



Flow cytometric characterization of human umbilical cord blood lymphocytes: immunophenotypic features

GIOVANNI D'ARENA, PELLEGRINO MUSTO, NICOLA CASCAVILLA, GIROLAMO DI GIORGIO,* SAVERIO FUSILLI,^o FEDERICA ZENDOLI,* MARIO CAROTENUTO

Division of Hematology,*Division of Obstetrics, and ^oDepartment of Pathology IRCCS "Casa Sollievo della Sofferenza" Hospital, San Giovanni Rotondo, Italy

ABSTRACT

Background and Objective. One of the most important potential advantages in the use of human umbilical cord blood (HUCB) for hematopoietic reconstitution after myeloablative therapy seems to be the lower occurrence of acute graft-versus-host-disease (GvHD) in recipients after allogeneic transplantation. Since mature T cells play an important role in GvHD pathogenesis, we tried to verify whether a different immunophenotypic pattern exists between HUCB and peripheral blood (PB) T cells.

Design and Methods. An immunophenotypic study on 40 HUCB and 40 PB samples from healthy adult volunteers was performed, by means of flow cytometry using a large panel of monoclonal antibodies in double labeling.

Results. The absolute lymphocyte count was greater in HUCB ($5233 \pm 1808 \mu\text{L}$) than in adult PB ($1941 \pm 378 \mu\text{L}$). Significant differences in percentage were found between cord and adult T-cells, respectively (CD3^+ : 59.9 ± 1.2 vs $74.9 \pm 4.6\%$), $\text{CD3}^- \text{CD16}^+$ and/or CD56^+ natural killer (NK) cells (23.8 ± 10.1 vs $10.8 \pm 5.3\%$) and $\text{CD3}^+ \text{CD16}^+$ and/or CD56^+ cytotoxic T lymphocyte subset (0.3 ± 0.3 vs $10.7 \pm 4.1\%$). There was no difference in CD4/CD8 ratio (1.7 ± 0.5 vs 1.6 ± 0.2) between the two groups. In absolute terms, HUCB contained an higher number of all lymphocyte subsets, with the exception of $\text{CD3}^+ \text{CD16}^+$ and/or CD56^+ T lymphocyte subpopulation, $\text{CD3}^+ \text{CD25}^+$ and $\text{CD3}^+ \text{HLA-DR}^+$ activated T-cells. CD38 , a marker of activation and immaturity, was present on virtually all cord T cells and on approximately half of the adult T cells. The large majority of HUCB T cells co-expressed CD45RA naive antigen ($\text{CD4}^+ \text{CD45RA}^+$: $87.6 \pm 5.2\%$, $\text{CD8}^+ \text{CD45RA}^+$: $93.5 \pm 7.8\%$; $\text{CD4}^+ \text{CD45RO}^+$: $12.3 \pm 5.2\%$; $\text{CD8}^+ \text{CD45RO}^+$: $6.4 \pm 7.8\%$) whereas in adult PB T cells an higher number of CD45RO^+ memory cells was detected ($\text{CD4}^+ \text{CD45RA}^+$: $44.8 \pm 9.6\%$; $\text{CD8}^+ \text{CD45RA}^+$: $71.5 \pm 8.1\%$; $\text{CD4}^+ \text{CD45RO}^+$: $55.2 \pm 9.6\%$; $\text{CD8}^+ \text{CD45RO}^+$: $28.5 \pm 8.1\%$). Finally, less than 14% of lymphocytes were shown to belong to B lineage in both sources, while, in absolute terms, they were more represented in HUCB with respect to adult PB.

Interpretation and Conclusions. In the present study we found significant differences between HUCB and adult PB lymphocytes in their immunophenotypic

profile. In particular HUCB showed T lymphocytes that appeared to be phenotypically immature. Indeed, as a likely consequence of poor antigenic experience during pregnancy, the majority of HUCB cells were naive, expressing the RA isoform of the CD45 molecule. These findings could justify the previously reported reduced cord blood lymphocyte alloreactivity when allogeneic transplantation is performed and require further functional studies in order to confirm the impairment of HUCB immune system response to alloantigens.

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Key words: human umbilical cord blood, graft-versus-host disease, flow cytometry, lymphocyte subpopulations, transplantation

Human umbilical cord blood (HUCB) collected immediately after delivery has been shown to contain multipotent hematopoietic progenitor cells at a higher frequency than bone marrow and mobilized peripheral blood (PB) cells.¹⁻⁸ Therefore, HUCB has been used as a source of stem cells for transplantation because of its ability to provide hematopoietic reconstitution after myeloablative therapy.⁹⁻¹³ Potential advantages of this procedure include: 1) absence of donor risk; 2) very low risk of transmissible infectious diseases; 3) a lower possible incidence of acute graft-versus-host disease (GvHD).^{12,13}

GvHD is a complication of allogeneic bone marrow and peripheral blood transplantation in which recipient mature T cells play an important role.^{14,15} Targets of T cell mediating GvHD reactions are surface molecules encoded by the major histocompatibility complex (MHC) and minor histocompatibility antigens. Both CD4^+ and CD8^+ T-lymphocytes are thought to be involved in GvHD. Natural killer (NK) cells appear to contribute to GvHD, but with an ancillary role with respect to T cells.¹⁶

A possible explanation of the infrequent occurrence of GvHD when using HUCB resides in the neonatal immune system immaturity.¹⁷⁻²⁰ In order to investigate the immunophenotype of HUCB lymphocyte subsets, and particularly T cells, we performed a flow cytometric study on HUCB and PB of healthy adult volunteer samples.

Materials and Methods

Forty unfractionated eparinized HUCB samples were collected from the umbilical vein immediately after vaginal delivery in uncomplicated pregnancies delivered at terms, at the Obstetrics Department of our hospital. Forty unfractionated eparinized PB samples were also collected from healthy adult volunteers, 20 males and 20 females, aged 20-56 years, as controls. There was no evidence of concurrent infections in these adult donors. Full blood counts were determined using a Coulter STKS (Coulter Diagnostics). The directly-labeled monoclonal antibodies (MoAbs), conjugated either to fluorescein isothiocyanate (FITC) or phycoerythrin (PE), and their double labeling combinations used in this study, are listed in Table 1.

Briefly, 100 μ L of whole HUCB or PB were simultaneously stained with 10 μ L of the appropriate FITC and PE MoAbs and incubated for 30 minutes at 4°C in the dark. After the red blood cells were lysed (Lysing Solution, Ortho Diagnostic) and washed twice by centrifugation in phosphate-buffered saline containing 0.1% sodium azide and 0.5% bovine serum albumin, the samples were analyzed by flow cytometry.

Data were acquired on a FACSort flow cytometer (Becton Dickinson Immunocytometry Systems, BDIS,

San José, CA, USA) equipped with a 15 mW argon laser emitting at 488 nm and Lysis II software. All channels were set for acquisition in the logarithmic mode. An acquisition gate was set according to side and forward light scattering cell properties to collect only the lymphoid population. Mouse IgG₁ and IgG_{2a} (BDIS) were used as isotypic controls to determine background fluorescence. Five thousand events were stored in list mode data files and analyzed for two-color fluorescence. The relative size of each lymphocyte subset was expressed as the percentage within the total lymphocyte population. The lymphocyte gate was checked for its purity and recovery by use of CD14 (Leu-M3)/CD45 (HLe-1) (BD) double staining. In fact, the lymphocytes express the highest levels of CD45 and little or no CD14. These cells have low forward light scatter and low right-angle light scatter. Erythrocytes, platelets, and non-hematopoietic cells do not express CD45. Before starting the sample acquisition, the recovery (> 85%) and the purity (> 85%) of lymphocytes in the light scatter gate was determined, adjusting the light scatter gate to include the maximum number of lymphocytes while reducing the other contaminating cells. Finally, the absolute size of each lymphocyte subset was calculated from the relative size of the lymphocyte subset, the relative size of the total lymphocyte population, and the absolute leukocyte count.

Table 1. Combinations of MoAbs used in this study.

MoAbs	Specificity
IgG-PE*/IgG-FITC*	Background staining
CD45 (HLe-1)-FITC*/CD14(Leu-M3)-PE*	Leukocytes/monocytes
CD19 (Leu-12)-FITC*/CD3(Leu-14)-PE*	B/T lymphocytes
CD20 (Leu-16)-FITC*/CD2(Leu-5b)-PE*	B/T lymphocytes and most NK cells
CD8 (OKT8)-FITC°/CD4(Leu-3a)-PE*	Suppressor/cytotoxic and helper/inducer T-cell subsets
CD45RA (Leu-18)-FITC*/CD4(Leu-3a)-PE*	T naive cells
CD45RO (Leu-45RO)-FITC*/CD4(Leu-3a)-PE*	T memory cells
CD45RA (Leu-18)-FITC*/CD8(Leu-2a)-PE*	T naive cells
CD45RO (Leu-45RO)-FITC*/CD8(Leu-2a)-PE*	T memory cells
CD3 (OKT3)-FITC°/CD38(Leu-17)-PE*	Immature/activated T lymphocytes
CD3 (OKT3)-FITC°/HLA-DR-PE*	Activated T lymphocytes
CD25 (IL2 receptor)-FITC*/CD3(Leu-14)-PE*	Activated T lymphocytes
CD3 (OKT3)-FITC°/CD16(Leu-11c)+ CD56 (Leu-19)-PE*	NK cells
CD10 (OKT10)-FITC°/CD19(Leu-12)-PE*	Immature B cells

Sources: *Becton Dickinson; °Ortho Diagnostics; #Immunotech.

Statistics

The Shapiro-Wilk's W-statistic was performed to evaluate the normality of data distributions. The Mann-Whitney non-parametric statistic was performed to compare the groups that didn't show the normally distribution of data; otherwise, the pooled t-test or the separate t-test for unmatched samples was performed, after the Levene's test to verify the homogeneity of the two sample variances.

Results

Automatized blood count

Table 2 shows the mean \pm standard deviations values of hematological parameters in the two groups. Macrocytosis, leukocytosis, lymphocytosis, and a greater Hb content in red cells, were the most relevant features of HUCB compared to adult PB.

In Table 3 the proportional and absolute mean values \pm standard deviation of T- and B-antigen expression on HUCB and PB lymphocytes, as evaluated by means of MoAbs tested in our study, are summarized.

B lymphocytes

No difference was found with respect to CD19⁺, CD20⁺ B lymphocyte percentage between the two sources, whereas a greater absolute number of B cells was observed in HUCB. In some cases (10 HUCB and 10 adult PB) we tested CD10 MoAb: a very low

Table 2. Automatized blood cell count.

	HUCB	Adult PB	p
Red cells (x10 ⁶ /μL)	5014±424 (4420-5780)	4626±317 (4210-5460)	< 0.003°
Hct (%)	47.8±3.4 (42.5-56.5)	41.9±3.1 (37.2-48.3)	< 0.0001°
MCv (fL)	103.3±6.5 (88.6-116)	83.9±2.1 (80.1-89)	< 0.0001°
MCH (pg)	33.7±1.5 (32.5-38.8)	27.7±0.9 (25-29.2)	< 0.0001°
MCHC (g/dL)	32±1.4 (32.5-34.7)	33.4±0.5 (28.1-33.6)	< 0.0001°
Hb (g/dL)	15.3±0.7 (14-16.6)	14.1±0.9 (12.6-16)	< 0.0001°
Platelets (x10 ⁹ /L)	0.256±0.63 (0.173-0.360)	0.228±0.059 (0.157-0.378)	NS°
White cells (x10 ⁹ /L)	13.696±3.520 (8.2-20.7)	5.887±1.265 (4.2-9.3)	< 0.0001°
Neutrophils (%)	50.8±14.2 (14-70)	56.9±3.8 (48-66)	< 0.05*
(/μL)	7,058±3,054 (1,974-14,076)	3,395±873 (2,310-5,580)	< 0,0001°
Lymphocytes (%)	38.3±10.8 (20-61)	33.2±3.5 (28-40)	< 0.03*
(μL)	5.233±1,808 (2.280-9,272)	1,941±378 (1,302-2,790)	< 0,0001°
Monocytes (%)	6.3±4.7 (1-20)	6.5±0.3 (4-10)	NS*
(/μL)	840±671 (121-2,820)	372±84 (244-576)	< 0.001°
Eosinophils (%)	3±1.7 (1-6)	2.3±1.6 (0-8)	NS*
(/μL)	381±187 (141-750)	138±104 (0-392)	< 0,0001°
Basophils (%)	0.7±0.7 (0-3)	1±1 (0-3)	NS°
(/μL)	102±111 (0-375)	59±63 (0-210)	NS°

Values are expressed as mean±standard deviation (range).

°Student t-test; °Mann-Whitney non parametric test.

expression of this marker was found (less than 0.6% of all lymphocytes) in both groups, without statistical difference.

T lymphocytes

In adult PB we found a higher percentage of T cells (CD2⁺, CD3⁺), while higher absolute levels of these cells were found in HUCB. In this setting, we must take in account that CD2 molecule may also be expressed on NK cells, thus explaining the differences

observed between the percentages of HUCB and PB CD3⁺ and CD2⁺ cells (see below). A greater percentage of CD8⁺, as well as of CD4⁺ T cells was also seen in adult PB (although, the latter is not significant); however, the total number was lower than in HUCB. Both percentage and absolute number of double labelled CD4⁺CD8⁺ cell were found to be greater in HUCB, while no difference in CD4⁺/CD8⁺ cell ratio was observed. In absolute terms, we found an increased number of helper/inducer, suppressor/cytotoxic T-lymphocyte subsets and double labelled CD4⁺CD8⁺ cells in HUCB.

In order to evaluate immature and activated T cells, we performed a biparametric analysis in double labelling of CD3 and CD38, CD3 and CD25, and CD3 and HLA-DR. CD3⁺CD38⁺ immature T cells were more abundant in HUCB than in adult PB both in propor-

Table 3. Lymphocyte subpopulations.

Subpopulations	HUCB	Adult PB	p
	mean % ±sd mean (/μL) ± sd	mean % ±sd mean (/μL) ± sd	
CD19 ⁺	13.7±8.7 718±519	10.3±2.6 192±76	NS° < 0.0003°
CD20 ⁺	12.3±8.5 723±419	11.6±3.0 225±85	NS° < 0.0001°
CD10 ⁺	0.5±1.8 10±12	0.3±0.9 5.6±10	NS° NS°
CD2 ⁺	72.1±8.9 3225±1032	80±5.2 1604±324	< 0.02° < 0.0001°
CD3 ⁺	59.9±12 2860±1168	74.9±4.6 1465±322	< 0.0001° < 0.0001°
CD4 ⁺	42.4±9.9 1972±798	43.9±4.3 864±201	NS* < 0.0001°
CD8 ⁺	24.8±4.7 1199±104	28.5±3.6 562±138	0.0005* < 0.0001°
CD4 ⁺ CD8 ⁺	1.1±0.1 49±5	1±0.1 19±2	NS° < 0.0001°
CD3 ⁺ CD25 ⁺	0.9±1.3 46±24	4.2±2.3 84±49	< 0.0001° < 0.0001°
CD3 ⁺ HLA-DR ⁺	1.1±1.7 58±28	5.3±2.6 95±49	< 0.0001° < 0.0001°
CD3 ⁺ CD16 ⁺ CD56 ⁺	23.8±10.1 1112±656	10.8±5.3 20±496	< 0.0001° < 0.0001°
CD3 ⁺ CD16 ⁺ CD56 ⁻	0.3±0.3 13.1±13.7	10.7±4.1 105±71.2	< 0.0001° < 0.0001°
CD4 ⁺ CD8 ⁺ ratio	1.7±0.5	1.6±0.2	NS°
CD3 ⁺ CD38 ⁺	94.1±2.4 2344±1075	49.9±10.6 643±307	0.0001* < 0.0001°
CD3 ⁺ CD38 ⁻	5.9±2.4 140±70	50.1±10 672±240	< 0.0001° < 0.0001°

CD3⁺CD38⁺ and CD3⁺CD38⁻ data are expressed as percentage number of CD3⁺ cells. *Student t-test; °Mann-Whitney non parametric test.

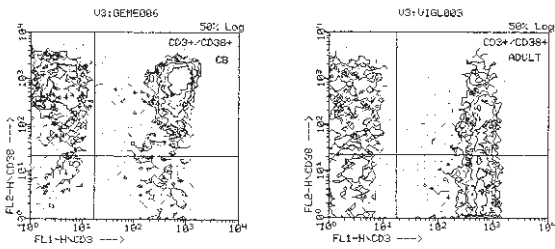


Figure 1. Representative contour plots showing examples of CD3-FITC co-expression with CD38-PE both on HUCB (left) and adult PB (right) lymphocytes.

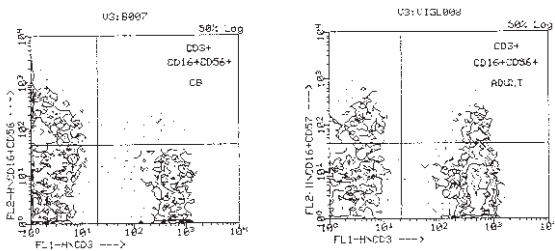


Figure 2. Representative contour plots showing the pattern of co-expression of CD3-FITC with CD16+CD56-PE both on HUCB (left) and adult PB (right) lymphocytes.

tional and absolute values. However, a greater number of CD3⁺HLA-DR⁺ and CD3⁺CD25⁺ cells was found in adult PB with respect to HUCB. Figure 1 shows the typical pattern of CD38 expression on T cells (CD3⁺) either in HUCB and adult PB.

Natural killer cells

Regarding to NK cells, both proportional and absolute number of *true* NK cells (CD3⁻/CD16⁺ and/or CD56⁺) were higher in HUCB compared to adult PB. However, a greater CD3⁺/CD16⁺ and/or CD56⁺ T-cell subtype number was observed in adult PB. Figure 2 shows the typical pattern of NK and CD3 antigens co-expression in double labelling.

Naive and memory T cells

A different distribution of CD45 isoforms was observed on CD4⁺ and CD8⁺ T cell subpopulations between HUCB and adult PB lymphocytes, as shown in Table 4.

In HUCB CD4⁺ and CD8⁺ T cells were predominantly found coexpressing CD45RA antigen (*naive* cells), while only very small subsets of *memory* CD45RO⁺ cells were observed. On the other hand, a greater number of CD4⁺CD45RO⁺ cells was found in adult PB, while more than 70% of CD8⁺ cells showed CD45RA⁺ phenotype. In absolute terms, an increased number of CD45RA⁺ T cells was observed in HUCB and a greater number of CD45RO⁺ T cells in adult PB.

Figure 3 shows the typical pattern of expression of CD45 isoforms both on CD4⁺ and CD8⁺ T mature cells in HUCB and adult PB.

Discussion

GvHD is a complication of allogeneic transplants in which donor mature T cells play an important role. In fact, depletion of T cells from the marrow or PB graft effectively prevents GvHD across both major and minor histocompatibility differences.²¹

A reduced risk of acute GvHD in HUCB transplantation has been previously described.⁹ Kurtzberg, and more recently Gluckman for Eurocord, reported data on HUCB transplantations from related and unrelated donors confirming successful hematopoietic reconstitutions and a lower incidence of GvHD than expected with bone marrow grafts.^{12,13} GvHD observed were not lifethreatening and the incidence of chronic GvHD was low. In particular, for unrelated donors, despite the HLA incompatibility, GvHD was mild (almost always less than grade III). In addition, it must be taken into account that HUCB grafts contain a total number of hematopoietic and immune cells significantly lower (10-100 times less) than bone marrow or PB progenitor cell grafts because of the little volume (about 100 mL) of HUCB that may be collected at the birth. The relevance of the fewer immunological total cell number infused in HUCB transplantation on the GvHD reactions must not be discarded. Whether such a lower incidence of GvHD results in a decrease in graft-versus-leukemia (or other diseases) activity and, in turn, in an increase in the risk of neoplastic relapse remain to be established.

Given this intriguing clinical scenario, efforts have been focused at better understanding the biology of HUCB lymphocytes.

HUCB differs from adult PB in morphological and immunophenotypic nucleated cell features. In the former, a greater number of red and white cells is commonly found when compared to the latter.

Table 4. Naive and memory cells.

Subpopulations	HUCB	Adult PB	p
CD4+CD45RA+ (%)	87.6±5.2	44.8±9.6	<0.0001°
(μ L)	1635±720	355±150	<0.0001°
CD4+CD45RO+ (%)	12.3±5.2	55.2±9.6	<0.0001°
(μ L)	219±117	421±130	<0.0001*
CD8+CD45RA+ (%)	93.5±7.8	71.5±8.1	<0.0001°
(μ L)	1320±640	445±172	<0.0001°
CD8+CD45RO+ (%)	6.4±7.8	28.5±8.1	<0.0001°
(μ L)	86±97	185±106	<0.0001°

Data are expressed as mean percentage values (\pm standard deviation) within CD4⁺ or CD8⁺ T cell population and absolute number (μ L).

°Student t-test; *Mann-Whitney non parametric test.

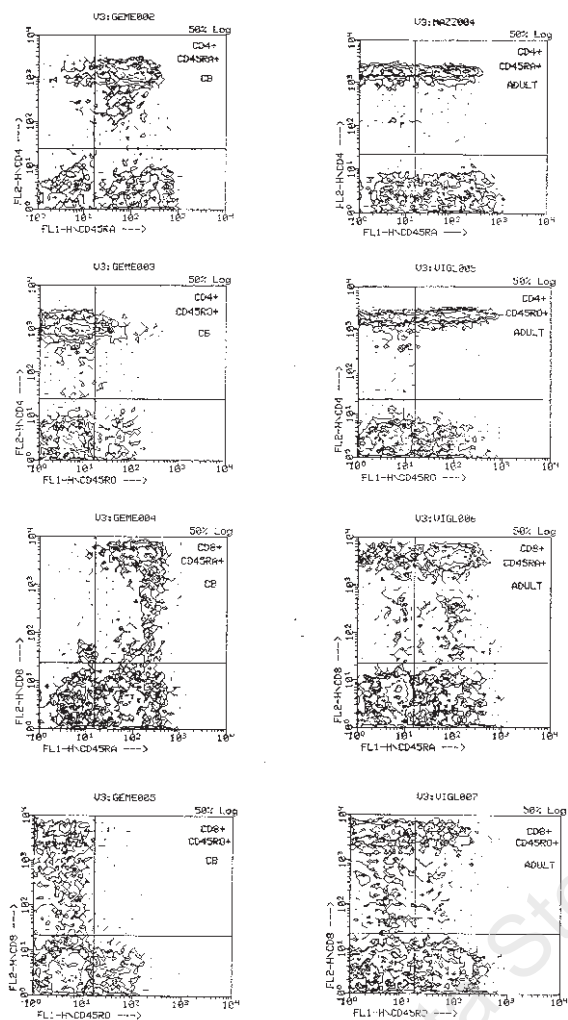


Figure 3. Representative contour plots showing the pattern of co-expression of CD4 and CD8-PE with CD45RA and CD45RO-FITC both on HUCB (left) and adult PB (right) lymphocytes.

Macrocytosis and nucleated red cells are also typical features of HUCB. All these findings were confirmed in our study.

Regarding to lymphocyte subpopulations, we did not find different proportions of B cells between the two groups, whilst, as HUCB has a higher lymphocyte count, the absolute B cell count resulted higher in these samples. HUCB also had a reduced proportion of mature T cells, as previously suggested by other investigators.^{22,23} No relevant differences were found with respect to CD4 and CD8 percentage cell number, as well as to CD4:CD8 ratio. However, a greater absolute number of CD4⁺, CD8⁺ and double labelled CD4⁺CD8⁺ cells was found in HUCB.

CD38 is a transmembrane glycoprotein frequently

expressed on *early* differentiating and activated cells. However, its real function is still unknown.²⁴ A greater number of *immature* (CD3⁺CD38⁺) T cells was found in HUCB. These cells seemed to be not activated as only a small number of CD3⁺HLADR⁺ and CD3⁺CD25⁺ cells was detected in HUCB. These findings likely confirm the cord blood lymphocytes functional immaturity.

The leukocyte common antigen (CD45) is a highly glycosylated cell surface protein expressed on all nucleated cells of the hemopoietic system. CD45 has been demonstrated to play a role in signal transduction and interaction with other cell surface molecules.²⁵⁻²⁸ Because of alternative splicing of CD45 mRNA results in the expression of different isoforms of CD45 glycoproteins on T cells, PB T-lymphocytes can be divided in two reciprocal subpopulations based on the molecular weight of the expressed isoforms: the CD45RA subpopulation, which has a molecular mass of 220 kD, and the CD45RO subpopulations, which has a molecular mass of 180 kD.^{29,30} CD45RA recognizes *naive* cells, whilst CD45RO recognizes *memory* cells within T lymphocytes. The CD45RO isoform gradually replaces the CD45RA isoform on the surface of the same T cells as a result of antigen exposure. In addition, a greater proliferative response to recall antigen is disclosed by *memory* CD45RO⁺ T cells.³¹⁻³⁴ The most striking feature displayed in both CD4⁺ and CD8⁺ HUCB lymphocytes was, as expected, their *naive* phenotype (CD45RA⁺CD45RO⁻) with respect to adult PB in which we reported a greater number of *memory* (CD45RA⁻CD45RO⁺) CD4⁺ lymphocytes; CD8⁺ cells were found to co-express CD45RA antigen. These observations further support the view that the immunological burden of the HUCB is in a relatively virgin status.

The CD3-FITC/CD16-PE and/or CD56-PE double staining allows to identify the *true* NK cells as CD3⁻CD16⁺ and/or CD56⁺ and a subset of T cell (CD3⁺CD16⁺ and/or CD56⁺) with cytotoxic activity.³⁵ CD3⁻CD16⁺ and/or CD56⁺ large granular lymphocytes are NK cells that do not express the CD3/T-cell receptor (TCR) complex or rearrange TCR or immunoglobulin genes. These cells can lyse some tumor and virus-infected cells, mediate acute rejection of bone marrow cell grafts and also contribute to GvHD, depending on the presence of CD4⁺ T cells.^{36,37} In the past the NK killing was termed non-class I MHC restricted because of the NK-mediated activity that was thought to be unrelated to the MHC haplotype of target cells. However, it is now clear that NK-mediated lysis is regulated by class I MHC molecules. Despite the fact that T cells recognize antigens such as peptide fragments bound to MHC molecules, NK cells become functional in the absence of class I proteins on target cells.^{38,39} In addition, T and NK cells are ontogenetically and functionally related, both being involved with cell-mediated cytolysis.⁴⁰⁻⁴² CD3⁺CD16⁺ and/or CD56⁺ T cells do express the CD3/TCR complex and rearrange TCR

genes. This T-cell subset is thought to be *in vivo* activated cytotoxic lymphocytes. In our hands, HUCB samples showed a significantly higher proportion and absolute number of *true* NK cells, while in adult PB a greater number of CD3⁺CD16⁺CD56⁺ cells was found.

In conclusion, although most of the HUCB recipients are children and the risk of GvHD is known to be low in recipients below 10 years of age, a reduced GvHD incidence in these transplants is recorded. Relevant differences were found in the immunophenotypic profile of HUCB with respect to adult PB lymphocytes. Whether these differences really account for a reduced capacity of transplanted cord blood cells to modulate GvHD remains to be determined by means of functional studies.

Contributions and Acknowledgments

GDA was the principal investigator, designed the study and was responsible for ethical approval, recruitment, day-to-day contact with participants, data handling and interpretation and the writing of the paper. He carried out the flow cytometric analyses. PM was responsible for the conception of the study with GDA, interpretation and the writing of the paper. GDG collaborated in the study design and provided samples of umbilical cord blood. NC designed the study and worked to flow cytometry. FZ collaborated in the study design and provided samples of umbilical cord blood. SF was responsible for data handling, statistical analysis and interpretation. MC formulated the study design and wrote the paper with GDA and PM.

All the authors contributed to the analysis and writing the paper. The order in which his/her names appear in the paper is related to the real contribution of each author, except for the last name (MC), that has been reserved to the Chief of our Department.

Disclosures

Conflict of interest: none.

Redundant publications: no substantial overlapping with previous papers.

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