# Comparative cytokine production by in vitro stimulated mononucleated cells from cord blood and adult blood
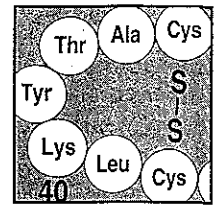
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## Abstract

Cord blood transplantations successfully reconstituted hemopoiesis in patients treated with myeloablative therapies. These transplantations were associated with a low rate of acute graft-versus-host disease (aGVHD), a major life-threatening complication of allo-transplantation. The physiopathology of aGVHD implies the recognition of host alloantigens by donor T cells but also involves a cytokine cascade. In this cascade, interleukin (IL)-1, IL-2, IL-6, tumor necrosis factor $\alpha$ (TNF-$\alpha$), and interferon $\gamma$ (IFN-$\gamma$) produced by donor T cells and monocytes/macrophages play a major effector role. Therefore, we investigated whether the lower percentage of aGVHD in cord blood transplants could be related to a lower ability to produce these cytokines in vitro compared with adult blood. Mononucleated cells (MNCs) isolated from term cord blood and adult peripheral blood were stimulated with a combination of lipopolysaccharide and phytohemaglutinin and incubated for 96 hours. Levels of IL-1$\beta$, IL-2, IL-3, IL-4, IL-6, TNF-$\alpha$, IFN-$\gamma$, and granulocyte-macrophage colony-stimulating factor (GM-CSF) were measured in the supernatants after various times of incubation. The productions of IL-1$\beta$, IL-6, and GM-CSF were similar in stimulated cord and adult blood and IL-3 levels, though lower and delayed in cord blood, were not statistically different. On the other hand, we found markedly lower levels of IFN-$\gamma$, TNF-$\alpha$, and IL-4 in cord blood throughout the incubation period. The stimulated levels of IL-2 were similar in cord and adult samples throughout the first 48 hours of incubation but became significantly lower in cord blood after 72 and 96 hours. We suggest that the cytokine production pattern that characterizes cord blood could provide an explanation for the lower occurence of aGVHD following cord blood transplants.

## Introduction

Primitive hemopoietic cells in cord blood from neonatal siblings or unrelated donors have the capacity to reconstitute hematopoiesis in patients treated with myeloablative therapies for genetic inherited diseases [1,2] or malignancies [3]. These transplantations were associated with a low incidence of aGVHD [4], though probably partly because they were mainly performed in children. As aGVHD is one of the most serious allo transplant-related complications, this feature of cord blood transplants could, therefore, be very important. The physiopathology of aGVHD implies the recognition of host allo-antigens by donor mature T cells. Several risk factors have been described, including older donors and recipients, sex mismatch, infections, and diagnosis. Most of the clinical manifestations, however, could be secondary to an inappropriate production of cytokines [5,6]. Several cytokines like IL-1 [7–9], IL-2 [5,6], IL-6 [7,8,10,11], TNF-$\alpha$ [8,10,12,13,14], and IFN-$\gamma$ [10,12,15] have been recognized as involved in the cytokine cascade that leads to aGVHD. Therefore, we investigated whether the lower clinical expression of aGVHD following cord blood transplantations could be related to a lower in vitro production of cytokines by cord blood cells. We used separated MNCs stimulated with a combination of lipopolysaccharide (LPS) and phytohemaglutinin (PHA). After various incubation periods, we measured the levels of IL-1, IL-2, IL-3, IL-4, IL-6, TNF-$\alpha$, IFN-$\gamma$, and GM-CSF in the supernatants of stimulated and unstimulated cord MNCs. These results were compared with those obtained with adult MNCs studied according to the same protocol.

## Materials and methods

### Samples

Cord blood samples from term babies were collected shortly after delivery through the umbilical vein into bags containing citrate-phosphate-dextrose A (Baxter Laboratories, Irvine, CA) as anticoagulant. Adult blood samples were collected from six healthy adult volunteers aged 25 to 58. Informed consent was obtained before blood collection from the adult volunteers and from the mothers. The investigations were approved by the Ethical Committee of the University of Liège. All samples were kept at room temperature before processing. The average delay between collection and incubation was 4 hours and never exceeded 8 hours.

For the first 11 cord and all adult blood samples the production of cytokines was measured both in 1:10 diluted whole blood and in separated MNCs. From cord blood sample #12, only separated MNCs were used.

Whole blood was diluted 1:10 in RPMI 1640 (Gibco BRL, Gaithersburg, MD) supplemented with L-glutamine (2 mM), penicillin (100 U/mL), and streptomycin (100 μg/mL).

MNCs were isolated from whole blood diluted 1:2 in PBS (Gibco BRL) using Ficoll-Paque (Pharmacia LKB, Uppsala, Sweden). MNCs were then washed twice in 5% autologous plasma-PBS before being resuspended in RPMI 1640 supplemented with L-glutamine (2 mM), penicillin (100 U/mL), streptomycin (100 µg/mL), and autologous plasma (5% v/v). The MNC concentration was adjusted to be equivalent to that of the corresponding 1:10 diluted whole blood and was ranging from 230 to 2100/µL with a mean value of 710/µL. From cord blood sample #12, the MNC concentration was adjusted to 400/µL. The white cell counts were obtained using a Technicon H1 cell counter (Tarrytown, NJ).

### Cell cultures

Cell suspensions were distributed (2 mL/well) into 24-well plates (Falcon, Bedford, MA). A combination of LPS (from Salmonella enteridis, Sigma, St. Louis, MO), 25 µg/mL final concentration and PHA (HA16, Wellcome Diagnostic, Dartford, UK), 5 µg/mL final concentration was added as a polyclonal activator to half of the wells. The remaining ones served as controls.

Plates were incubated at 37°C in 8% $CO_2$ in air. At each time point (0, 4, 24, 48, 72, and 96 hours of incubation), the contents of two test and two control wells per point were centrifugated at 800$g$ for 10 minutes. Supernatants were recovered and stored at –20°C until cytokine assays.

### Cytokine assays

Concentrations of IL-1β, IL-2, IL-3, IL-4, IL-6, GM-CSF, IFN-γ, and TNF-α were measured in the supernatants using specific ELISA assays (Medgenix Diagnostics, Fleurus, Belgium) according to the manufacturer's instructions. Minimal detectable levels of IL-1β, IL-2, IL-3, IL-4, IL-6, GM-CSF, IFN-γ, and TNF-α were 2 pg/mL, 0.1 IU/mL, 5 pg/mL, 2 pg/mL, 3 pg/mL, 3 pg/mL, 0.03 IU/mL, and 3 pg/mL, respectively. Intra-assays and inter-assays variabilities are below 5.7% and 9.9%, respectively. Results obtained per mL were divided by the MNC concentration and expressed per $1 \times 10^5$ MNCs.

### Statistical analysis

Mann-Whitney U test was used to compare cord vs. adult blood production of cytokines by stimulated or unstimulated MNCs. This analysis was performed using MINITAB statistical software (MINITAB, State College, PA). $p$ values <0.05 were considered significant.

## Results

In preliminary experiments we demonstrated that the production of cytokines from separated MNCs from cord and adult blood was always at least equal to that from 1:10 diluted whole blood and that their kinetics were very similar (data not shown). Therefore, the results presented here are those obtained from MNCs only.

Following stimulation with LPS + PHA, a rapid increase in the production of IL-1β, IL-6, and GM-CSF from cord and adult MNCs was observed (Fig. 1). Plateau levels were reached within 24 hours of stimulation and overall were maintained up to 96 hours. There was no appreciable difference in the production of these three cytokines in unstimulated and stimulated cord vs. adult blood.

The levels of TNF-α were not different between cord and adult unstimulated MNCs (data not shown). The production of TNF-α by stimulated cord MNCs, however, was significantly lower than by adult MNCs at all time points (Fig. 2). In both cord and adult, the main increase occurred during the first 24 hours and was followed by a plateau.

Baseline IFN-γ levels in cord blood were significantly lower than in adult blood (Median [range], 0.10 [0.02–0.21] vs. 0.44 [0.06–0.60] U/$10^5$ MNCs; $p$ = 0.0077) and no further production was evidenced in unstimulated samples. Maximum production of IFN-γ was obtained after 72 hours of stimulation in both cord and adult samples. Figure 3 shows that regardless of the duration of the stimulation, there was significantly less IFN-γ produced by cord than by adult MNCs.

IL-4 production by unstimulated cord and adult MNCs could not be detected with our ELISA test at any incubation time. Stimulated cord and adult cells produced detectable amounts of IL-4 after 24 hours and up to 96 hours, maximum titers being obtained after 48 hours. IL-4 production by cord MNCs was significantly lower compared with adult MNCs (Fig. 4).

In common with IL-4, IL-3 production by unstimulated cord and adult MNCs was undetectable. After stimulation, 24 hours were needed to detect IL-3 in adult blood and 48 hours in cord blood. Despite this delayed production in cord blood, we could not find any significant difference between IL-3 titers in stimulated cord and adult MNCs.

Compared to baseline levels, no further production of IL-2 was detectable in unstimulated cultures. Levels of IL-2 in the supernatant of stimulated cord and adult MNCs (Fig. 5) were not significantly different after 24 and 48 hours of incubation but, after 72 and 96 hours, cord blood levels dropped significantly, while those from adult blood continued to increase. Therefore, the difference between cord and adult samples became highly significant.

## Discussion

The kinetics of production of IL-1β and IL-6 as shown in Figure 1 are consistent with those described elsewhere [16–19]. As previously reported with a bioassay [20,21], we demonstrate with a specific ELISA method that IL-1β production is similar in cord and adult stimulated MNCs. Together with previous studies by Dinarello et al. [22] and Wilmott et al. [23], we assert that preterm and term cord blood MNCs are as capable as adult MNCs of producing IL-1β upon appropriate stimulation.

In our study, IL-6 production by LPS + PHA stimulated MNCs from cord and adult blood is not significantly different. Similar results were found by Pahwa et al. [3], who compared the levels of IL-6 production by adult and cord MNCs or T cells after stimulation with PHA + PMA, as well as by Yachie et al. [18], who used a whole blood culture technique with stimulation with ConA or LPS.

Constitutive titers of GM-CSF in cord and adult samples were close to our minimally detectable levels and remained unchanged with time. With or without stimulation, we could not find any difference in the production of GM-CSF between cord and adult MNCs. Stimulating cord whole blood or T cells with PHA + PMA, Pahwa et al. [3] also measured similar levels of production as compared with adults. Laver et al. [24] meas-
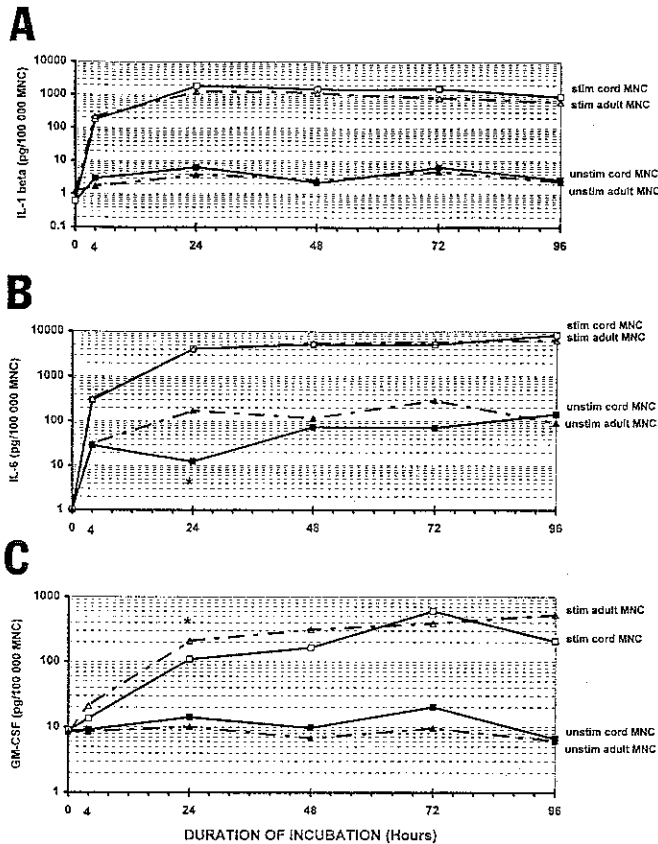
**Fig. 1.** Median production of IL-1β **(A)**, IL-6 **(B)** and GM-CSF **(C)** in stimulated (stim) and unstimulated (unstim) cord and adult MNCs after different incubation times.
* = $p < 0.05$ for cord vs. adult samples.



**Fig. 2.** Median production of TNF-α by stimulated (stim) cord vs. adult MNCs after 24, 48, 72, and 96 hours of incubation.

ured higher plasma GM-CSF titers in term neonates than in adults but their bioassay was not absolutely specific for GM-CSF. On the other hand, Cairo et al. [25] reported a lower GM-CSF production by PHA + PMA stimulated cord blood MNCs after 72 hours of incubation. They attributed this to a reduced stability of GM-CSF mRNA. In that study, after 72 hours of incubation, adult levels were similar to ours (400 pg/$10^5$ MNCs) while cord blood titers were 10 times lower (57 pg/$10^5$ MNCs). This could be due to a lower PHA concentration (2 µg/mL) compared with ours (5 µg/mL) and Pahwa's (20 µg/mL), suggesting that there could be a threshold of PHA to stimulate efficiently cord blood MNCs.

Like IL-1β and IL-6, TNF-α is known to reach high levels within 24 hours of stimulation [16,17,26]. It is mainly produced by monocytes and T cells [26] stimulated with LPS and the addition of PHA to LPS does not affect its production [16]. We found significantly lower levels of TNF-α in the supernatants of stimulated cord compared with adult MNCs. These findings support those by Weatherstone et al. [20] and English et al. [26], who reported respectively a lower production of TNF-α by LPS stimulated monocytes from preterm neonates and by PMA- or PHA-stimulated cord MNCs from term neonates compared with adults. English also mentioned a high subject to subject variability for both adult and cord samples as regards to TNF-α production.
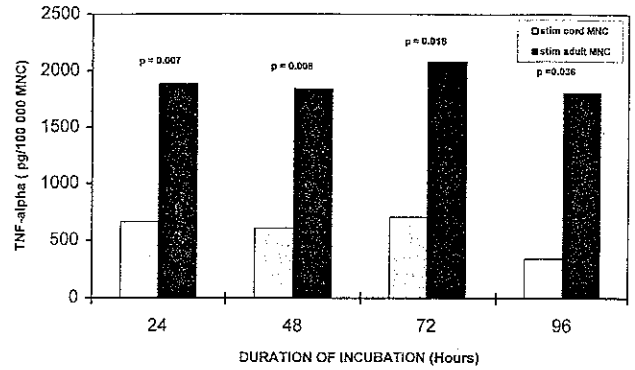
IFN-γ is a cytokine mainly produced by T cells through interactions with macrophages. As described previously [27–33], we report a lower production of IFN-γ by stimulated cord blood MNCs compared with adult MNCs. Taylor and Bryson [29] hypothesized that this defect was mainly because of functionally immature macrophages in cord blood, while Lewis et al. [34] found that it was secondary to a pretranslational defect intrinsic to T cells and was not linked to macrophages. English et al. [26] also reported lower levels of IFN-γ mRNA in stimulated cord blood. According to Wakasugi et al. [35], this impaired IFN-γ production could be a result of an increased cell sensitivity to the suppressive effects of PGE-2, and not to a defect in IL-2 production. This is consistent with our results demonstrating impaired IFN-γ production from the initiation of the culture period despite there being no defect in IL-2 production at the early time points. Finally, Kruse et al. [31] suggested recently that the absence of CD4+/CD45RO+ memory T cells could be partly responsible for the lower IFN-γ production.

We report undetectable levels of IL-4 in the supernatant of unstimulated cord and adult MNCs and a significantly lower production in stimulated cord than in adult MNCs. These results are consistent with those obtained by ELISA or bioassay after stimulation of cord MNCs by various procedures [27,32,36], as well as with the report of Andersson et al. [28],
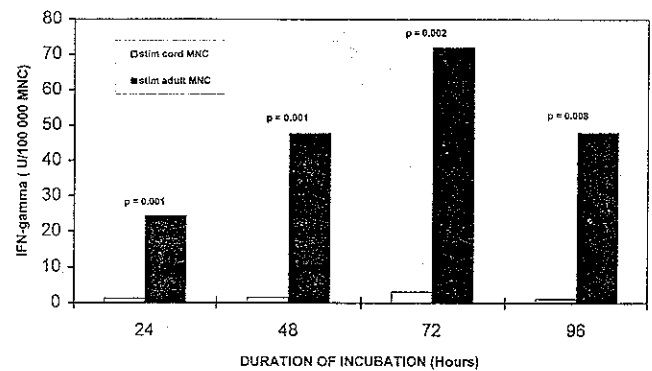


**Fig. 3.** Median production of IFN-γ by stimulated (stim) cord vs. adult MNCs after 24, 48, 72, and 96 hours of incubation.
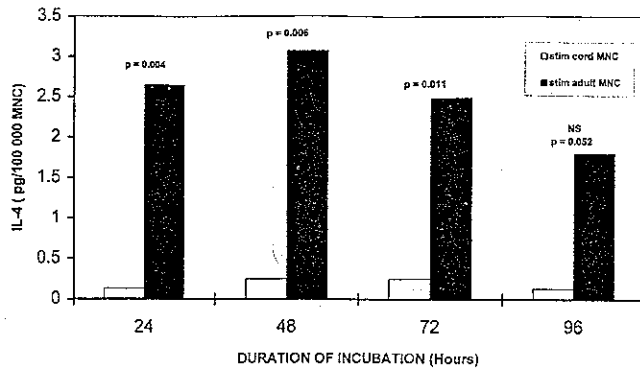
**Fig. 4.** Median production of IL-4 by stimulated (stim) cord vs. adult MNCs after 24, 48, 72, and 96 hours of incubation.
NS = nonsignificant.

who could not detect IL-4 producing cells among activated cord blood MNCs. The impaired IL-4 production could be a result of lower numbers of activated T cells in cord blood compared with adult blood [37].

We could not detect IL-3 in the supernatants of unstimulated MNCs and did not find any significant difference between stimulated production by cord and adult MNCs. However, IL-3 production tended to be decreased and delayed in cord blood. Other results in the literature are controversial. Cairo et al. [38] reported undetectable levels of IL-3 in unstimulated MNCs but also found lower IL-3 production and mRNA expression in stimulated cord compared with adult MNCs. Pahwa et al. [3] reported opposite results, with the supernatants of PMA + PHA stimulated cord MNCs producing twice as much IL-3 than adult MNCs after 48 hours of culture.

IL-2 production by stimulated cord blood cells has been described as lower [32,36], similar [28,39,40,41], or higher [27] than by stimulated adult cells. The higher levels obtained by Pastorelli et al. [27] could be explained by the use of different stimulators for only 48 hours and the different specificity of their bioassay. Our results have shown similar IL-2 production by cord and adult MNCs during the first 48 hours of stimula-
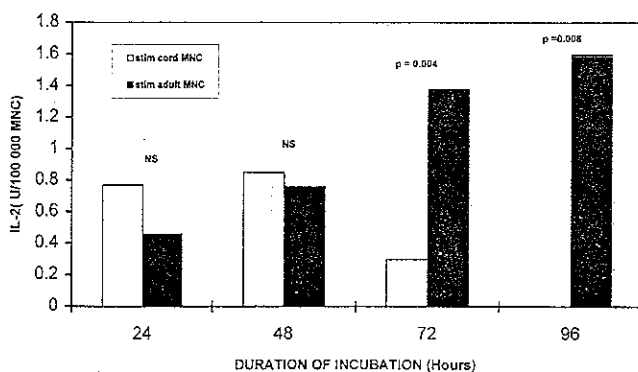


**Fig. 5.** Median production of IL-2 by stimulated (stim) cord vs. adult MNCs after 24, 48, 72, and 96 hours of incubation.
NS = nonsignificant.

tion. Afterwards, while IL-2 supernatant levels of adult MNCs continued to increase, IL-2 titers in cord blood decreased after 72 and 96 hours of stimulation. This is consistent with the results obtained by Kruse et al. [31] after 72 hours of incubation with Staphylococcus enterotoxin B. In other experiments, they reported maximum IL-2 levels after 2 or 3 days followed by a decrease at day 4 in both cord and adult samples [32]. The apparent late decrease of IL-2 production we observed could also be caused by an increased consumption of IL-2 by stimulated cord blood cells after 48 hours. It is clearly not caused by lower IL-1 levels, as they are similar to the adult ones throughout the stimulation period.

Taken together, our results describe different cytokine production patterns in cord and adult blood. Stimulated cord blood is characterized by lower levels of TNF-α, IFN-γ, IL-4, IL-2, and delayed IL-3 production, while that of IL-1β, IL-6, and GM-CSF is similar. Variations in the composition of cord and adult blood populations can be evocated to explain the differences observed. Similarly, it is also possible that Ficoll-Paque isolation of MNCs did not recover exactly the same populations in cord and adult blood. Also, as previously suggested for IL-2, the higher progenitor cell concentration in cord blood could account for some cytokine consumption and, therefore, lower levels at late time points as compared with adult blood. The involvement of TNF-α and IFN-γ in the physiopathology of aGVHD has been described in a skin explant model [12]. Other authors found increased levels of TNF-α in serum [7,10,13], or of TNF-α mRNA [8] in MNCs of patients with aGVHD. Higher serum levels of IFN-γ before and during aGVHD were also described [10,15]. The role of IL-1 has been evidenced in animal models [6] and in MNCs from patients with aGVHD, increased amounts of IL-1β and IL-1β mRNA were measured [7,8]. IL-2 plasma levels [10], as well as IL-2 mRNA in peripheral blood MNCs [8], were not found increased in patients with aGVHD. However, experimental data [6] supported an early role of IL-2 in aGVHD, probably through the stimulation of the production of other cytokines such as IL-1 and TNF-α. A role for IL-6 in the cytokine cascade has been implied [8,11] but remains controversial [7,42], while IL-4 seems to be mainly associated with chronic GVHD [6,43,44]. On the other hand, GM-CSF could have a favorable impact by reducing the effect of IL-1 through potentiation of an IL-1 receptor antagonist production [5]. Antin and Ferrara [5], in their schematic representation of the role of cytokines in aGVHD, proposed that the production of IFN-γ and IL-2 by donor T cells and that of IL-1 and TNF-α by donor MNCs play a central role. Therefore, we suggest that the reduced clinical manifestations of aGVHD observed after cord blood transplantation may be associated with a lower ability to produce IL-2, TNF-α, and IFN-γ and an equivalent production of GM-CSF despite an equivalent production of IL-1β and IL-6 compared with adult blood MNCs.

## Acknowledgments

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