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Early immune responses in skin and lymph node after skin delivery of Toll-like receptor agonists in neonatal and adult pigs

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ABSTRACT

The skin is potentially an important vaccine delivery route facilitated by a high number of resident antigen presenting cells (APCs), which are known to be stimulated by different Toll-like receptor agonists (TLRa). In this study, neonatal and adult pigs were vaccinated in the skin using dissolving microneedle patches to investigate the immuno-stimulatory potential of different TLRa and possible age-dependent differences early after vaccination. These patches contained TLR1/2a (Pam3Cys), TLR7/8a (R848) or TLR9a (CpG ODN) combined with inactivated porcine reproductive and respiratory syndrome virus (PRRSV) or with an oil-in-water stable emulsion. Vaccinated skin and draining lymph nodes were analysed for immune response genes using microfluidic high-throughput qPCR to evaluate the early immune response and activation of APCs. Skin pathology and immunohistochemistry were used to evaluate the local immune responses and APCs in the vaccinated skin, respectively.

In both neonatal and adult pigs, skin vaccination with TLR7/8a induced the most prominent early inflammatory and immune cell responses, particularly in the skin. Skin histopathology and immunohistochemistry of APCs showed comparable results for neonatal and adult pigs after vaccination with the different TLRa vaccines. However, in vaccinated neonatal pigs in the skin and draining lymph node more immune response related genes were upregulated compared to adult pigs. We showed that both neonatal and adult skin could be stimulated to develop an immune response, particularly after TLR7/8a vaccination, with age-dependent differences in regulation of immune genes. Therefore, age-dependent differences in local early immune responses should be considered when developing skin vaccines.

1. Introduction

The skin is the largest organ in the body and forms a physical and immunological boundary to protect from injury and pathogens [1,2]. Because of its immunological role, the skin is an attractive delivery route for vaccination. Skin vaccination can induce immune responses quantitatively equivalent or qualitatively enhanced compared to conventional intramuscular (i.m.) vaccination [3,4], sometimes with a reduction of the vaccine antigen-dose [5,6]. The epidermis and dermis are the most important layers that determine the immune responses within the skin [7,8]. These layers can be physically breached by dissolving microneedle (DMN)-patches, a needle-free delivery system for skin vaccination [9], also termed "microarray patches". DMN-patches can target the high numbers of quiescent or immature APCs present in both epidermis and dermis. After vaccination, activated APCs mature and transport the vaccine-antigen to the draining lymph nodes (LNs), where the antigen is presented to naïve T cells [10,11] to induce an immune response. Besides APCs, epidermal keratinocytes and newly recruited dermal immune cells also contribute to this skin vaccinated induced immune response [11,12].

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Dendritic cells (DCs) are professional APCs, which are essential to induce effective immune responses after vaccination [13,14]. We studied two different DC subsets in the skin of adult and neonatal pigs: Langerhans cells (LC) in the epidermis and dermal DCs (dDC) in the dermis, by detecting the expression of cell-surface markers (CD1a, MHCII and CD163) [7,15,16] combined with their localisation within the skin. In the dermis, macrophages (Mϕ), expressing cell-surface marker CD163, can also act as APCs. In the steady state, dermal resident Mϕ are rare in the porcine skin [7], but after injury, inflammation or vaccination circulating monocytes recruited from blood differentiate into mature Mϕ in the skin [15,17].

Inactivated porcine reproductive and respiratory syndrome virus (iPRRSV) was selected as vaccine-antigen to explore early immune response following skin vaccination. PRRSV is an important swine pathogen causing major worldwide economic losses [18–20]. As such, there is a need for effective PRRSV vaccines [21,22], which can already be administered to newborn or neonatal piglets [23]. Neonatal immune responses are directed towards a Th2 response, limiting both Th1- and B-cell responses in neonates compared to adults, often resulting in a reduced vaccine efficacy [24–26]. Moreover, murine studies have shown that neonatal LCs [27–29] were less effective APCs than adult LCs, which could influence skin vaccination in neonatal pigs. Therefore, it seems appropriate to apply a strategy that intensifies immune responses in the skin by stimulating APCs. It has been reported that potent immuno-stimulators, such as TLR agonists (TLRa), enhanced neonatal immune responses [30,31]. Therefore they could contribute to more effective vaccine responses in neonates after skin vaccination. In this study, three different TLRa, i.e. TLR1/2a (Pam3Cys), TLR7/8a (R848) and TLR9a (CpG ODN) were selected as they have shown potential to contribute to APC activation in blood of neonatal and adult pigs [32–34]. TLR7/8a and TLR9a have already proven efficacy after skin vaccination in adult pigs [35,36] and mice [37], but to our knowledge no studies have evaluated the efficacy of skin vaccination in neonatal pigs with TLRa.

In the present study, neonatal and adult pigs were administered vaccines to the skin using DMN-patches containing iPRRSV-antigen with different TLRa as adjuvant. We investigated the potential of different TLRa to induce early immune responses in the skin and draining LN and whether these early immune responses were age-dependent.

2. Materials and methods

2.1. Vaccines

The vaccine-antigen consisted of binary ethylenimine (BEI) inactivated PRRSV type 1 strain 07 V063 (iPRRSV, each dose is 1.0×10^8 $TCID_{50}$). This antigen was prepared as described previously [38]. Three different vaccines were prepared for the vaccination of neonatal and adult pigs, each containing a different TLRa as adjuvant: TLR1/2a, Pam3Cys L2000 from EMC Micro-collections; TLR7/8a, R848, Resiquimod from InvivoGen; and TLR9a, CpG ODN-type A sequence D32, ggTGCGTCGACGCAGggggg, from Eurofins Genomics. A full vaccine dose contained 250 μg of the individual TLRa mixed with iPRRSV.

Additionally, three different DMN-patches were produced for use in neonates only, that contained either a) the vaccine-antigen (iPRRSV) only or b) iPRRSV with an oil-in water (O/W) stable squalene (SE) emulsion [39] (29% volume per volume (v/v)) and a mixture of the three TLRa (SE + TLRa) or c) squalene emulsion without the TLRa mixture (SE). This TLRa combination mixture contained 80 μg of each of the three TLR agonists mentioned above. The SE and SE + TLRa patches were only evaluated for macroscopic and histopathologic changes. The DMN-patches were prepared as previously described [38,40], using trehalose and polyvinylalcohol (PVA) as excipients. The iPRRSV and TLRa were dispersed homogenously throughout the full volume of the microneedle pore. One full vaccine dose was contained in 200 microneedles, which were 500 μm in height. Placebo patches, containing excipients only, were also produced and administered.

2.2. Animals and experimental design

Six (n = 6) male twelve-week-old pigs and twelve (n = 12) male four-day-old piglets (Topigs Norsvin Z-line, commercial breed) from 4 different sows with the same parity were used. The pigs were purchased from a PRRSV-negative, defined high health status farm (van Beek SPF Varkens B.V., the Netherlands). The adult experiment started after one week of acclimatization, the neonatal experiment started after two days of acclimatization (neonatal pigs were weaned at 1–2 days of age). After weaning the piglets were fed *ad lib* with the Opticare™ milk programme (Swinco B.V., Helmond, the Netherlands). The animal experiment was conducted in accordance with the Dutch animal experimental and ethical requirements and the project was approved by the Dutch Central Authority for Scientific Procedures on Animals (CCD) (Permit number: ADV401002015356).

All of the pigs, adult and neonate, received one vaccine-dose with one specific TLRa on the medial side of each hind-leg (n = 4 for every TLRa, Table 1). In adults, on the skin of the the left leg two patches (200 DMN in total) containing iPRRSV and either TLR1/2a or TLR9a (250 μg) and one placebo patch (Table 1), were administered. On the right leg of adult pigs 2 patches (200 DMN in total) containing iPRRSV and either TLR9a or TLR7/8a (250 μg) and one placebo patch (Table 1 and supplementary Fig. 1SA), were administered. Due to the smaller size of neonates and more limited skin surface we used a different shape of patches in neonates. The total vaccine dose in neonates was the same as in the adult pigs. However, in neonates the placebo patch i.e. without vaccine, was administered to separate animals. In neonates, the left leg was administered with four patches (200 DMN in total) containing a full dose of iPRRSV or iPRRSV with TLR1/2a or TLR9a (250 μg). Neonatal right legs received 4 patches containing iPRRSV with TLR7/8a, (250 μg) or SE or SE + TLRa or they received placebo patches (Table 1 and supplementary Fig. 1SB).

To macroscopically evaluate the local reaction in the skin, six hours post-administration, half of the vaccine-patches were removed from each leg (one vaccine patch from adults or 2 vaccine patches from neonates) and one placebo patch was removed from each adult pig. The remaining patches were removed at 24 h, which was the time of necropsy. During necropsy, skin biopsies (8 mm punch biopsies) were taken from treated and untreated skin (non-vaccinated; NV). The skin of the caudal ventral abdomen on the right side (comparable skin thickness as medial side hind-leg) was selected as NV control skin. The right axillary lymph node was sampled as control lymph node next to the draining lymph node (superficial inguinal lymph node).

2.3. Assessment of post-vaccination reaction: Macroscopy and histology

After removal of the DMN-patches, at 6 h and 24 h after patch application, the skin was graded from 0 (no visible change) to 3 (discolouration and/or swelling of the skin) [38]. Skin biopsies taken 24 h after patch application were fixed in 10% neutral buffered formalin and routinely processed into paraffin-embedded tissue samples. Three consecutive 4 μm thick sections were cut and stained with haematoxylin and eosin (HE). HE-stained slides were semi-quantitatively analysed in a "blinded" manner per animal by

Table 1
Adjuvant overview of skin vaccines with iPRRSV.

Animal	Left leg		Right leg	
Adult				
#1, 2	TLR1/2a	Placebo	TLR9a	Placebo
#3, 4	TLR1/2a	Placebo	TLR7/8a	Placebo
#5, 6	TLR9a	Placebo	TLR7/8a	Placebo
Neonate				
#1 to 4	TLR1/2a		TLR7/8a	
#5 to 8	Antigen only		Placebo	
#9, 10	TLR9a		SE	
#11, 12	TLR9a		SE + TLRa	

The dissolving-microneedle (DMN) patches contained different Toll-like receptor agonists (TLRa) as adjuvant: TLR1/2a (Pam3Cys), TLR7a (R848) and TLR9a (CpG ODN), combined with inactivated porcine reproductive and respiratory syndrome virus (iPRRSV). Each pig received two different DMN-patches, one on the left leg and on one on the right leg. Placebo patches without vaccine were applied on each leg of the adult pig next to the vaccine. Only the neonates received patches with only iPRRSV (antigen only) and additionally patches with a stable emulsion (SE) with a mixture from the three TLRa (SE + TLRa) or without (SE).

a board-certified veterinary pathologist. Each section was graded for number of inflammatory cells, hyperaemia and dermal oedema, resulting in an overall score from 0 to 3. Detailed scoring of histology is described in supplementary data (Table S1A). Additionally, the NV neonatal and adult skin was compared by measuring the epidermal and dermal thickness (three measurements per animal, objective 2x) with cellSense software V1.16 from Olympus.

2.4. Identification of antigen presenting cells in the skin

For cell phenotyping skin biopsies were snap-frozen on dry-ice in Tissue-Tek® optimal cutting temperature compound (O.C.T.™). Cryosections (8 μm) were cut with a Leica CM3050S cryostat. Before processing for immunohistochemistry (IHC) with 3,3′-diaminobenzidine (DAB) or immunofluorescence (IF) microscopy, tissue sections were air-dried, fixed in cold acetone, and stained with mouse anti-pig CD1a (IgG2a, clone 76–7-4 from Southern Biotech). For IHC the anti-CD1a was incubated for 4 h at room temperature. The ChemMate Envision Detection kit™ /mouse with DAB from Dako was used according to manufactures' instruction for antigen visualisation. IHC slides were analysed semi-quantitatively in a "blinded" manner and graded based on the number of positive staining cells in epidermis (0–3 for LC) and dermis (0–3 for dDC). Detailed IHC-scoring is described in the supplementary data (Table S1B).

For IF microscopy, skin samples were stained using the three primary antibodies: CD1a (as earlier described), mouse-anti-pig MHCII (IgG1, clone K247.3G8 from Bio-rad) and mouse anti-pig CD163 (IgG1, clone 2A10/11 from Bio-rad). The following secondary antibodies were used: Alexa Fluor 647® goat anti-mouse (IgG2a from Bio-rad) and Alexa Fluor 568® goat anti-mouse (IgG1a from Bio-rad) (supplementary data Table S2). Hoechst 33,342 staining dye solution (Sigma-Aldrich) was applied for nuclear staining. From every skin sample, 3 different images were acquired using consistent microscope settings (Leica DM6b upright microscope, 20x objective), and positive cells were selected manually and counted with the Leica Application Suite X software (version 2.0.0.14332). For negative controls (IHC and IF microscopy), the primary antibodies were replaced by isotype controls or only the secondary antibodies were used.

2.5. Gene analysis with high throughput qPCR

2.5.1. Gene selection

Based on literature search and previous porcine expression studies of subcutaneous tissue [41], we selected 86 genes likely involved in the early immune response after skin vaccination. For 10 important genes, two different primer sets were selected. The genes were divided into 5 main clusters, which are presented in the results section. Primer sequences, amplicon lengths and if applicable literature reference for selected genes are described in the supplementary data. (Table S3).

2.5.2. RNA extraction and cDNA synthesis

Skin biopsies and lymph nodes were snap-frozen on dry-ice and stored at −80 °C. Before RNA extraction samples were homogenized in TRIZOL® and RNA was extracted using the Directzol® RNA MiniPrep (Zymo Research) according to the manufacturer's instructions. RNA quantity and purity were assessed with the NanoDrop 1000™ (Thermo Fisher Scientific). The purity was evaluated based on optical density (OD) using the A260/280 and A260/230 ratios. RNA-quality was assessed by measuring the RNA integrity number (RIN) on an Agilent Bioanalyzer (Agilent Technologies) using the RNA 6000 nano kit (Agilent Technologies). Samples with a RIN > 5 were considered appropriate for further processing. The lymph node samples had a mean RNA integrity of 7.9 ± 0.8 (SD), while the skin samples had a lower average RIN value of 6.6 ± 0.8 (SD) (results not shown). From each RNA sample, duplicate cDNA syntheses were made from 500 ng extracted RNA using the QuantiTect Reverse Transcription kit from Qiagen according to manufacturer's instructions. The cDNA was diluted 1:10 in low-ethylenediaminetetraacetic acid (EDTA) TE-buffer (Panreac AppliChem) before pre-amplification. 3 μl TaqMan PreAmp Master Mix (Applied Biosystems), 2.5 μl 200 mM mix of all primers used subsequently for qPCR, 24 μl low- EDTA TE-buffer (Panreac AppliChem) and 2.5 μl diluted cDNA was mixed and incubated at 95 °C for 10 min followed by 20 cycles of 95 °C for 10 s and 60 °C for four minutes. The pre-amplified cDNA was treated with 16 U Exonuclease I (New England Biolabs) for 30 min at 37 °C and stored at –20 °C until further processing.

2.5.3. High-throughput qPCR

qPCR was performed using 96.96 Dynamic array integrated fluid circuit (IFC) chips (Fluidigm) in the BioMark HD System (Fluidigm), combining 96 primer sets with 96 samples. Each primer mix contained 3 μl 2X Assay Loading Reagent (Fluidigm) and 3 μl of 20 mM forward and reverse primers suspended in low-EDTA TE-buffer (Panreac AppliChem). The sample mixes were prepared using 3 μl 2X TaqMan Gene Expression Master Mix (Applied Biosystems), 0.3 μl 20X DNA Binding Dye Sample Loading reagent (Fluidigm), 0.3 μl 20X Evagreen (Panreac AppliChem), 0.9 μl low-EDTA TE-buffer (Panreac AppliChem) and 1.5 μl pre-amplified diluted (1:10) cDNA. Before loading samples and primers, the chip was primed in an HX IFC controller (Fluidigm). After priming, 5 μl of each sample mix and primer mix were distributed into the appropriate compartments and loaded into the chip in the HX IFC controller. Thereafter the chip was inserted in the BioMark real-time PCR instrument (Fluidigm) and the following program

was used: two minutes at 50 °C and 10 min at 95 °C, next 35 cycles with 15 s at 95 °C and one minute at 60 °C. Non-template controls and non-reverse transcriptase controls were included to indicate problems with contamination, non-specific amplification or genomic DNA, respectively. Standard curves constructed from three separate dilution series of pooled cDNA of all samples to determine the efficiency of each primer. Based on the melting curves, standard curves and control samples 13 genes (HPRT1 (25), CCL2 (117), CCR7 (608), GZMA (758), IL8 (37), SAA (158), TNF (125), IL12A (44), IL13 (279), IL23A (195), TLR7 (164), TLR8 (127), TLR9(81) were excluded from the skin sample analysis and 7 genes (HPRT1 (25), CCL2 (117), GZMA (758), IL13 (279), IL23A (195), IFNL3(298), IFNB (223) from the lymph node sample analysis.

2.5.4. qPCR data analysis

Raw data were inspected using Fluidigm Real-Time PCR Analysis software (v. 4.1.3). GeneEx5 (v. 5.4.4.119) (MultiD) was used for data pre-processing. To compensate for variation between dynamic chips, three pooled samples were used as interplate calibrators. Out of six tested reference genes, actin beta (ACTB), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), peptidylprolyl isomerase A (PPIA) and tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta (YWHAZ), were found to have the most stable expression in porcine skin and lymph nodes using both GeNorm and NormFinder in (GeneEx5 software). Expression data were normalized following the approach of Pfaffl [42] with geometric averaging of relative quantities of reference genes [43]. Gene expression changes were normalized against the average expression of control samples (NV skin or right axillary lymph node) of the same animal. Relative expression of genes (fold-change) of test samples was calculated for individual replicate separately and passed to the following statistical analysis. Normalization was performed with 'in-house' script (Supplementary materials).

2.6. Statistical analysis

The data of the macroscopic changes, histology and IHC were analysed with a non-parametric Kruskal-Wallis test followed by a post-hoc Dunnett test for multiple comparisons. The data of the IF microscopy were analysed with a one-way ANOVA followed by a post-hoc Dunnett test for multiple comparisons. Statistical assessment of differences between NV samples for adults and neonates was performed with an unpaired *t* test (two-tailed with a Welch's correction). Statistical analysis was performed with GraphPad Prism 8.2.1 software. *P*- values <0.05 were considered significant statistically ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$).

Statistical assessment of expression changes of selected genes during the experiments was performed using one-way ANOVA test (one parameter, treatment) or two-way ANOVA (two parameters, treatment and age, and their interaction). Influence of individual treatments on gene expression was analysed with Mann-Whitney *U* test. Holm-Bonferroni correction was used to adjust p-value threshold for multiple testing. Statistical analysis was performed using Python 2.7.15 and modules sklearn, statsmodels (biopython version 1.70), pandas (version 0.23.4), numpy (version1.15.1) and matplotlib (version 2.2.3).

3. Results

3.1. Macroscopic and histopathological changes after skin vaccination

First, the skin histology of NV (control) adult (Fig. 1A and B) and neonatal pigs (Fig. 1C and D) was evaluated and compared to identify possible age-dependent differences, which could influence the effect of skin vaccination. The neonatal and adult skin had a similar structure. However, the epidermis (Fig. 1B, arrow) and especially the dermis (Fig. 1B, arrowhead) were significantly thicker (1.5-fold and 1.8-fold, respectively) in the adult skin compared to the neonatal skin (Table 2). For both adult and neonates, the skin thickness was larger (>500 μm) than the length of the DMN (500 μm). The adult and neonatal dermis contained a comparable variety of adnexal structures (i.e. hair follicle, sweat glands and sebaceous glands). However, the neonatal dermis was distinctly more cellular in the HE stain compared to the adult skin, most likely due to a larger number of fibroblasts and immune cells.

Macroscopic skin changes were evaluated 6 h and 24 h after DMN-patch application. Six hours after application of the TLR7/8a patch, a macroscopic grade 1 to 3 skin reaction was induced in all adult and neonatal pigs (results not shown), which continued with a similar intensity to 24 h after vaccination (Fig. 2A and B). In both age-groups the TLR1/2a and 9a patches induced only a mild and occasionally moderate skin reaction (grade 0 to 2), which was significantly less severe than the TLR7/8a patch and comparable to the placebo patch. The antigen-only patch and the SE patch, which were only used in neonates, induced no skin changes. While the SE + TLRa patch, also only used in neonates, induced mild skin changes (Fig. 2A and B)

The macroscopic findings were supported by the histopathologic changes, where in both adults (Fig. 2C) and neonates (Fig. 2D) the TLR7/8a patch induced mainly grade 3 changes. The histopathologic changes induced by the TLR1/2a and 9a patches were restricted to maximum grade 2 in both adult and neonatal pigs (Fig. 2C and D), where adult pigs showed more skin changes for TLR1/2a (macroscopy and histology) than neonatal pigs (Fig. 2-A-D). The antigen-only patch, which was only used in neonates, induced no skin changes in histology. While the SE patch and SE + TLRa patch, also only used in neonates, induced mild to moderate skin changes respectively. Histologically, the skin changes, especially grade 3, were characterized in both age groups by a focal extensive infiltration of mononuclear inflammatory cells and lower numbers of neutrophils in the superficial dermis extending to the epidermis (Fig. 2E and F, arrow adult skin) with pustule formation (Fig. 2G and H, asterisk neonatal skin). Focally, the dermal-epidermal junction was affected (Fig. 2F and H) in both age-groups.

3.2. In situ characterization of antigen presenting cells after skin vaccination

To characterize and quantify the number of APCs within the epidermis and dermis (Table 2), we used surface-marker CD1a in IHC (Fig. 3), and surface-markers CD1a, MHCII and CD163 in IF microscopy (Fig. 4). First we evaluated the normal NV skin to identify any age-dependent differences which could influence the skin vaccination. $CD1a^{+}$ cells (LC) in the epidermis were localized near the basement membrane as shown by IHC (Fig. 3A, arrowhead) and IF microscopy (Fig. 4A, arrowhead). IF microscopy showed that all $CD1a^{+}$ cells in the epidermis were $MHCII^{+}$ (data for CD1a and MHCII double stained sections are not shown) and $CD163^{-}$ (Fig. 4A). The number of LCs, *in situ* quantified by IHC (Fig. 3E) and IF (Fig. 4B and C), was similar in the NV epidermis of adult and neonatal pigs.

In the dermis, the dDCs were $CD1a^{+}$ in IHC (Fig. 3A, arrow) and $CD1a^{+}CD163^{+/-}$ (Fig. 4A, arrow) or $CD1a^{+}MHIIC^{+}$ in IF microscopy. All $CD1a^{+}$ cells in dermis also expressed MHCII, in both neonates and adults (results not shown). The dDCs were mainly located in the superficial dermis near the epidermal basement membrane in the proximity of small blood vessels. There was no significant difference in the number of dDCs in NV neonatal and adult pigs based on the results of the IHC (Fig. 3F) and IF microscopy (Fig. 4D). $CD1a^{+/-}CD163^{+}$ dermal macrophages (MΦ) were located

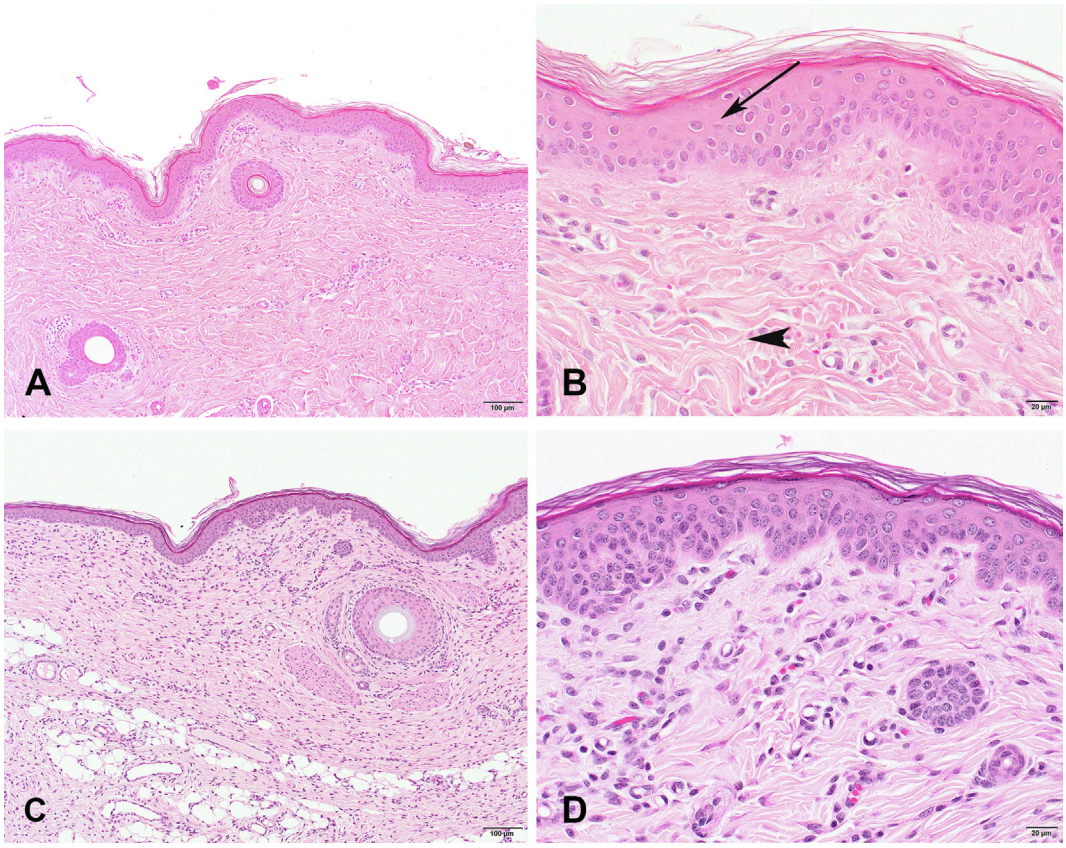


Fig. 1. Normal skin histology in adult and neonatal pigs. Skin biopsies of non-vaccinated (NV) adult (A and B) and neonatal (C and D) control skin were stained with haematoxylin and eosin (HE). The epidermis is indicated with an arrow and the dermis with an arrowhead (B). The NV neonatal control skin (C and D) showed a more cellular dermis than the adult control skin (A and B); For A and C we used objective 10x and for B and D objective 40x.

Table 2
Comparison non-vaccinated adult and neonatal skin.

Histology normal skin (HE)		
Thickness	**Adult**	**neonate**
Epidermis	62 μm ± 11	41 μm ± 5 *
Dermis	1263 μm ± 107	698 μm ± 160 ***
	Identification APCs skin	
	IFT	***IHC***
Epidermis *LC*	$CD1a^+$ $MHCII^+$ $CD163^-$	$CD1a^+$
Dermis *dDC*	$CD1a^+$ $MHCII^+$ $CD163^{+/-}$	$CD1a^+$
Dermis *Mϕ*	$CD1a^{-/+}$ $CD163^+$	Not evaluated

Adult (n = 6) and neonatal skin (n = 12) were compared for epidermal and dermal thickness with a haematoxylin and eosin (HE) stain. The neonatal dermis and epidermis were significantly thinner than the adult dermis and epidermis. The different antigen presenting cells (APC): Langerhans cell (LC), dermal dendritic cell (dDC) and dermal macrophages (Mϕ) were evaluated with immunohistochemistry (IHC) and immunofluorescence (IF) microscopy using different surface markers.

in the superficial and deeper dermis (Fig. 4A, arrowhead) and the number of macrophages was significantly higher in the dermis of NV neonatal pigs compared to NV adult pigs (Fig. 4E).

Both IHC and IF microscopy were used to quantify the number of DCs (LC and dDC) and dermal macrophages in the skin 24 h after DMN-patch application. In both the adult and neonatal pigs, the number of LCs in the epidermis (($CD1a^+$) in IHC (Fig. 3E) and $CD1a^+$/$MHCII^+$ or $CD1a^+CD163^-$ in IF microscopy (Fig. 4B and C)) did not significantly change in any of the used skin vaccines compared to the NV skin.

The number of dDCs in the vaccinated skin did not significantly change compared to the NV skin in the adult and neonatal pigs as evaluated with IHC for expression of CD1a (Fig. 3F) and with IF microscopy for dermal expression of $CD1a^+MHCII^+$ (Fig. 4D). Also, the number of dermal Mϕ ($CD163^+$) did not change significantly following vaccination (Fig. 4E). For both IHC and IF microscopy there was a large variation within the different samples of neonatal pigs and within the different samples of adult pigs.

3.3. Gene expression in skin and lymph node

The selected immune genes were grouped according to gene ontogeny (GO) in 6 different clusters (Table S3): (1) APC activation and migration, (2) acute phase inflammatory response; (3) anti-inflammatory response, (4) Th-directed response, (5) TLR expression and activation, and (6) other/epithelial junction. After data processing and removal of duplicate primers, valid expression data from 74 genes of the skin samples and 74 genes of the LN samples were obtained. Differences in gene expression were considered relevant when a statistically significant fold change of >2 or <0.5 compared to the reference sample was measured and only the genes with such a fold change are reported below. Gene expression of SE and SE + TLRa patches, only used in neonates, was not analysed.

3.3.1. Gene expression in non-vaccinated adult and neonatal skin and lymph node

To investigate potential age-dependent differences in basal gene expression in NV, control skin and control LN, we compared the level of expression in the neonatal samples with the level of expression in the adult samples for 74 genes (Table S4). The neonatal skin showed a relevant different expression level in 9 of 74 investigated genes. The main difference was the lower expression of APC activation/migration related genes (e.g. CD86 and SLA-

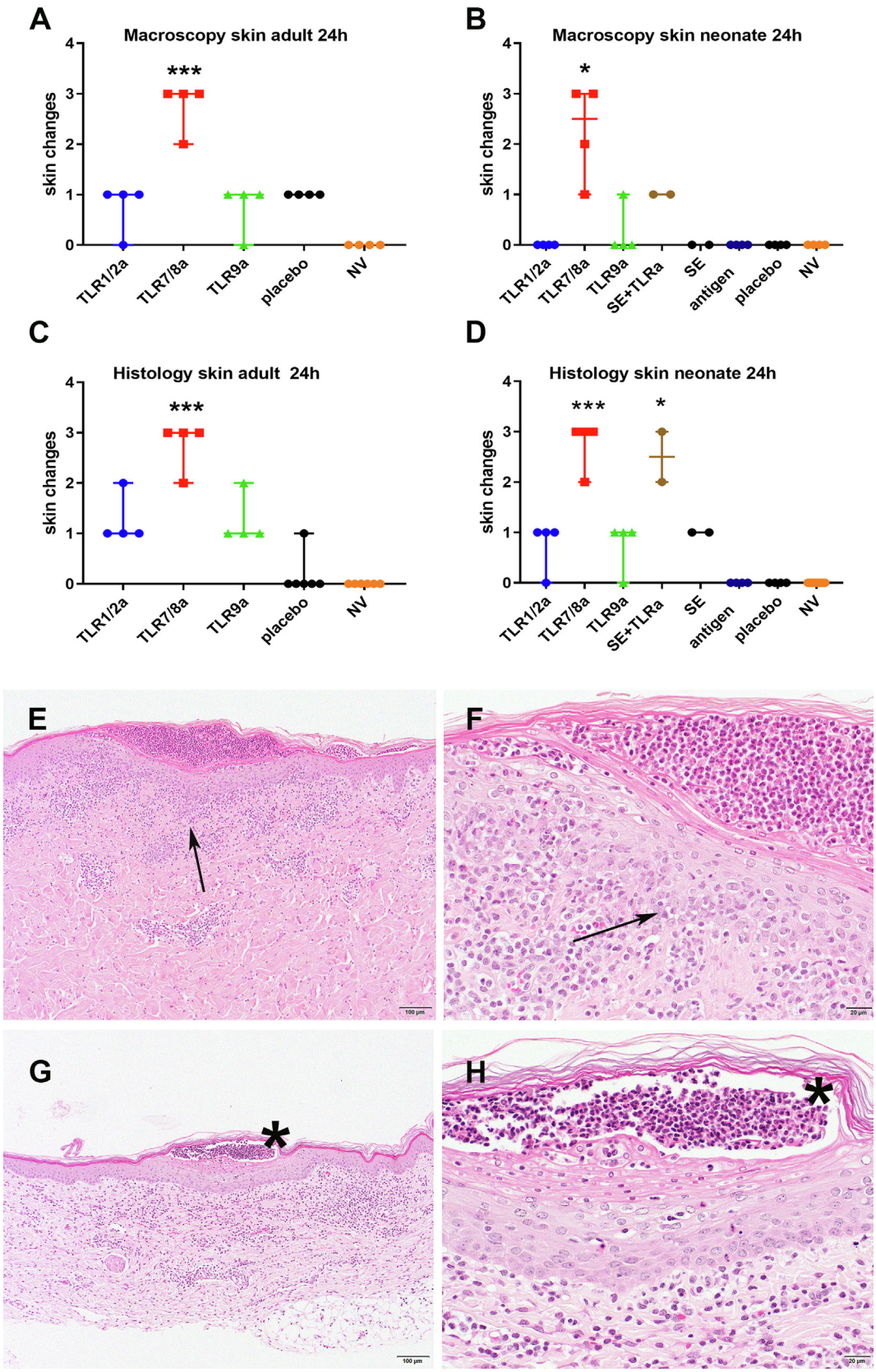


Fig. 2. Skin changes after vaccination with dissolving-microneedle (DMN) patches. The DMN patches contained vaccines with different Toll-like receptor (TLR) agonists, a squalene emulsion (SE), a mixture of TLR agonists and SE (SE + TLRa), antigen only, or a placebo patch without vaccine. (A) macroscopic skin changes in adult pigs graded from 0 to 3, 24 h after skin vaccination; (B) macroscopic skin changes in neonatal pigs; skin biopsies taken 24 h after vaccination were stained with haematoxylin and eosin (HE) and graded from 0 to 3 for adult (C) and neonatal (D) pigs. Skin vaccination with TLR 7/8 agonist (TLR7/8a) induced grade 3 changes in adult (E and F) and neonatal (G and H) skin characterized by a focal extensive infiltration of mononuclear inflammatory cells and lower numbers of neutrophils in the superficial dermis extending to the epidermis (E and F, arrow) with pustule formation (G and H, asterisk). Focally, the dermal-epidermal junction was affected (F and H). Each symbol represents one animal, line indicates the median of a group (n = 2–6) with the 95% CI. (*$p < 0.05$, **$p < 0.01$) compared to non-vaccinated (NV) control skin. For E and G we used objective 10x and for F and H objective 40x.

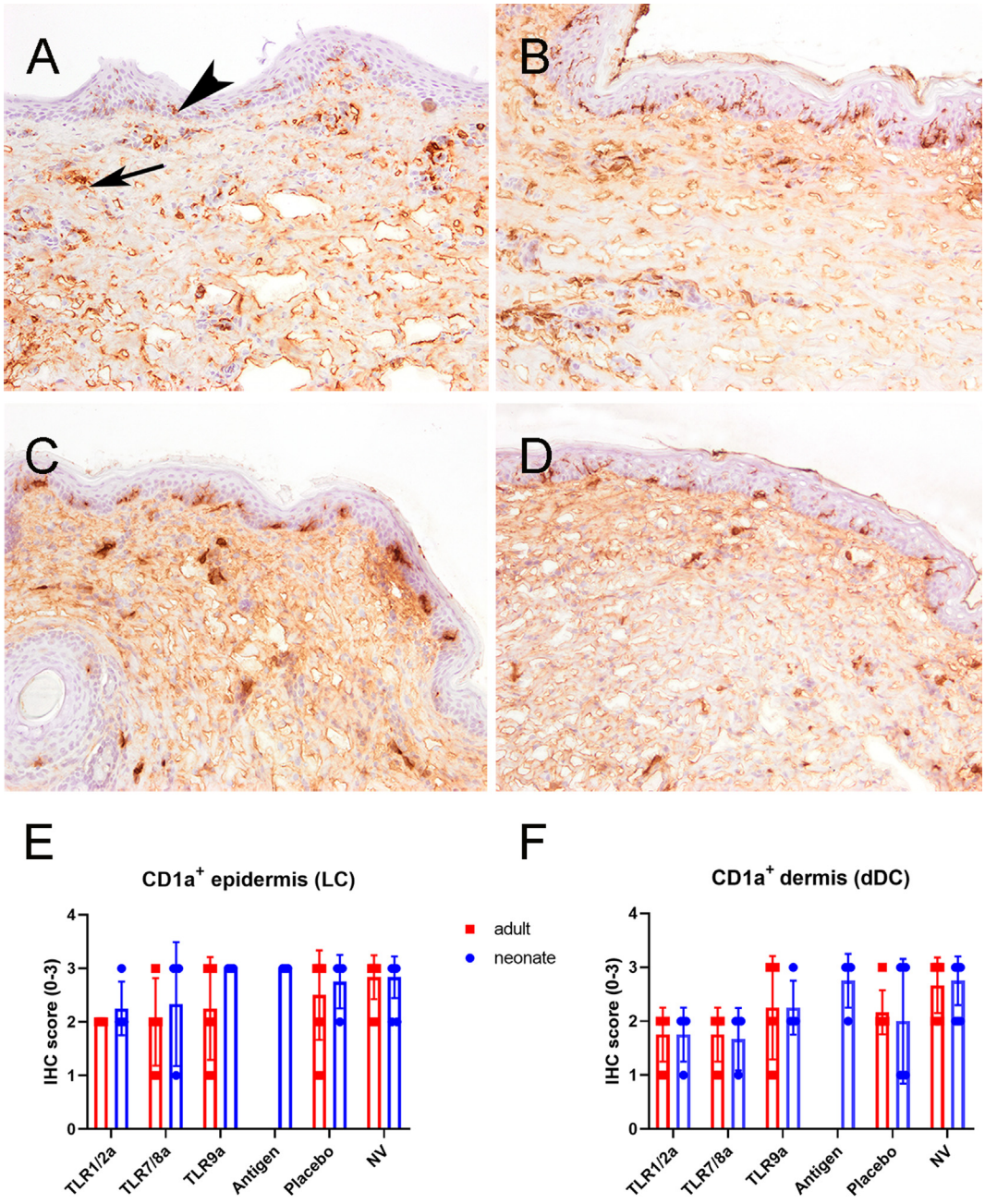


Fig. 3. CD1a expression in adult and neonatal skin measured with immunohistochemistry (IHC). Adult (A and B) and neonatal (C and D) skin was evaluated for CD1a expression (objective 20x) to identify Langerhans cells (LC) in the epidermis (A, arrowhead) and dermal dendritic cells (dDCs) in the dermis (A, arrow). The CD1a expression is comparable in non-vaccinated (NV) adult (A) and neonatal skin (C). After skin treatment with TLR7/8a vaccine (B, adult and C, neonate) the CD1a expression in epidermis or dermis was not affected. After vaccination (24 h) with different Toll-like receptor (TLR) agonists, antigen only or with placebo patches, the skin was evaluated for number of Langerhans cells (LCs) in the epidermis and dermal DCs (dDc) with IHC and graded from 0 to 3 (E and F). Each symbol represents one animal (adult = red square and neonate = blue circle). The line indicates the median with S.D. Statistical significance was calculated for the different vaccines compared to NV animals for every age-group or for NV neonatal samples compared to NV adult samples. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

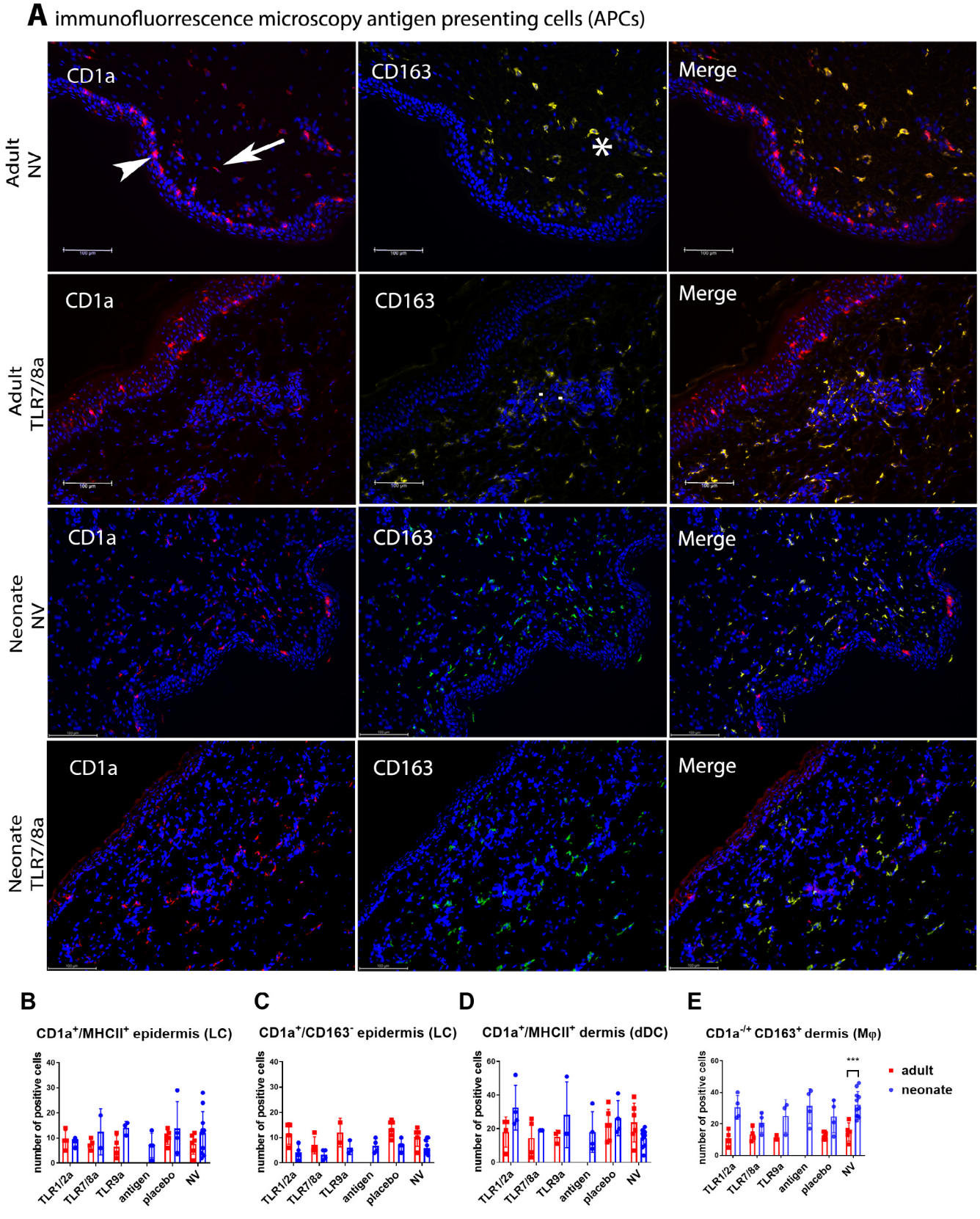
DRB1, surface-markers for antigen presentation) in the neonatal skin. The macrophage surface-marker CD163 was the only gene that was significantly higher expressed in the NV neonatal skin compared to the adult NV skin. We found no difference in TLR gene expression in the neonatal skin compared to the adult skin. Both adult and neonatal skin showed no expression of TLR9, while we did find TLR9 gene expression in the LN of both age-groups.

The neonatal control LN showed a relevant different expression level in 24 of the 74 investigated genes compared to the adult control LN. Genes related to DCs/ antigen presentation (CD1a and CCR7) were less expressed, while genes related to monocytes/-macrophages (CD14 and CD163) were more expressed in the neonatal control LN compared to the adult control LN. All data on biologically relevant genes in neonatal NV skin and control LN compared to adult control samples are provided in supplementary Table S4.

3.3.2. Gene expression after skin vaccination in adult and neonatal pigs

To investigate the effect of skin vaccination on expression of the selected genes in the vaccinated skin and draining LN, 24 h after

administrations, we calculated the fold-change difference in gene expression between the NV control skin and control LN and the vaccinated skin and vaccinated draining LN. For both age groups, TLR7/8a vaccinated skin showed the most prominent induction of gene expression compared to NV control skin, with the overall number of upregulated genes being higher in neonates (38 of 74



genes) compared to adults (21 of 74 genes). In both age groups, especially the genes in the clusters APC activation/migration (e.g. CCL20, CCR7, CD86 and CXCL10) and TLR expression/activation (e.g. IFNL3, IRF7, OAS1 and RNASEL) were significantly upregulated. Neonatal pigs showed a high upregulation (fold-change > 25) of gene expression for the pro-inflammatory genes IL8, GZMB and CASP1 in the skin. No upregulation of these genes was observed in the adult skin samples after TLR7/8a vaccination. In contrast, the overall gene expression in the TLR9a vaccinated skin contained a higher number of downregulated genes in adult and neonatal samples compared to the other TLRa vaccines (Table 3a). This downregulation in gene expression was observed in all immune response clusters, except for the APC cluster (e.g. CCL20, CD86 and CXCL10) in neonatal pigs which showed an upregulation. The TLR1/2a vaccine was the only skin vaccine which did not induce a significant change in gene expression in the Th clusters compared to the NV control skin (Table 3a). The placebo vaccination induced mainly a downregulation in gene expression particularly in the adult skin (12 of 74 genes) and to a lesser extend in the neonatal skin (4 of 74 genes), especially for the APC activation/maturation cluster (e.g. CCL3 and CXCR4) and for the acute phase inflammatory response cluster (e.g. DEFB1). The antigen-only vaccine (Ag), only administered in neonates, induced only a regulation in a limited number of genes (3 of 74 genes).

Draining LNs of skin vaccinated with TLR7/8a regulated the expression of a larger number of genes (13 of 74 genes neonate and 10 of 74 genes adult) compared to the other skin vaccines in both age groups (<9 of 74 genes neonate and <3 of 74 genes adult) (Table 3b). In both age groups, nearly all upregulated genes in the LN samples of the TLR7/8a vaccine showed a fold-change of <5, while a large number of upregulated genes in the TLR7/8a vaccinated skin samples showed a fold-change of >10. Thereby the response to vaccination was larger in the skin compared to the draining LN (Tables 3a and 3b).

Draining LNs of the TLR7/8 vaccine showed particularly a gene upregulation in the pro-inflammatory immune response cluster (TP53, GZMB and SAA) in both age-groups. The upregulation of gene expression in the APC activation and migration cluster (e.g. CCR7, CCL19 and MMP9) was comparable for all the skin vaccines in both age-groups, including the Ag-only vaccine. Overall, the neonatal LNs contained a higher number of regulated genes compared to the adult LNs after skin vaccination, especially for the TLR9a vaccine (7 of 74 genes neonate versus 1 of 74 genes adult) and for the TLR1/2a vaccine (10 of 74 genes neonate versus 3 of 74 genes adult). The fold-changes of all significantly regulated genes in the vaccinated skin and draining LN are presented in Table 3a and Table 3b, respectively. In Supplementary Fig. S 2–7 the *p*-value threshold corrected for multiple testing (MTC) is shown for all analysed genes.

4. Discussion

In this study, neonatal and adult pigs were vaccinated with DMN-patches containing different TLRa in combination with iPRRSV-antigen to investigate the immune-stimulatory potential of the TLRa and whether or not these immune responses were age-dependent. We compared the NV neonatal skin to the NV adult skin to investigate if differences in skin thickness, location and quantity of APC subsets or TLR expression were associated with the higher number of up-or downregulated genes in vaccinated neonatal skin compared to adult skin. The significantly thinner skin of the neonate, mainly caused by the thinner dermis, was also observed in other porcine [44] and human studies [45]. We consider it unlikely that the thinner skin had an impact on skin vaccination in our study, because the length of the microneedles of the DMN patch (500 μm) did not exceed the thickness of the skin. We showed that the neonatal skin contained an equivalent number of DCs compared to the adult skin as was also observed in human studies [29,46]. However, the gene expression profile of the NV neonatal skin compared to the NV adult skin showed a lower basal expression of cell-surface markers for antigen presentation (e.g. CD86, SLA-DRB1 (MHCII)) and for DC markers (CD101 and FLT3). On the other hand, the neonatal skin contained a higher number of macrophages, which was supported by the enhanced neonatal basal gene expression of CD163 compared to adults. Finally, we showed that there was no difference in basal TLR gene expression within the neonatal and adult skin. Therefore, we consider it likely that the significantly larger number of dermal macrophages and differences in basal immune gene expression contributed to the age-dependent differences after skin vaccination.

In both the adult and neonatal pigs, the TLR7/8a skin-vaccine induced the most prominent histopathological skin changes and skin immune response at the vaccination site. This was illustrated by the highest number of differentially expressed genes in the skin and draining LN. We observed not only the expected upregulation of pro-inflammatory genes, which is in accordance with the leucocyte influx in the dermis, but also a higher expression of genes regulating APC activation and migration (CD86, CCR7 and CD69). However, in this study potential activation of LCs did not lead to migration of LCs to the dermis. This absence of DC migration to the dermis was also observed in human *ex vivo* skin studies [11,47,48] with TLRa as immune-stimulator.

For the TLR1/2a and TLR9a vaccines the local skin responses were less evident than for the TLR7/8a vaccine. The limited local skin response and number of regulated genes after TLR1/2a vaccination showed that the potential of Pam3Cys as adjuvant for skin vaccination in this study was limited. However, with a different delivery route this potential could be different as shown in a murine study [49]. Interestingly, TLR9a containing vaccine resulted in more downregulated than upregulated immune response genes in the skin of both age-groups, compared to the TLR1/2a and TLR7/8 vaccines. The gene expression by the TLR9a vaccine was more comparable to the Ag-only vaccine (without TLRa). We could therefore speculate that TLR9 stimulation with the CpG type A we used was limited. Other CpG types could be more appropriate for skin application [50] as shown by a porcine study with CpG type C [35]. They showed that TLR9a (CpG) induced an upregulation of several IFN inducing genes (MX1, IRF7), chemokines (CCL5, CXCL10) and IL10 at 24 h after skin vaccination in pigs. These genes

Fig. 4. Antigen presenting cells (APCs) in adult and neonatal skin measured with immunofluorescence microscopy (IF). (A) APCs were localized with surface-markers CD1a (red, first column), with CD163 (green/yellow, second column) or CD1a with MHCII (results not shown). In non-vaccinated (NV) normal skin (control) and 24 h after vaccination with different Toll-like receptor agonists (TLRa), antigen only or with placebo patches, the skin was evaluated for number of APCs in the skin: $CD1a^+$ Langerhans cells (LCs) in the epidermis (A, arrowhead), dermal DCs (dDc) in the dermis (A, arrow) and $CD163^+$ macrophages (Mϕ) (A, asterisk) in the dermis. There was no clear difference in number of staining cells and staining intensity between control and vaccinated animals for both age-groups. Individual variation between animals was observed as shown in (A). Objective used 20x, scale bars = 100 μm; The number of APCs was counted in adult and neonatal skin within three different 20x objective fields: LCs ($CD1a^+/MHCII^+$ (B) or $CD1a^+CD163^-$ (C)) in the epidermis. dDCs ($CD1a^{+/}$ $MHCII^+$ (D) in the dermis and Mϕ ($CD1a^{+/-}/CD163^+$) in the dermis (E). Each symbol represents one animal (adult = red square and neonate = blue circle). The line indicates the mean with the 95%CI. Statistical significance was calculated for the different vaccines compared to NV animals for every age-group or for NV neonatal samples compared to NV adult samples. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 3a
Heat map of relative gene expression in skin after skin vaccination.

gene cluster	gene	TLR1/2a		TLR7/8a		TLR9a		Antigen (Ag)		Placebo	
		adult	neonate	adult	neonate	adult	neonate	adult	neonate	adult	neonate
APC (activation, migration)	CCL19(908)	0.417			17.431						
	CCL20(995)	14.062	16.036	32.822	99.659		5.538		2.696		6.89
	CCL3(236)		0.392		4.587	0.094	2.272		0.684	0.408	0.523
	CCL5(121)		2.604	11.733	54.578						
	CCL8(1066)									0.622	
	CCR7(607)				9.757		0.361				
	CD101(572)	0.259	0.164		4.677	0.212				0.14	
	CD14(614)				3.877				0.662	0.701	0.633
	CD163(150)									2.805	1.917
	CD1A(556)		0.482								
	CD209(586)										
	CD40(9)			4.649	6.202						
	CD69(267)			12.862	12.303						
	CD80(1063)										
	CD86(560)			10.169	22.409		2.864			0.601	
	CXCL10(111)			333.917	2281.509		19.906		3.104		6.26
	CXCR4(575)		1.51		2.611	0.422				0.384	0.525
	FLT3(580)				5.315						
	MMP2(332)	0.398			0.594				0.733	0.507	0.713
	MMP9(335)			9.555	39.372		3.146				
	MX1(501)			47.369	16.583					0.731	
	SLA-DRB1(593)				2.755					0.728	
acute -phase inflammatory respons	C3(10)	0.722	3.585	1.704	19.155	0.655					
	CASP1(184)			9.374	84.067						
	CASP3(383)										0.755
	CRP(12)					0.162	0.363			0.275	
	DEFB1(779)	0.256	0.3	0.277	0.684	0.358	0.402		0.144	0.188	0.269
	GZMB(208)				39.284						
	ICAM1(369)	0.233		2.703	5.666					0.324	
	IL8(36)				18.904		0.247				
	NOS2(102)										0.347
	SELE(106)				3.302		1.615		1.895		2.081
	SELP(461)									0.268	
	TNF(74)				4.518		4.556			0.158	
	TP53(493)		0.552						0.748		
	VCAM1(346)			7.064	2.723	0.547			0.658	0.762	0.569
anti-inflammatory response	IDO1(308)			218.731	140.552		2.353				
	IL10(133)						0.39				
	IL1RN(142)	2.379		31.741	10.631						2.52
	TGFB(860)	0.733	1.75	0.386	0.598	0.52				0.404	
Th response	CD4(326)										
	IFNA(201)										
	IFNB(223)						0.226				
	IFNG(50)						0.366				
	IL12B(45)				13.22		0.384				
	IL18(234)								0.627	0.492	
	IL1A(157)						1.523				6.229
	IL1B(233)			18.036	12.163	0.376	0.46				
	IL4(108)										0.354
	IL5(771)					0.321	0.439			0.539	
	IL6(232)				13.376						
TLR expression and activation	IFNL3(298)			76.119	14.196		0.436				
	IRF3(112)				1.609	0.463					
	IRF7(149)			22.026	13.087					0.464	
	IRF9(289)										
	MYD88(179)		0.611			2.457					1.734
	NFKB1(612)		1.61	3.39	3.167		1.351		0.701		
	OAS1(503)			6.426	12.399						
	PIK3R1(521)										
	RNASEL(487)			23.594	13.131					0.669	
	TLR1(188)										
	TLR2(100)				8.218						
	TLR3(123)		3.35	9.159	11.557						
	TLR4(235)	0.511		2.888							
	TLR5(364)	0.419		0.293		0.326				0.187	0.45
	TLR6(1303)										
	TLR7(124)		2.083		11.333						
	TLR8(183)				11.572						
	TLR9(99)										
Other	CDH1(1069)										
	CLDN1(360)										
	OCLN(358)										
	TNNC2(480)										
	TNNI2(485)		0.289		0.185				0.51		0.566
Total 74 genes	up	2	5	21	38	1	7		2	1	5
	down	6	5	3	1	9	11		1	12	4

For each vaccine, only the significant relative fold-changes are presented for immune response genes 24 h after skin vaccination. The relative fold-change of the individual genes in the vaccinated skin (n = 4) compared to the non-vaccinated skin (n = 4) is expressed in adult and neonatal pigs. The colour scheme for the relative gene expression is as follows: the yellow results are not significantly changed after vaccination and fold-change is not indicated or have a significant fold-change between 0.5 and 2; <0.5 is dark green (i.e. the respective gene is down regulated); between 2 and 10 is red (i.e. the gene is moderately upregulated) or >10 is dark red (i.e. the gene is strongly upregulated).

Table 3b
Heat map of relative gene expression in draining lymph node after skin vaccination.

gene cluster	gene	TLR1/2a		TLR7/8a		TLR9a		Antigen (Ag)	
		adult	neonate	adult	neonate	adult	neonate	adult	neonate
APC (activation, migration)	CCL19(908)	1.273	2.269		2.809		1.641		2.508
	CCL20(995)						2.123		
	CCL3(236)								
	CCL5(121)	1.373	1.266				0.569		1.367
	CCL8(1066)		0.766		1.201		0.7		0.716
	CCR7(607)	1.873	2.605	1.746	3.426		2.535		4.351
	CD101(572)		2.129		2.18		2.967		4.714
	CD14(614)	1.634		1.879			0.567		
	CD163(150)	2.247	0.799	2.632			0.606		
	CD1A(556)		2.166	1.372	1.906		2.082		2.018
	CD209(586)		0.728		0.749		0.482		0.752
	CD40(9)		1.389		1.429				1.165
	CD69(267)		1.465		1.733		1.935		1.18
	CD80(1063)	1.553		1.427	1.337	1.411			1.252
	CD86(560)				1.442				
	CXCL10(111)						0.824		0.538
	CXCR4(575)		2.029		1.732		1.757		1.95
	FLT3(580)		1.711		1.894		1.468		1.497
	MMP2(332)		1.375				0.812		1.147
	MMP9(335)	1.966	5.562		2.32		2.338		3.678
	MX1(501)	1.574	1.209	2.383	2.217		0.673		
	SLA-DRB1(593)		1.669		1.553				1.495
acute -phase inflammatory response	C3(10)	0.796		0.802		0.822			
	CASP1(184)		1.329	1.515	1.818		1.18		
	CASP3(383)			1.397	1.277	1.418			
	CRP(12)								0.679
	DEFB1(779)				3.679				
	GZMB(208)			3.346	1.639				
	ICAM1(369)	1.821		1.778			0.73		1.387
	IL8(36)					2.349	3.698		1.859
	NOS2(102)			1.657			0.696		
	SAA(158)			6.833					
	SELE(106)		1.509	2.036	3.57				
	SELP(461)		1.536	1.713	1.447		1.158		1.712
	TNF(74)	0.774		0.597		0.611	0.768		
	TP53(493)	1.853	2.467	2.097	1.951				1.352
	VCAM1(346)								0.857
anti-inflammatory response	IDO1(308)		2.39		2.991	1.451	1.644		
	IL10(133)		0.5		0.531		0.437		0.494
	IL1RN(142)		1.939	3.278	2.218		1.979		2.29
	TGFB(860)	1.392		1.267		1.364			
Th response	CD4(326)	1.637		1.336	1.217		0.705		1.506
	IFNA(201)	1.681		1.339	0.809				1.24
	IFNG(50)						1.574		
	IL12A(44)				2.654				
	IL12B(45)				1.547		1.535		1.259
	IL18(234)								0.765
	IL1A(157)		0.642		0.743				
	IL1B(233)				2.783		1.964		1.286
	IL4(108)	0.538		0.501		0.516			
	IL5(771)				1.665		1.413		
	IL6(232)			3.192		1.64			1.364
TLR expression and activation	IRF3(112)		1.29		1.144	0.864	0.878		1.097
	IRF7(149)		1.586	2.506	2.18		0.683		
	IRF9(289)						0.67		
	MYD88(179)				0.831		0.696		
	NFKB1(612)	1.223	1.316	1.375	1.311	1.247	0.803		1.274
	OAS1(503)	1.67	1.391		1.481		0.822		
	PIK3R1(521)	1.918		1.737		1.464	0.702		
	RNASEL(487)	1.509	1.269	1.313	1.45	1.354	0.904		
	TLR1(188)	1.83		1.567		1.445			
	TLR2(100)		0.732		0.73		0.735		0.789
	TLR3(123)		0.699						0.59
	TLR4(235)		0.798		0.641		0.607		
	TLR5(364)	1.788				1.501	0.764		
	TLR6(1303)	1.685	0.858	1.345	0.74		0.735		
	TLR7(124)		0.841		0.892				0.744
	TLR8(183)		0.775	1.363	0.863		0.617		0.808
	TLR9(99)		1.907		1.665	0.77			1.724
Other	CDH1(1069)	1.477			1.208				
	CLDN1(360)								
	OCLN(358)	2.171	1.57				0.862		
	TNNC2(480)								
	TNNI2(485)	1.363	2.3	1.548	2.347		2.095		2.502
Total genens 74 genes	up	2	9	9	13	1	7		7
	down	0	1	0	0	0	0		0

For each vaccine, only the significant relative fold-changes are presented for immune response genes 24 h after skin vaccination. The relative fold-change of the vaccinated draining lymph node (LN) (n = 4) compared to the non-vaccinated LN (n = 4) is expressed in adult and neonatal pigs. The colour scheme for the relative gene expression is as follows: The colour scheme for the relative gene expression is as follows: the yellow results are not significantly changed after vaccination and fold-change is not indicated or have a significant fold-change between 0.5 and 2; <0.5 is dark green (i.e. the respective gene is down regulated); between 2 and 10 is red (i.e. the gene is moderately upregulated) or >10 is dark red (i.e. the gene is strongly upregulated).

were not significantly differentially regulated in our study, except for CXCL10.

The early immune response in the draining LN, is essential to induce and modulate the subsequent adaptive immune response. In all draining LNs, except for the LNs of adult pigs vaccinated with TLR9a, the gene expression of CCR7 was significantly upregulated, which could be related to an increased expression on single DCs and DC maturation [51]. On the other hand, the fold-change in gene induction in the draining LN was clearly lower compared to the genes that were induced in the skin after vaccination. The duration needed for LN activation after skin vaccination could be the cause of this lower gene induction in the draining LNs. Significant gene activation in the draining LN with CpG vaccination in goats [52] required 72 h. On the other hand, several studies with pigs [53–55] and neonatal primates [56] reported significant gene upregulation in the draining LN already within 24 h following skin and i.m. vaccination. The overall lower gene induction of the draining LNs in our study could also be related to the poor immunogenic properties of the used vaccines, which was shown in a PRRSV vaccine study with similar skin vaccines [38].

Differences in basal gene expression in the neonatal control LN compared to adult control LNs could indicate a difference in immune development between adult and neonatal LNs contributing to age-dependent differences in gene induction after skin vaccination. Genes involved in B-cell activation (CD40/CD69) showed a lower basal expression in the neonatal LN compared to the adults, which is consistent with the limited protective B-cell responses described for neonates [25,57]. Additionally, lower gene expression of Th1 cytokines IFNγ and IL12 [58] in the neonatal LN support the limited neonatal cellular immune responses. These differences could limit the development of a specific protective immune response in neonatal pigs after vaccination.

The placebo patch was used to investigate the effects of mechanical stimulation and induced predominantly downregulation of pro-inflammatory genes in both adult and neonatal pigs compared to the NV pigs. Another porcine study showed also that placebo intradermal injection did not induce genes at 24 h after vaccination, although at 2 h after vaccination upregulation of pro-inflammatory genes (IL8, IL1β) and chemokines (CCL2, CCL3 and CCL20) was found [59]. Therefore, it is possible that the downregulation of e.g. CCL3 and DEFB1 observed in our study could have been preceded by an upregulation of these genes. Interestingly, more genes (e.g. chemokines CCL20 and CXCL10 in the APC cluster) were upregulated in the neonatal pigs compared to the adult pigs after placebo patch vaccination, indicating that the neonatal skin was more responsive to the mechanical stimulation than the adult skin.

The antigen-only (Ag) vaccination, which was administered in neonates only, induced minimal changes in gene expression in the vaccinated skin when compared to the vaccines in conjunction with the different TLRa. However, the changes in gene expression induced by the Ag-vaccine in the draining LN were comparable to the vaccines containing TLRa. This suggests that the TLRa were especially important for the local skin immune responses and that the Ag had a limited contribution to the local immune response in the skin.

This study investigated the skin as vaccine administration route in neonatal and adult pigs. We showed induction of significant immune responses in both neonatal and adult skin after application of DMN-patches loaded with iPRRSV and different TLRa. In both age-groups, skin vaccination with TLR7/8a induced the most prominent early immune responses compared to other TLRa vaccines, especially in the skin. However, age-dependent differences in immune responses should be considered when developing skin vaccines for pigs in different age-groups.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: [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. Joanne McCaffrey and Damien Collins are former employees of Xeolas Pharmaceuticals Limited and had no financial or other competing interests. The other authors have no competing interest to declare.]

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Appendix A. Supplementary data

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