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Human neonates display altered *ex vivo* monokine production related to healthy adults

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ABSTRACT

The inflammatory response plays an important role during the induction of several neonatal diseases. Previous studies have shown that during newborn infections, the natural imbalance between pro- and anti-inflammatory responses shifts toward the production of pro-inflammatory cytokines. In this study, we employed an array system to detect 9 pro- and anti-inflammatory cytokines, and performed ELISA for 6 other cytokines. We then compared the immune response profiling in umbilical cord blood (UV) plasma samples with circulating levels in otherwise healthy donors (HD). Concentrations of *ex vivo* monokine levels, such as interleukins (IL)-18, IL-23 and IL-27, were profoundly reduced in the UV in relation to the HD group (p-values of 0.003, 0.009 and <0.0001, respectively). Conversely, UV-plasmatic TGF-β1 levels displayed marked enhancement (p-value = 0.005) in relation to HD. Several factors may be implicated in these neonatal alterations, and additional characterization of a broader cytokine panel is warranted to reveal other possible candidates.

1. Introduction

Recently, there have been remarkable advances on developmental and functional mechanisms of the human neonatal immune system [1]. Several aspects of the comparison between adult and neonate features have been described, particularly in regards to the rapid maturation of neonatal immunity. Compared to adults, newborns exhibit increased susceptibility to infectious agents [2]. This reflects the generalized hypofunction of inflammatory and immune mechanisms in a number of protective systems, which significantly increases the risk of infection in this exposed population. This may be related to the natural dampening of the Th-1 associated immune response. However, during development the neonatal immune system is constantly maturing, since it is a continuous process where both accelerated and retarded development is deleterious [3]. Conversely, there are virtually no comparative studies concerning *ex vivo* broaden analysis addressing the role of monokines in the newborn vulnerable population.

This is a community based open-label cross-sectional population study of a Brazilian sample, and the rationale is to reveal critical aspects of *ex vivo* monokine and lymphokine profiles related to both innate and adaptive immunity. The study was undertaken to compare newborn (UV) and adult (HD) plasma samples using multiplex array and ELISA approaches, and we set out to investigate whether the quantitative detection of circulating biomarkers differs between these groups.

2. Methods

Between November 2010 and August 2012, two groups of donors were enrolled in this study at the Gaffrée Guinle State University Hospital of Rio de Janeiro. Samples were obtained from healthy donors (HD = 28) from a blood bank (≥18-years old), and from newborns' umbilical vein (UV = 28) after obtaining the assent of admitted healthy mothers who voluntarily participated in umbilical cords puncture procedures after full-term births. In the present study, the gestational age ranged from 39 weeks to term, and the most frequent delivery mode was elective cesarean section (80%; Table 1). Exclusion criteria for those individuals utilized HIV-seronegative status, a negative history of malignant, degenerative, or transmitted diseases, diabetes mellitus, and use of corticosteroids or other immunosuppressive

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Table 1
Characteristics of the neonate population.

Neonatal growth parameters	UV
Gestational age (weeks)	39.2 ± 0.07[a]
Birth weight (kg)	0.04 ± 0.02
Birth length (cm)	51.1 ± 0.2
Mode of delivery	**UV**
Induced vaginal	–
Vaginal	13%[b]
Elective cesarean	80%
Emergency cesarean	7%[c]

[a] Mean ± SEM.
[b] Duration: 5.3 h.
[c] Duration: 4 h.

agents at the time of the study. For each participant, 2 vials of plasma were kept frozen at −70 °C. Other details related to those individuals are described elsewhere [4]. Local ethical committee protocol approvals were achieved before enrolments, and all written informed consents were obtained from adult and parturient subjects. Subjects' identities were omitted. The study was approved by the Institutional Review Board of the State University Hospital (060/2009 & 089/2011).

Extensive evaluations of the cytokine levels involved in the pro- and anti-inflammatory pathways, which is thought to play a key role in particular conditions and might act as biomarkers aiding future clinical diagnosis, were performed by commercially available protein multiarray system (Bio-Rad, Hercules, CA, USA), which uses Luminex-based technology to quantify human IL-2, IL-4, IL-5, IL-10, IL-12, IL-13, GM-CSF, TNFα and IFNγ, and ELISA (DuoSet R&D, Minneapolis, MN, USA) to quantify human IL-1α, IL-18, IL-23, IL-27, IL-33 and TGF-β1 in parallel. Both immunoassays were carried out using thawed plasma samples in accordance with the manufacturers' instructions. Detection limits ranged from 1.8 to 7.7 pg/ml for multiarray, and from 0.012 to 0.06 ng/ml for ELISA.

Data were analyzed using GraphPad Instat software. Cytokine levels between groups were compared using the Mann–Whitney *U* test and correlation analysis intra-groups were performed by Spearman's rank coefficient test, and deemed to be significantly different at p-values ≤ 0.05.

3. Results

To study the critical aspects of *ex vivo* cytokine profiles related to both innate and adaptive immunity, we carried out two experiments comparing newborn (UV) and adult (HD) plasma samples using multiplex array and ELISA approaches. We set out to investigate whether the quantitative detection of circulating biomarkers differs between these groups. Plasma samples harvested from heparinized whole blood from HD and UV groups were carefully thawed out once and used for a range of analyte determinations. All experiments were carried out using the same lots of reagents.

As a whole, no significant differences were found between the UV and HD groups for the majority of cytokines tested (Table 2). In the ELISA evaluations, we found statistically significant lower plasma concentrations of IL-18 (Fig. 1A) and IL-23 (Fig. 1B), as well as IL-27 (Table 2), in neonates compared to healthy adults (p-value: 0.003, 0.009 and 0.0001, respectively).

Conversely, we observed a significant increase in TGF-β1 levels (p-value = 0.005) in newborn plasma samples when compared to circulatory levels in adult donors (Fig. 1C).

Of note, no correlation among those cytokines was found in the HD group, but IL-18 plasma levels were positively correlated with IL-23 ($\rho = 0.52$; p-value = 0.02) in the UV group only (Supplementary Fig. 1). No other correlations were found for the UV group.

Table 2
Ex vivo human cytokine levels (pg/ml) determined in thawed healthy donor adult plasma (HD = 28) and umbilical cord blood samples (UV = 28) using commercially available protein multiarray system and enzyme linked immunosorbent assay (ELISA).

Cytokines	UV	HD
IL-1α	0.07 ± 0.01[a]	0.06 ± 0.01
IL-2	7.7 ± 3.2	4.6 ± 2.1
IL-4	29.4 ± 9.0	17.1 ± 7.3
IL-5	27.3 ± 4.9	24.7 ± 3.6
IL-10	77.2 ± 23.9	27.6 ± 9.5
IL-12	11.7 ± 4.2	7.9 ± 1.5
IL-13	17.9 ± 2.2	14.3 ± 0.9
IL-33	0.02 ± 0.0	0.2 ± 0.1
IFNγ	69.8 ± 15.5	51.8 ± 12.0
TNFα	46.2 ± 12.8	32.4 ± 9.1
GM-CSF	22.3 ± 7.7	10.2 ± 4.0
IL-27	1.6 ± 0.5[*]	12.3 ± 3.4

[*] $p < 0.0001$, when compared to HD group and based on statistical significance using the Mann–Whitney *U* test.
[a] Mean ± SEM.

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When compared to the multiplex approach, it should be stated that the apparent superior performance of the ELISA approach was an anticipated bias, due to our selective choice to quantify monokines based on a previous trend (data not shown).

4. Discussion

In newborns' immune system, there is a natural disparity between pro- and anti-inflammatory responses. Previous studies suggest that this imbalance is essential to drive the production of pro-inflammatory cytokines in young individuals during common infections [5–7]. A recent study proposes that neonatal antigen-presenting cells also display functional alterations in terms of soluble factors released, namely monokines, which may involve additional impairments of adaptive immune responses [8].

Clinical studies are essential to infectious disease research. The clinical focus target was to uncover performance indicators (e.g., biomarkers, such as cytokines) on which a given cohort was altered. The first review comparing plasmatic cytokine levels between adults and newborns was only published in the turn of the century [9]. Therefore, in the last few years our group has been interested in original, leading studies comparing newborn *vs.* adult immunity by using plasma from both cohorts. Thus, by evaluating other *ex vivo* monokines and lymphokines related to both innate and adaptive immune responses, we have uncovered an important role played by monokines, such as IL-18, IL-23 and IL-27. These factors are typically produced primarily by dendritic cells, monocytes and macrophages. Levels of IL-33, which is constitutively and abundantly expressed in normal human endothelium, fibroblasts, skin keratinocytes and epithelial cells [10], also followed that trend (Table 2; p-value = 0.09).

Discrepancies in cytokine levels between adult and neonatal immune cells have been largely reported, particularly for IL-18 and IL-27. Recently, Fransson et al. [11] compared the *ex vivo* IL-18 production in pregnant women and infants, and found no significant differences in IL-18 plasma levels across the two groups. Strikingly, IL-18 and IL-27 have dual functions, as they are both able to induce Th1- and Th2-immune responses according to the *in vivo* context [12]. It is widely accepted that during pregnancy and at term, protection against severe infections is modulated by specific cells and molecules [1].

Elevated levels of IL-27 mRNA were detected in the murine model, and similar results were obtained with human cord-blood derived macrophages as well [2]. Our previous [13] and current