

Evaluation of cytotoxic function and apoptosis in interleukin (IL)-12/IL-15-treated umbilical cord or adult peripheral blood natural killer cells by a propidium-iodide based flow cytometry

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Both deficient natural killer (NK) cell effector function and increased propensity to apoptosis of neonatal NK cells contribute to the increased susceptibility to infection in the neonates. Interleukin (IL)-12 and IL-15 are two immunoregulatory cytokines known to enhance cytolytic function of neonatal NK cells. The present study aims to simultaneously investigate the effect of IL-12/IL-15 on K562 cytotoxicity as well as NK cells apoptosis of enriched umbilical cord blood (CB) and adult peripheral blood (APB) NK cells, using flow cytometric cytotoxicity assays. The results indicated that (i) prior to cytotoxicity assays, CB NK cells underwent greater degree of spontaneous apoptosis than did APB NK cells; (ii) both IL-12 and IL-15 inhibited the spontaneous apoptosis of CB NK cells, while IL-15 promoted the apoptosis in APB NK cells; (iii) the deficient K562 cytotoxicity of CB NK cells could be enhanced to levels comparable with that of APB NK cells by IL-15; (iv) IL-15 increased the percentages of apoptosis in NK–K562 conjugates in a dose-dependant manner in both CB and APB with a greater effect seen with APB NK cells; (v) target-induced apoptosis was observed with APB NK cells which were further enhanced with IL-15. However, CB NK cells, unstimulated or IL-15-activated, were resistant to K562-induced apoptosis. Thus, the multi-parameter flow cytometry analysis not only demonstrates better for the deficient CB NK function but also provides greater details for cytotoxic mechanisms of NK cells.

Syh-Jae Lin¹, Po-Jen Cheng², Yen-Ju Huang³ and Ming-Ling Kuo^{3,4}

¹Division of Allergy, Asthma and Rheumatology, Department of Pediatrics, ²Department of Obstetrics/Gynecology, ³Health Research Division, ⁴Department of Microbiology and Immunology, Graduate Institute of Basic Medical Research, Chang Gung University, Taoyuan, Taiwan

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Ming-Ling Kuo, PhD, Health Research Division, 12th floor, Building L, Chang Gung Children's Hospital, 5 Fu-Hsin Street, Kweishan, Taoyuan, Taiwan
Fax: 886-3-3286210
E-mail: mingling@adm.cgmh.org.tw

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Natural killer (NK) cells are a distinct lineage of lymphoid cells defined as being membrane CD3[−], CD16⁺ and/or CD56⁺, providing the first-line defense by lysing tumor and virus-infected cells in a non-major histocompatibility restricted fashion and without the need of prior sensitization (1, 2). Decreased graft-vs.-host disease (GVHD), easier accessibility, and sustained engraftment encourage the use of umbilical cord blood (CB) as an alternative source to bone marrow for immune reconstitution for children with immunological and hematological disorders

(3). Expansion and activation of CB NK cells using cytokines may speed the recovery of patients receiving stem cell transplants by providing resistance to viral infection during the post-transplant period. Suppression of GVHD and potential graft-vs.-leukemia (GVL) effect for prevention of tumor relapse can be achieved with NK activation in CB recipients (4).

Deficient NK function also predisposes the neonates to increased infectious morbidity and mortality from viral infections (5). Compared with adult NK cells, neonatal NK defects that

have been reported to date included low CD57 expression (6), decreased binding to targets (7), and decreased NK and antibody-dependant cell-mediated cytotoxicity (ADCC) (8, 9). A number of groups including the authors of the present study have confirmed up-regulation of neonatal NKC and ADCC to adult levels with IL-2-like cytokines, such as IL-12 and IL-15 (10). However, the above observations have all been elucidated from peripheral blood mononuclear cells (PBMC) experiments, with the NK population representing a small and variable minority of the cells.

On the contrary, cytokine-priming by IL-2, IL-12 and IL-15 has been shown to render adult NK cells susceptible to activation-induced cell death, representing a novel mechanism for NK cell regulation (11). NK cells can undergo apoptosis following contact with target cells, providing another mechanism for down-regulating the NK cell response (12). NK sensitive target K562 cells induced the death of IL-2 stimulated, but not unstimulated NK cells. The induction was shown to be a CD18-dependant process and this effect might contribute to the limited clinical benefit of lymphokine-activated killer (LAK) cells based therapy in cancer (13). It has not been examined, however, whether CB NK cells behave in the similar fashion to adult NK cells, and how IL-12 and IL-15 would affect the target binding and subsequent apoptosis of NK cells following contact with K562 cells.

Multi-parameter flow cytometry-based cytotoxicity assay that was determined with propidium iodide (PI) uptake produced results which correlated well with conventional ^{51}Cr (^{51}Cr) release assays in assessing NK activity (14). In addition to cytotoxicity assessment, it provides details simultaneously regarding NK-K562 interactions, such as binding capacity, apoptosis in conjugates and NK cells following binding, and the influence of cytokines. It serves as a promising tool to better dissect the defects of neonatal NK cell function on cellular and molecular levels and to ascertain the mechanism of cytokine-enhanced cytotoxicity in neonatal NK cells.

In the present study, we investigated the effect of IL-12 and IL-15, alone or in combination, on apoptosis of immunomagnetically enriched CB and adult peripheral blood (APB) NK cells by detecting PI uptake with flow cytometry. With the purified NK cells, the analyses obviated the need of post-culture immunostaining of CD16/56. We sought to determine whether different pattern exists between APB and CB

with regard to NK cell apoptosis and conjugates formation following K562 contact and the differential influence of IL-12 and IL-15 on these parameters.

Materials and methods

NK cell enrichment and cytokine treatment

Mononuclear cells (MNCs) were obtained from healthy adult volunteers ($n = 7$, four male and three female, mean age 28 ± 3 yr) and from term newborn umbilical cords ($n = 9$) following normal vaginal deliveries according to the guidelines of the Human Subjects Protection Committee of the Chang Gung Memorial Hospital and the informed consents were obtained from all of the donors and parents (for CB samples). Both CB and APB were collected in sterile tubes containing heparin (20 units/ml of blood) and was processed within 24 h of collection. MNCs were then separated from heparinized blood using Ficoll-Hypaque density gradient centrifugation. NK cells were enriched from MNCs by depleting CD3^+ cells (T lymphocytes), CD19^+ cells (B lymphocytes), and CD14^+ cells (macrophage/monocytes) using the magnetic antibody cell sorting (MACS) Cell Isolation Kits for NK cells (Miltenyi Biotec, Bergisch Gladbach, Germany) including bead-coupled monoclonal antibodies specific for CD3, CD4, CD19, and CD33. Through an MACS magnetic separator (Miltenyi Biotec), NK cells were enriched phenotypically $> 80\%$ of $\text{CD16}^+/\text{CD56}^+$, as determined by flow cytometry. The isolated NK cells were resuspended at $1 \times 10^6/\text{ml}$ in RPMI-1640 medium (Irvine Scientific, Santa Ana, CA, USA) supplemented with 10% heat-inactivated fetal calf serum in six-well plates without or with recombinant cytokines IL-15 (Pepro Tech EC, London, UK) at 10 ng/ml, and IL-12 (Pepro Tech EC) at 1 ng/ml, and their combination for 72 h at 37°C in 5% CO_2 . Because of the short half-life of the cytokines, the cytokines were added every other day to the cultures. The IL-15 and IL-12 concentrations have been previously determined to be optimal (15).

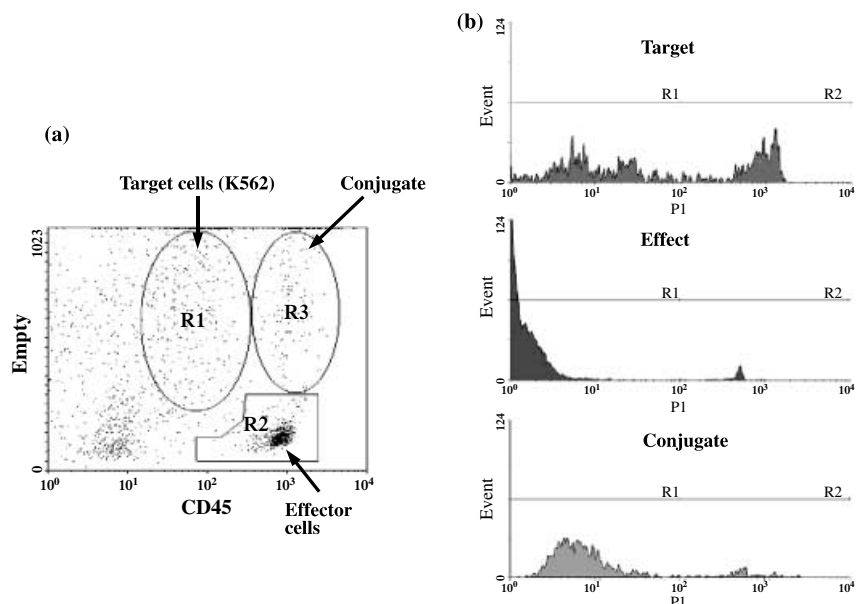
Flow cytometric cytotoxicity assay

Cytokine-cultured NK cells (effectors) and K562 cells (targets, an erythromyelocytic leukemia cell line) were added in duplicate to 12×75 mm round bottom polystyrene tubes to yield effector:target (E:T) ratios of 10:1, 5:1, and 2.5:1. Control tubes including only target or effector cells were assayed to determine the spontaneous

cell death. Tubes were mixed by gently tapping, centrifuged at $200 \times g$ for 1 min and incubated at 37°C in 5% CO_2 for 4 h. After the incubation, 10 μl of CD45-fluorescein isothiocyanate (FITC) (Becton-Dickinson, San Jose, CA, USA) were added to each tube. The tubes were then mixed gently and incubated for 20 min on ice. PI of 20 μl (Sigma Chemical Co., St Louis, MO, USA) at 1 $\mu\text{g}/\text{ml}$ were added to each tube 10–15 min before acquisition.

Flow cytometry was performed with a FAC-SCalibur cytometer (Becton-Dickinson) as previously described (14). Fig. 1 demonstrates a typical dotplot of FITC-conjugated antibody specific to CD45 (fluorescence 1, FL1) vs. side scatter, the intensity derived from the binding of anti-CD45 antibody resulted in a reliable separation between K562 cells and NK cells. With the control sample containing either the target cells or effector cells, the cell cluster that was designated as region 1 (R1) represented the target cells displayed on the dotplot and the cell cluster that was designated as region 2 (R2) represented effector cells. After 4 h of incubation of both cell populations, a third cell cluster that was identified as region 3 (R3) represented the conjugates between target and effectors. Percentages of dead cells in different regions are calculated from histograms showing PI uptake as measured by the intensity of fluorescence 3 (FL3). After the subtraction of background cell death, the percentages of live and dead cells in (i) free target, (ii) NK-target conjugates, and (iii) free NK cells in each mixed culture were obtained.

Fig. 1. Flow cytometric measurement of K562 cytotoxicity of enriched natural killer (NK) cells using FITC-conjugated anti-CD45 staining and side scatter profiles to identify different cell populations (a) and uptake of propidium iodide (PI) to detect death (b). Target cells, NK cells, and NK–K562 conjugates are identified by region 1 (R1), region 2 (R2), and region 3 (R3), respectively. Percentages of dead cells in different regions are calculated from histograms showing PI uptake.



Statistics

Student's *t*-test (paired and unpaired) was used to compare the reported mean values with standard deviations for the different variables. Groups being compared were considered to be significantly different if $p < 0.05$.

Results

Spontaneous NK cell apoptosis prior to cytotoxicity assay

We first examined the apoptotic cells in CB and APB purified NK cells after 72 h culture with and without IL-12 and/or IL-15. Freshly enriched NK cells from CB or APB are nearly 100% viable (data not shown). As shown in Table 1, without cytokines, a greater percentage of enriched CB NK cells underwent apoptosis as assessed by PI staining than corresponding APB NK cells ($18.8 \pm 4.3\%$ vs. $8.0 \pm 3.8\%$, $p < 0.05$). The percentages of apoptotic cells in CB NK cells significantly decreased on

Table 1. Effect of interleukin-12 (IL-12) and IL-15 on apoptosis of enriched natural killer (NK) cells from cord blood (CB) and adult peripheral blood (APB) using propidium iodide staining and analyzed with flow cytometry

	Media	IL-12 (1 ng/ml)	IL-15 (10 ng/ml)	IL-12 + IL-15
CB NK cells (n = 8)	$18.8 \pm 4.3^*$	$11.2 \pm 5.0^\dagger$	$8.4 \pm 2.2^{*\dagger}$	$10.2 \pm 4.4^\dagger$
APB NK cells (n = 6)	8.0 ± 3.8	8.4 ± 2.0	$15.0 \pm 7.8^\dagger$	$15.2 \pm 4.7^\dagger$

Data are expressed as percentage of cells (mean $\pm$ s.d.)

* $p < 0.05$, compared with corresponding APB NK cells.

$^\dagger p < 0.05$, compared with media.

incubation with IL-12 (1 ng/ml) ($11.2 \pm 5.0\%$, $p = 0.01$), IL-15 (10 ng/ml) ($8.4 \pm 2.2\%$, $p < 0.01$), and IL-12 + IL-15 ($10.2 \pm 4.4\%$, $p < 0.01$). In contrast to that observed with CB NK cells, the percentages of apoptotic APB NK cells was not influenced by IL-12 ($8.4 \pm 2.0\%$, $p = 0.34$), and was significantly increased with IL-15 ($15.0 \pm 7.8\%$, $p = 0.04$) and IL-12 + IL-15 ($15.2 \pm 4.7\%$, $p = 0.014$).

K562 cytotoxicity assessed by PI uptake

The cytotoxicity of untreated and cytokine-stimulated APB and CB NK cells against K562 targets after 72 h of incubation is shown in Fig. 2. Correlated well with chromium release assay (data not shown), K562 cytotoxicity of both CB and APB NK cells increased in an effector:target (E:T) ratio-dependant fashion. At an E:T ratio of 10:1, the CB NK activity in media alone was far less than APB NK activity ($5.1 \pm 2.1\%$ vs. $13.4 \pm 5.5\%$, $p < 0.05$). The addition of IL-12 enhanced the K562 cytotoxicity of CB compared with media alone

($10.5 \pm 6.7\%$, $p = 0.02$), the level of which was much lower than that of IL-12-treated APB NK cells ($20.9 \pm 12.6\%$). IL-15 enhanced the cytotoxicity of CB NK cells nearly eightfold ($40.4 \pm 18.2\%$), comparable with that of IL-15-treated APB NK cells. The combination of IL-12 + IL-15 did not, however, further enhance the cytotoxicity of CB NK cells ($40.6 \pm 19.5\%$) compared with IL-15 alone. Similar effect was observed at E:T ratios of 5:1 and 2.5:1.

Apoptosis in NK-K562 conjugates

From the cultures without cytokine treatment, the percentages of APB and CB NK cells that were engaged with K562 cells at the end of 4-h cytotoxicity assays were inversely correlated with the E:T ratios (Fig. 3). The percentage of CB NK cells conjugated with K562 was comparable with that of APB NK cells in each given E:T ratio tested (for E:T of 10:1, CB $9.0 \pm 5.1\%$ vs. APB $5.1 \pm 1.8\%$, $p = 0.12$; for E:T of 5:1, CB $14.7 \pm 8.3\%$ vs. APB $13.6 \pm 8.3\%$, $p = 0.81$; for E:T of 2.5:1, CB $22.2 \pm 11.4\%$ vs. APB $17.2 \pm 5.9\%$, $p = 0.36$). In addition, IL-12/IL-15 treatment did not influence the percentage of conjugated NK cells in CB or APB (data not shown).

Although IL-12 increases the apoptosis of APB NK-K562 conjugates compared with media, it did not affect the apoptosis of CB NK-K562 conjugates. Conversely, IL-15 and IL-12 + IL-15 greatly enhanced the apoptosis of CB and APB NK-K562 conjugates in an E:T ratio-dependant manner (Fig. 4). The apoptotic response of CB NK-K562 conjugates enhanced by IL-15 and IL-12 + IL-15 was lower than that of similarly tested APB NK cells, respectively.

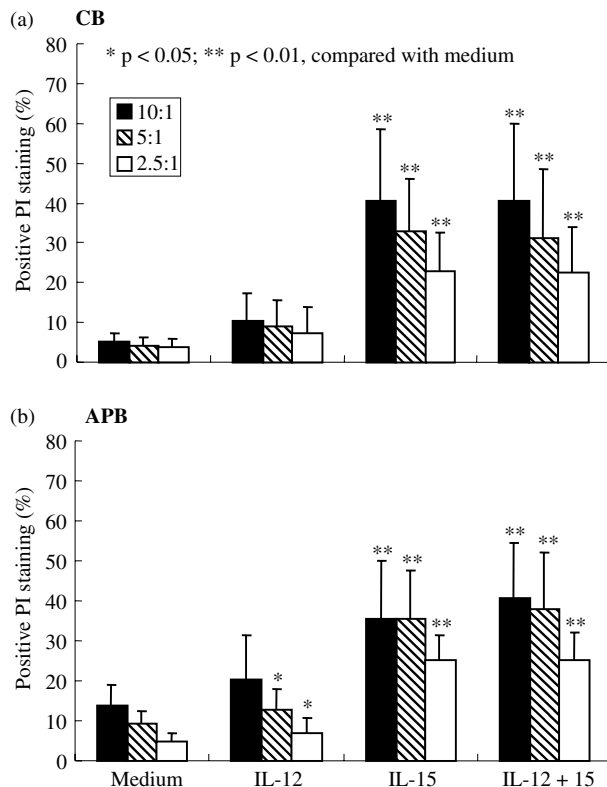


Fig. 2. Evaluation of cytokine-induced K562 cytotoxicity of enriched cord blood (CB; $n = 7$) (panel a) and adult peripheral blood (APB; $n = 6$) (panel b) NK cells as measured by the percentages of dead K562 cells in region 1 (R1) in Fig. 1 using effector/target (E:T) ratios of 10:1, 5:1, and 2.5:1.

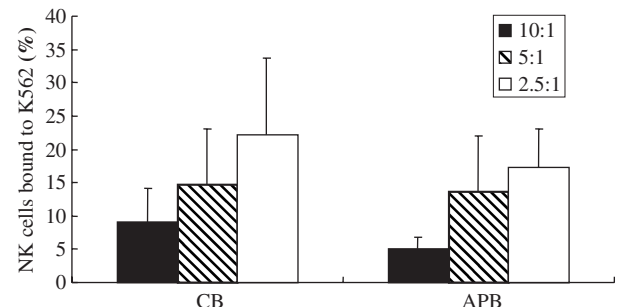


Fig. 3. Percentages of NK cells from cord blood (CB; $n = 5$) and adult peripheral blood (APB; $n = 4$) conjugated with K562 [cell number in region3/(cell number in region2 + cell number in region 3), as presented in Fig. 1] after 4-h culture using effector/target (E:T) ratios of 10:1, 5:1, and 2.5:1.

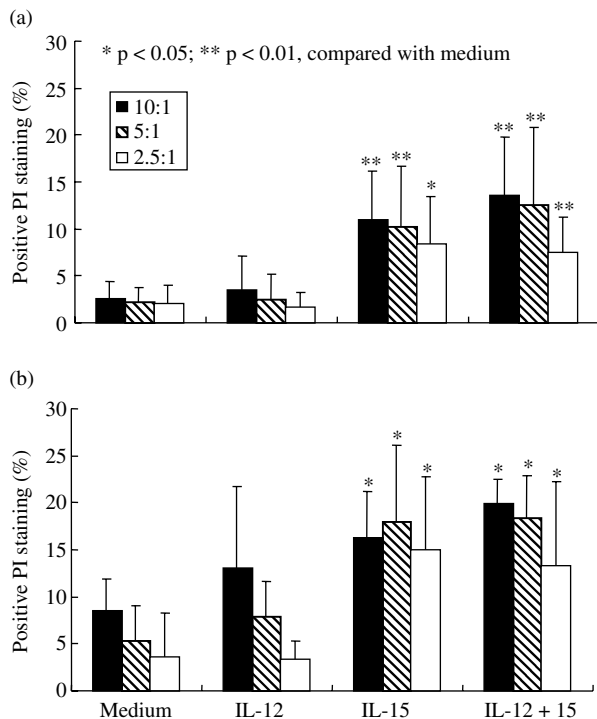


Fig. 4. Evaluation of cytokine-induced cell death from NK-K562 conjugates with either (a) cord blood (CB; $n = 7$) or (b) adult peripheral blood (APB) ($n = 6$) enriched NK cells, as measured by the percentages of dead cells in region 3 (Fig. 1) using effector/target (E:T) ratios of 10:1, 5:1, and 2.5:1.

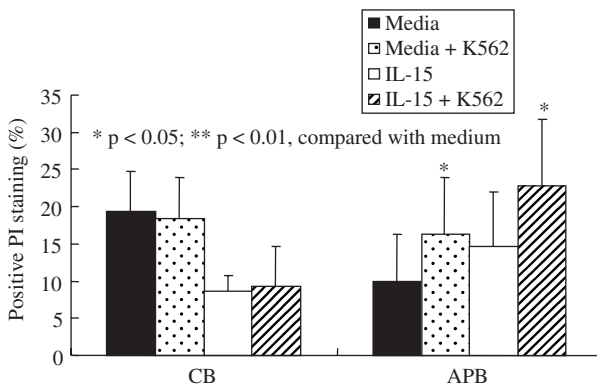


Fig. 5. Evaluation of K562-induced death cell of cord blood (CB; $n = 9$) and adult peripheral blood (APB; $n = 7$) enriched natural killer cells under the influence of IL-15.

Target-induced apoptosis in CB and APB NK cells

Natural killer cells underwent apoptotic process following contact with K562 targets. We therefore determined whether IL-12 and IL-15 would influence K562-induced apoptosis in CB and APB NK cells. This was assessed with the E:T ratio of 2.5:1, because most of the monoconjugates predominated in the cell mixture with this

ratio after a 4-h incubation (Fig. 3). As shown in Fig. 5, the percentages of apoptotic cells in both unstimulated and IL-15-stimulated APB NK cells increased following contact with K562 (for unstimulated APB NK cells, $14.2 \pm 6.7\%$ vs. $8.1 \pm 3.9\%$, $p = 0.03$; for IL-15-treated APB NK cells, $22.8 \pm 9.8\%$ vs. $13.0 \pm 6.1\%$, $p = 0.01$). The greatest degree of apoptosis was observed with IL-15-treated APB NK cells following contact with K562. In contrast, neither unstimulated nor IL-15-activated CB NK cells showed increased apoptosis following contact with K562 cells (for unstimulated CB NK cells, $19.4 \pm 5.3\%$ vs. $18.3 \pm 5.6\%$, $p = 0.59$; for IL-15-treated CB NK cells, $9.3 \pm 5.4\%$ vs. $8.7 \pm 2.0\%$, $p = 0.79$). IL-12, alone or in combination with IL-15, had little effect on K562-induced apoptosis in both CB and APB NK cells (data not shown).

Discussion

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 that in adult MNCs (16, 17). We have previously demonstrated the ability of IL-12 and IL-15 in enhancing various NK cytolytic function and CD16/56 expression in CB and human immunodeficiency virus-infected subjects (15). The effect of IL-12 and IL-15 on apoptosis and proliferation in CB mononuclear cells have also been demonstrated in PBMC cultures (18). Cytokine immunotherapy using IL-2, IL-12, and IL-15 may greatly benefit newborn at risk for severe infections because of their unique NK enhancing ability. The present study sought to characterize the CB NK functional defects by using purified NK population and observe their interaction with K562 cells under flow cytometry. We also examined how IL-12 and IL-15 would affect the NK cytotoxic process in CB and APB NK cells including target lyses, apoptosis in conjugated population, and the death in NK cells following contact with K562 cells.

No studies to date have examined the apoptosis of CB NK cells. The present study is the first to report that CB NK cells, like CB $CD4^+$ $CD45RA^+$ cells (19), underwent greater degree of spontaneous apoptosis than their adult counterparts. Expression of Fas was markedly lower on CB lymphocytes than on APB lymphocytes (20). Fas expression on CB NK cells was also far less than that on APB NK cells (our unpublished observation). Altered profiles of cytokine expression and cytokine deprivation may contribute in

part to enhanced CB NK loss by apoptosis. Although IL-15 functions as an NK cell survival factor by maintaining bcl-2 expression (21, 22), our data showed that adult NK cells exhibited cytokine-induced apoptosis with IL-12/IL-15 (Table 1). In agreement with Ross et al. (11), the increased percentage of apoptotic cells were observed in NK cells treated with IL-15 and IL-12 + IL-15, which would be involved in the autocrine release of tumor necrosis factor- α , by a Fas-independent apoptotic pathway. In contrast to the findings with APB NK cells, IL-12/IL-15 resulted in decreased apoptotic cells in CB NK populations. It may be due to the expansion of the viable CB NK cell pool by IL-12/IL-15 through their potent stimulation of NK proliferation. Consequently, this effect counterbalanced the cell death associated with cytokine-induced activation. Another possibility would be that CB NK cells responded more readily to IL-12/IL-15 with regard to certain anti-apoptotic molecules (such as bcl-2, Bcl-xL) up-regulation than did their adult counterparts.

The utilization of multi-parameter flow cytometry to evaluate K562 cytotoxicity was demonstrated to have strong correlation with the conventional ^{51}Cr release assays (14). It may prove to be even more sensitive as the changes in plasma membrane permeability allowing uptake of PI take place earlier than the changes required for the release of ^{51}Cr -bound intracellular proteins (14). IL-15 greatly enhanced the deficient K562 cytotoxicity of CB NK cells to levels comparable with that of adult NK cells similarly treated while IL-12 appeared to have less of an effect. Our findings support that IL-15, but not IL-12, is essential for both survival and differentiation for the less mature NK cells in CB (23), and are in agreement with Arment et al. who demonstrated the greater ability of IL-15 than IL-12 to protect NK cells from programmed cell death (24). IL-15-activated CB NK cells may therefore have an increased potential for a GVL effect compared with bone marrow NK cells (25, 26).

Previous studies on NK-K562 binding kinetics of PBMC using flow cytometry has demonstrated that the majority of the NK cells bind to K562 and quickly dissociated within the first hour and the conjugates contain no dead cells (14, 27). We found that the percentages of CB NK cells conjugated with K562 after 4-h contact, whether untreated or cytokine stimulated, were not inferior to those of APB NK cells, in agreement with Kohl et al. showing intact binding machinery of neonatal NK cells (28). Minimal apoptosis was observed within conjugates with unstimu-

lated CB and APB NK cells. An E:T ratio dependant increment of percentage of apoptosis in NK-K562 conjugates was observed with IL-15 and IL-12 + IL-15-treated CB NK cells, and to a greater extent, with APB NK cells, suggesting that the apoptosis involved mainly K562 cells. Thus, IL-15-activated NK cells delivered a more powerful hit resulting in the membrane changes of K562 cells before they dissociated from NK cells. Further studies at earlier time point will be warranted to investigate the cytokine effect on CB NK binding and killing of K562 target.

Target-induced apoptosis of CB NK cells had not been previously examined. IL-15-treated APB NK cells following 4-h exposure to K562 exhibited the greatest degree of apoptosis, confirming previous reports (13, 29). In contrast, CB NK cells, resting or IL-15 stimulated, were found to be relatively resistant to K562-induced apoptosis. Numerous surface ligands including CD16 (30) and Fas (31) are believed to be involved in NK-K562 interactions. Our previous studies have shown that CB NK cells responded more readily to IL-15 than did APB NK cells in terms of CD16 (32) and intercellular adhesion molecule-1 (CD54) (33) up-regulation. Additional work will be needed to elucidate the underlying mechanism explaining the resistance of IL-15-treated CB NK cells to target-induced death. The disadvantage of IL-2-activated NK cells (LAK cells) as an adoptive immunotherapy for malignancy due to increased NK apoptosis (13) may well be overcome by IL-15. NK cells recover early in CB recipients and serve to regulate GVHD and provide GVL activity (34). In view of the ability of IL-15-treated CB NK cells to boost K562 cytotoxicity to adult levels while at the same time exhibiting decreased tendency for target-induced apoptosis, IL-15 might prove useful as an immunomodulator in patients receiving CB transplantation and as an adjuvant for NK/LAK cell-based therapies for malignancies.

Acknowledgments

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