

Phenotypic changes of T-lymphocyte subsets induced by interleukin-12 and interleukin-15 in umbilical cord vs. adult peripheral blood mononuclear cells

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The decreased incidence of graft-vs.-host disease found following umbilical cord blood (CB) transplantation, and the increased susceptibility of newborns to infections, have been attributed, in part, to functional and phenotypic immaturity of neonatal T cells. We investigated the phenotypic changes of CB T cells induced by two immunoregulatory cytokines, interleukin (IL)-12 and IL-15, alone or in combination. Adult peripheral blood (APB) mononuclear cells (MNCs) were also tested for comparison. Prior to culture, the percentages of CD3⁺ CD8⁺, CD3⁺ CD25⁺, and CD3⁺ CD56⁺ cells were significantly lower in CB MNCs than in APB MNCs. IL-15, but not IL-12, significantly increased CD3⁺ CD8⁺ expression among the CB MNCs after 1 week of culture. Combining IL-12 and IL-15, however, resulted in decreased CB CD3⁺ CD8⁺ expression compared with IL-15 alone. The percentage of CD3⁺ CD25⁺ cells in CB MNCs spontaneously increased in the absence of cytokines, while that of CD3⁺ CD56⁺ cells in CB MNCs could not be enhanced with cytokines. In contrast, the percentages of CD3⁺ CD25⁺ and CD3⁺ CD56⁺ cells among the APB MNCs could be increased with IL-12, IL-15, and further with IL-12 and IL-15 combined. Thus, different patterns of T-cell subset changes were demonstrated between CB MNCs and APB MNCs in response to IL-12 and/or IL-15. These data may serve as a foundation for using cytokine therapy in newborns and children receiving CB transplants.

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Because of its high composition of hematopoietic stem cells, umbilical cord blood (CB) has been increasingly used as an alternative to bone marrow for transplants in children with immunological and hematological disorders (1,2). The reduced incidence and severity of graft-vs.-host disease (GvHD) in patients transplanted with CB compared with bone marrow has been attributed to the reduced immunological response of CB T lymphocytes (3–5). CB mononuclear cells (MNCs) have a reduced capacity to produce cytokines, particularly interleukin (IL)-2 and interferon- γ , and a decreased cytotoxic response following allogeneic stimulation, as compared to adult peripheral blood (APB) MNCs (6,7). These

decreased immune responses may, in part, explain the increased susceptibility of newborns to infection. Expansion and activation of CB cells by cytokines might greatly benefit newborns at risk for severe infection and patients undergoing stem cell transplantation, by increasing resistance to viral infections and providing a graft-vs.-leukemia (GvL) effect through enhancement of cytotoxic T-lymphocyte function and natural killer (NK) activity (8,9).

IL-12 is a 75-kDa heterodimeric protein that is secreted by macrophages, monocytes and dendritic cells, and which stimulates NK and T cells (10). Another recently cloned cytokine, IL-15, also plays an important role in enhancing various

lymphocyte functions, including T and NK cytotoxicity (11,12). IL-15, mainly produced by macrophages and monocytes, uses the β - and γ -chains of the IL-2 receptor, and a unique α -chain, for binding and signal transduction. The level of mRNA expression and production of both IL-12 and IL-15 have been shown to be decreased in neonatal MNCs compared with adult MNCs (13,14). The role of IL-12 and IL-15 in enhancing various NK cytolytic functions and CD16⁺ CD56⁺ expression in CB and peripheral blood MNCs from human immunodeficiency virus (HIV)-infected subjects has been previously demonstrated (15–17). Previous studies have also suggested that a combination of low-dose IL-12 and IL-15 may augment NK activity and minimize toxicity (18).

The CD25 antigen, α -chain of the IL-2 α receptor, was expressed at lower levels in neonatal T cells than in adult T cells (19). The un-responsiveness of cord cells in the autologous mixed lymphocyte reaction has been correlated with a lack of up-regulation of CD25 (20). Thus, increased CD25 expression by cytokines could be used to assess T-cell immune responses of CB lymphocytes. CD3⁺ CD56⁺ (NK-T) cells, comprising a rare subset of peripheral blood lymphocytes (<5%) in normal individuals (21), are extra-thymically differentiated T cells with potent non-major histocompatibility complex (MHC)-restricted cytolytic capability, which might provide protection against viral infection, including human immunodeficiency virus (HIV) (22).

The aim of this study was to investigate the effect of long-term culture with IL-12 and IL-15, alone or in combination, on the expression of CD3⁺ CD4⁺ (helper T cells), CD3⁺ CD8⁺ (suppressor T cells), CD3⁺ CD25⁺ (activated T cells), and CD3⁺ CD56⁺ (non-MHC-restricted killer cells) of CB MNCs, using two-color flow cytometry. We sought to determine whether different activation patterns, in response to cytokines, existed between CB and APB MNCs.

Materials and methods

Preparation of MNCs and culture with cytokines

MNCs were obtained from normal adult volunteers and from term newborn umbilical cords following normal vaginal deliveries, according to guidelines established by the Human Subjects Protection Committee of Chang Gung University. CB was collected in sterile tubes and processed within 24 h of birth. MNCs were separated from heparinized blood using Ficoll-Hypaque density-gradient centrifugation. The

isolated cells were resuspended at 1×10^6 /ml in RPMI-1640 (Irvine Scientific, Santa Ana, CA, USA) supplemented with 10% heat-inactivated fetal calf serum (FCS). MNCs were then placed in 25-cm² tissue culture flasks with no cytokines or with the recombinant cytokines IL-15 (10 ng/ml) and IL-12 (1 ng/ml) (both from Pepro Tech EC, London, UK), individually or in combination, for 18 h (overnight), or for 7 days, at 37°C in 5% CO₂. The IL-15 and IL-12 concentrations were chosen according to a previous study which reported that incubating CB MNCs with IL-15 at 1, 10, and 100 ng/ml produced a dose-dependent increase in NK activity with a maximum effect that plateaued at 100 ng/ml (15). IL-12 at 1 ng/ml was comparable to the peak serum levels that could be achieved by *in vivo* administration of IL-12 in patients with advanced renal cell carcinoma (23). Because of the short half-life of the cytokines, they were added on alternate days to the 7-day cultures.

Flow cytometric analysis

CB and APB MNCs were washed in cold phosphate-buffered saline (PBS) with 2% FCS and 0.1% sodium azide, and then stained with fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-conjugated mouse anti-human monoclonal antibodies. The following FITC/PE paired antibodies: anti-CD3/CD4, anti-CD3/8, anti-CD3/25, and anti-CD3/CD56 (Becton-Dickinson Worldwide Inc, Taiwan branch, Taiwan), were used in two-color flow cytometric analyses. The fluorescence was analyzed by using a fluorescence-activated cell sorter (FACS Calibur; Becton-Dickinson) flow cytometer. Electronic gates were set to enable analysis of the fluorescence of the lymphocyte or monocyte in each preparation. The percentage of cells staining with each monoclonal antibody was determined by comparing each histogram with one from control cells stained with FITC- or PE-labeled anti- γ -1 or - γ -2 (isotype control) monoclonal antibodies.

Statistics

The Student's *t*-test was used to compare the reported means with standard deviation (SD) for the different variables; *n* refers to the number of experiments performed. Groups being compared were considered significantly different if the *p*-value was <0.05. The statistical calculations were performed using StatView 4.0 (Abacus Inc., CA, USA).

Table 1. Percentages and absolute numbers of mononuclear cells with various T-cell (CD3⁺) subsets expressing CD4, CD8, CD25, and CD56 surface markers prior to culture

Surface markers	Adult	Cord
CD3/CD4	40.5±5.7 0.45±0.13	28.8±11.5* 0.97±0.45*
CD3/CD8	27.7±7.8 0.31±0.12	12.4±4.5** 0.43±0.23
CD3/CD25	8.1±3.7 0.07±0.01	2.1±1.7* 0.08±0.01
CD3/CD56	5.6±2.8 0.06±0.03	0.6±0.5** 0.02±0.02

For each surface marker the first row of values represents the percentage of double-positive cells, shown as mean ± SD. The second row of values represents the absolute lymphocyte numbers (×10³ cells/μl).

*Significant effect (p <0.05); **highly significant effect (p <0.005), compared with the adult counterpart. The age effect was determined by p-values calculated according to the Student's *t*-test.

Results

Comparison of T-cell subsets in CB and APB MNCs prior to culture

Table 1 compares the percentages and absolute numbers of cord and adult MNCs exhibiting various T-cell subset surface markers prior to culture. Because the number of CB MNCs far exceeded that of APB MNCs ($3.4 \pm 0.8 \times 10^3$ cells/μl vs. $1.1 \pm 0.2 \times 10^3$ cells/μl, $p < 0.01$), the absolute numbers of CD3⁺ CD4⁺ and CD3⁺ CD8⁺ cells are increased in CB compared to APB, despite the lower percentages of marker expression in CB MNCs. The percentage of CD3⁺ CD8⁺ cells in CB MNCs was significantly lower than that in APB MNCs ($12.4 \pm 4.5\%$ vs. $27.7 \pm 7.8\%$, $p < 0.005$). The percentages of CD3⁺ CD25⁺ and CD3⁺ CD56⁺ cells were significantly lower in fresh CB MNCs than in APB MNCs.

Cell recovery and viability after 1 week of culture

Incubation for 18 h with IL-15 and/or IL-12 did not influence cell recovery and viability in either CB or APB MNCs (data not shown). The recovery of cells after 1 week of culture, with or without cytokines, is shown in Table 2. IL-15 significantly increased cell recovery in both CB and APB MNCs ($p < 0.05$, compared with unstimulated cells), with a much greater effect seen in CB MNCs (>2-fold increase compared with media alone). IL-12, however, did not increase

the cell yield as compared to cultures with no cytokines (Media), and the combination of IL-12+IL-15 resulted in decreased cell recovery when compared with IL-15 alone. The viability of CB and APB MNCs after 1 week of culture was 90–95% for all culture conditions, as determined by trypan blue exclusion.

Phenotypic changes after 1 week of culture with IL-12 and IL-15

The percentage of CD3⁺ CD4⁺-expressing cells in both CB and APB MNCs did not change significantly after 7 days of culture with IL-12 (1 ng/ml) or IL-15 (10 ng/ml), but did increase in CB MNCs cultured with IL-12+IL-15 ($51.8 \pm 21.3\%$ vs. $40.4 \pm 16.2\%$ with media alone, $p = 0.012$, Fig. 1a). The level of CD3⁺ CD8⁺ expression in CB MNCs, but not in APB MNCs, was significantly increased after 7 days of culture with IL-15 ($33.1 \pm 6.1\%$ vs. $13.3 \pm 3.1\%$ with media alone, $p < 0.01$, Fig. 1b). Seven-day culture with IL-12 (1 ng/ml) alone did not significantly increase the percentage of CD3⁺ CD8⁺ cells among either CB or APB MNCs. The combination of IL-12+IL-15, however, resulted in much lower level of CD3⁺ CD8⁺ expression on CB MNCs ($8.6 \pm 3.4\%$) than that seen with IL-15 alone ($33.1 \pm 6.1\%$). Similarly, the percentage of CD3⁺ CD8⁺ cells among the APB MNCs was decreased after 1 week of incubation with IL-12+IL-15 (Fig. 1b).

Table 2. Effect of 1 week of cytokine incubation on the recovery† of mononuclear cells (MNCs) from cord blood (CB) and adult peripheral blood (APB)

Subjects	Cytokines added			
	Media alone	IL-12 (1 ng/ml)	IL-15 (10 ng/ml)	IL-12+IL-15
CB MNCs	64±18‡	72±22	140±32*	102±19*
APB MNCs	60±12	74±22	95±34*	73±24

†% Recovery = (cell count of day 7 ÷ cell count of day 1)×100.

‡Data are expressed as mean ± SD, obtained from 15 cords and 9 adult blood samples.

* $p < 0.05$, compared with media alone, determined by using the Student's *t*-test.

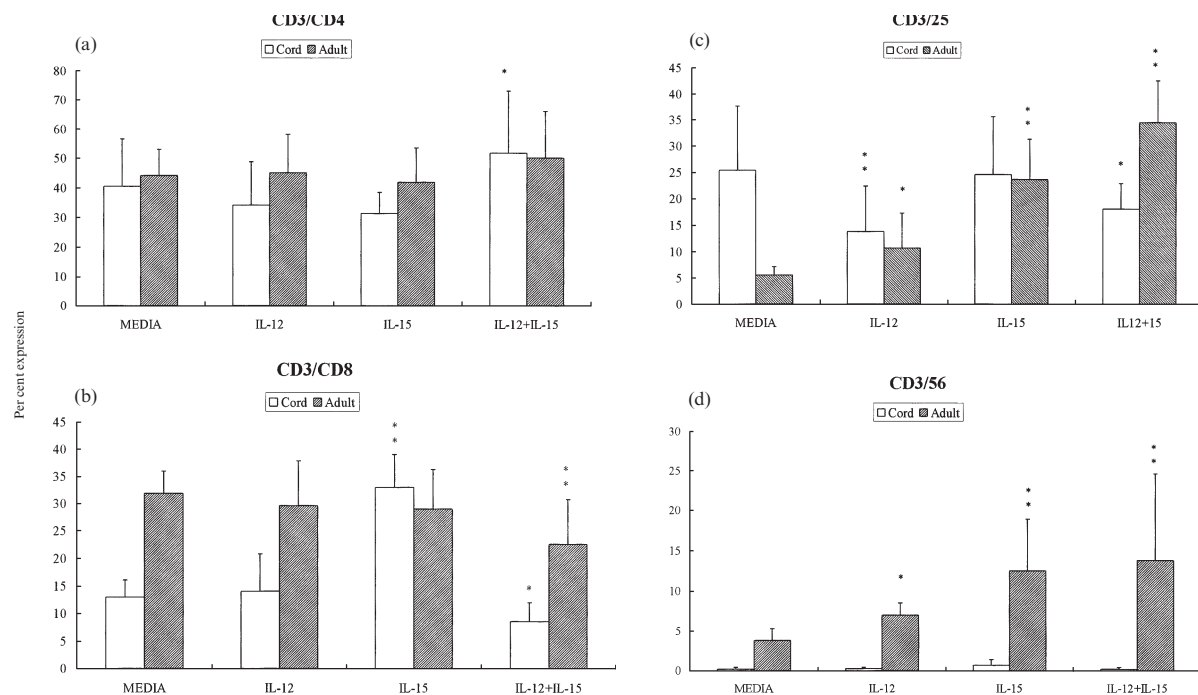


Fig. 1. Effect of 1 week of culture with interleukin (IL)-12 (1 ng/ml) and IL-15 (10 ng/ml) on lymphocyte subsets of umbilical cord blood mononuclear cells (MNCs) (Cord, n=13) and adult peripheral blood MNCs (Adult, n=8) defined by double-positive staining of the following monoclonal antibodies using flow cytometry: (a) CD3/CD4; (b) CD3/CD8; (c) CD3/CD25; (d) CD3/CD56. *Significant effect ($p < 0.05$); **highly significant effect ($p < 0.01$), compared with media, calculated by using the Student's *t*-test.

The percentage of CD3⁺CD25⁺ cells in CB MNCs increased significantly after 1 week of culture, even in the absence of cytokines ($25.4 \pm 12.3\%$, Fig. 1c), and was not increased further on addition of IL-15 ($24.6 \pm 11\%$). In contrast, IL-12 decreased the expression of CB CD3⁺CD25⁺ when it was added alone ($13.8 \pm 8.6\%$) or in combination with IL-15 ($18.1 \pm 4.8\%$). APB CD3⁺CD25⁺ expression, however, remained low in the absence of cytokines ($5.6 \pm 1.6\%$), but could be enhanced with IL-12 ($10.7 \pm 6.6\%$), IL-15 ($23.7 \pm 7.6\%$), and further with IL-12+IL-15 combined ($34.5 \pm 8.0\%$). CD3⁺CD56⁺ expression on APB MNCs could be increased by IL-12 ($7.0 \pm 1.5\%$), IL-15 ($12.5 \pm 6.4\%$), and further with IL-12+IL-15 combined ($13.8 \pm 10.8\%$). In contrast, the addition of IL-12, IL-15, or both, had no effect on CB CD3⁺CD56⁺ expression, which remained very low after 1 week of culture.

Discussion

Immunological and functional differences between CB and APB MNCs have been well characterized (24–27). Several studies have suggested that CB T cells represent an immature transitional T cell by showing that expression of CD38 (a marker of activation and immaturity)

and CD45RA (an un-primed, naive T-cell marker) were higher in CB T cells than in APB T cells (9,26). In this work it was found that CB MNCs contained a significantly lower percentage of CD8⁺ cytotoxic T cells than APB MNCs, confirming previous reports (26,27). This study further indicated that the expression both of CD3⁺CD25⁺ and CD3⁺CD56⁺ were lower in CB MNCs than in APB MNCs, contributing to the phenotypic immaturity of CB T cells.

IL-15, but not IL-12, preferentially increased the expression of CD3⁺CD8⁺ on CB MNCs without affecting CD3⁺CD4⁺ expression; this was not observed with APB MNCs. Combining IL-12 and IL-15, however, resulted in decreased CD3⁺CD8⁺ expression compared with IL-15 alone, an effect more evident on CB MNCs than on APB MNCs (Fig. 1b). Our results indicate that CB cytotoxic T cells are particularly responsive to stimulation with IL-15, while IL-12 had little or even an inhibitory effect on CB CD3⁺CD8⁺ expression in long-term (7-day) cultures. The combination of IL-12+IL-15 also suppressed cell recovery as compared with IL-15 alone (Table 2), confirming the anti-proliferative nature and apoptosis-inducing ability of IL-12 (28,29). Similar results have been observed with CB CD16⁺CD56⁺ expression in previous studies (15).

Cytotoxic (CD8⁺) T lymphocytes are important in the host defense against a variety of intracellular infections, in allograft rejection, and in tumor cell surveillance. Expansion of CD8⁺ T cells in CB MNCs has been shown to contribute to the lymphokine-activated killer (LAK) activity that is responsible for the GVL effect that prolonged disease-free survival in children receiving a CB transplant (8). Our results suggest IL-15 may be superior to IL-12 in generating LAK and GVL activity by its ability to up-regulate the originally deficient CD3⁺ CD8⁺ expression on CB MNCs.

The decreased CD3/25 expression found in newborns may reflect a lack of prior stimulation. In the present study, the level of CD3/CD25 expression on CB MNCs readily increased in the absence of cytokines, possibly responding to FCS present in the culture media. IL-12, alone or in combination with IL-15, appeared to inhibit CB CD3⁺ CD25⁺ expression. In fact, the CB CD3⁺ CD25⁺ population was greatly increased with IL-12 after 7 days, as compared to media alone (data not shown). Thus, IL-12 might preferentially activate CD25 expression on CB NK cells. CD3⁺ CD25⁺ expression on APB MNCs could be increased by IL-12 and IL-15 alone, and further with IL-12+IL-15, suggesting an additive effect of IL-12 and IL-15 on T-cell activation.

Musha et al. indicated that CB NK-T (CD3⁺ CD56⁺) cells could be expanded when cultured with IL-2 (100 U/ml) for 14 days (30). However, we found that CB CD3⁺ CD56⁺-expressing cells were not expanded after 1 week of culture with IL-12 and/or IL-15; this may have been because of the shorter duration of culture in our study. In contrast, CD3⁺ CD56⁺ expression on APB MNCs responded well to cytokines, particularly to IL-15 and IL-12+IL-15. Warzynski et al. reported two children with acquired immune deficiency syndrome (AIDS) who had an unusually large percentage of CD3⁺ CD56⁺ lymphocytes. They experienced no severe opportunistic infections even though they had possessed almost no CD4 cells for 3 years (31). Therefore, the deficient CD3⁺ CD56⁺ expression on CB MNCs and the lack of response to IL-12 and/or IL-15 might contribute to the morbidity of HIV-infected newborns and infants.

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