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Original

Regulatory T cells in human cord blood of preterm and term infants

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Abstract

Regulatory T (Treg) cells are defined based on expression of CD4, CD25 and the transcription factor forkhead box P3 (FoxP3). FoxP3 is a reliable marker of Treg cells. However, intracellular localization prohibits its use to isolate viable Treg cells. Low-level surface expression of CD127 is a characteristic of Treg cells, facilitating the use of CD127 as an alternative to FoxP3 for isolating viable Treg cells. However, the expression of CD127 in preterm cord blood (CB) is not fully understood. We analyzed $CD4^+CD25^+FoxP3^+$ ($FoxP3^+$ Treg) and $CD4^+CD25^+CD127^-$ ($CD127^-$ Treg) cells in CB obtained from 36 preterm neonates and 67 term neonates. A strong correlation was found between the proportions of $FoxP3^+$ and $CD127^-$ Treg cells ($r=0.96$). Preterm CB contained a significantly higher proportion of $FoxP3^+$ and $CD127^-$ Treg cells than did term CB. The absolute number of $FoxP3^+$ and $CD127^-$ Treg cells did not differ between preterm and term CB. CD127 is preferable to FoxP3 as a marker of Treg cells in the CB at various gestational weeks. The high Treg proportion during early gestation suggests that naive T cells of the fetus at early gestation have a high propensity to differentiate into Treg cells in response to antigenic stimulation.

Key words : *regulatory T cells, FoxP3, cord blood, preterm infants, flow cytometry*

I. Introduction

Regulatory T (Treg) cells are a subset of $CD4^+$ T cells that play a role in suppressing excessive immune responses and maintaining immune tolerance [1]. In the $CD4^+$ T cell subpopulation, Treg cells express high levels of CD25 (interleukin-2 receptor [IL-2R] α-chain) on the surface [2, 3]. Moreover, it has been revealed that the transcription factor forkhead box P3 (FoxP3) controls the development and function of $CD4^+CD25^+$ T cells and can be used as a reliable marker of Treg cells [4, 5]. Because FoxP3 is an intracellular protein, it cannot be used to separate viable human Treg cells for use in functional studies or ex vivo expansion for cellular therapy. Cell surface expression of CD127 (IL-7R α-chain) enables the isolation of viable populations of Treg cells because the expression of FoxP3 and CD127 is inversely correlated within the $CD4^+CD25^+$ T-cell population in adult peripheral blood (PB) [6]. However, the pattern

of CD127 expression by Treg cells in the cord blood (CB) of preterm and term infants during gestation remains unknown.

The proportion of $CD25^+FoxP3^+$ cells within the $CD4^+$ T-cell population in the CB of term newborns is significantly lower than in adult PB [7]. In contrast to term CB, human preterm CB contains a high proportion of $CD4^+CD25^+$ [8] and $CD4^+CD25^+FoxP3^+$ [9] T cells, which declines with advancing gestational age. Elucidating the proportion and number of Treg cells in preterm CB would enhance our understanding of immunological tolerance during fetal development.

In this study, we analyzed and compared the expression of FoxP3 and CD127 in $CD4^+CD25^+$ T cells in preterm and term CB and evaluated factors that might affect the expression of these Treg cell markers.

II. Materials and methods

1. Study groups

CB samples were obtained at Iwate Medical University and affiliated hospitals from preterm and term neonates after obtaining written informed consent from mothers. These studies were performed in accordance with the policies of and approval of the Ethics Committee of Iwate Medical University School of Medicine and affiliated hospitals. All of the neonates had no hereditary disorders, hematologic abnormalities, or immunodeficiency disease. Of 103 total samples, 36 were obtained from preterm (24-36 gestational weeks) neonates and 67 were obtained from term (37-41 gestational weeks) neonates. The number of samples in the 24-26, 27-29, 30-32, 33-36, 37-38, and 39-41 gestational-week groups was 7, 13, 7, 9, 39, and 28, respectively. None of the mothers involved in the study had any specific underlying diseases.

2. Blood sample collection

Immediately after delivery of placenta, CB samples were collected from the umbilical vein of all neonates. Ten pairs of CB samples were obtained from the umbilical artery and vein. CB mononuclear cells (CBMCs) were isolated within 6 h of CB sampling by density gradient centrifugation over Histopaque-1077 (Sigma-Aldrich, St. Louis, MO, USA) and then frozen at −80℃ in KM Banker Ⅱ (Cosmo Bio, Tokyo, Japan), a cell storage solution for lymphoid cells. Frozen CBMCs were thawed in a 37℃ water bath. All samples were analyzed within 2 weeks after freezing of CBMCs.

3. Flow cytometry

CBMCs were stained in media with the following anti-human monoclonal antibodies and conjugates for 20 min at room temperature: CD4-FITC, CD25-PE-Cy5, and CD127-PE (all from Becton Dickinson [BD] Biosciences, San Jose, CA, USA). Intracellular staining was then performed with FoxP3-PE monoclonal antibody (BD Biosciences) for 30 min at room temperature after fixation and permeabilization of the cells using Human FoxP3 Buffer Set (BD Biosciences) according to the manufacturer's instructions. All stained samples were examined on a FACSCalibur flow cytometer, and data were analyzed using CellQuestPro software (BD Biosciences) to determine $CD4^+CD25^+FoxP3^+$ ($FoxP3^+$ Treg) or $CD4^+CD25^+CD127^-$ ($CD127^-$ Treg) staining within the total lymphocyte population. The absolute numbers of $FoxP3^+$ and $CD127^-$

Treg cells were calculated by multiplying the Treg frequency by the absolute lymphocyte count obtained by automated differential analysis. Because laboratory staff who could use the automated differential analyzers were occasionally absent at night and during holidays, we could not analyze 14 out of 103 CB samples. Therefore, we measured the absolute lymphocyte counts of 89 CB samples from 30 preterm infants and 59 term infants.

4. Effect of maternal factors on CB Treg cells

We analyzed the effects of delivery mode and maternal allergic disease status on the proportion of Treg cells in term CB. Of 67 term infants, 43 were delivered by caesarean (C)-section and 24 were delivered vaginally. Of 43 C-sections, 5 (11.6%) were emergency deliveries. Of the 67 mothers of term infants, 17 had a history of allergic disease, such as asthma, hay fever, or food or drug allergy. None of the mothers who had allergic disease received glucocorticoids or other drugs before delivery.

We also analyzed the effects of the presence of histological chorioamnionitis (CAM) and pregnancy-induced hypertension (PIH), and the drugs the mothers received before delivery (steroid, ritodrine hydrochloride, or magnesium sulfate) on the proportion of Treg cells in preterm CB.

5. Differences in the proportion and number of Treg cells in the umbilical vein and artery

Treg cells are typically examined in CB obtained from the umbilical vein. To understand the Treg cell distribution more completely, we analyzed the proportion and the absolute number of Treg cells in blood obtained from both the umbilical vein and artery. Ten pairs of CB samples from neonates at 27-40 gestational weeks were analyzed.

6. Proportion of Treg cells in fresh versus frozen CBMCs

We evaluated the effects of freezing on CBMCs because it was not possible to analyze CB immediately after sampling due to the complicated process of evaluating FoxP3 expression. The proportion of Treg cells in fresh and frozen CBMCs obtained from 12 term infants was determined soon after CBMC isolation and 7, 14, 21, and 28 days after the CBMCs were frozen.

7. Statistical analysis

The correlation between the proportions of $FoxP3^+$ and $CD127^-$ Treg cells in CB was evaluated using the Pearson's correlation test. The Kruskal-Wallis test and multiple comparisons with the Steel-Dwass method were used to compare the proportions or the absolute numbers of Treg cells of each gestational-week groups, and the fresh to frozen samples. The Mann-Whitney test was performed to compare the median values of two proportions of Treg cells. Differences of maternal characteristics between preterm and term infants were analyzed by Fischer's exact test. A p-value of < 0.05 was considered significant.

III. Results

1. Expression of FoxP3 or CD127 by CB $CD4^+CD25^+$ T cells

No differences were observed in the proportions of $FoxP3^+$ (median 4.5%) and $CD127^-$ (median 5.0%) Treg cells. A strong correlation was found between the proportions of $FoxP3^+$ and $CD127^-$ Treg cells ($r = 0.96$;

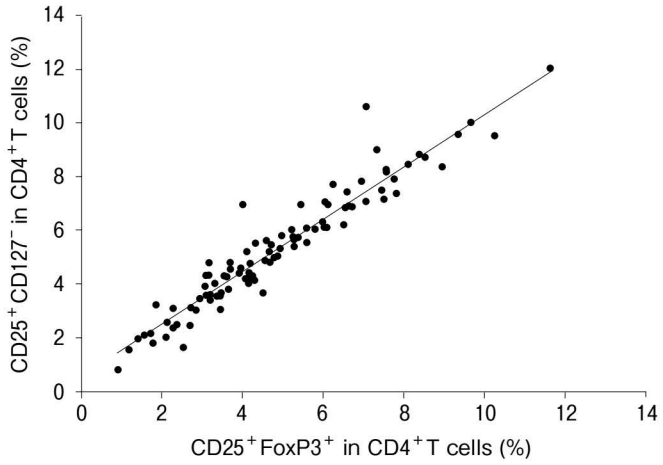

Fig. 1. Correlation between the proportions of $CD25^+FoxP3^+$ and $CD25^+CD127^-$ cells in the $CD4^+$ T-cell population in cord blood. Regression equation, $y=0.98x+0.54$; Coefficient of determination, $R^2=0.915$; Correlation coefficient, $r=0.96$.

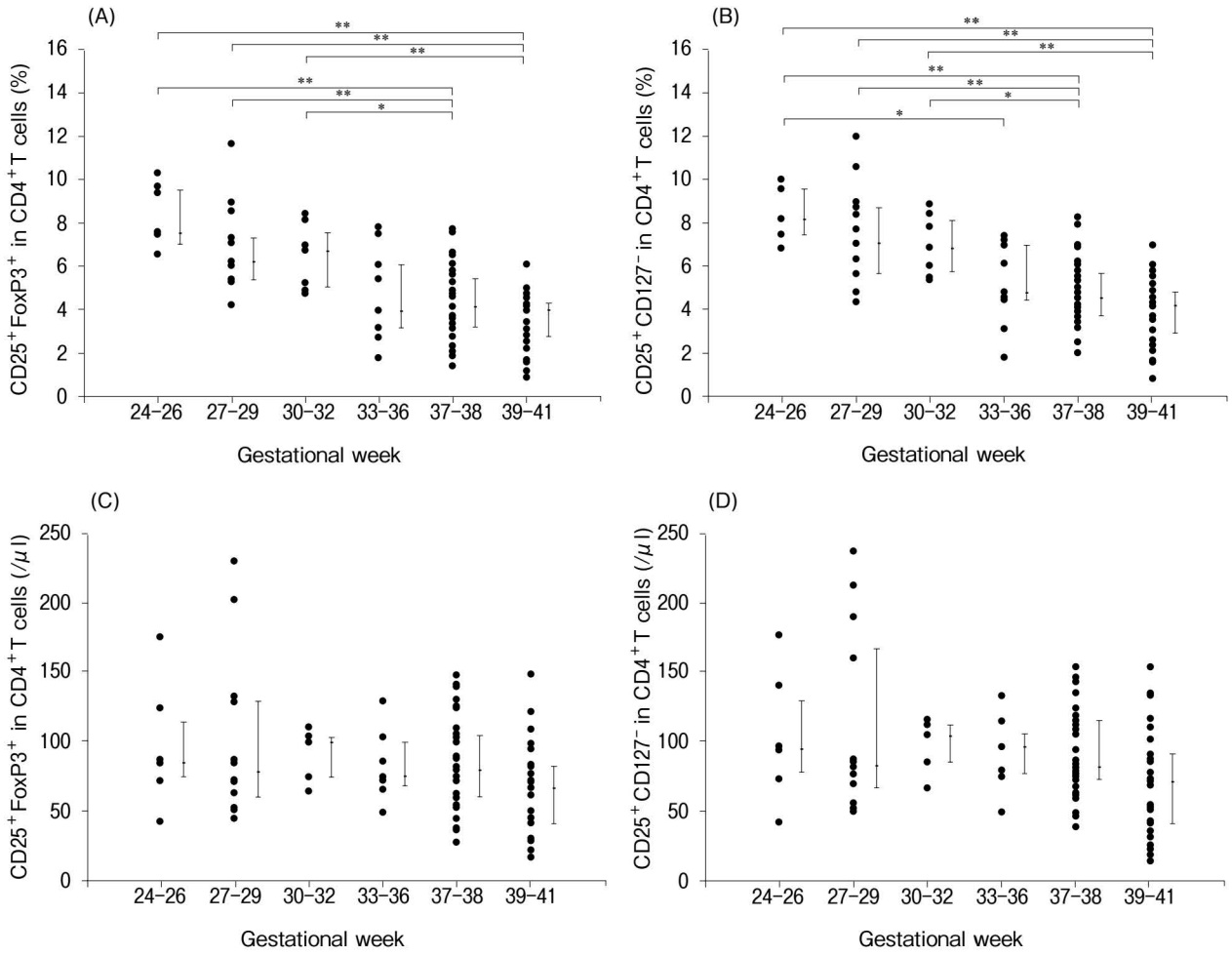

Fig. 2. The proportions and absolute numbers of $FoxP3^+$ and $CD127^-$ Treg cells in cord blood of infants at various gestational ages.
(A) Proportion of $CD25^+FoxP3^+$ cells in the $CD4^+$ T-cell population (n=103).
(B) Proportion of $CD25^+CD127^-$ cells in the $CD4^+$ T-cell population (n=103).
(C) Absolute number of $CD4^+CD25^+FoxP3^+$ cells (n=89).
(D) Absolute number of $CD4^+CD25^+CD127^-$ cells (n=89).
Each bar indicates the median with range from the 25th to 75th percentiles.
*, $p < 0.05$; **, $p < 0.01$

Table 1. Maternal characteristics

	Preterm (n=36)	Term (n=67)	p-value
Mode of delivery			
C-section	32 (88.9%)	43 (64.2%)	0.009
Maternal allergic disease	3 (8.3%)	17 (35.8%)	0.040
Prenatal drug use			
Steroid	29 (80.6%)	6 (9.0%)	< 0.001
Ritodrine hydrochloride	27 (72.2%)	0	< 0.001
Magnesium sulfate	15 (41.7%)	0	< 0.001
Histological chorioamnionitis	10 (30.6%)	1 (1.5%)	< 0.001
Pregnancy-induced hypertension	4 (11.1%)	0	0.013

Value indicates n (%).

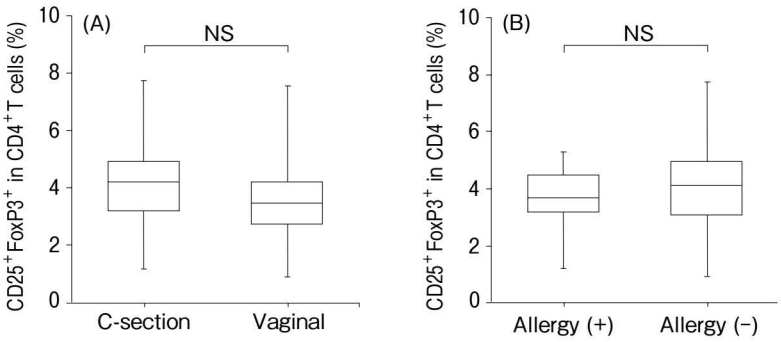

Fig. 3. Effect of maternal factors on the proportions of Treg cells in term cord blood.
(A) Proportion of $CD25^+FoxP3^+$ cells in the $CD4^+$ T-cell population of infants delivered by C-section (n=43) and vaginally (n=24).
(B) Proportion of $CD25^+FoxP3^+$ cells in the $CD4^+$ T-cell population of infants whose mothers had allergic disease (n=17) and those whose mothers did not have allergic disease (n=50).
NS, not significant.

Fig. 1). The proportion of $FoxP3^+$ Treg cells in the CB of neonates at 24-26, 27-29, and 30-32 gestational weeks was significantly higher than in the CB of neonates at 37-38 and 39-41 gestational weeks (Fig. 2A). The proportion of $CD127^-$ Treg cells in the CB of neonates at 24-26, 27-29, and 30-32 gestational weeks was also higher than in the CB of neonates at 37-38 and 39-41 gestational weeks (Fig. 2B). The absolute number of $FoxP3^+$ and $CD127^-$ Treg cells did not differ between the CB of preterm and term neonates (Fig. 2C, D). The absolute counts of lymphocytes were significantly lower in the CB of preterm infants (median 2,910/μl) than in term infants (median 3,380/μl) ($p < 0.05$).

2. Effect of maternal factors on CB Treg cells

The maternal characteristics of preterm and term infants are shown in Table 1. In the CB of term infants, there was no significant difference between the two modes of delivery with respect to the proportion of $FoxP3^+$ Treg cells (Fig. 3A). However, the proportion of $FoxP3^+$ Treg cells tended to be higher in infants delivered vaginally than in those

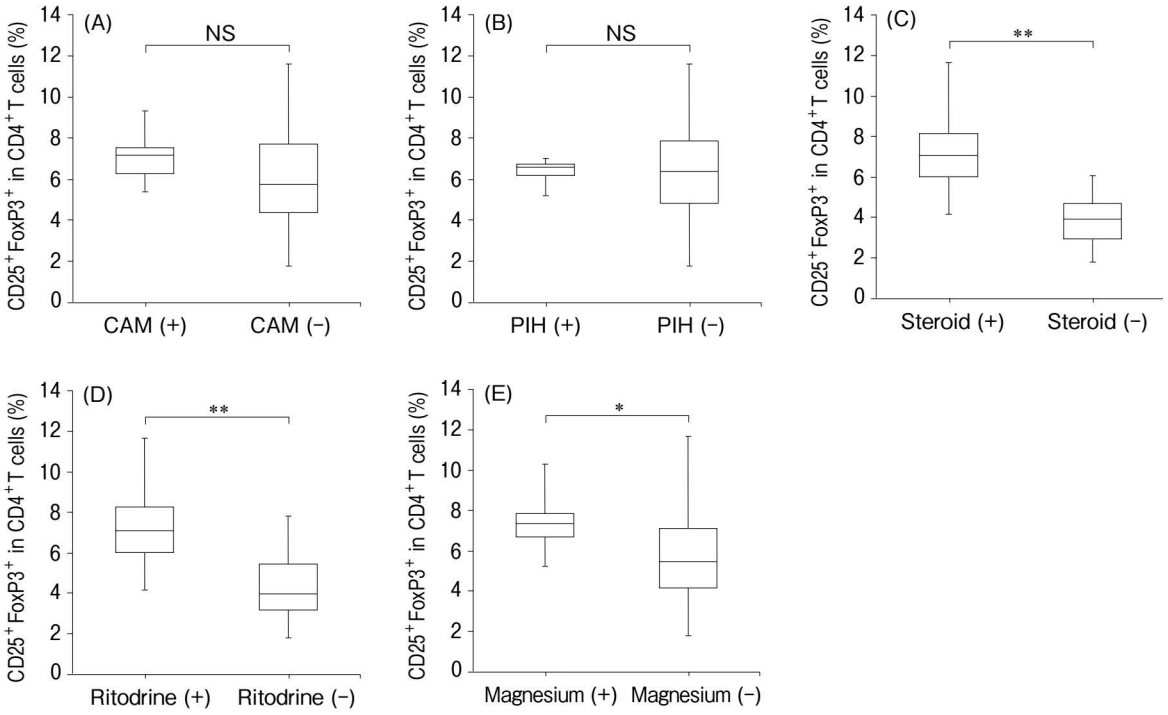

Fig. 4. Effect of maternal factors on the proportions of Treg cells in preterm cord blood.
(A) Proportion of $CD25^+FoxP3^+$ cells in the $CD4^+$ T-cell population of infants whose mothers were diagnosed with chorioamnionitis (CAM) histologically (n=10) and those whose mothers were not diagnosed (n=26).
(B) Proportion of $CD25^+FoxP3^+$ cells in the $CD4^+$ T-cell population of infants whose mothers had pregnancy-induced hypertension (PIH) (n=4) and those whose mothers did not have (n=32).
(C) Proportion of $CD25^+FoxP3^+$ cells in the $CD4^+$ T-cell population of infants received steroid (n=29) and did not receive (n=7).
(D) Proportion of $CD25^+FoxP3^+$ cells in the $CD4^+$ T-cell population of infants received ritodrine hydrochloride (n=27) and did not receive (n=9).
(E) Proportion of $CD25^+FoxP3^+$ cells in the $CD4^+$ T-cell population of infants received magnesium sulfate (n=15) and did not receive (n=21).
NS, not significant; *, $p < 0.05$; **, $p < 0.01$

delivered by elective C-section (p=0.11). The proportion of $FoxP3^+$ Treg cells did not differ between infants whose mothers had allergic disease and those whose mothers did not have allergic disease (Fig. 3B). Similar results were obtained from the analyses of the proportion of $CD127^-$ Treg cells (data not shown).

In the CB of preterm infants, there were no significant differences between the proportion of $FoxP3^+$ Treg cells depending on the presence of CAM and PIH (Fig. 4A, B). The proportion of $FoxP3^+$ Treg cells was significantly higher if mothers received prenatal steroid, ritodrine hydrochloride, or magnesium sulfate (Fig. 4C, D, E). Similar results were obtained from the analyses of the proportion of $CD127^-$ Treg cells (data not shown).

3. Differences in the proportion and number of Treg cells in the umbilical vein and artery

There was no difference between the umbilical

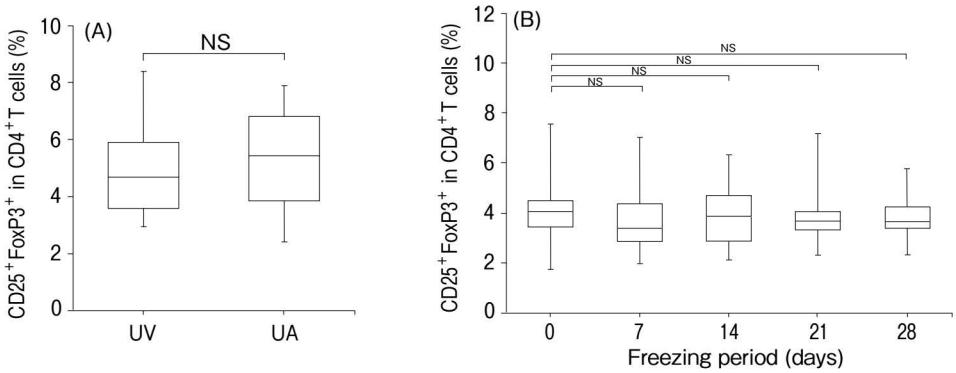

Fig. 5. Treg cells in the pair of CB from umbilical vein and artery and frozen samples.
(A) Proportion of $CD25^{+}FoxP3^{+}$ cells in the $CD4^{+}$ T-cell population in the umbilical vein (n=10) and umbilical artery (n=10).
(B) Proportion of $CD25^{+}FoxP3^{+}$ cells in the $CD4^{+}$ T-cell population in cord blood mononuclear cell samples frozen for varying lengths of time (n=12).
NS, not significant; UV, umbilical vein; UA, umbilical artery.

vein and artery in terms of the proportions of $FoxP3^{+}$ Treg cells (Fig. 5A) and $CD127^{-}$ Treg cells. No difference was observed between the umbilical vein and artery with respect to the absolute numbers of $FoxP3^{+}$ (median 82 vs 76 / μl, p=0.37) and $CD127^{-}$ (median 105 vs 90 /μl, p=0.37) Treg cells.

4. Proportion of Treg cells in fresh versus frozen CBMCs

There were no differences between fresh and frozen CBMCs with respect to the proportions of $FoxP3^{+}$ Treg cells (Fig. 5B) and $CD127^{-}$ Treg cells at any time point examined.

IV. Discussion

In this study, a strong correlation was observed between the proportions of $FoxP3^{+}$ and $CD127^{-}$ Treg cells. Therefore, CD127 is preferable to FoxP3 as a marker for identifying Treg cells in the CB of various gestational weeks because CD127 would be useful for separating Treg cells for therapeutic applications.

We showed in this study that the proportions of $FoxP3^{+}$ and $CD127^{-}$ Treg cells in the CB of preterm infants are significantly higher than in the CB of term infants. It was previously reported that the proportions of $CD4^{+}CD25^{+}$ T cells (correlation coefficient [CC] = −0.57)[8] and $CD4^{+}$ $CD25^{+}FoxP3^{+}$ T cells (CC = −0.41)[9] were inversely correlated with gestational age; however, to our knowledge, our results are the first demonstration of FoxP3 and CD127 expression in CB $CD4^{+}CD25^{+}$ T cells throughout gestation. The absolute numbers of $FoxP3^{+}$ and $CD127^{-}$ Treg cells did not differ between the CB of preterm and term infants, and the absolute counts of lymphocytes were significantly lower in the CB of preterm infants than in the CB of term infants. The functional implications of the high proportion of Treg cells in preterm CB are unknown but may be important for the suppression of undesirable alloimmune reactions to maternal derivatives in utero and the maintenance of tolerance during pregnancy.

Several Treg cell types have been characterized, the most prominent of which include natural and adaptive Treg cells. Natural Treg cells arise in the thymus and are exported to the periphery. Adaptive Treg cells are derived from naive $CD4^+$ T cells in the periphery under certain conditions, such as antigen exposure [10], oral tolerance [11], and glucocorticoid treatment [12]. Other reports have indicated that there is no difference in the frequency of $CD4^+CD25^+FoxP3^+$ T cells between fetal and infant thymus [13]. In contrast, the frequency of $CD4^+CD25^+FoxP3^+$ T cells in preterm CB were higher than in term CB. Most of the preterm CB $CD4^+CD25^+$ T cells were of the naive phenotype [14] and fetal $CD4^+$ T cells show greater expression of CD25 and FoxP3 than adult $CD4^+$ T cells after stimulation with alloantigens [13]. The high proportion of Treg cells found during early gestation suggests that naive T cells of the fetus at early gestation have a high propensity to differentiate into adaptive Tregs in response to antigenic stimulation, such as by maternal alloantigens.

A recent study showed that the percentage of Treg cells and the serum cortisol level are higher in the CB of infants delivered vaginally [15] and by emergency C-section [16] than in the CB of infants delivered by elective C-section. Our results showed that mode of delivery did not affect the proportion of Treg cells in the CB of term infants. However, the proportion of Treg cells in infants delivered vaginally tended to be higher than in those delivered by elective C-section. Although the small number of samples from the infants delivered by emergency C-section is a limitation of our study, our results may support previous studies.

A previous investigation found that maternal hay fever is related to lower Treg numbers in CB and that lower Treg numbers at birth are associated with a higher risk of developing atopic dermatitis [17]. In our study, the proportion of Treg cells was similar in the CB of infants, irrespective of the allergic disease status of the mother. This discrepancy between our study and others may be related to the extent of symptoms; in our study, the mothers with allergic disease had few symptoms during pregnancy. It has also been reported that maternal allergy has no significant effect on the proportion of Treg cells in CB, but elevated proportions of IL-4-producing Th2 cells in CB increase the risk of atopic dermatitis [18]. Analyzing only the frequency of Treg cells in CB may thus be inadequate for estimating the risk of allergic disease.

In this study, CAM and PIH did not affect the proportion of Treg cells in the CB of preterm infants, and the proportion of Treg cells in the CB was higher in the preterm infants who received steroids, ritodrine hydrochloride and magnesium sulfate than in the CB of preterm infants who did not received these drugs. FoxP3 expression has been shown to increase after systemic glucocorticoid treatment in patients with asthma [12]. However, the effects of the dose, frequency, and time of steroid treatment remain unclear. In our study, some infants were delivered a few hours after steroid treatment and others were delivered a few weeks after treatment. We need further investigation about the effect of the time on steroid treatment. Steroids and tocolytic drugs

were more predominant in early preterm infants, thus the high proportion of Treg cells could be related to gestational weeks differences.

Because CB samples are generally obtained from the umbilical vein, the proportion and absolute number of Treg cells in CB from the umbilical artery remain unclear. We report here for the first time the results of analyses of the proportion and the absolute number of Treg cells in CB from the umbilical artery compared with CB from the umbilical vein. No differences were observed between the umbilical artery and vein with respect to the proportions and the absolute numbers of $FoxP3^+$ and $CD127^-$ Treg cells. These results suggest that the distribution of Treg cells in the circulation of a fetus without any inherited abnormalities is homogeneous.

Because analyzing the expression of FoxP3 requires time-consuming cell fixation and permeabilization steps, we could not analyze the expression of FoxP3 soon after CB sampling. Therefore, we stored the CBMCs in human PB lymphocyte solution at −80℃, and examined the effect of freezing on the proportion of Treg cells. We found no significant differences in the proportions of CB $FoxP3^+$ and $CD127^-$ Treg cells between fresh samples and samples that had been frozen for varying periods, indicating that Treg cells can be maintained frozen in human PB lymphocyte storage solution for at least 28 days.

Conflict of interest: The authors have no conflict of interest to declare.

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早産児と正期産児の臍帯血中制御性 T 細胞の測定

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要旨

制御性 T 細胞（regulatory T cells，以下 Treg と略）は CD4 と CD25 および転写因子である FoxP3 を発現している．FoxP3 は Treg の特異的マーカーであるが，細胞内に局在するため，これを測定しようとすると生細胞を得ることができない．一方，Treg の細胞表面には CD127 の発現が低く，この分画を分離すれば生細胞を得ることが可能となるが，早産児の臍帯血中 Treg の CD127 発現に関する報告はない．本研究では，早産児 36 例，正期産児 67 例を対象に，在胎週数別の臍帯血中の $CD4^{+}CD25^{+}FoxP3^{+}$ 細胞（$FoxP3^{+}$ Treg）と $CD4^{+}CD25^{+}CD127^{-}$ 細胞（$CD127^{-}$ Treg）を測定した．その結果，$FoxP3^{+}$ Treg と $CD127^{-}$ Treg の割合には強い相関がみられた（$r = 0.96$）．$FoxP3^{+}$ Treg と $CD127^{-}$ Treg の割合は早産児が正期産児よりも有意に高値であったが，それらの数は両群間で有意差がなかった．以上から，CD127 は在胎期間にかかわらず Treg のマーカーとして有用であると考えられた．Treg の割合が早産児で高値であったのは，在胎の短い胎児のナイーブ T 細胞が抗原刺激で Treg へ分化しやすいことが示唆された．