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Short Communication

Analysis of regulatory T cells in cord blood and in adults

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ABSTRACT

Immune tolerance is of critical importance for the development of pregnancy and control of autoimmunity, the prevention of transplant rejection and reduction of the strength of immune reaction against infectious pathogens. It is a result of a multicomponent network where regulatory T cells (Tregs) are shown to play a very important role. The aim of our study is to analyse Tregs population in cord blood (CB) in comparison to peripheral blood (PB). Peripheral blood mononuclear cells (PBMCs) from 10 cord blood samples and from 14 healthy adult individuals were analysed by flow cytometry. Our results demonstrated that the proportion of Treg ($FoxP3^+CD4^+$) cells in CB is similar to those in PB. Like in peripheral blood, CB Treg population consisted of $CD45RA^+$ and $CD45RA^-$ cells. Conversely to PB, in CB around 60% of Tregs were with naïve ($CD45RA^+$) and 40% with memory ($CD45RA^-$) phenotype. The analysis of CD25 expression demonstrated that only in CB almost all Tregs are CD25-positive, while in adults they declined with age. It may be concluded that although the proportion of Tregs is stable in cord and peripheral blood, the profile differs significantly with regard to both CD45RA and CD25. Probably this is a consequence of proliferation, either homeostatic or antigen-driven. Our results contribute to the better understanding of the maturation and age-related changes in the subset of regulatory T cells.

KEYWORDS: regulatory T cells, FoxP3, phenotype, cord blood, adults.

INTRODUCTION

The state of tolerance is a fascinating feature of the immune system characterized by an attenuation of immune response or complete unresponsiveness towards particular antigens. Among multiple players in the tolerance-developing network, regulatory T cells are of specific importance for pregnancy, transplantation, autoimmune conditions, etc. Their population in the periphery is ensured by both the thymus-derived or natural Tregs (nTregs) and those that were induced from the naïve pool of T cells (iTregs), [1, 2]. Both subsets are defined by the expression of the transcription factor FoxP3 (forkhead box protein 3) [3] and α-subunit of IL-2R (CD25) [4-6].

CD45RA is considered as a marker of naïve T cells, including Tregs [7, 8]. $CD45RA^+$ Tregs are described as resting Tregs, because of the low/ negative expression of the proliferation marker Ki-67. After T-cell receptor (TCR) stimulation *in vivo* and *in vitro*, Tregs change from naïve $CD45RA^+$ to memory $CD45RA^-$ phenotype [9, 10].

The specific conditions and molecular mechanisms for iTregs generation are intensively studied and now well elucidated. On the contrary, analysis of nTregs in humans is still challenging because of their complex ontogenesis and limited number in peripheral blood. Intensive studies in reproductive immunology showed an important role of nTregs in the fetus as early as the 2nd trimester [11-13], with fetal $CD4^+$ and $CD8^+$ T cells capable of triggering effective immune response being already present at gestational week 10 [14, 15]. Thus, the role of Tregs in immune tolerance during pregnancy seems to be dependent on both mother and fetus.

In the present work we focused on differencies between cord blood (CB) and peripheral blood (PB) Tregs obtained from adults with regard to CD45RA and CD25 expression. Our results demonstrate that CB Tregs are composed of $CD45RA^+$ and $CD45RA^-$ cells. The proportion of both subsets differs between study groups: $CD45RA^+$ Tregs prevailed in CB but not in peripheral blood. The analysis of CD25 shows that only in CB almost all Tregs are CD25-positive. The results indicate differences in Tregs populations in CB and PB that might be associated with ontogenesis and post-thymic development of Tregs.

MATERIALS AND METHODS

Study participants

Twenty-four individuals were included in the study. Ten CB and 14 PB samples were collected in sodium-heparin BD Vacutainer® tubes from healthy individuals. Cord blood samples were collected directly after normal delivery (n = 6) or planned section cesarean (n = 4). All newborns were in healthy condition, with female newborns (7 out of 10) being overrepresented within our cohort. All participants included in the study gave informed consent according to Declaration of Helsinki.

Cell staining and flow cytometry

Extracellular labeling of freshly isolated PBMCs at concentration of 1 x 10^7 cells/ml was performed at 4 °C for 30 min using anti-CD3-HV450 (clone SP34-2), anti-CD4-HV500 (clone RPA-T4) (both obtained from BD Biosciences, San Jose, CA, USA), anti-CD45RA-PerCP (clone HI100) and anti-CD25-PE-Cy7 (clone M-A251) (both obtained from Biolegend, San Diego, CA, USA) according to the manufacturer's recommendations. Cells were washed and forwarded to intracellular labeling using anti-FOXP3-PE (clone PCH101) (eBiosciences, San Diego, CA, USA) according to the manufacturer's recommendations. Rat serum was applied to block the nonspecific bindings. Appropriate PE-labeled rat IgG2a isotype controls were used for FoxP3 analysis. Data were acquired on BD FACS CANTO II (BD Biosciences, San Jose, CA, USA) and analysed by FlowJo V10 software (TreeStar Inc.).

Statistical analysis

Student's t-test was used to determine the significance of differences between study groups. P values < 0.05 were considered as statistically significant. The data was analyzed with GraphPad Prism version 7.

RESULTS

Participants characteristics

The present study included only healthy participants without signs or symptoms of autoimmune diseases, chronic inflammation, and proliferative diseases. Furthermore, no individual was under corticosteroid therapy or other conditions with potential modulating effects on immune system. No complications during the pregnancies were observed and the gestational age was in the normal range for all samples (38-42 weeks of gestation). Vitamin therapy was included where it was necessary (n = 4).

Tregs in cord blood are composed of two subsets according to CD45RA expression

The suppressive function of Tregs is strongly dependent on the transcription factor FoxP3 [16]. Although FoxP3 was also found in other populations such as $CD8^+$ T cells [17], $CD3^+CD4^+$ T cells represent the major population of FoxP3 positive cells. The percentage of Tregs was almost identical in CB samples and PB of adult participants (mean ± SD = 5.6% ± 2.4 vs 5.7% ± 2.3), ($p > 0.05$), (Figure 1A).

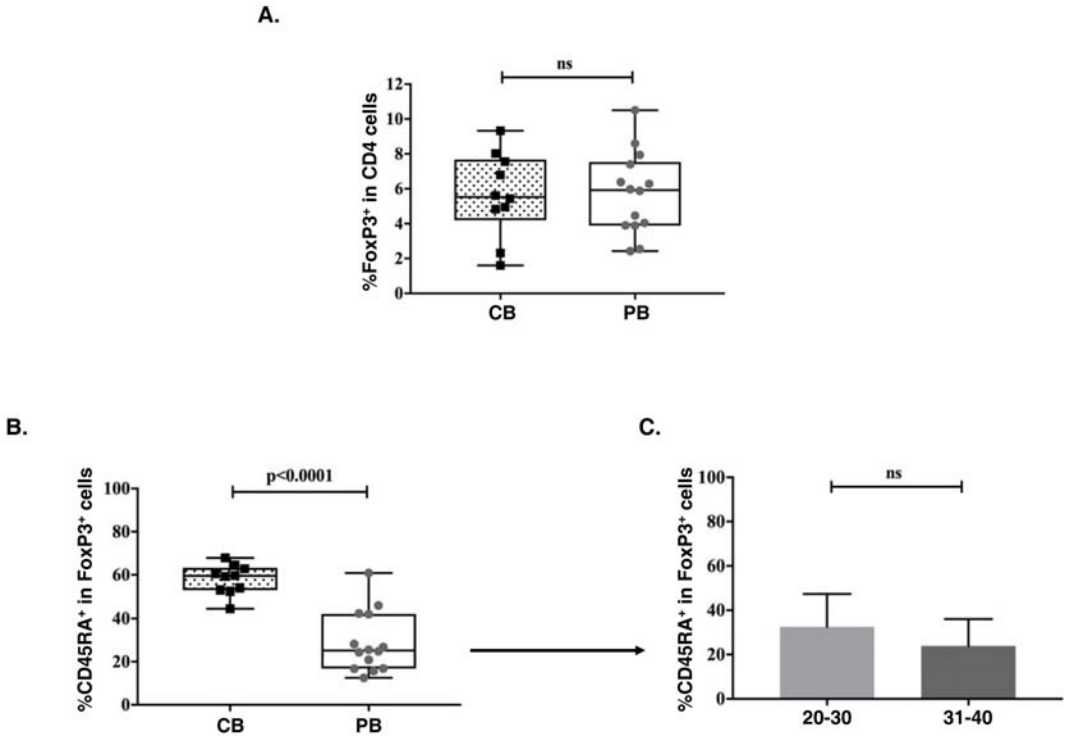

Figure 1. Analysis of Tregs in cord blood and peripheral blood from adults.
A) There is no difference in the percentage of $FoxP3^+$ cells in the $CD3^+CD4^+$ T lymphocyte population between cord blood (black dots), and peripheral blood (grey dots)-derived samples, respectively. (B) The percentage of $CD45RA^+$ cells in the $FoxP3^+$ population is significantly higher in samples obtained from cord blood compared to peripheral blood. (C) $CD45RA^+$ Tregs showed a trend of decrease with age (group 20-30 yrs: n = 8; group 31-40 yrs: n = 6). Mean values ± SD are shown. Student's t-test was used to determine the significance of differences.

In adults the $FoxP3^+$ population is not homogeneous as it can be further subclassified into $CD45RA^+$ and $CD45RA^-$ Tregs. We expected that CB Tregs would be almost entirely naïve ($CD45RA^+$), but similarly to PB ones, the analysis revealed two subsets. Although CD45RA-Tregs in CB were significantly lower as compared to the adult PB samples they still accounted for 42.0% ± 6.9 vs PB: 71.1% ± 13.92 of the $FoxP3^+$ cells (see Figure 1B). As the group of adult controls covered approximately two decades of age ranging from 23-39 years, we checked for an age-dependent expression level of $CD45RA^+$. As it is shown in Figure 1C, a trend of decrease with age was detected (Figure 1C). We did not observe associations of our results with the gestational week of parturition and mother's age (data not shown). Thus, it might be concluded, that although being relatively preserved in CB and PB, Tregs undergo an age-dependent reshape of $CD45RA^+$ and $CD45RA^-$ subsets.

Analysis of CD25 expression in Tregs from cord blood

CD25 expression is important for the stability of FoxP3 and viability of Tregs [16]. In our study, CD25 was evaluated in both $CD45RA^+$ and $CD45RA^-$ Tregs in cord blood samples and peripheral blood from adult volunteers. Additional analysis was done to identify age-related differences in CD25 expression. In cord blood, almost all cells were $CD25^+$ (75.1% ± 21.00 and 78.9% ± 14.8, for $CD45RA^+$ and $CD45RA^-$, respectively; $p > 0.05$)). Although high individual variability was observed, no correlations were found with the gestational week, mother's age or the sex of the baby.

Contrarily, in PB nTregs the percentage of $CD25^+$ cells was decreased (42.4% ± 19.10) as compared to iTregs (58.0% ± 11.75), ($p = 0.002$). We also observed that the proportion of $CD25^+$ Tregs in adults is relatively stable across ages between

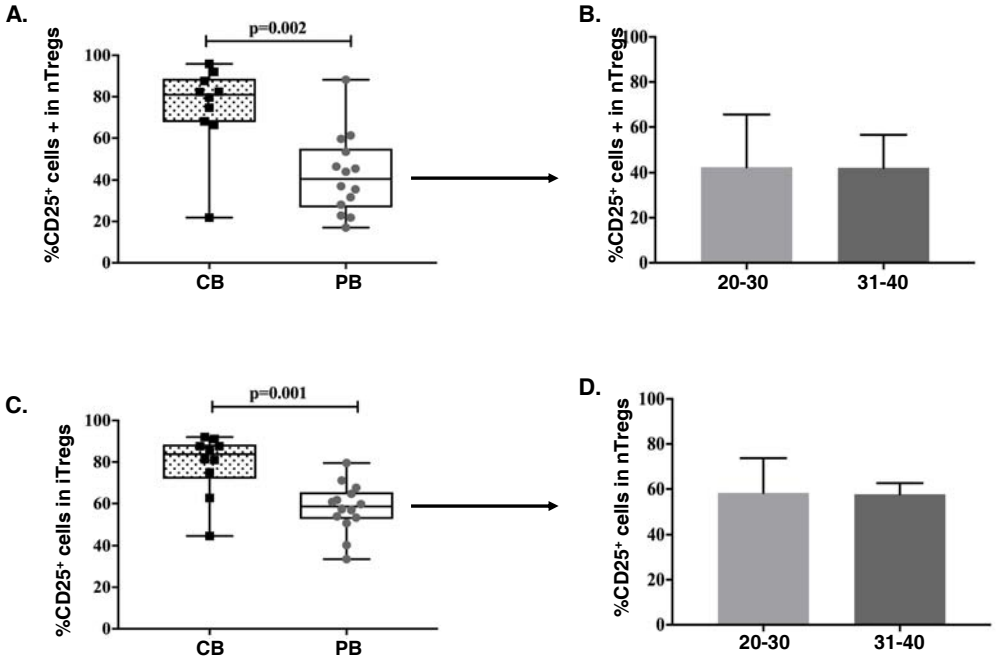

Figure 2. Proportion of CD25$^+$ in Tregs from cord blood and peripheral blood from adults.
Left panel shows %CD25$^+$ cells in CD45RA$^+$ (A) and CD45RA$^-$ (C) Treg subsets. The right panel evidences the proportion of CD25$^+$ Tregs in adults in relation to their age (B and D). Mean values ± SD are shown. Student's t-test was used to determine the significance of differences.

23-39 years. Indeed, CD25$^+$ Tregs from CB exceeded approximately two times those in PB Tregs ($p = 0.002$ and $p = 0.001$ for nTregs and iTregs, respectively), (Figure 2).

Therefore, CB and PB Tregs differ significantly from each another with regard to CD25 expression, suggesting the influence of normal physiologic processes like age-related changes in the endocrine milieu or scenescence.

DISCUSSION

During the last decades, there is a constantly growing interest in the role of regulatory T cells with respect to the control of immune reactions and the development of immune-based clinical conditions. Despite recent results, the question about their age-related variations and suppressive function development still remains a fascinating challenge.

In our study, we evaluated Treg populations in cord blood and peripheral blood from healthy adults. Our analysis showed that the percentage of FoxP3$^+$CD4$^+$ cells in cord blood did not differ significantly from peripheral blood. This topic is still under discussion in the literature and there is still no consensus - some studies showed data supporting our findings while others evidenced substantial increase in the percentage of FoxP3$^+$ Tregs [17, 14].

The analysis within groups of CB and PB samples revealed that Tregs are composed of CD45RA positive and CD45RA negative subpopulations. Moreover, we found significant differences in the proportion of CD45RA$^+$ and CD45RA$^-$ Tregs among CB and PB. This fact indicates a remodeling of the profile of both subsets with time that supports the relatively stable percentage of Tregs. Indeed, the percentage of CD45RA$^-$ subset found by us was unexpected as we assumed almost all cells to be either naïve, recent thymic emigrants or both. Supporting information comes from observations that T cells, including Tregs, loose CD45RA expression following homeostatic proliferation [18]. Probably it begins around the end of the 1st - 2nd trimester when the fetal T cells appear [13]. Another explanation

might be the exposure to non-inherited maternal alloantigens (NIMA) [19, 20].

The decrease in nTregs, observed in adults could be a result of the descendent thymic function. It has been shown that the capacity of the thymus to produce new T cells declines with age [21]. Here, we confirm these findings even for a short age range (23-39 yrs).

Additionally, in CB but not in PB, almost all Tregs were $CD25^+$. CD25 is of critical importance for Tregs' viability, function and phenotype [22-24]. During nTregs ontogenesis, the upregulation of CD25 is guided by intercellular intrathymic interactions, cytokines such as IL-7 and IL-2, and transcription factors [25, 2, 26, 27]. Therefore, our results show that fetal Tregs are fully functional, with stable FoxP3 expression and suppressive capacity at the time of birth. However, in adults after puberty, the hormonal milieu can change Tregs population [28, 29]. We suggest that the decrease in nTregs and CD25 expression in the group of adult controls might be a result of a complex physiological processes.

CONCLUSIONS

1. The proportion of regulatory T cells did not differ between cord blood and peripheral blood of adults.
2. In cord and peripheral blood, Tregs population consists of cells with naïve ($CD45RA^+$) and memory ($CD45RA^-$) phenotype, showing different distribution in postnatal stage and in adulthood.
3. CB Tregs are characterized by abundant expression of CD25, while in PB it declined with age. These findings offer possibility for new studies on the maturation and aging process in regulatory T cells.

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CONFLICT OF INTEREST STATEMENT

The authors certify that they have no affiliations with or involvement in any organization or entity with any financial interest or non-financial interest in the subject matter or materials discussed in this manuscript.

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