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Original Article

Lymphocyte Phenotypic Subsets in Umbilical Cord Blood Compared to Peripheral Blood from Related Mothers

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Umbilical cord blood (UCB) is a peripheral whole blood obtained from the umbilical cord at the time of childbirth. The interest in UCB has increased since the discovery that cord blood is a good source of hematopoietic stem cells, useful for patients who are in need of a transplant, but do not have a sibling donor. This evidence has led in recent years to the building up of blood banks to store UCB for future use in situations where the reconstitution of the hematopoietic system is required (1). Cord blood has been demonstrated to be an effective alternative source of stem cell transplantation in both children and adult recipients; however, in this context analysis of UCB is limited to flow cytometry determination of CD34+ progenitors (2). Notwithstanding, pre-birth selection of UCB donors may be of paramount importance. The investigation of predictors useful for early identification of suitable units would allow a reduction of costs for the collection, storage, and characterization of UCB with insufficient volume or cell numbers (3). Phenotypic characterization of UCB should be therefore a main issue. As a result of this increased use, it is now of fundamental importance to have a full picture of the UCB units to be transplanted and of the cellular populations that they contain. Normally UCB showed two-lymphoid populations defined by different levels of CD45 expression, the $CD45^{dim}$ population likely included naïve cells with elevated proliferative activity, T, B, NKT, and NKs besides to the majority of CD34+ cells while $CD45^{bright}$ cells distribution was similar to that of adult peripheral blood (APB) (4). The components of a UCB unit can influence its ability to engraft after transplantation and hence best flow cytometry protocols for cell identification have been forwarded (5). In this context two main issues could be addressed from literature, the phenotypic differences between UCB and adult peripheral blood with new cytomic approaches (5) and the need for an intelligent software data warehouse to collect immune system data and to outline complex multiparametric readings of different cell subpopulations, owing to their functional relationships (6). Sex, age, race, and genetic background of UCB donors are important issues but most of studies concerning lymphocyte population in umbilical cord blood evaluated the comparison of UCB with peripheral blood taken from general healthy adult individuals, both males and females, while, at best of our knowledge, no

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work comparing UCB with APB from related mothers was addressed. A preliminary evaluation of phenotypic differences between UCB and APB from mothers in childbirth by applying a routinely used flow cytometry multi-parametric protocol is presented here.

METHODS

Subjects Enrollment and Sampling

Caucasian mothers recruitment was randomized (mean age 32.6 ± 5.4 SD) and based on pre-selective criteria and UCB collection guidelines agreed in the unit (3): the study was approved by ethical commission and subjects signed an informed consent (7). None of the mothers enrolled in the study had any autoimmune or chronic disease. Subjects underwent peripheral blood withdrawal (3 ml) by venipuncture 3 days after childbirth and samples collected as K2-EDTA anticoagulated blood for analysis. Umbilical cord blood samples were obtained by puncture of the umbilical cord vein of term neonates (after 37–39 weeks) born in normal or cesarean delivery and collected into K2-EDTA vials for analysis within 3 h. Whole blood cell counts were performed by an automated hematology blood analyzer (Technicon H.3RTX or ADVIA 120, Bayer, Tarrytown, NY) and processed for immunological staining with monoclonal fluorochrome-labeled antibodies.

Flow Cytometry Instrument Details

Flow analysis was performed using a 488–633 nm two-laser BD FACScanto flow cytometer: the instrument had a 10,000 events/s capability, six-color detection and 0.1% sample carryover. Compensation followed cytometer manufacturer's instruction according an off-line procedure by applying automated electronics algorithms and preset templates, by using biparametric logarithmic dot plots, gate-specific tubes and single-tube data analysis, and optimizing FSC threshold and fluorochrome voltage as set up parameters by specific controls. To reduce standard deviation due to positive fluorescent cells respect to negative or dimly ones, a logarithmic scale and a coefficient of variation to measure variability dispersion were used. The alignment of the instrument was checked daily using fluorescent microbeads. When the intensity of the homogeneity of signals deviated from standardized and acceptable limits the alignment was adjusted accordingly. Also the accuracy of volumetric apparatus was addressed by using check commercial fluorescent beads having predetermined acceptable ranges in three different levels during the study.

Flow Samples and Specimen

To qualify UCB units several flow cytometry multiparametric tests have been proposed (5,7). Fifty microliters of undiluted whole blood for each sample underwent staining process with monoclonal antibodies within 3–4 h from the withdrawal and contaminating erythrocytes were lysed with (BD-FACS Lysing Solution, BD Biosciences, USA) according to previously published reports (6,8). Polychromatic flow cytometry panels used in this study included a six-color protocol routinely applied in the Immunology and Allergology Service for lymphocyte phenotyping in adult bloods, using the following fluorochrome-labeled monoclonals (BD-Pharmigen, USA) (8–10): Panel A CD4-FITC, CD56-PE, CD45-PerCP, CD8-PECy7, CD3-APC, CD16-APCCy7; Panel B CD27-FITC, CD23-PE, CD45-PerCP, CD5-PECy7, CD3-APC, CD19-APCCy7; Panel C CD57-FITC; CD4-PerCP, HLA-DR-APC, CD8-APCCy7, CD25-PECy7, CD38-PECy7; Panel D CD45RA-FITC, CD27-PE, CD4-PerCP, CD8-APCCy7, CD62L-APC. To evaluate fluorochrome unspecific staining, isotype controls for anti-IgG_1 and anti-$IgG_{2\alpha}$ were introduced in the preliminary procedure to set up photomultiplier and instrument technical parameters; a control which used a staining procedure carried on without introducing in the assay system the fluorochrome of interest was also performed.

Gating and Data Filtering

Gating strategies were established as follows, in order to define different subsets of lymphocytes: B cells: CD19+; T cells: CD3+; NK-T cells: CD3+/CD56+ and/or CD16+; NK cells: CD3-/CD56+ and/or CD16+. In addition, B lymphocytes (CD19+ cells) were also differentiated in CD5+ (B1 cells), CD5- (conventional B cells), CD23+ (transitional B cells) and CD27+ (memory B cells); T lymphocytes (CD3+ cells) were distinguished in CD4+ (T helper/inducer cells), CD8+ (T suppressor/cytotoxic cells), and T CD4/CD8 double negative, the latter mostly thought to pertain to γδ T cells (11). To evaluate the functional state of activation of CD4+ and CD8+ cells, surface expression of HLADR, CD45RA, CD27, CD38, and CD57 was further investigated. Analysis was performed using the FACSDIVA software (Becton Dickinson BD Biosciences, Milan, Italy). Both logarithmic and logical display methods to visualize cell subsets with minimal fluorescence were applied (12). The SSC and FSC settings were done with linear amplification (1,024 channels) and the logarithmic amplification scale (5 log decades) was used for the fluorescence channels and for dot plot analysis. Panels were adjusted for the parameter voltage and for the overlapping spectra. For the assay, threshold was set to a forward scatter (FSC) parameter to exclude debris. Sequential gating strategies using physical parameters (SSC vs. FSC) and/or immunological parameters were applied to recover and phenotype lymphocyte populations.

Statistics

Statistical evaluation was performed with a SPSS v.11.0 program and plotted with a Sigma Plot v.10 software; data were described as mean ± standard deviation (SD) and/or as box plot reporting medians and 95% confidence intervals; normality distribution was evaluated by a Shapiro-Wilk's test. Comparison of the frequency and phenotype of lymphocyte cells between UCB and APB of related mothers were evaluated by a one-way ANOVA test and LSD-post hoc analysis. Two-sided *P*-values < 0.05 were considered statistically significant.

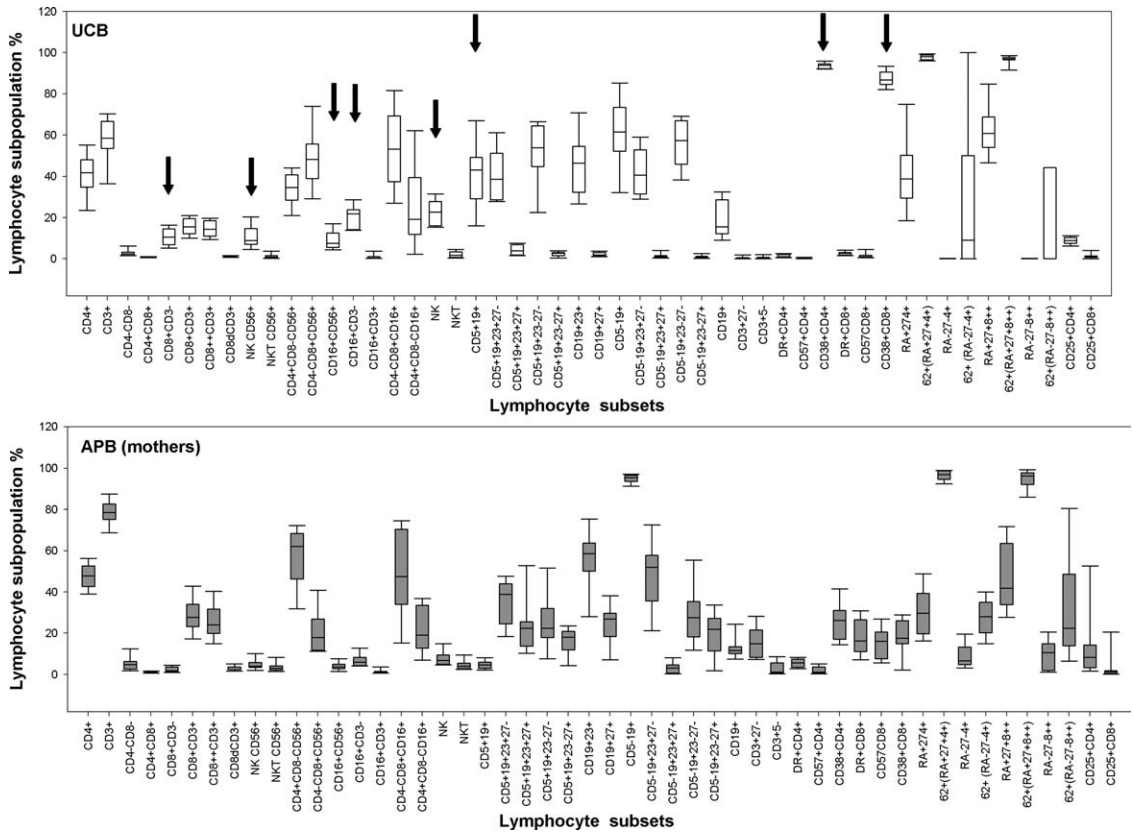

FIG. 1. Box plots of 25–75% confidence limits with medians of 50 different lymphocyte subpopulations in umbilical cord blood (UCB) and in related mothers adult peripheral blood (APB). Data are referred to 24 independent evaluations and to 50 different cell subsets/phenotypes. Arrows indicate main significant differences between UCB and newborns'-related mothers APB described in the text.

RESULTS

A first overall picture of UCB and APB cell subsets of 50 different lymphocyte phenotypes is described in Figure 1. Data analysis showed that both the populations had comparable total white blood cells (WBC) counts (14743.33 ± 3915.68 SD for UCB and 14591.82 ± 5236.44 SD for APB): an evidence that argues for a good pre-analytical procedure of sampling, which has been provided by the standardized protocol used in the study. In this graph main differences (>50%) are focused by arrows within the plots. Table 1 reports main % values of lymphocyte subsets as mean ± standard deviation (SD) and the ratio UCB/APB to evaluate up or down-regulation of the different phenotype frequencies with relative statistics. Absolute lymphocyte counts in UCB (5728.7 ± 2827.4 SD) and APB (2255.3 ± 705 SD) confirmed previously reported difference in which UCB/APB ratio was ≥2.0 and assessed also that APB has a higher CD3+ phenotype frequency compared to UCB but this result was not significant ($P > 0.05$), maybe because of sample variability (UCB CV% = 19.10; APB CV% = 7.35) (13). γδ-T cells are highly expressed in mothers, as expected (14). CD4+ subset was similarly represented in both the populations (UCB 40.7 ± 10.39 SD; APB = 47.6 ± 5.85 SD), while mothers' APB appeared to have a slightly higher CD8+ frequency. Most of CD4+ and CD8+ T-cells in UCB express CD38 (93.71 ± 1.32 and 87.71 ± 3.76, respectively). Table 1 reports that CD3+CD8+ in UCB are much more frequent than as for the $CD45^{dim}$ population has been previously reported (4). CD4+CD25+ T-reg as well as CD8+CD25+ in UCB and in APB have comparable frequency while previous evidence has shown a clear preponderance of these subsets in UCB (4). B-cell compartment was evaluated on the basis of CD19 binding, as elsewhere (4) but cells were also characterized for the expression of B1-lymphocyte marker CD5, of c-lectin CD23 (mature B-cells) and of the tumor necrosis factor receptor CD27, which plays a key role in regulating B-cell activation (B-memory marker). B1 lymphocytes CD5+CD19+ are highly represented in UCB (more than seven-fold compared to APB), while B2-cells (CD5-CD19+) is highly frequent in APB, thus confirming previously published results from ours (8,15). As far as with natural killer (NK) cells are concerned, UCB has a higher CD3-CD56+ phenotype than APB while CD3+CD56+ are highly represented in related mothers. Data of memory CD45RA-CD27- lymphocytes expressing L-selectin (CD62L) in UCB samples are widely dispersed due their complex distribution within both the investigated populations. Most of naïve T-cells CD45RA+CD27+ co-expressed also CD62L, both in UCB than in APB, while

Table 1
Main Differences Between Lymphocyte Subsets in Umbilical Cord Blood and Peripheral Blood from Related Mothers

Cell type	CD phenotype	Mean UCB ± SD	Mean APB ± SD	Ratio UCB	Ratio APB	*P*
Leukocytes	CD3+	57.38 ± 10.96	78.98 ±5.81	↓0.273	↑0.370	0.219
CD3+ T-cells	CD8+CD3+	15.79 ± 3.94	28.46 ± 7.97	↓0.445	↑0.802	<0.0001
	$CD8^{bright}CD3+$	14.74 ± 3.89	25.72 ± 8.04	↓0.427	↑0.745	<0.0001
CD4+	CD4+CD38+	93.71 ± 1.32	25.29 ± 8.83	↑2.705	↓0.730	<0.0001
	CD4+CD25+	8.85 ± 1.63	6.13 ± 3.50	↑1.440	↓0.603	0.023
CD8+	CD8+CD38+	87.71 ± 3.76	18.37 ± 8.16	↑↑3.775	↓0.790	<0.0001
	CD8+CD25+	8.85 ± 1.63	1.27 ± 1.20	↓0.866	↑1.154	0.674
γδCD3+	CD4−CD8−	2.86 ± 1.56	5.15 ± 3.46	↓0.445	↑0.800	0.048
B cells	CD5+CD19+	40.73 ± 15.82	4.71 ± 1.93	↑↑7.651	↓0.884	<0.0001
	CD5−CD19+	61.61 ± 15.93	95.03 ± 1.91	↓0.352	↑0.542	<0.0001
B cells 23/27 positive	CD19+CD23+	46.50 ± 14.37	56.03 ± 14.27	↓0.170	↑0.205	0.117
	CD19+CD27+	2.12 ± 0.96	24.21 ± 9.53	↓0.912	↑↑10.392	<0.0001
CD5+CD19+ B-cells	CD5+CD19+CD23+CD27+	4.28 ± 2.19	23.5 ± 12.79	↑0.821	↑↑4.491	<0.0001
	CD5+CD19+CD23−CD27−	52.07 ± 14.69	25.06 ± 12.67	↑1.078	↓0.518	<0.0001
	CD5+CD19+CD23−CD27+	2.42 ± 1.08	16.27 ± 6.15	↓0.851	↑↑5.723	<0.0001
	CD5+CD19+CD23+CD27−	40.57 ± 12.78	35.12 ± 10.52	↑0.155	↓0.865	0.266
CD5−CD19+ B-cells	CD5-CD19+CD23+CD27+	1.28 ± 1.15	3.25 ± 2.71	↓0.606	↑1.539	0.030
	CD5-CD19+CD23−CD27−	56.15 ± 11.13	28.99 ± 13.14	↑0.937	↓0.484	<0.0001
	CD5-CD19+CD23−CD27+	0.85 ± 0.77	19.95 ± 10.29	↓0.957	↑↑22.470	<0.0001
	CD5-CD19+CD23+CD27−	41.72 ± 10.97	47.80 ± 15.57	↓0.127	↑0.457	0.281
Naïve T-cells	CD45RA+CD27+CD4+	40.97 ± 16.73	30.20 ± 11.38	↑1.356	↓0.737	0.079
	CD45RA+CD62+CD27+CD4+	97.69 ± 1.26	96.62 ± 2.33	↑1.011	↓0.989	0.176
	CD45RA+CD27+CD8++	62.20 ± 11.58	46.78 ± 16.01	↑1.329	↓0.752	0.013
	CD45RA+CD62+CD27+CD8++	96.61 ± 2.05	94.91 ± 4.31	↑1.021	↓0.979	0.230
Memory T-cells	CD45RA−CD27−CD8++	0.11 ± 0.09	9.26 ± 5.76	↓↓0.012	↑↑↑84.180	<0.0001
	CD45RA−CD27−CD4+	0.07 ± 0.03	9.89 ± 6.81	↓↓0.001	↑↑↑141.28	<0.0001
	CD45RA−CD27−CD8++CD62L+	48.55 ± 12.09	30.40 ± 23.70	↑1.597	↓0.626	0.321
	CD45RA−CD27−CD4+CD62L+	66.67 ± 28.87	27.60 ± 8.57	↑↑2.415	↓ 0.414	0.086
NK cells	NK	22.19 ± 6.12	7.78 ± 3.46	↑1.851	↓0.649	<0.0001
	CD8+CD3−	10.77 ± 3.87	2.32 ± 1.24	↑↑3.651	↓0.785	<0.0001
	CD56+	10.68 ± 5.24	4.90 ± 2.54	↑1.180	↓0.541	0.002
	CD16+CD56+	8.85 ± 4.30	4.13 ± 1.90	↑1.143	↓0.533	0.002
	CD16+CD3−	20.03 ± 5.61	6.76 ± 3.06	↑1.964	↓0.662	<0.0001
NKT cells	NKT CD56+	1.21 ± 1.18	3.52 ± 2.21	↓0.657	↑1.917	0.004
	CD4+CD8-CD56+	34.01 ± 7.55	57.80 ± 13.55	↓0.411	↑0.699	<0.0001
	CD4−CD8+CD56+	48.75 ± 13.67	20.71 ± 10.32	↑1.353	↓0.575	<0.0001
	CD4+CD8−CD16+	25.17 ± 19.57	21.28 ± 10.72	↑0.182	↓0.154	0.552
	CD4−CD8+CD16+	53.84 ± 18.89	48.73 ± 20.41	↑0.105	↓0.949	0.531
	CD16+CD3+	1.03 ± 1.24	1.40 ± 1.02	↑0.964	↑0.355	0.433

CD62L expression in CD45RA- memory cells was highly variable in UCB reaching more than 40–50% of CD45RA-CD27- while in APB only one third of CD4+ or CD8+T-memory cells expressed CD62L. Very few differentiated T-cells (as low as 1–2%), both in UCB and in APB are terminated effector T-cells expressing CD57.

DISCUSSION

Taken together the results here discussed confirm many previous findings and raise new ones, so few differences can be observed between UCB and APB. Actually, other recently published reports have pointed out the existence of both phenotypic differences and similarities between cord and adult blood. The approach here presented has not taken into account CD34+CD38- cells, having the simple preliminary purpose to trace a comparison between mother APB and UCB: our investigation was quite different to many other studies aiming at showing that pluripotent hematological stem cells, having greater expansion and differentiation capacities, can be obtained from the UCB: these studies need lineage negative (Lyn-)/CD34+ cells to be considered.

Our data confirm that cells co-expressing CD45RA naïve antigens was the vast majority (13) and almost UCB whole naïve cells expressed CD62L-selectin. However, distribution of naïve cells between UCB and mothers' APB was surprisingly the same, while memory cells are highly frequent in peripheral blood: this may suggest a closer relationship between fetus and mother CD4+ and CD8+ naïve cells distribution compared to other works in which APB was from heterogeneous adult population (4,13).

Our data show also that the expression of immature/activation marker CD38 in adult T-cells is yet lower than an expected previously reported 50%, though confirming the high expression of this marker on UCB cells (13). These differences were probably due to APB individual distribution and sampling (4,13,16,17).

The absence of CD38 is incompatible with human life (18). In UCB percentage of T-cells co-expressing CD4/CD38 or CD8/CD38 was higher than 85–90%. In umbilical cord blood $CD4^+$ T-cells express high levels of this 45 kDa Type II transmembrane glycoprotein (19), but in adult peripheral organs CD38 is not present in all $CD4^+$ T-cells and is preferentially expressed by $CD45RO^-$ ones

(20). The expression of membrane cyclic ADP ribose hydrolase (CD38) is age-related and actually the presence of CD38 in T-cells, either CD4+ or CD8+, in cord blood is extremely high up until 2 years of age (18). The expression of CD38 on UCB $CD4^+$ T cells is a log order more intense than APB and is more homogeneous than that on APB $CD45RO^-CD4^+$ T cells (18). Other evidence reported that UCB CD3+CD8+ and CD3+CD4+ express high level of CD25 (4). UCB contained a high proportion of CD25+CD4+ T cells that declined with gestational age to the level of APB: CD25+ or CD25+CD4+ T cells in UCB had a higher frequency of CD45RA+ and CD38+ cells than in APB (21). CD4+/CD45RA+ naïve T cells express CD38 within the circulating pool, as does a subset of regulatory CD4+/CD25+ T cells, at least in mouse model (18). The significance of CD38 expression CD3+CD8+ is still controversial, as it is also readily expressed at high levels by the majority of peripheral blood mononuclear cells upon *in vitro* and *in vivo* activation: expression of CD38 on T cells residing in the tissues mainly depends on the degree of their activation (18). Further issues about the role of CD38 and CD25 in UCB CD3+ cells should therefore be addressed.

The expression of CD23, a low affinity receptor for IgE, and of CD27 in B lymphocyte has recently reviewed by the authors (10): the present data confirm that memory CD23-CD27+ B2-cells are highly represented in APB (more than 22-fold than UCB) (8). B2 cells development occurs from late neonatal life onwards: our data show that a significant percentage of B2 CD5-CD19+ cells are present in UCB (Table 1). Almost all CD19+ B-cells are B2 in APB while B1/B2 in UCB is approximately equally distributed. Most of UCB B-cell subtypes do not express CD27, as expected. It is generally admitted that UCB and APB NK cells have many similarities (21): this is true for all those papers which compared UCB to peripheral blood from a general healthy cohort of adults. Previously reported evidence has shown this trend only in the $CD45^{dim}$ fraction of UCB lymphocytes, while in the $CD45^{high}$ population CD3-CD56+ cells are equally distributed both in UCB than APB (4); however CD16+ subset is significantly higher in UCB than in APB (Table 1). UCB CD4+ CD56+NKT are less and CD56+CD4- NKT are more present than APB, despite to previously published reports (4). Most of differences in CD3-NK and CD3+NK cells depend, however, on pre-analytical conditions. There is considerable variability in the reports describing the phenotypic and functional characteristics of UCB NK cells. While differences in reagents and laboratory techniques probably account for some of this variation, other factors typically not available to laboratory investigators (and specific to UCB) should also be considered. For instance, a number of issues influence the NK cell content of UCB, including the race and sex of the donor, the gestational age of the UCB donor, vaginal delivery, or caesarean section with general anesthesia (21). In conclusion, UCB and mother APB, appear to present much more similarities than differences. Notwithstanding, UCB NKT distribution, which should be related to maternal-fetal interface (22), is quite different to previous reports. Establishment of the maternal-fetal interface is characterized by the influx of maternal NK cells, macrophages, and T cells into the decidua. Although a great deal has been learned about the function of NK cells in the decidua, comparatively little is known of decidual T cell function.

This is the first study to compare lymphocyte subsets of UCB with that of PBL of related mothers. Some differences to previous studies comparing UCB lymphocyte subsets with healthy unrelated adults may be explained by this fact. Genetic kinship should play a significant role in lymphocyte function that routinely cytomics usually could not outline. This issue claims for the need that UCB should be compared to related mothers' APB in order to address further insights on UCB lymphocyte distribution and immune physiology in newborns.

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