

## Differences in Circulating Dendritic Cell Subtypes in Pregnant Women, Cord Blood and Healthy Adult Women

Different subtypes of dendritic cells (DC) influence the differentiation of naïve T lymphocytes into T helper type 1 (Th1) and Th2 effector cells. We evaluated the percentages of DC subtypes in peripheral blood from pregnant women (maternal blood) and their cord blood compared to the peripheral blood of healthy non pregnant women (control). Circulating DC were identified by flow cytometry as lineage (CD3, CD14, CD16, CD19, CD20, and CD56)-negative and HLA-DR-positive cells. Subtypes of DC were further characterized as myeloid DC (CD11c<sup>+</sup>/CD123<sup>±</sup>), lymphoid DC (CD11c<sup>-</sup>/CD123<sup>+++</sup>) and less differentiated DC (CD11c<sup>-</sup>/CD123<sup>±</sup>). The frequency of DC out of all nucleated cells was significantly lower in maternal blood than in control ( $P<0.001$ ). The ratio of myeloid DC/lymphoid DC was significantly higher in maternal blood than in control ( $P<0.01$ ). HLA-DR expressions of myeloid DC as mean fluorescence intensity (MFI) were significantly less in maternal blood and in cord blood than in control ( $P<0.001$ , respectively). The DC differentiation factors, TNF- $\alpha$  and GM-CSF, released from mononuclear cells after lipopolysaccharide stimulation were significantly lower in maternal blood than in control ( $P<0.01$ ). The distribution of DC subtypes was different in maternal and cord blood from those of non-pregnant women. Their role during pregnancy remains to be determined.

**Key Words :** Cord Blood; Dendritic Cells; Flow Cytometry; Pregnancy

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## INTRODUCTION

Pregnancy requires the establishment of selective immunologic tolerance to allow implantation and growth of the fetus. Recent investigations have suggested that, during pregnancy, maternal circulating immune cells undergo important changes that result in alterations in their cell counts, phenotypes, functions and ability to produce soluble factors (1). During this physiological state, immune regulation involves a shift from T-helper 1 (Th1) to T-helper 2 (Th2) activity (2-4). It has been also observed that neonates are deficient in the Th1 response and that cord blood transplantation results in less graft-versus-host disease (GvHD) (5-7).

The dendritic cell (DC), an important antigen-presenting cell, plays a key role in adaptive immune responses. Depending on their developmental origin, cytokine activators, surface antigens and functional capacity, dendritic cells can be subdivided into two major distinct populations, referred to as myeloid and lymphoid DC (8). Myeloid DC are CD11c<sup>+</sup>/CD123<sup>±</sup>, and induce Th1 cell differentiation, whereas lymphoid DC are CD11c<sup>-</sup>/CD123<sup>+++</sup> and induce Th2 cell differentiation (8, 9). A recently reported third population of dendritic cells, referred to as CD11c<sup>-</sup>/CD123<sup>±</sup>, is thought to be a less differentiated DC population (10-12). Although a number of studies have tried to correlate the changes in immune status during pregnancy or in neonates with changes

in DC subtype populations in the peripheral blood of pregnant women or in their cord blood, the results have been inconsistent (12-19) and there is no available data in Korean population.

In this study, in order to minimize the *in vitro* effect of cell-preparation, we employed a direct immunofluorescence technique on whole blood samples and four-color flow cytometry (19, 20) to evaluate the proportion of each DC subtype in the peripheral blood of pregnant women at the time of delivery, in their umbilical cord blood, and in the peripheral blood of age-matched healthy, non-pregnant women in Korean population.

## MATERIALS AND METHODS

### Study populations

Thirty pregnant women with uncomplicated labor were enrolled in the study. They provided written informed consent before being admitted for delivery at the Gynaecology-Obstetrics Department of Seoul National University Boramae Hospital. Cord blood was collected in plastic bags containing citrate phosphate dextrose adenine (CPDA) solution before placental delivery. Maternal peripheral blood was collected in tubes containing CPDA solution (Becton Dickinson, Franklin Lakes, NJ, U.S.A.) at the time of delivery. Twenty nine age-matched healthy, non-pregnant volunteer women were used as a control group. Complete blood counts were obtained for all the collected samples considering the ratio of CPDA solution to collected blood. The study design was accepted by the Institutional Review Board of Seoul National University Boramae Hospital.

### Immunophenotyping

The subtypes of dendritic cells from whole blood samples were analyzed as previously described (19, 20). A total of 50  $\mu$ L of whole blood was incubated with optimum amounts (as indicated in the instructions of the manufacturer) of mouse anti-human CD123-PE, CD11c-APC, HLA-DR-PerCP and a lineage cocktail 1-fluorescein isothiocyanate (Lin1-FITC) (containing a mixture of FITC-conjugated anti-CD3, anti-CD14, anti-CD16, anti-CD19, anti-CD20 and anti-CD56 antibodies) (BD Biosciences, San Jose, CA, U.S.A.) for 25 min at room temperature in the dark. Mouse anti-human IgG isotype control for each monoclonal antibody was used to determine background fluorescence levels. After the incubation period, 2 mL of FACS lysing solution (BD Biosciences) diluted 1:10 in distilled water was added and the samples were incubated under the same conditions for another 15 min to fix leukocytes and to lyse nonnucleated red cells. Cells were washed twice with phosphate-buffered saline (PBS), and then resuspended in 0.5 mL of PBS.

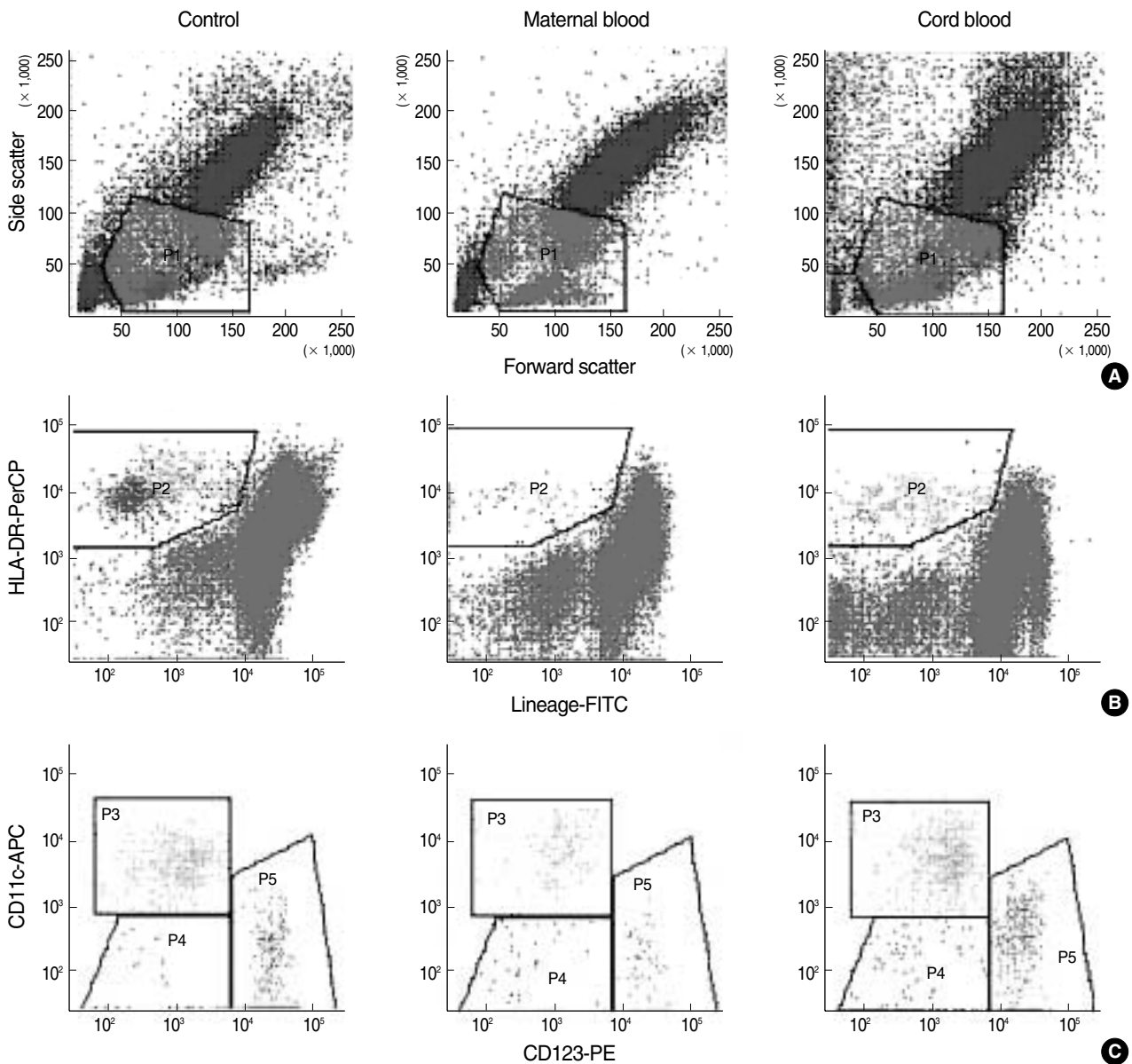
Labeled cells were analyzed with a FACSAria flowcytometer (Becton Dickinson) using FACSDiva™ Software (Becton Dickinson). From each sample, 100,000 events were acquired and analyzed. A gate on mononuclear cells was established and drawn (Fig. 1; P1 gate). Dendritic cells are negative for lineage cocktail but express HLA-DR (Fig. 1; P2 gate). Among the DC population, cells expressing CD11c were characterized as myeloid DC: CD11c<sup>+</sup>/CD123<sup>±</sup> (P3 gate), whereas cells expressing CD123 with high intensity were characterized as lymphoid DC: CD11c<sup>-</sup>/CD123<sup>+++</sup> (P5 gate). Cells expressing CD123 with low intensity were characterized as less differentiated DC: CD11c<sup>-</sup>/CD123<sup>±</sup> (P4 gate), as previously described (12, 19). Results were expressed as a percentage of total DC to ANC (all nucleated cells) and PBMC (peripheral blood mononuclear cells). Absolute numbers of DC were calculated from complete and differential blood cell counts. Subpopulations of myeloid DC, lymphoid DC, and less differentiated DC were expressed as percentages of total DC. The intensity of HLA-DR expression by each DC subtype was estimated from the mean fluorescence intensity (MFI).

### Measurement of cytokine release

Mononuclear cells (MNC) from cord blood and peripheral blood of pregnant (n=6) and non-pregnant women (n=6) were obtained by Ficoll-Hypaque (Amersham Biosciences, Uppsala, Sweden) density gradient centrifugation. MNC ( $2.5 \times 10^6$  cells/mL) were cultured with RPMI 1640 (Gibco BRL, Life Technologies, Paisley, U.K.) containing 10% heat-inactivated fetal calf serum in 96-well plate in the presence of lipopolysaccharide (LPS) (Sigma Aldrich, St. Louis, MO, U.S.A.) for 16 hr at 37°C, and supernatants were taken for cytokine measurement. Procarta Cytokine Multiplex Assay kit (Panomics, Fremont, CA, U.S.A.) analyzed with Bio-Plex Assay System (Bio-Rad, Hercules, CA, U.S.A.) was used to measure IL-1 $\beta$ , IL-6, IL-10, TNF- $\alpha$  and GM-CSF.

### Statistical analysis

Results are expressed as the mean  $\pm$  standard deviation (SD). Differences in frequency of dendritic cells and the MFI of HLA-DR between healthy, non-pregnant women (control) and pregnant women (maternal blood) or cord blood were tested by Student's t test. Those between maternal and cord blood were tested by Student's t test for paired samples. Differences of cytokine release between control and maternal blood or cord blood were analyzed by Mann-Whitney U test, and those between maternal and cord blood were analyzed by Wilcoxon signed rank test. The correlations of the characteristics between maternal blood and their paired cord blood were evaluated using the Spearman R test. The statistical analysis was performed with SPSS V 12.0 (SPSS Inc., Chicago, IL, U.S.A.) and all comparisons and correlations were considered significant if the *P* value was less than 0.05.



**Fig. 1.** Identification of circulating dendritic cells (DC) by flow cytometry. (A) The mononuclear cell analysis gate was applied to light scatterers (P1). (B) The gated events were analyzed for lineage cocktail (a mixture of FITC-conjugated anti-CD3, anti-CD14, anti-CD16, anti-CD19, anti-CD20 and anti-CD56 antibodies) and HLA-DR. P2 gate represented total DC (lineage<sup>-</sup>/HLA-DR<sup>+</sup>). (C) The P2-gated events were analyzed for CD123 and CD11c staining. Myeloid DC (lineage<sup>-</sup>/HLA-DR<sup>+</sup>/CD11c<sup>+</sup>/CD123<sup>+</sup>) were represented in gate P3, lymphoid DC (lineage<sup>-</sup>/HLA-DR<sup>+</sup>/CD11c<sup>-</sup>/CD123<sup>+++</sup>) in gate P5, and less differentiated DC (lineage<sup>-</sup>/HLA-DR<sup>+</sup>/CD11c<sup>-</sup>/CD123<sup>+</sup>) in gate P4. Healthy, non-pregnant women (control), left panel; peripheral blood of pregnant women (maternal blood), middle panel; cord blood, right panel.

## RESULTS

### Comparison of the dendritic cell subtypes of pregnant and non-pregnant women

The frequency of dendritic cells (lineage<sup>-</sup>/HLA-DR<sup>+</sup>) in all nucleated cells was markedly decreased in the peripheral blood of pregnant women (maternal blood) compared to healthy, non-pregnant women (control) ( $0.34 \pm 0.21\%$  vs.  $1.15 \pm$

$0.31\%$ ,  $P < 0.001$ ) (Table 1). The percentage of lymphoid DC was lower in maternal blood than in the control ( $42.6 \pm 13.6\%$  vs.  $58.9 \pm 11.8\%$ ,  $P < 0.001$ ) (Fig. 2A) and the myeloid DC/lymphoid DC ratio was higher in maternal blood than in that of control ( $0.75 \pm 0.53$  vs.  $0.44 \pm 0.26$ ,  $P < 0.01$ ) (Fig. 3). The percentage of less differentiated DC was also higher in maternal blood than in the control ( $30.7 \pm 13.0\%$  vs.  $17.5 \pm 8.8\%$ ,  $P < 0.001$ ) (Fig. 2A). The MFI of HLA-DR on the myeloid DC was significantly lower in maternal blood than in the control

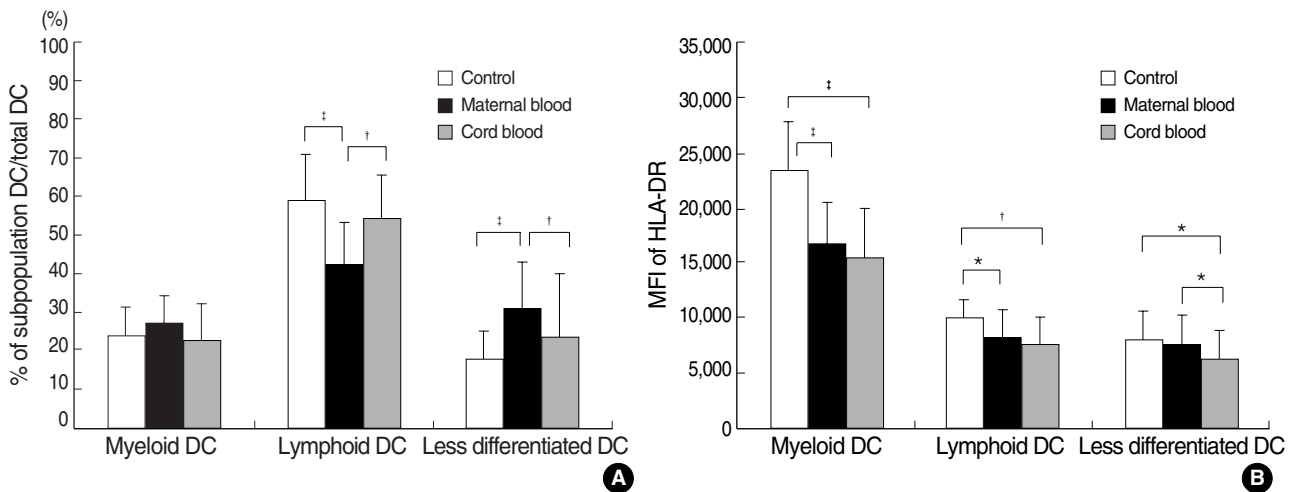


Fig. 2. (A) The percentage of myeloid dendritic cells (DC), lymphoid DC and less differentiated DC in peripheral blood of healthy, non-pregnant women (control), peripheral blood of pregnant women (maternal blood) and cord blood. (B) The expression of HLA-DR as mean fluorescence intensity (MFI) in subtypes of DC of each group. Error bars represent standard deviations. Differences between peripheral blood of healthy, non-pregnant women (control) and pregnant women (maternal blood) and cord blood were tested by Student's *t*-test. Differences between peripheral blood of pregnant women (maternal blood) and cord blood were tested by Student's test for paired samples. \* $P < 0.05$ ; † $P < 0.01$ ; ‡ $P < 0.001$ .

Table 1. Frequency of dendritic cells in peripheral blood of healthy, non-pregnant women (control) and pregnant women (maternal blood) and their cord blood

	Control (n=29)	Pregnant women (n=30)	
		Maternal blood	Cord blood
WBC count	6,253 ± 1,166	8,996 ± 2,690 <sup>†</sup>	11,886 ± 3,570 <sup>‡</sup>
% DC/ANC	1.15 ± 0.31	0.34 ± 0.21 <sup>†</sup>	0.85 ± 0.35 <sup>‡*</sup>
% DC/PBMC	2.07 ± 0.70	1.31 ± 0.53 <sup>†</sup>	2.10 ± 0.81 <sup>†</sup>
DC/ $\mu$ L of blood	72.6 ± 24.1	29.9 ± 24.7 <sup>†</sup>	102.8 ± 53.7 <sup>*‡</sup>

Results were expressed as mean ± standard deviation. WBC, white blood cells; DC, dendritic cells; ANC, all nucleated cells; PBMC, peripheral blood mononuclear cells; Differences between healthy, non-pregnant women (control) and pregnant women (maternal blood) or cord blood were tested by Student's *t* test. \* $P < 0.01$ ; † $P < 0.001$ . Differences between maternal blood and cord blood were tested by Student's *t* test for paired samples. ‡ $P < 0.001$ .

(16,605 ± 4,004 vs. 23,435 ± 5,007,  $P < 0.001$ ) (Fig. 2B). TNF- $\alpha$  and GM-CSF secretion of mononuclear cells (MNC) after LPS stimulation were significantly lower in maternal blood than in the control ( $P < 0.01$ , respectively) (Fig. 4). The production of IL-1 $\beta$ , IL-6 and IL-10 of MNC from maternal blood was not different from the control.

#### Comparison of the dendritic cell subtypes of cord blood and the blood of non-pregnant women

The frequency of dendritic cells among all nucleated cells was also significantly less in cord blood than in the control (0.85 ± 0.35% vs. 1.15 ± 0.31%,  $P < 0.001$ ) (Table 1). The percentages of myeloid DC and lymphoid DC in cord blood were not different from the control (Fig. 2A), and the myeloid

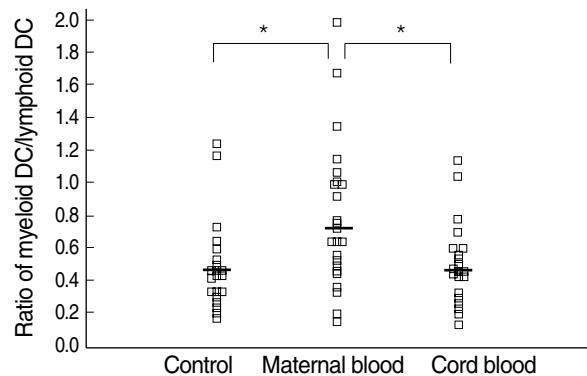
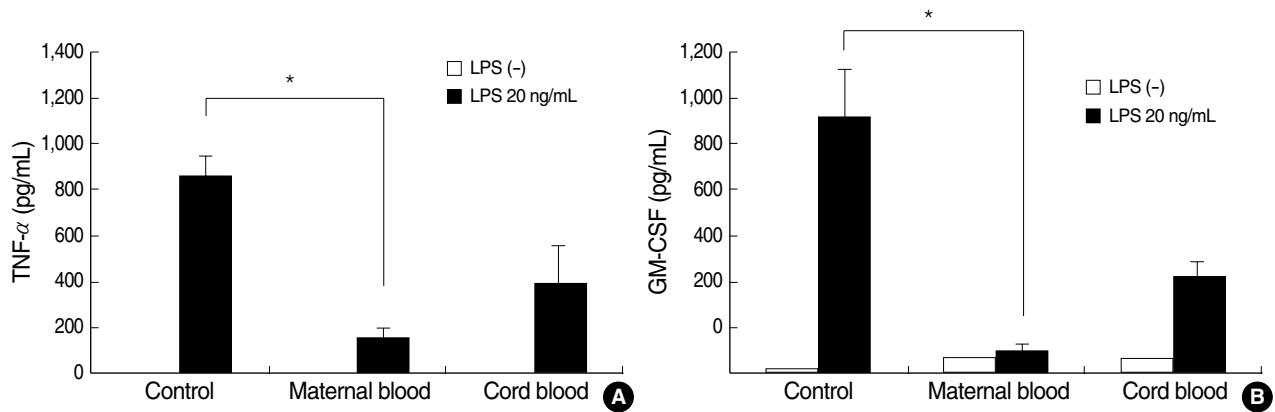


Fig. 3. The scatter plot shows ratio of myeloid dendrite cell (DC)/lymphoid DC in peripheral blood of healthy, non-pregnant women (control), peripheral blood of pregnant women (maternal blood) and cord blood. Horizontal bars represent the mean ratio of myeloid DC/lymphoid DC. Differences between peripheral blood of healthy, non-pregnant women (control) and peripheral blood of pregnant women (maternal blood) or cord blood were tested by Student's *t* test. Differences between maternal blood and cord blood were tested by Student's *t* test for paired samples. \* $P < 0.01$ .

DC/lymphoid DC ratio in cord blood was also not different from the control (Fig. 3). The MFI of HLA-DR on myeloid DC from cord blood was significantly less than in blood of healthy, non-pregnant women (15,873 ± 4,274 vs. 23,435 ± 5,007,  $P < 0.001$ ) (Fig. 2B). TNF- $\alpha$  and GM-CSF released from MNC of cord blood after LPS stimulation showed a tendency of decreasing in cord blood compared to control but did not reach statistical significance (Fig. 4). The production of IL-1 $\beta$ , IL-6 and IL-10 of MNC from cord blood was not different from control.



**Fig. 4.** The TNF- $\alpha$  (panel A) and GM-CSF (panel B) secretion of mononuclear cells with or without lipopolysaccharide (LPS, 20 ng/mL) stimulation in peripheral blood of healthy, non-pregnant women (control), peripheral blood of pregnant women (maternal blood) and cord blood. Error bars represent standard deviations. Differences between peripheral blood of healthy, non-pregnant women (control) and pregnant women (maternal blood) and cord blood were tested by Mann-Whitney U test. Differences between maternal blood and cord blood were tested by Wilcoxon signed rank test. \* $P < 0.01$

**Table 2.** Correlations between maternal blood and paired cord blood ( $n=30$ )

	Spearman r	<i>P</i> value*
WBC count	0.378	0.04
% DC/PBMC	0.449	0.01
DC/ $\mu$ L of blood	0.407	0.03
MFI of IDC	0.753	<0.001

\*Only statistically significant correlations have been shown.

WBC, white blood cells; DC, dendritic cells; PBMC, peripheral blood mononuclear cells; MFI, mean fluorescent intensity; IDC, lymphoid dendritic cells; Correlations between maternal blood and their paired cord blood were evaluated by Spearman correlation coefficient (*r*) test.

### Correlations of characteristics between maternal blood and paired cord blood

Among all the characteristics tested on thirty maternal blood and their paired cord blood samples, there were significant correlations in total WBC count ( $r=0.378$ ,  $P=0.04$ ), the frequency of dendritic cells among PBMC ( $r=0.449$ ,  $P=0.01$ ), the absolute number of dendritic cells ( $r=0.407$ ,  $P=0.03$ ) and MFI of lymphoid dendritic cells ( $r=0.753$ ,  $P < 0.001$ ) between maternal blood and their paired cord blood (Table 2).

## DISCUSSION

A significant decrease in the amount of DC in maternal blood was observed in our study. It has been suggested that during pregnancy, maternal dendritic cells take up apoptotic placental syncytiotrophoblast debris, present fetal HLA class II antigens to maternal T cells, and induce immunologic tolerance to the fetus (21). The hypothesis could partly explain the marked decrease in the frequency of dendritic cells in ma-

ternal blood seen in our study.

The immunological features of pregnancy and of neonates are known to be shifted to the Th2 response (2-5, 7). As different DC subtypes are known to play an important role in eliciting Th1 and Th2 responses, considerable effort have been made to identify differences in the DC subtypes of cord blood and maternal blood. Despite this, however, the results have been conflicting (12-19). This can be partly explained by the use of different detection procedures and antibodies (22, 23). An increase in the lymphoid DC population was reported in cord blood by the negative selection method and with anti-CD11c/CD123 antibodies (13, 14), but no significant difference was observed in whole blood samples with the direct immunofluorescence technique using same anti-CD11c/CD123 antibodies (12, 15, 18, 19). In our study, though we also used whole blood samples with direct immunofluorescence technique, the myeloid DC/lymphoid DC ratio was significantly increased in maternal blood. Therefore, the Th2 shift during pregnancy cannot be explained by the increase in the proportion of lymphoid DC.

Although the myeloid DC and lymphoid DC subtypes have been reported to primarily induce Th1 differentiation and the Th2 response, respectively (8, 9), recent evidence suggests that the opposite may also be true depending on various factors, such as the maturational status, the cytokine microenvironment, and the type of pathogen to which dendritic cells are exposed (24-26). In our study, although the myeloid DC/lymphoid DC ratio was significantly higher in maternal blood than non-pregnant women, HLA-DR expression level of dendritic cells and DC differentiation cytokines (TNF- $\alpha$  and GM-CSF) production of MNC after LPS stimulation were significantly lower in maternal blood than non-pregnant women. The present data suggests that the Th2 dominant status seen in pregnant women and neonates is more a consequence of the immature status of the dendritic cells by the decrease of DC differentiation cytokines rather

than the increased proportion of lymphoid DC.

It is also noteworthy that the less differentiated DC subtype constituted a major fraction of the DC population in maternal blood, which is in accordance with some previous reports (12, 19). The existence of less differentiated DC, characterized as CD11c<sup>-</sup>/CD123<sup>±</sup>/HLA-DR<sup>+</sup> cells in maternal or cord blood, has recently been recognized, but their role in eliciting immunological responses has not been elucidated yet (12, 19). In cancer patients, the frequency of the newly defined HLA-DR<sup>+</sup> immature cells (characterized as CD11c<sup>-</sup>/CD123<sup>-</sup>/HLA-DR<sup>+</sup> cells showing a similar immunophenotype to the less differentiated DC) is significantly increased, and these cells show a reduced capacity to capture antigens, as well as poor induction of the proliferation and IFN- $\gamma$  secretion of T-lymphocytes (27). The relevance of the less differentiated dendritic cell subtype in maternal blood seems worthy of further investigation.

In our study, many characteristics of cord blood were similar to those of maternal blood however they did not reach statistical significance. Similar features have been reported (18, 19). Interestingly, there are significant correlations in some characteristics such as the frequency of dendritic cells among PBMC or MFI of lymphoid dendritic cells between maternal blood and paired cord blood, which implies some biological effects of maternal status on those of cord blood. We also need further studies to elucidate it.

In summary, our study revealed that the myeloid DC/lymphoid DC ratio was not decreased in maternal or cord blood. Instead, expression of HLA-DR on myeloid DC and DC differentiation cytokines (TNF- $\alpha$  and GM-CSF) released from MNC were decreased in maternal and cord blood, which implicates that the maturation status of dendritic cells is more of a critical factor in the regulation of immunologic tolerance during pregnancy. Further studies are needed to clarify the functional relevance of each subtype of dendritic cells in the regulation of tolerogenic immune responses of pregnancy.

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