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ORIGINAL ARTICLE

Characterization of Regulatory T Cells in Preterm and Term Infants

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Abstract

Our study aimed to study regulatory T cells (Tregs) and their expression of CD45RA, HLA-DR, and CD39 in preterm and full-term infants. In an observational study, we used a three-color flow cytometry for determination of Tregs and their expression of CD45RA, HLA-DR, and CD39 in preterm and full-term infants. The percentages of $CD4^+CD25^{+high}Foxp3^+$, $CD39^+$ Tregs, $HLA\text{-}DR^+$ Tregs and the expression of $Foxp3^+$ in $CD4^+CD25^{+high}$Foxp3 Tregs cells were significantly lower in neonates when compared to healthy adult controls. The levels of naïve resting Tregs ($CD45RA^+$Tregs) were significantly higher in neonates than controls. The percentages of $CD4^+CD25^{+high}Foxp3^+$Tregs, total $CD4^+CD25^+$ and $CD4^+CD25^{+high}$ were significantly higher in preterm infants when compared to the full-term group. Moreover, $CD45RA^+$Tregs were significantly higher in preterm than in term infants. We found significant inverse correlations between the gestational age and the levels of both Tregs ($r=-0.395$, $p=0.017$) and $CD45RA^+$Tregs ($r=-0.422$, $p=0.010$). Relative to full-term, the frequencies, and phenotypes of Tregs were affected by prematurity. A larger longitudinal study with a sufficient number of newborns is needed to investigate the Treg pool of term and preterm infants thoroughly and to explore the association between the Treg pool and clinical variables.

Keywords Regulatory T cells · Preterm · Full-term newborn

Introduction

The immune system of the fetus develops in an aseptic environment in utero and so lacks any antigenic experience. The immunity in term and preterm infants is mainly influenced by many factors including mother's immune status and in utero inflammations (Shankaran et al. 2002). The whole performance of the neonatal immune system after birth is diminished in many vital elements. Consequently, preterm infants are more susceptible than other children to severe bacterial, viral, and fungal infections. Neonatal sepsis is one of the most common causes of death in preterm infants (Shankaran et al. 2002; Stoll et al. 2010). Regulatory T cells (Tregs) are a subtype of $CD4^+$ T cells that play a vital role in the peripheral tolerance to harmless elements and decrease the excessive or improper immune responses to these molecules. Besides, they control the development of allergic reactions by suppressing the activation of T-helper 2 (Th2) cells and blocking the migration of the effector T lymphocytes to the inflamed sites (Saad et al. 2018). Tregs constituted about 5–10% of $CD4^+$ population and are characterized by the expression of the CD25 surface marker and transcription factor forkhead box protein 3 (Foxp3). Tregs increased during pregnancy as they have a key role in the maternal-fetal tolerance (Saad et al. 2018; Sakaguchi 2005). Based on the expression of CD45RA and Foxp3, Tregs were divided into three subpopulations: (1) $CD4^+Foxp3^{low}CD45RA^+$ cells as naïve or resting Tregs, (2) $CD4^+Foxp3^{+high}$CD45RA—cells which are the fully

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functional Tregs and, (3) $CD4^+Foxp3^{low}CD45RA^-$—non-Treg cells which are cytokine-secreting, non-suppressive T cells (Miyara et al. 2009). The first group (naïve, resting Tregs) are newly generated T cells which freshly released from thymus with no antigen exposure experience (Valmori et al. 2005). Tregs express numerous molecules linked with their suppressive role. About 40% of Tregs showed expression of surface HLA-DR. HLA-DR^+ Tregs induce more rapid and intense suppression of T cells than the Tregs with negative HLA-DR expression (Baecher-Allan et al. 2006). Tregs also suppress the effector T cells directly by transferring cyclic adenosine monophosphate (cAMP) into the responder cells. cAMP is an inhibitor of differentiation, proliferation, and synthesis of IL-2 in T lymphocytes. CD39 is an ectoenzyme that may be involved in the suppressive functions of Tregs by degrading ATP to AMP. All Tregs express CD39 in mice but to a minor extent in human Tregs (Borsellino et al. 2007).

There were few studies on the expression of activation and memory markers (CD25, CD69, and HLA-DR) on the total cord blood $CD4^+$ cells in newborns (Crespo et al. 2012; Luciano et al. 2011). However, there are no studies about the expression of these markers on cord blood Tregs. Therefore, our study aimed to study Tregs and their expression of CD45RA, HLA-DR, and CD39 in preterm and full-term infants.

Patients and Methods

Our study was approved by Assiut University Ethical Scientific Committee, Assiut, Egypt and was conducted under the code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans. Written informed consent from caregivers of all participants was obtained following Assiut University Hospital Ethical Committee guidelines.

Study Design

This was a case-controlled study undertaken in Assiut University hospitals Assiut, Egypt.

Participants

We prospectively recruited a subset of newborns of mothers attending Assiut university hospitals, Assiut, Egypt, where all laboratory work was conducted. Cord blood samples were collected from infants after obtaining written informed consent from mothers. Subjects included healthy term infants (gestational age ≥ 37 weeks, $n = 24$) and preterm infants (gestational age ≤ 36 weeks, $n = 17$). In addition to 30 healthy adults as a control group. Subjects were enrolled from January 2017 to June 2017. After delivery, umbilical cord blood samples were collected from the umbilical vein. Peripheral venous blood was obtained from all adult control group enrolled in the study. All samples were handled within 24 h.

Flow Cytometric Detection of Tregs Phenotype

Phycoerythrin conjugated anti CD25, fluoroisothiocyanate-conjugated anti-Foxp3 and peridinium-chlorophyll-protein conjugated anti CD4 (Becton Dickinson Bioscience, USA) and allophycocyanin (APC)-conjugated anti HLA-DR, APC-conjugated anti CD39, APC-conjugated anti CD45RA were used to detect regulatory T cells and their phenotype. All monoclonal from Becton Dickinson Biosciences (USA) expect CD25 (IQ Product the Netherland) and anti-Foxp3 (Bioscience, USA). Hundred µl of blood sample was incubated for 20 min at room temperature in the dark with 10 µl of anti-CD4, anti-CD25, and anti-CD45RA; anti-CD4, anti-CD25, anti-CD39; anti-CD4, anti-CD25, anti-HLA-DR in separate tubes. Then red blood cells lysis and washing with phosphate buffer saline (PBS) were done. Addition of a fixed solution to fix the cells and incubation for 10 min was then done. After incubation, cells were washed with PBS, and then the permelized solution and 10 µl of Foxp3 were added to each tube and incubated for 20 min at room temperature. After washing, the cells were resuspended in PBS and analyzed by FACSCalibur flow cytometry with CellQuest software (Becton Dickinson Biosciences, USA). An isotype-matched negative control was used for each sample. Forward and side scatter histogram was used to define the lymphocytes population (R1). Then $CD4^+$ cells were gated. Total $CD4^+CD25^+$, $CD4^+CD25^{+low}$, $CD4^+CD25^{+high}$ and $CD4^+CD25^{+high}$ $Foxp3^+$ Tregs were evaluated as a percentage of $CD4^+$ cells. $Foxp3^+$ in $CD4^+CD25^{+high}$ cells were expressed as the geometric mean of fluorescence intensity. The expressions of CD39, CD45RA, and HLA-DR were evaluated as a percentage of $CD4^+CD25^{+high}$ $Foxp3^+$ cells as shown in Fig. 1.

Statistical Analysis

Statistical analyses were performed using the Statistical Package for Social Sciences, version 19 (SPSS Inc., USA). Data are expressed as mean ± standard error of mean (SEM) for continuous variables and percentages for categorical variables. Differences between groups were examined by using Mann–Whitney analysis. A p value of ≤ 0.05 was considered significant. Spearman's correlation coefficient was used to examine the correlations among different studied parameters.

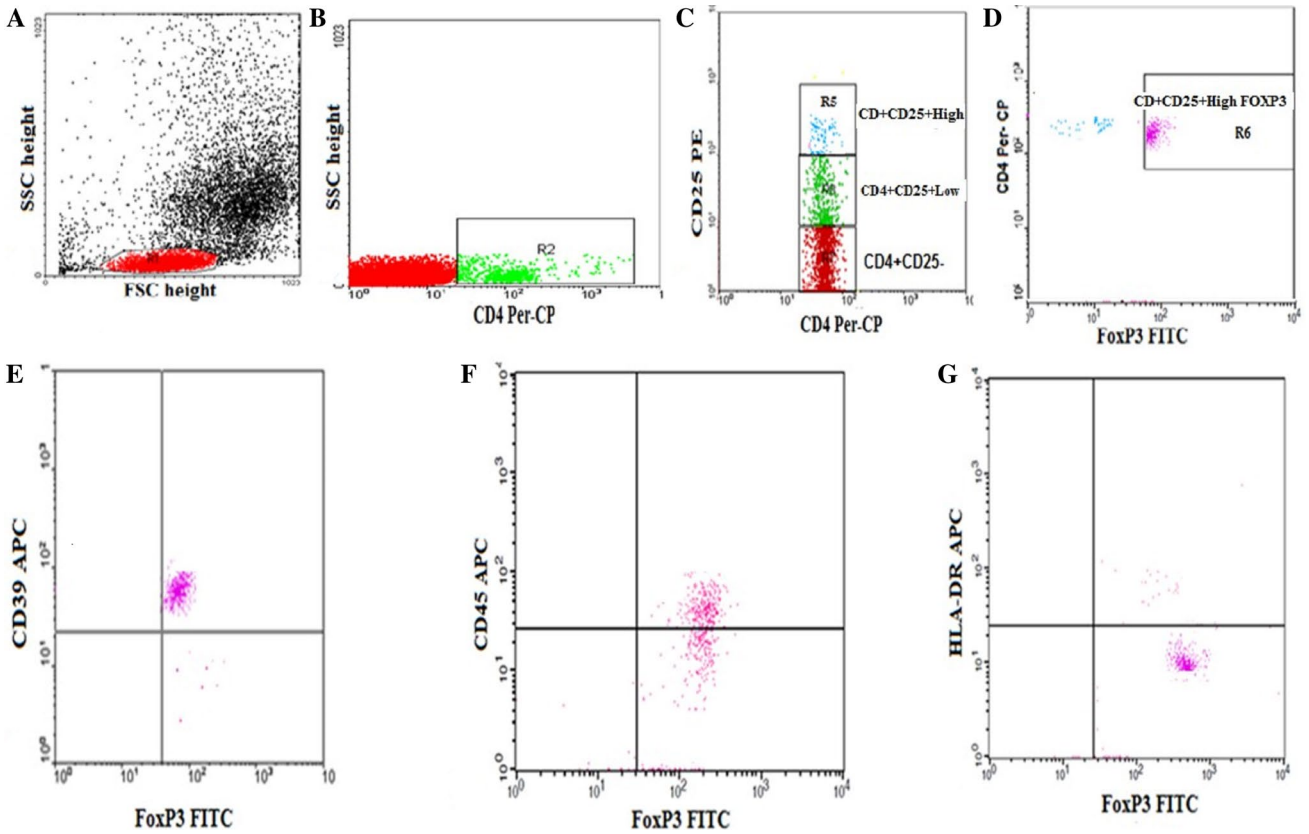

Fig. 1 Flow cytometric detection of regulatory T cells phenotype. **a** Forward and side scatter histogram was used to define the lymphocytes population (R1). **b** The expression of CD4 in the lymphocytes population was detected, and then $CD4^+$ cells were gated for further analysis of CD25. **c** Different gates were drowning to define $CD4^+CD25^-$ cells, $CD4^+CD25^{+low}$ cells and $CD4^+CD25^{+high}$ cells. **d** The percentage of $CD4^+CD25^{+high}$ $Foxp3^+$cells (regulatory T cells) was then detected. **e–g** The expression of CD39, HLA-DR and CD45RA was then detected in regulatory T cells (R6)

Results

Forty-one cord blood samples were obtained: 24 full-term and 17 preterm. Table 1 shows all participants' characteristics. There were insignificant differences between full-term and preterm groups for sex, gravidity, parity, maternal age, and prenatal use of antibiotics. There were significant differences between term and preterm for the mode of delivery ($p < 0.001$), gestational age ($p < 0.001$), and weight at birth ($p < 0.0001$) and prenatal steroids ($p < 0.001$).

Table 1 Baseline characteristics of newborns in the study

	Term infants ($n=24$)	Preterm infant ($n=17$)
Gender		
Male *N* (%)	14 (58.3%)	11 (64.7%)
Female *N* (%)	10 (41.7%)	6 (35.3%)
Prenatal use of steroids *N* (%)	4 (16.6%)	6 (35.3%)
Prenatal use antibiotics *N* (%)	4 (16.6%)	3 (17.6%)
NICU admissions	3 (12.5%)	9 (52.9%)
Gestational age (weeks; mean ± SD)	39 ± 1	31 ± 4
Birth weight (grams; mean ± SD)	3176 ± 339	1897 ± 463
Mode of delivery (%)		
Vaginal	18 (75%)	5 (29.4%)
Caesarian section	6 (25%)	12 (70.6%)

The percentages of $CD4^+CD25^{+high}Foxp3^+$ (Tregs) were significantly lower in neonates when compared to healthy adult controls, while there were no significant differences between neonates and adults for the percentages of total $CD4^+CD25^+$, $CD4^+CD25^{+high}$, and $CD4^+CD25^{+low}$. The expression of $Foxp3^+$ in $CD4^+CD25^{+high}Foxp3$ (Tregs) cells was significantly decreased in neonates than adults. The level of naïve resting Tregs ($CD45RA^+$Treg) was significantly higher in neonates than adults. On the one hand, the levels of $CD39^+$ Tregs and HLA-DR^+Tregs were significantly lower in neonates when compared to the healthy adults (Table 2).

Regarding the comparison between full-term and preterm infants' groups, the percentages of $CD4^+CD25^{+high}Foxp3^+$ Tregs, total $CD4^+CD25^+$ and $CD4^+CD25^{+high}$ were significantly higher in preterm infants when compared to the full-term group. Besides, the expression of $Foxp3^+$ in $CD4^+CD25^{+high}Foxp3$ cells was significantly increased in preterm than term infants. Moreover, naïve resting Tregs ($CD45RA^+$Treg) were significantly higher in preterm than in term infants. On the other hand, the percentages of $CD4^+CD25^{+low}$, $CD39^+$Tregs, and HLA-DR^+Tregs were not significantly different between full-term and preterm groups (Table 3). We found significant inverse correlations between the gestational age and the levels of both Tregs ($r = -0.395$, $p = 0.017$) and $CD45RA^+$Tregs ($r = -0.422$, $p = 0.010$). In addition, there were significant negative correlations between the frequencies of $CD39^+$Tregs, and HLA-DR^+Tregs and the level of naïve $CD45RA^+$Tregs.

Discussion

$CD4^+CD25^{+high}Foxp3^+$ Tregs have a vital role in balancing the immune responses and preserving tolerance against allergens and antigens. Tregs that are specific for self-antigens are naturally created in the thymus, while Tregs that are specific for exogenous antigens are most likely induced from non-regulatory T-cell precursors in the periphery (Sakaguchi 2005). Our study aimed to characterize Tregs phenotypes in an Egyptian cohort of neonates and to compare Treg cells from newborns to those of healthy adult control. Our study showed that the frequency of Tregs in

Table 2 Tregs in adult and neonates

	Neonates ($n=41$)	Adults ($n=30$)	p value
$CD4^+CD25^+/CD4^+$ (%)	14.24 ± 0.30	13.77 ± 0.33	0.247
$CD4^+CD25^{low}/CD4^+$ (%)	10.18 ± 0.23	9.83 ± 0.21	0.272
$CD4^+CD25^{+high}/CD4^+$ (%)	3.91 ± 0.28	3.94 ± 0.30	0.981
$CD4^+CD25^{+high}$ $Foxp3^+$Tregs (%)	1.92 ± 0.16	3.57 ± 0.25	0.001
MFI of $Foxp3^+$ expression in $CD4^+CD25^{+high}$	99.62 ± 2.25	129.30 ± 28.40	0.001
$CD45RA^+$ Tregs	70.11 ± 1.85	28.11 ± 1.08	0.001
$HLADR^+$ Tregs	8.11 ± 086	30.5070 ± 0.89814	0.001
$CD39^+$ Tregs	16.03 ± 1.06	41.46 ± 1.36	0.001

Data represented as means ± SEM; $p \leq 0.05$ is significant

Mann–Whitney Test

MFI mean fluorescence intensity, *Tregs* regulatory T cells

Table 3 Tregs in preterm and full-term infants

	Preterm infant ($n=17$)	Term infants ($n=24$)	p value
$CD4^+CD25^+/CD4^+$ (%)	15.16 ± 0.42	13.68 ± 0.37	0.016
$CD4^+CD25^{low}/CD4^+$ (%)	10.33 ± 0.34	10.07 ± 0.31	0.575
$CD4^+CD25^{+high}$/CD4+ (%)	4.86 ± 0.54	3.26 ± 0.24	0.005
$CD4^+CD25^{+high}Foxp3^+$Tregs (%)	2.51 ± 0.21	1.52 ± 0.19	0.010
MFI of $Foxp3^+$ expression in $CD4^+CD25^{+high}$	110.69 ± 8.25	92.24 ± 12.43	0.027
$CD45RA^+$ Tregs	75.56 ± 3.03	66.48 ± 2.07	0.008
HLA-DR^+ Tregs	8.9 ± 1.48	8.00 ± 1.08	0.912
$CD39^+$ Tregs	16.15 ± 1.83	15.72 ± 1.31	0.802

Data represented as means ± SEM; $p \leq 0.05$ is significant

Mann–Whitney test

MFI mean fluorescence intensity, *Tregs* regulatory T cells

neonates was lower than that of adults; this is consistent with other reports (Luciano et al. 2014; Ly et al. 2009; Rueda et al. 2015a).

We found significantly higher levels of Tregs in preterm infants than in full-term in this study, with a gestational age-dependent significant difference in the percentage of total Tregs, in which there is an inverse correlation between the Tregs level and the infant gestational age. Our finding could be explained by the fact that during the fetal life, maternal cells cross the placenta to reside in lymphoid tissues, inducing the production of Tregs, which preserve the maternal-fetal tolerance (Burt 2013). These results are matched with previous studied (Dirix et al. 2013; Luciano et al. 2014). In contrast to our findings, Rueda et al. (2015b) reported that the frequencies of Tregs were comparable among the preterm and term infant groups.

In our study, we also found that the frequency of naïve Tregs (expressing CD45RA) was higher in neonates than in adults and the highest level was reported in preterm; this may be due to the absence of external antigen exposure. Our results are supported by the result of previous studies (Dirix et al. 2013; Ly et al. 2009; Ng et al. 2001; Schlossberger et al. 2013; Takahata et al. 2004). Also, the frequencies of $CD39^{+}$Tregs, $CD69^{+}$Tregs, and HLA-DR^{+}Tregs were lower in neonates when compared to healthy adults, but with no significant difference between term and preterm groups. The proportion of naïve Tregs primarily explains the difference between adult and neonate Tregs expressing these markers. This is reinforced by the fact that the frequencies of $CD39^{+}$Tregs, $CD69^{+}$Tregs, and $HLA^{-}DR^{+}$Tregs are negatively correlated with naïve Tregs. Our results agree with other studies (Dirix et al. 2013; Ng et al. 2001; Takahata et al. 2004).

Our study analyzed only one cord blood sample, providing a snapshot of the Tregs in preterm and term infants. Besides, our study has a relatively small sample size; so, it has not enough power to make any conclusions about the relationship between Tregs and the clinical outcomes.

The results of our study have implications for the understanding of neonatal immune regulation since Tregs are essential for immune tolerance and autoimmunity. Relative to full-term, the frequencies and phenotypes of Tregs were affected by prematurity. A larger longitudinal study with a sufficient number of newborns is needed to investigate the Treg pool of term and preterm infants thoroughly and to explore the association between the Treg pool and clinical variables.

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Compliance with Ethical Standards

Conflict of interest All authors declare no potential conflicts of interest concerning the research, authorship, and/or publication of this article. All authors approved the final manuscript as submitted and agree to be accountable for all aspects of the work.

Ethical approval The protocol of this study was under the regulations of the relevant clinical research ethics committee and with those of the code of ethics of the world medical association Declaration of Helsinki.

Informed consent Written informed consents of caregivers of all children were taken according to the Ethical Committee of Faculty of Medicine, Assiut University, Egypt.

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