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Analysis of selected aspects of inflammasome function in the monocytes from neonates born extremely and very prematurely

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ABSTRACT

Background: Inflammasomes regulate activation of caspase-1, which cleaves and activates interleukin (IL)-1β and IL-18, the cytokines that trigger pro-inflammatory and antimicrobial responses. There is very little known about inflammasome function in the subsets of monocytes (MO) isolated from preterm neonates born extremely and very prematurely.

Methods: A group of 76 very low birth weight patients without early-onset sepsis was divided into extremely preterm (< 28 gestational week) or very preterm (28–32 gestational week) neonates. The first blood sample was collected on the 5th day of life (5th DOL) to analyse MO subsets as well as the intracellular IL-1β expression and supernatant concentration of IL-1β and IL-18. Secondary blood samples were collected within 24 h of late-onset sepsis (LOS) development and analysed as above.

Results: On the 5th DOL, the extremely preterm neonates were characterized by a significantly higher absolute count of MO, in particular in the classical and intermediate subsets, as compared to the very preterm group. The counts of the intermediate and non-classical MO subsets increased during LOS in all neonates. We did not observe significant differences in the intracellular IL-1β expression between the analysed groups. Furthermore, the levels of the analysed cytokines in the MO supernatants were comparable between the extremely and very preterm neonates on the 5th DOL. Finally, a higher level of IL-18 was observed in the supernatant of the extremely preterm group during LOS.

Conclusions: During LOS, extremely preterm neonates excrete a higher level of IL-18 cytokines compared to very preterm neonates. Further studies are required to determine whether this observation is a result of a higher count of the circulating MO or is a true reflection of increased inflammasome function in this particular group of newborns.

1. Introduction

There has been substantial progress in neonatal intensive care over the decades, yet systemic infections still remain an important cause of neonatal morbidity and mortality, especially in those born prematurely (Shane and Stoll, 2014). Invasive infections can lead to irreversible organ damage and impaired neurodevelopment in early childhood but can also be fatal within the first weeks of life (Stoll et al., 2004). The increased risk of sepsis in preterm infants can be attributed to prolonged intensive care stays and invasive medical treatments including mechanical ventilation, parenteral feeding and intravenous catheters. Additionally, the underdeveloped immune system of a neonate helps to facilitate rapid colonization by various hospital pathogens (Siegrist and Aspinall, 2009).

From birth, neonates are heavily reliant on their innate immune system for protection against invasive infections (Goenka and Kollmann, 2015). Monocytes (MO) play an important role in both innate and adaptive immune responses and constitute approximately 10% of the peripheral leukocytes in humans (Yang et al., 2014). In addition to being specialized phagocytic and antigen presenting cells, MOs produce pro-inflammatory interleukins (IL): IL-1β and IL-18, which regulate the innate and adaptive immunity, and play key roles in host defence against infection (Krumm et al., 2014). These cytokines are released in response to the presence of a pathogen. The regulation of cytokine release from MOs is tightly controlled by multi-protein complexes called inflammasomes. Each inflammasome is composed of a pattern recognition receptor, an adaptor molecule and a caspase-1

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enzyme, which in MOs is constitutively activated (van de Veerdonk et al., 2011; Netea et al., 2009). The stimulation of a membrane toll-like receptor (TLR) by a bacterial lipopolysaccharide (LPS) such as the TLR4 ligand – a component of the outer membrane of Gram-negative bacteria, or a synthetic analogue of bacterial lipoproteins, *N*-Palmitoyl-*S*-[2,3-bis(palmitoyloxy)-(2*RS*)-propyl]-*R*-cysteinyl-(*S*)-seryl-(*S*)-lysyl-(*S*)-lysyl-(*S*)-lysyl-(*S*)-lysine x 3 HCl (Pam3Cys – a TLR2 ligand) leads to the activation of the MyD88-dependent intracellular signaling pathway. This results in the activation of nuclear factor-B pathway, which increases the expression of the inflammasome related protein genes, pro-IL-1β and pro-IL-18 (Belge et al., 2002; Segovia et al., 2012; Kawasaki and Kawai, 2014). The caspase-1 enzyme within the inflammasome cleaves the cytokine precursors pro-interleukin 1β (pro-IL-1β) and pro-interleukin 18 (pro-IL-18) into their mature active forms, IL-1β and IL-18 respectively (Franchi et al., 2009). It is important to note that over-stimulation or malfunction of inflammasomes or MOs may lead to excessive inflammation and consequent organ damage (Huttenlocher et al., 1995).

Monocytes can be categorized into three subsets based on their surface expression of two antigens, namely CD16, a low affinity Fc receptor for IgG, (FcγIII) and CD14, a lipopolysaccharide receptor. These MO subsets include, classical = $CD14^{++}CD16^{-}$, intermediate = $CD14^{++}CD16^{+}$ and non-classical = $CD14^{+}CD16^{++}$ types (Ziegler-Heitbrock et al., 2010). Every MO subset differs in phenotype, size, nuclear morphology, cytoplasmic granularity, intracellular gene expression profiles and functionality (Gren et al., 2015; Yona and Jung, 2010). Furthermore, each MO subset is also present in distinctive amounts at any particular stage of human development. In a healthy adult, the classical subset accounts for up to 80% of the circulating MO pool, whereas the intermediate and non-classical subsets constitute 10% each (Wong et al., 2012).

Neonates are heavily reliant on the production of cytokines (such as IL-1β and IL-18) and the phagocytic and antigen-adhesion abilities of these circulating MOs to eliminate bacteria. Evidence suggests that MOs of preterm neonates are more functionally deficient, leading to the insufficient production of inflammatory mediators and therein, increasing the risk of infection (Iliodromiti et al., 2013). Overall, the mechanism of these functional impairments and how they relate to gestational age remains poorly understood.

The goal of our study was to determine the distribution of MO subsets, as well as certain aspects of inflammasome function, namely the expression of intracellular IL-1β and the levels of excreted IL-1β and IL-18 in neonates who were born very preterm (28–32 weeks gestation) or extremely preterm (< 28 weeks gestation). Within these groups, we also wanted to further assess whether monocyte distribution and inflammasome function varied between healthy preterm neonates and those who developed late-onset sepsis (LOS).

2. Materials and methods

2.1. Patients

The study was approved by the Jagiellonian University Ethics Committee. Patients enrolled in the study were recruited on an ongoing basis from the Neonatal Intensive Care Unit, Department of Paediatrics, University Children Hospital in Krakow, Poland. All parents signed informed consent about the patients' participation in the study.

Enrolled newborns were divided into two groups according to their gestational age: neonates born between 28 and 32 gestation weeks were classified as very preterm (VP group); whereas neonates born at < 28 weeks gestation were classified as extremely preterm (EP group). The exclusion criteria were: multiple congenital malformations and early-onset sepsis (EOS) defined as a blood culture-positive clinical sepsis within the first 72 h of life. Once enrolled, all the subjects were carefully monitored for symptoms of late-onset sepsis. LOS was diagnosed based on an initial clinical suspicion of the attending neonatologist and confirmed by a positive blood culture in children who were ≥72 h of age.

Two blood samples (2 × 700 µl) were obtained from all the study participants on the 5th DOL. When a patient's clinical condition changed and LOS was suspected, blood was collected again within 24 h of sepsis onset and analyses were repeated.

2.2. Flow cytometry analysis of monocyte subpopulations

Blood samples were drawn into ethylene-diamine-tetra-acetic acid (EDTA) containing tubes (Vacutainer System®; Becton Dickinson Biosciences, San Jose, California), and were washed by adding 3 ml of 0.9% sodium chloride (NaCl) in polypropylene round-bottom tubes (Becton Dickinson Biosciences, San Jose, California) and centrifuged (1000 × *g*). Then, each blood sample was put into a TruCOUNT™ Tube (Becton Dickinson Biosciences, San Jose, California) and stained on ice for 30 min with the following: anti-CD45-APC, anti-HLA-DR-PerCP, anti-CD14-FITC, anti-CD16-PE (Becton Dickinson Biosciences, San Jose, California) monoclonal antibodies (mAbs).

The samples were then treated with FACS Lysing Solution (Becton Dickinson Biosciences, San Jose, California) to lyse the erythrocytes and the cells were then immediately processed in the FACSCanto flow cytometer (Becton Dickinson, San Jose, California) along with 10.000 beads per tube. Data analysis was performed using FlowJo software (Tree Star, Inc, Ashland, Oregon). The absolute numbers of $CD14^{++}CD16^{-}$ (classical), $CD14^{++}CD16^{+}$ (intermediate) and $CD14^{+}CD16^{++}$ (non-classical) MO were calculated with reference to the bead count. The gating strategy and analysis of MO subsets was previously described by us (Siedlar et al., 2011) and others (Heimbeck et al., 2010) and is shown on Fig. 1.

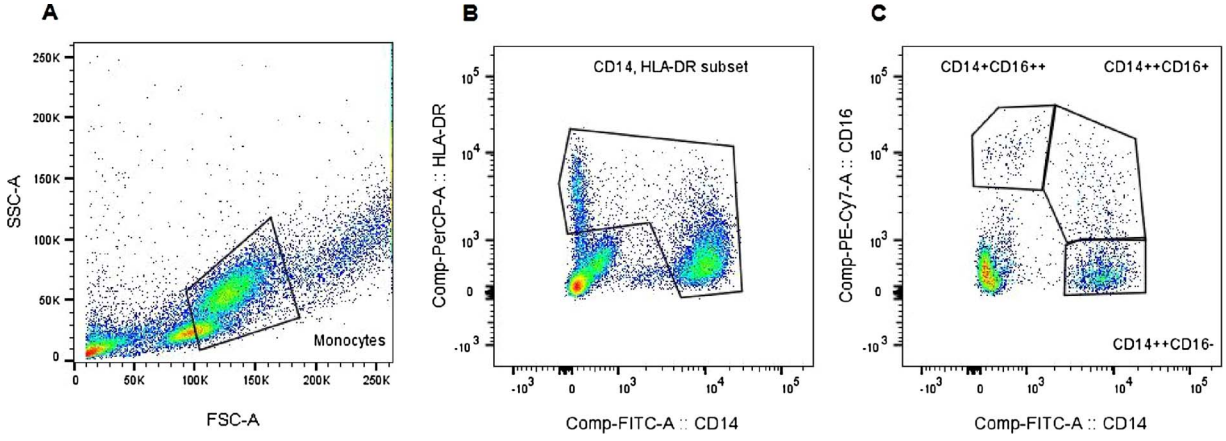

Fig. 1. Gating strategies of monocyte subsets. Monocytes were gated according to FSC and SSC parameters together with adjacent lymphocytes (A). The cells were then gated to exclude $CD14^{-}HLA\text{-}DR^{-}$NK cells (B) and finally divided into $CD14^{++}CD16^{-}$ (classical), $CD14^{++}CD16^{+}$ (intermediate) and $CD14^{+}CD16^{++}$ (non-classical) monocytes (C).