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Research paper

Immune responses induced by inactivated porcine reproductive and respiratory syndrome virus (PRRSV) vaccine in neonatal pigs using different adjuvants

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ABSTRACT

Vaccination of neonatal pigs could be supportive to prevent porcine reproductive and respiratory syndrome virus (PRRSV), which is an important porcine pathogen causing worldwide welfare and health problems in pigs of different age classes. However, neonatal immunity substantially differs to adults, thus different vaccines may be required in neonateal pigs. We examined if the immunogenicity and efficacy of inactivated PRRSV (iPRRSV) vaccines in neonatal pigs could be improved with adjuvants containing oil-in water (O/W) emulsions with or without Toll-like receptor (TLR) agonists and by altering the delivery route from intramuscular (i.m.) to the skin. Three-day-old PRRSV-naïve piglets (n = 54, divided in 6 groups) received a prime vaccination and a booster vaccination four weeks later. The vaccine formulations consisted of different O/W emulsions (Montanide™ ISA28RVG (ISA28)), a squalene in water emulsion (SWE) for i.m. or a Stable Emulsion (SE) with squalene for skin vaccination) and/or a mixture of TLR1/2, 7/8 and 9 agonists (TLRa) combined with iPRRSV strain 07V063. These vaccines were delivered either i.m. (ISA28, SWE, TLRa or SWE + TLRa) or into the skin (skiSE + TLRa) with dissolving microneedle (DMN)-patches. All animals received a challenge with homologous PRRSV three weeks after booster vaccination. Specific antibodies, IFN-γ production and viremia were measured at several time-points after vaccination and/or challenge, while lung pathology was studied at necropsy. After booster vaccination, only ISA28 induced a specific antibody response while a specific T-cell IFN-γ response was generated in the SWE group, that was lower for ISA28, and absent in the other groups. This suggests that prime vaccination in neonates induced a specific immune response after booster vaccination, dependent on the emulsion formulation, but not dependent on the presence of the TLRa or delivery route. Despite the measured immune responses none of the vaccines showed any efficacy. Further research focused on the early immune response in draining lymph nodes is needed to elucidate the potential of TLR agonists in vaccines for neonatal pigs.

1. Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV) is occurring globally causing health and welfare problems with severe economic losses (Holtkamp et al., 2013; Nathues et al., 2017). This positive-stranded RNA virus of the *Arteriviridae* causes abortions in sows, respiratory diseases and increased susceptibility to other infections in nursery and fattening pigs. Modified-live PRRSV based vaccines induce weak immune responses, however lack of cross-protection and reduced safety, because of spreading the vaccine virus strain, are points of shortcomings (Renukaradhya et al., 2015). Therefore, inactivated PRRSV (iPRRSV) vaccines are preferred, however these iPRRSV vaccines

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induce little or no cellular and humoral immunity and induce only limited protection against infection (Geldhof et al., 2012; Vanhee et al., 2009; Zuckermann et al., 2007). Thus, there is a need for strong immuno-stimulators (adjuvants) to increase the efficacy of these iPRRSV vaccines (Charerntantanakul, 2009).

Vaccination of neonate pigs (< 1-week-old) could be an effective strategy to increase protection at an early age and thereby decrease the number of PRRSV infections in young piglets (Balasch et al., 2018; Jeong et al., 2018). New-born piglets depend heavily on their innate immune defence and need to develop their specific immune system after birth. The neonatal immune response in mammals is characterized by Th2 directed cytokine patterns and limited Th1 and cytotoxic T-cell responses (Kumar and Bhat, 2016; Levy, 2007; Saso and Kampmann, 2017), combined with limited germinal centre B-cell and plasma-cell responses (Kollmann et al., 2009; Siegrist and Aspinall, 2009). Furthermore, milk-derived maternal antibodies (Poonsuk and Zimmerman, 2017), could interfere with the development of an active immune response in different ways (Chappuis, 1998; Siegrist, 2003; Zinkernagel, 2003). All these aspects could complicate iPRRSV vaccination of neonatal pigs. New adjuvants and adjuvant formulations could enhance the immunogenicity of inactivated vaccines, but must be suitable for the neonatal immune system (Kollmann and Marchant, 2016; Mohr and Siegrist, 2016).

Toll-like receptor (TLR) agonists and combination of TLR agonists with oil-in water (O/W) emulsions (de Brito et al., 2009), have shown to increase the Th1 directed immune response to improve vaccine efficacy in neonates (Morris and Surendran, 2016) and adults (Maisonneuve et al., 2014). *In vitro* stimulation of porcine PBMCs with various TLR agonists demonstrated that TLR1/2, 7/8 and 9 agonists induced activation of antigen presenting cells (APCs), including dendritic cells (DCs), monocytes and B-cells in adult pigs (Auray et al., 2016; Braun et al., 2017) and TLR1/2 and 9 agonists activated APCs in neonatal pigs (Vreman et al., 2018). In our previous vaccine study in 6-week-old pigs (Vreman et al., 2019), the iPRRSV vaccine was combined with individual TLR1/2, 7/8 or 9 agonists as adjuvant. In that study, the individual TLR agonists did not generate a PRRSV-specific immune response when the vaccines were applied i.m. or by skin vaccination, however the O/W emulsion Montanide™ ISA28R VG (ISA28) induced humoral and cellular immunity and reduction of viremia. Thus, we selected ISA28 as reference O/W emulsion for this neonatal iPRSSV study. An O/W emulsion (SWE) combined with a mixture of TLR1/2, 7/8 and 9 agonists (TLRa) has been shown to elicit a clear Th1 and innate immune response in adult pigs (Matthijs et al., 2019) and therefore we hypothesised that this may be a candidate adjuvant for neonatal pigs.

Next to the antigen and the choice of adjuvant, the route of vaccine delivery can be important to induce an effective immune response (McKay et al., 2016; van Aalst et al., 2018). The skin contains a large amount of APCs, which could be directly activated with skin vaccination to transport the antigen to the draining lymph node. This may result in an equal or enhanced immunological effectiveness compared to traditional i.m. delivery (Ferrari et al., 2013; Vrdoljak et al., 2016; Wang et al., 2016). Other suggested advantages of skin vaccination are dose-sparing (Eble et al., 2009), needle-free application (Marshall et al., 2016) and the induction of mucosal immune responses, which is preferred for respiratory diseases like PRRSV (Le Luduec et al., 2016; Martelli et al., 2014).

In this study we assessed *in vitro* the cytokine production of porcine neonatal PBMCs that were stimulated with the individual TLR1/2 (Pam3Cys), 7/8 (R848) or 9 (CpG) agonists or the TLRa mixture. We then examined the immune responses and the protection after challenge in 3-day-old PRRSV-naïve piglets that were vaccinated with iPRRSV vaccines. The vaccines contained different adjuvant formulations (O/W emulsion with or without TLRa) and were applied i.m. and through skin vaccination to overcome the potential weak immune response of neonatal pigs.

2. Materials and methods

2.1. Animal procedures and ethics

All scientific procedures on animals in this study were executed according to the Dutch animal experimental and ethical requirements and the Dutch Central Authority for Scientific Procedures on Animals (CCD) has approved the project license application under Permit number: ADV401002015356.

2.2. PBMC isolation and stimulation with Toll-like receptor agonists in neonate pigs

Heparin stabilized blood was collected from four (n = 4) three-day-old male piglets. Stabilized blood was diluted 1:1 with PBS within 2 h after sampling and added to a Leucosep™ tube containing a 60 % FICOLL-PAQUE™ Plus density- gradient for PBMC isolation. Remaining red blood cells, which are often present after PBMC isolation in neonatal porcine blood, were lysed with Gibco™ ACK lysing buffer. Cells were inserted in 96-well plates with 0.5×10^6 cells/well in RPMI 1640 medium (Gibco®) with 10 % foetal bovine serum. After 1 h of incubation, cells were stimulated with the same TLR agonists as used in the vaccine adjuvant: TLR1/2 agonist (10 μg/mL Pam3Cys L2000 from EMC microcollections), TLR7/8 agonist (5 μg/mL R848, Resiquimod from InvivoGen), TLR9 agonist (5 μg/mL CpG ODN-type A sequence D32, 5′-ggTGCGTCGACGCAGggggg-3′, from Eurofins Genomics.), the TLR1/2a, 7/8 and 9 agonist mixture (with 10 μg/mL, 5 μg/mL and 5 μg/mL, respectively), the TLR1/2 and 9 agonist mixture (10 μg/mL and 5 μg/mL, respectively) or cells were left unstimulated as negative control.

Due to limited amount of neonatal blood and subsequently isolated PBMC, we were restricted in the number of TLR agonist combinations. We decided to select the single TLR agonists, the TLR1/2 + TLR7/8 + TLR9 combination, which was recently used in an adult vaccine study (Matthijs et al., 2019) and the TLR1/2 + TLR9 combination, which we used in a previous *in vitro* study comparing neonatal and adult blood (Vreman et al., 2018).

2.3. In vitro cytokine production measured by multiplex immunoassay

PBMCs were stimulated for 24 h and supernatants of PBMC cultures were collected and frozen at −80 °C until analysis within 2 months after collection. Cytokine protein were measured with a multiplex Cytometric Bead Array (Cytokine & chemokine 9-Plex Porcine ProcartaPlex® Multiplex Immunoassay from eBioscience) according to the manufacturer's instructions and read on a Luminex®200™ (Luminex Corporation). Cytokine concentrations were determined using xPONENT® software (Luminex Corporation). The detection limits of the measured cytokines were 0.72 pg/mL (IFN-α), 4.96 pg/mL (IFN-γ), 7.57 pg/mL (TNF), 8.20 pg/mL (IL-10), 35 pg/mL (IL-12p40), 3.54 pg/mL (IL-1β), 1.55 pg/mL (IL-4), 6.32 pg/mL (IL-6) and 12 pg/mL (IL-8).

2.4. Vaccines

All the vaccines (i.m. and DMN-patches) contained the same dose of binary ethylenimine (BEI)-inactivated PRRSV 07V063 ($1.0 \times 10^8 TCID_{50}$) and were formulated with different adjuvants (Table 1). The iPRRSV-antigen was manufactured as described previously (Vreman et al., 2019). Within the study we used three different O/W emulsions. Commercial available Montanide™ ISA 28R VG (kindly provided by SEPPIC), an O/W emulsion containing a combination of a mineral and non-mineral oil, was applied i.m. (ISA28). Two different O/W emulsion with squalene both with a similar chemical composition, however with different quantities and based on a different manufacturing process. The squalene based emulsion (SWE), used for the i.m vaccination, is developed and produced by the Vaccine Formulation Laboratory, and composed of 3.9 % weight per volume (w/v)

Table 1
Experimental design: vaccine formulations, administration route and treatment days.

Group [n = 9]	Vaccine formulation[1]	Adjuvant		Antigen[2] [yes/no]	Delivery route/ Dose (ml)	Prime/boost/challenge /necropsy [study day]
		TLR agonist	O/W emulsion			
1	**Non Vaccinated (NV)**	– PBS	–PBS	No PBS	i.m/1.0 mL	0/28/49/70
2	**ISA28**	–	Montanide™ ISA 28R VG 15 % v/v	Yes	i.m/1.0 mL	0/28/49/70
3	**SWE**	–	Squalene based emulsion (SWE) 42 % v/v	Yes	i.m/1.0 mL	0/28/49/70
4	**TLRa**	80ug Pam3Cys 80ug R848 80ug CpG	–	Yes	i.m/1.0 mL	0/28/np /49
5	**SWE + TLRa**	80ug Pam3Cys 80ug R848 80ug CpG	Squalene based emulsion (SWE) 42 % v/v	Yes	i.m/1.0 mL	0/28/49/70
6	**skiSE + TLRa**	80ug Pam3Cys 80ug R848 80ug CpG	Stable emulsion (SE) 29 % v/v	Yes	Skin DMN-patch 0.2 mL	0/28/49/70

Abbreviations: oil-in-water (O/W emulsion); volume by volume (v/v); Toll-like receptor agonist mixture (TLRa); intramuscular (i.m.); skin vaccination (ski); dissolving microneedle (DMN)-patch; not performed (np).
[1] Described based on used adjuvant.
[2] Type and dose of used antigen: iPRRSV 07V063 10^8TCID$_{50}$.

squalene, 0.5 % (w/v) Tween 80 and 0.5 % (w/v) Span 85) (Ventura et al., 2013). The O/W stable emulsion (SE) with squalene used in the DMN-patch was made according to previously described methods (Shah et al., 2015) and is composed of 3.5 % volume per volume (v/v) squalene, 1% (v/v) Tween 80 and 0.5 % (v/v) Span 85).

Three groups were i.m immunised with an O/W emulsion with or without TLR agonists mixture and iPRRSV. The ISA28 group received a solution containing 15 % volume per volume (v/v) Montanide™ ISA 28R VG with iPRRSV. The SWE group received iPRRSV in combination with 42 % (v/v) SWE. The third group received SWE with the three TLRa by the i.m. route (SWE + TLRa). For this vaccine 42 % (v/v) SWE was mixed with 80 μg of each of the three TLR agonists: Pam3Cys, R848, Resiquimod and CpG ODN-type A (25 % (v/v) for the TLRa). Alternatively, animals received iPRRSV with the TLR agonist mixture only (TLRa).

For the skin vaccination group (skiSE + TLRa), the formulation used to make the DMN patches contained 29 % (v/v) of SE, 14 % (v/v) trehalose, 1.25 % (v/v) polyvinyl alcohol (PVA), along with iPRRSV and the same dose of TLRa as described above. One dose of vaccine was administered in 2 patches and each patch contained 120 microneedles and was 8cm^2 in area (240 microneedles in total). Patches were fabricated as described previously (Vreman et al., 2019).

2.5. Animals and housing

For this study fifty-four (n = 54) male three-day-old piglets (Topigs Norsvin Z-line, commercial breed) were purchased from a PRRSV-negative (confirmed with a commercial antibody ELISA) high health status farm (van Beek SPF Varkens B.V., the Netherlands). The piglets received colostrum from the sow and were weaned at 1–2 days of age. After weaning, the piglets were stratified based on their weight and family background (pigs were from 8 different sows) followed by a randomisation to six groups (n = 9 for each group). The different groups were housed in separate stables in an isolation unit with HEPA filtered air. Stables were enriched with straw and different toys.

2.6. Experimental design vaccination experiment and sampling

All the pigs except for the non-vaccinated (NV) group received a prime vaccination at 3-days of age (D0) followed by a booster vaccination (D28) at 4.5-weeks of age (groups are described in Table 1). The NV animals received 1.0 mL of PBS i.m. All i.m vaccines (1.0 mL) were administered in the lateral side of the right hind-leg, whereas the DMN-patches were applied at the medial side of both hind legs. The DMN-patches were removed after 24 h.

Three weeks after the booster vaccination (D49; 7.5 weeks of age) the animals from groups 1, 2, 3, 5 and 6 received an intranasal challenge with PRRSV 07V063 (10^5 TCID$_{50}$) in PBS (1.0 mL per nostril). The challenge virus was prepared as described previously (Vreman et al., 2019). At this time point, the pigs from the TLRa group were dissected and lungs were used as reference representing non-PRRSV infected lungs. Three weeks post challenge (D70) the pigs of groups 1, 2, 3, 5 and 6 were dissected. as previously described (Vreman et al., 2019).

Serum was sampled at D21, D28 (post-prime), D35, D42, D48 (post-boost), D52, D54, D57, D59, D63, D66 and D70 (post-challenge) to determine antibody responses and virus titres. Heparin stabilized blood samples (approximately 15 mL) were obtained at D21, D42, D56 and D63 for IFN-γ ELISpot assay or flow cytometry (FCM).

2.7. Monitoring of post-vaccination and challenge reaction

After vaccination the injection site or DMN-patch application area was monitored over 4 days for local effects. For skin vaccination we evaluated redness and swelling of skin, graded from 0 to 3 (no changes, mild, moderate or severe changes) for each hind leg (maximum total score of 6). For the i.m. vaccination we palpated and observed the injection site for increased consistency, redness and local temperature (grade 0–3). Around vaccination and challenge the body temperature and clinical signs were monitored as described before (Vreman et al., 2019).

2.8. PRRSV-specific immune responses

PRRSV-specific IgG antibodies in serum samples were assessed with an indirect antibody ELISA (Ingezim PRRS 2.0) according to the manufacturer's instruction. A sample-to-positive (S/P) ratio of equal or greater than 0.4 was considered positive.

The specific cellular immune response was evaluated with an enzyme-linked immunospot assay (ELISpot assay) kit (Porcine IFN-γ ELISpot PLUS (ALP) from Mabtech) (Mateu de Antonio et al., 1998). PBMC were stimulated with iPRRSV with a MOI of 0.1 for 24 h as described before (Vreman et al., 2019). Next to the ELISpot, we analysed the specific cellular responses against PRRSV in PBMCs by FCM. On D21, D42 and D56 percentages of intracellular TNF or IFN-γ staining cells

were determined in T-cell subsets and NK-cells. On D56 and D63, unstimulated PBMCs were analysed for relative proportions of T-cell subsets, NK-cells and B-cells within the total PBMC population. For the intracellular staining, PBMC were re-stimulated with iPRRSV, as described for the ELISpot. For the last 4 h of stimulation, we added Brefeldin A (BD Bioscience) to each well to inhibit cytokine release and allow intra-cellular detection of cytokines. As positive control we added leucocyte activation cocktail (containing ionomycin and phorbol-12-myristate-13-acetate (PMA)) with BD GolgiPlug™ (BD Biosciences) according to manufacturer's instruction and as negative control we left samples unstimulated with Brefeldin A. Cells were then harvested and the cytokine-production of T-cell subsets and NK-cells was determined using a 4-step 6-colour staining protocol. Cells were first incubated with the BD Horizon™ Fixable Viability Stain 450 (FVS450 from BD Biosciences) according to the manufacturer's instructions. The cells were then incubated with directly labelled PE-Cy™7 mouse anti-pig CD3ε (clone BB23-8E6-8C8 from BD Biosciences), PerCP-Cy™5.5 mouse anti-pig CD4a (clone 74-12-4 from BD Biosciences) and Alexa Fluor 647® anti-CD8α (clone PG164A, WSU, Pullman, WA, USA) antibodies. Following surface staining, cells were fixed in 4 % paraformaldehyde and after a wash with 0.1 % saponin (Panreac Applichem), cells were incubated with directly labelled Alexa Fluor 647® anti-human TNF-α (clone MAb11 from BioLegend), PE mouse anti-pig IFN-γ (clone P2G10 from BD Biosciences) in 0.3 % saponin followed by another 0.1 % saponin wash. On D56 and D73 unstimulated PBMCs were stained directly after PBMC isolation with the same surface markers as described for the stimulated PBMCs and directly labelled Alexa Fluor 647® mouse anti-pig CD21 (clone BB6-11C9.6 from SouthernBiotech) was added as additional surface marker for B-cells.

PBMCs were analysed on a FACSVERSE™ (BD Biosciences) using the BD FACSsuite™ software. The flow cytometry data were analysed with Flowjo™ software version 10.0. The gating strategy is depicted in the supplementary data (Supp. Fig. 3). First doublets and dead cells were excluded and cells were gated for PBMCs. After this cells were classified by expression of the following combinations of surface markers (Gerner et al., 2015; Sinkora et al., 2013): T cells ($CD3^+$), T helper (Th) cells ($CD3^+CD4^+$), Ag-experienced T-cells (Tm) ($CD3^+CD4^+CD8\alpha^+$), cytotoxic T cells (Tcyto) ($CD3^+CD4^-CD8\alpha^+$), NK-cells ($CD3^-CD8\alpha^+$) and B-cells ($CD3^-CD21^+$) and this classification is used throughout this study. The proportion of the different subpopulations was measured as percentage (relative level) within the live PBMC population. For the Th, Tm, Tcyto and NK cells, we gated the percentage of TNF or IFN-γ positive cells in these specific populations.

2.9. PRRSV viremia and lung pathology

Virus titres in serum ($TCID_{50}$) were determined by virus titration as described before (Vreman et al., 2019). Briefly, porcine alveolar macrophages (PAM) were cultured and ten-fold dilution series of the serum samples (six dilutions for each sample) were added to the PAM, after 3 days the monolayers were stained by immune peroxidase monolayer assay (IPMA) to visualize infection in the cells.

PRRSV-associated lung pathology was assessed by a veterinary pathologist (SV) as described previously (Vreman et al., 2019). Briefly, macroscopic lung lesions were scored to estimate the percentage of affected lung tissue. For histology, hematoxylin and eosin (H&E) stained lung tissues (three sections per lung) were scored for the presence of perivascular and peribronchiolar inflammatory infiltrate, and for the alveolar wall infiltrate from 0 (no findings) to 5 (extended manifestation).

2.10. Statistical analysis

GraphPad Prism 8.1.1. was used for statistical analysis. The distribution of the data was investigated with descriptive statistics. Normal distributed data (*in vitro* cytokines, ELISA, FCM, viremia, body temperature and FCM-TNF,) were analysed with a one-way ANOVA followed by a Tukey's post hoc correction for multiple comparisons. Non-parametric data (ELISpot, skin changes, clinical signs and lung pathology,) were analysed with a Kruskal-Wallis test followed by a post-hoc Dunn's multiple comparisons test. P- values less than 0.05 were considered statistical significant and $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$ denotes a significant difference of a vaccine formulation compared to the NV animals. Vaccine groups displaying different letters (a, b and c) show a significant difference between these groups.

3. Results

3.1. In vitro cytokine responses after TLR stimulation in neonate PBMCs

Single TLR agonists (TLR1/2, 7/8 or 9) and TLR agonist combinations (TLR1/2 + 9 and TLR1/2 + 7/8 + 9) were used to stimulate neonatal PBMCs to determine their *in vitro* potency to elicit cytokine responses. IFN-γ and IL12p40 were mainly produced after stimulation of TLR7/8 and 9 and after stimulation with both combinations (TLR1/2 + 9 and TLR1/2 + 7/8 + 9), where high levels of IL-12p40, but lower levels of IFN-γ were observed compared to unstimulated control samples (Fig. 1A and B). High levels of IFN-α were only observed after stimulation of TLR9 and the TLR1/2 + 9 combination. Surprisingly, the response diminished when TLR7/8 was added to the TLR9 stimulation (Fig. 1C).

The overall IL-4 response was low but significantly increased for all stimuli, except for stimulation of TLR1/2 (Fig. 1D). Significantly elevated levels of IFN-γ, IL-12p40, IL-4, IL-1β, IL-6 and IL-10 were induced when TLR7/8 was present (Fig. 1A, B, D–G) compared to the unstimulated control samples. Notably, only TLR7/8-containing stimuli induced significant increases in IL-1β, IL-6 and IL-10. In contrast, the TLR1/2 agonist, Pam3Cys, was the least potent of the three TLRa to induce this panel of cytokines. Elevated levels for IL-8 and TNF were observed with a mixed pattern in all groups, albeit not significant (Fig. 1H and I). Overall, the TLR1/2 + 7/8 + 9 agonist combination stimulated neonateal PBMC to produce significant levels of IFN- γ, IL-12p40, IL-4, IL-1β, IL-6 and IL-10 compared to unstimulated control samples and the TLR1/2 agonist showed minimal potential to induce cytokine production compared to the TLR7/8 and 9 agonists.

3.2. Local vaccine responses and challenge reaction

After prime and booster vaccination, none of the tested vaccine formulations induced any systemic adverse effects, such as raise in body temperature, loss of appetite and activity, or reduced weight gain (data not shown). A mild local skin reaction (grade 1) was observed in most of the animals after prime DMN-patch application, characterized by a variable local redness (Supp. Fig. 1A and D). However, after the booster vaccination (4.5 weeks of age) we observed in nearly all animals significantly more skin reaction (grade 2 and 3) (Supp. Fig. 1B and D) than after prime vaccination. This moderate to severe skin reaction was still visible 4 days after vaccination (Supp. Fig. 1C), but completely disappeared two weeks after booster vaccination. No local reaction was observed after i.m. administration, as observed by palpation or macroscopic muscle changes during necropsy.

After challenge (from 3 to 11 days post infection (dpi)) there was a raise in body temperature in all the groups. This increased body temperature appeared to be related to PRRSV-induced clinical signs. The main observed signs were loss of appetite, reduced liveliness and a few animals were coughing or showed skin changes of the ears. There was no significant difference between the various vaccine groups, or between the vaccine groups and NV group regarding to body temperature or PRRSV-related clinical symptoms (Supp. Fig. 2A and B). At the time of the necropsy (21 dpi), none of the pigs displayed any clinical symptoms.

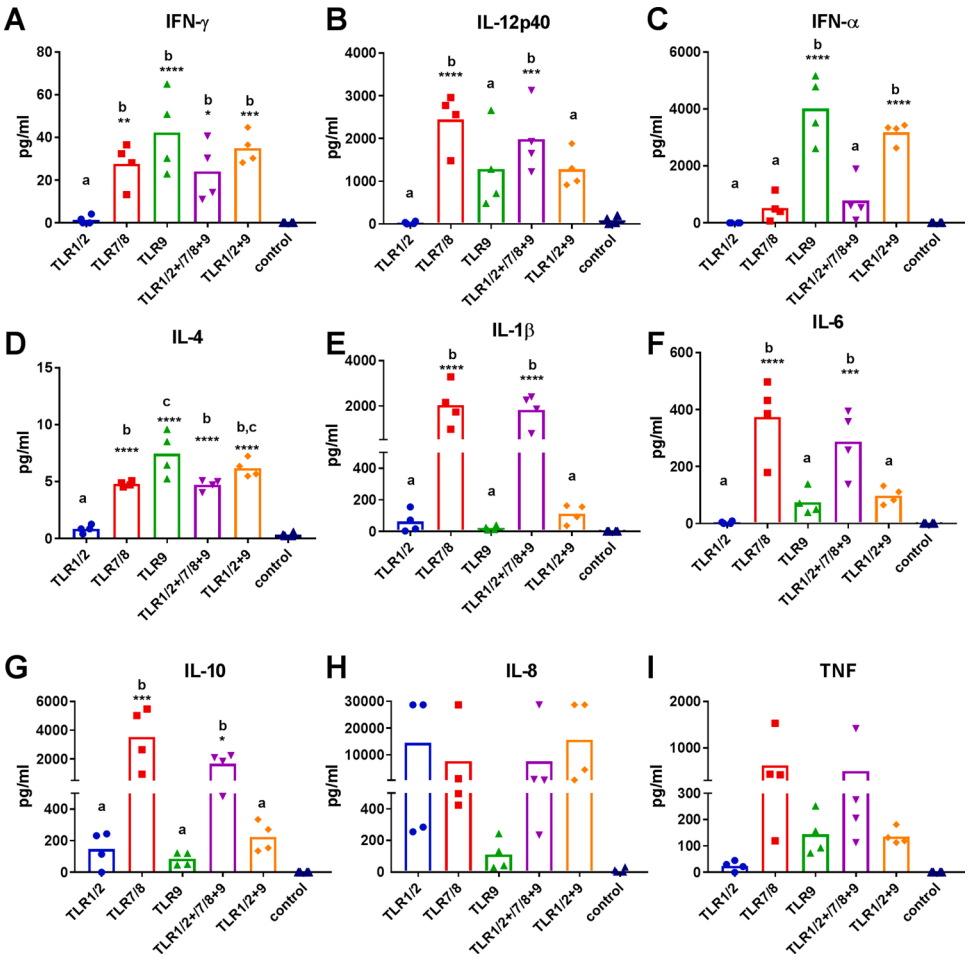

Fig. 1. Cytokine response after TLR agonist stimulations in neonatal pigs. PBMCs of 3-day-old pigs (n = 4) were stimulated with different Toll-like receptor agonists (TLR1/2, TLR7/8 or TLR9) and combinations of TLR agonists (TLR1/2 + 7/8 + 9 and TLR1/2 + 9). Supernatants were tested for (A) IFN-γ, (B) IL-12p40, (C) IFN-α, (D) IL-4, (E) IL-1β, (F) IL-6, (G) IL-10, (H) IL-8 and (I) TNF by Luminex multiplex. Each symbol represents one animal and the mean of the group is indicated by the bar.

There were no significant differences in weight or weight gain between the groups after challenge (results not shown).

3.3. PRRSV-specific humoral immune response

PRRSV-specific IgG antibodies were analysed in the serum to investigate the specific humoral immune response. Two weeks after booster vaccination (D42) only the ISA28 group showed a detectable PRRSV antibody response (S/P > 0.4) in 4 of the 9 animals (Fig. 2A), while none of the animals in the other vaccine groups developed a detectable specific antibody response before challenge.

From ten days after challenge (D59 or 10 dpi) onwards all vaccinated groups developed a PRRSV-specific antibody response, which was significantly higher on D59 compared to the NV animals. The ISA28 group showed a significantly higher specific antibody response compared to the other vaccine groups. From D63 (14 dpi) also the NV animals showed a specific antibody response (Fig. 2A).

The area under the curve (AUC) value, representing the total antibody response after challenge (D52-D70) (Fig. 2B), was significantly higher for all vaccinated groups compared to the NV group. However, a significant higher specific antibody response was found for ISA28 as compared to the other adjuvant groups.

3.4. PRRSV-specific cellular immune response (ELISpot)

The cellular immune response was evaluated by the number of IFN-γ secreting cell (SCs) in the PBMCs after re-stimulation with the challenge strain. There was no detectable increase in the number of IFN-γ SCs three weeks after prime vaccination (D21) in any of the measured groups (NV, ISA28 and SWE + TLRa) (results not shown).

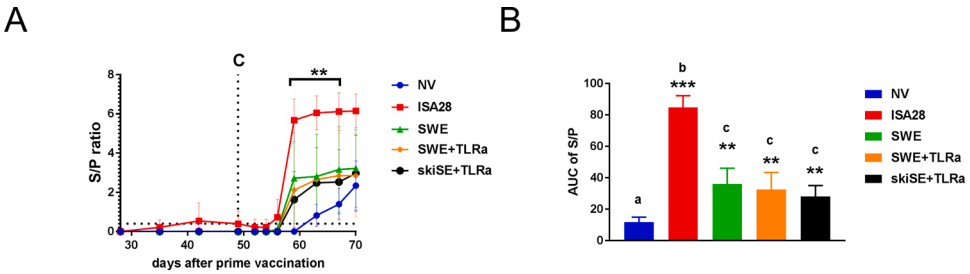

Fig. 2. Kinetics of PRRSV-specific antibody response after booster vaccination and challenge. PRRSV antibody titres were measured with ELISA. (A) PRRSV-specific IgG titres after booster vaccination (D28), in non-vaccinated (NV) or iPRRSV vaccinated animals using different adjuvants (ISA29, SWE, TLRa, SWE + TLRa) and skin vaccination (skiSE + TLRa); each data point represents the mean of 9 animals ± SD; the dotted line marks the limit of detection for the S/P ratio; C indicates challenge at D49; (B) Area under the curve (AUC) for specific antibody production after challenge.

Two weeks after booster vaccination (D42) the SWE group showed a significant increase in antigen-specific IFN-γ SCs. This antigen-specific cellular response was lower (although not significant) in the ISA28 group, but absent in the other groups (Fig. 3A). One week after challenge (D56) all vaccine groups and the NV group had large numbers of antigen-specific IFN-γ SCs, however there was no significant difference between the NV group and the vaccine groups (Fig. 3B).

3.5. Lymphocyte responses measured by flow cytometry

We also evaluated the cellular immune response after vaccination and challenge by the percentage of positive IFN-γ or TNF staining cells in the different T-cell subsets (Th, Tm, Tcyto) and NK-cells within *in vitro* re-stimulated PBMCs. Three weeks after the prime vaccination (D21) we detected no significant intracellular staining for TNF and IFN-γ after PRRSV stimulation in the different T-cell subsets and NK-cells in any of the measured groups (NV, ISA28 and SWE + TLRa) (results not shown). Two weeks after booster vaccination (D42) a significant increase in the percentage of IFN-γ positive cells after PRRSV stimulation in all T-cell subsets and NK-cells was observed in the ISA28 and SWE groups compared to NV animals. No response was observed in the other groups (Fig. 4A). The overall percentage of cells with TNF staining (average < 0.05 %) was significantly lower than for the IFN-γ staining (average 2.0–5.0 %). Only the Th and Tm subsets in PBMCs of pigs from the ISA28 and SWE groups showed a significant increase in the percentage of TNF positive cells compared to the NV group (Supp. Fig. 4A).

Seven days after challenge (D56) all of the vaccinated animals showed a significantly lower percentage of IFN-γ positive cells after PRRSV stimulation in the Th, Tm and Tcyto subsets compared to the NV animals (Fig. 4B). This reduction was not significant for the NK-cells. No significant intracellular TNF responses were observed in any of the groups at seven days after challenge. (Supp. Fig.4B).

We also analysed the percentages (relative level) of the different T-cell subsets (Tm and Tcyto), NK-cell and B-cells within unstimulated PBMCs 7 and 13 days after challenge (Fig. 4C). The ISA28 group was the only group that showed a significant increase in the percentage of B-cells between 7 and 13 days after challenge (Fig. 4C). The percentage of Tm cells significantly increased between 7 and 13 days after challenge in the ISA28, SWE and SWE + TLRa groups. Tcyto cells significantly increased in the ISA28 and SWE groups. None of the vaccine groups showed a significant increase in the percentage of NK-cells within the PBMCs between 7 and 13 days after challenge.

3.6. PRRSV viremia

Before challenge we detected no PRRSV in the serum as measured by virus titration (Fig. 5A). The TLRa group was not challenged with PRRSV and was used as reference for non-PRRSV infected lungs. At D52 (3 dpi) PRRSV was detected in the serum of 88 % of the animals and at 5 dpi all challenged animals were viremic. From 10 dpi onwards the virus titre declined in all the groups. None of the vaccinated groups showed a reduced viremia compared to the NV group as measured on different time-points after challenge or determined as AUC (Fig. 5A and B). At the end of the study 21 dpi (D70) 95 % of the animals were still viremic.

3.7. PRRSV induced lung pathology

The lungs of infected pigs showed mild to moderate macroscopic pathological changes 21 days after challenge in all groups, but there were no significant differences between the NV animals and vaccinated animals (Supp. Fig. 5A). No macroscopic lesions were observed in the unchallenged TLRa group. Individual lesion extension ranged from 0 % to 15 % of affected lung surface. The macroscopic changes were mainly observed in the cranial and the middle lung lobe and were characterized by multifocal, irregular, slightly sunken red to tan areas.

There was no significant difference in severity of the lung histopathology between the vaccine groups and NV groups (Supp. Fig. 5B, C and D). The lungs of the unchallenged pigs from the TLRa group displayed no significant changes related to PRRSV-infection (Supp. Fig. 5E). The lungs of all PRRSV challenged pigs showed mild to moderate interstitial pneumonia characterized by a mononuclear infiltrate of mainly macrophages and lymphocytes in the alveolar septa and around the blood vessels and bronchi and bronchioles. Dispersed alveolar lumina were occluded by the expanded alveolar walls often combined with a similar mononuclear infiltrate occasionally admixed with cellular debris (Supp. Fig. 5F and G).

4. Discussion

Efficacy of inactivated and subunit vaccines strongly depends on the immuno-stimulating properties of the selected adjuvant in combination with the specific antigen and the age of the vaccine recipient (Leroux-Roels, 2010). Porcine neonates can be vaccinated effectively since studies with inactivated vaccines (O'Neill et al., 2011; Wang et al., 2016) showed specific cellular and humoral immune responses combined with partial or full protection after challenge, although these studies used different adjuvants and antigens. As the immunogenic potential of the iPRRSV is minimal, even in studies with adult pigs (Geldhof et al., 2012; Vanhee et al., 2009), there is a strong immuno-stimulator needed for neonatal vaccines. Here, we investigated if vaccinated neonatal pigs developed a protective immune response with the weak iPRRSV antigen using specific adjuvants and different delivery routes. Porcine *in vitro* studies have shown that neonates can elicit an adult-like DC responses after effective stimulation with TLR1/2, 7/8 and 9 agonists (Auray et al., 2013; Vreman et al., 2018).This potentially contributes to increased antigen-presentation and subsequent differentiation of T-cells (Medzhitov, 2001) and thereby increasing vaccine efficacy. A recent porcine study has used these TLR1/2, 7/8 and 9 agonists in a mixture in combined with O/W emulsion (SWE) where this adjuvant formulation induced Th1 and innate immune response, although in adult pigs and

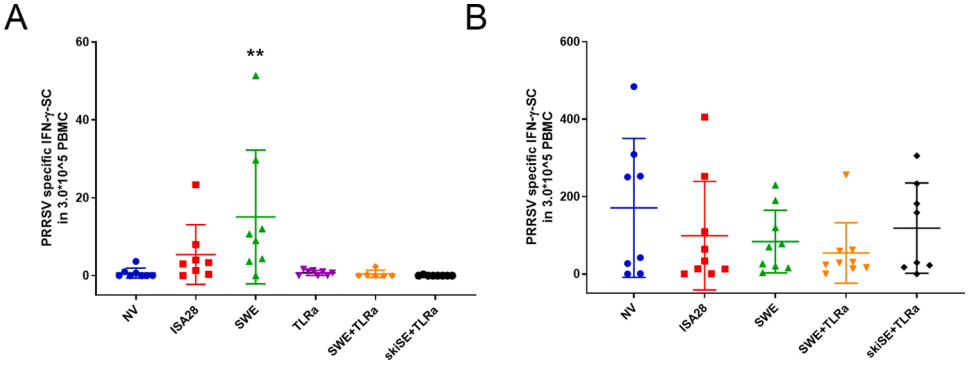

Fig. 3. PRRSV-specific T-cell IFN-γ response after booster vaccination and challenge (ELISpot). PBMCs were stimulated with PBS (background correction) or PRRSV 07V063.(A) Number of PRRSV-specific IFN-γ secreting cells (SC), in non-vaccinated (NV) or iPRRSV vaccinated animals using different adjuvants (ISA29, SWE, TLRa and SWE + TLRa) and skin vaccination (skiSE + TLRa); (A) two weeks after booster vaccination (D42) and (B) 7 days after challenge (D56); Each symbol represents one animal (mean of triplicate) and the median of the group set (n = 8) is indicated by the bar.

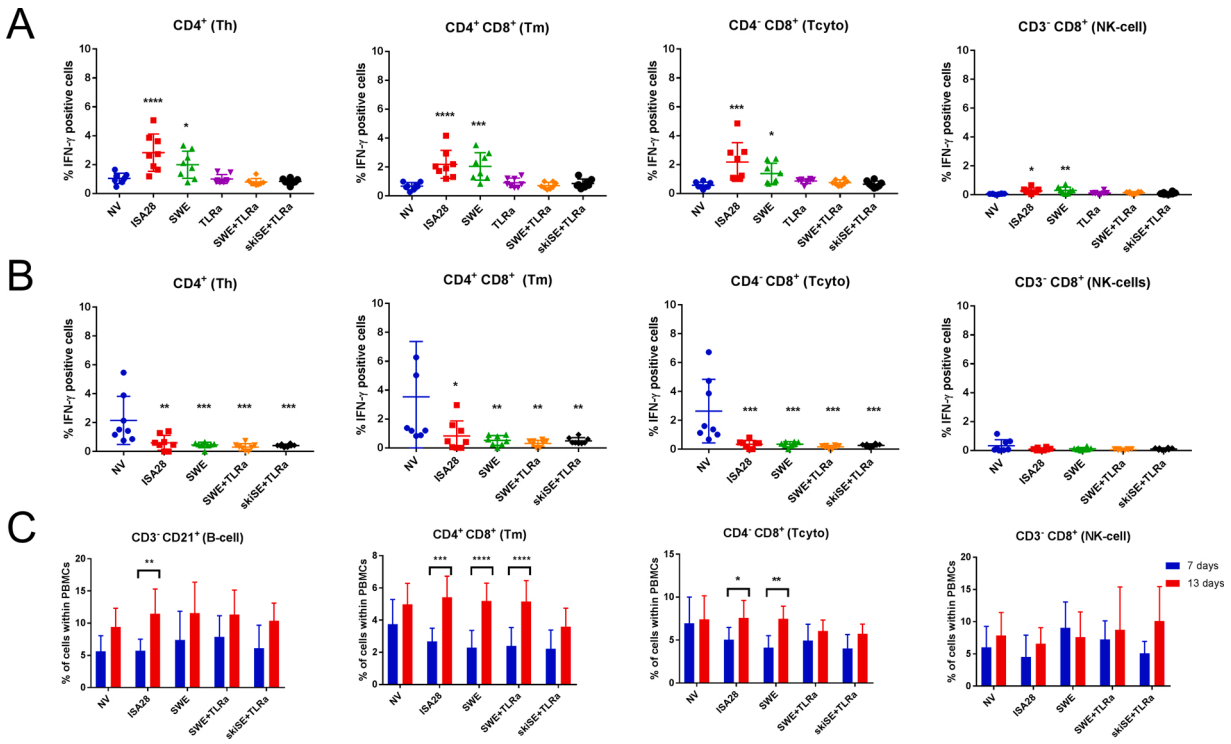

Fig. 4. PRRSV-specific intracellular IFN-γ responses and lymphocyte subsets (flow cytometry). PRRSV-specific IFN-γ response after *in vitro* re-stimulation of PBMCs in non-vaccinated (NV) or iPRRSV vaccinated animals using different adjuvants (ISA29, SWE, TLRa and SWE + TLRa) and skin vaccination (skiSE + TLRa). The percentage of positive IFN-γ cells within different T-cell subsets ($CD4^+$ (Th), $CD4^+CD8^+$ (Tm) $CD4^-CD8^+$ (Tcyto)), and NK-cells ($CD3^-CD8^+$) were analysed (A) 2 weeks after booster vaccination (D42) and (B) one week after challenge (D56). Each symbol represents one animal and the mean of the group set (n = 8) is indicated by the bar; (C) the relative proportion of B-cells ($CD3^-CD21^+$), T-cell subsets and NK-cells were determined within the live PBMC population 7 (D56) and 13 (D62) days after challenge; $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$ denotes a significant difference of the vaccine groups compared to the NV control (A+B) or a significant difference between 7 and 13 days after challenge (C).

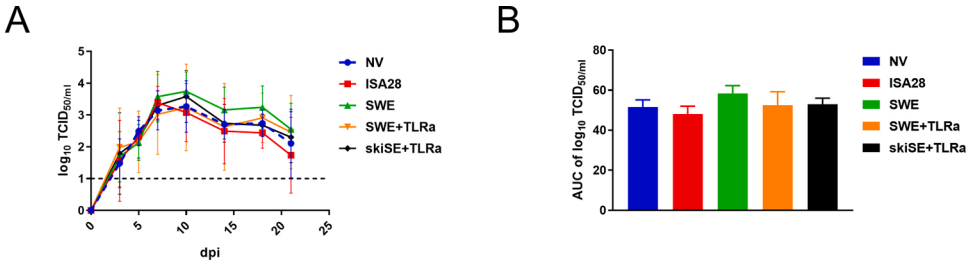

Fig. 5. Clearance of PRRSV in serum after challenge. Virus titres in serum (log_{10} $TCID_{50}$/mL) were determined by virus titration at different time-points after challenge (days post infection (dpi)). (A) serum virus titres in non-vaccinated (NV) or iPRRSV vaccinated animals using different adjuvants (ISA29, SWE and SWE + TLRa) and skin vaccination (skiSE + TLRa). Each data point represents the average of 9 animals ± S.D. The dotted line marks the detection limit for virus titration; (B) Area under the curve (AUC) calculated for the whole group with the S.D.

with a different antigen (Matthijs et al., 2019).

PBMC stimulation with the TLRa mixture without PRRSV-antigen induced significant levels of the Th1 directed cytokines IFN-γ and IL12p40, where we consider that IL12p40 reflects the IL12p35/IL-12 production (Auray et al., 2016). This response was accompanied by increased levels of Th2 directed IL-4 and pro-inflammatory cytokines like IL-1β and IL-6. When using TLR7/8 stimulation, but also the TLRa mixture, high levels of IL-10 were observed similar to what has been described after stimulation of human cord blood with TLR7/8 agonists (Kollmann et al., 2009). This IL-10 response could contribute induction and/or modulation to B-cell and antibody responses (Rojas et al., 2017) and could potentially avoid harmful inflammatory responses. Interestingly, the type I IFN-α cytokine response, which was measured after stimulation with a single TLR9 or TLR1/2 + 9 agonist, was abrogated when the TLR7/8 agonist was added to TLR9 containing stimuli, suggesting that this combination is less effective in inducing type I IFN responses. A similar decrease of IFN-α production when combining TLR7/8 and 9 agonists was observed, when using human PBMCs from adults, but not in cord blood and it was speculated that both TLR agonists would compete for endosomal signalling (Surendran et al., 2018). For future research, it would be interesting to investigate the potential of the TLR7/8 + TLR9 combination in neonatal pigs. Despite the limited IFN-α response, we anticipated that the induced cytokine responses would contribute to a Th1 polarizing vaccine response in neonates as shown in adult pigs (Matthijs et al., 2019). Therefore, we selected the TLR1/2 + 7/8 + 9 agonist mixture (TLRa) as immuno-stimulator for our neonatal study with iPRRSV. We used PRRSV-naive piglets, to allow us to distinguish adjuvant-related effects without interference from maternal antibodies.

In the vaccine study, neonatal pigs developed a detectable specific immune response after booster vaccination in the O/W emulsion groups: ISA28 (humoral and cellular) and SWE (only cellular), but not in the vaccines containing the TLRa mixture. Surprisingly, the SWE induced IFN-γ T-cell response was not initiated when TLRa were co-administered

as observed in both FCM and ELISpot. This suggests that administration of the TLRa mixture modulated the development of PRRSV-specific cellular immune responses, despite the fact that we anticipated that the TLRa would enhance the overall immune response. A similar observation was made in mice using a peptide-based vaccine where a water-in-oil (W/O) emulsion abrogated the CpG (TLR9a) induced immune response, while a squalene-based O/W emulsion enhanced the cellular immune response (Makinen et al., 2016). We speculate that a TLR agonist induced state of tolerance of the innate immune system after prime vaccination, or a downregulation of TLR-expression could mitigate the response to the booster vaccination with the TLRa mixture. Also in mouse macrophages, tolerance was induced along with a reduced expression of TLR7 and 9 upon the use of a TLR7 and 9 agonist-supplemented vaccine (Lee et al., 2015). In addition, the total cytokine response and its profile induced by SWE + TLRa could inhibit the immune response similar to a neonatal non-human primate study in which co-administration of a TLR5 agonist with a TLR7/8 agonist did not enhance the vaccine protection, but induced elevated C-reactive protein (CRP) levels after vaccination (Holbrook et al., 2016). Finally, the limited type I IFN response *in vitro* when using the TLRa mixture could contribute to less effective induction of the adaptive immune response (Ke and Yoo, 2017) in vaccine formulations containing TLRa.

Only the ISA28 group induced a specific humoral immune response after booster vaccination, where only post-challenge a specific response was observed in the SWE, SWE + TLRa and the skiSE + TLRa groups, based on the kinetics of antigen-specific antibodies in the serum The absence of significant differences between the vaccine groups with a memory-antibody response indicates that the humoral immune response was not influenced by TLRa in any positive or negative manner. In general, O/W emulsion induce a strong but short-term immune response, especially humoral (Aucouturier et al., 2001; Martinon et al., 2019), but also cellular immune response as observed for ISA28 in pigs (Lee et al., 2014; Vreman et al., 2019) and SWE in pigs (Matthijs et al., 2019) and mice (Younis et al., 2018). Presumably, the B-cell response after vaccination in the SWE, SWE + TLRa and the skiSE + TLRa groups was restricted to a memory B-cell response, without detectable production of PRRSV-specific IgG by plasma cells. This could be related to the neonatal immune status, as it was shown in humans that neonates are more primed for the induction of memory B-cell formation rather than plasma cell differentiation (Siegrist and Aspinall, 2009). Only ISA28 induced PRRSV-specific plasma cells after vaccination in neonates, supported by a significantly increased B-cell proportion between 7 and 13 days after challenge, which was not observed in other vaccine groups. Differences in emulsion composition, *e.g.* in mineral oil or in the surfactants, or in antigen-adjuvant interaction could be related to this humoral disparity between ISA28 and SWE in neonates. For future research, it will be valuable to determine the PBMC frequencies longitudinally from D0 to obtain information about the proportions of stimulated immune cells and the modus of immunisation by the different adjuvants.

With respect to the induction of cellular immunity after challenge, we were mainly interested in the differences between the adjuvant groups. We anticipated that vaccine induced cellular immune responses would be limited or abrogated between the adjuvant groups when analysed more than two weeks after challenge (D63 and D70). At these time-points immune responses due to the PRRSV infection would be more prominent than immune responses due to protection against challenge. Therefore, we only analysed the cellular immune response 7 days after challenge. At this time-point we expected an increased IFN-γ T-cell response in the vaccinated groups compared to the NV group. However, 7 days after challenge no significant changes between vaccinated and NV animals for IFN- SCs (ELISpot) were observed and even a reduced percentage of IFN-γ positive-specific T-cells compared to the NV animals was observed in the FCM. This could be related to the time-point of analysis as other studies (Ferrari et al., 2013; Martelli et al., 2009) detected a maximal IFN-γ response in PRSSV vaccinated animals more than 3 weeks after challenge. Our results would suggest that the T-cells of the vaccinated animals have become anergic to further *in vitro* stimulation. Alternatively, in vaccinated animals a more effective homing to peripheral tissues of activated antigen-specific T-cells might occur, thereby reducing the number of IFN-γ producing cells within the PBMC population. Moreover, the IFN- γ response in the NV animals confirmed that animals infected with the PRRSV strain 07V063 were able to develop a distinct specific cellular immune response within one week after challenge. This is quicker than the two or three weeks that has been described in other studies (Meier et al., 2003; Weesendorp et al., 2013; Zuckermann et al., 2007).

The delivery route is also an important factor contributing to the induction of an effective vaccination response. We showed a lack of immunogenicity and efficacy after i.m. as well as after skin-based vaccination. However, the exacerbated skin reaction after booster vaccination with DMN-patch could indicate a delayed type hypersensitivity (DTH) reaction induced by a strong local Th1 response (Black, 1999) suggesting that a T-cell response was induced by skin immunisation, however these responses were restricted to the skin and/or non-peripheral blood components. Despite the induction of a DTH reaction in the skin, the clinical signs and lung pathology after challenge were not intensified in these or any of the vaccinated groups (Heinen et al., 2002), which makes a PRRSV-related reaction less likely. In our previous vaccine study (Vreman et al., 2019) using 6-week-old pigs with similar DMN-patches we did not observe this exacerbated skin response after booster vaccination. However that study did not test the delivery of a PRRSV vaccine with a combination of a SWE and a mixture of TLR agonists and moreover used a shorter DMN (500 μm in adults, compared to 600 μm in neonates).

None of the vaccines used in this study showed any efficacy *in vivo* as measured by reduction of viremia, mitigation of lung pathology or decreased clinical signs after PRRSV challenge. However, the measured immune responses provided insights for future porcine neonatal research. Based on the PBMC stimulation and our *in vivo* results, we hypothesize that an O/W emulsion such as ISA28 VG in combination with TLR7/8 agonist could be an effective adjuvant in neonates, as neonatal studies have shown that TLR7/8 agonist in mice and non-human primates (Dowling et al., 2017; Ganapathi et al., 2015; Holbrook et al., 2016) enhanced the Th1 directed response and B-cell activation. Also, a next step would be to investigate the immune responses in the skin and draining lymph node shortly after vaccination to study in more detail, how the different TLR agonists contribute to the development of the immune response in neonatal pigs and the DTH reaction after skin booster vaccination.

5. Conclusion

The O/W emulsions ISA28 or SWE combined with iPRRSV induced a vaccine-specific immune response after booster vaccination in PRRSV-naïve neonatal pigs, this specific immune response was not observed in the vaccines containing TLRa mixture. However, none of the vaccines were able to reduce the viremia and lung pathology when prime vaccination was applied at three-days of age. Further research focussed on the early immune response is needed to elucidate the potential of TLR agonists in vaccines for neonatal pigs.

Declaration of Competing Interest

Anne Moore is an inventor of patents that have been or may be licensed to companies developing microneedle-based products. This potential competing interest has been disclosed and is being managed by University College Cork. Dennis McDaid is Chief Operating Officer and current director and owns stock in Xeolas Pharmaceuticals Limited. Damien Collins is a former employee of Xeolas Pharmaceuticals Limited and have no financial or other competing interests. The other authors have no competing interest to declare.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetimm.2020.110170.

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