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Efficient priming of CD4 T cells by Langerin-expressing dendritic cells targeted with porcine epidemic diarrhea virus spike protein domains in pigs

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Highlights

- Targeting PEDV spike protein domains to Langerin-expressing dendritic cells through a single chain antibody
- Transdermal Langerin-targeting of PEDV S antigen significantly enhanced CD4 T cell immunity
- Systemic Langerin-targeting of PEDV S antigen significantly augmented serum IgG and IgA responses

Abstract

Porcine epidemic diarrhea virus (PEDV) first emerged in the United States in 2013 causing high mortality and morbidity in neonatal piglets with immense economic losses to the swine industry. PEDV is an alpha-coronavirus replicating primarily in porcine intestinal cells. PEDV vaccines are available in Asia and Europe, and conditionally-licensed vaccines recently became available in the United States but the efficacies of these vaccines in eliminating PEDV from swine populations are questionable. In this study, the immunogenicity of a subunit vaccine based on the spike protein of PEDV, which was directly targeted to porcine dendritic cells (DCs) expressing Langerin, was assessed. The PEDV S antigen was delivered to the dendritic cells through a single-chain antibody specific to Langerin and the targeted cells were stimulated with cholera toxin adjuvant. This approach, known as "dendritic cell targeting," greatly improved PEDV S antigen-specific T cell interferon-γ responses in the $CD4^{pos}CD8^{pos}$ T cell compartment in pigs as early as 7 days upon transdermal administration. When the vaccine protein was targeted to $Langerin^{pos}$ DCs systemically through intramuscular vaccination, it induced higher

serum IgG and IgA responses in pigs, though these responses require a booster dose, and the magnitude of T cell responses were lower as compared to transdermal vaccination. We conclude that PEDV spike protein domains targeting Langerin-expressing dendritic cells significantly increased CD4 T cell immune responses in pigs. The results indicate that the immunogenicity of protein subunit vaccines can be greatly enhanced by direct targeting of the vaccine antigens to desirable dendritic cell subsets in pigs.

Keywords: porcine epidemic diarrhea virus (PEDV); porcine Langerin; dendritic cell targeting; T cell immunity; subunit vaccine

1. Introduction

Porcine epidemic diarrhea (PED) is a transboundary disease of pigs that is endemic primarily in Asia and Europe (Song et al., 2015). In 2013, the disease emerged for the first time in the United States and quickly spread to more than 30 states (Stevenson et al., 2013). The disease mainly affects neonatal piglets often presented with watery diarrhea, vomiting, and 90-95% mortality in naïve populations (Stevenson et al., 2013) causing immense economic losses to the swine industries (Schulz and Tonsor, 2015). The causative agent of the disease is *porcine epidemic diarrhea virus* (PEDV), an alpha-coronavirus in the family *Coronaviridae* (Chasey and Cartwright, 1978; Tobler et al., 1993). PEDV encodes four structural proteins: Spike (S), Envelope (E), Membrane (M) and Nucleocapsid (N) (Brian and Baric, 2005). PEDV S protein is a type I transmembrane protein. The extracellular portion of S protein contains major antigenic determinants, and is made up of three domains, N-terminal domain (NTD), S1 domain and S2 domain. The S1 and S2 domains contain potential virus neutralizing epitopes (Huang et al., 2013; Song and Park, 2012). A recombinant, spike-based (NTD-S1) vaccine induces virus-neutralizing antibody responses in pregnant sows and protected their offspring from mortality (Oh et al., 2014). However, the cellular immune responses of this protein subunit vaccine have not been determined. Recent studies showed that PEDV infection or vaccination enhanced the antigen presenting capabilities of porcine DCs *in vitro* and *in vivo* which subsequently stimulated T cell proliferation efficiently (Gao et al., 2015; Gao et al., 2016).

In mouse and monkey models, *Human immunodeficiency virus gag* antigens were targeted to specific dendritic cell (DC) subsets expressing c-type lectin receptors (CLRs) in order to generate effective cellular immune responses even at low antigenic doses (Trumpfheller et al., 2012). More recently, we successfully targeted *porcine reproductive and respiratory syndrome*

virus (PRRSV) antigen to a pig DC subset expressing DC-SIGN that effectively primed CD4 T cells in pigs at a low antigenic dose (Subramaniam et al., 2014). However, the effects of antigen targeting to additional porcine CLRs are entirely unknown.

Langerin is a CLR expressed in DC subsets residing in the epidermis, dermis, spleen, peripheral lymph nodes, and peripheral tissues (Idoyaga et al., 2008; Maisonnasse et al., 2015; Marquet et al., 2014). Porcine Langerin is expressed in epidermal Langerhans cells (LCs), in a subset of dermal $CD163^{low}$ DCs and in type 2 conventional DCs in lung (Maisonnasse et al., 2015; Marquet et al., 2014). Mouse LCs and $Langerin^{pos}$ DCs targeted with Ovalbumin antigen presented both MHC-I and MHC-II products to stimulate Th1 and cytotoxic T lymphocytes, respectively (Idoyaga et al., 2008). $Langerin^{pos}$ $CD8\alpha^{neg}$ DCs were differentiated from bone marrow DC precursors in the skin in response to transcutaneous immunization of mice with tetanus toxoid and cholera toxin, which then accumulated in mesenteric lymph nodes and efficiently induced gut secretory IgA responses (Chang et al., 2008).

The protection against PEDV infection in piglets is mainly provided by lactogenic immunity through colostrum and milk of sows previously exposed to low pathogenic or live-attenuated virus (Goede et al., 2015; Oh et al., 2014; Song and Park, 2012). Intraperitoneal administration of PEDV-specific antibodies obtained from sera of PEDV-infected sows reduced disease severity and increased survival of piglets upon virus challenge (K.Poonsuk, 2015). Prior exposure of 11 day-old piglets with PEDV showed reduced morbidity upon re-challenge with virus three weeks later (de Arriba et al., 2002). The reduced morbidity strongly correlated with the frequencies of PEDV-specific antibody-secreting cells in intestinal tissues as well as with serum PEDV-specific antibody levels at the day of challenge (de Arriba et al., 2002). On the other hand, PEDV induces significantly higher CD4 T cell responses but not CD8 T cell

responses in ileal tissues of suckling piglets as compared to the weaned piglets. (Annamalai et al., 2015) However, the role of these CD4 T cell immune responses induced by PEDV to protection in pigs remains unknown.

In this study, we evaluated the immunogenicity of PEDV spike protein domains NTD-S1 targeted directly to Langerin-expressing DC subsets in the skin and in systemic sites. The cholera toxin was co-administered as adjuvant, which acts on mouse $CD11b^{pos}$ DCs through stimulating the alpha-subunit of stimulatory heterotrimeric G protein (Gsα) in order to promote Th1 and Th2 responses (Mattsson et al., 2015). Pigs were vaccinated through transdermal or intramuscular routes during priming followed by intramuscular route during boosting in order to evaluate the effects of different vaccination routes on priming of cellular and humoral immune responses due to the Langerin targeting of PEDV S antigen.

2. Materials and Methods

2.1. Cell lines, virus stock and antibodies

Vero cells and Chinese Hamster Ovary (CHO-K1) cells (ATCC) were maintained in Eagle minimum essential medium (MEM) or Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Human embryonic kidney (293T) cells were maintained in Pro293aCDM (Lonza) supplemented with 4% FBS, and after transient transfections, they were maintained in Iscove's modified Dulbecco's Medium (IMDM) supplemented with 2% ultra-low IgG FBS (Gibco). PEDV-CO/13 strain (Marthaler et al., 2013) was propagated in Vero cells with MEM maintenance medium supplemented with 0.02% yeast extract, 0.3% tryptose phosphate broth and 2 μg/mL trypsin. The antibodies used for flow cytometry analysis were described previously (Subramaniam et al., 2014).

2.2. Production of PEDV vaccine antigens

Mouse monoclonal antibodies specific to porcine Langerin were generated as described previously (Subramaniam et al., 2014) and evaluated using Pierce Rapid Antibody Isotyping kit. The variable (V) domains of mAb 3B3 (isotype G1, κ) were PCR-amplified and genetically linked through a flexible linker to make a single-chain-variable-fragment (scFv). The 3B3scFv was subsequently cloned in pFUSE-pIgG_2Fc2 (Subramaniam et al., 2014) and pFUSE-hIgG_2Fc2 (Invivogen) to make porcine and human F_c fusion antibodies, respectively. The S protein domains (amino acids 21-737) of the PEDV MN strain (Huang et al., 2013) (GenBank accession no. KF468752.1) was genetically combined with the carboxy terminus of porcine F_c or 3B3scFv-porcine F_c in pFUSE vectors. Recombinant vaccine antigens were produced by large-scale transient transfections of 293T cells and purified using protein A agarose beads as described previously (Subramaniam et al., 2014).

2.3. Experimental design of the pig immunogenicity study

A total of 31 four-week-old PEDV-negative piglets were divided into four treatment groups: Adjuvant control (group 1), non-targeted (group 2), transdermal DC-targeted (group 3), and intramuscular DC-targeted (group 4) **(Table 1)**. On day 0, pigs were administered with 200 μg recombinant antigen mixed with 50 μg cholera toxin (CT) transdermally in groups 2 and 3 but intramuscularly in group 4, while pigs in group 1 received 50 μg CT only through transdermal route. The transdermal vaccination was performed as previously described (Subramaniam et al., 2014) using a needle less injector that delivered the antigens in skin using 65 psi compressed CO_2. On day 28, pigs received a second dose of antigen-adjuvant mixtures (groups 2, 3, and 4) or adjuvant only (group 1) through the intramuscular route. Blood and fecal samples were collected at weekly time points **(Fig S1)**.

2.4. Intracellular IFN-γ staining and flow cytometry

Antigen-specific T cell responses were analyzed in T cell populations by *in vitro* antigen stimulation of mononuclear cells isolated from peripheral blood followed by intracellular staining of IFN-γ and subsequent flow cytometry analysis (Subramaniam et al., 2014).

2.5. Immunofluorescence assay

CHO-K1 cells were transfected with plasmids expressing porcine Langerin or CD137 (irrelevant gene control) using Lipofectamine LTX (Thermo Fisher Scientific). After 48 h, cells were fixed with 3.2 % paraformaldehyde, permeabilized with Triton X-100, and blocked with 3% bovine serum albumin (BSA). Cells were subsequently stained with Langerin-specific antibodies (1μg: 100μL) in blocking buffer followed by incubation with fluorochrome-conjugated secondary antibodies. DAPI (4', 6-diamidino-2-phenylindole) was used to stain the cell nucleus, and cells were imaged in Nikon Eclipse TE 300 fluorescence microscope.

2.6. Serum virus neutralization test

Vero cells were seeded in 96 well cell culture plates one day prior to testing. Pig serum samples were heat-inactivated at 56°C for 30 min and serially diluted two-fold with MEM. PEDV CO/13 strain was prepared at a concentration of 2,000 $TCID_{50}$/mL MEM and added to diluted sera at 1:1 ratio. We chose PEDV CO/13 strain for serum neutralizing test considering the fact that there are only two amino acid differences between S protein of PEDV-MN (Huang et al., 2013) and PEDV-CO/13 (Marthaler et al., 2013). These amino acid differences are located in NTD-S1 domains outside the neutralizing epitope containing region of PEDV S protein (Chang et al., 2002; Sun et al., 2008). The virus-serum mixtures were then incubated at 37°C for 1 h, and then transferred to Vero cell monolayers on 96 well cell culture plates (100 μL per well) which had been washed with MEM once. Cells were incubated at 37°C for 1 h and subsequently

washed with MEM three times. Maintenance medium containing trypsin (2μg/mL) was added to the cells and further incubated at 37°C for 5 days. Serum virus neutralization (SVN) titers were calculated as the reciprocals of the highest serum dilution resulting in complete inhibition of PEDV-induced cytopathic effect. Geometric means were calculated for triplicate as well as for groups.

2.7. ELISA

ELISA plates (96 well) were coated with PEDV NTD-S1 antigen (2 μg/mL) overnight at 4° C. Plates were washed and blocked with 150μL 5% non-fat milk for 2 h at 37° C. Diluted sera (1:400) and undiluted fecal extracts were applied in duplicate and incubated at 37°C for 1 h. Pig fecal extracts were prepared as previously described (Cordes et al., 2012). Wells were extensively washed and incubated with 100 μL secondary conjugates (Horse Radish Peroxidase (HRP)-conjugated anti-pig IgG (Santa Cruz Biotech) or anti-pig IgA (Bethyl) at 37°C for 1 h. Wells were extensively washed and incubated with 100 μL HRP substrate (KPL) for 5 min, and the reaction was stopped with 1N HCl. Absorbance was measured at 450 nm in microplate reader (Tecon or Promega Glomax). Sample-to-positive (S/P) ratio was calculated as: (sample mean – negative control mean) / (positive control mean – negative control mean) where "mean" is the average optical density (OD) at 450 nm of the duplicate samples.

2.8. Thiocyanate elution ELISA

To measure the relative affinities of Langerin mAbs, a thiocyanate elution based ELISA was carried out with purified mAbs and Langerin antigens as described elsewhere (Macdonald et al., 1988).

2.9. Statistical analysis

The significance in the difference between means of two treatment groups was determined by unpaired student 't' test assuming Gaussian distribution of variables. GraphPad Prism version 6 and Microsoft Excel programs were used to perform statistical analyses. A *p* value of less than 0.05 was considered statistically significant when the sample size n was ≥ 7.

3. Results

3.1. Characterization of a recombinant single chain antibody specific to porcine Langerin

We generated mouse mAbs (clones 3B3 and 5G7) specific to porcine Langerin by following conventional hybridoma technology. In thiocyanate elution ELISA analysis, the relative affinity of the 3B3 mAb (affinity index > 3.5) was found to be much higher than that of the 5G7 mAb (affinity index ≈ 0.4) **(Fig 1A)**. Since higher affinity antibodies are desired for "dendritic cell targeting" (Cheong et al., 2010), the 3B3 mAb was chosen for further analysis. The 3B3 mAb was shown to specifically stain the native porcine Langerin but not an irrelevant porcine cellular receptor; both were expressed exogenously on CHO-K1 cells **(Fig 1B).** The recombinant 3B3 single chain antibody, which is a human Fc fused with 3B3scFv, also specifically stained the over-expressed porcine Langerin on CHO-K1 cells comparable with that of a positive control antibody 929F3.01 **(Fig 1B).** The Langerin-targeting PEDV S antigen, which is porcine Fc fused with 3B3scFv at the amino terminus and with PEDV S antigen at the carboxy terminus (3B3scFv-pFc-PEDVsAg) **(Fig 1C)**, was detected as an approximately 210 KDa protein in SDS-PAGE analysis of transiently-transfected 293T cell lysates **(Fig 1D).** The porcine F_c fused with the PEDV NTD-S1 only (pF_c-PEDVsAg) served as non-targeting antigen and identified as an approximately 180 KDa protein in the SDS-PAGE analysis **(Fig 1C and**

1D). In short, the recombinant 3B3 single chain antibody specifically binds porcine Langerin which can be produced as a fusion protein with PEDV S domains.

3.2. T cell immune responses against Langerin-targeted PEDV spike protein domains in pigs

Subsequently, we evaluated whether Langerin-targeting of PEDV NTD-S1 enhanced the vaccine immunogenicity in pigs. First, we measured T cell immune responses against DC-targeted PEDV NTD-S1s in pigs. The peripheral blood mononuclear cells were isolated, stimulated *in vitro* with the recombinant NTD-S1 antigen, and stained for intracellular IFN-γ. Flow cytometry analyses showed that the $CD4^{pos}CD8^{pos}$ T cell subset was the only subset showing increased mean frequencies of IFN-γ producing cells in response to PEDV NTD-S1 **(Fig 2).** As early as 7 days after the prime vaccination in pigs, the antigen-specific CD4 T cell frequencies in the transdermal DC-targeted group were significantly higher (0.969±0.201, $p \leq 0.025$) than those in the non-targeted group (0.395±0.158) and in the intramuscular DC-targeted group (0.483±0.067) **(Fig 2B).** The CD4 T cell priming sustained in a proportion of pigs (2/8) in the transdermal DC-targeted group ($p=0.027$) until 28 days after prime vaccination **(Fig 2C).** At day 7 after booster vaccination, IFN-γ-specific CD4 T cell responses increased significantly in the transdermal DC-targeted group (0.391±0.107, $p=0.016$) as compared to the non-targeted group (0.108±0.052) **(Fig 2D)**. Our results indicate that transdermal delivery of Langerin-targeted PEDV S antigen significantly increased cellular responses in the $CD4^{pos}CD8^{pos}$ T cell compartment in pigs.

3.3. Effect of Langerin targeting of PEDV S antigen on humoral responses in pigs

We evaluated antibody responses against PEDV S antigen targeted to $Langerin^{pos}$ DCs. The first vaccine dose induced detectable levels of antigen-specific serum IgG but not IgA

starting at 21 days after vaccination in all vaccinated groups but not in the control group **(Fig 3)**. However, serum IgG levels specific to PEDV NTD-S1 increased considerably upon the second vaccine dose **(Fig 3A)**. Particularly, at day 7 after boosting, the mean IgG S/P ratio was significantly higher in the intramuscular DC-targeted group (1.372±0.039, p=0.037) than in the transdermal DC-targeted group (1.230±0.059) **(Fig 3A)**. The higher serum IgG levels in the intramuscular DC-targeted group sustained until 14 days after boosting (mean S/P ratio: 1.337±0.052, p=0.005) as compared to transdermal DC-targeted group (mean S/P ratio: 1.048±0.076) at the same time point **(Fig 3A)**. In contrast, substantial IgA levels were detected only from day 7 after the booster vaccination in all vaccinated groups but not in the control group. The mean IgA S/P ratio was much higher in the intramuscular DC-targeted group (0.346±0.118, p=0.001) than transdermal DC-targeted group (0.053±0.007) **(Fig 3B)**. However, there was no significant difference in both IgG and IgA antibody levels between the non-targeted and transdermal DC-targeted groups **(Fig 3A, 3B).** Our data suggest that pigs primed intramuscularly with DC-targeted PEDV NTD-S1 showed significantly higher IgG and IgA responses as compared to those primed through the transdermal route though these responses require an intramuscular booster vaccination.

3.4. Serum virus neutralizing antibody responses

We tested whether the serum antibodies induced against Langerin-targeted PEDV S antigen can neutralize the virus under *in vitro* conditions. The virus neutralizing antibodies were measured in serum samples by *in vitro* serum virus neutralizing test with PEDV CO/13 strain. Strong neutralizing antibody (Nab) responses were detected in all groups vaccinated with PEDV NTD-S1 at 7 days post-boosting, without significant differences, while the adjuvant control group did not show serum neutralizing activities **(Fig 5A)**. The mean PEDV-specific Nab titers

ranged between 32 and 64 in the groups vaccinated with PEDV NTD-S1 irrespective of DC targeting **(Fig 5A)**. There was a strong positive correlation between PEDV-specific virus neutralizing antibody and IgG levels in serum ($r = 0.88$, $p < 0.0001$) but there was no evidence of correlation between virus neutralizing antibody and IgA levels in serum ($r = 0.34$, $p = 0.062$) **(Fig 5B, 5C).** These results demonstrate convincingly that PEDV spike NTD-S1 induced a robust serum virus neutralizing antibody response in pigs, and the levels were not influenced by Langerin targeting of the antigen.

3.5. Gut IgA responses in vaccinated pigs

Finally, we examined the secretory levels of IgAs in pig feces at 35 days post-prime vaccination when the serum IgA responses were maximal in individual vaccination groups. There was no significant level of fecal IgAs in the intramuscular and the transdermal Langerin-targeted groups despite significant higher serum IgA levels were observed after booster vaccination **(Fig 4 and 3B)**. Similarly, no significant numbers of PEDV NTD-S1-specific IgA secreting cells were detected in ileal tissues of pigs in Langerin-targeted groups at necropsy (data not shown). These results imply that Langerin-targeting of PEDV NTD-S1 did not significantly induce secretory IgA responses in the gastro-intestinal tract of pigs regardless of the vaccination route.

4. Discussion

The ongoing PEDV outbreaks in the United States have devastated the swine industry and currently there is no fully-licensed PEDV vaccine in the United States. Modified live PEDV vaccines are available in Asia, but the efficacy of these vaccines is questionable as they do not completely prevent virus shedding and outbreaks of PED continue in Asia (de Arriba et al.,

2002; Oh et al., 2014). Therefore, more effective vaccines are needed to eradicate PEDV altogether from neonates, growing and adult pigs particularly in breeding herds. In this study, we developed a novel spike-based PEDV subunit vaccine candidate that was directly targeted to pig Langerin-expressing dendritic cells through a single chain antibody. This vaccine candidate enhanced the cellular immune responses, particularly in the $CD4^{pos}$ $CD8^{pos}$ T cell compartment if the vaccine was injected transdermally. This is important since traditional subunit protein-based vaccines typically do not induce strong cell-mediated immune responses. However, enhanced antibody predominantly IgG responses but reduced cellular immune responses were observed if the PEDV NTD-S1 was targeted to $Langerin^{pos}$ DCs in systemic sites in pigs. Our study shows that the "dendritic cell targeting" approaches could be incorporated into PEDV subunit vaccine developments to improve the efficacy of protein-based vaccines by modulating the cellular and humoral immune responses against PEDV.

Antibodies binding to Langerin on DCs are rapidly internalized and co-localized within intracellular antigen presenting compartments in those cells (Nfon et al., 2008; Valladeau et al., 2000). The strong affinity and high specificity of the mAb 3B3 toward porcine Langerin is ideal for inducing rapid internalization of Langerin molecules in porcine DCs. The 3B3scFv-based single chain recombinant antibody showed a binding capacity similar to the mAb 3B3 which might mean an added advantage of monocistronic expression.

Consistent with previous studies in mouse models (Idoyaga et al., 2008; Idoyaga et al., 2011), our study demonstrate that Langerin targeting of PEDV NTD-S1 in skin enhanced T cell immune responses, though restricted only to the $CD8^{pos}CD4^{pos}$ T cell compartment that provides both cytolytic and helper immune responses to fight viral infections in pigs (C. Chung, 2015; Gerner et al., 2009) by swine leukocyte antigen (SLA) class II restricted manner (Gerner et al.,

2009). The results of our study also indicate that the memory immune responses are indeed developed effectively in pigs due to Langerin-targeting of PEDV antigen as demonstrated by the increased frequencies of antigen-specific $CD4^{pos}$ $CD8^{pos}$ T cells which are considered as the effector memory T cells in pigs (Gerner et al., 2009). The overall magnitude of CD4 T cell responses against Langerin-targeted PEDV NTD-S1 was comparatively higher in pigs vaccinated by the transdermal route, when compared to the intramuscular route, which is possibly mediated by either pig $Langerin^{pos}$ dermal DCs or LCs or both (Marquet et al., 2014; Romani et al., 2012). This is consistent with reports that mouse $Langerin^{pos}$ dermal DCs are capable of inducing strong T cell immune responses in skin draining lymph nodes (Wang et al., 2008), while human LCs are capable of engulfing measles virus particles through Langerin molecules to present viral antigens to CD4 but not CD8 T cells (van der Vlist et al., 2011).

Interestingly, in our study, the antibody responses against Langerin-targeted antigen were influenced by the route of antigen delivery during priming. Our data suggest that IgG responses were slightly stronger against intramuscular administered targeted PEDV NTD-S1 when compared to transdermal administered antigen, however both groups showed this difference only after intramuscular boosting, which is quite contrary to the observed T cell responses after priming. This difference may be explained by the fact that pig $Langerin^{pos}$ DCs in systemic sites are a fraction of $CD11c^{pos}$ DCs which are classified as conventional type 2 DCs biased toward inducing Th2 responses (Bigley et al., 2015; Maisonnasse et al., 2015). Therefore, it is warranted to examine Th2 immune responses against porcine Langerin-targeted antigens in systemic sites in future studies. In addition, when compared to the transdermal vaccination, the intramuscular vaccination route made the antigen more readily accessible in mucosal lymphoid tissues producing significant IgA responses against PEDV NTD-S1. However, the significant

differences in antibody responses between the transdermal and the intramuscular vaccination were not recognizable at the serum neutralizing antibody levels at one week after booster vaccination.

Consistent with the previous study (Paudel et al., 2014), there was a positive and strong correlation between serum IgG levels specific to PEDV NTD-S1 and the serum virus neutralizing antibody levels further confirming that the spike protein is a determinant of PEDV neutralization (Song and Park, 2012). Unlike the murine immune system (Chang et al., 2008), there was no evidence of cross-talk between porcine skin and gut immune system through Langerin^{pos} DCs in the presence of cholera toxin, as we did not observe significant gut IgA responses against the PEDV NTD-S1 delivered transdermally.

The efficacies of DC-targeted vaccines have been extensively investigated in mouse and primate models, and have successfully advanced up to phase III clinical trials in humans as potential cancer therapeutic vaccines (Apostolopoulos et al., 2014). In this study, we demonstrate the immunogenicity of spike-based protein vaccines against PEDV can be manipulated toward better cellular immune responses if the vaccine antigen is targeted to Langerin^{pos} DCs in the pig skin. However, when the PEDV NTD-S1 is targeted to Langerin^{pos} DCs in pig systemic sites, enhanced humoral immune responses can be observed, however these responses require a booster dose. The protective efficacy of these enhanced immune responses against PEDV need to be investigated in the future specifically in the pregnant gilt/sow vaccination - piglet challenge model.

Conflict of interest

The authors declare that there is no conflict of interest. SQU and GL are employees of Elanco Biological R&D, Eli Lilly and Company.

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Figure legends

Figure 1. Characterization of porcine Langerin-specific antibodies. **(A)** Relative affinities of mouse monoclonal antibodies (mAb) toward Langerin as measured by thiocyanate elution ELISA. The affinity index ('a') is the measure of ammonium thiocyanate molar concentration that produced 50% decrease in the initial absorbance corresponding to the y-axis value 1.7 (dotted line). **(B)** Immunofluorescence analysis. CHO-K1 cells were transfected with plasmids expressing porcine Langerin or an irrelevant cellular receptor. After 48 h, cells were fixed, permeabilized and stained with indicated antibodies followed by corresponding fluorochrome-conjugated secondary antibodies. DAPI blue staining indicated the cell nucleus. 929.F3.01 is an anti-human Langerin rat mAb known to cross-react with porcine Langerin**. (C)** Schematic illustration of vaccine antigen constructs. pF_c-PEDVsAg and 3B3scFv-pF_c-PEDVsAg are the non-targeting and Langerin-targeting PEDV spike-based vaccine antigens. "IL-2 ss" is the signal sequence derived from human IL-2 gene. V_H and V_L represent the variable domains of heavy and light chains of the 3B3 mAb. NTD-S1 represents n-terminal domain together with the S1 domain of the PEDV spike protein. **(D)** SDS-PAGE analysis. Vaccine antigens were prepared from the large scale 293T transient transfections and resolved in 4 -20 % SDS-PAGE followed by Coomassie blue staining. **Lane 1:** pF_c-PEDVsAg; and **Lane 2:** 3B3scFv-pF_c-PEDVsAg. The molecular sizes were determined by protein ladder in a separate well.

Figure 2. T cell immune responses specific to Langerin-targeted PEDV vaccine antigen in pigs. **(A-D)** Flow cytometry analysis. Peripheral blood mononuclear cells were collected from vaccinated pigs at indicated timepoints, stimulated with PEDV NTD-S1 *in vitro*, stained for surface T cell receptors and intracellular IFN-γ. Data presented herein is for $CD8^{pos}CD4^{pos}$ T cell

compartment only as the percentage of CD3 gated T cell population. The frequencies of antigen-specific T cells were calculated by subtracting the values of unstimulated cells from that of stimulated cells. (t/d), transdermal; (i/m), intramuscular. Data on the y-axis represent arithmetic mean of antigen-specific T frequencies ± standard error of mean, $n \geq 7$. (*$p < 0.05$; **$p < 0.01$).

Figure 3. Serum antibody responses specific to a Langerin-targeted PEDV vaccine antigen in pigs. **(A)** IgG and **(B)** IgA ELISA analyses. Microplates were coated with PEDV NTD-S1 followed by blocking with non-fat milk. Diluted serum samples were incubated with coated antigen, and after several washings, the bound antibodies were detected with enzyme-conjugated secondary conjugates either anti-porcine IgG (**A**) or anti-porcine IgA (**B**) together with corresponding substrate. . The sample-to-positive (S/P) ratios were calculated as described in the methods section. Serum samples from PEDV negative pigs served as negative control while those collected from PEDV-infected pigs at 44 days post-infection served as positive control. Each sample was measured in duplicate. Data on the y-axis represent the arithmetic mean of S/P ratio values ± standard error of mean, $n \geq 7$. (*$p < 0.05$; **$p < 0.01$).

Figure 4. Serum virus neutralizing antibody responses specific to Langerin-targeted PEDV vaccine antigen in pigs. **(A)** Serum virus neutralization (SN) test on Vero cells. Heat-inactivated serum samples were mixed with PEDV CO/13 strain and incubated to allow for virus neutralization. Virus-serum mixtures were laid on Vero cell monolayer and incubated to allow for virus binding. Cells were washed three times with plain culture medium and incubated with serum-free maintenance medium containing trypsin for 5 days. Each sample was tested in triplicate and the SN titer was calculated as the geometric mean (GM) of titer values of each

sample. Data on the y-axis represent the geometric mean of SN titers ± standard error of mean, n ≥ 7 (n. s. non-significant). **(B and C)** Correlation analysis of serum neutralizing antibodies with serum IgG **(B)** or serum IgA **(C)** levels at 35 days post-prime vaccination. The Pearson correlation co-efficient (r) was calculated assuming Gaussian distribution of variables. A '*p*' value of less than 0.05 was considered significant to consider for a true correlation (n = 30).

Figure 5. IgA levels in feces of pigs at 35 days post-vaccination. Fecal samples were freeze-dried and resuspended in extraction buffer (PBS with Tween-20 and protease inhibitors). Fecal extracts were clarified by centrifugation and the supernatants were collected to measure fecal IgAs. The undiluted fecal extracts were incubated with PEDV NTD-S1 antigen coated on 96 well plates. After several washes, the bound antibodies were detected with horseradish peroxidase (HRP)-conjugated anti-porcine IgA secondary antibody followed by HRP substrate incubation. The enzyme reaction was stopped with HCl and the absorbance was measured at 450 nm in a microplate reader. Each sample was measured in duplicate. Data on the y-axis represent the arithmetic mean of the optical density at 450 nm ± standard error of mean, n ≥ 7.

Figure 1

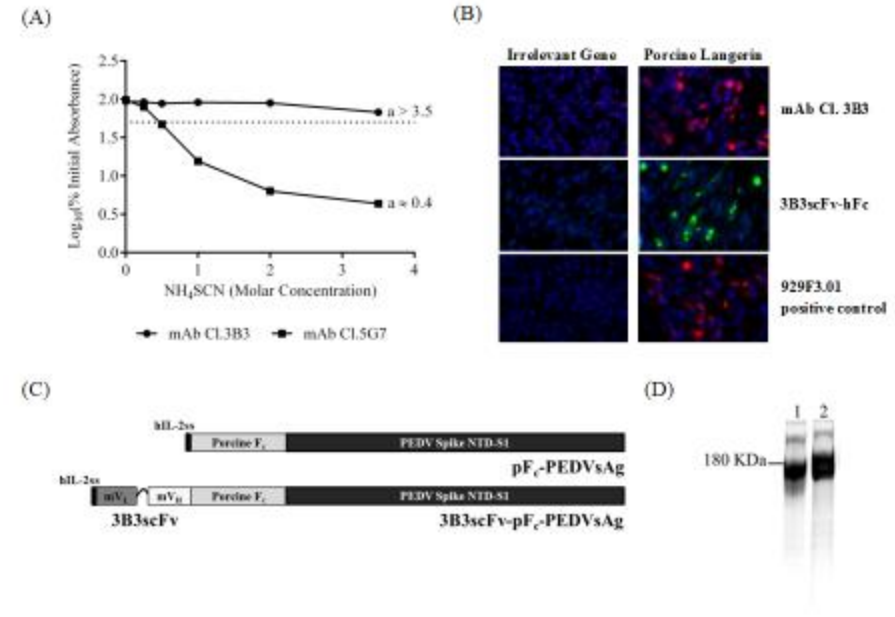

Figure 2

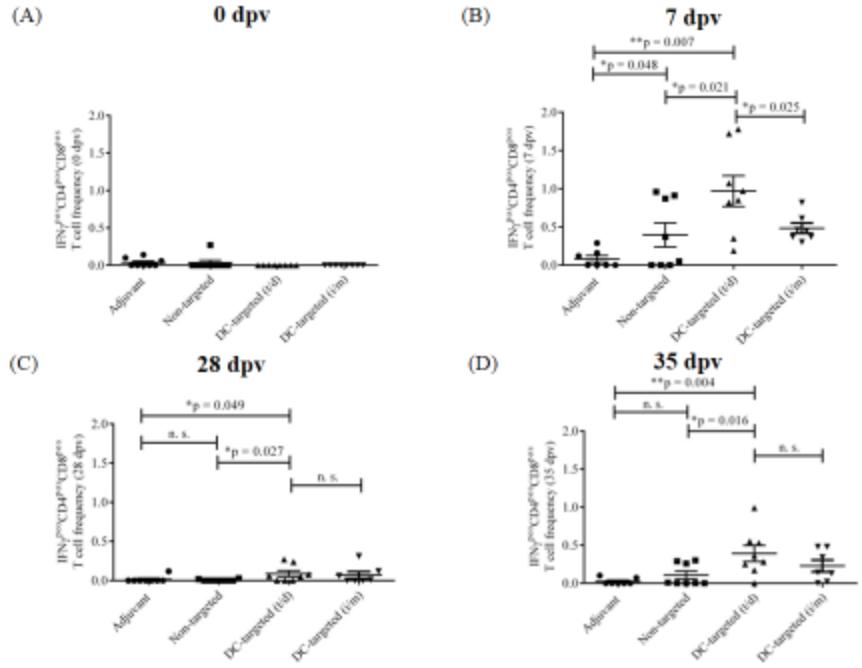

Figure 3

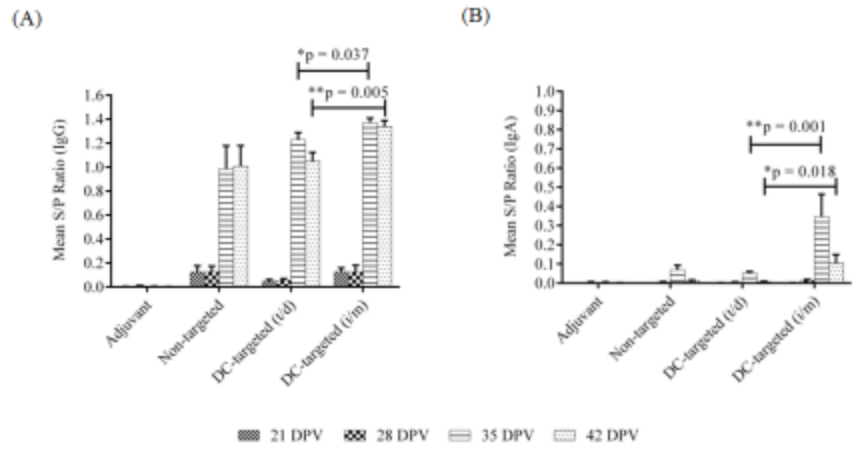

Figure 4

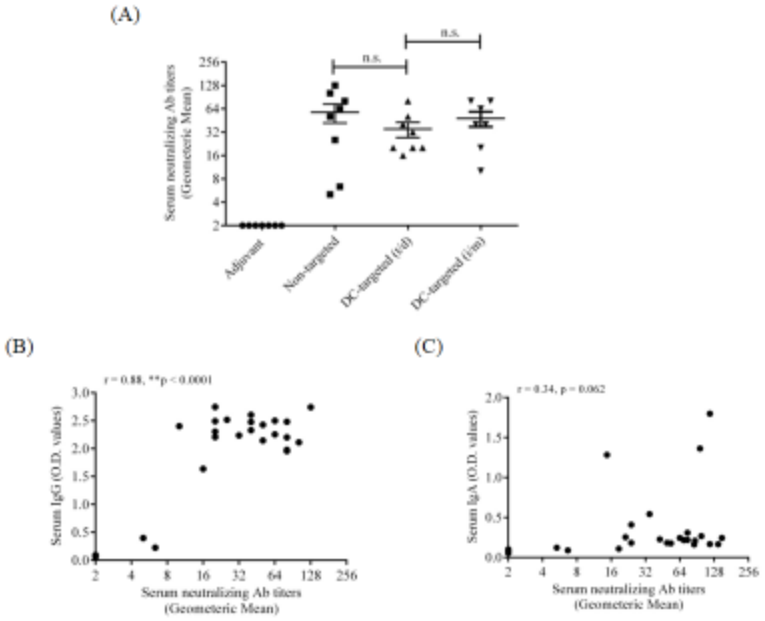

Figure 5

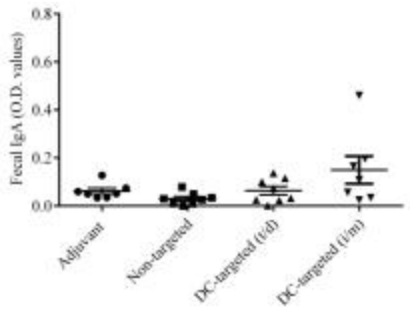

Table 1. Experimental design for the immunogenicity study in pigs of Langerin-targeted PEDV vaccine

No.	Treatment groups	Antigen dose	Adjuvant dose	Vaccine Volume	Prime route (single site)	Booster route (single site)	No. of pigs
1	Adjuvant Control	-	50μg	250μL	Transdermal	Intramuscular	8
2	Non-targeted PEDVsAg	200μg	50μg	250μL	Transdermal	Intramuscular	8
3	Langerin-targeted PEDVsAg	200μg	50μg	250μL	Transdermal	Intramuscular	8
4	Langerin-targeted PEDVsAg	200μg	50μg	250μL	Intramuscular	Intramuscular	7