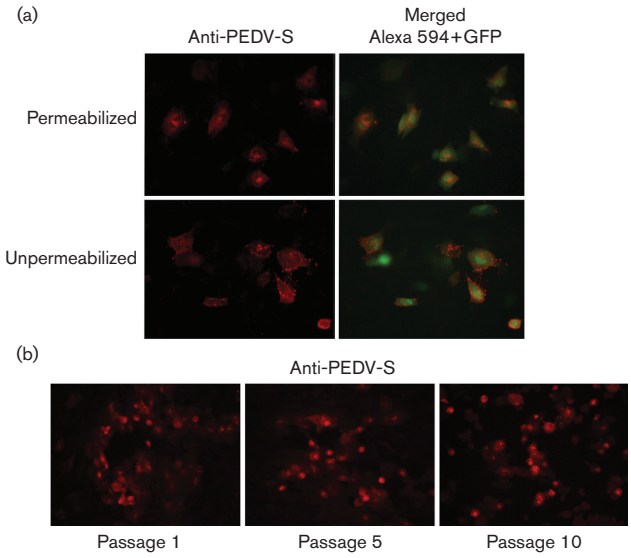

Fig. 3. Expression of PEDV S by the recombinant ORFV-PEDV-S. (a) IFA performed in primary OFTu cells infected (m.o.i. of 1) with the recombinant ORFV-PEDV-S. Cells were fixed with formaldehyde (3.7 %) at 24 h post-infection, permeabilized or not with Triton X-100, stained with anti-PEDV-S mAb (SD37-11) and visualized under a fluorescence microscope. Left panels show expression of the PEDV S intracelularlly (top) or on the cell surface (bottom), and right panels represent a merged image showing specific expression of PEDV S by ORFV-PEDV-S-infected cells (as evidenced by expression of GFP). (b) IFA showing expression of PEDV S by the recombinant ORFV-PEDV-S after serial passages in cell culture. Primary OFTu cells were infected with passages 1, 5 or 10 of ORFV-PEDV-S (m.o.i. of 3) and fixed, stained and permeabilized as described in (a).

Group 4 (4/4; 100 %) were positive on day 3 p.c., only 1/4 animal in Group 3 (25 %) was positive for PEDV (Table 2). Animals in Group 3 shed PEDV in faeces between days 7 and 9 p.c.; however, they ceased shedding virus earlier than animals from Groups 2 and 4 (Table 2). While on day 11 p.c. only 1/4 (25 %) animal from Group 3 was positive, all 4/4 (100 %) animals in Groups 2 and 4 were still shedding PEDV.

Quantitation of PEDV genome copy numbers in faecal swabs revealed a lower amount of the virus being shed in faeces by animals from Group 3, compared to animals from Groups 2 and 4 (Fig. 5b). Together these results indicate that immune responses elicited by IM immunization with the recombinant ORFV-PEDV-S led to protection from clinical PED and reduced virus shedding in faeces.

Serological responses after challenge

The serological responses post-challenge with PEDV were evaluated by S IgG and IgA ELISAs, virus neutralization assays and N IgG ELISA. Only animals in Group 3 presented detectable anti-S and PEDV NAs in serum (Fig. 5a–c and 6a–c) at the day of challenge (day 60 p.i.). Serological responses of each group differed markedly following challenge infection. Animals from Group 2 (which did not seroconvert after TC immunization) and from Group 4 (control sham-immunized group) developed lower levels of S-specific IgG and IgA and NAs (days 10 and 14 p.c.) when compared to animals in Group 3 (which seroconverted after IM immunization with ORFV-PEDV-S) (Fig. 6a–c). Notably, levels of NAs detected in animals from Group 3 were 3–4-fold higher than those in animals in Groups 2 and 4 (Fig. 6c). In contrast, serum IgG responses against PEDV N were more robust in animals from Groups 2 and 4 when compared to animals from Group 3 (Fig. 6d). These differences were more pronounced at early times p.c. (day 7 p.c.; $P<0.05$), with comparable levels of anti-NAs being detected at later times post-challenge (Fig. 6d). These results indicate an efficient priming of B cells by IM immunization with the recombinant ORFV-PEDV-S, and further demonstrate a robust and typical secondary response, with high level antibody production following challenge with PEDV.

DISCUSSION

The immunogenicity and protective efficacy of ORFV-based vectored vaccine candidates have been demonstrated in

Table 1. Experimental design of animal immunization challenge infection

Group*	Vector construct	Route†	Immunization days	Challenge infection‖	
				Inoculum	Day p.i.
1	ORFV-GFP	TC‡+IM§	0, 21, 45	MEM	60
2	ORFV-PEDV-S	TC	0, 21, 45	PEDV CO13	60
3	ORFV-PEDV-S	IM	0, 21, 45	PEDV CO13	60
4	ORFV-GFP	TC+IM	0, 21, 45	PEDV CO13	60

*Each group consisted of four 3-week-old weaned piglets.
†All control animals (Groups 1 and 4) were immunized with half dose via the TC route and half dose via the IM route.
‡Virus suspension was applied topically on a 4 cm^2 area of scarified skin.
§Virus suspension was injected intramuscularly.
‖Each animal was challenged orally with a virus suspension containing 2×10^5 $TCID_{50}$ of PEDV strain CO13.

multiple animal species (Amann *et al.*, 2013; Dory *et al.*, 2006; Fischer *et al.*, 2003; Rohde *et al.*, 2011, 2013; Voigt *et al.*, 2007). Notably, ORFV recombinants, based on the highly attenuated ORFV strain D1701, expressing the PRV gC and gD or the CSFV E2 glycoproteins have been shown to protect pigs against challenge with PRV and CSFV, respectively (Dory *et al.*, 2006; Fischer *et al.*, 2003; van Rooij *et al.*, 2010; Voigt *et al.*, 2007). Here we explored the potential of ORFV as a vaccine delivery vector for an enteric pathogen of swine. Using a well-characterized ORFV strain (OV-IA82) (Delhon *et al.*, 2004; Diel *et al.*, 2010, 2011a, b) and the locus of a recently identified virulence determinant of ORFV (*ORFV121*) as insertion site, we generated a recombinant ORFV expressing the full-length PEDV S protein.

The S is the major envelope glycoprotein of PEDV and has been shown to be the main target of NAs (Chang *et al.*, 2002; Cruz *et al.*, 2008; Sun *et al.*, 2007). Additionally, subunit PEDV vaccine candidates based on the S protein have been shown to induce protective immune responses in pigs (Oh *et al.*, 2014). Here, the coding sequences of the full-length PEDV S were inserted into the *ORFV121* gene locus (Diel *et al.*, 2011b) of the ORFV genome. The recombinant ORFV-PEDV-S was successfully generated (Fig. 1b–d), and sequencing of the resultant recombinant virus genome confirmed the insertion of PEDV S and deletion of the *ORFV121* gene from the ORFV genome. These results demonstrate the feasibility of *ORFV121* gene locus as an insertion site for heterologous antigens in ORFV-based recombinant vectors. Notably, the DNA fragment inserted into the *ORFV121* locus is ~5.2 kbp in length (full-length S and GFP coding sequences, and promoter sequences), demonstrating that the *ORFV121* locus may accommodate large fragments of heterologous DNA. Successful expression of both PEDV S and the GFP proteins in cells infected with the recombinant ORFV-PEDV-S (Fig. 1c), confirmed the

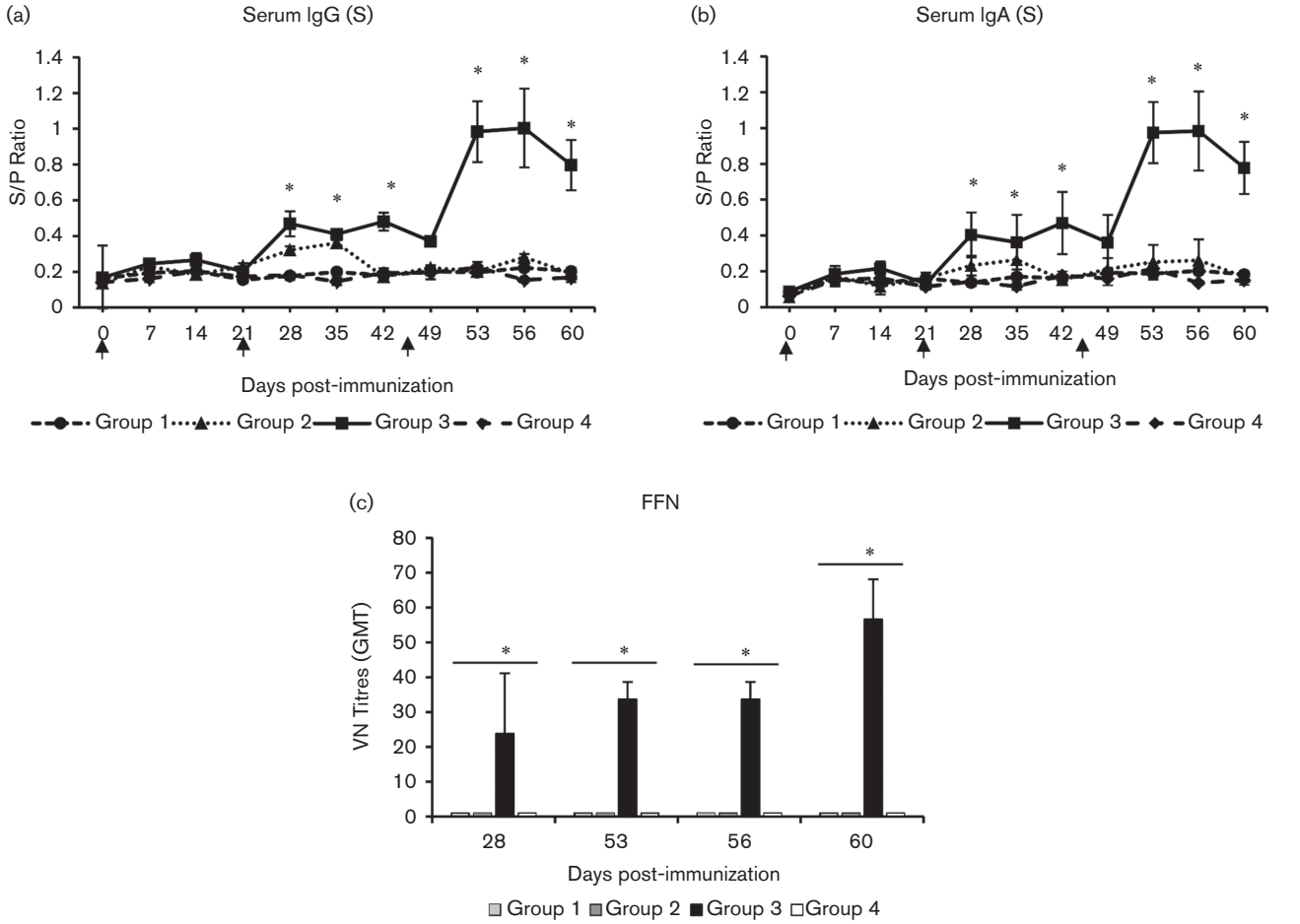

Fig. 4. Immunogenicity of recombinant ORFV-PEDV-S in pigs. (a) Isotype ELISA demonstrating serum IgG antibody responses specific to the PEDV S protein. (b) Isotype ELISA demonstrating serum IgA antibody responses specific to the PEDV S protein. (c) Virus NA responses elicited by immunization with recombinant ORFV-PEDV-S. S/P, sample to positive ratio; GMT, geometric mean titre. Arrow heads represent immunization/booster immunizations (days 0, 21 and 45). Error bars represent SEM. Statistical significance was determined using one-way ANOVA and Tukey's honest significant difference (HSD). *Statistically significant at the 0.05 level when the mean of Group 3 was compared to the mean of Groups 1, 2 and 4.

large payload capacity of the *ORFV121* gene locus and of ORFV-based vectors.

The expression and genetic stability of PEDV S carried by the recombinant ORFV-PEDV-S virus were assessed in infected cell cultures. IFAs performed in cell cultures infected with the recombinant ORFV-PEDV-S and permeabilized or not with Triton X-100 revealed high levels of PEDV S expression intracellularly and on the cell surface (Fig. 3a). While intracellular expression of PEDV S may allow for antigen processing and presentation through the MHC I pathway, expression of the protein on the surface of infected cells may allow for direct recognition and uptake of the protein by antigen presenting cells (APCs) or by B lymphocytes, thus potentially leading to stimulation of both cellular and humoral immune responses (Blum *et al.*, 2013). An important requirement for viral vectors is the stability of heterologous genes within their genome (Wang *et al.*, 2010). As shown in Fig. 3b, serial passage of ORFV-PEDV-S in cell cultures *in vitro* did not affect expression of PEDV S. High levels of the PEDV S protein were detected in cell cultures infected with passages 1, 5 and 10 of the recombinant ORFV-PEDV-S, demonstrating the genetic stability of the insert into the *ORFV121* gene locus.

The immunogenicity of the recombinant ORFV-PEDV-S was evaluated in pigs following IM or TC immunizations. While IM immunization has been shown effective for other ORFV-vectored antigens in pigs (Dory *et al.*, 2006; Voigt *et al.*, 2007), proof-of-concept TC immunization was used given its efficacy in inducing mucosal immune responses in other animal species (Lawson *et al.*, 2012). Notably, animals immunized via the IM route developed robust antibody responses (IgG, IgA and NA) against PEDV, whereas no seroconversion was detected in animals immunized via the TC route (Fig. 4a– c). These results corroborate the findings of previous studies (Dory *et al.*, 2006; Voigt *et al.*, 2007), demonstrating that the IM route is an effective route to deliver ORFV-vectored antigens in swine. Although no NAs against ORFV were detected in any of the immunized animals (data not shown), it is possible that local innate/inflammatory responses elicited by skin scarification may have affected the delivery and/or expression of PEDV S by ORFV-PEDV-S in the skin, thus, potentially preventing the development of immune responses against PEDV S in animals immunized via the TC route. Additionally, the dose delivered following skin scarification and topical application of the recombinant vector may not have been sufficient to prime and boost the immune system against PEDV S in animals from the TC immunized group. Given the natural tropism of ORFV for keratinocytes in natural hosts (sheep and goats), it would still be interesting, however, to explore more precise methods of TC delivery of ORFV-vectored antigens in the future.

To assess the protective potential of ORFV-PEDV-S-elicited immune responses, animals from Groups 2 (TC) and 3 (IM), and sham-immunized Group 4 (TC+IM), were challenged orally with a virulent PEDV strain CO13 (2×10^5 $TCID_{50}$). Notably, while 3 out of 4 (3/4; 75 %) animals from Group 2 (which did not seroconvert after TC immunization; Fig. 4a–c) and 2/4 (50 %) animals from control Group 4 developed characteristic signs of PED, none of the animals from Group 3 (IM, which developed serum antibody responses to PEDV) were affected. Additionally,

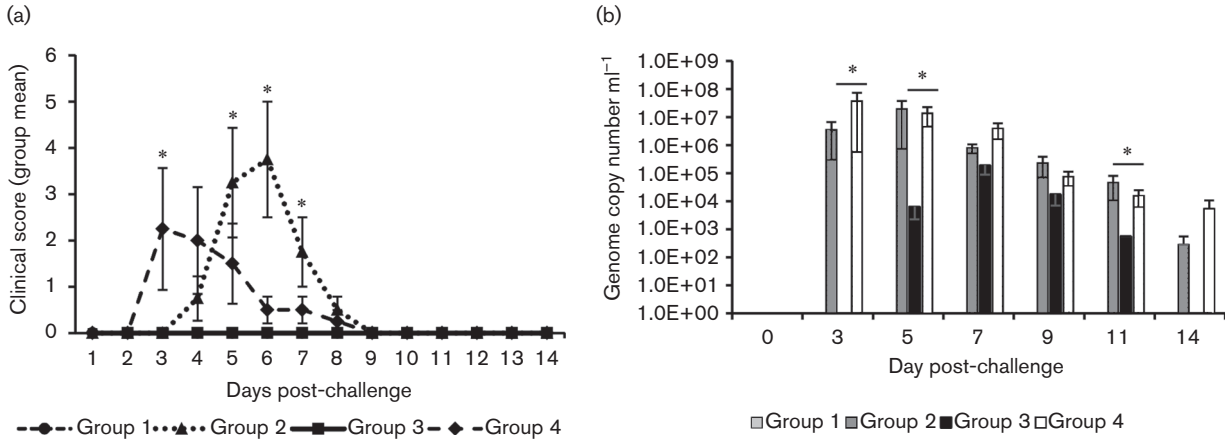

Fig. 5. Protective efficacy of ORFV-PEDV-S against PEDV challenge. (a) Average group clinical scores recorded post-challenge infection with PEDV strain CO13. Clinical signs were recorded and individual daily scores assigned to all animals based on the following criteria: 0, normal faeces; 1, pasty faeces; 2, moderate diarrhoea (semi-liquid); 3, diarrhoea (liquid); 4, severe diarrhoea (very liquid); 5, watery diarrhoea (profuse diarrhoea). Error bars represent SEM. Statistical significance was determined using one-way ANOVA and Tukey's HSD. *Statistically significant at the 0.05 level when the mean of Group 3 was compared to the mean of Groups 2 or 4. (b) Virus shedding in faeces was measured and expressed as $\log_{10}$ genome copy numbers per millilitre. Error bars represent SEM. Statistical significance was determined using non-parametric Kruskal–Wallis test between groups. *Statistically significant at the 0.05 level when the mean of Group 3 was compared to the mean of Groups 2 and 4.

animals from Group 3 presented reduced virus shedding in faeces when compared to animals from Groups 2 and 4 (Fig. 5a, b, Table 2). Results from the RT-qPCR performed in rectal swabs show a delayed onset and short duration shedding of PEDV by animals from Group 3 (Fig. 5b, Table 2). Together, these results demonstrate that IM immunization with the recombinant ORFV-PEDV-S protected pigs from clinical PED and reduced virus shedding following oral challenge infection. Although results here show a strong correlation between PEDV-specific antibodies in serum, protection from clinical disease and decreased virus shedding in faeces, the precise immunological mechanisms underlying these findings were not defined in our study. In future studies, it would be interesting to assess, for example, whether IM immunization with the recombinant ORFV-PEDV-S elicits secretory IgA (sIgA) responses at mucosal surfaces, or perhaps, homing of effector B or T lymphocytes to the intestinal mucosa.

Serological responses that followed challenge infection with PEDV varied significantly between immunized groups. While animals from Group 3 presented a robust serological response, typical of a secondary immunological response, characterized by high levels of S-specific and NA responses to PEDV (Fig. 6a–c), animals from Groups 2 and 4 developed delayed antibody responses, typical of primary exposure to PEDV (Fig. 6a–c). In contrast, antibody responses to the N protein (structural protein not present in the ORFV-PEDV-S construct) were lower in animals from Group 3 (day 7 p.c.), suggesting an early inhibition of PEDV infection/replication in animals from Group 3 (Fig. 6d). Together, these results suggest that IM immunization with the recombinant ORFV-PEDV-S virus efficiently primed B cells, which rapidly and effectively responded upon exposure to the virus in the intestinal mucosa, leading to anamnestic antibody responses in immunized animals. Additional studies involving larger numbers of animals with sequential sampling of mucosal and lymphoid tissues would be necessary, however, to dissect the immunological mechanisms underlying the phenotype observed here.

Although correlate(s) of protection for PEDV remain unknown, neutralizing secretory IgA (sIgA) antibodies are thought to play a major role in protection (de Arriba *et al.*, 2002a; Liu *et al.*, 2012; Poonsuk *et al.*, 2016; Saif, 2015). sIgA seem especially important in providing lactogenic immunity and protection during the first days of life of newborn piglets (Chattha *et al.*, 2014; Saif, 2015). One of the main obstacles in eliciting lactogenic immunity to PEDV, however, is the need for local gut stimulation of IgA secreting cells (plasmablasts) and their subsequent migration to the mammary gland where they produce sIgA antibodies which are secreted in the colostrum and milk and ultimately transferred to suckling piglets (gut-mammary-sIgA axis) (Langel *et al.*, 2016). This has been achieved by natural infection, oral vaccination of pregnant sows with live PEDV (de Arriba *et al.*, 2002b; Goede *et al.*, 2015; Song *et al.*, 2007) or, more recently, by a subunit vaccine candidate (S1; Makadiya *et al.*, 2016). Results here show partial protection of nursery pigs to oral PEDV challenge after IM immunization with the recombinant ORFV-PEDV-S. Whether this vector construct is capable of eliciting lactogenic immunity and protection in neonatal piglets remains

Table 2. Virus shedding in faeces after challenge infection with PEDV

Group	Animal ID	Virus shedding (day post-challenge)*						
		0	3	5	7	9	11	14†
1	1	–	–	–	–	–	–	–
	2	–	–	–	–	–	–	–
	3	–	–	–	–	–	–	–
	4	–	–	–	–	–	–	–
2	5	–	+	+	+	+	+	+
	6	–	+	+	+	+	+	–
	7	–	–	+	+	+	+	–
	8	–	+	+	+	+	+	–
3	9	–	+	+	+	+	+	–
	10	–	–	–	+	+	–	–
	11	–	–	–	+	+	–	–
	12	–	–	+	+	+	–	–
4	13	–	+	+	+	+	+	–
	14	–	+	+	+	+	+	–
	15	–	+	+	+	+	+	+
	16	–	+	+	+	+	+	–

*Virus shedding was assessed by real-time PCR in faecal swabs.
†Experiment was terminated on day 14 p.c.

to be determined. Nevertheless, the fact that IM immunization with the recombinant ORFV-PEDV-S led to protective immune responses in naïve animals indicates that this virus vector could be a useful tool for the control of PEDV in endemic areas. The recombinant ORFV-PEDV-S could be used to immunize naïve gilts prior to their introduction to PEDV positive farms or to boost the immunity of pregnant gilts/sows that have been naturally exposed to the virus.

In summary, here we show the successful generation of a recombinant ORFV containing the full-length S gene of PEDV into the *ORFV121* gene locus. Characterization of the recombinant ORFV-PEDV-S virus *in vitro* demonstrates efficient and stable expression of the heterologous protein in cell cultures infected with the recombinant virus. Immunization challenge studies in pigs, show that IM delivery of the recombinant ORFV-PEDV-S elicits robust serum antibody responses in immunized animals that correlated with protection against clinical PED and decreased virus shedding in faeces. These results may have important implications for future development of ORFV-vectored vaccines for prevention of infectious diseases of swine.

METHODS

Cells and viruses. Primary OFTu, porcine kidney (PK15; ATCC PTA-8244) and Vero-76 cells (ATCC® CRL-1587™) were cultured at 37 °C with 5 % CO_2 in minimum essential medium (MEM) supplemented with 10 % FBS, 2 mM L-glutamine and containing penicillin

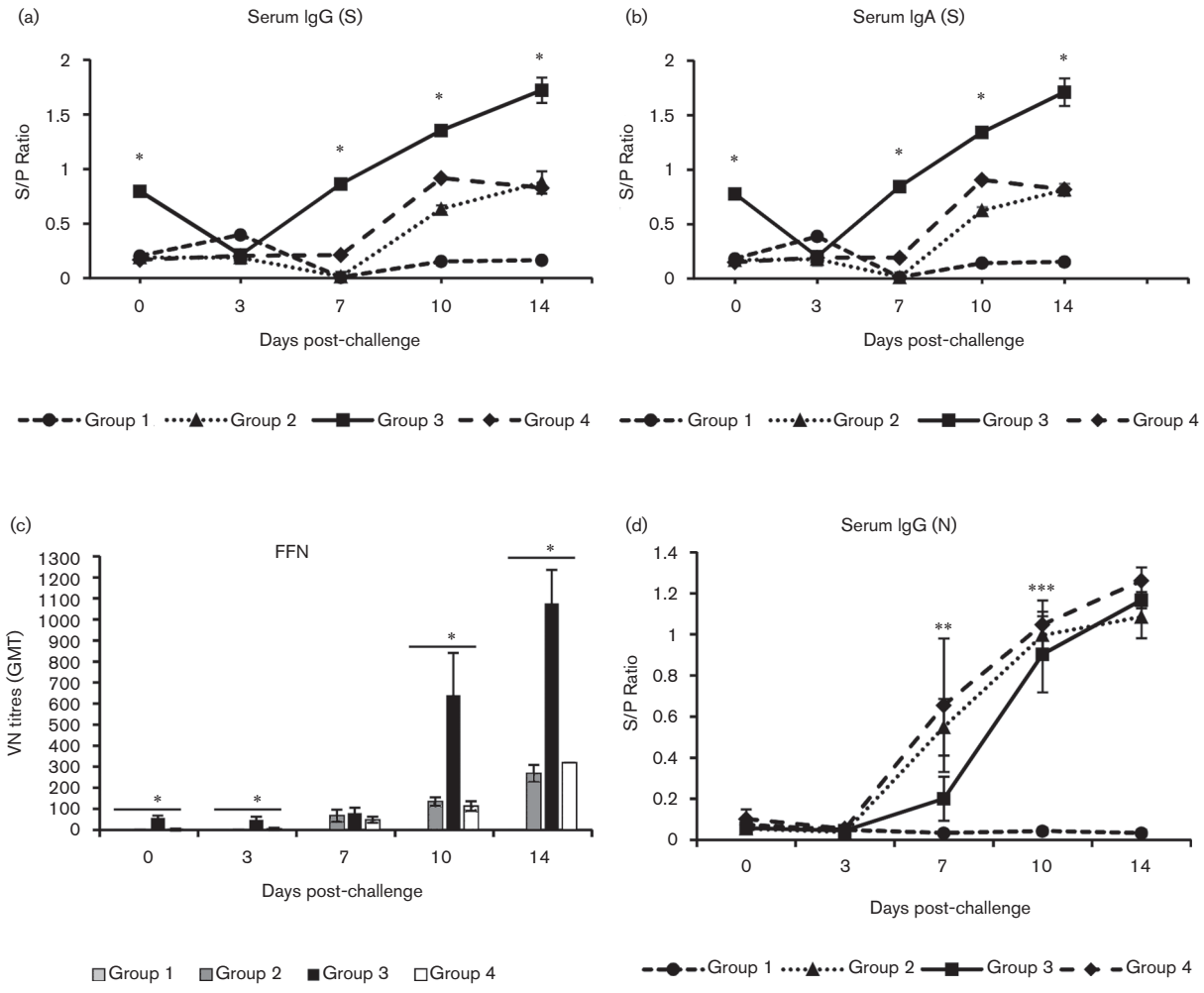

Fig. 6. Serological responses following challenge infection with PEDV. (a) Isotype ELISA demonstrating serum IgG antibody responses specific to the PEDV S protein. (b) Isotype ELISA demonstrating serum IgA antibody responses specific to the PEDV S protein. (c) Virus NA responses. (d) Isotype ELISA demonstrating serum IgG antibody responses specific to the PEDV N protein. S/P, sample to positive ratio; GMT, geometric mean titre. Error bars represent SEM. Statistical significance was determined using one-way ANOVA and Tukey's HSD. *Statistically significant at the 0.05 level when the mean of Group 3 is compared to the mean of Groups 1, 2 and 4; **statistically significant at the 0.05 level when the mean of Group 3 was compared to the mean of Groups 2 and 4; ***statistically significant at the 0.05 level when the mean of Group 3 was compared to the mean of Group 4.

(100 U ml^{-1}), streptomycin (100 µg ml^{-1}) and gentamicin (50 µg ml^{-1}).

ORFV strain IA82, a well-characterized ORFV strain (Delhon *et al.*, 2004; Diel *et al.*, 2010, 2011a, b), was used as the parental virus to construct the recombinant ORFV expressing the PEDV S protein (ORFV-PEDV-S) and in all experiments involving the use of wild-type ORFV. Wild-type and recombinant ORFV viruses were amplified in primary OFTu cells. PEDV strain USA/CO/2013 (CO13) was obtained from the National Veterinary Services Laboratory (NVSL) and propagated in Vero-76 cells in the presence of 1.5 µg ml^{-1} L-1-Tosylamide-2-phenylethyl chloromethyl ketone (TPCK)-treated trypsin (Sigma-Aldrich, St. Louis, MO).

Construction of ORFV-PEDV-S recombination plasmid. The full-length coding sequence of the spike gene of PEDV strain CO13 (GenBank accession no. KF267450) was analysed, and restriction endonuclease sites required for insertion into the ORFV genome [*ORFV121* locus (Diel *et al.*, 2011b)] were removed through silent nucleotide substitutions. In addition, early poxviral transcription termination signals (TTTTTNT) present within the coding sequence of PEDV S were removed by introducing silent nucleotide mutations. Coding sequences of the His-tag epitope (6xHis) were added to the 5′ and 3′ ends of the S coding sequence. The sequence of the VV7.5 early/late poxviral promoter (Chakrabarti *et al.*, 1997) was added to the 5′ end of the PEDV S coding sequence. Additionally, HindIII and SalI restriction sites were added to the 5′ and 3′ ends of the VV7.5-PEDV-S construct, respectively. A single DNA fragment containing the full-length PEDV S coding sequences under the control of the VV7.5 early/late poxviral promoter was chemically synthesized (GenScript®, Piscataway, NJ) and subcloned into the poxviral transfer vector pZippy-EGFP (Ning *et al.*, 2011) using HindIII and SalI restriction enzymes (pZGFP-PEDV-S).

To insert the PEDV-S coding sequences into the *ORFV121* genome locus (Diel *et al.*, 2011b), a recombination cassette was constructed. *ORFV121* left (LF, 1016 bp) and right (RF, 853 bp) flanking regions were PCR amplified from the ORFV strain IA82 genome with primers 121LF-Fw(SpeI)-5′-ATTCTTATGCGGCCGCGCAGCACTGCTCGGAGGAG TGCTC-3′; 121LF-Rv-(HindIII)-5′-CAGAATTCGCAAGCTTGGTTG TGTGGGCCACAGAGTTGAG-3′; 121RF-Fw-(NotI)-5′-ATTCTTA TGCGGCCGCGGAGCACTGCTCGGAGGAGTGCT-3′ and 121RF-Rv-(BglII)-5′-CAGAATTCGCAGATCTATCATGCGCAGCGACGACATCA TC-3′ and cloned into the vector pZGFP-PEDV-S resulting in the recombination vector pZGFP-121PEDV-S. Restriction enzymes indicated on each primer were used to clone ORFV121 LF and RF PCR amplicons into the plasmid pZGFP-PEDV-S (pZGFP-121PEDV-S). Correct cloning of ORFV121 LF and RF and of PEDV-S were confirmed by restriction enzyme analysis.

Generation and characterization of the ORFV-PEDV-S recombinant virus. The full-length PEDV S coding sequences were inserted into the *ORFV121* locus (Diel *et al.*, 2011b) of the ORFV genome by homologous recombination between the parental ORFV strain IA82 and the recombination cassette pZGFP-121PEDV-S. OFTu cells cultured in 6-well plates were infected with OV-IA82 (m.o.i. of 1) and 3 h later transfected with 2 µg of pZGFP-121PEDV-S DNA using Lipofectamine 3000 (Life Technologies) according to the manufacturer's instructions. At 72 h post-infection/transfection cell cultures were harvested, subjected to three freeze-and-thaw cycles and cell lysates used for recombinant virus selection by limiting dilution followed by plaque assay. Briefly, OFTu cells cultured in 96-well plates were infected with 10-fold serial dilutions of the cell lysates (10^{-1} to 10^{-3}), incubated at 37 °C for 72 h and screened under a fluorescence microscope. Wells containing viral foci expressing the GFP were harvested and subjected to one additional round of limiting dilution. GFP-positive wells from the second limiting dilution were subjected to plaque purification. OFTu cells cultured in six-well plates were infected with 10-fold serial dilutions (10^{-1} to 10^{-3}) of cell lysates from GFP positive wells (obtained during the limiting dilutions selection) and overlaid with culture medium containing 0.5 % agarose (SeaKem GTC agarose, Lonza, Alpharetta, GA). Fluorescent plaques were subjected to five additional rounds of plaque purification. The presence of PEDV-S and absence of *ORFV121* sequences in the purified recombinant virus were confirmed by PCR screening. Primers used for PCR amplification of PEDV-S sequences were PEDV-intS-Fw-5′-CGTGGTGGGTTTGGTTGATT-3′ and PEDV-intS-Rv-5′-CTGCACGTGGACCTTTTCAA-3′; and 121int-Fw-5′-GGCGGACTAC-CAGAGACATC-3′ and 121int-Rv-5′-GTCTTCCGGGATGTCGTAGA-3′, respectively. PCR amplicons were analysed by electrophoresis in 1 % agarose gels. Insertion and integrity of the PEDV full-length spike sequences were confirmed by whole genome sequencing using the Nextera XT DNA library preparation kit (Illumina, San Diego, CA) followed by sequencing on the Illumina Mi-Seq sequencing platform (Illumina, San Diego, CA).

Immunofluorescence. Expression of PEDV S by the ORFV-PEDV-S recombinant virus was assessed by IFA. OFTu cells were infected with the ORFV-PEDV-S recombinant virus (m.o.i. of 1) and fixed with 3.7 % formaldehyde at 24 h post-infection. After fixation cells were washed three times with PBS and permeabilized with 0.2 % PBS-Triton X-100 for 10 min at room temperature (RT). Unpermeabilized cells were kept as controls to assess expression of PEDV S on the membrane of ORFV-PEDV-S-infected cells. Cells were washed three times with PBS and incubated with a PEDV S-specific mouse mAb (SD37-11; 1 : 25 in PBS-1 % BSA) for 1 h at RT. After primary antibody incubation, cells were washed as above and incubated with goat anti-mouse IgG (H+L) secondary antibody (1 : 250 in PBS/1 % BSA; Alexa Fluor® 594 conjugate; Life Technologies, Carlsbad, CA) for 1 h at RT. Cells were washed three times with PBS and visualized under a fluorescence microscope.

Western blot. Expression of PEDV S by the ORFV-PEDV-S recombinant virus was assessed by Western blot. OFTu cells cultured in 6-well plates were infected ORFV-PEDV-S recombinant virus (m.o.i. of 10) and harvested at 48 h post-infection. Cells infected with parental ORFV strain IA82 were used as controls. Cells were lysed with M-PER mammalian extraction reagent (Thermo Scientific, Waltham, MA) containing protease inhibitors (RPI, Mount Prospect, IL). Fifty micrograms of whole cell protein extracts was resolved by SDS-PAGE in 7 % acrylamide gels and transferred to nitrocellulose membranes. Blots were incubated with 5 % non-fat dry milk TBS-Tween 20 (0.1 %; TBS-T) solution for 1 h at RT and probed with anti-His epitope tag antibody (His.H8, Thermo Scientific, Waltham, MA) overnight at 4 °C. Blots were washed three times with TBS-T for 10 min at RT and incubated with a goat anti-mouse IgG-HRP conjugate secondary antibody for 2 h at RT. Blots were washed three times with TBS-T for 10 min and developed by using a chemiluminescent substrate (Clarity, ECL; Bio-Rad, Hercules, CA).

Growth curves. Replication properties of ORFV-PEDV-S recombinant virus were assessed *in vitro*. OFTu and PK15 cells were cultured in 6-well plates, inoculated with ORFV-PEDV-S [m.o.i. of 0.1 (multistep growth curve) and m.o.i. of 10 (single-step growth curve)] and harvested at various time points post-infection (6, 12, 24, 48 and 72 h). Virus titres were determined on each time point using the Spearman and Karber's method (Hierholzer & Killington, 1996) and expressed as tissue culture infectious dose 50 ($TCID_{50}$) per millilitre.

Antigens for ELISAs. A truncated form of PEDV S protein was expressed as a recombinant protein in *Escherichia coli*, and used in indirect ELISAs to assess antibody responses in animals immunized with the ORFV-PEDV-S virus. A fragment of the PEDV S protein corresponding to nucleotides 1891–2400 was amplified from the genome of PEDV strain CO13 using standard reverse transcriptase and PCR amplification methods. Primers used for PCR amplification were PED-SPikS-1891 (BamHI)-F-5′-CGCGGATCCACGCCTAAACCATTTGAAG-3′ and

PED-SpikS-2400(XhoI)R-5′-CACACTCGAGGTAAAGCTGTAAATA TTCTGTCC-3′. The PCR amplicon was cloned into the bacterial expression plasmid pET-28a (EMD Millipore – Novagen, Billerica, MA) using the restriction enzymes indicated on each primer sequence (underlined). The recombinant S protein was expressed in *E. coli* as a 6xHis-tagged fusion protein (Okda *et al.*, 2015) and purified using nickel-charged agarose resin (Qiagen, Valencia, CA) according to the manufacturer's recommendations. Affinity purified recombinant protein was refolded and used as antigen on indirect ELISAs. DNA sequencing was used to confirm the identity and in frame cloning of PEDV S with 6xHis-tag.

Animal immunization challenge studies. The immunogenicity and protective efficacy of the ORFV-PEDV-S recombinant virus were assessed in pigs. The efficacy of the vector was evaluated by two immunization routes: TC and IM. The TC route was explored here because it has been shown to induce mucosal and systemic immunity in mice and humans (Lawson *et al.*, 2012), and because of the natural tropism of ORFV for keratinocytes in the natural host (Jenkinson *et al.*, 1990). Sixteen 3-week-old pigs, seronegative for PEDV, were randomly allocated to four experimental groups as follows: Group 1, sham-immunized/mock-challenged (n=4); Group 2, ORFV-PEDV-S-immunized/PEDV challenged (n=4); Group 3, ORFV-PEDV-S-immunized/PEDV challenged (n=4) and Group 4, sham-immunized/PEDV challenged (n=4) (Table 1). Animals from Groups 1 and 4 were immunized with a control ORFV vector expressing GFP (ORFV-GFP) and received half dose via the TC (1 ml) and the other half via the IM route (1 ml). Animals from Group 2 were immunized with the ORFV-PEDV-S recombinant virus via the TC route only, while animals in Group 3 were immunized by the IM route. IM immunization was performed by injection of 2 ml of a virus suspension containing $10^{7.38}$ $TCID_{50}$ ml^{-1} in MEM into the neck. For the TC immunization the skin of the inguinal region was scarified with a sterile scalpel blade and the virus suspension containing $10^{7.38}$ $TCID_{50}$ ml^{-1} in MEM was applied topically to the scarified skin area using a sterile cotton swab (~4 cm^2). All animals were immunized on day 0 and received two booster immunizations on days 21 and 45 post-primary immunization. Booster immunizations (TC and IM) were performed by scarification or infection in sites adjacent (3–5 cm) to original immunization sites. Serum samples were collected on days 0, 7, 14, 21, 35, 42, 49, 53, 56 and 60 p.i.

Challenge infection was conducted to evaluate the efficacy of recombinant ORFV-PEDV-S virus. On day 60 p.i., animals from Group 1 received 2 ml of MEM orally (mock challenge), while animals from Groups 2, 3 and 4 were challenged orally with a virus suspension containing 2×10^5 $TCID_{50}$ of PEDV strain CO13. Animals were monitored daily for clinical signs of PED. Clinical signs were recorded and individual daily scores assigned to all animals based on the following criteria: 0, normal faeces; 1, pasty faeces; 2, moderate diarrhoea (semi-liquid); 3, diarrhoea (liquid); 4, severe diarrhoea (very liquid) and 5, watery diarrhoea (profuse diarrhoea) (de Arriba *et al.*, 2002b). Mean daily group scores were calculated and compared among different treatment groups. Faecal swabs were collected on days 0, 3, 5, 7, 9, 11 and 14 post-challenge to assess virus shedding in faeces. Serum samples were collected on days 0, 3, 7, 10 and 14 post-challenge to evaluate humoral and cell-mediated responses. All animals were euthanized on day 14 post-challenge. Animal immunization challenge studies were conducted at SDSU Animal Resource Wing (ARW), following the guidelines and protocols approved by the SDSU Institutional Animal Care and Use Committee (IACUC approval no. 15-063A).

Antibody isotype ELISAs. PEDV-specific IgG and IgA antibody responses elicited by immunization with the recombinant ORFV-PEDV-S virus were assessed by S indirect ELISAs, while responses post-challenge infection were assessed by the S and nucleoprotein (N) (Okda *et al.*, 2015) ELISAs. Optimal assay conditions (amount of antigen, serum and secondary antibody dilutions) were determined by a checkerboard titration. Polystyrene microtitre plates (Immunolon 1B, Thermo Scientific, Waltham, MA) were coated with the appropriate antigen [S, 100 ng $well^{-1}$; and N, 25 ng $well^{-1}$ (Okda *et al.*, 2015)] in bicarbonate/carbonate coating buffer (15 mM sodium carbonate, 35 mM sodium bicarbonate, pH 9.6) in alternate wells. After incubation at 37 °C for 1 h, plates were washed four times with PBS Tween 20 (PBS-T, 0.05 %) and blocked overnight at 4 °C with PBS-T 5 % non-fat dry milk. Blocking reagent was removed and plates washed three times with PBS-T (300 µl). Test and control serum samples were diluted (1 : 50) in PBS-T 5 % non-fat dry milk, and 100 µl of diluted samples were added to paired coated and uncoated control wells and incubated at room temperature for 1 h. Unbound antibodies were washed with PBS-T (three times) and plates incubated with biotinylated secondary antibodies against swine IgG or IgA (Bethyl Laboratories, TX) followed by incubation with streptavidin-HRP conjugate (Pierce, Rockford, IL). Reactions were developed with 3,3′,5,5′-tetramethylbenzidine substrate (TMB) (KPL, Gaithersburg, MA) and OD values determined at 450 nm using a microplate reader (BioTek Instruments, Winooski, VT). OD values for each test and control samples were normalized to the OD value of uncoated wells and results expressed as sample to positive (S/P) ratios that were calculated as follows: S/P=optical density (OD) of sample – OD of buffer/OD of positive control – OD of buffer. All assay formats were pre-validated at the SD Animal Disease Research and Diagnostic Laboratory (ADRDL) using serum samples from animals of known serological status.

Fluorescent focus neutralization assay. NA responses elicited by immunization with the recombinant ORFV-PEDV-S were assessed by FFN assay as previously described (Okda *et al.*, 2015). Endpoint NA titres were determined as the highest dilution of serum capable of reducing 90 % of PEDV fluorescent foci relative to negative control samples. A FFN titre <20 was considered negative.

Viral RNA extraction and real-time reverse transcription PCR. Viral nucleic acid was extracted from faecal swabs using the MagMAX Viral RNA/DNA Isolation Kit (Life Technologies, Carlsbad, CA) following the manufacturer's instructions. Shedding of PEDV in faeces was assessed using a commercial multiplex (RT-qPCR) kit targeting the spike gene of PEDV, and other enteric coronaviruses including transmissible gastroenteritis virus (TGEV) and porcine deltacoronavirus (PDCoV) (EZ-PED/TGE/PDCoV MPX 1.0, Tetracore, Rockville, MD). Genome copy numbers per millilitre were determined using the relative standard curve method. All calculations were performed using a four-parameter logistic regression curve. RT-qPCR tests were performed at the SDSU ADRDL.

Statistical analysis. Statistical analysis was performed using SPSS 14 software. One-way ANOVA with Tukey's HSD multiple comparison test was performed on all groups, using harmonic mean sample size of 4 to account for equal group sizes. Nonparametric Kruskal–Wallis test was used to assess differences in virus shedding between treatment groups. Differences between groups were considered significant at P<0.05.

ACKNOWLEDGEMENTS

We thank our colleagues from the SD ADRDL Molecular Diagnostics Section for excellent technical assistance and Dr Daniel Rock (University of Illinois, Urbana-Champaign) for providing the wild type ORFV strain used in our study. We also thank the staff and students from the SDSU Animal Resource Wing (ARW) for their invaluable help with the animal experiments. This work was supported by the National Pork Board project number 15-034, the USDA National Institute of Food and Agriculture Hatch project SD00H517-14, and by the SDSU Scholarly Excellence Funds to D. G. D. The authors

D. G. D and E. F. F. declare that there is a patent pending related to this work (US patent pending, P11703US00).

REFERENCES

Amann, R., Rohde, J., Wulle, U., Conlee, D., Raue, R., Martinon, O. & Rziha, H. J. (2013). A new rabies vaccine based on a recombinant ORF virus (parapoxvirus) expressing the rabies virus glycoprotein. *J Virol* **87**, 1618–1630.

Blum, J. S., Wearsch, P. A. & Cresswell, P. (2013). Pathways of antigen processing. *Annu Rev Immunol* **31**, 443–473.

Chakrabarti, S., Sisler, J. R. & Moss, B. (1997). Compact, synthetic, vaccinia virus early/late promoter for protein expression. *Biotechniques* **23**, 1094–1097.

Chang, S. H., Bae, J. L., Kang, T. J., Kim, J., Chung, G. H., Lim, C. W., Laude, H., Yang, M. S. & Jang, Y. S. (2002). Identification of the epitope region capable of inducing neutralizing antibodies against the porcine epidemic diarrhea virus. *Mol Cells* **14**, 295–299.

Chattha, K. S., Roth, J. A. & Saif, L. J. (2014). Strategies for design and application of enteric viral vaccines. *Annu Rev Anim Biosci* **3**, 375–395.

Cruz, D. J., Kim, C. J. & Shin, H. J. (2006). Phage-displayed peptides having antigenic similarities with porcine epidemic diarrhea virus (PEDV) neutralizing epitopes. *Virology* **354**, 28–34.

Cruz, D. J., Kim, C. J. & Shin, H. J. (2008). The GPRLQPY motif located at the carboxy-terminal of the spike protein induces antibodies that neutralize Porcine epidemic diarrhea virus. *Virus Res* **132**, 192–196.

de Arriba, M. L., Carvajal, A., Pozo, J. & Rubio, P. (2002a). Isotype-specific antibody-secreting cells in systemic and mucosal associated lymphoid tissues and antibody responses in serum of conventional pigs inoculated with PEDV. *Vet Immunol Immunopathol* **84**, 1–16.

de Arriba, M. L., Carvajal, A., Pozo, J. & Rubio, P. (2002b). Mucosal and systemic isotype-specific antibody responses and protection in conventional pigs exposed to virulent or attenuated porcine epidemic diarrhoea virus. *Vet Immunol Immunopathol* **85**, 85–97.

Deane, D., McInnes, C. J., Percival, A., Wood, A., Thomson, J., Lear, A., Gilray, J., Fleming, S., Mercer, A. & Haig, D. (2000). Orf virus encodes a novel secreted protein inhibitor of granulocyte-macrophage colony-stimulating factor and interleukin-2. *J Virol* **74**, 1313–1320.

Debouck, P., Pensaert, M. & Coussement, W. (1981). The pathogenesis of an enteric infection in pigs, experimentally induced by the coronavirus-like agent, CV 777. *Vet Microbiol* **6**, 157–165.

Delhon, G., Tulman, E. R., Afonso, C. L., Lu, Z., de la Concha-Bermejillo, A., Lehmkuhl, H. D., Piccone, M. E., Kutish, G. F. & Rock, D. L. (2004). Genomes of the parapoxviruses ORF virus and bovine papular stomatitis virus. *J Virol* **78**, 168–177.

Diel, D. G., Delhon, G., Luo, S., Flores, E. F. & Rock, D. L. (2010). A novel inhibitor of the NF-{kappa}B signaling pathway encoded by the parapoxvirus orf virus. *J Virol* **84**, 3962–3973.

Diel, D. G., Luo, S., Delhon, G., Peng, Y., Flores, E. F. & Rock, D. L. (2011a). A nuclear inhibitor of NF-kappaB encoded by a poxvirus. *J Virol* **85**, 264–275.

Diel, D. G., Luo, S., Delhon, G., Peng, Y., Flores, E. F. & Rock, D. L. (2011b). Orf virus ORFV121 encodes a novel inhibitor of NF-kappaB that contributes to virus virulence. *J Virol* **85**, 2037–2049.

Dory, D., Fischer, T., Béven, V., Cariolet, R., Rziha, H. J. & Jestin, A. (2006). Prime-boost immunization using DNA vaccine and recombinant Orf virus protects pigs against Pseudorabies virus (Herpes suid 1). *Vaccine* **24**, 6256–6263.

Fischer, T., Planz, O., Stitz, L. & Rziha, H. J. (2003). Novel recombinant parapoxvirus vectors induce protective humoral and cellular immunity against lethal herpesvirus challenge infection in mice. *J Virol* **77**, 9312–9323.

Fleming, S. B., McCaughan, C. A., Andrews, A. E., Nash, A. D. & Mercer, A. A. (1997). A homolog of interleukin-10 is encoded by the poxvirus orf virus. *J Virol* **71**, 4857–4861.

Fleming, S. B., Anderson, I. E., Thomson, J., Deane, D. L., McInnes, C. J., McCaughan, C. A., Mercer, A. A. & Haig, D. M. (2007). Infection with recombinant orf viruses demonstrates that the viral interleukin-10 is a virulence factor. *J Gen Virol* **88**, 1922–1927.

Goede, D., Murtaugh, M. P., Nerem, J., Yeske, P., Rossow, K. & Morrison, R. (2015). Previous infection of sows with a "mild" strain of porcine epidemic diarrhea virus confers protection against infection with a "severe" strain. *Vet Microbiol* **176**, 161–164.

Henkel, M., Planz, O., Fischer, T., Stitz, L. & Rziha, H. J. (2005). Prevention of virus persistence and protection against immunopathology after borna disease virus infection of the brain by a novel Orf virus recombinant. *J Virol* **79**, 314–325.

Hierholzer, J. C. & Killington, R. A. (1996). Virus isolation and quantitation. In *Virology Methods Manual*, pp. 25–46. San Diego, CA: Academic Press Inc.

Jenkinson, D. M., Mcewan, P. E., Moss, V. A., Elder, H. Y. & Reid, H. W. (1990). Location and spread of Orf virus antigen in infected Ovine skin. *Vet Dermatol* **1**, 189–195.

Langel, S. N., Paim, F. C., Lager, K. M., Vlasova, A. N. & Saif, L. J. (2016). Lactogenic immunity and vaccines for porcine epidemic diarrhea virus (PEDV): Historical and current concepts. *Virus Res.* (in press) Doi:10.1016/j.virusres.2016.05.016.

Lawson, L. B., Clements, J. D. & Freytag, L. C. (2012). Mucosal immune responses induced by transcutaneous vaccines. *Curr Top Microbiol Immunol* **354**, 19–37.

Lee, C. (2015). Porcine epidemic diarrhea virus: an emerging and re-emerging epizootic swine virus. *Virol J* **12**, 193.

Liu, D. Q., Ge, J. W., Qiao, X. Y., Jiang, Y. P., Liu, S. M. & Li, Y. J. (2012). High-level mucosal and systemic immune responses induced by oral administration with Lactobacillus-expressed porcine epidemic diarrhea virus (PEDV) S1 region combined with Lactobacillus-expressed N protein. *Appl Microbiol Biotechnol* **93**, 2437–2446.

Madson, D. M., Magstadt, D. R., Arruda, P. H. E., Hoang, H., Sun, D., Bower, L. P., Bhandari, M., Burrough, E. R., Gauger, P. C. & other authors (2014). Pathogenesis of porcine epidemic diarrhea virus isolate (US/Iowa/18984/2013) in 3-week-old weaned pigs. *Vet Microbiol* **174**, 60–68.

Makadiya, N., Brownlie, R., van den Hurk, J., Berube, N., Allan, B., Gerdts, V. & Zakhartchouk, A. (2016). S1 domain of the porcine epidemic diarrhea virus spike protein as a vaccine antigen. *Virol J* **13**, 57.

Marsland, B. J., Tisdall, D. J., Heath, D. D. & Mercer, A. A. (2003). Construction of a recombinant orf virus that expresses an Echinococcus granulosus vaccine antigen from a novel genomic insertion site. *Arch Virol* **148**, 555–562.

McInnes, C. J., Wood, A. R. & Mercer, A. A. (1998). Orf virus encodes a homolog of the vaccinia virus interferon-resistance gene E3L. *Virus Genes* **17**, 107–115.

Mercer, A. A., Schmidt, A. & Weber, O. (2007). *Poxviruses.* Edited by A. A. Mercer, A. Schmidt & O. Weber. Berlin: Birkhäuser Verlag.

Meyer, M., Clauss, M., Lepple-Wienhues, A., Waltenberger, J., Augustin, H. G., Ziche, M., Lanz, C., Büttner, M., Rziha, H. J. & Dehio, C. (1999). A novel vascular endothelial growth factor encoded by Orf virus, VEGF-E, mediates angiogenesis via signalling through VEGFR-2 (KDR) but not VEGFR-1 (Flt-1) receptor tyrosine kinases. *EMBO J* **18**, 363–374.

Ning, Z., Peng, Y., Hao, W., Duan, C., Rock, D. L. & Luo, S. (2011). Generation of recombinant Orf virus using an enhanced green fluorescent protein reporter gene as a selectable marker. *BMC Vet Res* **7**, 80.

Oh, J., Lee, K. W., Choi, H. W. & Lee, C. (2014). Immunogenicity and protective efficacy of recombinant S1 domain of the porcine epidemic diarrhea virus spike protein. *Arch Virol* **159**, 2977–2987.

Okda, F., Liu, X., Singrey, A., Clement, T., Nelson, J., Christopher-Hennings, J., Nelson, E. A. & Lawson, S. (2015). Development of an indirect ELISA, blocking ELISA, fluorescent microsphere immunoassay and fluorescent focus neutralization assay for serologic evaluation of exposure to North American strains of Porcine epidemic Diarrhea virus. *BMC Vet Res* **11**, 180.

Paudel, S., Park, J. E., Jang, H. & Shin, H. J. (2014). Comparison of serum neutralization and enzyme-linked immunosorbent assay on sera from porcine epidemic diarrhea virus vaccinated pigs. *Vet Q* **34**, 218–223.

Pensaert, M. B. & de Bouck, P. (1978). A new coronavirus-like particle associated with diarrhea in swine. *Arch Virol* **58**, 243–247.

Poonsuk, K., Giménez-Lirola, L. G., Zhang, J., Arruda, P., Chen, Q., Correa da Silva Carrion, L., Magtoto, R., Pineyro, P., Sarmento, L. & other authors (2016). Does circulating Antibody play a role in the protection of piglets against porcine epidemic Diarrhea virus? *PLoS One* **11**, e0153041.

Rohde, J., Schirrmeier, H., Granzow, H. & Rziha, H. J. (2011). A new recombinant Orf virus (ORFV, Parapoxvirus) protects rabbits against lethal infection with rabbit hemorrhagic disease virus (RHDV). *Vaccine* **29**, 9256–9264.

Rohde, J., Amann, R. & Rziha, H. J. (2013). New Orf virus (Parapoxvirus) recombinant expressing H5 hemagglutinin protects mice against H5N1 and H1N1 influenza A virus. *PLoS One* **8**, e83802.

Saif, L. J. (2015). Lactogenic immunity and vaccines for porcine epidemic diarrhea virus (PEDV): Revising transmissible gastroenteritis virus (TGEV) vaccine concepts. In *AASV Annu Meet*, Orlando, pp. 403–406.

Seet, B. T., McCaughan, C. A., Handel, T. M., Mercer, A., Brunetti, C., McFadden, G. & Fleming, S. B. (2003). Analysis of an orf virus chemokine-binding protein: shifting ligand specificities among a family of poxvirus viroceptors. *Proc Natl Acad Sci U S A* **100**, 15137–15142.

Song, D. & Park, B. (2012). Porcine epidemic diarrhoea virus: a comprehensive review of molecular epidemiology, diagnosis, and vaccines. *Virus Genes* **44**, 167–175.

Song, D. S., Oh, J. S., Kang, B. K., Yang, J. S., Moon, H. J., Yoo, H. S., Jang, Y. S. & Park, B. K. (2007). Oral efficacy of Vero cell attenuated porcine epidemic diarrhea virus DR13 strain. *Res Vet Sci* **82**, 134–140.

Sun, D., Feng, L., Shi, H., Chen, J., Cui, X., Chen, H., Liu, S., Tong, Y., Wang, Y. & Tong, G. (2008). Identification of two novel B cell epitopes on porcine epidemic diarrhea virus spike protein. *Vet Microbiol* **131**, 73–81.

Sun, D. B., Feng, L., Shi, H. Y., Chen, J. F., Liu, S. W., Chen, H. Y. & Wang, Y. F. (2007). Spike protein region (aa 636789) of porcine epidemic diarrhea virus is essential for induction of neutralizing antibodies. *Acta Virol* **51**, 149–156.

Suo, S., Ren, Y., Li, G., Zarlenga, D., Bu, R. E., Su, D., Li, X., Li, P., Meng, F. & other authors (2012). Immune responses induced by DNA vaccines bearing Spike gene of PEDV combined with porcine IL-18. *Virus Res* **167**, 259–266.

van Rooij, E. M., Rijsewijk, F. A., Moonen-Leusen, H. W., Bianchi, A. T. & Rziha, H. J. (2010). Comparison of different prime-boost regimes with DNA and recombinant Orf virus based vaccines expressing glycoprotein D of pseudorabies virus in pigs. *Vaccine* **28**, 1808–1813.

Voigt, H., Merant, C., Wienhold, D., Braun, A., Hutet, E., Le Potier, M. F., Saalmüller, A., Pfaff, E. & Büttner, M. (2007). Efficient priming against classical swine fever with a safe glycoprotein E2 expressing Orf virus recombinant (ORFV VrV-E2). *Vaccine* **25**, 5915–5926.

Wang, Z., Martinez, J., Zhou, W., La Rosa, C., Srivastava, T., Dasgupta, A., Rawal, R., Li, Z., Britt, W. J. & Diamond, D. (2010). Modified H5 promoter improves stability of insert genes while maintaining immunogenicity during extended passage of genetically engineered MVA vaccines. *Vaccine* **28**, 1547–1557.

Weber, O., Knolle, P. & Volk, H.-D. (2007). Immunomodulation by inactivated Orf virus (ORFV) - therapeutic potential. In *Poxviruses*, 1st edn, pp. 297–310. Edited by A. A Mercer, A. Schmidt & O. Weber. Boston/Basel/Berlin: Birkhaeuser Verlag.

Weber, O., Mercer, A. A., Friebe, A., Knolle, P. & Volk, H. D. (2013). Therapeutic immunomodulation using a virus – the potential of inactivated orf virus. *Eur J Clin Microbiol Infect Dis* **32**, 451–460.

Wise, L. M., Veikkola, T., Mercer, A. A., Savory, L. J., Fleming, S. B., Caesar, C., Vitali, A., Makinen, T., Alitalo, K. & Stacker, S. A. (1999). Vascular endothelial growth factor (VEGF)-like protein from orf virus NZ2 binds to VEGFR2 and neuropilin-1. *Proc Natl Acad Sci U S A* **96**, 3071–3076.