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Age related innate responses to different TLR ligands in pig in vitro

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EXECUTIVE SUMMARY

Background	Neonates show major differences with adults regarding their innate and acquired immune responses, both due to interferences of maternal derived antibodies (MDA) and to the maturation development stage of the immune system. Most knowledge in neonatal immunity has been obtained in mice, in which the immature immune system was shown to be biased to Th2 immunity. In livestock, young animals present a critical period of sensitivity to pathogens when the MDA decrease beyond protective levels and when self-immunity has not yet been triggered by antigen exposition or vaccination. To reduce this gap of immunity, vaccine strategies that include the use of adequate adjuvants need to be developed taking into account the specificity of the young age immune system.
Objectives	To analyse and compare in vitro the innate immune cell responses, i.e. dendritic cells to different immunostimulants in neonatal and immunocompetent pigs.
Methods	The *in vitro* response of dendritic cells present in the peripheral blood mononuclear cell population (PBMCs) of neonatal and immunocompetent (adult) pigs were analysed and compared after stimulation with two different synthetic Toll-like receptor (TLR) ligands.
Results & implications	*In vitro* stimulation of PBMCs in adult (immunocompetent) and neonatal pigs with TLR 2/6 ligand (Pam3) and TLR 9 ligand (CpG) results in activation of pDCs compared to the control animals in both groups. In neonatal animals we see a more pronounced activation of pDCs after stimulation with TLR 2/6 ligand (Pam3) compared to the activation after stimulation with TLR 9 ligand (CpG). The immunocompetent (adult) animals show a better activation of pDCs after stimulation with TLR 9 ligand (CpG) compared to the activation of pDCs after stimulation with TLR 2 ligand (Pam3). These results of the adult animals are in line with the results of Task 9.1. The conclusion of Task 12.1 is that both ligands for TLR 2/6 (Pam3) and TLR 9 (CpG) give an activation of DCs in neonatal and immunocompetent (adult) animals and could be an adjuvant of choice in further vaccination trials. For neonatal animals TLR 2/6 (Pam3) would be the adjuvant of preference and for adult animals TLR 9 (CpG) which indicates that age could be an important factor in adjuvant selection.

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Introduction

Innate and adaptive immunity

The immune system is composed of an innate and an adaptive part. The innate immune response, uses receptors in germline configuration e.g. pathogen recognition receptors (PRR), such as toll-like receptors (TLR), NOD, scavenger receptors, recognizing conserved molecular patterns on viral, bacterial and fungal infectious particles (pathogen-associated molecular patterns (PAMP)) that are essential for microbial recognition. It has a limited specificity and generates a rapid response (hours), which is generally sufficiently effective to stop infections at an early stage. The innate immune response consists of particular cells (neutrophils, dendritic cells (DC), macrophages and natural killer (NK) cells) and soluble molecules like cytokines, chemokines, acute phase proteins, and the complement system.

The adaptive immune system is highly antigen specific by using recombined antigen-specific receptors on T-cells and B-cells, it is, however, slower in onset (days to weeks) and directed towards recently or previously exposed pathogens for which it also develops long-lasting immunological memory. The adaptive immune system relies on antigen presenting cells (APCs) like DC and macrophages and T- and B-lymphocytes, and humoral factors like (different) cytokines and chemokines, and antigen-specific antibodies.

The adaptive immune response can roughly be divided in a humoral B-cell response characterized by the production of antigen-specific antibodies, and a cellular T-cell response, which releases a.o. cytokines. It shows a profound degree of specificity down to the level of single amino acids on protein epitopes, develops long-term specific memory, and for antibodies displays an amplification and increased functionality by affinity maturation and heavy chain isotype switching. This reactivity ensures the final clearance of an infection and prolonged protection, due to memory T- and B-cells, which represents the hallmark of vaccination.

A special subpopulation of the APCs of the innate immune system, the DCs play an important role in presenting internalized and processed antigen to specific T-cells of the adaptive immune system and thereby bridge the adaptive to the innate immune system. These DCs are present in the blood and mucosal tissues (skin and mucosal surfaces in the airways and gastro-intestinal tract) where they display PRR that are able to bind and identify a large number of pathogens (PAMP) as well as parts of cells associated with necrosis (damage-associated molecular pattern (DAMP)).

The survival of an organism depends on an innate immune system that can quickly recognize and respond to microbial and viral products. TLRs are an important family of these PRR on the cell membrane of DCs and are responsible for much of that recognition and consequently have vital roles in survival. Activation via TLRs causes activation and maturation of the DCs in combination with production of various cytokines[1] and couples innate immunity with the adaptive immunity provided by lymphocytes.

Vaccines and adjuvants

The selection of vaccine antigens combined with a specific adjuvant for effective vaccination is still mostly an empirical process. Adjuvants can enhance the immunogenicity of vaccine antigens by eliciting a pro-inflammatory environment that recruits and promotes the infiltration of phagocytic cells, particularly APCs, to the site of injection.

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Adjuvants may also exert their immunopotentiating effects by enhancing antigen presentation, inducing cytokine expression, or by activating APCs.

Adjuvants can function in different ways. Of most interest in current adjuvants development for vaccines are adjuvants that contain molecules (like synthetic TLR ligands) that can bind to specific receptors including TLR expressed on DCs in blood and in tissue causing stimulation and maturation of the DCs resulting in induction of early protection, enhanced duration of immunity, induction of a broader immune response (stimulation of T helper cell 1 (Th1) response in neonatal animal [2]) efficacious against antigenic variants, reduction of individual variability to assure better vaccine coverage, and reduction of antigen dose in vaccines making them more economical[3].

Neonatal immunity

Neonates show major differences with adults regarding their innate and acquired immune responses, both due to interferences of maternal derived antibodies (MDA) and to the maturation development stage of the immune system. It is well known that in the neonatal period the immune response has a T helper cell 2 (Th2) bias which is more directed towards the development of humoral immunity [4].

The Th1 response which is more directed towards cell mediated immunity is less well developed and would be more beneficial for protection to invading pathogens. To reduce this gap of immunity, vaccine strategies that include the use of adequate adjuvants and optimal delivery systems need to be developed taking into account the specificity of the young age immune system and alleviating the Th1 response.

The selective expression of TLR by different APC subsets[5] and their reactivity to TLR ligands (TLR-L) differ among species [6, 7] and most likely between different age groups[7]. Several TLR have been recognized in neonatal and adult pigs on DCs. In humans it is known that the different subsets of DCs display different TLRs. Facci et. al.[8] show that porcine monocyte derived DCs (MoDCs) and blood derived DCs (BDs) express different levels of TLR and react different after stimulation with TLR-agonists. Further investigation is needed to provide new insights in the use of adjuvants (TLR-L) and their effects on different age groups regarding the enhancement of vaccine efficacy. The results of Task 9.1 indicate that stimulation of DCs from immunocompetent pigs with TLR 2/6, TLR 7 and TLR 9 give a significant upregulation of cytokines (TNF-alfa and INF-alfa) and maturation markers (CD40 and CD80/86) compared to other TLR ligands.

In this study on behalf of Task 12.1 and in close link to Task 9.1, we compare the effects of two promising different synthetic TLR-L (TLR 2/6 and TLR 9), on the activation and the maturation of neonatal and adult DCs and peripheral blood mononuclear cells (PBMCs) to see if there is a difference between the neonatal and adult animal and if the reaction of one of the ligands is more favourable.

The results of Task 12.1 will be used to develop further in vivo vaccination studies with these TLR ligands in adult and neonatal pigs.

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1. Material and Methods

1.1. Animals

Three days old piglets (neonatal) or twelve week old pigs (adult) were supplied by a high health status pig farm. All experiments were conducted in accordance to the ethical guidelines of the Central Veterinary Institute (CVI). This specific protocol was approved by the Centrale Commissie Dierproeven (CCD) (Permit number: ADV401002015356).

1.2. Stimulation of PBMCs

Whole PBMCs were stimulated for 5 h with TLR 2/6 ligand (10 µg/ml Pam3Cys-SKKKK (Pam3Cys L2000, EMC microcollections) or TLR 9 ligand (5 µg/ml CpG oligodeoxynucleotide (CpG, sequence D32, ggTGCGTCGACGCAGggggg, Eurofins genomics) or were left unstimulated as control. Cells were then harvested and the DC subset staining was performed as described in the flow cytometry (1.3).

For the intracellular cytokine staining PBMCs were stimulated 1h with the different TLR ligands or left unstimulated as controls. Brefeldin A (eBioscience) was then added to the culture for 4h to stop the cytokine secretion. For IFN-alfa intracellular staining, PBMCs were stimulated 3h before adding Brefeldin A for an extra 4h.

1.3. Flow cytometry

After 5 hours of stimulation for the maturation markers and TNF-alfa and 7 hours of stimulation for INF-alfa cells were harvested and resuspended in FACS buffer and stained with the antibodies for the DC subsets [9] and maturation markers..

For the intracellular staining the cells were first stained with the antibodies for the DC subsets and after this fixed in 4% paraformaldehyde. After a wash with 0.1% saponin (Panreac Applichem) cells were incubated with the antibodies for the intracellular staining.

PBMCs were analysed in the FACSCANTO (BD Biosciences) with a minimum of 100.000 events gated on the larger cells in the PBMCs without the lymphocytes using the FACS software. The flow cytometry data were analysed with the Flowjo™ software. The DCs were phenotyped with a four colour flow cytometry with a focus on the plasmacytoid DCs (pDC) which are known for their production of cytokines [9]. The pDCs are the CD14-, CD172a +, CADM-1-, CD4+ subset of the PBMCs and form with the conventional or classical DCs (cDC: CD14-, CD172a+, CADM-1-), the DC population in the PBMCs. The expression of the intracellular cytokines (TNF-alfa and INF-alfa) and the maturation markers (MHCII and CD80/86) was measured in this particular subset of DCs the pDCs.

1.4. Reverse transcription-quantitative real-time polymerase chain reaction (RT-qPCR)

After 7 hours of stimulation PBMCs were harvested and depleted from lymphocytes (CD3+ cells) using the Magnetic Activated Cell Sorting system (MACS®, Miltenyi Biotec). The used antibodies are described in table 1. The PBMCs without the CD3+ cells were harvested in TRIZOL ® and stored at -80 °C till mRNA extraction was performed. mRNA extraction was performed using the Direct-zol™ RNA MiniPrep and mRNA quality and quantity was accessed using the NanoDrop (Thermo Fisher Scientific). The SuperScript® II Reverse Transcriptase (Invitrogen™) was used to generate cDNA.

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Quantification of cDNA by SYBR green incorporation was performed using the Applied Biosystems 7500 for the Th1 cytokines TNF-alfa, INF-alfa and IL-12p40 and the Th2 cytokines IL-4 and IL-6. 18S was used as reference gene.

	Primary antibody	Clone	Secondary antibody
DC subset			
CD14	Anti-porcine CD14-FITC	MIL2, AbD Serotec	NA
CADM-1	Anti SynCAM/TSLC1/CADM1	3E1, MBL	Goat anti-chicken IgY, Jackson Immunoresearch Streptavidin V500, BD Biosciences
CD172a	Anti-porcine CD 172a	BL1H7, AbD Serotec 74-22-15A, Kingfisher	Anti-mouse IgG PE, Southernbiotech
CD4	Anti-porcine CD4 PerCP-Cy5.5	74-12-4, BD Biosciences	NA
Maturation markers			
MHCII	Mouse anti-pig SLA class II DQ	AbD Serotec, K247.3G8	Anti-mouse Alexafluor 647, Molecular Probes
CD80/86	Human CD152 (CTLA-4) mulg fusion protein	Ancell, 501-020	
Intracellular cytokines			
TNF-alfa	Alexa Fluor 647-conjugated anti-human	clone Mab11, Biolegend	NA
INF-alfa	Anti-porcine IFN-α	clone F17, PBL Assay Science	Anti-mouse Alexafluor 647, Mo-lecular Probes
MACS			
CD3	Anti-porcine CD3epsilon	PPT3, Southernbiotech	Anti-mouse IgG1 microbeads, Miltenyi Biotec

Table 1: Overview antibodies used in Flow cytometry and Magnetic Activated Cell Sorting.

2. Results

2.1. Flow cytometry

Both neonatal and adult animals show an increased number of pDCs with TNF-alfa expression (% TNF-alfa positive pDC) after stimulation with TLR 2/6 ligand (Pam3) and TLR 9 ligand (CpG) compared to the non-stimulated control animals (Fig.1a). In neonatal animals there is a stronger increase in the number of TNF-alfa positive cells after stimulation with Pam3 compared to the increase after stimulation with CpG. Where in adult animals the increase of TNF alfa positive pDC is stronger after stimulation with CpG compared to the increase after stimulation with Pam3. These result of the increase of TNF-alfa positive pDCs in adult animals stimulated with Pam3 and CpG are in line with the results of Task 9.1.

There is no clear increase in number of pDCs with INF-alfa expression after stimulation in both the animal groups for the two ligands compared to the non-stimulated control.

In regard to the maturation markers there is an increased number of pDCs with CD80/86 expression (% CD80/86 positive pDC) (Fig.1b) after stimulation compared to the non-stimulated cells in both the neonatal and adult group. Where the increase in the neonatal animals is more pronounced after Pam3 stimulation compared to the increase after stimulation with CpG, in the adult animals there is a stronger increase after CpG stimulation compared to the increase after Pam3 stimulation (figure 1b).
There is no increase in the number of pDCs with MHC II expression after stimulation with both ligands compared to the non-stimulated control groups in both neonatal and adult animals.

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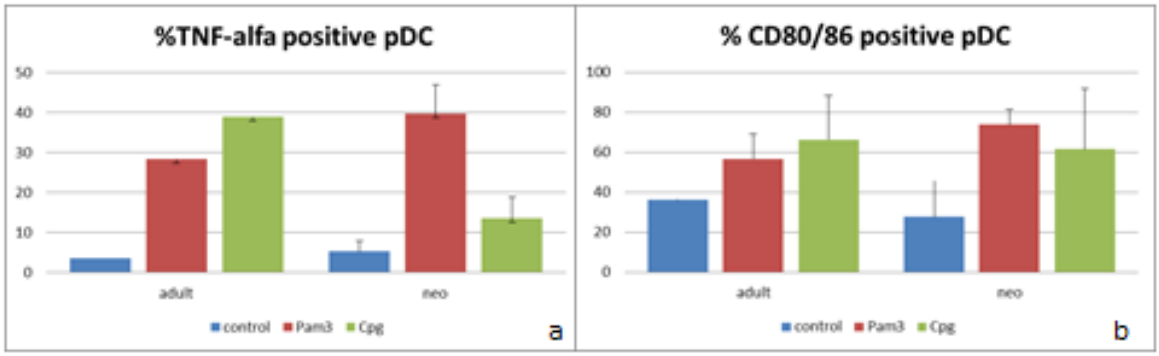

Figure 1: percentage of plasmacytoid Dendritic Cells (pDC) positive for intracellular cytokine TNF-alfa and maturation marker CD80/86 measured with flow cytometry in PBMCs in controls or after stimulation with TLR2/6 agonist (Pam3) or TLR9 (CpG) agonist. Error bars represent standard deviations (n=2 for adult and neonatal group, except for adult TNF-alfa, n=1)).

2.2. RT-qPCR

In general and independent of the age group expression levels of mRNA of the Th1 (TNF-alfa, INF-alfa and IL-12) and Th2 related cytokines (IL-4 and IL-6) show an upregulation after stimulation with TLR 2/6 ligand (Pam3) and TLR 9 ligand (CpG) compared to the non-stimulated controls in the non–lymphocytic (CD3 negative) PBMCs (Fig.2).

TLR2/6 agonist Pam3 induces a stronger upregulation of TNF-alfa and IL12 in neonates compared to immunocompetent (adult) animals, whereas IFN-alfa was more or less absent in cells of neonate pigs after stimulation. Also, mRNA levels of Th2 related cytokines (Il-4 and IL-6) were stronger upregulated after Pam3 stimulation in neonates than in adults.

TLR 9 agonist CpG induces an upregulation after stimulation in both neonatal and adult animals compared to the control for nearly all cytokines except for INF-alfa which shows no upregulation compared to the non-stimulated control in neonatal animals (Fig.2).

The levels of mRNA upregulation after CpG stimulation are comparable in adult and neonatal animals. CpG induces a more pronounced upregulation in adult animals compared to Pam3 where in neonatal animals Pam3 induces a stronger upregulation compared to CpG (Fig.2).

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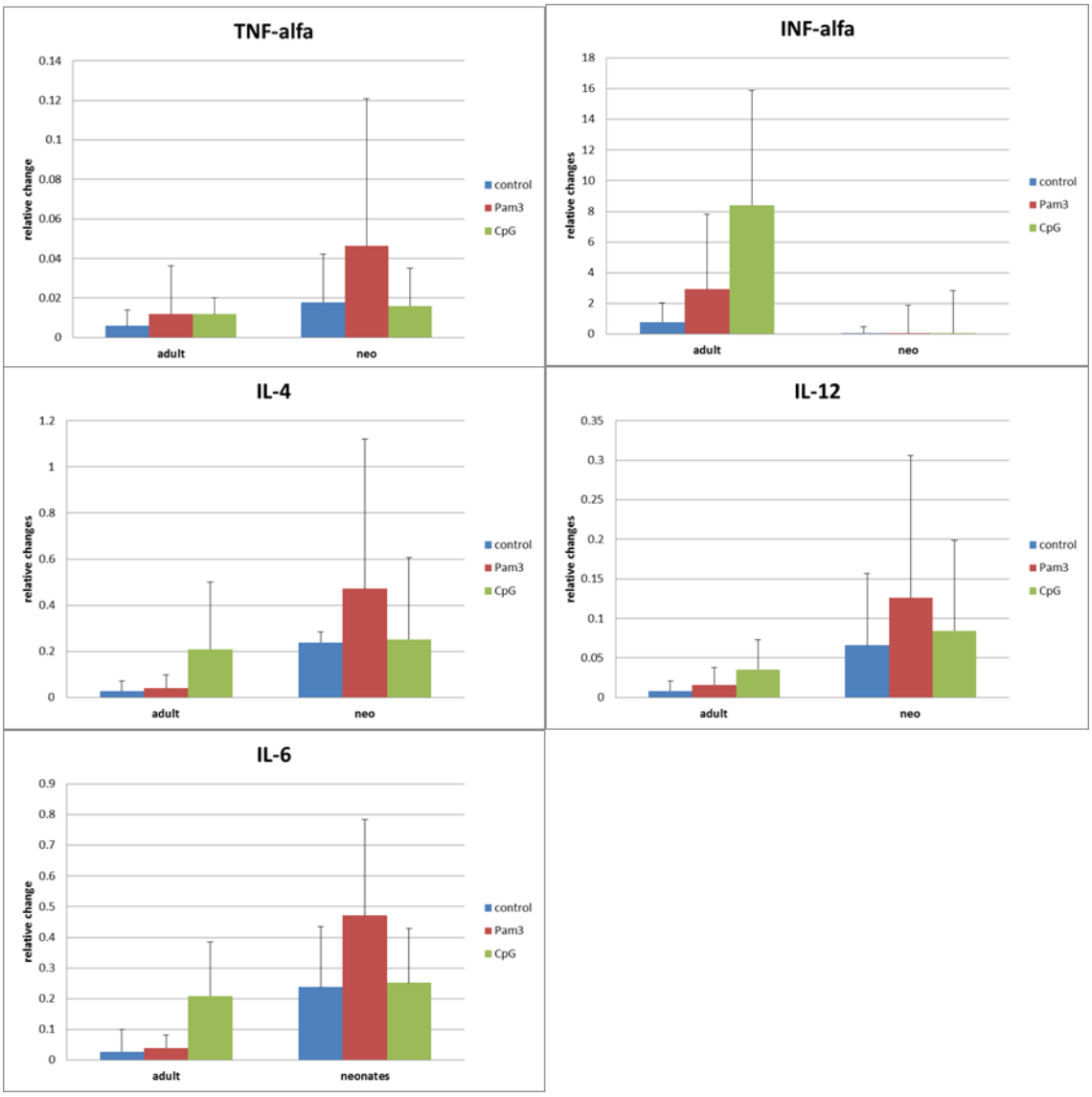

Figure 3: mRNA expression of cytokines TNF-alfa, INF-alfa, IL-4. IL-12 and IL-6 in non-lymphocytic (CD3 negative) PBMCs in controls or after stimulation with TLR2/6 agonist (Pam3) or TLR9 (CpG) agonist. Expression is calculated on basis of relative changes compared to household genes (18S), error bars represent standard deviations (n=3 for the adult and neonatal group).

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3. Implication

In Task 12.1 the in vitro response of DCs present in the PBMCs of neonatal and immunocompetent (adult) pigs was analysed and compared after stimulation with two different synthetic toll like receptor (TLR) ligands.

To measure the response of the DCs in the PBMCs, the stimulated and non-stimulated PBMCs were analysed by flow cytometry for the expression of intracellular cytokines (TNF-alfa and INF-alfa) and maturation markers (CD80/86 and MHC II) on pDCs, a subset of the DC population known for their secretion of cytokines. mRNA expression for Th1 cytokines (TNF-alfa and INF-alfa and IL-12) and Th2 cytokines (IL-4 and IL-6) was measured in the non-lymphocytic PBMC population of stimulated and non-stimulated control animals.

In vitro stimulation of PBMCs in immunocompetent (adult) and neonatal pigs with TLR 2/6 ligand (Pam3) and TLR 9 ligand (CpG) results in activation of pDCs compared to the control animals in both groups. In neonatal animals we see a more pronounced activation of pDCs after stimulation with TLR 2/6 ligand (Pam3) compared to the activation after stimulation with TLR 9 ligand (CpG). The adult animals show a better activation of pDCs after stimulation with TLR 9 ligand (CpG) compared to the activation of pDCs after stimulation with TLR 2 ligand (Pam3). These results of the adult animals are in line with the results of Task 9.1.

Based on the results of the upregulation of the mRNA expression of cytokines after stimulation compared to the non-stimulated controls neonatal animals seem to show a stronger response after stimulation with the ligands especially for TLR 2/6 ligand (Pam3) compared to the adult animals.

The conclusion of Task 12.1 is that both ligands for TLR 2/6 (Pam3) and TLR 9 (CpG) give an activation of DCs in neonatal and immunocompetent (adult) animals and could be an adjuvant of choice in further vaccination trials. The results indicate that age could be an important factor in adjuvant selection to achieve the best efficacy of a vaccine. For neonatal animals TLR 2/6 (Pam3) would be the adjuvant of preference and for adult animals TLR 9 (CpG).

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Glossary

APC: Antigen Presenting Cell
CCD: Centrale Commissie Dierproeven
CpG: TLR 9
CVI: Central Veterinary Institute
DAMP: Danger Associated Molecular Pattern
DC: Dendritic Cell
MDA: Maternal Derived Antibodies
PBMC: Peripheral Blood Mononuclear Cell
PAMP: Pathogen Associated Molecular Pattern
Pam3: TLR 2/6
PRR: Pathogen Recognition Receptor
Th1 response: T helper cell 1 response
Th2 response: T helper cell 2 response
TLR: Toll-Like Receptor
TLR-L: Toll-Like Receptor ligand

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