

Interleukin-15 enhances CD4⁺ CD45RA⁺ expression on umbilical cord blood mononuclear cells

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The reduced incidence of graft-vs.-host disease following umbilical cord blood (CB) transplantation may be related to the functional immaturity of newborn T cells expressing mainly the naive CD45RA phenotype. Expansion of CD4⁺ CD45RA⁺ T cells using cytokines may benefit neonates and infants with human immunodeficiency virus (HIV) infection, as a preferential decline in CD4⁺ CD45RA⁺ cells has been noted as HIV disease progresses. The aim of the study was to investigate the effect of interleukin (IL)-15, a novel cytokine similar to IL-2 in biological activities, on CD45RA/RO expression and apoptosis in umbilical cord blood (CB) and adult peripheral blood (APB) mononuclear cells (MNCs). Prior to culture, CB MNCs contained a greater number of CD4⁺ CD45RA⁺ cells and fewer CD4⁺ CD45RO⁺ cells than did APB MNCs. When incubated with RPMI-1640 containing 10% fetal calf serum for 7 days, the percentage of CD45RA⁺ cells within CD4⁺ T cells (%CD45RA⁺/CD4⁺) significantly decreased compared to that of fresh CB MNCs. IL-15 exerted a dose-dependent increase of %CD45RA⁺/CD4⁺ and a corresponding decrease of %CD45RO⁺/CD4⁺ in CB MNCs, an effect not observed with APB MNCs treated with IL-15. The percentages of CD45RA⁺ and CD45RO⁺ expression within CD8⁺ cells, however, were not influenced by IL-15, in either CB or APB MNCs. A greater number of CB MNCs underwent apoptosis than did APB MNCs after 7 days of culture in RPMI-1640 containing 10% fetal calf serum. IL-15 did not inhibit apoptosis but induced proliferation comparable to that achieved in APB MNCs. The ability of IL-15 to preferentially enhance the proliferation of CD4⁺ CD45RA⁺ cells in CB MNCs suggests a role for immunomodulative therapy in HIV-infected newborns and infants.

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The reduced incidence and severity of graft-vs.-host disease (GvHD) in patients transplanted with umbilical cord blood (CB) compared to bone marrow has been attributed to the functional immaturity of CB T lymphocytes (1,2). The expression of CD45 isoforms in human T cells distinguishes naïve (CD45RA⁺) from memory (CD45RO⁺) T cells (3). The majority of CB T cells (80–90%) express CD45RA antigens, which would convert to CD45RO phenotype upon antigen stimulation and acquire memory-cell characteristics, such as helper activity for B cells and production of interleukin (IL)-4 and interferon- γ (IFN- γ) (4).

IL-15, a recently cloned cytokine that is mainly produced by macrophages, uses the β and γ chains of the IL-2 receptor and a unique α chain for binding and signal transduction (5). IL-15 has several biological functions, similar to those of IL-2, that play an important role in enhancing various lymphocyte functions, including T- and natural killer (NK)-cell cytotoxicity (5,6). Recent studies also suggest that IL-15 could induce CB stem cells to differentiate into NK cells (7), making *in vivo* administration or *in vitro* expansion of lymphoid cells using IL-15 a feasible means to hasten recovery following stem cell transplant. We have previously demonstrated the

ability of IL-15 to enhance various NK cytolytic functions and CD16/56 expression in CB MNCs and MNCs from human immunodeficiency virus (HIV)-infected subjects (8,9). IL-15 has also been shown to enhance T-cell proliferative ability in response to mitogens and recall antigens (2,6), and inhibits apoptosis of neutrophils (10) and NK cells (11).

Maintenance of a naive T-cell repertoire, as found in CB using cytokines, may benefit newborn infants who are immunodeficient for various reasons, including HIV infection. A preferential decrease in the number of naive CD4⁺ T cells was noted as HIV disease progressed (12), and was considered important in the pathogenesis of terminal HIV infection, according to a cohort of 347 HIV-infected subjects (13). In the present study, we investigated the effect of IL-15 on CD45RA/RO expression of both CB and adult peripheral blood (APB) mononuclear cells (MNCs). Using propidium iodide (PI) staining under flow cytometry, we also sought to determine whether different apoptosis patterns existed between CB and APB MNCs, and their response to IL-15 stimulation.

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 the Chang Gung University. CB samples were collected in sterile tubes and were processed within 24 h of birth. MNCs were separated from heparinized blood using Ficoll-Hypaque (Amersham, Pharmacia, Biotech AB, Uppsala, Sweden) density-gradient centrifugation. The isolated cells were resuspended at a concentration of 1×10^6 /ml in RPMI-1640 (Irvine Scientific, Santa Ana, CA, USA) supplemented with 10% heat-inactivated fetal calf serum (FCS) (Gibco BRL, Gaithersburg, MD, USA). MNCs were then placed in 25-cm² tissue culture flasks with no cytokines, or with recombinant IL-15 (Pepro Tech EC, London, UK) at 1 and 10 ng/ml, for 7 days at 37°C in an atmosphere containing 5% CO₂. Because of the short half-life of IL-15, the cytokine was added every other day to the 7-day cultures.

Determination of CD45RO/RA expression

CB and APB MNCs (both fresh and cultured) were washed in cold phosphate-buffered saline (PBS) containing 2% FCS and 0.1% sodium azide

and then stained with fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-conjugated mouse anti-human monoclonal antibodies (mAbs). Ten microlitres of the appropriate fluorescent reagent was incubated with 5×10^5 cells for 20–30 min at 4°C in the dark. The antibodies used were anti-CD4 (FITC or PE), anti-CD8 (FITC or PE), anti-CD45RA (FITC), and anti-CD45RO (PE) (Becton-Dickinson, Immunocytometry Systems, San Jose, CA, USA). The cells were then washed twice with buffer and analyzed on a fluorescence-activated cell sorter (FACS Calibur; Becton-Dickinson) to determine the CD45RA/RO expression on CD4⁺ or CD8⁺ cells. Electronic gates were set to enable analysis of the fluorescence of the lymphocytes or monocytes in each preparation. The percentage of cells staining with each mAb was determined by comparing each histogram with one from cells stained with fluorescein-labeled anti- γ_1 (for CD4, CD8, and CD45RA) and anti- γ_{2a} (for CD45RO) mAbs (isotype-matched controls).

Analysis of viability and apoptosis of cultured cells

For quantitation of apoptosis, DNA staining with PI and flow cytometric analysis were performed as described previously (12). Briefly, 1×10^6 cells were pelleted at 200 *g* and gently resuspended in 2 ml of ice-cold 70% ethanol/30% PBS, and stored for at least 6 h at 4°C. Cells were then harvested and resuspended in a 300- μ l solution of 40 μ g/ml PI (Sigma, St Louis, MO, USA) and 100 μ g/ml of RNase (New England Biolabs, Beverly, MA, USA) in PBS, then incubated at room temperature for 30 min. Samples were then applied to a FACS Calibur (Becton-Dickinson) for analysis. The percentage of apoptotic cells was calculated according to the following equation:

$$\% \text{ Apoptotic cells} =$$

$$\frac{(\% \text{ of cells with subdiploid DNA content} \times 100)}{(\% \text{ of all cells positive for PI staining})}$$

Positive controls of 5×10^5 /ml Jurkat cells (American-Type Culture Collection [ATCC], Rockville, MD, USA), incubated at 37°C for 18–24 h with 200 ng/ml of anti-human Fas (Upstate Biotechnology Inc., Waltham, MA, USA), were also included.

Statistics

Paired and unpaired Student's *t*-tests were used to compare the reported means with standard deviation (SD) for the different variables. 'n' refers to the number of experiments performed.

Table 1. Percentages and absolute numbers of mononuclear cells with various T-cell subsets expressing CD45RA/RO prior to culture

Surface markers	Cord	Adult
% CD45RA in CD4 ⁺	85.0±10.0**	46.0±8.00
CD4 ⁺ CD45RA ⁺ count	0.82±0.37**	0.21±0.12
% CD45RO in CD4 ⁺	32.0±9.00**	56.0±16.0
CD4 ⁺ CD45RO ⁺ count	0.19±0.12**	0.28±0.15
% CD45RA in CD8 ⁺	90.0±3.00**	83.0±5.00
CD8 ⁺ CD45RA ⁺ count	0.37±0.12*	0.31±0.05
% CD45RO in CD8 ⁺	17.0±4.00*	23.0±8.00
CD8 ⁺ CD45RO ⁺ count	0.07±0.05	0.08±0.02

Data are shown as mean ± SD from 12 cords and eight adults. For each surface marker the first row of values are percentages of CD45RA⁺ or CD45RO⁺ cells in CD4⁺ or CD8⁺ cells. The second row of values are absolute lymphocyte numbers (cells/μl) of double-positive staining cells.

*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 by using the Student's *t*-test.

Groups being compared were considered significantly different if the p-value was less than 0.05. The statistical calculations were performed using SigmaPlot (SPSS Science, Chicago, IL, USA).

Results

CD45RA/RO expression in CD4⁺ and CD8⁺ cells in CB and APB MNCs prior to culture

Table 1 compares the percentages of CD45RA/RO expression in CD4⁺ and CD8⁺ cells, and the absolute numbers of CD4⁺ CD45RA⁺, CD4⁺ - CD45RO⁺, CD8⁺ CD45RA⁺, and CD8⁺ - CD45RO⁺ cells, in freshly isolated CB and APB MNCs. The percentage of CD45RA-expressing cells within CD4⁺ cells (%CD45RA⁺/CD4⁺) and the absolute numbers of CD4⁺ CD45RA⁺ cells in CB MNCs, far exceeded those of APB MNCs (p<0.001), while the reverse was true with regard to %CD45RO⁺/CD4⁺ and the numbers of CD4⁺ CD45RO⁺ cells (p<0.001). The majority of CD8⁺ cells expressed CD45RA instead of CD45RO, in CB and APB MNCs alike. Higher percentages of CD45RA⁺ cells (p=0.008) and lower percentages of CD45RO⁺ cells (p<0.001) were found within CB CD8⁺ cells, compared to those within APB CD8⁺ cells.

IL-15 restored the decreased %CD45RA⁺/CD4⁺ in cultured CB MNCs

As shown in Fig. 1(a) and Fig. 1(b), %CD45RA⁺/CD4⁺ cells decreased from a mean of 85% (SD 10%) among freshly isolated CB MNCs (n=12) to 48% (SD 19%) among cells cultured with media (RPMI-1640 +10% FCS) for 7 days (p<0.001), while the %CD45RO⁺/CD4⁺ of CB MNCs increased from 32% (SD 9%) to 49% (SD 15%) (p<0.001). In contrast, there were no significant changes in the percentages of CD45RA and CD45RO expression on APB CD4⁺ T cells before and after culture (p=0.81, and p=0.88, respectively; Fig. 1c, Fig. 1d). Incubating CB MNCs with IL-15 for 7 days resulted in a dose-dependent increase in %CD45RA⁺/CD4⁺ cells, from 48% (SD 19%) (media only) to 57% (SD 14%) with IL-15 at 1 ng/ml (p=0.02), and to 76% (SD 16%) with IL-15 at 10 ng/ml (p<0.001). A reciprocal decrease in %CD45RO⁺/CD4⁺ was also seen with CB MNCs (Fig. 1b, p=0.005 for IL-15 at 1 ng/ml and p<0.001 for IL-15 at 10 ng/ml, compared to media only). However, IL-15 had little effect on %CD45RA⁺/CD4⁺ of APB MNCs (Fig. 1c, p=0.34 for IL-15 at 1 ng/ml and p=0.25 for IL-15 at 10 ng/ml, compared to media only). The percentage expression of CD45RA/RO within CD8⁺ lymphocytes did not alter when CB MNCs or APB MNCs were cul-

Table 2. Effect of interleukin-15 (IL-15) on apoptosis and proliferation of cord blood (CB) and adult peripheral blood (APB) mononuclear cells (MNCs) using propidium iodide staining under flow cytometry

	CB MNCs (n=12)		APB MNCs (n=7)	
	Media	IL-15	Media	IL-15
% Apoptotic cells (M2)	9.3±3.7*	8.6±2.7*	3.9±1.9	3.70±2.1
% Proliferating cells (M3)	2.1±1.7	9.8±4.2†	1.7±1.2	10.5±3.6†

Data are expressed as mean ± SD

*p<0.001, compared to the corresponding APB MNCs.

†p<0.001, compared to media.

IL-15 was used at a concentration of 10 ng/ml.

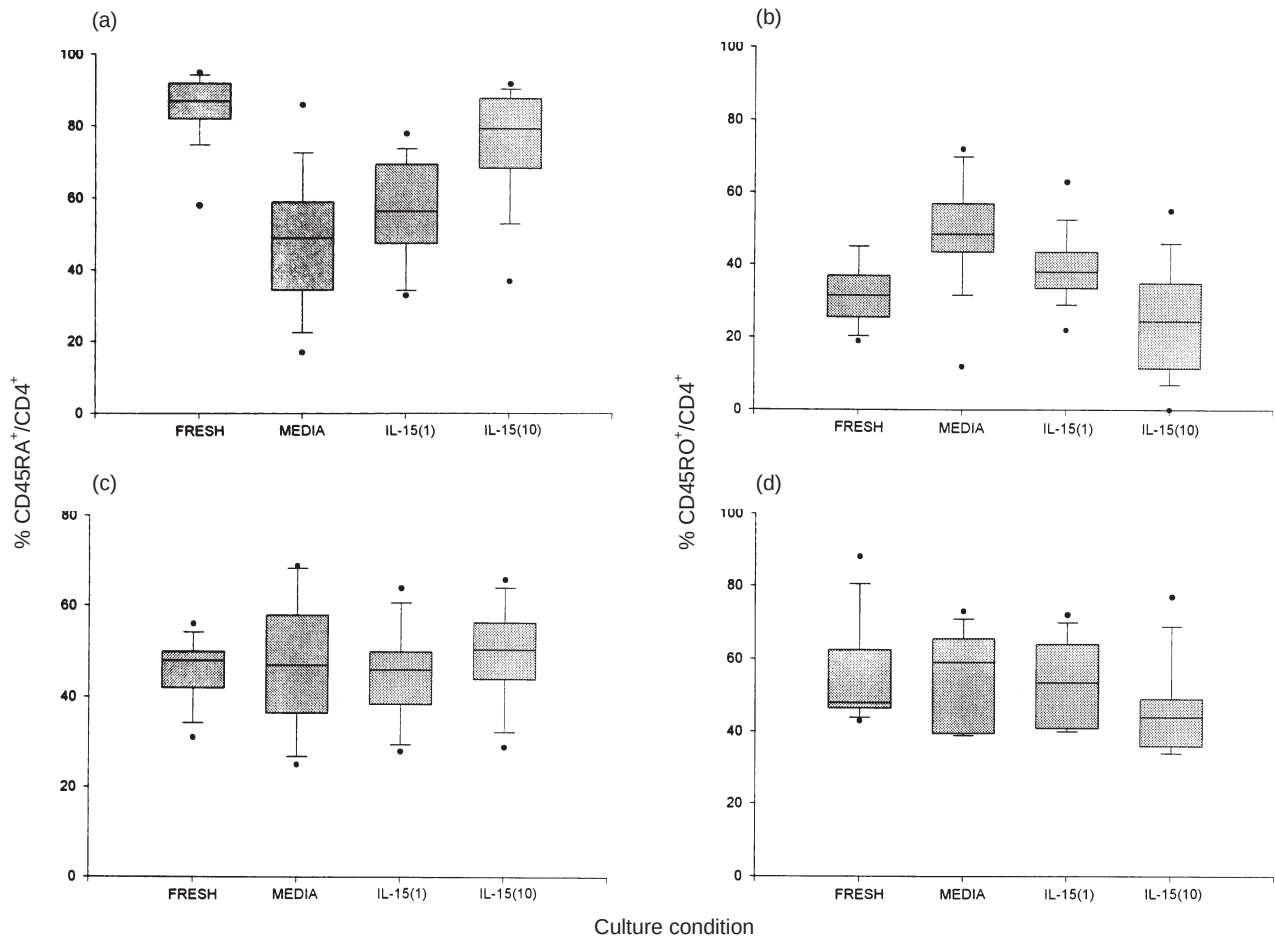


Fig. 1. Box-and-Whisker plot showing the effect of 7 days of culture with medium, in the presence or absence of interleukin-15 (IL-15), on the percentage of CD45RA/RO expression within CD4⁺ lymphocytes from umbilical cord blood (CB) mononuclear cells (MNCs) (n=12) and adult peripheral blood (APB) MNCs (n=8). (a) Percentage of CD45RA in CB CD4⁺ cells; (b) percentage of CD45RO in CB CD4⁺ cells; (c) percentage of CD45RA in APB CD4⁺ cells; (d) percentage of CD45RO in APB CD4⁺ cells. FRESH, freshly isolated cells; MEDIA, RPMI-1640 + 10% fetal calf serum (FCS) only; IL-15 (1); IL-15 at 1 ng/ml; IL-15 (10), IL-15 at 10 ng/ml. The horizontal line in the box represents the median, the lower and upper quartiles (25% and 75%). The 10th and 90th percentiles are used as the whiskers, with values outside this range plotted individually.

tured with media for 7 days, nor was it affected by addition of IL-15 (data not shown).

Effect of IL-15 on apoptosis and proliferation in CB and APB MNCs

We next examined the effect of IL-15 on apoptosis and proliferation in CB and APB MNCs using PI staining under flow cytometry (Table 2). When the cells were cultured with media (RPMI-1640 + 10% FCS) for 7 days, a greater number of CB MNCs underwent apoptosis (mean $\pm$ SD: $9.3 \pm 3.7\%$, n=12, M2 fraction, Fig. 2c) than did corresponding APB MNCs cultured under identical conditions ($3.9 \pm 1.9\%$, $p < 0.001$, compared with CB MNCs). After addition of IL-15 (10 ng/ml) for 7 days, the percentage of apoptotic cells in CB and APB MNCs did not alter significantly ($p = 0.48$ and $p = 0.98$, respectively, compared to media only).

However, the addition of IL-15 greatly increased the proportion of proliferating cells (cells in the M3 fraction, Fig. 2d) in CB MNCs, from $2.1 \pm 1.7\%$ to $9.8 \pm 4.2\%$ ($p < 0.001$), comparable to that achieved with APB MNCs treated similarly ($10.5 \pm 3.6\%$).

Discussion

The study of CB T-cell function and its enhancement with cytokines is becoming increasingly important. Transplant-related mortality owing to infectious complications after CB transplantation may be a result of the functional immaturity of lymphocytes transplanted with CB. In addition, the identification of factors that maintain a naive T-cell repertoire may be important, as some studies have indicated that CD4⁺ CD45RA⁺ cells from HIV-1-infected neonates and children are relatively resistant to HIV infection (14,15). *Ex*

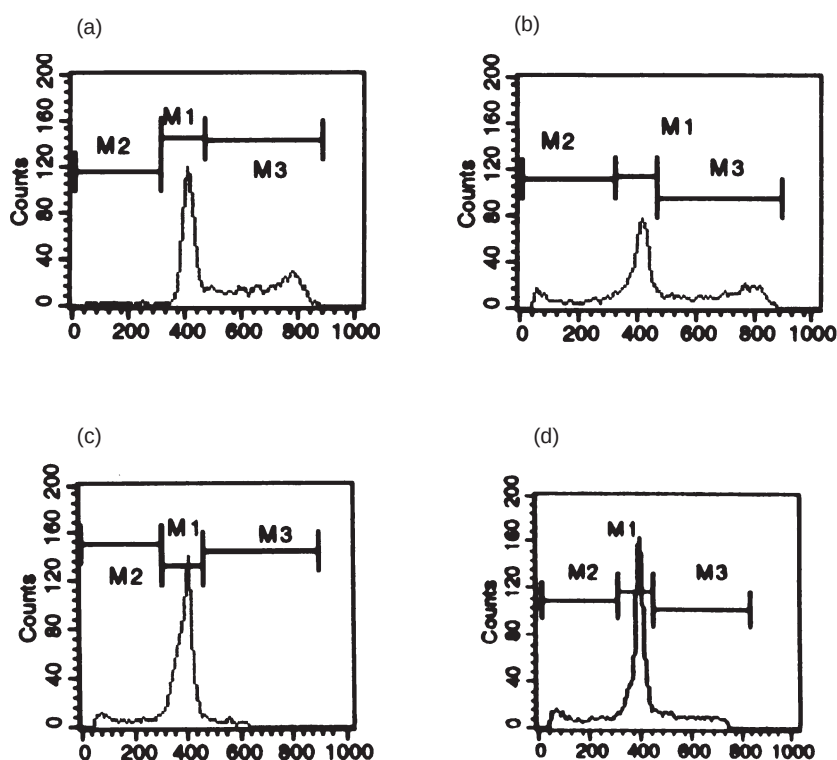


Fig. 2. Representative profiles obtained after propidium iodide (PI) staining and flow cytometric analysis. (a) Negative control using 5×10^5 /ml Jurkat cells cultured in media for 24 h; (b) positive control using 5×10^5 /ml Jurkat cells cultured for 24 h in media containing 200 ng/ml of anti-Fas; (c) cord blood mononuclear cells (CB MNCs) cultured in media for 7 days; (d) CB MNCs cultured in media containing 10 ng/ml of interleukin-15 (IL-15) for 7 days. M1 contains resting viable lymphocytes, M2, apoptotic cells, and M3, proliferating cells.

vivo expansion and re-infusion of naive T cells may benefit HIV-infected children (16). The present study addressed CB CD45RA⁺RO expression within CD4/CD8 lymphocyte subsets after stimulation with IL-15. We found that IL-15 preferentially increased the percentage of CD4⁺ CD45RA⁺ cells within CD4⁺ lymphocytes of CB MNCs, but not in that of APB MNCs.

We found that the percentage of CD4⁺ - CD45RA⁺ cells in CB MNCs cultured with media only for 7 days decreased significantly, while those in APB MNCs were not affected, in agreement with a previous report that neonatal CD45RA⁺ T cells were more susceptible to spontaneous apoptosis than adult CD45RA⁺ cells (17). We further demonstrated that IL-15 preferentially up-regulated CD4⁺ CD45RA⁺ expression on CB MNCs in a dose-dependent manner, an effect not observed in APB MNCs. Although it has been reported that the IL-2R γ chain (a part of the functional IL-15 receptor) is expressed at lower levels on CB than on APB lymphocytes (18,19), the results of our study and those of others (8,20) indicate that CB lymphocytes readily responded to IL-2-like cytokines (IL-12 or IL-15), and that CB CD4⁺ CD45RA⁺ T cells are even more responsive to IL-15 stimulation than their adult counterparts. Several investigators have also reported that CB CD4⁺ CD45RA⁺ T cells behave differently in magnitude of activation (21) and antigen-independent adhesion

(22) compared with APB CD4⁺ CD45RA⁺ T cells. Based on our results, we speculate that IL-15-induced expansion of CB CD4⁺ CD45RA⁺ T cells may be one of the mechanisms responsible for maintenance of the naive T-cell repertoire in adulthood after repeated antigen stimulation.

Evaluation of spontaneous apoptosis using PI staining revealed that when un-stimulated, a greater number of CB MNCs underwent apoptosis than did APB MNCs, in agreement with El Ghalbzouri A *et al.* (23) but in contrast to Aggarwal *et al.* (24). Previous studies have reported that IL-15 inhibits activation-induced apoptosis using APB lymphocytes (6,25). Our study is the first to examine the effect of IL-15 on spontaneous apoptosis of CB and APB MNCs. We demonstrated that IL-15 had no effect on apoptosis of CB MNCs, but enhanced their proliferation (cells in the M3 fraction, Fig. 2d) to a degree comparable to that achieved with APB MNCs. Thus, exogenous IL-15 may compensate for the immaturity of CB MNCs, and enhance the immune reconstitution after CB transplantation.

Previous studies have demonstrated the unique ability of IL-15 in enhancing CB cytolytic functions, including NK cytotoxicity, lymphokine-activated killer activity (26), and antibody-dependent cellular cytotoxicity (8), suggesting that IL-15 might be useful as a form of anti-tumor immunotherapy after CB transplantation. IL-15 could also enhance the immune functions of HIV-

infected patients by expanding the number of their NK cells (27) and HIV-specific cytotoxic T lymphocytes (28). Our finding, that IL-15 exerted a greater protective effect for CB naive T cells than for APB CD4⁺ CD45RA⁺ cells, implies the possible use of IL-15 for immunomodulative therapy in HIV-infected newborns.

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