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ORIGINAL ARTICLE

Immune response of toddlers with history of prematurity

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Abstract
Background: It is not quite well established how immune responses differ in term and preterm infants beyond the first year of life. This study aimed to evaluate aspects of the innate and adaptive immune responses in a group of preterm infants in comparison with their term peers.
Methods: In this cross-sectional study peripheral blood mononuclear cells (PBMC) were isolated from preterm and term children at age three years. Innate immune response was evaluated by the analysis of TLR receptors expression on $CD11c^{+}HLADR^{high}$ cells and inflammatory cytokine production after PBMC stimulation with Toll like receptors (TLR) ligands. Adaptive immune response was evaluated by T cells' phenotyping and function after stimulation with polyclonal conventional T cell stimulus.
Conclusion: We have found that the patterns of innate and adaptive immune responses at 3 years of age were not affected by the fact of the children having being born preterm or at term.

Abbreviations: CBA, Cytometric Bead Array; CPG ODN, CpG oligodeoxynucleotide; DC, dendritic cells; LPS, lipopolysaccharide; PBMC, peripheral blood mononuclear cell; PGN, peptidoglican; Poly I:C, Polyinosinic-polycytidylic acid; PRR, pattern-recognition receptor; TLR, Toll-like receptor.

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Introduction

Eleven percent of all infants are born premature (fewer than 37 weeks of gestation), and this represents almost 13 million children each year worldwide.[1] In general terms, newborn babies have an increased susceptibility to infections due to the functional immaturity of their immune system.[2] Preterm neonates present greater risk for morbidity and mortality, and this is in general associated with infection, since the immune system of preterm neonates is thought to be less developed at birth in comparison with term babies.[3] However, this greater risk rapidly and significantly decreases during infancy,[4] due to the progressing maturation process of the immune system. Protection against infectious pathogens is achieved through the coordinated actions of innate and adaptive immunity. There are few studies looking at leukocytes from preterm children and even less data available concerning the development of innate and adaptive immune systems comparing term and preterm babies in the first years of life.

Innate immune function is activated by sensors expressed by leukocytes. The best known innate sensors are pattern-recognition receptors (PRRs) of the Toll-like receptors (TLRs) family. Recent studies have demonstrated that the expression of TLRs in mononuclear cells of healthy infants over the first five years of life appears to be stable and to occur at adult-like levels.[5–7] The cytokines released upon TLR stimulation have a potent regulatory effect, on both innate and adaptive immune cells. Dendritic cells (DC) are antigen presenting cells (APC), linking innate and adaptive immune response, controlling T cells differentiation. After contact with cognate MHC-peptide complexes on APCs, naïve T cells became effectors cells or memory T cells. It was previously demonstrated that the numbers of CD4 and CD8 T cells were lower in cord blood of preterm compared to term infants.[8] Interestingly, the proportion of memory T cells ($CD3^+CD45RO^+$) was higher in peripheral blood of preterms compared to term babies at birth.[9] However, reports on the development of adaptive immune response are scarce during this early maturation stage of the first years of life. The main objective of this study is to determine patterns of innate immune responses in term and preterm infants at age three years by characterising the TLR expression on dendritic cells and adaptive immune responses evaluating CD4 and CD8 T cells in peripheral blood mononuclear cells.

Materials and methods

Ethics statement

All procedures involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Written informed consent was obtained from all children's parents or guardians. This study was reviewed and approved by Research Ethics Committee of Pontifícia Universidade Católica do Rio Grande do Sul (CEP/PUCRS) under protocol number CEP10/04999.

Subjects

This is a cross-sectional study with data collected at a mean age of three years from children born preterm (mean gestational age 32.18 weeks) and full-term (mean gestational age > 37 weeks). Patients were enrolled from 2011 to 2012. Exclusion criteria were viral respiratory infection or any other infection in the previous four weeks, chronic diseases (heart disease, neurological diseases, immunodeficiencies, allergic rhinitis and asthma) or the use of oral steroids or medications that could affect the results. Five millilitres of blood was collected from healthy preterm ($n = 28$) and term children ($n = 9$). Clinical data reported by parents or guardians and physical examination were collected by one of the investigators. Healthy term children were recruited among those undergoing an elective surgery, during the preoperatory evaluation.

Mononuclear cell isolation

Peripheral blood mononuclear cells (PBMCs) were purified from 5 mL of whole blood using a Histopaque-1077 (Sigma–Aldrich, Saint Louis, MO, USA) gradient. Cells were frozen at $-80\,^{\circ}C$ until use. PBMCs were seeded at 2×10^5 cells/well in 96 well-culture plates with RPMI medium (Cultilab, Campinas, SP, Brazil), containing 2% of foetal calf serum (Cultilab, Campinas, SP, Brazil) and were stimulated with 2 μg/mL of purified, no azide, low endotoxin, anti-CD3 (clone UCHT1) and anti-CD28 (clone CD28.2) antibodies (BD Bioscience, USA) or with TLR ligands: LPS from *E. coli* O111:B4 (10 μg/mL) (Sigma–Aldrich, USA), PGN (peptidoglycan) from *B. subtilis* (4 μg/mL) (Invivogen, San Diego, CA, USA), Poly I:C (Polyinosinic-polycytidylic acid) (1 μg/mL) (Invivogen, San Diego, CA, USA) or left unstimulated for 24 h. The culture supernatant was collected for cytokine analysis.

Flow cytometry

To analyse TLR expression and activation markers on DC, PBMCs were stained with the antibodies: anti-CD11c PE-Cy7 (clone B-ly6), anti-HLADR PE-Cy5 (clone TU36), anti-CD86 PerCP-Cy5.5 (2331 (FUN-1)), anti-TLR4 Biotin (clone HTA 125) followed by FITC Streptavidin, and anti-CD282 (TLR2) Alexa Fluor® 647 (clone 11G7). To analyse memory T cells, PBMCs were stained with: anti-CD3 APC (clone UCHT1), anti-CD4 PECy7 (clone SK3), anti-CD8 FITC (clone RPA-T8), anti-CD45RO PerCP Cy 5.5 (clone UCHL1), and anti-CD27 APC-H7 (clone M-T271) antibodies. To evaluate the expression of intracellular TLR and granzyme B, cells were permeabilised using Cytofix/Cytoperm kit and stained with anti-TLR9 PE (clone eB72 1665) and anti-Granzyme B Alexa Fluor® 647 (clone GB11). All antibodies were from BD Bioscience (USA). Data were accessed by flow cytometry using FACS Canto II (BD Bioscience) and analysed using Flow Jo software (Tree Star). Cytokine analysis from cell culture supernatant was performed using CBA (Cytometric Bead Array) Inflammation kit (BD Bioscience, USA) followed by analysis on FCAPArray 3.0 (Soft Flow).

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