Decreased Interleukin-12 (IL-12) From Activated Cord Versus Adult Peripheral Blood Mononuclear Cells and Upregulation of Interferon-γ, Natural Killer, and Lymphokine-Activated Killer Activity by IL-12 in Cord Blood Mononuclear Cells

By Sun Min Lee, Yu Suen, Leh Chang, Vivian Bruner, John Qian, Jeff Indes, Eva Knoppel, Carmella van de Ven, and Mitchell S. Cairo

Interleukin-12 (IL-12) is a critical cytokine regulating natural killer (NK) and T-cell function. We hypothesized that the impaired ability of cord blood (CB) to produce normal adult levels of IL-12 in response to stimulation may contribute to the immaturity of CB immunity. Furthermore, exogenous IL-12 may compensate for the immaturity in CB cellular immunity and have the potential for immunotherapy post cord blood transplantation. We compared the expression and production of IL-12 from activated cord versus adult mononuclear cells (MNC), regulatory mechanisms associated with IL-12 expression in CB MNC, and the effects of IL-12 on induction of CB interferon (IFN)-γ production, NK, and lymphokine-activated killer (LAK) cytotoxicity. Northern analysis and enzyme-linked immunosorbent assay were performed in lipopolysaccharide (LPS)-stimulated CB and adult peripheral blood (APB) MNC. IL-12 mRNA expression was induced within 6 hours with LPS (10 μg/mL) and reached peak levels at 12 hours in both CB and APB MNC. However, IL-12 mRNA expression and protein accumulation in CB MNC were 35.8% ± 4.84% (12 hours, n = 11, P < .05), and 17.6% ± 1.7% (24, 72, 96 hours, n = 9, P < .05) respectively, when compared with APB MNC. Nuclear run-on assays showed no differences between CB and APB MNC in both the basal levels of transcription and the degree of transcriptional activation.

However, the half-life of IL-12 p40 mRNA was approximately threefold lower in activated CB MNC than in activated APB MNC (CB: 114 ± 3.0 minutes v APB: 353 ± 7.8 minutes, n = 3, P < .05). Exogenous IL-12 (10 U/mL) induced a significant increase of IFN-γ from both CB and APB MNC (24 hours, 72 hours, P < .05, n = 3). The stimulated CB IFN-γ level reached comparable levels produced by unstimulated APB. IL-12 treatment also significantly enhanced CB NK cytotoxicity against K562 and NB-100 cell lines to the comparable levels of APB (P < .05, n = 4). CB MNC was more responsive to IL-12 stimulation with respect to IFN-γ production, NK, and LAK cytotoxicity when compared with APB. The present study suggests that IL-12 mRNA and protein expression is decreased in activated CB. This discrepancy in IL-12 production is secondary, at least in part, to the altered posttranscriptional regulation. The impaired ability of CB MNC to produce IL-12 in response to stimulation may contribute to the decrease in IFN-γ production and NK cytotoxicity. However, IL-12 enhanced IFN-γ and NK activity in CB MNC up to the comparable levels of APB MNC. These findings suggest that reduced expression and production of IL-12 from activated CB may contribute to the immaturity in CB cellular immunity and contribute, in part, to decreased graft-versus-host disease following CB stem cell transplantation.

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IL-12 is a novel cytokine purified from the conditioned media of an Epstein-Barr virus (EBV)-transformed human cell line. IL-12 is a heterodimer of 70 kD (p70) formed by two covalently-linked glycosylated chains of 40 kD (p40) and 35 kD (p35). Both p35 and p40 cDNA are required for secretion of the biologically active p70 heterodimer. The heterodimeric form of IL-12 is produced mainly by phagocytic cells, B cells, and possibly other peripheral blood accessory cells in response to bacteria, parasites, or their products. IL-12 induces cytokine secretion, activation of macrophages, and enhancement of cell-mediated cytotoxicity. These diverse functions suggest that IL-12 may play an important role in facilitating cell-mediated immune responses.

The antitumor effect of IL-12 has been demonstrated with a number of human tumors in mice. IL-12 treatment markedly reduced experimental metastases and resulted in a significant increase in survival. In vivo data suggests that the importance of T-cell responses and IFN-γ production are critical in these tumor suppressive activities of IL-12. IL-12 appears to enhance tumor cytotoxicity by inducing IFN-γ production from NK cells and enhancement of Th1 responses. The qualitative and quantitative deficiency in neonatal immune defense mechanisms may be, in part, due to a defect in IL-12 production and/or function.

In this study, we sought to compare the expression and production of IL-12, regulatory mechanisms associated with IL-12 expression in CB MNC, and to determine if IL-12 can induce CB IFN-γ production, NK, and LAK cytotoxicity. We hypothesize that the impaired ability of activated CB MNC to produce normal adult levels of IL-12 may contribute, in part, to immaturity of CB immunity. Furthermore, depending on the toxicity profile of phase I and II clinical trial study results, exogenous IL-12 administration may compensate for the immaturity in CB cellular immunity and have the potential for immunotherapy post CBT.

MATERIALS AND METHODS

Isolation and stimulation of MNC. Peripheral blood was obtained by venipuncture from healthy adult volunteers, in accordance with the principles of the Declaration of Helsinki. Blood samples were also obtained from the umbilical cords of the placentas of normal, full-term, nonstressed infants immediately after scheduled caesarean section. The samples were collected in heparinized syringes. CB and APB MNC were isolated from whole blood by sedimentation on Ficoll-Hypaque gradients (density 1.077 g/mL) (Sigma Chemical Co, St Louis, MO) for 30 minutes. The MNC at the interface were collected, washed twice, and resuspended in RPMI-1640 (GIBCO, Grand Island, NY) culture medium supplemented with 10% heat-inactivated human AB serum (Sigma). MNC isolated by this density gradient separation were purified to greater than 98% homogeneity, and cell viability as measured by trypan blue exclusion was more than 99%. There was no difference in the MNC differential between CB and APB (CB: 82% ± 8.0% lymphocytes and 8.8% ± 4.0% monocytes; APB: 86% ± 4.0% lymphocytes and 7.2% ± 3.0% monocytes). The cells were plated at a density of 1 × 10⁶ cells/mL in culture medium.

To determine IL-12 mRNA expression and protein production, CB and APB MNC (20 × 10⁶ cells) were stimulated with lipopolysaccharide (LPS from Escherichia coli 0127:B8 at 1, 2, 5, and 10 μg/mL) (Sigma) for up to 96 hours. Cells were harvested and lysed in a buffer containing guanidium thiocyanate, sodium citrate, sarcosyl, and mercaptoethanol for RNA analysis. Tissue culture supernatant samples were collected for enzyme-linked immunosorbent assay (ELISA) to detect IL-12 protein levels. To determine IFN-γ protein production, CB and APB MNC (1 × 10⁶ cells/mL) were stimulated with IL-12 (10 U/mL) (kindly provided by Dr S. Wolf, Genetics Institute, Cambridge, MA) for up to 144 hours. Tissue culture supernatant samples were collected for ELISA to detect IFN-γ protein levels.

To determine the effect of IL-12 with or without IL-2 to enhance NK, LAK cytotoxicity, CB and APB MNC at 5 × 10⁶ cells/mL were adhered to plastic tissue culture flasks for 1 hour to isolate monocyte-depleted MNC (MD MNC). Both CB and APB MD MNC were then stimulated with either IL-12, IL-2 or the combination of IL-12 and IL-2.

IL-12 ELISA. The IL-12 protein levels from unstimulated and LPS-stimulated CB and APB MNC culture supernatants were measured by a sandwich ELISA (R & D Systems, Minneapolis, MN). The immunoassay was developed with a capture antibody that recognizes only the IL-12 p70 heterodimer and not the individual subunits, thus eliminating the potential for interference by the subunits. A monoclonal antibody specific for human IL-12 was coated onto the microtiter plate. Standards and samples were added and presented to the immobilized coating antibody. After washing, an horseradish peroxidase (HRP)-linked polyclonal antibody for human IL-12 was added as substrate for colorimetric reaction. The optical density (OD) was then measured at 450 nm with a Bio-Rad EIA reader (Bio-Rad Lab, Richmond, CA). All samples were run in duplicate and data was presented as mean ± standard error of mean (SEM). The sensitivity of the assay was ≥5 pg/mL.

IFN-γ ELISA. The IFN-γ protein levels from unstimulated and IL-12 (10 U/mL) stimulated CB and APB MNC culture supernatants were measured by a sandwich ELISA (Biosource, Camarillo, CA). An antibody specific for human IFN-γ was coated onto the microtiter plate. Standards and samples were added and presented to the immobilized coating antibody. After washing, an HRP-linked antibody for human IFN-γ was added. Following washes, TMB was added as substrate for colorimetric reaction. The OD was then measured at 450 nm with a Bio-Rad EIA reader (Bio-Rad Lab). All samples were run in duplicate and data was presented as mean ± SEM. The sensitivity of the assay was ≥5 pg/mL.

RNA isolation and Northern blotting. Total cellular RNA was extracted from stimulated and unstimulated cells by the method of Chomczynski and Sacchi and electrophoresed on 1% agarose, 5% formaldehyde gels. The samples were heated in 40% formamide, 14% formaldehyde at 65°C for 15 minutes and then cooled before the addition of 1 μg/mL ethidium bromide. RNA was transferred to nitrocellulose and baked for 2 hours. Hybridization was performed at 42°C in 50% formamide, 5× sodium chloride, sodium citrate (SSC), 1× Denhardt’s, 50 mmol/L sodium phosphate (pH 6.5), 0.1% sodium dodecyl sulfate (SDS), 250 μg/mL salmon sperm DNA, and 10% dextran sulfate. RNA blots were hybridized sequentially with DNA probes using random oligonucleotide priming. The template for 32P-labeled probe was the human IL-12 p40 cDNA, which was a 2.3-Kb fragment (entire sequence) cloned into pED (EMC) vector at the Xba I site (provided as a generous gift by Dr S. Wolf, Genetics Institute). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) 775 bp PstI/Xba I fragment from pchGAP (ATCC) was used as an internal standard. Filters were washed to a stringency of 0.1× SSC at 65°C and exposed to Kodak XAR-5 film (Eastman Kodak, Rochester, NY). The hybridization signals were quantified by densitometry of autoradiographs. The levels of mRNA were calculated by normalizing signal optical densities to those of GAPDH mRNA. CB and APB Northern blot analyses were performed simultaneously.
under identical hybridization conditions and with the same amount of exposure time of the blot.

**Nuclear run-on transcription assay.** Cultures of three different samples were stimulated with LPS (10 μg/mL) (Sigma) for 12 hours to obtain maximal induction of IL-12. Nuclei isolation and nuclear run-on assays were performed as previously described and involved modification procedures described by Weber et al and Grouinie and colleagues. Nuclear run-on assays were performed with cDNA and shorter-sized cDNA fragments as target to avoid cross-hybridization artifacts, as suggested by Bronson et al. Target DNA samples used in the hybridization include IL-12 p40, 2.3 Kb cDNA (Dr S. Wolf, Genetics Institute), 1.0 Kb Xba I/EcoRI fragment (F-I) of cDNA; 754 bp Xba I/EcoRI fragment (F-II) of cDNA; GAPDH, 775 bp PstI/Xba I fragment from pBGAP (ATCC); and negative control pUC18 (GIBCO). The amount of full-length cDNA per slot was 1 μg. The amount of truncated cDNA fragments (F) was adjusted to equal the full-length cDNA molar concentration. The hybridization mixture contained 2.5 to 5 × 10^6 cpm/mL. Run-on signal strengths were determined by densitometry of autoradiograms. The density of the bands was calculated by normalizing values with respect to the signals of internal standards (GAPDH).

**mRNA half-life.** CB and APB MNC (20 × 10^6 cells) were stimulated with LPS (10 μg/mL) for 12 hours before exposure to the transcriptional inhibitor actinomycin D (10 μg/mL). Cells were harvested at intervals of 60, 120, 240, and 360 minutes. Cytoplasmic RNA was extracted and performed as described in Northern blot analysis for IL-12 p40 mRNA regulation. The amount of IL-12 p40 mRNA was normalized to the amount of GAPDH mRNA in each sample and then expressed as a percentage, setting the amount of mRNA at time 0 equal to 100%. The data were plotted against the time after addition of actinomycin D, and the half-life of each transcript was calculated based on the resultant graphs.

**NK and LAK cytotoxicity assay.** MD MNC were resuspended in a serum-free media, Progenitor 34 (GIBCO) or RPMI 1640 with 10% fetal calf serum (FCS) at 1 × 10^6 cells/mL. For NK cytotoxic activity, cells were stimulated with either 10 U/mL of IL-12 or a combination of 10 U/mL IL-12 with 1 U/mL IL-2, or cytokine-free media control in a 6-well tissue culture plate at 37°C, 5% CO2 humidified incubator for 18 hours before running the assay. For LAK cytotoxic activity, cells were stimulated with 100 U/mL of IL-2, 10 U/mL of IL-12, or a combination of 10 U/mL IL-12 with 1 U/mL IL-2 and a cytokine-free media control in a 6-well tissue culture plate at 37°C, 5% CO2, humidified incubator for 72 hours before running the assay. At the end of the incubation, cells were harvested, washed, and resuspended in RPMI 1640 supplemented with 10% FCS at 1 × 10^6 cells/mL. A 3-hour standard 3^5^Cr-release assay was performed with 20:1, 10:1, and 5:1 effector:target ratios. K562 target, a human erythroleukemic cell line (ATCC, Rockville, MA); Daudi target, a human Burkitt’s lymphoma cell line (ATCC); SK-N-MC and NB-100 target, human pediatric neuroblastoma cell lines (ATCC) were labeled with 100 μCi/1 × 10^6 cells of sodium 3^5^Chromate (Amersham, Arlington Heights, IL) for 2 hours. Before the assay, cells were washed twice with cold assay media and incubated for at least 1 hour at 37°C. A total of 100 μL of effector and 100 μL of 3^5^Cr-labeled targets were added together in a V-bottom 96-well culture plate. All assays were run in triplicate. For spontaneous release (SR) control, 100 μL of assay media was added with 100 μL of targets; for total release (TR) control, 100 μL of 1% Triton X was added with targets. After cells were added, plates were centrifuged at 1,800 rpm for 1 minute and then incubated for 3 hours at 37°C. At the end of the 3-hour incubation, 150 μL of culture supernatant was harvested from each well, and the radioactivity was measured in a Beckman LS 1800 Liquid Scintillation Counter (Beckman, Fullerton, CA). Percent (%) lysis was calculated and then converted to lytic units (LU) using computer-assisted regression analysis.

**RESULTS**

**Accumulation of IL-12 protein from stimulated CB and APB MNC.** ELISA was performed to compare the IL-12 protein in CB versus APB MNC before and after LPS stimulation. Unstimulated MNC from both CB and APB had undetectable production of the IL-12 heterodimer (<5.0 pg/mL) in the tissue culture supernatant samples. A total of 10 μg/mL of LPS significantly induced less IL-12 protein production in CB compared with APB MNC (24 hours: CB 5.0 ± 2.0 pg/mL, APB 27.0 ± 6.0 pg/mL, P = .03; 72 hours: CB 6.3 ± 0.7 pg/mL, APB 31.3 ± 13.7 pg/mL, P = .028; 96 hours CB 5.0 ± 0.1 pg/mL, APB 35 ± 11.3 pg/mL, P = .0286) (n = 3) (Fig 1). The IL-12 protein level in CB MNC was only 17.6% ± 1.7% (n = 9, P < .05) at 24, 72, and 96 hours when compared with similar levels in APB MNC.

**Reduced IL-12 mRNA expression in stimulated CB versus APB MNC.** Northern blot analysis of CB and APB MNC was performed simultaneously under identical conditions to compare the IL-12 mRNA expression before and after LPS stimulation. Unstimulated MNC from both CB and APB had undetectable expression of IL-12 p40 mRNA. In a dose-response study of MNC with LPS stimulation (concentrations = 1, 2, 5, and 10 μg/mL, 12 hours), we demonstrated a concentration-dependent upregulation of IL-12 p40 mRNA expression in both CB and APB (Fig 2). A total of 10 μg/mL of LPS induced a twofold increase of IL-12 mRNA expression compared with 1 μg/mL of LPS in both CB and APB MNC (10 μg/mL v 1 μg/mL, n = 3, P < .05). However,
ILE ET AL

LPS (μg/mL)

IL-12 (pg/10)

GAPDH CB APB

0 1 2 5 10 0 1 2 5 10

Fig 3. Time course of induction of IL-12 mRNA expression in cord (CB) and adult (APB) MNC. CB and APB MNC were isolated, cultured in RPMI, and stimulated with 10 μg/mL LPS for 0, 2, 4, 6, 12, 18, 24, and 48 hours. Cells were harvested and RNA samples were analyzed by Northern blot analysis of IL-12 p40 mRNA (2.3 kb). Results shown are representative of three different CB and APB RNA blot hybridizations, normalized to GAPDH signal (n = 3, P < .05).

IL-12 mRNA half-life. Because the transcriptional activity of the p40 gene was virtually the same for both CB and APB MNC, the stability of p40 mRNA was compared in stimulated CB and APB by blocking mRNA synthesis with actinomycin D to determine whether the differential regulation was occurring at the posttranscriptional level. CB and APB MNC were stimulated with LPS (10 μg/mL) for 12 hours before actinomycin D (10 μg/mL) was added for various time periods (0 to 360 minutes). Northern blots of total cytoplasmic RNA were hybridized to 32P-labeled p40 cDNA. The levels of p40 mRNA progressively decreased during actinomycin D exposure in both CB and APB. Transcripts were quantitated by densitometric scanning of the autoradiographs. As shown in Fig 5, the measured mRNA half-life of p40 from stimulated CB MNC was approximately threefold lower than that from stimulated APB MNC (t1/2: 114 ± 3.0 minutes v 353 ± 7.8 minutes, CB v APB, n = 3, P < .05).

upregulation of IL-12 p40 mRNA from CB MNC was significantly lower than that from APB MNC at each concentration of LPS stimulation (1, 2, 5, 10 μg/mL, CB v APB, P < .05, n = 3). We also performed a time course study of IL-12 mRNA expression following LPS (10 μg/mL) stimulation. As shown in Fig 3, IL-12 mRNA expression was induced within 6 hours upon LPS stimulation and reached a peak level at 12 hours in both CB and APB MNC, returning to basal levels after 48 hours. However, IL-12 mRNA expression in CB MNC was only 35.8% ± 4.84% (n = 11, P < .05) compared with the level in APB MNC after 12 hours of stimulation with LPS (Fig 3, lower panel).

Transcriptional activity of IL-12 gene. Nuclear run-on transcription analysis was performed to determine if the low amount of IL-12 mRNA in stimulated CB MNC was due to lack of transcription of the IL-12 gene. Nuclear run-on transcripts from nuclei isolated from CB MNC that were stimulated with LPS for 12 hours were compared with run-on transcripts isolated from similarly-treated APB MNC. As shown in Fig 4, unstimulated CB and APB MNC showed negligible basal level signals of p40 transcript (OD <0.1), which was approximately the same in both CB and APB. After stimulation with LPS (12 hours), the transcriptional rate of the p40 gene was significantly increased in both CB and APB MNC (CB: 20 ± 0.72 fold, APB: 19 ± 1.18 fold, n = 3, P < .05). However, there was no appreciable differences between activated CB and APB MNC in the degree of transcriptional activation (P > .05). The result was approximately the same either with the full-length cDNA or with the truncated cDNA fragment as a target (data not shown).

IL-12 mRNA half-life. Because the transcriptional activity of the p40 gene was virtually the same for both CB and APB MNC, the stability of p40 mRNA was compared in stimulated CB and APB by blocking mRNA synthesis with actinomycin D to determine whether the differential regulation was occurring at the posttranscriptional level. CB and APB MNC were stimulated with LPS (10 μg/mL) for 12 hours before actinomycin D (10 μg/mL) was added for various time periods (0 to 360 minutes). Northern blots of total cytoplasmic RNA were hybridized to 32P-labeled p40 cDNA. The levels of p40 mRNA progressively decreased during actinomycin D exposure in both CB and APB. Transcripts were quantitated by densitometric scanning of the autoradiographs. As shown in Fig 5, the measured mRNA half-life of p40 from stimulated CB MNC was approximately threefold lower than that from stimulated APB MNC (t1/2: 114 ± 3.0 minutes v 353 ± 7.8 minutes, CB v APB, n = 3, P < .05).
Accumulation of IFN-γ protein from CB and APB MNC. ELISA was performed to compare the IFN-γ protein production in tissue culture supernatant from CB versus APB MNC before and after IL-12 stimulation. Unstimulated MNC from CB had undetectable production of IFN-γ (≤5.0 pg/mL) for up to 72 hours of incubation. However, unstimulated APB MNC produced significantly higher levels of IFN-γ than CB after 72 hours incubation (CB vs APB: 72 hours, 6.0 ± 1.0 v 466.7 ± 71.3 pg/mL, n = 3, P = .0229). IL-12 (10 U/mL) induced a significant increase of IFN-γ protein production from both CB and APB MNC after 24 and 72 hours stimulation. However, the IFN-γ production from IL-12-stimulated CB was only 24% (24 hours) and 50% (72 hours) when compared with the level produced by IL-12-stimulated APB (control vs IL-12 stimulation: CB, 24 hours, 6.0 ± 1.0 v 116.7 ± 18.5 pg/mL, n = 3, P = .0198; 72 hours, 6.0 ± 1.0 v 496.7 ± 98.4, n = 3, P = .0379; APB, 24 hours, 13.7 ± 4.7 v 506.7 ± 28.5 pg/mL, n = 3, P = .0125; 72 hours, 466.7 ± 71.3 v 996.7 ± 73.6 pg/mL, n = 3, P = .014).

IFN-γ production from both CB and APB MNC reached maximum levels after 72 hours of IL-12 stimulation and remained upregulated for the duration of the experiment (144 hours) (data not shown) (Fig 6). IL-12-stimulated CB MNC accumulated comparable levels of IFN-γ when compared with unstimulated APB after 72 hours of incubation (IL-12-stimulated CB vs unstimulated APB: 496.7 ± 71.3 pg/mL, n = 3, P = ns) (Fig 6).

Activation of NK and LAK cytotoxic activity. The NK cytotoxicity of CB and APB MNC was determined after 18 hours of incubation in the absence of cytokines. The ability of CB to kill K562, NB-100, and SK-N-MC was significantly lower than APB MNC (CB vs APB, K562: P = .038; NB-100: P = .0286; SK-N-MC: P = .0297, n = 4). The APB NK lytic activity against K562 and NB-100 was significantly increased up to comparable levels of the APB (control vs IL-12 stimulation, K562: P = .0312; NB-100: P = .0286, n = 4). The APB NK lytic
CSF, M-CSF, IL-3, IL-8, and IL-2. The rapid induction of IFN-γ production by IL-12 both in vitro and in vivo indicates that it may be involved in the differentiation of Th1 cells during the normal immune response. IL-12 has also been demonstrated as a facilitating factor for the IFN-γ producing Th1 cell development and as a suppressing factor for the Th2 cell development in both humans and mice. Similar to IFN-γ and IL-2, IL-12 rapidly enhances the cytotoxic ability of resting and activated NK cells. The effect of IL-12 directly enhances NK cellular activity against SK-N-MC was also significantly increased (control vs IL-12-stimulated, P < .0322, n = 4). The combination of IL-12 (10 U/mL) with low-dose IL-2 (1 U/mL) further enhanced the NK cytotoxicity from CB (IL-12 + IL-2, K562: P < .021; NB-100: P < .0428; SK-N-MC: P < .0073, n = 4) (Table 1).

The LAK cytotoxicity of CB and APB MD MNC was determined after 72 hours of incubation. As shown in Table 2, incubation of MD MNC in the absence of IL-2 did not have any effect on the levels of LAK lytic activity against Daudi, NB-100, and SK-N-MC. IL-2 (100 U/mL) stimulation of both CB and APB MD MNC induced significant and comparable levels of LAK lytic activities against all three tumor cell lines (control vs IL-2, Daudi: CB, P < .0286; APB, P < .0079; NB-100: CB, P < .0286; SK-N-MC: CB, P < .0286; APB, P < .0286, n = 4). Although, IL-12 (10 U/mL) alone did not induce comparable levels of lytic activity when compared with IL-2 at 100 U/mL, the combination of IL-12 (10 U/mL) with low-dose IL-2 (1 U/mL) stimulated LAK activity up to the IL-2 (100 U/mL) induced level (control vs IL-2 + IL-2, Daudi: CB, P < .0312; APB, P < .00719; SK-N-MC: CB, P < .05; APB, P < .0448, n = 4) (Table 2).

### DISCUSSION

IL-12 induces production of IFN-γ and other lymphokines that activate phagocytic cells and induce inflammatory responses. The physiological functions of IL-12 are exhibited by inducing NK and T cells to secrete IFN-γ, TNF-α, GM-CSF, M-CSF, IL-3, IL-8, and IL-2. The rapid induction of IFN-γ production by IL-12 both in vitro and in vivo indicates that it may be involved in the differentiation of Th1 cells during the normal immune response. IL-12 has also been demonstrated as a facilitating factor for the IFN-γ producing Th1 cell development and as a suppressing factor for the Th2 cell development in both humans and mice. Similar to IFN-γ and IL-2, IL-12 rapidly enhances the cytotoxic ability of resting and activated NK cells. The effect of IL-12 directly enhances NK cellular cytotoxicity and does not require the participation of accessory cells. In addition to short-term activation of NK cells, IL-12 also induces generation of LAK cells in the culture of peripheral blood leukocytes (PBL) or purified NK cells and generation and activation of alloreactive CTL. During the early stage of infection, the production of IL-12 and the induction of diverse lymphokine production suggest that IL-12 might have a major regulatory effect in the subsequent antigen-specific adaptive immune responses.

In an analysis of IL-12 mRNA accumulation in B cells, T cells, NK cells, monocytes, and leukemic cell lines, all expressed p35 transcripts. By contrast, expression of p40 transcripts is more restricted and present only in cells producing p70 heterodimers with biological activities. APB MNC levels are demonstrated to produce constitutively low levels of p40 transcript and IL-12 protein. Enhancement of IL-12 protein production and p40 mRNA expression from APB MNC occurs in response to Staphylococcus aureus Cowan Strain 1 (SAC) and to LPS. Further studies suggest that,

### Table 1. Effect of IL-12 + IL-2 on NK Cytotoxicity of CB and APB MNC Against K562, NB-100, and SK-N-MC

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<th>K562 (LU)</th>
<th>NB-100 (LU)</th>
<th>SK-N-MC (LU)</th>
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<tr>
<td>CB (control)</td>
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<td>APB (control)</td>
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<tr>
<td>CB (IL-12 10 U/mL)</td>
<td>136.2 ± 32.8</td>
<td>31.5 ± 13.7</td>
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<td>APB (IL-12 10 U/mL)</td>
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<td>CB (IL-12 10 U/mL + IL-2 1 U/mL)</td>
<td>235.83 ± 54.36</td>
<td>58.5 ± 17.91</td>
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<td>APB (IL-12 10 U/mL + IL-2 1 U/mL)</td>
<td>312.75 ± 43.41</td>
<td>280.7 ± 72.7</td>
<td>310.7 ± 45.8</td>
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Abbreviations: CB, cord blood MNC; APB, adult peripheral blood MNC.

* K562 CB control v APB control P = .0381; NB-100 CB control v APB control P = .0286; SK-N-MC CB control v APB control P = .0297.
† K562 CB IL-12 + IL-2 P = .0312; NB-100 CB control v CB IL-12 P = .0286.
‡ K562 CB IL-12 + IL-2 P = .021; NB-100 CB IL-12 + IL-2 P = .0428; SK-N-MC CB IL-12 + CB IL-12 + IL-2 P = .0073.

### Table 2. Effect of IL-12 + IL-2 on LAK Cytotoxicity of CB and APB MNC Against Daudi, NB-100, and SK-N-MC

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<th>Daudi (LU)</th>
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<th>SK-N-MC (LU)</th>
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<td>CB (control)</td>
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<td>APB (control)</td>
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<tr>
<td>CB (IL-2 100 U/mL)</td>
<td>280.7 ± 20.2</td>
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<td>APB (IL-2 100 U/mL)</td>
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<td>CB (IL-12 10 U/mL)</td>
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<td>APB (IL-12 10 U/mL)</td>
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<tr>
<td>CB (IL-2 1 U/mL + IL-12 10 U/mL)</td>
<td>114.8 ± 50.4</td>
<td>238.0 ± 81.4</td>
<td>128.0 ± 39.4</td>
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<tr>
<td>APB (IL-2 1 U/mL + UL-12 10 U/mL)</td>
<td>543.4 ± 147.1</td>
<td>71.8 ± 29.7</td>
<td>91.0 ± 24.0</td>
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</table>

Abbreviations: CB, cord blood MNC; APB, adult peripheral blood MNC.

* Daudi CB control v CB IL-12 + IL-2 P = .0312; SK-N-MC CB control v CB IL-12 + IL-2 P = .05.
while the p40 transcript is highly regulated, p35 is expressed constitutively and minimally regulated in APB MNC.50

During the states of increased demand, mononuclear phagocytes and B cells contribute significantly to the production of IL-12 in both CB and APB. However, our results indicate that CB MNC have an impaired ability to express and produce IL-12 in response to bacterial stimulation (Figs 1 and 3). The decreased mRNA expression in CB versus APB MNC are not secondary to alteration in IL-12 gene transcription (Fig 4). Alteration in posttranscriptional stability appears to account for the decrease in IL-12 mRNA expression in CB versus APB MNC (Fig 5).

Control of mRNA stability is not well understood, but the process is thought to involve various factors interacting with specific mRNA sequences.51,52 The adenosine + uridine (AU)-rich, or AUUUA-repeat elements (ARE) in the 3'-untranslated regions (UTR) of many cytokine and proto-oncogene transcripts are known to be the targets of a pathway for selective processing and mRNA degradation.53-57

Analyses of the published IL-12 sequence.58,59 indicate the presence of several AUUUA-repeats in the 3'-UTR of both p35 and p40 subunit, which may contribute to the instability of the IL-12 mRNA. We have previously reported the complex regulatory mechanism of several cytokines. The decreased accumulation of GM-CSF and M-CSF mRNA in CB MNC is associated with a reduction in its half-life compared with APB MNC, whereas the rate of gene transcription remains comparable in CB and APB MNC.6,10 The GM-CSF, as well as M-CSF mRNA 3' UTR, have multiple copies of AUUUA-elements.58,60 A reduced mRNA half-life and comparable transcription rates for GM-CSF, as well as M-CSF, in CB versus APB MNC indicate that these ARE-containing transcripts may also be less stable in CB MNC. Translational inhibition by cycloheximide (CHX) after stimulation of cells causes a super-induction of GM-CSF, as well as M-CSF mRNA, which is approximately 2.5-fold greater in CB versus APB MNC.11,61 Increased transcript stabilization in stimulated CB MNC after CHX treatment suggests that before translational inhibition, higher levels of a translational-dependent nucleas may be present in CB MNC. An ARE-directed nuclease and several ARE-binding factors have been identified. One of these is a 37-kD protein, designated AUFl. We have recently reported that the decreased GM-CSF mRNA stability in CB versus APB MNC was inversely correlated with AUUUA-element binding activity and with the levels of AUFl binding factor.61 It seems likely that any alteration in the expression and/or biological activities of these various factors in stimulated CB MNC could also contribute to the reduction of IL-12 mRNA. Further studies are required to test these possibilities.

In light of the important biological functions of IL-12, the reduced expression and production of IL-12 from stimulated CB MNC, as demonstrated in this study, may contribute to the immaturity of CB cellular immunity. The functional effects of IL-12 on the CB cellular immunity was evaluated by measuring the induction of IFN-γ production and the enhancement of NK and LAK cytotoxic activities. IL-12 induced a significant increase of IFN-γ from both CB and APB MNC. Although the levels of IFN-γ produced by IL-12-stimulated CB were still lower than the levels produced by IL-12-stimulated APB, they reached comparable levels produced by unstimulated APB (Fig 6). IFN-γ production from phorbol myristate acetate (PMA) and staphylococcal enterotoxin A stimulated CB MNC have been demonstrated to be significantly lower than stimulated APB.68 However, Lau et al69 reported that unlike other stimulants, IL-12 at a concentration as low as 10 U/mL induced similar levels of IFN-γ production from both CB and APB MNC. Wu et al60 reported that IL-12 can induce IFN-γ synthesis by neonatal CD4 T cells isolated from CB. IL-2 can further synergize with IL-12 in triggering IFN-γ production in neonatal cells. They proposed that IL-12 is capable of inducing the maturation of resting naive CD4 T cells into cells capable of producing Th1 cytokines including IFN-γ that might contribute to activating NK cells for cytotoxic activity in CB. Our data suggest that the IFN-γ-producing cell population in APB does not seem to exist in unstimulated CB. IL-12 may stimulate the maturation of naive non-IFN-γ producing cells into IFN-γ-producing cells in CB.

Harris et al70 reported that CB possesses similar concentrations of NK cells (CD16+) comparable to APB. However, we have previously observed that the baseline NK cytotoxicity of CB MD MNC against K562 and two pediatric neuroblastoma cell lines, NB-100 and SK-N-MC was significantly lower than APB. IL-12 significantly enhanced CB NK cytotoxicity against K562 and NB-100 up to the comparable levels of APB. The combination of IL-12 with a low dose of IL-2 can further enhance NK cytotoxicity from CB (Table 1). Chin et al71 demonstrated that IL-2–induced LAK cytotoxicity was elevated in both CB and APB MNC against multiple pediatric tumor cell lines, eg, Wilms’ tumor NEP-1, Ewing’s sarcoma SK-ES-1, Rhabdomyosarcoma A204, and Neuroblastoma SH-SY-5y, SK-N-MC. Both CB and APB MNC have comparable levels of LAK activity against NEP-1, SK-ES-1, and A204. However, IL-2–induced CB LAK activity against SH-SY 5y was significantly higher than APB. In this study, we have demonstrated that IL-12 alone did not induce comparable levels of LAK activity when compared with IL-2 against Daudi, SK-N-MC, and NB-100 from both CB and APB MD MNC. However, the combination of IL-12 with low dose IL-2 stimulated LAK activity up to the IL-2–induced level against Daudi and SK-N-MC (Table 2). Interestingly, IL-2, IL-12, and the combination of IL-12 and IL-2 induced significantly higher CB LAK cytotoxicity than APB against NB-100. The increased LAK cytotoxicity in CB versus APB against SH-SY5Y and NB-100 suggests a possible cellular immune mechanism to the improved prognosis for advanced congenital neuroblastoma (Stage IV S) compared with similar advanced disease in older children.72

Furthermore, these findings suggested that CB MD MNC were more responsive to IL-12 stimulation with respect to IFN-γ production, and NK and LAK cytotoxicity when compared with APB. Wu et al60 reported that CD4 T cells isolated from CB demonstrate phenotypic features of naive cells, which mainly secrete IL-2 upon activation by PMA and can be stimulated by IL-12 to produce a high level of IFN-γ.
Beverly et al.\(^2\) reported that circulating CD4 cells of healthy individuals are comprised of two distinct populations; CD45 RA and RO isoforms. Clement et al.\(^3\) reported that greater than 90% CB CD4 T cells expressed CD45 RA+/RO\(^-\), whereas only 40% to 60% of APB CD4 T cells express CD45 RO+/RA\(^-\). Upon stimulation, CD45 RA+/RO\(^-\) CD4 T cells produce only IL-2, whereas the CD45 RO+/RA\(^-\) population contains cells producing high levels of IL-2, IL-4 and IFN-\(\gamma\).\(^4,5\) The higher percentage of naive T cells carrying CD45 RA+/RO\(^-\) phenotype in CB MNC may explain the lower constitutive level and higher inducibility of IFN-\(\gamma\) production, NK and LAK cytotoxicity in CB. The impaired ability of CB to produce IL-12 and the decreased population of CB CD45 RO+/RA\(^-\) CD4 cells might both contribute to the immune deficiency, including IFN-\(\gamma\)-production and NK cytotoxicity. Exogenous IL-12 might induce the maturation of naive CB cells, IFN-\(\gamma\)-production, and the enhancement of CB NK and LAK cytotoxicity.

In summary, the present study demonstrated that IL-12 mRNA and protein production is decreased in activated CB compared with APB MNC. This discrepancy in IL-12 production is secondary, at least in part, to the altered posttranscriptional regulation. The impaired ability of CB MNC to produce IL-12 in response to stimulation may contribute to the decrease in IFN-\(\gamma\)-production, NK and LAK cytotoxicity in CB compared with APB. However, IL-12 stimulation enhanced IFN-\(\gamma\), NK and LAK activity in CB MNC and MD MNC up to the comparable levels of APB MNC. These findings suggest that reduced expression and production of IL-12 from activated CB may contribute to the immaturity of CB cellular immunity and contribute, in part, to a decrease in GVHD following both unrelated and related CB stem cell transplantation; that exogenous IL-12 stimulation can compensate for the immaturity in CB cellular immunity; and that the combination of IL-12 with or without low-dose IL-2 post CBT may enhance NK and LAK activities.

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Decreased interleukin-12 (IL-12) from activated cord versus adult peripheral blood mononuclear cells and upregulation of interferon-gamma, natural killer, and lymphokine-activated killer activity by IL-12 in cord blood mononuclear cells

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