Research paper

Toll-like receptor agonists as adjuvants for inactivated porcine reproductive and respiratory syndrome virus (PRRSV) vaccine

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ABSTRACT

Toll-like receptor (TLR) agonists can effectively stimulate antigen-presenting cells (APCs) and are anticipated to be promising adjuvants in combination with inactivated vaccines. In this study, the adjuvant potential of three different TLR-agonists were compared with an oil-in-water (O/W) adjuvant in combination with inactivated porcine reproductive and respiratory syndrome virus (iPRRSV) applied by different administration routes: intramuscular (i.m.) or into the skin using dissolving microneedle (DMN) patches. Pigs received a prime vaccination followed by a booster vaccination four weeks later. TLR1/2 (Pam3Cys), TLR7/8 (R848) or TLR9 (CpG ODN) agonists were used as adjuvant in combination with iPRRSV strain 07V063. O/W adjuvant (Montanide™) was used as reference control adjuvant and one group received a placebo vaccination containing diluent only. All animals received a homologous challenge with PRRSV three weeks after the booster vaccination. Antibody and IFN-γ production, serum cytokines and viremia were measured at several time-points after vaccination and/or challenge, and lung pathology at necropsy. Our results indicate that a TLR 1/2, 7/8 or 9 agonist as adjuvant with iPRRSV does not induce a detectable PRRSV-specific immune response, independent of the administration route. However, the i.m. TLR9 agonist group showed reduction of viremia upon challenge compared to the non-vaccinated animals, supported by a non-antigen-specific IFN-γ level after booster vaccination and an anamnestic antibody response after challenge. Montanide™-adjuvanted iPRRSV induced antigen-specific immunity after booster combined with reduction of vireamia. Skin application of TLR7/8 agonist, but not the other agonists, induced a local skin reaction. Further research is needed to explore the potential of TLR agonists as adjuvants for inactivated porcine vaccines with a preference for TLR9 agonists.

1. Introduction

Porcine reproductive and respiratory syndrome (PRRS) is an important viral disease affecting swine health and welfare. The swine industry reports large economic losses due to the PRRS virus (PRRSV) (Nathues et al., 2017; Nieuwenhuis et al., 2012) and vaccination is essential to prevent PRRSV infection and transmission. The disease is widespread globally, and after infection there is a variable morbidity and mortality with reproductive disorders in sows and respiratory symptoms often with co-infections in growing and finishing pigs.

PRRSV is an enveloped, positive-stranded RNA virus of the *Arteriviridae* family of which two distinct viral species are known (King et al., 2018): PRRSV-1, most relevant in Europe and divided in three subtypes (Stadejek et al., 2008) with a different pathogenicity, and PRRSV-2, mostly occurring in North America and South East Asia (Brar et al., 2015; Nelsen et al., 1999). PRRSV-1 and PRRSV-2 are constantly evolving and new strains appear, which complicates the development of a protective vaccine (Kappes and Faaberg, 2015). Several commercial vaccines have been licenced for the different types of PRRSV (Nan et al., 2017). In general, modified-live-virus vaccines induce a good protection against homologous infections, however cross protection and safety are points of concern (Renukaradhya et al., 2015). Conversely,

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inactivated PRRSV (iPRRSV) vaccines are safe to use, but provide limited protection (Geldhof et al., 2012; Renukaradhya et al., 2015; Vanhee et al., 2009; Zuckermann et al., 2007). Safe and effective iPRRSV vaccines are needed for virus control and adjuvants such as Toll-like receptor (TLR) agonists could help to increase the vaccine efficacy.

TLRs are a family of conserved pattern recognition receptors (PRRs) that recognize specific parts of microbial pathogens and activation of these receptors links the innate and adaptive immune response by activation of APCs (Takeda and Akira, 2005). Vaccines can benefit from this property by using specific synthetic TLR agonists (TLRa) as adjuvant to increase the magnitude and the efficacy of the immune response (Maisonneuve et al., 2014). TLRa have been extensively investigated in mice and humans for their immunostimulatory properties (Steinhagen et al., 2011; Toussi and Massari, 2014) and their applications as adjuvants. However, functional differences of specific TLRs and variation between species (Akira and Takeda, 2004; Uenishi and Shinkai, 2009) necessitates investigation of the efficacy of individual TLRa in porcine vaccines. *in vitro* stimulation of various porcine TLRs demonstrated that predominantly TLR2a, 7a and 9a (Auray et al., 2016; Vreman et al., 2018) induced activation of APCs, which is important for antigen uptake and transport to the draining lymph nodes. Also *in vivo*, individual administration of TLR2a (Basto and Leitao, 2014), 7a or 9a (Scheiermann and Klinman, 2014; Steinhagen et al., 2011; Van der Stede et al., 2002) enhanced the quality and quantity of the host adaptive immune response in several vaccine studies in humans and mice. Therefore, TLR2a, 7a and 9a are interesting candidates as adjuvants for iPRRSV vaccine development.

The search for an optimal antigen-adjuvant combination is a critical step in the development of inactivated vaccines (Knudsen et al., 2016). TLRa have been previously studied in combination with PRRSV-2. CL097, a TLR7a, combined with iPRRSV-2 enhanced the immune response and protection against PRRSV in pigs (Zhang et al., 2013), and the effect could be mimicked in mice by using the TLR7a SZU101 combined with PRRSV-2 (Du et al., 2016). Other porcine studies have shown that iPRRSV-2 in combination with a TLR9a (CpG ODN) enhanced protection after challenge with a highly pathogenic PRRSV-2 strain (Linghua et al., 2007; Quan et al., 2010). To our knowledge, TLR2a have not been used in recent PRRSV vaccination research. In this study we focussed on PRRSV-1, which is most common in Western Europe and is known to have large genomic differences compared to PRRSV-2 (Stadejek et al., 2013).

Additionally to the use of adjuvants, the route of administration can be crucial to achieve optimal protection after vaccination. Different delivery routes with the same vaccine can induce variation in immune response and efficacy (Vrdoljak et al., 2016; Zhang et al., 2015). The efficacy of TLR9a CpG ODN with live-attenuated PRRSV vaccine was route-dependent: i.m. administration reduced the viral shedding more than the intranasal route (Ouyang et al., 2016). The magnitude of the immune response and the cytokine profile were differentially affected using intradermal or intranasal vaccination routes in a mouse model with a TLR7/8a (R848) as adjuvant (van Aalst et al., 2018). Dissolving microneedle (DMN) patches deliver the vaccine directly into the skin and possibly thereby target high numbers of APCs that are constitutively present in the skin. Skin-based vaccination may thus result in equivalent or stronger immune responses compared to traditional i.m or subcutaneous routes (Ferrari et al., 2013; Marshall et al., 2016), especially when combined with appropriate adjuvants (Krejci et al., 2013). Other reported advantages of skin vaccination are dose reduction (Eble et al., 2009) and generation of mucosal immune responses, which is especially important for respiratory diseases (Le Luduec et al., 2016; Martelli et al., 2014). Previous studies with TLRa showed that intradermal injection of TLR9a in pigs may promote recruitment of innate immune cells and Th1 cytokine production (Magiri et al., 2016), while a

TLR7a can augment antigen-specific serum responses in mini-pigs (McKay et al., 2016). Overall, the skin seems a promising administration site to combine with TLRa in iPRRSV vaccines. However, to our knowledge, no studies to date have evaluated the microneedle-based skin delivery of TLRa to non-mouse species (Weldon et al., 2012).

In this study we investigated the immunogenicity and efficacy of TLR1/2a (Pam3Cys), TLR7/8a (R848) or TLR9a (CpG ODN) as adjuvant with iPRRSV-1 administered by i.m. injection or skin-based DMN patches. The antigen-specific PRRSV antibody response, IFN-γ production, reduction of viremia, serum cytokines and lung pathology after challenge were assessed to define the immune responses of the pigs.

2. Material and methods

2.1. Virus

PRRSV strain Belgium A (07V063) is a low pathogenic subtype 1 PRRSV-1 isolate. This strain was isolated from an aborted foetus from a Belgian farm, by inoculation of porcine alveolar macrophages (PAM) (Van Doorsselaere et al., 2011). This strain was used in recent studies describing viral, clinical and pathological data (Karniychuk et al., 2010; Weesendorp et al., 2013) and as inactivated virus for vaccination (Geldhof et al., 2012).

For challenge, MARC-Sn grown stocks were prepared of the isolates 07V063 (4th passage on MARC-Sn cells). For vaccine preparation, MARC-Sn cell culture supernatants of 07V063 (2nd passage on PAM + 2 passages on MARC-Sn), were purified via ultracentrifugation as described previously (Vanhee et al., 2009).

2.2. Virus inactivation and quality control

Purified virus (07V063) was suspended in RPMI 1640 (Invitrogen) to a titer of $10^{9.7}$ $TCID_{50}$/mL. Subsequently, the virus was inactivated using binary ethylenimine (BEI) as described before (Vanhee et al., 2009), and inactivated virus was stored at $-80\,°C$. To confirm that all virus was completely inactivated, a complete vaccine dose of 07V063 was inoculated on MARC-145 cells and subsequently passaged twice. As a positive control, MARC-145 cells were inoculated with 1 ml of non-inactivated 07V063. The MARC-145 cells were routinely checked for cytopathic effect (CPE) and ultimately stained for the PRRSV nucleocapsid protein via an immunoperoxidase monolayer assay (IPMA) using monoclonal antibody 13E2 (Van Breedam et al., 2011). No CPE or positive nucleocapsid staining was detected in cells that were inoculated with inactivated virus, while clear CPE and nucleocapsid staining were observed in cell cultures that were inoculated with non-inactivated virus. Since conservation of entry of inactivated virus may serve as a quality control for the preservation of antigenic properties, the effect of BEI inactivation on virus attachment and internalization into macrophages was examined as described previously (Vanhee et al., 2009). Non-inactivated virus suspensions were included as positive controls. The entry experiment showed that the binding and internalization kinetics of all BEI-inactivated virus stocks are similar to those observed for the non-inactivated virus stocks.

2.3. Vaccines

All the vaccines (i.m. and DMN-patches) contained the same dose of BEI-inactivated PRRSV 07V063 ($1.0 × 10^{8.0} TCID_{50}$) with different adjuvants. Montanide™ ISA 28 R VG (kindly provided by SEPPIC), an oil-in-water (O/W) emulsion compromising a blend of a mineral and non-mineral oil, was selected as reference control adjuvant, as a previous study with PRRSV 07V063 (Geldhof et al., 2012) showed a clear antibody response with a similar O/W adjuvant. Montanide™ was administered only i.m., as 15% volume per volume (v/v) in a final volume
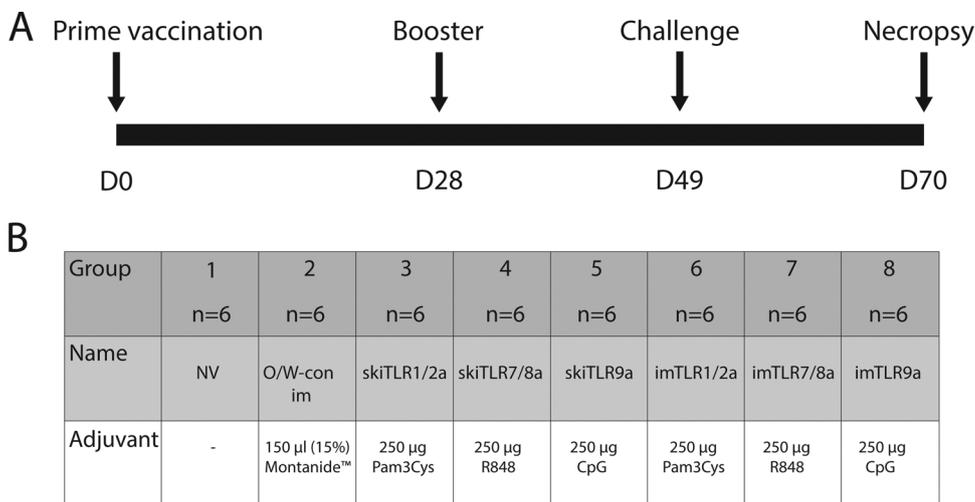
A

Prime vaccination	Booster	Challenge	Necropsy
D0	D28	D49	D70

Fig. 1. Experimental design and group overview. (A) Study design with prime vaccination at D0, booster vaccination at D28 followed by a homologous challenge at D49 and necropsy at D70; (B) Group overview: non-vaccinated animals (NV); in all vaccinated groups iPRRSV 07V063 antigen was used either with Montanide ™ ISA 28R VG (O/W con) or Toll-like receptor agonist (TLR1/2a, TLR7/8a or TLR9a); im, indicates groups with i.m. vaccination; ski indicates groups with skin vaccination with dissolving microneedle (DMN)-patch.

B

Group	1	2	3	4	5	6	7	8
	n=6	n=6	n=6	n=6	n=6	n=6	n=6	n=6
Name	NV	O/W-con im	skiTLR1/2a	skiTLR7/8a	skiTLR9a	imTLR1/2a	imTLR7/8a	imTLR9a
Adjuvant	-	150 µl (15%) Montanide™	250 µg Pam3Cys	250 µg R848	250 µg CpG	250 µg Pam3Cys	250 µg R848	250 µg CpG

of 1 ml according to manufacturer's instruction. The adjuvants of the experimental groups were composed of different TLRa: TLR1/2a; Pam3Cys L2000 from EMC micro-collections, TLR7/8a; R848, Resiquimod from InvivoGen or TLR9a; CpG ODN-type A sequence D32, 5′-ggTGCGTCGACGCAGggggg-3′, from Eurofins Genomics (Auray et al., 2016; Vreman et al., 2018). All vaccines (i.m. and DMN-patches) contained 250 µg of the individual TLRa (Fig. 1b). This adjuvant dose was based on study results, which were obtained in different animal species and using different delivery routes, for Pam3Cys (Shakya et al., 2011), R848 (McKay et al., 2016; Salabert et al., 2016), CpG ODN (Linghua et al., 2006; Quan et al., 2010) and the maximum dose which could be incorporated in the DMN- patches. The i.m. vaccines were freshly prepared on the day of vaccination and mixed with PBS to a volume of 1 ml.

Each DMN patch contained 225 microneedles, 500 µm in length, in a 9 cm^2 area. They were prepared as previously described (Vrdoljak et al., 2016), using trehalose and polyvinyl alcohol (PVA) as excipients. The iPRRSV and TLRa was dispersed homogenously throughout the full volume of the microneedle. Briefly, formulation was delivered directly onto the water-filled microneedle cavities in a polydimethylsiloxane (PDMS) mould at a rate of 1–3 µL/min. Formulation in the moulds was dried overnight at room temperature and then pulled from the mould onto medical grade adhesive tape (1525 l Poly Med tape, 3 M). The patches were immediately packaged and sealed in moisture-barrier bags containing desiccant and stored at 2–8 °C prior to shipping and administration.

2.4. Animals and housing

Forty-eight (n = 48) male six-week-old pigs (Topigs Norsvin Z-line, commercial breed) were purchased from a PRRSV-negative, high health status farm (van Beek SPF Varkens B.V.) in the Netherlands. The seronegative-PRRSV status of the pigs was confirmed upon arrival at the research facility with a commercial antibody ELISA. After arrival, the pigs were stratified based on their weight and family background followed by a randomisation to eight groups (n = 6 for each group). Before challenge the animals were housed in eight separated boxes in one stable. One week before challenge the animals were moved to 8 separate rooms in an isolation unit with HEPA-filtered air. All the stables were enriched with straw and toys. Standard feed for finishing pigs was provided twice a day and the pigs had unlimited access to water. The experiment started after one week of acclimatization. The animal experiment was conducted in accordance with the Dutch animal experimental and ethical requirements and the project license application was approved by the Dutch Central Authority for Scientific Procedures on Animals (CCD) (Permit number: ADV401002015356).

2.5. Experimental design

All the pigs except for the non-vaccinated (NV) group received a prime vaccination (D0) with iPRRSV and different adjuvants (Fig. 1A and B) at 7 weeks of age followed by a booster vaccination (D28) at 11 weeks of age. The NV control group received 1.0 ml of PBS i.m. in the right hind leg. The reference control group (O/W con) group received O/W adjuvant Montanide® i.m. (1.0 ml) in the right hind leg. The six experimental groups with the different TLR agonists were divided in three i.m groups (imTLR1/2a, 7/8a and 9a) and three groups with DMN-patches (skiTLR1/2a, 7/8a and 9a).

The lateral right hind leg was used for the i.m. injection (1.0 ml) and the DMN-patch was applied at the medial side of the left hind leg. Before vaccination with a DMN-patch the pigs were sedated i.m. with a mixture of Zoletil® (4 mg/kg, Virbac) and Sedamun® (2 mg/kg, Dechra) to ensure correct and equal application and by this optimize standardisation of skin vaccination. Pigs receiving an i.m vaccination were not sedated. The DMN-patches were removed after 24 h. Three weeks after the booster vaccination (D49; 14 weeks of age) all animals were challenged intranasally with PRRSV 07V063 (10^6 TCID$_{50}$) in PBS (1.0 ml per nostril). Three weeks post challenge (D70) the animals were euthanized with pentobarbital (150 mg/kg intravenous) after i.m. sedation with a mixture of Zoletil® (4 mg/kg, Virbac) and Sedamun® (2 mg/kg, Dechra), followed by exsanguination. During necropsy different lung lobes were collected for pathological examination.

2.6. Monitoring of post-vaccination and challenge reaction

After vaccination the injection site or DMN-patch application area were monitored over 4 days for local effects, such as redness and swelling of skin, graded from 0 (no changes) to 3 (clear redness and swelling of skin). Rectal temperature and clinical signs were recorded daily from two days before vaccination until 4 days after vaccination, and twice a day from one week before challenge until the end of the experiment respectively. Fever was defined as body temperature higher than 40 °C. Pigs were monitored twice a day for PRRSV-related clinical signs: liveliness, coughing, breathing, skin changes ears and appetite, as previously described (Weesendorp et al., 2013). Pigs were weighed upon arrival (D-7), three weeks after booster vaccination (D48) and during the challenge (D56, D63 and D70).

2.7. Blood sample collection and pre-treatment

Serum samples were collected at D-4, D21, D28, D35, D42, D48, D52, D54, D57, D59, D63 and D66 to determine virus titres and antibody levels. Serum samples from D52 (3 days after challenge) were

used for cytokine determination. The samples were stored directly at $-80\,^{\circ}C$ until analysis.

Heparin stabilized blood samples (approximately 15 ml) were collected at D21 and D42 for isolation of PBMCs for IFN-γ ELISpot assay. Collected blood was diluted 1:1 with PBS within 2 h of collection and transferred to a Leucosep® tube using a 60% FICOLL-PAQUE™ Plus density- gradient to isolate the PBMCs. Remaining red blood cells were lysed with ACK lysis buffer. PBMC were rested overnight at $4\,^{\circ}C$ before further analysis or freezing. Additionally, on D-4 we collected whole blood samples in Paxgene® RNA tubes for future analysis on genetic markers.

2.8. PRRSV serology

Serum samples were thawed on ice and the PRRSV-specific IgG antibodies in serum samples were tested with an indirect antibody ELISA (IDEXX PRRS X3 enzyme immunoassay) designed to detect PRRSV IgG antibodies using a recombinant nucleocapsid (N) protein as the coating antigen and an anti-pig immunoglobulin (Ig)G-HRP conjugate in a second step (Diaz et al., 2012). The kit was used according to the manufacturer's instruction and a sample-to-positive ratio of equal or greater than 0.4 was considered positive. Samples that tested negative were consequently given a numerical value of 0.00.

2.9. IFN-γ ELISpot assay

The number of antigen-specific IFN-γ secreting cells (SCs) was determined with an enzyme-linked immunospot assay) ELISpot assay kit (Porcine IFN-γ ELISpot [PLUS] (ALP) from Mabtech) according to the manufacturer's instruction. Briefly, 0.5×10^6 PBMCs were added to antibody pre-coated 96 well plates in RPMI 1640 medium (Gibco®) supplemented with 10% FBS and 1% Penicillin-Streptomycin (Gibco®). Cells were stimulated with PRRSV 07V063 with a multiplicity of infection (MOI) of 0.1 based on previous ELIspot results with PRSSV-1 stimulation (Mair et al., 2015; Mokhtar et al., 2016; Weesendorp et al., 2013). Cells without stimulus (medium only) were used as negative control. ConA (3 μg/ml from Sigma-Aldrich) stimulated cultures were used as positive control. All samples were analysed in triplicate. After incubation at $37\,^{\circ}C$ and 5% CO_2 for 24 h, PBMCs were discarded and secondary IFN-γ antibody and streptavidin was added according to manufacturer's instruction. Spot development was stopped by washing plates under tap water. The number of specific IFN-γ SCs, as determined using an Immunospot® S4 Analyzer (Cellular Technology Ltd.), were calculated as the average number of spots in the triplicate PBMC cultures stimulated with virus minus the average number of spots in the triplicate PBMC cultures exposed to culture medium only (non-specific response or background).

2.10. Cytokine production measured by multiplex assay

Protein concentrations of IFN-α, TNF, IL-4 and IL-6 were measured in the serum at 3 days after challenge. A custom-designed multiplex Cytometric Bead Array (PorcineProcartaplex®; eBioscience) was used according to the manufacturer's instructions and read on a Luminex machine (Luminex®200™). Cytokine concentrations were determined using xPONENT® software. The detection limits of the cytokines were 0.72 pg/ml (IFN-α), 7.57 pg/ml (TNF), 1.55 pg/ml (IL-4) and 6.32 pg/ml (IL-6), respectively.

2.11. PRRSV titers in serum measured by virus titration

Virus titers in serum were determined by virus titration on PAM, obtained from 3 to 5 week old piglets from a PRRSV and PCV2 -free herd in the Netherlands. The PAM were cultured in 24-well plates (Greiner) at a concentration of 5×10^5 cells/well in 1 ml RPMI 1640 medium (Gibco®) supplemented with 10% FBS and 1% Penicillin-

Streptomycin (Gibco®). 10-fold dilution series of the serum samples (four dilutions for each sample) were inoculated to the PAM (250 ụl) and plates were incubated at $37\,^{\circ}C$ in a 5% CO_2 humidified atmosphere. After 3 days, the monolayers were washed in 0.15 M NaCl solution, dried and frozen. The monolayers were stained by IPMA to visualize infection in the cells (van der Linden et al., 2003) using a 1:500 dilution of the monoclonal antibody SDOW17-A (Rural Technologies) against the nucleocapsid protein of PRRSV. Virus titers were calculated as $TCID_{50}$ as described by (Reed and Muench, 1938). The detection limit of the virus titration was $1.0 \log_{10}TCID_{50}/$ ml. To check the sensitivity of the PAM, all cell batches were assessed in the virus titrations with a PRRSV stock (07V063) with a known virus titer.

2.12. Pathology

A complete necropsy was performed on all pigs. An ECVP board-certified veterinary pathologist (SV) performed the macroscopic examination of the lung lesions. Macroscopic (or gross) lung lesions were scored to estimate the percentage of affected lung tissue by pneumonia according to (Halbur et al., 1995). For histology, samples were taken from three predefined locations of the right lung: anterior lobe, middle lobe and caudal lobe. Tissues were fixed in 4% neutral-buffered formaldehyde and routinely processed and embedded in paraffin. Consecutive 4 ụm thick sections were cut and stained with hematoxylin and eosin (H&E).

H&E stained slides were analysed semi-quantitively in a "blinded" manner by the same veterinary pathologist. The lung histology was scored according to (Weesendorp et al., 2014) with slight modifications. Briefly, the three lung sections of the right lobe were scored for the presence of a perivascular and peribronchiolar inflammatory infiltrate from 0 (no findings) to 5 (extended manifestation) and for the alveolar wall infiltrate (0–5). To compare the histological findings between the groups, the perivascular and peribronchiolar scores of the three sections per lung were added to an overall score, which could add up to a maximum of 30.

2.13. Statistical analysis

Data were analysed using SPSS Statistics version 23. The distribution of the data was explored with the descriptive statistics. Data with an overall normal distribution (ELISA and virus titration; Figs. 3 and 6, respectively) were analysed with a one-way ANOVA followed by a post-hoc Dunnett test (2-sided) using non-vaccinated animals and O/W control group as reference groups. Non-parametric data (skin changes, ELISpot, cytokines, an lung pathology; Figs. 2, 4, 5 and 7, respectively) were analysed with a Kruskal-Wallis test followed by a post-hoc Mann-Whitney U test for multiple comparisons. Association analysis was performed with a Pearson correlation test (2-tailed). *P*- values less than 0.05 were considered statistical significant (*$p < 0.05$).

3. Results

3.1. Vaccine safety and challenge reaction

None of the vaccines induced any systemic adverse effects, such as raise in body temperature, loss of appetite and activity or reduced weight gain (data not shown). However, after application of TLRa and iPRRSV in DMN patches, a transient local reaction was observed in some animals, characterized by a variable local redness and swelling of the skin graded from 0 (no change) to 3 (swelling and redness of the skin) (Fig. 2A). The skiTLR7/8a group showed significantly more skin reactions than skiTLR1/2a or 9a group (Fig. 2B and C) up to 2 days after primary and 1 day after the booster immunization. There was no difference in severity between the prime and booster vaccination. All of the local skin reactions disappeared within 4 days post vaccination. No local reaction was observed after i.m. administration.
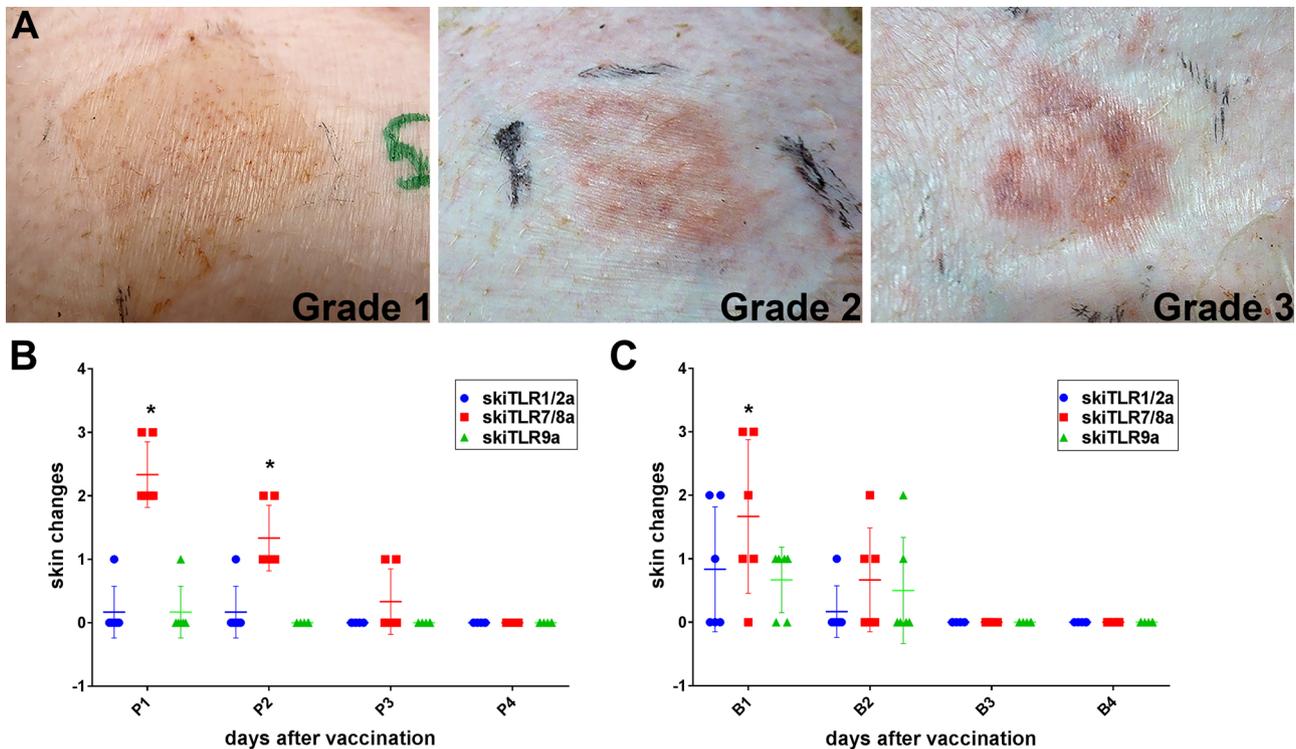
Fig. 2. Skin reaction after dissolving microneedle (DMN) patch application with different Toll-like receptor (TLR) agonists. (A) Examples of grading of skin changes (grade 1–3) based on redness and swelling of skin; normal skin (grade 0) is visible around the skin changes (B) Skin reaction overview from day 1 till day 4 after prime vaccination (P) and after booster vaccination (C). Each bar represents the median of a group of 6 animals with the 95% CI. (*$p < 0.05$ compared to other groups).

Following challenge, none of the pigs in any of the study groups exhibited PRRSV-related clinical signs or raise in body temperature. Additionally, there was no significant difference in weight or weight gain between the groups (results not shown).

3.2. PRRSV specific IgG antibody response

PRRSV-specific IgG antibodies were measured in the serum to determine the humoral immune response. After booster vaccination (D35) only the O/W control group showed a detectable PRRSV antibody response (in 4 of the 6 animals) (Fig. 3A and B), while none of the animals in the experimental TLRa groups were able to develop a specific antibody response after booster vaccination.

From eight days after challenge (D57 or 8 days post infection (dpi)) the imTLR1/2a, 7/8a and 9a groups showed a specific antibody response at a comparable level to the O/W-control group (anamnestic antibody response) with no significant difference between the different TLRa groups (Fig. 3A). On D57 and D59 (8 and 10 dpi) the skiTLRa groups showed a significantly lower antibody response compared to the O/W control group (Fig. 3B). The skiTLRa groups developed a specific antibody response from 14 dpi (D63) in nearly all animals, with no significant difference between the TLRa groups.

3.3. T-cell IFN-γ response

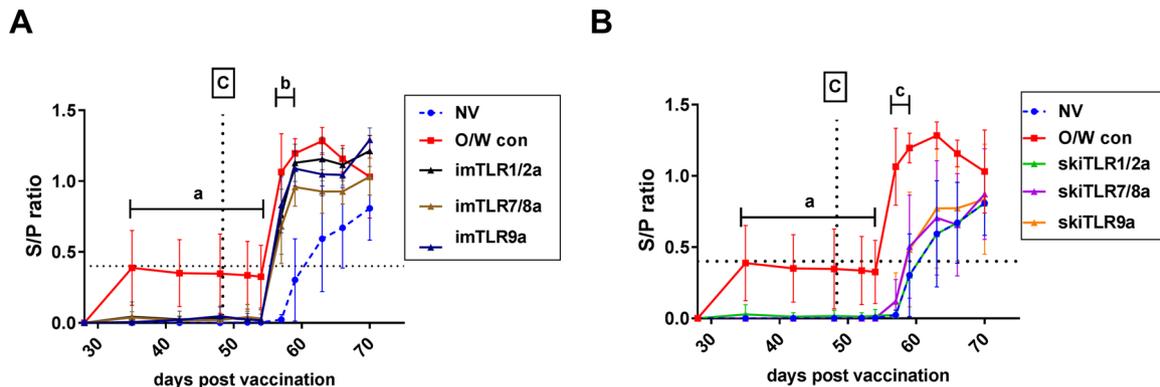After prime and booster vaccination the cellular immune response

Fig. 3. Kinetics of PRRSV specific antibody response after booster vaccination. PRRSV antibody titers from booster vaccination (D28) till the end of the study determined by ELISA. (A) i.m. vaccinated Toll-like receptor agonist (imTLRa) groups compared to non-vaccinated (NV) group and oil-in-water group control (O/W con); (B) skin vaccinated skiTLRa groups compared to NV and O/W con groups; "C" indicates challenge at D49; The dotted line marks the detection limit for the ELISA; Each data points represents the average of 6 animals ± S.D. "a" indicates a significant difference (*$p < 0.05$) between the O/W con group and the imTLRa/ skiTLRa/ NV groups; "b" indicates a significant difference between the NV group and the imTLRa/ O/W con groups; "c" indicates a significant difference between the O/W con group and the skiTLRa/ NV groups.
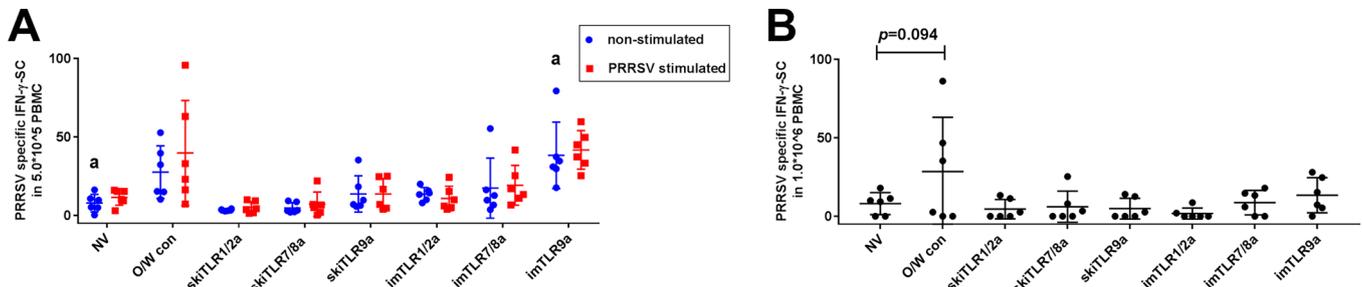
Fig. 4. PRRSV specific T-cell IFN-γ response two weeks after booster vaccination. Number of IFN-γ secreting cells (SC) after PBMC stimulation for 24 h with PBS (non-stimulated) or PRRSV(07V063). Response of non-vaccinated (NV) group and oil-in-water control (O/W con) group compared to the i.m. vaccinated Toll-like receptor agonists (imTLRa) groups and skin vaccinated skiTLRa groups measured two weeks after booster vaccination. Each symbol represents one animal (mean of triplicate wells) and the median of the group set (n = 6) is shown as a line; (A) Number IFN-γ SCs in non-stimulated (● background) and PRRSV stimulated samples (■); (B) Net IFN-γ SCs with background subtracted from stimulated samples; "a" indicates a significant difference (*p* < 0.05) between non-stimulated samples of NV group and imTLR9a group.

was evaluated as number of IFN-γ SCs in the PBMCs after homologous *in vitro* re-stimulation with the challenge strain. Overall, in all groups there was a large individual variation between the animals in number of IFN-γ SCs in non-stimulated and PRRSV stimulated samples. A large number of non-stimulated control samples contained IFN-γ SCs, indicating a non-antigen-specific IFN-γ production. Only after booster vaccination (D42) the imTLR9a group showed significantly more non-specific IFN-γ spots compared to the NV animals (Fig. 4A), additionally this was observed as a trend, albeit not significant (0.05 < p < 0.10) in the O/W control group compared to the NV animals. This increased non-specific response compared to the NV group was not detectable in the skiTLR9a group, TLR1/2a and 7/8a experimental groups of either delivery routes.

There was no detectable antigen-specific cellular immune response on D21 (3 weeks after prime vaccination) as PBMCs produced no specifically induced IFN-γ SCs (results not shown). On D42 (2 weeks after booster vaccination) only in the O/W control group, 3 of the 6 animals showed an increase in IFN-γ SCs (Fig. 4B) compared to the non-stimulated control samples (p = 0.094), albeit not significant. None of the animals in the experimental TLRa groups showed a significant increase in IFN-γ SCs compared to the control samples.

3.4. Serum cytokines 4 days after challenge

To assess the influence of vaccination on the host response to viral challenge the serum IFN-α (anti-viral cytokine), IL-4 (Th2-type cytokine) levels and TNF and IL-6 levels (pro-inflammatory cytokines) were analysed 3 days after challenge. All animals showed a detectable IFN-α response with no significant difference between the groups (Fig. 5A). The O/W control group showed significant increased IL-4 level compared to the NV group with no significant difference between the two administration routes (Fig. 5B).

The median levels and number of animals producing the pro-inflammatory cytokines TNF and IL-6 were highest in the skiTLR7/8a group (Fig. 5C and D). TNF and IL-6 were equivalent to the non-vaccinated groups in all other groups. However, no significant differences in TNF and IL-6 between groups were detected.

3.5. Viremia in serum

PRRSV 07V063 was not detected in the serum before challenge. At D52 (3 dpi) PRRSV was detected in the serum of 92% of the animals and at 5 dpi all animals were viremic. At D54 (5dpi) the virus titer of only the skiTLR9a group was significantly lower than the NV group (Fig. 6A), this reduction of viremia was not observed for other time points in this group. At D63 (14 dpi) the O/W control and imTLR9a group showed a significantly lower virus titer compared to the NV animals (Fig. 6A and B); this decline was not observed in the other
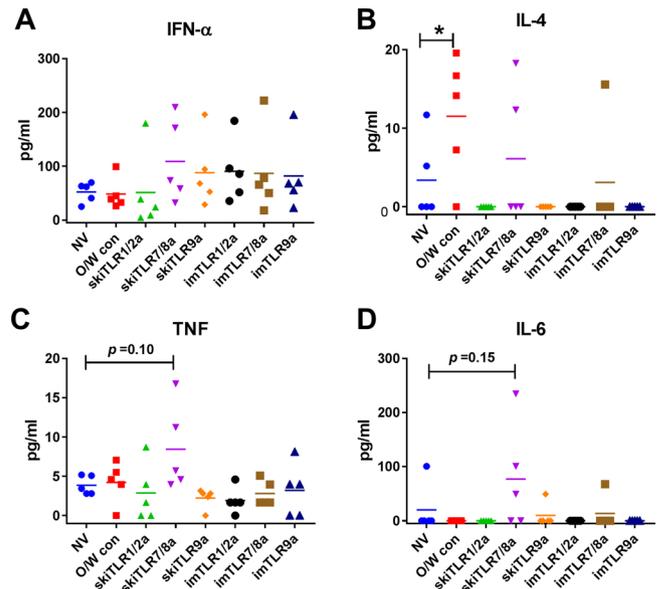

Fig. 5. Serum cytokine concentrations 3 days after PRRSV challenge. Serum cytokines (pg/ml) in non-vaccinated (NV) group and oil-in-water control (O/W con) group compared to the experimental i.m. vaccinated Toll-like receptor agonist (imTLRa) groups and skin vaccinated skiTLRa groups for IFN-α (A), IL-4 (B), TNF (C) and IL-6 (D). Each symbol represents one animal and the median is shown for the data set (n = 5); (*p* < 0.05).

experimental TLRa groups. The imTLR9a group showed as well a significantly lower virus titer at 8 and 10 dpi.

The area under the curve (AUC) value was significantly lower for the imTLR9a and O/W control group compared to the NV animals (Fig. 6C), the other experimental TLRa groups did not show this decline.

3.6. Pathology

There were no or minimal gross pathological changes in the lungs 21 days after challenge in all groups. The O/W control group did not show any macroscopic changes in the lungs. In the other groups, individual scores ranged from 0% to 4% of affected lung surface (Fig. 7A). There was no significant difference in lung histopathology between the groups (Fig. 7B, C and D). The gross lesions were predominantly located in the cranial and the middle lobe and were characterized by multifocal, irregular, slightly sunken red to tan areas. The lungs of all pigs displayed mild to moderate histopathological changes, although the gross changes were not evident. Compared to normal lung tissue histology of a pig with the same age and genetic background (Fig. 7E), we observed
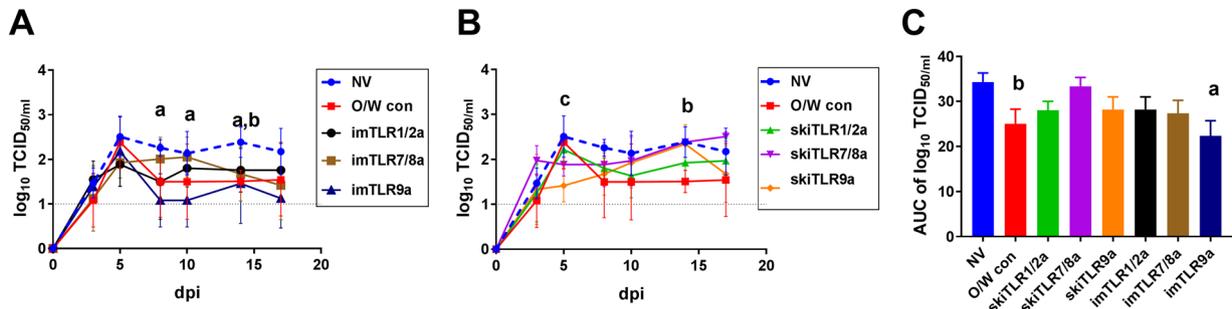
Fig. 6. Clearance of PRRSV in serum after challenge. Virus titers in serum ($\log_{10}$ TCID$_{50}$/ml) determined by virus titration. (A) serum virus titers of i.m. vaccinated Toll-like receptor (imTLRa) groups compared to non-vaccinated (NV) group and oil-in-water control (O/W con) group (B); serum virus titers of skin vaccinated skiTLRa groups compared to non-NV group and O/W con group. Each data point represents the average of 6 animals ± S.D. The dotted line marks the detection limit for virus titration; (C) Area under the curve (AUC) calculated for the whole group with the S.D. "a" indicates a significant difference (*$p < 0.05$) between imTLR9a group and NV group; "b" between O/W con group and NV group and "c" between the skiTLR9a group and the NV group.

an 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 (Fig. 7F and G). Also, dispersed the alveolar lumina contained a similar mononuclear infiltrate, which was occasionally admixed with cellular debris.

3.7. Association between specific humoral and cellular immune response and viral clearance

The time-points with the most prominent immune response (humoral D35 and D57; cellular D42) and the time-point with the most significant reduction of vireamie (D63) were used to investigate the association between specific immune response and viral clearance. This association between immunogenicity and efficacy was independent of

the vaccine adjuvant and administration route (all animal were included, as well the NV group). There was a significant association of the humoral immune response one week after booster vaccination (D35) and one week after challenge (D57) with the cellular immune response two weeks after booster vaccination (D42) (Fig. 8A and B). However, no significant association was found between the viral clearance (D63) and the specific humoral or cellular immune response (Fig. 8C and D).

4. Discussion

PRRSV is an important pathogen resulting in large scale health issues in pigs with major economic impact, therefore development of effective inactivated vaccines is highly desirable. Inadequate specific immune responses, especially cell-mediated responses, are often
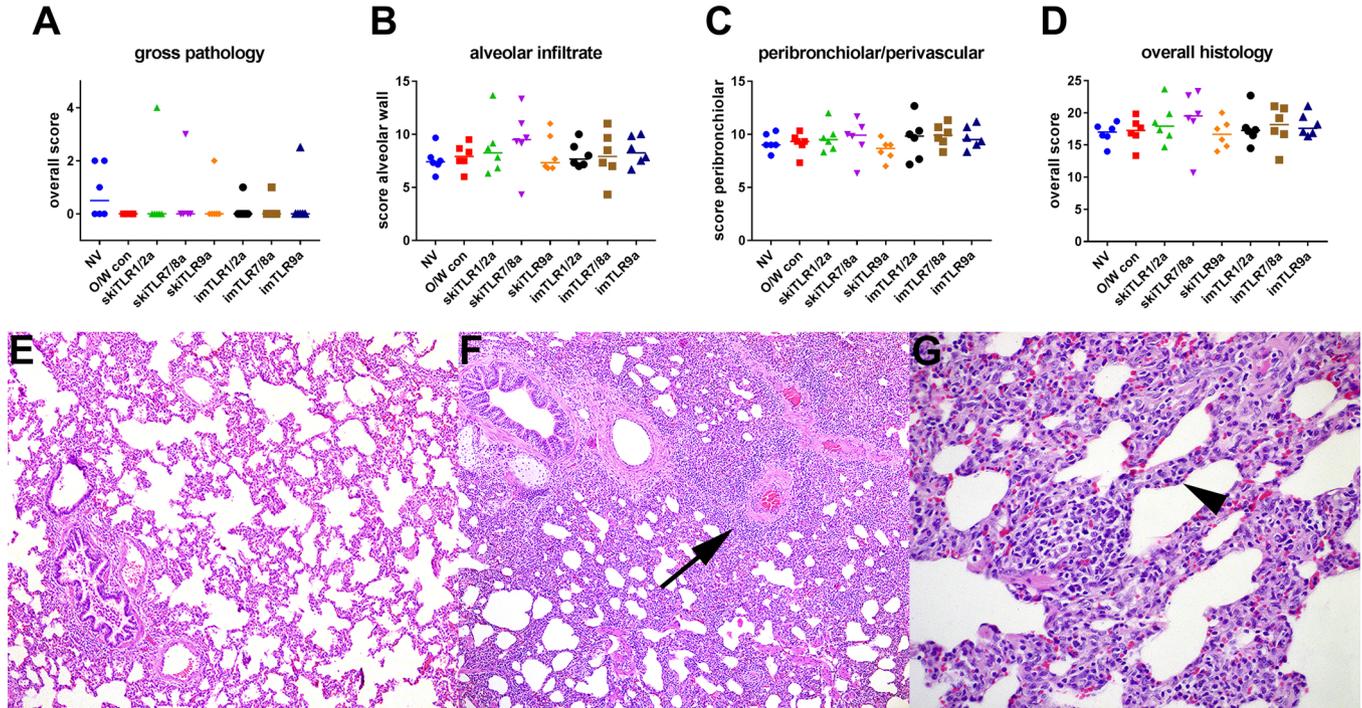

Fig. 7. PRRSV lung pathology 21 days after challenge. Lung tissue of non-vaccinated (NV) group and oil-in-water control (O/W con) group compared to the experimental i.m. vaccinated Toll-like receptor agonist (imTLRa) groups and skin vaccinated skiTLRa groups. (A) The extension of gross lung lesions calculated as proportion of total lung area; (B) Histologic lesions evaluated in the left cranial, medial and caudal lung lobe for infiltrate of mononuclear inflammatory cells in alveolar walls (score 0–5 for each lobe), (C) and around blood vessels, bronchi and bronchioli (score 0–5 for each lobe) (D) combined to an overall histology score (score 0–30); Each symbol represents one animal and the median is shown for the data set (n = 6). (E) Normal lung tissue histology of a control pig with the same background and age (Haematoxylin and Eosin (H&E) stain; magnification 200x); (B) interstitial pneumonia 21 days after PRRSV infection with a significant mononuclear infiltrate of mainly macrophages and lymphocytes around blood vessels and bronchi and bronchiole (*arrow*) (magnificantion 200x); (C) alveolar walls are thickened by the same type of inflammatory cells (*arrowhead*) (magnification 400x).
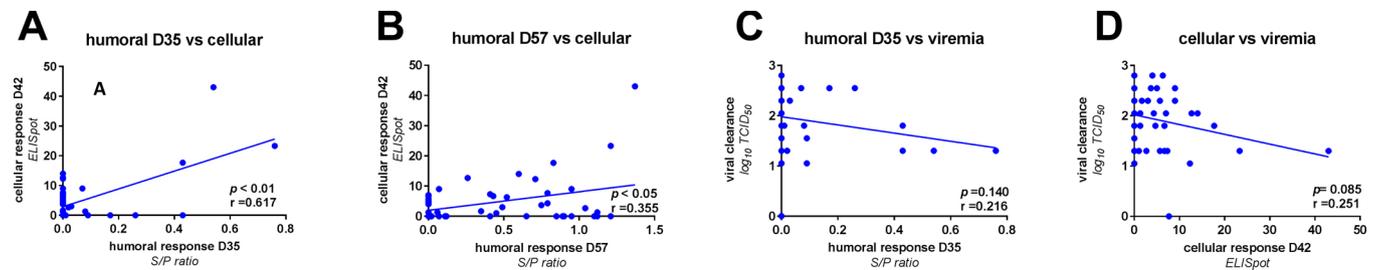
Fig. 8. Association between PRRSV specific humoral and cellular immune response and viral clearance. Scatter plots of humoral immune response on D35 (one week after booster vaccination) and D57 (one week after challenge) versus cellular immune response on D42 (two weeks after booster vaccination)(A and B); Scatter plots of viral clearance on D63 (15 days after challenge) versus cellular and humoral immune response (C and D). All animals indepent of vaccination an administration route, were included in this association.

described for iPRRSV vaccines as a cause for limited protective efficacy (Charerntantanakul, 2009; Geldhof et al., 2012; Renukaradhya et al., 2015; Vanhee et al., 2009). TLRa activity links the innate and adaptive immune response by activation of APCs and has a potency to enhance cell-mediated responses and therefore could be a suitable method to improve the efficacy of iPRRSV vaccines. Three different TLRa were selected based on porcine *in vitro* study results (Auray et al., 2016; Vreman et al., 2018) and this selection was supported by positive immunogenicity results from *in vivo* studies with inactivated vaccines in pigs, mice and humans (Basto and Leitao, 2014; Linghua et al., 2007; Zhang et al., 2013). The skin, which harbours a large number of APCs, was included as administration route to explore the potency of the different TLRa to a broader extent. Here, we examined the adjuvant potency of TLR1/2a (Pam3Cys), TLR7/8a (R848) or TLR9 (CpG ODN) to increase the iPRRSV vaccine response compared to an established O/W control adjuvant.

We found that none of the TLRa was able to induce a detecable specific immune response after booster vaccination for either delivery routes. However, the imTLR9a showed reduction of vireamia, indicating efficacy for CpG ODN as an injected adjuvant. As we only observed a reduction of the viremia in the i.m. TLR9a group, we consider that this result was most likely induced by the TLR9a as it was not observed in the other experimental groups with TLRa and the iPRRSV antigen. In the study design we did not include an iPRRSV only group as we were interested in the vaccine formulation. We anticipated that the iPRRSV antigen would be insufficient to show efficacy and/or an immune response on its own, as it is common, that inactivated viral vaccines are administered with an adjuvant.

Vaccine immunogenicity was measured by production of specific IgG antibodies in the serum and specific IFN-γ + T-cell response after vaccination. The ELISA showed only in the O/W control group a specific antibody response before challenge. These early antibodies often appear within one week after booster vaccination, however there is evidence that they play no significant role in PRRSV protection (Labarque et al., 2000). Virus neutralizing antibodies appear at a later time-point and their presence before challenge appears to correlate with passive protection (Loving et al., 2015). In our study the experimental TLRa groups lacked the induction of any measurable specific antibody response before challenge, therefore the neutralizing antibodies were not measured.

Three days after challenge the IL-4 serum level was measured as an indicator of a Th2 or humoral immune response. Only in the O/W control group 4 of the 5 animals showed a clear IL-4 response at 3 days after challenge, supporting the specific antibody response after booster vaccination in this group as observed in other PRRSV vaccine studies (Chen et al., 2013; Wang et al., 2011). However, serum IL-4 is often difficult to detect and the significance of this cytokine response for porcine B-cell stimulation is under debate (Murtaugh et al., 2009). In our study 2 of the 5 non- vaccinated animals also showed detectable IL-4 levels and in addition the serum before challenge was not analysed. Our study did not include an uninfected control group to measure

baseline cytokines as our aim was to investigate response differences between vaccines. Therefore the IL-4 results should be interpreted with caution as a reflection of the humoral response.

An enhanced specific IFN-γ response after booster vaccination and IFN-α response after challenge could contribute to more effective iPRRSV vaccines as insufficient T-cell responses and cytokine responses most likely play a pivotal role in the delayed adaptive immune response and clearance of PRRSV (Loving et al., 2015; van Reeth and Nauwynck, 2000). In our study the experimental TLR agonist groups did not enhance antigen-specific IFN-γ cell-mediated response after booster and the anti-viral IFN-α response after challenge. However, an increase in non-specific IFN-γ production in the imTLR9a group was noticed in the ELISpot, which was most likely induced by activation of innate immune cells. This non-specific Th1-skewed response was also observed in a iPRRSV study with a different adjuvant system (Zuckermann et al., 2007), which induced no protective immunity. Interestingly, in our study only the imTLR9a group with this non-specific IFN-γ production also showed a reduction of viremia. This suggests that this group was able to induce a protective immune response, most likely supported by the non-specific stimulation as measured in the ELISpot and the anamnestic antibody response after challenge. We consider this anamnestic antibody response suggestive for the added effect of the TLR agonist, although an iPRRSV only vaccination group was not included and we cannot fully exclude the contribution of iPRRSV antigen on its own.

Besides reduction of viremia, vaccine efficacy was assessed after challenge by reduction of both clinical signs and lung pathology. Within 6 days after challenge all animals were viremic. However, the PRRSV challenge did not induce any clinical signs or fever in the non-vaccinated animals. This mild clinical course has been described before for the 07V063 strain (Geldhof et al., 2012; Weesendorp et al., 2013). The lung pathology was determined 21 days after challenge and the histologic changes were generally mild to moderate and characteristic for PRRSV infection (Weesendorp et al., 2014). This mild lung pathology could be due to the time of necropsy (day 21 post-infection), as lung lesions are most prominent at 7–10 days post infection (Halbur et al., 1995). Overall, we can conclude that the reduction of the viremia in the O/W control and imTLR9a group did not result in reduced lung pathology compared to NV group at 21 days post-infection. However, we cannot exclude that at an earlier time-point after infection there could have been differences in lung pathology.

Skin delivery with DMN-patches did not influence the vaccine immunogenicity compared to the i.m route. However, the imTLRa groups were able to induce an anamnestic antibody response after challenge, which was lower in the skiTLRa groups and only the imTLR9a group showed significant vaccine efficacy after challenge. We did observe a transient, but significant reduction of viremia at 5 dpi in the skiTLR9a group. However, lower viral titers were not detected at other time-points after challenge and AUC for the skiTLR9a was not significantly reduced compared to the NV group. We interpret this finding that an immune response was induced in this group that was capable of retarding early virus growth, however it was insufficient to protect the

host over time from continued and increased virus proliferation. A porcine influenza study with skin vaccination demonstrated as well that TLR9a (CpG ODN) (Bernelin-Cottet et al., 2016) intradermally in pigs promoted the antigen-specific Ab responses, significantly but quite weakly. Overall, it is an interesting finding that TLR9a by both the i.m. and skin routes demonstrated the strongest efficacy potential.

It is unknown why the skin route was not able to benefit from the high number of accessible APCs in the skin to induce a comparable immune response and vaccine efficacy as seen in the imTLRa groups. One explanation could be a lower vaccine bioavailability by the skin route due to partially inserted DMN. It is commonly appreciated in the microneedle field that less than 100% of the cargo is delivered into skin; some reports where antigen is coated onto silicon microneedles report as little as 7% being delivered into skin (Crichton et al., 2010). A change in patch design, whereby the DMN contain the vaccine and adjuvant in the tips of the DMN only, compared to homogenous distribution throughout the length of the DMN could facilitate an increased dose delivery in future studies.

Another route-dependent finding was the local skin redness in skiTLR7a group, which was less prominent or absent in the skiTLR1/2a and 9a groups. This skin reaction is a strong indication for overall successful DMN-patch application and as well a side effect for topical administration of TLR7a Resiquimod (R848) as observed in other studies (Hengge and Ruzicka, 2004; Meyer et al., 2013). These side effects were considered negligible compared to the immunostimulatory properties (Sauder, 2000; van Aalst et al., 2018) of the TLR7/8a. In our study this difference in local immune response between the different TLR agonist did not result in a more prominent antigen-specific immune response, as assessed by antibody ELISA or IFN-γ ELISpot or anamnestic immune response after challenge. However, other specific immune responses, such as tissue-specific T and/or B memory or mucosal responses, which did not contribute to protection, may have been induced but were not evaluated.

No correlation was observed between the immune responses that were evaluated and protection against virus challenge. This indicates that other immune responses, which were not evaluated, were responsible for efficacy. These responses could include non-antigen specific innate immunity that may have been still active 2 weeks after the booster immunization and/or memory immune responses that were quickly re-activated after challenge but not assessed in our assays. Interestingly, we found in the serum of skiTLR7a group a trend for a higher level for the pro-inflammatory cytokines TNF and IL-6, post-challenge, which could be correlated to the skin reaction. Overall, the two different delivery routes showed only post-challenge differences in efficacy for TLR9a.

We anticipated that selected TLR agonists would have stimulated the production of pro-inflammatory cytokines/chemokines and type I IFNs supporting the development of an adaptive immune response in combination with the iPRRSV antigen. TLR7a and TLR9a combined with high pathogenic iPRRSV-2 antigen in a comparable setting (Linghua et al., 2007; Zhang et al., 2013) although with a different TLR7a, were able to enhance these specific immune responses and showed enhanced protection after homologous challenge. PRRSV-1 and PRRSV-2 are highly different strains (around 60% homologous at genome level) (Van Doorsselaere et al., 2012) and the use of iPRRSV-1 antigen might have provoked that our selected TLRa-antigen combinations were less successful. As well, we have to consider that the innate immune response directly after vaccination was not evaluated and it is unclear to what extent actual TLR activation occurred. We cannot exclude that the dose of the different TLRa was not optimal for the i.m/ and or the skin vaccination. As especially for the patches, no previous experiments have been performed and our reference dose for the different TLRa was based on different animal species and delivery routes combined with the maximum dose which could be incorporated in the DMN- patches. However, the O/W reference adjuvant was able to induce a specific immune response with iPRRSV-1 in combination with

reduction of viremia. Possibly a different adjuvant system where different combinations of TLR agonists are combined with e.g. O/W adjuvant, liposomes (McKee and Marrack, 2017; Neeland et al., 2014) or plasmids (Quan et al., 2010) could enhance the vaccine properties and would be a direction for further research.

5. Conclusion

TLR 1/2a, 7/8a or 9a incorporated as adjuvants in iPRRSV 07V063 antigen based vaccine were unable to induce a measurable specific immune response after booster vaccination and only the imTLR9a group induced reduction of viremia after homologous challenge, supported by non-antigen-specific IFN-γ production after booster vaccination and an anamnestic antibody response after challenge. Further research is needed with different adjuvant systems, dosing regimens and combinations of TLR agonists and delivery routes to explore the potentials of TLRa as adjuvant for porcine vaccines with a preference for TLR9a.

Conflict of interest statement

None of the authors have any potential conflict of interest regarding or related to this work.

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