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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