CHARACTERIZATION OF THE INFLAMMATORY STATUS OF PRETERM INFANTS

PhD thesis

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1. INTRODUCTION

The hallmark of the immune system is that it recognizes invading foreign organisms, prevents their spread, and ultimately clears them from the body. It also plays an important role in the regulation of the inflammatory response often related to complications affecting preterm infants. Preterm birth, defined as birth at less than 37 completed weeks of gestation, occurs in around 12% of deliveries worldwide with major implications for the long term health of the child. The preterm immune system is not fully developed compared to a term neonate or an adult individual, and seems to be compromised leading to different long term complications due to perinatal and postnatal factors and events.

Both the innate and adaptive immunity are compromised in preterm neonates, and they also have deficiencies in the interaction between these two systems.

Several inflammatory disorders have been described in neonates which lead to an exaggerated release of inflammatory mediators, amongst the most important and common are BPD, NEC and sepsis. These disorders also have a multi-factorial pathogenesis.

A variety of prenatal factors and events may lead to preterm birth, further modulating and compromising the immune system of the newborn. Amongst the most important

complications during pregnancy are hypertensive disorders, such as preeclampsia (PE), gestational diabetes, premature rupture of membranes (PROM) and of vitamin D deficiency. These perinatal complications seem to have a serious impact on different immune cell subpopulations, cytokines and lymphocyte activation markers in the maternal and fetal immune system. The use of antenatal steroid prophylaxis for the prevention of surfactant deficiency and respiratory distress syndrome (RDS) may also have long-term consequences on neonatal immunity.

Our research group has a longstanding interest in the investigation of the neonatal and preterm immune response and function. The results obtained in such studies do not only help us better understand the basic principles of the development of the human adaptive immune system, but also play a key role in identifying diagnostic and therapeutic targets that may provide better care and reduction of lifelong adverse immune consequences for this vulnerable population in the very near future.

2. AIMS

Our aim was to characterise the inflammatory status of preterm infants at birth and during the first week of life and its association with perinatal complications as well as the influence of maternal factors.

The specific aims for our investigations were:

1. To assess the plasma 25(OH)D concentrations from cord blood of 28 preterm infants born before the 30th gestational week and to determine its correlation with cellular and soluble indicators of the inflammatory status. Effects of other factors, such as gestational age and plasma cortisol levels were also assessed.

2. To address the hypothesis that PE impacts the fetal immune system, we analysed the prevalence of distinct lymphocyte subsets and plasma cortisol and cytokine levels in preterm neonates of PE mothers during the first week of life (at birth and on the 1st, 3rd and 7th postnatal days) and compared them to preterm neonates with comparable clinical characteristics born from pregnancies not complicated by PE.

3. To assess the association of gender, gestational and

postnatal age, preeclampsia (PE), premature rupture of membranes (PROM) and prenatal steroid treatment (PS) with the frequency of activated T lymphocyte subsets (CD69+, CD25+, CD62L+, HLA-DR) and major T lymphocyte subpopulations (CD4, CD8, Th1, Th2, naïve, memory) in peripheral blood during the first postnatal week in preterm neonates. Since data on the physiological frequency of these cell subsets is challenging to obtain, we aimed to gather preliminary data to describe the dynamic postnatal alteration of these parameters in preterm neonates affected by different perinatal factors.

3. MATERIALS AND METHODS

Blood samples for all three studies were collected at the Neonatal Intensive Care Unit of the 1st Department of Obstetrics and Gynecology, Semmelweis University, Budapest, Hungary. Flow cytometry measurements were performed at the Research Laboratory of the 1st Department of Pediatrics, Semmelweis University, Budapest, Hungary. Some of the patients fulfilling the inclusion criteria for more than one study were overlapping across multiple studies.

3.1 PATIENTS

In the study on vitamin D levels, venous cord blood samples were taken from 28 preterm infants (gestational age: 29 (27-30) weeks, median (range); birth weight: 1080 (920-1550) grams, median (range)). All infants had a highly suspected or proven intrauterine infection based on standard criteria. However, no ongoing inflammatory reaction was detected in patients at the time of sampling, as indicated by normal IL-6 levels. Of note, since no suggestive clinical signs of chorioamnionitis were present, placental histology was not performed. Based on plasma vitamin D levels, patients were divided into two groups (below and above median, 23.3 ng/mL), between which all comparisons were made.

In the study on the effects of preeclampsia, we enrolled 14 preterm neonates born to PE mothers and 14 preterm neonates born to healthy mothers as controls. Median gestational age was 30 weeks in the PE group and 29 weeks in controls, while birthweight was 985 g in the PE group and 1180 g in controls, respectively. All neonates had a highly suspected intrauterine infection based on standard criteria and went through partial septic screen, however, CRP and IL-6 levels were within the normal range at birth and on the 1st and 3rd postnatal days.

In the study on activation markers, we enrolled 43 preterm infants. Gestational age was 30 (25-33) weeks, while birthweight was 1300 (490-1980) g at birth. The suspected ground for preterm birth was PE in 8 cases, PROM in 13 cases, and could not be settled in 22 cases. PS treatment was applied in 25 cases. All infants had a highly suspected or proven intrauterine infection based on standard criteria. PROM cases were coupled with elevated IL-6 levels (256.2 (64.8-2358.9) pg/ml) measured in cord blood, while IL-6 levels in cord blood of infants who had no PROM were normal (11.6 (2.9-45.1) pg/ml).

3.2 ETHICAL CONSIDERATIONS

Written informed consent was obtained from parents of subjects, and our study was reviewed and approved by an

independent ethical committee of the institution (Semmelweis University, Budapest). The study was adhered to the tenets of the most recent revision of the Declaration of Helsinki.

3.3. CBMC ISOLATION

Cord blood mononuclear cells (CBMCs) were separated by a standard density gradient centrifugation (Ficoll Paque, Amersham Biosciences AB, Uppsala, Sweden, 25 minutes, 400 g, 22 °C) from freshly drawn blood collected in lithium heparin-treated tubes (BD Vacutainer, BD Biosciences, San Jose, CA, USA). Peripheral blood samples were taken on the 1st, 3rd, and 7th postnatal days of life. Plasma was handled separately and frozen until further analysis.

3.4. FLOW CYTOMETRY

CBMCs and peripheral whole blood were stained for 30 min at room temperature in the dark with specific monoclonal antibodies.

In the study on vitamin D levels we used the following monoclonal antibodies: PE Cy7-conjugated CD4, APC-Cy7-conjugated CD8, FITC-conjugated CD25, PerCP-conjugated

CD62L, APC-conjugated CXCR3, PE-conjugated CCR4, APC-conjugated CD69, PerCP-conjugated HLA-DR, PerCP-conjugated CD3, APC-conjugated CD161, PE-conjugated 6B11, APC-conjugated CD11c, PE-conjugated CD123 and FITC conjugated Lin 1 cocktail in separate tubes, respectively (all from BD Biosciences).

Th1 cells were defined as CD4+ CXCR3+ CCR4-, while Th2 cells were defined as CD4+ CXCR3- CCR4+. NK cells were identified as CD3- CD161+, while NKT cells were identified as CD3+ CD161+ and invariant NKT (iNKT) cells as CD3+ 6B11+. For the determination of DC, pDC and mDC subsets, samples were stained with a lineage cocktail containing antibodies against CD3, CD14, CD16, CD19, CD20, and CD56 (Lin 1). After gating on Lin 1 negative cells, DCS were determined as HLA-DR+, pDCs were determined as the CD123+ HLA-DR+ subset, while mDCs were determined as the CD11c+ HLA-DR+ subset.

After washing, cells were analyzed on a BD FACSAria flow cytometer (BD Biosciences) equipped with 488 nm and 633 nm excitation lasers. Data were processed using the FACSDiVa software.

For the study on the effects of preeclampsia we used the following monoclonal anibodies:

PE Cy7-conjugated CD4, APC-Cy7-conjugated CD8, FITC-

conjugated CD25, PerCP-conjugated CD62L, APC-conjugated CXCR3, PE-conjugated CCR4, APC-conjugated CD69, PerCP-conjugated HLA-DR, PerCP-conjugated CD3, APC-conjugated CD161, PE-conjugated 6B11, APC-conjugated CD11c, PE-conjugated CD123 and FITC conjugated Lin 1 cocktail in separate tubes, respectively (all from BD Biosciences).

Th1 cells were defined as CD4+ CXCR3+, while Th2 cells were defined as CD4+ CCR4+. Naïve T cells were defined as CD4+ CD45RA+, while memory T cells were defined as CD4+ CD45RO+. Myeloid dendritic cells (mDCs) were defined as Lin 1- CD11c+, while plasmacytoid dendritic cells (pDCs) were defined as Lin 1- CD123+.

After lysing red blood cells and washing, samples were analyzed on a BD FACSAria flow cytometer (BD Biosciences) equipped with 488 nm and 633 nm excitation lasers. Data were processed using the FACSDiVa software.

In the study on activation markers we used the following monoclonal antibodies: PE-Cy7-conjugated CD4, APC-Cy7-conjugated CD8, FITC-conjugated CD25, PerCP-conjugated CD62L, APC-conjugated CXCR3, PE-conjugated CCR4, APC-conjugated CD69, PerCP-conjugated HLA-DR, FITC-conjugated CD45RA, PE-conjugated CD45RO in separate tubes, respectively (all from BD Biosciences).

Th1 cells were defined as CD4+ CXCR3+, while Th2 cells were defined as CD4+ CCR4+. Naïve T cells were defined as CD4+ CD45RA+, while memory T cells were defined as CD4+ CD45RO+.
After lysing red blood cells and washing, CBMCs and PBMCs were analyzed on a BD FACSAria flow cytometer (BD Biosciences) equipped with 488 nm and 633 nm excitation lasers. Data were processed using the FACSDiVa software.

3.5. PLASMA CORTISOL AND VITAMIN D LEVELS

Plasma cortisol levels and vitamin D levels were measured with commercially available Roche kits on an Elecsys automated analyzer.

3.6. PLASMA CYTOKINE LEVELS

Cytokine levels were measured using the Bio-Plex Pro Human Cytokine 17-Plex Panel (M50-00031YV, Bio-Rad, Hercules, CA, USA) following the manufacturer's instructions. Samples were read using a Bio-Plex reader (Bio-Rad).

3.7. STATISTICS

Data are expressed as median and range. Since Kolmogorov–Smirnoff analysis indicated non-normal distribution of data, the Mann-Whitney test was used to make comparisons between the study groups in the study on the effects of PE. The sample size was estimated to achieve 80% power with 0.45 effect size to detect differences between the patient groups.

The independent effects of gestational and postnatal age, PE, PROM, PS and gender were analyzed using the ‘mixed effect model’ method in the study on activation markers. This is a statistical model containing both fixed effects and random effects. It is particularly used in settings where repeated measurements are made on the same statistical units (ie. longitudinal studies), or where measurements are made on clusters of related statistical units.

The mixed effect model was also used to assess the effect of factors other than plasma vitamin D levels on the analyzed inflammatory parameters in the study on vitamin D levels. These factors were plasma cortisol levels as continuous variables and gestational age as categorical variables (27 weeks, 28-29 weeks and 30 weeks).

Statistics were calculated at 5% significance level ($p = 0.05$) using the GraphPad Prism 5 software (La Jolla, CA, USA) and the SAS software (Cary, NC, USA).

4. RESULTS

4.1. THE ROLE OF VITAMIN D LEVELS AT BIRTH IN PRETERM INFANTS

In the study on vitamin D levels, we included 28 preterm infants with a median gestational age of 29 weeks at birth. In the majority of them, vitamin D levels were higher than normal in cord blood (23.3 [9.9–45.4] ng/ml (median [range])). Based on vitamin D levels, two patient groups were created: below median (<23.3 ng/ml) and above median (>23.3 ng/ml).

First, we compared the prevalence of pro- and anti-inflammatory cell subsets and plasma cytokine levels between these groups. In infants with vitamin D level below median the prevalence of CD4+ CXCR3+ (Th1) and CD8+ CXCR3+ cell subsets was higher, while the prevalence of CD4+ CCR4+ (Th2), CD8+ CCR4+ and pDC cell subsets was lower than in infants with a plasma vitamin D level above median. No difference was detected in the prevalence of other cell subsets between the two groups, including lymphocyte activation markers (CD25, CD62L, CD69, HLA-DR) and NK cell subtypes.

Of note, no difference was detected in the plasma cytokine levels investigated. Since the inflammatory status might also be influenced by gestational age and plasma cortisol levels, we used the mixed effect model to assess the effects of these factors.

According to our analysis, CD4+ CXCR3+ (Th1) lymphocytes were also influenced by both gestational age (at the gestational age of 28–29 weeks) and plasma cortisol levels, while CD8+ CXCR3+ and CD8+ CCR4+ lymphocytes were affected by gestational age only (in the 28–29 weeks and ≥30 weeks categories, respectively). Plasmacytoid dendritic cells (pDCs) and CD4+ CCR4+ (Th2) lymphocytes are the only cell subsets from the current study which were only influenced by vitamin D levels.

4.2 THE IMPACT OF PREECLAMPSIA IN PRETERM NEONATES

In the study on the effects of PE, we first compared the prevalence of distinct lymphocyte, NK and dendritic cell subsets between the two groups.

The prevalence of CD4+ T lymphocytes and CD4+HLA-DR+ T cells was significantly lower in preterm neonates of PE mothers on postnatal day 3 (p= 0.0159 and p= 0.0348, respectively) when compared with preterm neonates born to non-PE mothers. In contrast, memory T cells (CD4+CD45RO+) were found to have a significantly higher prevalence in PE on day 7 (p= 0.0308) when compared with the control group. The prevalence of CD8+CXCR3+ cells was significantly lower in PE on postnatal days 1 and 7 (p= 0.0009 and p= 0.0163, respectively) when

compared with control subjects. CD8+CD69+ T lymphocytes had a lower prevalence on days 0 and 1 (p= 0.0109 and p= 0.0015, respectively) in preterm neonates born to PE mothers. Furthermore, CD8+HLA-DR+ T cells had a significantly lower prevalence in PE on days 0, 3 and 7 (p= 0.0084, p= 0.0308 and p= 0.0019, respectively). mDCs had a lower prevalence on days 1 and 3 (p= 0.0011 and p= 0.0538, respectively) in PE neonates when compared with controls.

No significant difference was detected in the prevalence of other investigated cell subsets between the two groups, including CD3+ T cells, cytotoxic T cells, naïve T cells, CD25+ and CD62L+ activated T cells, Th1 and Th2 cells, NKT, iNKT, NK cells or pDCs. We also compared plasma cytokine levels between the two groups. Interestingly, the indicated cytokine levels are significantly higher in preterm neonates of PE mothers on days 1, 3 and 7 and significantly lower on day 0 when compared to the control group. Of note, monocyte chemotactic protein-1 (MCP-1) and IL-4 had significantly higher levels on all 3 days (1, 3 and 7) (MCP-1: p= 0.0069, p= 0.0089 and p= 0.0178, respectively; IL-4: p= 0.0013, p= 0.0339 and p= 0.0106, respectively) in preterm neonates of PE mothers. Cortisol levels were found to be significantly lower in PE neonates on day 1 and 7 (p= 0.0370 and p= 0.0471, respectively).

4.3 EARLY AND LATE ACTIVATION MARKER EXPRESSION IN T CELLS OF PRETERM NEONATES

The frequency of CD4+ CD25+ and CD8+ CD25+ activated T lymphocytes was higher in case with PROM at all time points. We observed a decrease in the frequency of CD4+ and CD8+ T lymphocytes as well as the CD4+/CD8+ T cell ratio in PE compared to infants not affected by PE at all time points. The frequency of CD4+CD62L+ and CD8+ CD62L+ T lymphocytes was higher in male infants when compared to female infants at all time points. None of the investigated factors had an effect on the expression of the HLA-DR and CD69 activation markers,or the frequency of Th1 (CD4+ CXCR3+), Th2 (CD4+ CCR4+), naïve (CD45RA+) and memory (CD45RO+) T cell subsets. The frequency of Th1 (CD4+ CXCR3+) lymphocytes was higher in infants born before the 29th gestational week compared to those born on the 29-30th gestational week on postnatal days 1 and 3. When we looked at the effect of postnatal age (day 1, 3 and 7 of life) on the frequency of the investigated markers and subsets, we detected several changes. CD4+ T cells have a higher frequency on postnatal days 0 and 3 when compared to day 7. CD4+ CD25+ cells had a lower frequency on postnatal day 0 than on day 7. Of note, Th2 (CD4+ CCR4+) lymphocytes also had a lower frequency on postnatal days 1 and 3 when compared to day 7.

5. CONCLUSIONS

1. Low plasma vitamin D levels are associated with higher Th1, lower Th2 and lower pDC peripheral cell prevalences in the preterm infant.

2. pDCs and Th2 lymphocytes are the only cell subsets which were solely influenced by vitamin D levels, but not by plasma cortisol levels or gestational age.

3. The prevalence of CD4+ T lymphocytes and CD4+HLA-DR+ T cells was significantly lower in preterm neonates of PE mothers on postnatal day 3 when compared with preterm neonates born to non-PE mothers.

4. Memory T cells (CD4+CD45RO+) were found to have a significantly higher prevalence in PE infants on day 7.

5. Investigated cytokine levels are significantly higher in preterm neonates of PE mothers on days 1, 3 and 7 and significantly lower on day 0 when compared to the control group.

6. Cortisol levels were found to be significantly lower in PE neonates on day 1 and 7 of life.

7. Higher expression of CD25+ T lymphocytes is associated with PROM.

8. The expression of CD62L+ T lymphocytes was higher in male compared with female infants.

9. Antenatal steroid prophylaxis did not affect the frequency of the investigated markers.

6. PUBLICATIONS

Publications directly related to the PhD dissertation:

1. **Sava F,** Toldi G, Treszl A, Hajdú J, Harmath Á, Tulassay T, Vásárhelyi B. (2016) Expression of lymphocyte activation markers of preterm neonates is associated with perinatal complications. BMC Immunol, 17(1):19
IF: 2.161

2. **Sava F,** Treszl A, Hajdú J, Toldi G, Rigó J Jr, Tulassay T, Vásárhelyi B. (2016) Plasma vitamin D levels at birth and the inflammatory status of preterm infants. Immunobiol, 221(11): 1289-1292.
IF: 2.781

3. **Sava F,** Toldi G, Treszl A, Hajdú J, Harmath A, Rigó J Jr, Tulassay T, Vásárhelyi B. (2017) Immune cell subsets, cytokine and cortisol levels during the first week of life in neonates born to preeclamptic mothers. Am J Reprod Immunol, accepted: DOI: 10.1111/aji.12659.
IF: 2.916

Publications not related to the PhD dissertation:

1. Garanto A, Riera M, Pomares E, Permanyer J, de Castro-Miró M, **Sava F**, Abril JF, Marfany G, Gonzàlez-Duarte R. (2011) High transcriptional complexity of the Retinitis pigmentosa CERKL gene in human and mouse. Invest Ophthalmol Vis Sci, 52(8): 5202-14.
IF: 3.597

2. Dulic S, Vásárhelyi Z, **Sava F**, Berta L, Vásárhelyi B, Toldi G, Kovács L, Balog A. (2017) The impact of biological therapies on CD4+ and CD8+ cell subsets in rheumatoid arthritis: a long term follow-up study. PLoSONE, submitted.

3. Stanciu AE, **Sava F,** Toldi G. (2016) Polyglandular autoimmune syndrome type IIIc with primary antibody failure. Med Princ Pract, submitted.