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Skewed pattern of Toll-like receptor 4-mediated cytokine production in human neonatal blood: Low LPS-induced IL-12p70 and high IL-10 persist throughout the first month of life

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Abstract Newborns are highly susceptible to infectious diseases, which may be due to impaired immune responses. This study aims to characterize the ontogeny of neonatal TLR-based innate immunity during the first month of life.
Cellularity and Toll-like receptor (TLR) agonist-induced cytokine production were compared between cord blood obtained from healthy neonates born after uncomplicated gestation and delivery ($n=18$), neonatal venous blood obtained at the age of one month ($n=96$), and adult venous blood ($n=17$). Cord blood TLR agonist-induced production of the Th1-polarizing cytokines IL-12p70 and IFN-α was generally impaired, but for TLR3, 7 and 9 agonists, rapidly increased to adult levels during the first month of life. In contrast, TLR4 demonstrated a slower maturation, with low LPS-induced IL-12p70 production and high IL-10 production up until the age of one month. Polarization in neonatal cytokine responses to LPS could contribute to neonatal susceptibility to severe bacterial infection.

Introduction

Neonates have an increased susceptibility to infection, causing significant morbidity and mortality. The incidence of infections is particularly high in the first weeks of life, and rapidly decreases thereafter [1]. Common causes of infection in neonates include commensal bacteria such as group B streptococci and coagulase negative staphylococci, and Gram-negative organisms like *Escherichia coli* [1].

Susceptibility to infection appears to be due to immaturity of the neonatal immune system. Neonatal adaptive immune responses are hampered by a lack of pre-existing memory and decreased Th1-type responses [2]. In addition, the innate immune system of newborns is also impaired [3]. Toll-like receptors (TLRs) are highly conserved components of the

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innate immune system and are involved in the recognition of microbial pathogen-associated molecular patterns. TLR activation triggers intracellular signalling cascades, resulting in production of inflammatory mediators that modulate the primary immune response and instruct the adaptive immune system. Thus, TLRs are essential in initiating and orchestrating the immune response. Studies of neonatal cord blood suggest that neonatal responses to multiple TLR agonists are impaired at birth. Neonatal cord blood monocytes demonstrate lower *in vitro* production of tumor necrosis factor-α (TNF-α) after stimulation with several TLR agonists, including bacterial lipopeptides (TLR2) and lipopolysaccharide (LPS; TLR4) [4,5]. TLR-mediated responses in human cord blood dendritic cells (DC) are also distinct. Upon *in vitro* LPS stimulation, neonatal monocyte-derived DC (moDC) showed a significantly lower expression of activation markers CD40 and CD80 and decreased production of interleukin-12p70 (IL-12p70) and interferon-β (IFN-β) compared to adult moDC [5,6]. Thus, impairments in the newborn TLR system may predispose for infections. The importance of the TLR system in newborns and infants is exemplified by patients with defects in the TLR-MyD88-IRAK4 pathway, who tend to present with severe infections early in life and clinical disease lessens with age [7–9].

Most studies assessing neonatal TLR responses used cord blood, which is more readily available than neonatal venous blood. However, the rapidly changing physiology at birth leads to significant changes to the blood compartment in the first hours and days of life. Because of the critical role of TLRs in the developing neonatal immune system, insight into the development of TLR function during the first months of life will likely contribute to a better understanding of the host defence against infection during this critical period in life. Here we show that unlike responses to agonists for TLR3, 7 and 9, neonatal responses to LPS are impaired throughout the first month of life, suggesting a TLR-pathway selective impairment that could contribute to susceptibility to particular infections.

Materials and methods

Blood

The research protocol was approved by the local Medical Ethical Committee of the University Medical Centre Utrecht and written informed consent was obtained from parents of all participants. Blood was obtained from healthy newborns participating in an ongoing birth cohort study on the role of neonatal TLR responses in the pathogenesis of respiratory tract infections and asthma. Cord blood was collected directly after uncomplicated vaginal delivery ($n=18$). Peripheral venous blood was obtained by venipuncture at the age of 1 month ($n=96$), or from healthy adult volunteers ($n=17$). Exclusion criteria for blood collection at birth or at the age of one month were preterm delivery, a complicated obstetric history, perinatal use of antibiotics by mother or child or any type of medical intervention. To investigate the timing of TLR4 maturation, a third group of children was included from whom venous blood was collected 5 days (range 1–7) after delivery ($n=22$). In the latter group, we allowed for minor medical issues, such as macrosomy or low temperature warranting glucose control. None of the participants had any sign or symptom of infectious disease, such as respiratory tract complaints or fever, in the two weeks prior to sampling. Due to practical considerations, we were unable to obtain repeated blood samples in the same children. Baseline characteristics are shown in Table 1. Blood was collected in sterile tubes and anticoagulated with EDTA for differential blood count, or with sodium heparin for flow cytometry and *in vitro* TLR stimulation assays. Limited blood volume and technical issues prevented us from performing all measurements in all subjects. The exact *N* for each experiment can be found in Supplementary Table 1.

Flow cytometry

Expression of cell surface antigen was determined by incubating whole blood samples with fluorescence-labeled monoclonal antibodies for 15–30 min. Antibodies were conjugated to fluorescein isothiocyanate (FITC) (CD8, CD14, CD45RA, CD56, lineage cocktail), phycoerythrin (PE) (CD5, CD16, CD45RO, CD62L, CD123), allophycocyanin (APC) (CD3, CD11c, CD19) or peridinin–chlorophyll–protein complex (PerCP) (CD4, HLA-DR). All antibodies were obtained from Becton and Dickinson Biosciences, Franklin Lakes, NJ.

After incubation, red blood cells were lysed using 1× lysing solution (BD Biosciences). Cell pellets were washed in phosphate-buffered saline and fixed using 1% paraformaldehyde. Flow cytometry was performed using the FACS Calibur system (BD Biosciences) and data were analyzed using CellQuest pro software (BD Biosciences). Whole blood concentrations of lymphocytes and neutrophils were determined by total and differential leukocyte count using the Cell-Dyn Sapphire haematology analyzer (Abbott diagnostics, Abbott Park, IL). Manual leukocyte differential was performed in

Table 1 Characteristics of participants.

Characteristic	Group		
	Birth ($n=18$)	1 wk ($n=22$)	1 mo ($n=96$)
Gender, male, *n* (%)	10 (56)	13 (59)	49 (51)
Gestational age, wk, mean (95% CI)	40.0 (39.3–40.6)	39.9 (39.3–40.6)	39.8 (39.5–40.0)
Birth weight, g, mean (95% CI)	3650 (3443–3857)	3643 (3354–3931)	3583 (3487–3678)
Mode of delivery, *n* (%)			
Vaginal	18 (100)	6 (27)	67 (70)
Cesarean section	0 (0)	16 (73)	29 (30)
Siblings, *n* (%)	13 (72)	16 (73)	59 (61)