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Immune cell subsets at birth may help to predict risk of late-onset sepsis and necrotizing enterocolitis in preterm infants

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ABSTRACT

Background: Parameters predicting late-onset sepsis (LOS) and necrotizing enterocolitis (NEC) in preterm infants would be valuable. Ten-color flow-cytometry enables the estimation of cellular immune status requiring only small sample volumes.
Aims: Identifying predictive parameters for LOS and NEC in the cellular immune status of preterm infants.
Study design and subjects: In this prospective study in 40 preterm infants (week 26 + 0 to 30 + 6) and 10 healthy full-term newborn infants (control group, week 37 + 0 to 40 + 6), flow cytometric analyses of lymphocyte subpopulations were performed between the 2nd and the 6th day of life, with a follow-up until the preterm infant reached the calculated gestational age of week 40. Patients' episodes of infections and NEC were analyzed according to the NEO-KISS criteria of the German National Reference Center.
Results: Ten preterm infants showed events within the first week of life and were excluded from the analysis. Of the other 30, five developed NEC, twelve LOS. In patients with LOS, the proportion of double-negative (DN) T cells was significantly elevated compared to patients without LOS, while immune-regulatory CD56bright and CD56negCD16+ NK cells were significantly decreased ($p < 0.05$). Patients with NEC showed a reduction in the NK cell proportion (<3.7%) and significantly decreased naïve cytotoxic CD45RA+CD62L+ T cells ($p < 0.05$).
Conclusion: NK cells and DN-T cell counts within the first week of life may be predictors for NEC and LOS in preterm infants. In order to identify patients at risk early, further analysis of these populations might be of interest.

1. Introduction

Neonatal late-onset sepsis (LOS) has a high mortality and a major influence on neurodevelopmental outcome and increases costs for the public health system [1]. LOS is defined as neonatal sepsis occurring after three days of age. Early and correct diagnosis of LOS in preterm infants is challenging because present signs are subtle and non-specific [2,3]. Various risk factors have been described for both, LOS and necrotizing enterocolitis (NEC) including low gestational age, exposure to antenatal antibiotics, central venous catheters, total parenteral nutrition and sepsis [4–6]. Only a few of these factors may be influenced by treatment strategies on the neonatal intensive-care unit (NICU). The immature immune system of preterm infants has been discussed largely as a factor promoting sepsis [7]. El-Sameea et al. showed that in LOS the NK cell functionality is reduced [8]. The major population of $CD56^{+}CD3^{-}$ NK cells in the peripheral blood (PB) can be subdivided into a major $CD56^{dim}CD16^{+}$ population which is highly cytotoxic and a smaller immune regulatory $CD56^{bright}CD16^{dim/-}$ population with a potent cytokine producing capacity [9]. However, a dysfunctional NK cell population named $CD56^{neg}CD16^{+}$ emerges, characterized by the loss of CD56, while the expression of CD16 is maintained [10,11]. Also the T cell compartment especially the main T cell subpopulations, Helper and cytotoxic T cells, and their differentiation bearing T cell receptor γδ were investigated in preterm infants with sepsis [12–15]. The rare T cell population $CD3^{+}$ which expressed neither CD4 nor CD8 namely double negative (DN) T cells has immune-regulatory functions and has been studied for their contribution to peripheral tolerance to discriminate between self and non-self [16]. DN-T cells do not undergo positive selection and consequently lack MHC restriction [17].

NEC is most common in the ileum, and is characterized by inflammation and coagulation necrosis [18]. Current treatment strategies for NEC

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are antibiotic administration and, if required, intestinal surgery to remove the affected part of the intestine. There are several indications that regulatory T cells (T_{reg}) are essential for intestinal immune homoeostasis. It is described that in acute NEC directly in the ileum of rats T_{reg} cells are significantly reduced [19]. Lu et al. showed an altered T cell differentiation in acute NEC in humans and there was a trend towards increased FoxP3+ cells in the ileum of NEC patients who underwent reanastomosis [20]. Furthermore, an increase in T_{reg} levels could be the mechanism for protective effects on nosocomial sepsis [21].

Naïve T cells are defined conventionally by the co-expression of the transmembrane phosphatase CD45RA isoform and the lymph node homing molecules L-selectin (CD62L) and CCR7, reflecting their predominant residence in lymphoid tissue to probe APCs for cognate antigen and to respond to activating signals that give rise to more differentiated memory and effector progeny [22]. At birth, most T cells in peripheral blood are naïve, and memory T cells which develop over time in response to diverse antigen exposure [23]. Memory T cells are subdivided into CD45RO+CD62L+ central memory T (T_{CM}) cells which traffic to lymphoid tissues, and CD45RO+CD62L− effector memory T (T_{EM}) cells which are able to migrate to multiple peripheral tissue sites [24].

The advantages in the development of multicolor 10-color flow cytometry made it possible to analyze many subgroups of lymphocytes only requiring a small amount of blood with the aim to predict life-threatening events, e.g. LOS or NEC and enable early therapeutic intervention.

Lab parameters distinguishing between patients with normal and high risk for LOS might be of use for clinicians. Prophylaxis and prevention of NEC and LOS have been discussed in many publications [25–30]. Defining subgroups of patients at risk could help to initiate prophylaxis programs in a rational manner.

As only few publications deal with the cellular immune status and its influence on LOS and NEC, we initiated a prospective study on preterm infants by analyzing their cellular immunity within their first week of life. Thereby, we analyzed immune cell subpopulations within the clinical course of the patients until the age of a mature neonate.

2. Materials and methods

2.1. Patients and setting

The study was designed as a single-center prospective, non-randomized epidemiological monitoring of the cell-mediated immune system of 40 preterm infants and ten full-term infants. Preterm infants (week 26 + 0 to 30 + 6) and healthy full-term newborn infants (control group, week 37 + 0 to 40 + 6) were enrolled consecutively and were born between August 2010 and June 2012 in our university hospital. Peripheral Blood (PB) samples were taken from both groups between the second and the sixth days of life (median: 4th day of life). The healthy full-term neonates had control venipunctures for icterus exclusion. Patients with a sepsis or NEC within the first seven days of life were not further evaluated. The preterm infants were subdivided by gestational age into five subgroups (Table 1). Informed consent was obtained from the legal guardian and pseudonymization was performed according to the guidelines of the medical ethics committee of the Frankfurt University Hospital (Ethic No: 91/10, Protocol: NEOIMMUN-FFM 2010).

2.2. Definitions

Patients' episodes of infections and NEC were analyzed according to the NEO-KISS criteria of the German National Reference center for Surveillance of nosocomial infections (NRZ). The project collects nationwide reference data about the frequency of nosocomial infections among preterm infants. All children with a birthweight (BW) of less than 1500 g are included until their hospital discharge, death or weight of over 1800 g. Special definitions regarding development are applied for the diagnosis of the three kinds of infections tracked: pneumonia, primary bloodstream infections and NEC.

Clinical sepsis: administration of antibiotics for at least five days, no microbial agents are detectable in the peripheral blood, no signs of local infection and two of the following conditions are visible: fever, tachycardia, prolonged recapillarization, apnea, acidosis, hypoglycemia or other signs of sepsis.

Microbiological sepsis: if additionally a microbial agent is found in blood or liquor (not coagulase negative Staphylococcus).

NEC is defined by one radiological sign (pneumoperitoneum, pneumatosis intestinalis, paralytic ileus) and two of the following conditions: vomiting, chyle transportation problems, swollen belly, rubor at the flanks or rectal bleeding. Clinical Risk Index for babies (CRIB) score was enquired based on the CRIB score defined by the International Neonatal Network [31].

2.3. Laboratory processes

PB EDTA samples were collected in the morning, kept at room temperature and analyzed within 8 h (median 4 h). Flow cytometry of immune cell subsets in the PB was performed as single-platform analysis using a Navios™ 10-color/three laser flow cytometer (Beckman Coulter, Krefeld, Germany). Monoclonal IgG1, [1]IgG2b and [2]IgG2a antibodies against CD45, CD56, CD19, CD3, [2]CD14, CD4, CD16, [2]CD25, CD45RA, CD62L, [2]CD45RO, [1]CD69, CD4, CD127, CD3, [1]HLA-DR, and CD8[#] ([#]Invitrogen, all others from Beckman Coulter, Immunotech, Marseille, France) were conjugated with fluorescein isothiocyanate (FITC), phycoerythrin (PE), phycoerythrin-Texas-Red-Tandem (ECD), phycoerythrin-cyanine-5.5 (PC5.5), phycoerythrin-cyanine-7 (PC7), Allophycocyanine (APC), APC-Alexa Fluor (APC-A700 or 750), Pacific Blue (PacB), and KromeOrange (KO) for staining. In total a maximum of 250 μl peripheral blood was needed. In brief, 50–100 μl whole blood was mixed with 1–10 μl of the respective antibody and incubated at room temperature for 15 min in the dark. An automated lyse/no wash procedure with a fixation step followed with the ImmunoPrep® reagent using TQ-Prep™ Workstation (Beckman Coulter, Krefeld, Germany) was done. Immediately before measurement, with the same pipette as used for the sample accurately 100 μl Flow-Count Fluorospheres (Beckman Coulter) was added after gently shaking. A median of 250,000 events with a medium flow rate of approximately 30 μl/min was used for assessment. The precise gating was done with the CXP- and Kaluza® Software (Beckman Coulter, Krefeld, Germany).

The optical alignment, fluidic stability and accuracy of the flow cytometer were tested with Flow Check Pro Fluorospheres (Beckman Coulter) and Immuno-Trol™ Cells (stabilized leukocytes as positive process controls) daily, before measurements. Flow set Fluorospheres (Beckman Coulter) served to set up the photomultiplier tube values weekly. Peripheral blood of healthy donors and stained Cyto-Comp cells (Beckman Coulter) was used to compensate the fluorescence overlap.

The first tube examined the quantity of leukocytes, lymphocytes, $CD14^+$ monocytes, $CD3^+$ T cells, $CD3^+CD4^+$ helper T cells (T4), $CD3^+CD8^+$ cytotoxic T cells (T8), $CD3^+CD4^+CD8^+$ double-positive (DP) T cells, $CD3^+CD4^-CD8^-$ double-negative (DN) T cells, $CD56^+CD3^-$ NK cells, NK incl. $CD56^-$ cells, $CD3^+CD56^+$ NK-T cells, and $CD19^+$ B cells. The second panel allowed the differentiation of the cytotoxic T and helper T cell compartment in naïve ($CD45RA^+CD62L^+$, $T_{Naïve}$), central memory ($CD45RO^+CD62L^+$, T_{CM}), effector memory ($CD45RO^+CD62L^-$, T_{EM}) and effector memory RA ($CD45RA^+CD62L^-$, T_{EMRA}) as well as the early ($CD69^+$) and late ($HLA\text{-}DR^+$) activation status. Furthermore, the regulatory T cell amount ($CD4^+CD25^{++}$ $CD127^{dim/neg}$, T_{reg}) and NK subpopulations, $CD3^-CD56^{bright}CD16^{-/+}$ ($CD56^{bright}$), $CD3^-CD56^{dim}CD16^+$ ($CD56^{dim}$) and $CD3^-CD56^{-negative}CD16^+$ were analyzed (overview summarized in Fig. Fig. 1).