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Neonatal porcine blood derived dendritic cell subsets show activation after TLR2 or TLR9 stimulation

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ABSTRACT

The present study investigated the innate immune response *in vitro* to determine porcine neonate responses with Toll-like receptor (TLR)2 ligand (Pam3Cys) or TLR9 ligand (CpG) and compared these with adults. We identified the same phenotypically defined dendritic cell (DC) subsets and DC proportions in porcine neonate and adult blood by flow cytometry, which were plasmacytoid DCs (pDCs): $CD14^{-}CD4^{+}CD172a^{+}CADM1^{-}$) and conventional DCs (cDCs), being further divided into a cDC1 ($CD14^{-}CD4^{-}CD172a^{low}CADM1^{+}$) and a cDC2 ($CD14^{-}CD4^{-}CD172a^{+}CADM1^{+}$) subset. With neonatal cells, the TLR2 ligand induced a stronger TNF expression in monocytes and pDCs, and a stronger CD80/86 upregulation in cDC1, when compared to adult cells. Furthermore, in neonatal mononuclear cells TLR9 ligand was more potent at inducing *IL12p40* mRNA expression. These results indicate clear responses of porcine neonatal antigen presenting cells after TLR2 and TLR9 stimulation, suggesting that corresponding ligands could be promising candidates for neonatal adjuvant application.

1. Introduction

The early-life is a critical period characterized by high exposure to pathogens and subsequent development of infectious diseases. In swine farms diseased newborns and young piglets show a high mortality rate, causing severe welfare problems and economic losses. Vaccination is one of the most powerful strategies to protect against early-life infections (Murtaugh, 2014; Rose and Andraud, 2017). However, due to differences in both innate and adaptive immune response in neonates and adults (Kollmann and Marchant, 2016; Mohr and Siegrist, 2016) and the presence of maternal derived antibodies (Siegrist, 2003) vaccines are less effective in neonates.

Newborns have experienced minimal antigen exposure *in utero* and depend heavily on their innate immune system. The neonatal innate response is characterized by cytokine patterns that are different from the response in adults (Kumar and Bhat, 2016; Levy, 2007), often resulting in a less effective Th1 response (Holt and Jones, 2000; Kollmann et al., 2009). Stimulation of the neonate innate immune system can provide insights for application of novel adjuvants, such as synthetic Toll like receptor (TLR) ligands (Savelkoul et al., 2015; Toussi and Massari, 2014), and can be a promising strategy to strengthen the immune response to vaccination of different age groups and induce a more effective Th1 or Th1/Th2 balanced response.

In vitro studies with human blood cells have already demonstrated that neonates respond differently after specific TLR stimulation (Kollmann et al., 2009). In general, neonate cord blood cells produce less IFN-α after stimulation (Aksoy et al., 2007; Danis et al., 2008); while IL-6 response of PBMCs appears stronger in neonates compared to adults, (Angelone et al., 2006). Nonetheless, adult-like responses after TLR stimulation in human cord blood and in goat kids PBMCs can also be reached when cells are appropriately stimulated (Nguyen et al., 2010; Schuller et al., 2016; Tourais-Esteves et al., 2008). Therefore, TLR ligands are interesting candidates for adjuvant application in neonatal vaccination.

The main target of vaccine adjuvants are dendritic cells (DCs),

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potent antigen presenting cells (APCs), which are an essential link between the innate and adaptive immune response (Dutertre et al., 2014; McCullough and Summerfield, 2009). After stimulation, DCs mature and migrate to the draining lymph node to activate Th cells and induce an adaptive immune response. Different DC subsets have recently been described in porcine adult PBMCs: plasmacytoid DCs (pDCs: $CD14^{-}CD4^{+}CD172a^{+}CADM1^{-}$) and conventional DCs (cDCs), being further divided into a cDC1 ($CD14^{-}CD4^{-}CD172a^{low}CADM1^{+}$) and a cDC2 ($CD14^{-}CD4^{-}CD172a^{+}CADM1^{+}$) subset (Auray et al., 2016; Summerfield et al., 2015). pDCs are especially important for antiviral responses, as they are the main producers of type I interferon (IFN-α) (Reizis et al., 2011; Summerfield et al., 2003) and other cytokines, such as TNF and IL-12, especially after TLR7 and TLR9 stimulation in pigs (Auray et al., 2016). cDCs are most efficient in presenting antigen and activating naïve T-cells by using MHC-class II molecules and costimulatory molecules, such as CD40, CD80 or CD86 (Summerfield et al., 2015).

In this study, we focus on two synthetic TLR ligands that recently have proven to be efficient in stimulating the different adult porcine DC subsets: TLR2 ligand Pam3Cys and TLR 9 ligand CpG ODN and could as well stimulate the porcine neonate DC subsets (Auray et al., 2016). *Auray* et al. showed that TLR2 ligands were a potent activators of monocytes and DCs for pro-inflammatory responses in many monocytic cells, while TLR9 ligands typically induced strong IFN-α and IL-12 responses in pDCs. With these ligands we therefore covered to different types of innate immune responses and ensured to have responses in all DC subsets and monocytes. First we identified the DC subsets and proportions of DC subsets and monocytes in the neonate pig and compared these with adult pigs. Innate immune responses after TLR2 or TLR9 stimulation were compared between neonate and adult pigs. DC activation was assessed by upregulation of cell-surface molecule MHCII and costimulatory molecule CD80/86, combined with intracellular staining for TNF. The same parameters were measured in monocytes. In the supernatant we measured the overall PBMC cytokine production and we evaluated the cytokine and TLR mRNA expression in an enriched mononuclear cell population.

2. Materials and methods

2.1. Animals

Blood was collected from four-day-old female piglets (neonates, n = 12) or twelve-week-old female immunocompetent pigs (considered adults, n = 7). All pigs were purchased from the same high health status pig farm in the Netherlands, reared under controlled conditions and were having the same genetic background. (Topigs Norsvin: Z-line (sow) x Tempo line (boar)). The neonates were randomly selected from different sows and received sufficient colostrum. In total we used 12 neonates and 7 adults, as we were not able to perform all different assays on one animal due to small blood volume of the neonates.

Pigs were euthanized with Euthasol® and immediately exsanguinated. The blood was collected aseptically using 0.1% heparin (Heparine LEO, 5.000 I.E/ml) as anticoagulant. All experiments were conducted in accordance to 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).

2.2. PBMC isolation and TLR specific stimulation

Collected blood was diluted 1:1 with PBS containing 0.5 mM EDTA within 2 h after collection and converted to a Leucosep® tube using a 60% FICOLL-PAQUE™ Plus density-gradient to isolate the PBMCs. Cells rested overnight at 4 °C on ice and were plated in 12-well plates with 2.5×10^6 cells/well in 1 ml RPMI 1640 medium (Gibco®) supplemented with 10% fetal bovine serum and 1% Penicillin-Streptomycin (Gibco®). Cells were stimulated with 10 μg/ml Pam3Cys-SKKKK (TLR2 ligand), (PAM3Cys L2000, EMC microcollections) or 5 μg/ml CpG oligo-deoxynucleotide type A (TLR9 ligand) (CpG, sequence D32, ggTGCGTCGACGCAGggggg, Eurofins genomics), or they were left unstimulated as control. The TLR ligands from the same batch were diluted with PBS at the beginning of the study and stored in small aliquots of 100 μl (1 mg/ml) at −20 °C. For each independent experiment new aliquots from the same batch were used to stimulate the samples.

2.3. PBMC cytokine production measured by multiplex immunoassay

PBMCs were stimulated for 7 h with TLR2 or TLR9 ligand, or were left unstimulated as control. Supernatants of PBMC cultures were collected and frozen at −80 °C until analysis. Protein concentration of IFN-α, TNF, and IL-6 in the supernatant were measured in duplicate with a custom-designed multiplex Cytometric Bead Array (PorcineProcartaplex®; eBioscience) 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) and 6.32 pg/ml (IL-6), respectively.

2.4. Cytokine and TLR mRNA expression in enriched innate mononuclear cell population

The $CD3^{-}$ fraction was generated as an enriched innate mononuclear cell population. For CD3 depletion, stimulated PBMCs were first incubated with anti-porcine CD3 ε antibody (clone PPT3, from SouthernBiotech) and then with anti-mouse IgG1 microbeads (Miltenyi Biotec). The $CD3^{-}$ fraction was then sorted using the Magnetic Activated Cell Sorting system (MACS®, Miltenyi Biotec) with LD columns. Purity of the isolated cells was confirmed by flow cytometry (>95% purity). This $CD3^{-}$ fraction was harvested in TRIZOL® and stored at −80 °C until mRNA extraction was performed. mRNA was extracted by using the Directzol® RNA MiniPrep according to the manufacturer's instructions. mRNA quantity was accessed with the NanoDrop 1000™ (Thermo Fisher Scientific) by evaluating the optical density (OD) at 260 nm and the OD 260/280 ratio was then used to evaluate the quality.

The mRNA expression of *TLR2* and *TLR9*, and the cytokines *IFN-α, TNF, IL-6* and *IL12p40* extracted from the PBMC/$CD3^{-}$ fraction was detected by RT-qPCR as previously described (Wichgers Schreur et al., 2011). Briefly, the SuperScript II Reverse Transcriptase® (Invitrogen) was used to generate cDNA according to the manufacturer's instructions. cDNA was quantified by SYBR green incorporation by using the Applied Biosystems 7500/7500 standard™. All primer sequences were obtained from (de Greeff et al., 2010).

Peptidyl-prolyl cis-trans isomerase A(*PPIA*) was used for normalisation of the results given the stable expression in adult and neonatal PBMC/$CD3^{-}$ fraction. Quantification was done using serial dilutions of a plasmid with the gene of interest, which were used as internal standards. The efficiency of the PCR reaction was 90–100% for all reactions.

2.5. DC subsets identification by flow cytometry

After stimulation, PBMCs were harvested and resuspended in staining buffer (PBS containing 0.5 mM EDTA and 0.25% BSA) and

stained with antibodies (Abs) for DC subset identification as described previously (Summerfield et al., 2015). Briefly, PBMCs were first incubated with anti-CD172a (clone 74-22-15A from KINGFISHER) and anti-CADM1 (clone 3E1 from MBL) Abs. Cells were then incubated with the corresponding secondary Abs goat anti-mouse IgG2b PE (SouthernBiotec) and biotin-conjugated goat anti-chicken IgY (JacksonImmunoResearch) respectively. Ig blocking was then performed with whole mouse IgG molecules (JacksonImmunoResearch). Finally, cells were incubated with the following conjugated Abs: anti-CD14-FITC (clone MIL2 from Abd Serotec) and anti-CD4-PerCP-Cy5.5™ (clone 74-12-4 from BD Bioscience) and V500-coupled streptavidin (BD Bioscience). PBMCs were then run on a FACSCANTO™ (BD Biosciences) using the BD FACSDiva™ software. The flow cytometry data were analysed with the Flowjo™ software version 10.0.

2.6. Assessment of cell-surface markers and intracellular TNF staining in DC subsets and monocytes by flow cytometry

For the assessment of the cell-surface markers and intracellular TNF staining, PBMCs were harvested 5 h after stimulation. Cell were then stained as described before for DC subsets and to assess the cell surface molecule expression, anti-CD80/86 (anti-CD152 Ig Fusion protein, clone BHK from Ancell) or anti-MHCII (anti-SLA class II DQ, clone K274.3G8 from AbD Serotec) were added in the first step of the DC subset staining. In the second step the corresponding secondary Ab was added: goat anti-mouse IgG2a secondary antibody AlexaFluor® (Invitrogen) for CD80/86 or goat anti-mouse IgG1 secondary antibody AlexaFluor® (Invitrogen) for MHCII.

For the intracellular staining, Brefeldin A (1 ng/ml eBioscience) was added 1 h after stimulation to the cultures to stop the cytokine secretion. Cells were further cultured for 4 h before being harvested for flow cytometry staining. For the intracellular staining, the cells were first stained with the antibodies for the DC subsets as described previously and after this fixed in 4% paraformaldehyde. After a wash with 0.1% saponin (Panreac Applichem), cells were incubated with anti-TNF AlexaFluor®647 (clone Mab11 from BioLegend) in 0.3% saponin followed by another 0.1% saponin wash.

2.7. Statistical analysis

All statistics were calculated in GraphPad Prism 7.02 software. The two-way ANOVA followed by a Tukey's multiple comparisons test was used to assess significance for flow cytometry surface molecule expression and intracellular cytokine production in the DC subsets and the cytokine protein production of the PBMCs. The non-parametric Mann-Whitney U test was used to assess statistical significance of the RT-qPCR data and to evaluate the proportions of the DC subsets and monocytes (***$p < 0.001$, **$p < 0.01$ and *$p < 0.05$).

3. Results

3.1. Blood collection, PBMC isolation and number of animals

From 12 neonates an average volume of 62 ± 14 ml blood was collected and subsequently $42 \pm 29 \times 10^6$ PBMCs were isolated. Due to this limited and variable amount of PBMCs more than one neonate was used to perform the different assays (flow cytometry, cytokine production and mRNA expression). From the 7 adult animals we collected sufficient blood and PBMCs (average of 120 ml blood with $200 \pm 100 \times 10^6$ PBMCs). For the identification of the DC subsets and proportions by flow cytometry we used 6 animals from each age-group. For flow cytometry analysis for DC maturation and cytokine production measurement 4 animals from each age-group were used. For the mRNA expression (cytokines and TLRs) we used the data of 7 neonates and 4-5 adults. All analysis were performed in duplicate, which showed a high correlation ($R^2 = 0.96 \pm 0.05$).

3.2. PBMC cytokine production following TLR stimulation

To investigate the impact of the neonatal and adult environment on monocytic cell and DC responses, we evaluated the cytokine production capacity of the whole PBMC population after TLR2 (Pam3Cys) and TLR9 (CpG) stimulation. Stimulation of PBMCs with CpG induced a significant increase of IFN-α, TNF and IL-6 production in PBMCs of adults and a significant increase in IFN-α production in neonates, with IFN-α being the most abundant cytokine produced when compared to TNF and IL-6. IFN-α and TNF production was significantly higher in the adult group compared to the neonates after CpG stimulation (Fig. 1A and B).

PBMC stimulation with Pam3Cys induced a similar increase in IL-6, but not IFN-α production compared to unstimulated control PBMCs in all animals of both age groups (Fig. 1C). There was only one adult and one neonate that showed a significant higher level of TNF production from Pam3Cys stimulated PBMC compared to the average control (Fig. 1B).

3.3. Cytokine mRNA expression in enriched mononuclear cells following TLR stimulation

The PBMC/CD3$^-$ fraction was generated to represent an enriched innate mononuclear cell population by removing the lymphocytes. Only CpG induced a clear upregulation (fold change) of mRNA *IFN-α* expression in both age groups with no significant difference between the adults and the neonates (Fig. 2A). Pam3Cys and CpG induced upregulation of *TNF* and *IL-6* mRNA expression, but there was no significant difference in upregulation between the age groups (Fig. 2B and C). CpG induced only in neonates a significant upregulation of *IL-12p40* mRNA expression (Fig. 2D).

3.4. TLR mRNA expression in enriched mononuclear cells following TLR stimulation

To investigate if any differences observed between neonates and adults were related to TLR mRNA expression, we assessed this expression in the enriched mononuclear cells (PBMC/CD3$^-$ fraction). Basal *TLR2* mRNA expression in the control PBMC/CD3$^-$ fraction showed no significant difference between neonates and adults and was not induced by the TLR ligands in any age group. Nevertheless, *TLR2* mRNA expression was significantly higher in neonates compared to adults after Pam3Cys and CpG stimulation (Fig. 3A).

Basal *TLR9* mRNA expression also showed no age-dependent difference in the control PBMC/CD3$^-$ fraction. However, only adult *TLR9* mRNA expression showed a trend (although not significant) for downregulation after Pam3Cys and CpG stimulation and there was a significant higher expression for neonates after CpG stimulation (Fig. 3B).

3.5. Identification of DC subsets in neonate and adult PBMCs

The phenotype of the different DC subsets and monocytes in adults were determined by flow cytometry as previously described (Auray et al., 2016) (Fig. 4A). After doublet discrimination, gates were set on cells with high forward and side scatter (big cells; excluding the lymphocytes) and the four surface markers CD14, CD172a, CADM1 and CD4 were used to identify the different DC subsets. The CD14$^+$ cells were monocytes whereas pDCs were CD14$^-$CD4$^+$CD172a$^+$CADM1$^-$, cDC1 were

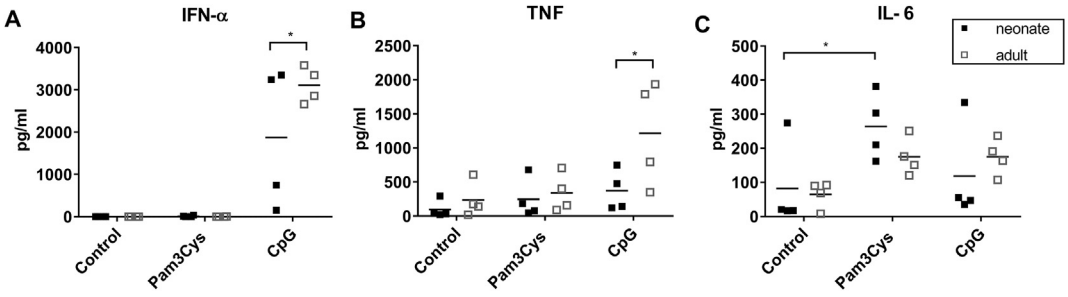

Fig. 1. PBMC cytokine production following TLR stimulation. PBMCs of neonates (■) and adults (□) were stimulated for 7 h with 10 μg/ml Pam3Cys or 5 μg/ml CpG or were left unstimulated as control. INF-α, TNF, and IL-6 production in pg/ml are presented from neonates and adults (n = 4). Each symbol represents the average of the duplicate from one animal and the mean is shown for each data set. The results shown are from 4 independent experiments, which were performed on different days. Statistical significance was calculated using a two-way ANOVA followed by a Tukey's multiple comparisons test. (**p < 0.01, *p < 0.05).

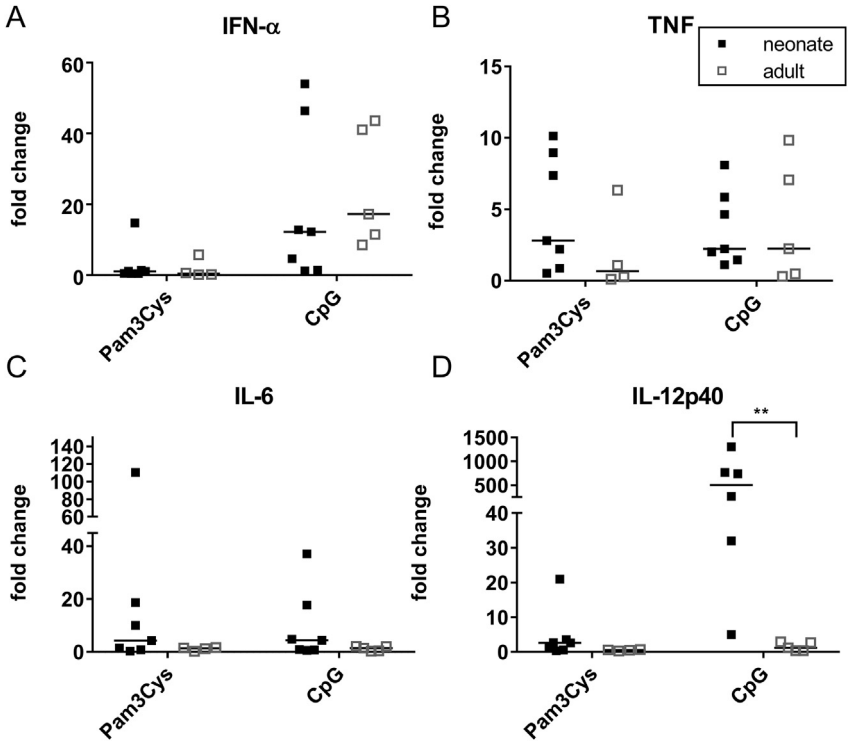

Fig. 2. Cytokine mRNA expression in enriched mononuclear cells following TLR stimulation. PBMCs of neonates (■) and adults (□) were stimulated for 7 h with 10 μg/ml Pam3Cys or 5 μg/ml CpG or were left unstimulated as control. The fold change of *IFN-α* (**A**), *TNF* (**B**), *IL-6*(**C**) and *IL12p40* (**D**) expression in PBMC/CD3$^-$ fraction is shown in the Pam3Cys and in the CpG stimulated animals. Each symbol represents one animal and the median is shown for each data set. Cytokine expression was assessed with RT-qPCR using *PPIA* expression to normalize the data. The results shown are from 6 independent experiments (neonates n = 7 and adults n = 4–5), which were performed on different days. Statistical significance was calculated using a Mann- Whitney U test,(**$p < 0.01$, *$p < 0.05$).

CD14$^-$CD4$^-$CD172a^{low}CADM1$^+$; and cDC2 were CD14$^-$CD4$^-$CD172a$^+$CADM1$^+$. The same markers were used to identify these subsets in PBMCs isolated from neonatal pigs (Fig. 4B). The neonatal DC subsets presented similar levels of these markers compared to their adult counterparts. To investigate the possible difference in proportions of the DC subsets in neonates and adults, the percentage of pDCs, cDC1 and cDC2 cells in the whole PBMC population was calculated. Compared to monocytes these DC frequencies were very low (<0.5%) and there were no significance differences in the proportion of the different DC subsets in neonates compared to adults (Fig. 4C–E). However, the neonatal PBMCs contained significantly more monocytes (median 17.18%) compared to the adult PBMCs (median 6.75% monocytes in PBMCs) (Fig. 4F).

3.6. CD80/86 and MHCII expression in DC subsets and monocytes following TLR stimulation

The maturation of the different DC subsets after specific TLR stimulation was investigated by the expression of the cell-surface molecule MHCII and costimulatory molecules CD80 and CD86 in PBMCs of neonates and adults. Monocytes were assessed for the same parameters as the DC subsets. Cell-surface expression of CD80/86 was most prominent in the neonate and adult cDC1 and cDC2 subset and a lower expression was found in the pDCs and monocytes (Fig. 5A and B). Only in the adult cDC1 and cDC2 subsets, CpG induced a significant CD80/86 upregulation (Fig. 5A and B). For Pam3Cys (TLR2), there was a significant CD80/86 upregulation in neonates and adults for the cDC1 subset, where the neonatal cDC1 were more responsive to Pam3Cys stimulation compared to adults

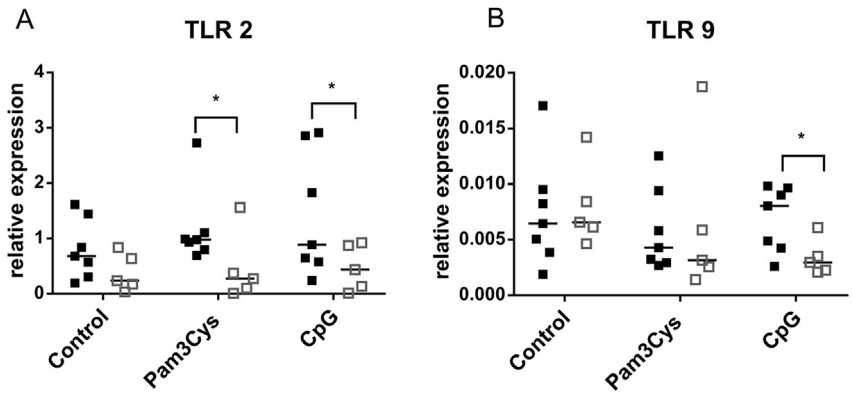

Fig. 3. TLR mRNA expression in enriched mononuclear cells following TLR stimulation. PBMCs of neonates (■) and adults (□) were stimulated for 7 h with 10 μg/ml Pam3Cys or 5 μg/ml CpG or were left unstimulated as control. The relative expression of *TLR2* (**A**) and *TLR9* (**B**) in PBMC/CD3-cells is shown in the unstimulated control samples (basal expression), in the Pam3Cys and in the CpG stimulated animals. Each symbol represents one animal and the median is shown for the data set. Expression of *TLR2* and *TLR9* was assessed with RT-qPCR using *PPIA* expression to normalize the data. The results shown are from 6 independent experiments (neonates n = 7 and adults n = 5), which were performed on different days. Statistical significance was calculated using a Mann- Whitney U test (*$p < 0.05$).

age groups (Fig. 5C). The adults showed also a CD80/86 upregulation in the cDC2 subset after Pam3Cys stimulation.

MHCII expression was observed in cDC1, cDC2 and pDC subset in the neonatal and adult PBMCs with no difference between unstimulated control and TLR specific stimulated samples (Fig. 5D and E). The neonate cDC1 subset showed a higher expression of MHCII in the control samples compared to adults (Fig. 5F).

3.7. TNF expression in DC subsets and monocytes following TLR stimulation

Another characteristic of activated DCs is their capacity to produce cytokines. We stimulated PBMCs and performed intracellular flow cytometry staining to assess TNF production of the different DC subsets and monocytes. Following TLR2 stimulation, pDCs and monocytes were the only subsets to produce TNF in both age groups. pDCs were the only cell type responding to CpG. In addition, stimulated pDC clearly had the highest percentage of TNF expressing cells as well as TNF mean fluorescence intensity (MFI, reflecting the amount of cytokine) (Fig. 6A–D).

When comparing the responses of neonates with adults we found that Pam3Cys, but not CpG induced a significant higher percentage of TNF expressing pDCs as well as TNF MFI in neonates (Fig. 6E and F). Interestingly, all neonates tested showed a significant TNF response in monocytes after Pam3Cys stimulation, while monocytes from adult pigs generally not responded (Fig. 6G and H).

4. Discussion

The innate immune response was investigated in mononuclear cells of four-day-old neonate pigs who received sufficient colostrum and were reared under the same conditions with the same genetic background as the adult pigs in the study. We aimed to identify neonate responsiveness after specific TLR stimulation to explore strategies for optimisation of neonatal responses to vaccination. To this end, we decided to stimulate TLR2 and TLR9 with synthetic ligands in neonates, which have proven to be effective in adult porcine blood DC subsets: TLR2 ligand Pam3Cys and TLR 9 ligand CpG ODN (Auray et al., 2016). TLR2 targeting as adjuvant gave promising, yet contradictory results for vaccine application in mice and humans (Basto and Leitao, 2014). TLR9 stimulation generally induces a Th1 biased immune response (Scheiermann and Klinman, 2014), which could be beneficial to redirect the Th2 skewed response in neonates.

Using whole PBMCs or enriched mononuclear cells (PBMC/CD3$^-$ fraction), we found that Pam3Cys induced similar levels of IL-6 and TNF, but no IFN-α production in both age groups relating to previous observations (Auray et al., 2016). Nevertheless, neonates showed a consistent trend, albeit not significant, of producing more IL-6 and expressing more *IL-6* mRNA after Pam3Cys stimulation compared to adults, which was also found in moDCs derived from human cord blood monocytes (Nohmi et al., 2015). Together with the corresponding TNF response in adults and neonates, this suggest that the TLR2 mediated immune response in neonate PBMCs is at least at an adult level.

Whereas CpG stimulation induced IFN-α and TNF production in neonates and adults, only the neonates showed a significant increase of *IL12p40* mRNA expression in the enriched mononuclear cells after CpG stimulation. This higher *IL-12p40* mRNA expression in neonates was described before in pigs (Auray et al., 2013) and goats (Ferret-Bernard et al., 2011). Auray et al. (2016) have shown that only pDCs produce IL12p40 and that this is associated with IL12p35 expression, which would suggest that the IL12p40 expression measured in the present study reflects IL12 production. For *IFN-α* we found an equal mRNA expression level of the enriched mononuclear cells in adults and neonates, indicating that neonates are able to upregulate the expression of this cytokine after CpG stimulation as found in other studies on cord blood for neonate horses (Vendrig et al., 2013) and humans (Schuller et al., 2016). Together, this clear IFN-α- and IL12p40 response would reflect the ability of CpG to induce a Th1 type cytokine response in porcine neonates.

To investigate cell-specific differences, we focused on the innate immune cells of the mononuclear phagocyte system: cDC1, cDC2, pDCs and monocytes. The present study demonstrated that neonates have similar DC subsets and proportion of pDCs, cDC1 and cDC2 in the total PBMC population as adult animals, although we noticed larger individual variation in the subsets of the neonates compared to adults. Our results are in agreement with an earlier study demonstrating this for pDCs in pigs (Auray et al., 2013). The larger proportion of monocytes found in the neonatal PBMCs compared to adults was also found in human studies with cord blood (Prabhu et al., 2016; Zenarruzabeitia et al., 2016) and could reflect a higher rate of monocytopoiesis in the bone marrow. We also investigated expression of cell surface markers on DC subsets at steady-state. In our study the porcine neonates expressed higher levels of MHC II on cDC1 and no differences were seen concerning the levels of the costimulatory molecules CD80/86. In contrast,

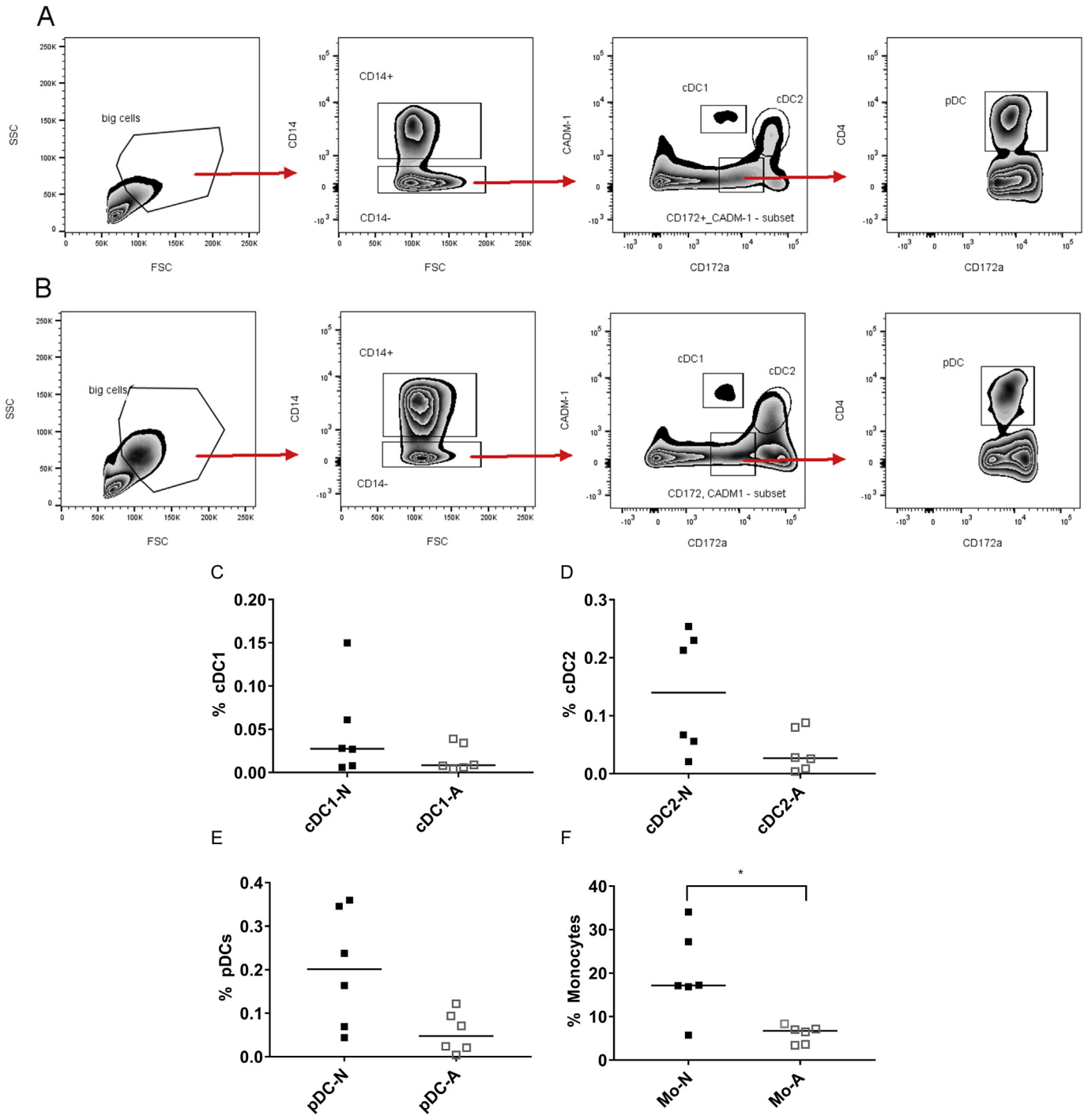

Fig. 4. Phenotype and proportions of DC subsets and monocytes. Gating strategy following five-colour flow cytometry by using antibodies against CD14, CD172a, CADM1 and CD4 in adult **(A)** and neonate **(B)** PBMCs. After doublet discrimination, gates were set on cells with high forward and side scatter (big cells). Monocytes were defined as $CD14^+$ cells and DC subsets ($CD14^-$) were defined as pDCs for $CD4^+$ $CD172a^+CADM1^-$ cells; cDC1 for $CD4^-CD172a^{low}CADM1^+$ cells; cDC2 for $CD172a^+CADM1^+$ cells. The proportions of the different DC subsets **(C–E)** and monocytes **(F)** in neonate (■) and adult (□) PBMCs were determined in duplicate, each symbol represents the average of two measurement from one individual animal and the median is shown for each data set. The results shown are from 4 independent experiments (n = 6), which were performed on different days. Statistical significance was calculated using a Mann-Whitney U test, (*$p < 0.05$).

human studies have demonstrated lower steady state expression of MHCII and CD80/86 on APC in neonates (Velilla et al., 2006) but these studies used foetal cord blood, which is different from the neonatal blood of four-day-old piglets we used in our study.

The ability of neonatal porcine DCs to get efficiently activated was measured and compared to adults by the expression of costimulatory molecules and cytokine production. As already shown in adult pigs, neonatal pDCs were the main producers of TNF (Auray et al., 2016). In neonates TLR2 stimulation with Pam3Cys induced a stronger TNF expression in pDCs, and upregulation of CD80/86 in cDC1 compared to what was observed in adult cells. These results suggest an increased maturation capacity of porcine

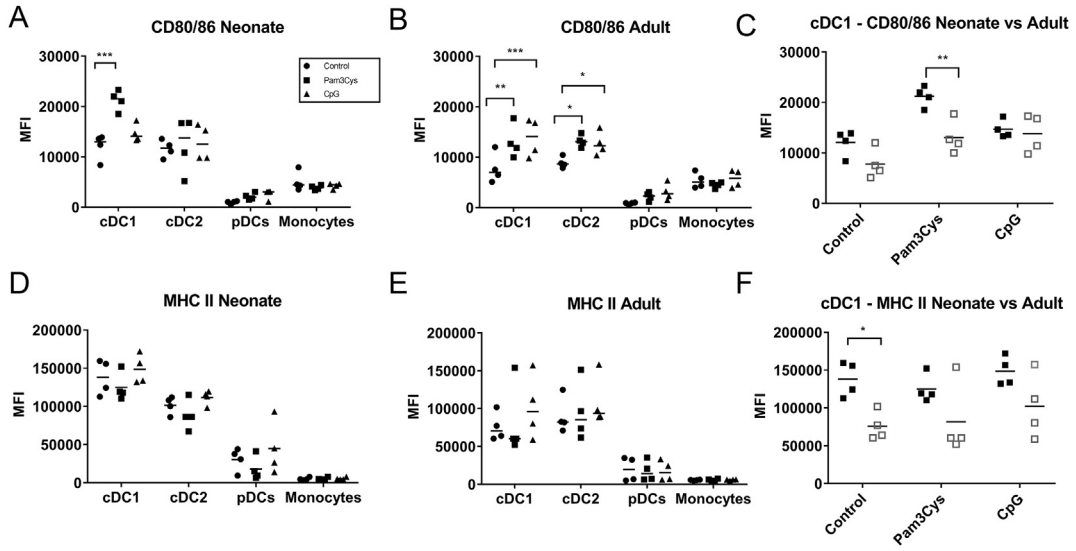

Fig. 5. CD80/86 and MHCII expression in DC subsets and monocytes following TLR stimulation. Whole PBMCs were stimulated for 5 h with 10 μg/ml Pam3Cys or 5 μg/ml CpG or left unstimulated as control. The mean fluorescence intensity (MFI) for CD80/86 and MHCII expression was measured in duplicate for neonates (**A and D**) (n = 4) and adults (**B and E**) (n = 4), each symbol represents the average of two measurement from one individual animal and the mean is shown for each data set. For the cDC1 subset the MFI for CD80/86 (**C**) and MHC II (**F**) was compared between neonates (■) and adults(□). The results shown are from 4 independent experiments, which were performed on different days. Statistical significance was calculated using a two-way ANOVA followed by a Tukey's multiple comparisons test. (***$p < 0.001$, **$p < 0.01$, *$p < 0.05$).

neonate DCs after Pam3Cys stimulation compared to adults. Studies on human cord blood DCs (Nohmi et al., 2015), horse PBMCs (Vendrig et al., 2013) and human naïve T-cells (Sinnott et al., 2016) have also reported this potency of TLR2 stimulation in neonates for vaccination. A remarkable finding was that only after Pam3Cys stimulation neonatal porcine monocytes responded with high numbers of TNF expressing cells. This response was not observed in adult porcine monocytes as demonstrated by (Auray et al., 2016). However, in humans, both neonate and adult monocytes showed increased TNF expression after Pam3Cys stimulation (Dreschers et al., 2016) suggesting development-related species differences for Pam3Cys stimulation.

After TLR9 stimulation with CpG the different DC subsets of neonates and adults showed an equivalent response in terms of TNF production by pDCs and CD80/86 expression by cDC1, demonstrating that *in vitro* DCs of neonates are able to mount an adult-like response (Zhang et al., 2013). Auray et al. (2013) showed that porcine neonatal BDCs after CpG stimulation displayed a more mature phenotype (CD80/86 expression) compared to adults, while other studies in human and horse cord blood (Nguyen et al., 2010; Schuller et al., 2016) found a lower responsiveness after CpG stimulation in neonates compared to adults. These differences in upregulation of CD80/86 between studies could be a result of disparities between age-groups, experimental conditions, DC populations and species, which complicates the comparison of neonate *in vitro* experiments, especially where DCs are involved (Auray et al., 2010; Facci et al., 2010).

Finally, we investigated the TLR2 and TLR9 expression in the enriched mononuclear cells to see if any difference in response between neonates and adults could be correlated to differences in TLR expression. At steady-state we found no significant difference in *TLR2* and *TLR9* mRNA expression between neonates and adults in the PBMC/CD3$^-$ fraction, as previously shown for TLR9 expression in porcine monocytes (Auray et al., 2013). This suggest that microenvironment and downstream NF-κβ activation play an important role in this differential responsiveness between neonates and adults after specific TLR stimulation as suggested before in other studies (Kollmann et al., 2012; Shao et al., 2016). Nonetheless, after stimulation we found age-dependent differences, which were most likely caused by the not significant trend of neonatal upregulation of TLR2 expression and downregulation of adult TLR9 expression in our study. Another study in neonate and adult horses has demonstrated this upregulation of TLR2 expression and downregulation of TLR9 expression after Pam3Cys stimulation as well (Vendrig et al., 2013). Further research is needed to elucidate the mechanism of this age-dependent response in porcine DCs and monocytes after TLR2 stimulation.

Transcribing our results to possible adjuvant application, TLR2 stimulation with Pam3Cys provided in neonates a stronger activation of DCs and monocytes compared to adult pigs. Nonetheless, this neonatal Pam3Cys response was without IFN-α and IL-12p40 induction and with a higher Il-6 response compared to CpG and by this not necessarily Th1 directed. TLR9 stimulation with CpG generated a distinct Th1 skewed response, in neonates as found by other studies in goats (Tourais-Esteves et al., 2008) and humans (Gay et al., 2011; Zhang et al., 2014). CpG administration has induced *in vivo* also a Th1 skewed response in neonatal piglets (Cai et al., 2014), supporting the application as adjuvant in neonate vaccine research.

Because both TLRs showed different properties, co-stimulation of TLRs 2 and 9 could be an interesting option, as suggested by other studies for different TLRs (Hu et al., 2016; Sugitharini et al., 2016; van Haren et al., 2016).

Individual variation, as commonly seen in outbred animals and a small blood volume in neonates reducing the number of assays per animal, are potential limitations of our study. However, our consistent *in vitro* findings, despite the individual variation, contribute to the further understanding of the porcine neonate immune response. Our data demonstrate that porcine neonates comprised the same DC subsets and proportions and are not generally incompetent in responding to TLR2 and TLR9 ligands. However, the DC response in our study cannot explain why neonates are generally less immunocompetent when compared to adults and it cannot be concluded that DC or APC functionality is not of importance for this difference. For instance, it could be that other functions of the APCs are less potent in neonates or that the

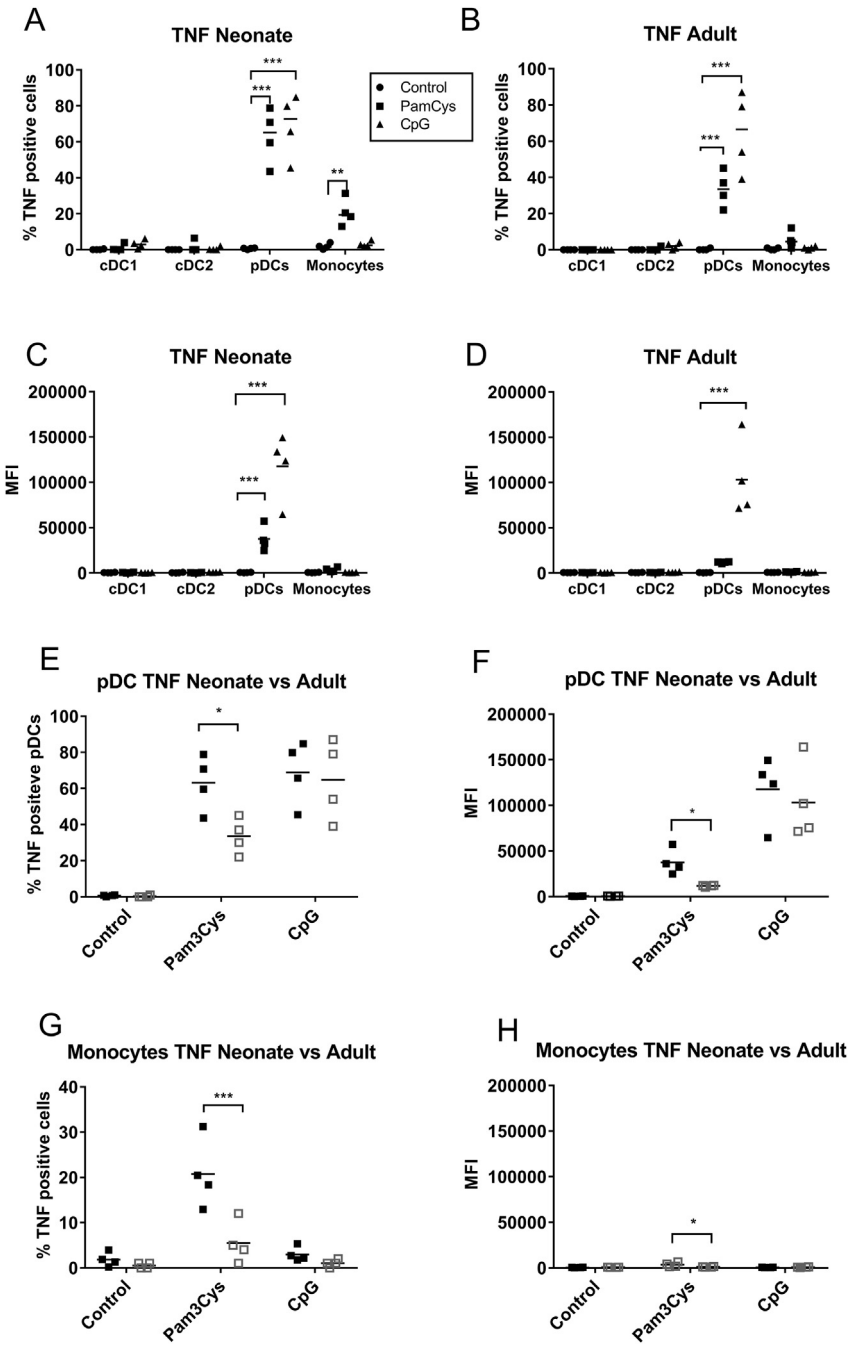

Fig. 6. Intracellular TNF expression in DCs subsets following TLR stimulation. Whole PBMCs were stimulated with 10 μg/ml Pam3Cys or 5 μg/ml CpG or left unstimulated as control. Results are expressed either as the percentage of TNF positive cells (**A,B,E and G**) or as the mean fluorescence intensity (MFI) of the cell subset (**C, D, F and H**). The TNF was measured in duplicate for neonates (n = 4) and adults (n = 4), each symbol represents the average of the duplicate from one animal and the mean is shown for each data set. The TNF response of the pDCs (**E and F**) and monocytes (**G and H**) was compared between neonates (■) and adults(□). The results shown are from 4 independent experiments, which were performed on different days. Statistical significance was calculated using a two-way ANOVA followed by a Tukey's multiple comparisons test. (***$p < 0.001$, **$p < 0.01$, *$p < 0.05$).

frequency of the DCs in neonatal lymphoid tissue is different. Next steps could be to have a closer look at APCs in the lymphoid tissue combined with a focus on the lymphoid structure and stromal components.

We also have to be aware that the *in vitro* selection process cannot entirely reflect the complex interactions occurring *in vivo*, because of the lack of the essential microenvironment (neonatal plasma and the organs and tissues essential in the immune system). This is especially true for the pDC fraction in neonates resulting in different outcomes for comparable *in vivo* experiments (Belnoue et al., 2013). So caution is needed for projection of our results to possible *in vivo* applications.

5. Conclusion

We showed that the porcine neonate PBMCs comprised the same DC subsets and similar proportions and a higher proportion of monocytes, as their adult counterparts. Porcine neonate pDCs, cDC1

and monocytes were more responsive after TLR2 stimulation with respect to TNF expression (pDC and monocytes) and CD80/86 upregulation (cDC1). Furthermore TLR9 stimulation induced increased *IL12p40* mRNA expression only in neonate mononuclear cells. Overall, our results indicate responsiveness of neonates after TLR2 and TLR9 stimulation with age-dependent differences in porcine innate immune response, as well suggesting that CpG and Pam3Cys could be interesting components to stimulate the neonate innate immune system for future vaccine research.

Conflicts of interest

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

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