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Intracellular cytokine profile of cord blood T-, and NK- cells and monocytes

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ABSTRACT

Background and Objectives. **Many functional peculiarities of cord blood (CB) lymphocytes and antigen presenting cells, including cytokine production, are associated with low intensity of innate and acquired (cellular and humoral) responses. These peculiarities may have implications both for immunologic maturation in post-natal life and for immune functions after CB transplantation [e.g. the lower incidence of graft-versus-host disease (GvHD) in comparison with after bone marrow transplantation (BMT)]. The aim of our study was to compare the intracellular production of cytokines involved both in phagocyte-dependent immunity/inflammation and in humoral immune responses in CB and adult peripheral blood (PB).**

Design and Methods. **Intracellular single cell analysis by flow cytometry was used to detect, following aspecific *in vitro* stimulation, the production of interferon (IFN)-γ, interleukin (IL)-2, IL-1α, IL-1β, IL-8, tumor necrosis factor (TNF)-α and -β, IL-4, IL-5, IL-6, IL-10 and IL-13 by T-cell subsets, NK-cells and monocytes obtained from 10 CB and 10 PB samples. The cytokine production was expressed as the percentage of positive cells.**

Results. **Significantly lower numbers of CD4+ T-cells producing IFN-γ (p<0.001), TNF-α (p=0.012) and TNF-β (p=0.03) and of CD8+ T-cells producing IFN-γ (p<0.001), IL-2 (p=0.005) and TNF-α (p<0.001) were found in CB as compared to PB. In CB we also found a lower number of NK cells and monocytes producing TNF-α (p<0.001 and p=0.001, respectively). In contrast, the number of IL-1α$^+$ monocytes was higher in CB than in PB (p=0.03).**

Interpretation and Conclusions. **Our data confirm that the cytokines which normally sustain the phagocytic-dependent T helper/cytotoxic 1-type immune responses (IFN-γ, IL-2 and TNF-α) and the NK-cell/monocyte-dependent aspecific responses (TNF-α) are reduced in CB. Since these cytokines are also involved in acute GvHD pathogenesis, these results are in keeping with the evidence of a low incidence of acute GvHD after CB transplantation.**
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Key words: intracellular cytokines, cord blood, T-cells, NK-cells, monocytes

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Cord blood (CB) has been used as a source of hematopoietic stem cells as an alternative to allogeneic bone marrow transplantation (BMT) in a variety of malignant and non-malignant hematologic disorders.[1-5] CB transplantation appears to be associated with a lower incidence of acute graft-versus-host disease (GvHD) as compared to BMT,[1-10] even if donor and recipient are HLA-mismatched.[1-6,8,10] This is likely due to a number of particular features of CB, including decreased reactivity of CB T-, B- and NK- cells,[11-25] impaired antigen presentation and cytokine production by CB antigen presenting cells (APC)[21,26-28] and NK-activity inhibition by soluble factors within placental serum.[24]

Intracellular single cell analysis by flow cytometry has been recently used to investigate the cytokine profile of T-cell subsets, thus contributing to a better definition of their functional patterns.[29,30] Several methodologic approaches have shown that CD4$^+$ and CD8$^+$ T-cells can be classified into three types according to their cytokine pattern: T-helper (Th)/cytotoxic (Tc) 1 (IFN-γ, IL-2 and TNF-producing), Th(c)2 (IL-4, IL-5, IL-6, IL10 and IL-13-producing) and Th(c)0 [Th(c)1- and Th(c)2-type cytokine-producing].[31-33] The different cytokine production is associated with the involvement of T-cells in different forms of immunity, i.e. phagocyte-dependent [Th(c)1] or phagocyte-independent [Th(c)2] immune responses.[31-33]

Recent data generated using the flow cytometry approach confirm defined peculiarities of CB immune cells, including the lower production of IL-2,[34,35] IFN-γ,[34-36] IL-4 and TNF-α[34] by CB lymphocytes in comparison to those in adult peripheral blood (PB). Conversely, the ability of monocytes to produce TNF-α and IL-6 appears to be similar in CB and adult PB.[35] Some data, however, are still controversial, i.e. IL-2 production by CB lymphocytes, which has been found to be normal[36] by some authors and reduced[34,35] by others. No extensive data are available on the intracellular cytokine production by NK-cells or monocytes, or about the production and possible significance of a number of other cytokines by T-cells.

In the present study we used flow cytometry to investigate the production of a broad panel of cytokines, including Th(c)1-type (IFN-γ, IL-2, TNF-α and TNF-β), Th(c)2-type (IL-4, IL-5, IL-6, IL-10 and IL-13) and inflammatory (IL-1α, IL-1β, IL-8) cytokines by CD4$^+$ and CD8+ T-cells, NK-cells and monocytes in 10 CB samples from full-term normal deliveries. Results were compared with those obtained in PB samples from 10 normal adults. We found some differences in cytokine production consistent with the lower incidence of unfavorable immune events after CB transplantation.

Design and Methods

Sample collection and cell culture

Samples were obtained, after informed consent, from the CB of 10 full-term normal deliveries and PB of 10 healthy adults, collected into preservative-free heparin. Mononuclear cells (MNCs) were aseptically separated by density gradient centrifugation (Lymphoprep, Nycomed Pharma, Oslo, Norway) and plated at 2x10^6 cell/mL concentration in RPMI-1640 culture medium (GIBCO Laboratories, Grand Island, NY, USA) containing 10% heat-inactivated fetal calf serum (FCS), 1% L-glutamine and antibiotics (culture wells were previously coated with FCS in order to reduce the monocyte adhesion after activation). We stimulated MNCs for 4 hours at 37°C, in 5% CO_2 with 30 ng/mL phorbol 12-myristate 13-acetate (PMA), 1 μg/mL ionomycin (both from Sigma Chemical Co, St. Louis, MO, USA) and 10 μg/mL brefeldin A (Sigma) to block cytoplasmic cytokine transport. MNCs were then collected, washed with 1% PBS solution and prepared at a final concentration of 10x10^6 cells/mL.

Intracellular cytokine detection

T-cell subsets were stained by Tricolor-conjugated anti-CD4 and -CD8 (Caltag, South S. Francisco, CA, USA) monoclonal antibodies (mAbs). The NK-cell population was identified by negativity for CD3 (R-PE-Cy5-CD3, Dako, Glostrup, Denmark) and expression of CD16 and CD56 [FITC-conjugated mAbs, both from Becton Dickinson (BD), S. José, CA, USA]. Monocytes were identified by physical parameters and by staining with anti-CD14 and anti-CD4 (R-PE-conjugated mAbs, BD). Permeabilization of cell membranes was obtained by FACS permeabilizing solution (BD, 0.5 mL/sample for 10 minutes, room temperature). After washing [1% PBS, 1% bovine BSA, 0.1% sodium azide], samples were first incubated for 15 minutes with mouse immunoglobulins (10 μg/10^6 cells, by Sigma) to prevent aspecificity, and then for 30 minutes at +4°C in the dark with conjugated anti-cytokine mAbs: FITC-conjugated anti-IFN-γ and R-PE-conjugated anti-IL-1α, -IL-1β, -IL-2, -IL-4, -IL-6, -IL-8, -IL-13, -TNF-α, all from BD; R-PE-conjugated anti-IL-5, -IL-10 and -TNF-α from Pharmingen, S. Diego, CA, USA. Specificity controls were performed using appropriate isotypic mAbs. After washing, immunophenotypic patterns were investigated by a flow cytometer equipped with an argon-ion laser (488 nm, FACScan, BD), using Cell Quest software. At least 50,000 events were acquired and positive cells were expressed as percentages among the different subsets (CD4$^+$ and CD8$^{bright+}$ T-cells, NK-cells and monocytes).

Table 1. Flow cytometric detection of intracellular cytokines in cord blood and adult peripheral blood T cell subsets.

	Cord blood (n=10)		*Adult PB (n=10)*		*stats (M-W U test)*
CD4+ T cells	*%*	*median*	*%*	*median*	
IFN-γ+	0.8±0.4	0.8	16.4±8.2	14.5	p<0.001
IL-2+	13.6±10.8	11.5	15.9±5.5	16.6	p=n.s.
TNF-α+	19.1±19.6	10.4	43.8±8.9	43.3	p=0.012
TNF-β+	1.4±1.9	0.5	2.9±2.7	2.1	p=0.03
IL-4+	1.2±1.0	1.2	0.9±0.4	1	p=n.s.
IL-5+	<1		<1		p=n.s.
IL-6+	<1		<1		p=n.s.
IL-10+	<1		<1		p=n.s.
IL-13+	<1		<1		p=n.s.
IL-1-α+	<1		<1		p=n.s.
IL-1-β+	<1		<1		p=n.s.
IL-8+	6.5±6.8	3	1.2±0.8	0.9	p=n.s.
CD8+ T cells	*%*	*median*	*%*	*median*	
IFN-γ+	5.0±5.6	2.4	41.4±16.4	38.1	p<0.001
IL-2+	1.6±1.7	0.8	5.1±2.7	3.9	p=0.005
TNF-α+	5.2±5.5	2.9	31.0±17.1	28.8	p<0.001
TNF-β+	1.7±0.7	1.8	2.2±1.6	1.9	p=n.s.
IL-4+	1.9±2.0	1.9	1.0±0.9	0.6	p=n.s.
IL-5+	<1		<1		p=n.s.
IL-6+	<1		<1		p=n.s.
IL-10+	<1		<1		p=n.s.
IL-13+	<1		<1		p=n.s.
IL-1-α+	<1		<1		p=n.s.
IL-1-β+	<1		<1		p=n.s.
IL-8+	<1		<1		p=n.s.

Note: the positive cells were expressed as percentages among the different subsets; a percentage <1% was considered as negative.

Statistics

Statistical evaluation of data from intracellular cytokine detection in CB and PB mononuclear cells was performed by the Mann-Whitney U-test. Differences were considered as statistically significant when the *p*-value was <0.05.

Results

MNC subpopulations

The percentages of MNC subpopulations collected after stimulation *in vitro*, which we studied for intracellular cytokine production, were as follows: CD4$^+$ T-cells 40.5±8.7% and 50.7±13.1% in CB and adult blood, respectively; CD8$^{bright+}$ T-cells 19.8±1.2% and 19.6±3.6%; NK-cells 14.2±4.6% and 7.0±4.2%; monocytes 22.1±3.9% and 15.1±7.2%.

Intracellular cytokines

We found a statistically significant difference in percentages of CD4+/IFN-γ$^+$ (0.8±0.4%, median 0.8% in CB; 16.4±8.2%, median 14.5% in PB, *p*<0.001), CD4$^+$/TNF-β$^+$ (19.1±19.6%, median 10.4% in CB; 43.8±8.9%, median 43.3% in PB, *p*=0.012), CD4$^+$/TNF-α$^+$ (1.4±1.9%, median 0.5% in CB; 2.9± 2.7%, median 2.1% in PB, *p*=0.03), CD8$^+$/IFN-γ$^+$ (5.0±5.6%, median 2.4% in CB; 41.4±16.4%, median 38.1% in PB, *p*<0.001), CD8$^+$/IL-2$^+$ (1.6±1.7%, median 0.8% in CB; 5.1±2.7%, median 3.9% in PB, *p*=0.005) and CD8$^+$/TNF-α$^+$ (5.2±5.5%, median 2.9% in CB; 31.0±17.1%, median 28.8% in PB, p<0.001). The percentages of CD4$^+$/IL-2$^+$ and CD8$^+$/TNF-β$^+$ were equivalent in the two groups. We did not find any difference in percentages of Th(c)2-type cytokine (IL-4, IL-5, IL-6, IL-10 and IL-13)-producing cells. No significant production of inflammatory cytokines was observed. Likewise, IL-8 production by CD4$^+$ T-cells,

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Table 2. Flow cytometric detection of intracellular cytokines in cord blood and adult peripheral blood NK-cells and monocytes.

	Cord blood (n=10)		*Adult PB (n=10)*		*stats (M-W U test)*
NK cells	*%*	*median*	*%*	*median*	
IFN-γ+	10.6±8.9	11	16.6±8.9	15	p=n.s.
IL-2+	<1		<1		p=n.s.
TNF-α+	5.1±5.2	3.7	23.6±10.3	21.6	p<0.001
TNF-β+	3.0±3.3	1.3	1.3±0.8	1	p=n.s.
IL-4+	<1		<1		p=n.s.
IL-5+	<1		<1		p=n.s.
IL-6+	<1		<1		p=n.s.
IL-10+	<1		<1		p=n.s.
IL-13+	<1		<1		p=n.s.
IL-1-α+	<1		<1		p=n.s.
IL-1-β+	<1		<1		p=n.s.
IL-8+	<1		<1		p=n.s.
Monocytes	*%*	*median*	*%*	*median*	
IFN-γ+	<1		<1		p=n.s.
IL-2+	<1		<1		p=n.s.
TNF-α+	3.0±3.7	2	9.0±5.6	7.6	p=0.001
TNF-β+	<1		<1		p=n.s.
IL-4+	<1		<1		p=n.s.
IL-5+	<1		<1		p=n.s.
IL-6+	11.1±12.3	7.4	5.5±5.1	4.2	p=n.s.
IL-10+	1.6±1.0	1.2	0.7±0.3	0.6	p=n.s.
IL-13+	<1		<1		p=n.s.
IL-1-α+	28.8±24.0	25.2	10.9±13.3	3.3	p=0.03
IL-1-β+	36.9±25.3	36.4	44.1±18.9	42.4	p=n.s.
IL-8+	33.8±25.4	34.3	36.7±18.5	31.4	p=n.s.

Note: the positive cells were expressed as percentages among the different subsets; a percentage <1% was considered as negative.

although higher in CB than in PB (6.5±6.8%, median 3%, vs 1.2±0.8%, median 0.9%, respectively) was not significantly increased (*p*=0.15) (Table 1).

The NK-cell population was characterized by a similar production of IFN-γ (10.6±8.9%, median 11% in CB and 16.6±8.9%, median 15% in PB, *p*=n.s.) and TNF-β (3.0±3.3%, median 1.3% in CB; 1.3±0.8%, median 1% in PB, *p*=n.s.). In contrast, TNF-α production by NK-cells was significantly lower in CB (5.1±5.2%, median 3.7% in CB; 23.6±10.3%, median 21.6% in PB, *p*<0.001). No detectable levels of the other cytokines were observed in the NK-cell population (Table 2).

We found in CB and PB similar percentages of monocytes producing IL-1β (36.9±25.3%, median 36.4% in CB; 44.1±18.9%, median 42.4% in PB, *p*=n.s.), IL-6 (11.1±12.3%, median 7.4% in CB; 5.5±5.1%, median 4.2% in PB, *p*=n.s.), IL-8 (33.8±25.4%, median 34.3% in CB; 36.7±18.5%, median 31.4% in PB, *p*=n.s.), and IL-10 (1.6±1.0%, median 1.2% in CB; 0.7±0.3%, median 0.6% in PB, *p*=n.s.). By contrast, TNF-α production was significantly lower in CB than in PB monocytes (3.0±3.7%, median 2% in CB; 9.0±5.6%, median 7.6% in PB, *p*=0.001). A statistically significant increase of IL-1α$^+$ monocytes was observed in CB (28.8±24.0%, median 25.2% in CB; 10.9±13.3%, median 3.3% in PB, *p*=0.03). No detectable levels of the other cytokines were observed in monocytes (Table 2).

Discussion

In this study we used flow cytometry to analyze the cytokine pattern of CB and adult PB T-cell subsets, NK-cells and monocytes. The main purpose of our study was to evaluate in these cells the expression of cytokines involved in both aspecific inflammatory reactions (IL-1α, IL-1β, IL-8) and acquired Th(c)1-type (IFN-γ, IL-2, TNF-α, TNF-β) and Th(c)2-type (IL-4, IL-5, IL-6, IL-10, IL-13) immune responses. We found in CB: i) defective production of some Th(c)1-type cytokines (IFN-γ, TNF-α and TNF-β by CD4$^+$ T-cells and IFN-γ, IL-2 and TNF-α by CD8$^+$ T-cells; IL-2, by contrast, was similarly expressed in CB and adult PB CD4$^+$ T-cells); ii) no significant differences in the Th(c)2-type cytokine production; iii) a peculiar pattern of pro-inflammatory cytokines (lower TNF-α production by NK-cells and monocytes and higher IL-1α production by monocytes); iv) a trend, although not statistically significant, towards a higher number of IL-8-producing CD4$^+$ T-cells.

The low expression of Th(c)1-type cytokines we found in CB CD4$^+$ and CD8$^+$ T-cells is in keeping with previous reports[15,19-24,34-36] and confirms that this kind of immune response, which is prevalent in adult PB, is less efficient in CB. We previously demonstrated that a progressive maturation of the Th(c)1 pattern occurs along with aging, having observed that the percentages of circulating IFN-γ$^+$ and IL-2$^+$ CD4$^+$ and CD8$^+$ T-cells progressively increase in post-natal life.[37] In accordance with other authors,[36] we showed detectable levels of IL-2 in both CB and adult PB CD4$^+$ T-cells. Since IL-2 is the main immune response-initiating cytokine,[31-33] we can suppose that other defects in Th(c)1-type cell polarization (leading to IFN-γ, TNF-α and TNF-β production) could be present in CB T-cells before the antigen priming, independently of IL-2. This phenomenon does not appear to be related to a Th(c)2-type shift. In fact, we did not find, at variance with other authors,[34] a significant production of Th(c)2-type cytokines, despite the stronger stimulus used (PMA 30 versus 5 ng/mL), thus suggesting the non-constitutive expression of these cytokines in normal conditions. Other factors, such as weak activation of CB T-cells by APCs and cells involved in aspecific inflammatory reactions, could be associated with the defect of Th(c)1-polarization. Also in partial disagreement with other studies,[35] we observed significantly lower TNF-α production by both monocytes and NK-cells. The deficiency of this cytokine, which contributes to the induction of Th(c)1-responses[31-33] appears to be a specific feature of CB. The percentages of CB cells producing other cytokines (IL-6, IL-10, IL-1β and IL-8 for monocytes; IFN-γ and TNF-β for NK-cells) did not significantly differ from those in PB. Only IL-1α$^+$ monocytes are increased in CB, possibly suggesting a substitutive mechanism for the TNF-α defect. In fact, IL-1α usually remains in the cytosol after translation and plays a role as an intracellular autocrine molecule regulating normal cellular differentiation.[38] In addition, the

trend, although not statistically significant, towards a higher production of IL-8 by CB CD4+ T-cells appears of interest. IL-8 has a role not only in the aspecific inflammatory response,[39] but also in various maturation patterns, including myeloid and monocytic differentiation.[40-42]

Taken together, our data further support the evidence for a low reactivity of CB T-, NK-cells and monocytes, in terms of cytokine production, after aspecific stimulation. This appears an interesting issue as far as BMT and CB transplantation are concerned. The cytokine cascade, triggered by tissue damage induced by conditioning treatment, plays a role in the development of acute GvHD. The effect of pro-inflammatory molecules (such as IL-1 and TNF-α) released by APCs, in the context of foreign histocompatibility tissue antigen recognition by alloreactive donor T-cells, induces a dysregulation of the T-cell cytokine network leading to predominance of the Th(c)1-type responses.[43-45] This Th(c)1 polarization is known to be a primary cause of induction and maintenance of the typical tissue damage of acute GvHD. In fact, under stimulation by Th(c)1-type cytokines, NK-cells and monocytes/histiocytes become the main effectors of tissue damage.[46,47] Thus, the defective TNF-α production by CB monocytes and NK-cells and the absence of a Th(c)1-shifting may be biologically relevant in CB transplantation. The low incidence of acute GvHD after CB transplantation could reflect a partial immaturity of both the afferent (APC and T-cell-mediated) and the efferent (NK-cell and monocyte-mediated) phases of GvHD.

Contributions and Acknowledgments

MK designed the study and, together with LT, analyzed the samples, prepared the manuscript and managed the statistical data; FB, GN and GP were involved in critically revising the intellectual content of the manuscript; GP also gave the final approval for its submission. The order of the names reflects the importance and the contribution of each author to the work. The authors thank Mariangela Fornalè for her collaboration in the collection of samples, Carlo Vincenzi for his technical assistance and Fabrizio Vinante for his fruitful suggestions.

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Disclosures

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Potential implications for clinical practice

- Our data further support the evidence of a low reactivity of cord blood T-, NK- and monocytic cells, in terms of cytokine production, after aspecific stimulation. These findings are in keeping with the low incidence of acute GvHD following cord blood transplantation. These features could be the basis for a biological modulation of GvHD effector cells after bone marrow transplantation by inhibiting cytokines.