The effects of natural killer-cell depletion on *ex vivo* expansion of hematopoietic progenitor cells from umbilical cord blood

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Background and Objectives. The small total number of hematopoietic progenitor cells (HPC) in cord blood limited its use in adult recipients. Since the hematopoiesis might be controlled by both positive and negative factors, the finding of negative cellular components and thereafter depletion of them would be of importance for further expansion of HPC from cord blood *in vitro*. The role of natural killer cells (NK cells) in hematopoiesis remains unclear and needs to be elucidated.

Design and Methods. Cord blood mononuclear cells were co-cultured in a liquid culture system containing the hematopoietic cytokines interleukin (IL)-1, IL-3, IL-6, stem cell factor (SCF), granulocyte-monocyte colony-stimulating factor (GM-CSF) and G-CSF for a total of 20 days with or without depletion of NK cells.

Results. The percentage of $CD34^+$ cells was significantly higher in the NK-cell-depleted group at each time point (day 5, day 10, day 15, day 20). This finding was further confirmed by examination of functional HPC including CFU-GM and CFU-GEMM. The intracellular interferon (IFN)-γ and tumor necrosis factor (TNF)-α content of $CD56^+$ NK cells increased more than 10-fold after incubation with hematopoietic cytokines (day 4, day 8). Neutralization of IFNγ or TNFα, present in liquid culture, also significantly increased CFU-GM formation and $CD34^+$ cell content.

Interpretation and Conclusions. The results indicate that depletion of NK cells may be beneficial in producing hematopoietic progenitor cells from cord blood in *ex vivo* expansion systems. The effect of NK-cell depletion on *ex vivo* expansion of hematopoietic stem cells should be further assessed by a repopulating cell assay in NOD/SCID mice.

Key words: NK cells, hematopoiesis, cord blood, *ex vivo*.

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The remarkable expansion and cell renewal in the human hematopoietic system are thought to be supported by a small population of hematopoietic progenitor cells (HPC).[1,2] Because of its high content of HPC, umbilical cord blood (CB) has been extensively used as an alternative to bone marrow for transplants in patients with immunologic and hematologic disorders.[3,4] So far, more than 1600 CB transplants have been performed world-wide. Although clinical results are promising, around 80 % of CB recipients are pediatric patients.[5–8] At present the small number of HPC in CB limits the use of this strategy in most adult recipients. To overcome this limitation, several protocols for *ex vivo* expansion of HPC from CB have been developed in the last few years.[9–12] Establishing culture systems that facilitate *in vitro* maintenance and augmentation of stem cell activity is a major challenge in the expansion of HPC from CB. This is of great importance not only for HPC transplantation but also for gene therapy. It is also an important step towards cellular and molecular understanding of the regulatory mechanisms that mediate the commitment and self-renewal decisions of stem cells. A number of hematopoietic cytokines have been identified in the last two decades. It has been also revealed that hematopoietic cytokines, alone or in combination, support HPC proliferation and that it is possible to increase the number of progenitors in culture.[13] Recently, several groups of investigators have reported that combinations of early-acting cytokines support the maintenance or expansion of progenitor cells and even of transplantable stem cells in human systems.[14–17] On the other hand, the importance of stromal cells in the maintenance and expansion of primitive progenitor cells including transplantable stem cells has also been repeatedly demonstrated.[18–21] Until now, most research has been focused on the importance of supportive factors including soluble factors and stromal cells in *ex vivo* expansion of HPC from CB, while few studies have looked for inhibitory factors, especially cellular components with a negative regulatory function on hematopoiesis. In view of their inhibitory effects, a few soluble factors, such as interferon (IFN)-γ and tumor necrosis factor (TNF)-α,[22–26] and cellular components, such as $CD8^+$ Tc cells[27–29] were reported to exert a negative regulation on the hematopoietic process. Since the hematopoietic process might be controlled by both positive and negative factors, the finding of negative cellular components in hematopoiesis and depletion of such negative components would have important consequences for further expansion of HPC from CB.

In the present study, we addressed whether NK cells represent an inhibitory cellular component in the hematopoiesis of CB by depleting NK cells during the *ex vivo* expansion of HPC of CB. The rest of the cellular components of mononuclear cells (MNC) from CB are also investigated and compared with NK-cell depletion in the hematopoiesis of CB.

Design and Methods

Cytokines and antibodies

Recombinant human (rh) interleukin (IL)-1, rhIL-3, rhIL-6, rh stem cell factor (SCF), rh granulocyte colony-stimulating factor (G-CSF) and rh granulocyte-monocyte colony-stimulating factor (GM-CSF) were purchased from R&D Systems (Minneapolis, MN, USA). The concentrations of the cytokines were 10 ng/mL as previous reported.[9,15–17] Anti-IFNγ monoclonal antibody (mAb), anti-TNFα mAb, anti-CD3 mAb, anti-CD14 mAb, anti-CD19 mAb and anti-CD56 mAb (clone B159 or NCAM16.2) for flow cytometry were purchased from BD Pharmingen (San Diego, CA, USA). The concentrations of mAb used were those recommended by the manufacturer.

Liquid culture of cord blood mononuclear cells (CB-MNC), cytokine neutralization and depletion of cells

CB was collected, after the mothers' informed consent, from full-term neonates according to guidelines established by the Human Subjects Protection Committee of our institution. After delivery of the baby, the umbilical cord was clamped and disinfected and CB was recovered with the placenta into sterile CB collection bags containing 29 mL of citrate-phosphate dextrose (CPD) as anticoagulant. Cord blood was collected in sterile tubes and was processed within 24 h of birth. After sedimentation of RBC by incubating CB samples with the same volume of 6% (w/v) hydroxyethyl starch dissolved in Ringer's solution (Veen-D Inj., Nikken Chemical, Tokyo, Japan) at room temperature for 30 min, low density (<1.077 g/mL) mononuclear cells were collected with Ficoll-Paque (Pharmacia Biotech, Uppsala, Sweden). A total of 2×10^4 $CD34^+$ cells (around 2×10^6 CB-MNC cells) were cultured in quadruplicate in flat-bottomed 24-well plates in 1 mL of Iscove's modified Dulbecco's medium (IMDM; GIBCO BRL, Grand Island, NY, USA) supplemented with 10% FCS (Hyclone, Logan, UT, USA), 10% HS and conditions containing cytokines including IL-1, 3, 6, SCF, G-CSF and GM-CSF at a concentration of 10 ng/mL for each cytokine. Because of the short half-life of the cytokines, these were added every other day to the cultures. In order to neutralize cytokines released by cells into the culture supernatant, anti-IFNγ mAb (10 μg/mL) and/or anti-TNFα mAB (10 μg/mL) were added to the liquid culture, according to the manufacturer's guidance based on the antibody-antigen (i.e. IFNγ and TNFα here) reaction, at the beginning of the experiment and again when half the medium was replaced each week to maintain the concentration of antibodies. At initiation of the culture the number of $CD34^+$ cells, and the CFU-GM and CFU-GEMM content in 1 mL were determined. Each week half of the medium was removed and replaced with fresh medium and growth factors. Aliquots were removed from the medium and the cells were counted and analyzed for $CD34^+$, CFU-GM and CFU-GEMM.

Regarding NK-cell depletion, CB-MNC cells were incubated with anti-CD56 mAb (clone AF12-7H3) and rabbit complement for 60 minutes at 37°C in 5% CO_2 in order to deplete $CD56^+$ cells by complement-dependent cellular cytotoxicity (CDCC). The treated cells were then further depleted of $CD56^+$ cells using a modification of the positive immunoselection technique with Ab-coated magnetic beads according to the manipulation manual supplied by MACS Company (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). The monoclonal antibody AF12-7H3 produced by the MACS Company recognizes a CD56 epitope which is distinct from the epitopes recognized by the CD56-specific mAbs NCAM16.2 and B159 produced by BD Pharmingen. Briefly, the cells (anti-CD56 mAb plus complement-treated cells) were incubated with anti-CD56 mAb (clone AF12-7H3) for 30 min and cells combining with magnetic microbeads were retained in the column during magnetic separation, while the CD56 negative cells (NK-cell-depleted CB-MNC) passed through the column. These NK-cell-depleted CB-MNC were used for liquid culture in the presence of hematopoietic cytokines. $CD3^+$ T cells, $CD14^+$ monocytes and $CD19^+$ B cells were depleted using similar procedures as those used for NK-cell depletion.

Colony assays

Colony assays were performed as previously reported by us.[30] Briefly, the purified CB-MNC cells and those obtained after liquid culture were plated in duplicate in 35 mm tissue culture plates at concentrations of 2,000 cells/mL. The medium contained 0.9% methylcellulose, 30% FBS, 1% BSA, 10^{-4} M 2-mercaptoethanol, 50 ng/mL SCF, 10 ng/mL GM-CSF, and 10 ng/mL IL-3 (StemCell Technologies, Vancouver, Canada). Cultures were incubated for 14 days at 37°C in a 5% CO_2 fully humidified atmosphere and colony-forming units (CFU)-GM, identified as colonies of >50 translucent cells, were scored by microscopy. For CFU-GEMM, IL-3 (10 ng/mL), GM-CSF (10 ng/mL), erythropoietin (3 U/mL) and SCF (50 ng/mL) were added. The next steps were the same as those for CFU-GM.

Flow cytometric analysis

Flow cytometry was performed as previously reported by us.[31] Initial and cultured cells were evaluated for the expression of CD34 antigen. Cells were labeled with the monoclonal antibody anti-CD34 PE (HPCA-2; Becton Dickinson, San Jose, CA, USA). Labeling was allowed for 30 min in the dark at 4°C. The cells were then washed twice in PBS + 1% BSA and analyzed with a FACScalibur flow cytometer (Becton Dickinson). At least 5,000 events were acquired for each analysis. When fluorescent cells represented only a minority of the total population, many more events were considered (20,000). Analysis was performed with Cell Quest software (Becton Dickinson). The depleting efficiency of NK cells, T cells, B cells and monocytes from CB-MNC cells was investigated by phenotypic analysis using flow cytometry. Aliquots of cells were stained with FITC- and PE-conjugated monoclonal antibodies in PBS + 0.1% BSA at 4°C for 30 min. Antibodies used were as follows: FITC-conjugated antibodies to CD3 and CD19, and PE-conjugated antibodies to CD56 and CD14. FITC- and PE-conjugated mouse IgG1 antibodies (DAKO) were used as isotype-matched controls. Dead cells were gated out with a forward vs side scatter window and propidium iodide staining. Intracellular staining for IFNγ and TNFα expression was performed by means of the Pharmingen Intracellular Staining Kit. The surface staining was first performed with PE-conjugated anti-CD56, and then cells were permeabilized by a saponin-based method (Pharmingen) and stained with CyChrome-anti-IFNγ or anti-TNFα.

Statistical analysis

The Wilcoxon signed-rank sum test was used to determine the significance of differences between paired groups. Values of p lower than 0.05 were considered as statistically significant. Results are reported as median and range. Experiments were performed at least 3 times with a representative experiment being shown.

Results

NK-cell depletion enhanced expansion of hematopoietic progenitor cells of cord blood

In order to obtain a new *ex vivo* amplification system of cord blood mononuclear cells (CB-MNC) without contamination by NK cells, the CB-MNC were depleted by two steps of treatment: treatment of CB-MNC by anti-CD56 antibodies plus rabbit complement (CDCC) and further treatment of anti-CD56-depleted CB-MNC by passing them through the column containing CD56-positive magnetic beads. The results of the NK-cell depletion were then analyzed by flow cytometry and are shown in Figure 1. The two anti-CD56 antibodies, one for CDCC and positive selection with magnetic beads (clone AF12-7H3, MACS company) and another one for flow cytometric analysis (clone NCAM16.2, BD Pharmingen), are from two different hybridoma clones and target the separate epitopes on the CD56 molecule. CD56+ NK cells were totally depleted from CB-MNC. The NK-cell-depleted or -undepleted (control) CB-MNC, which were regulated to the same concentration of CD34+ cells, were then co-cultured in a liquid culture system containing 6 kinds of cytokines (IL-1, IL-3, IL-6, SCF, GM-CSF and G-CSF) for a total of 20 days. As shown in Figure 2A, the percentage of CD34+ cells increased from 21.7% to 37.5% after five days' incubation (day 5), from 22.1% to 35.7% at day 10, from 12.3% to 32.5% at day 15 and from 3.7% to 23.6% at day 20. The absolute amounts of CD34+ cells in the NK-cell-depleted group were 1.88, 1.75, 1.84 and 1.63 fold higher than those in the control group at day 5, day 10, day 15 and day 20, respectively, when the starting concentration of CD34+ cells was regulated to 2×10^4/mL (Figure 2B). The flow cytometric analysis of CD34+ cells, including percentages and absolute numbers, was further confirmed after examination of functional hematopoietic progenitor cells (HPC) including CFU-GM (Figure 2C) and CFU-GEMM (Figure 2D). The results indicate that NK cells in CB play an inhibitory role in hematopoiesis, and depletion of NK cells in our liquid culture system benefited the *ex vivo* expansion of HPC from CB.

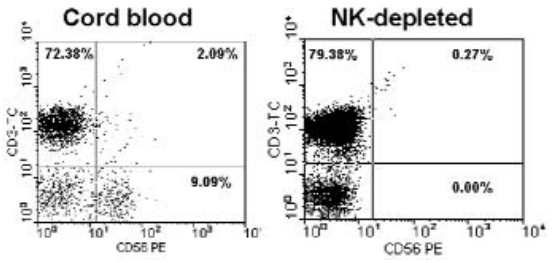

Figure 1. Depletion of NK cells from cord blood mononuclear cells. Cell depletion was performed in two steps: the first step was to add anti-CD56 mAb together with rabbit complement into CB-MNC and incubate for one hour (CDCC-treated); the second step was to load the CDCC-treated cells into a column containing CD56 Ab-coated magnetic beads according to instructions in the MACS manipulation manual. The pass-through cells were examined by flow cytometry using double staining with anti-CD56-PE and anti-CD3-FITC. The mAbs for NK-cell depletion and flow cytometry targeted a distinct epitope on the CD56 molecule.

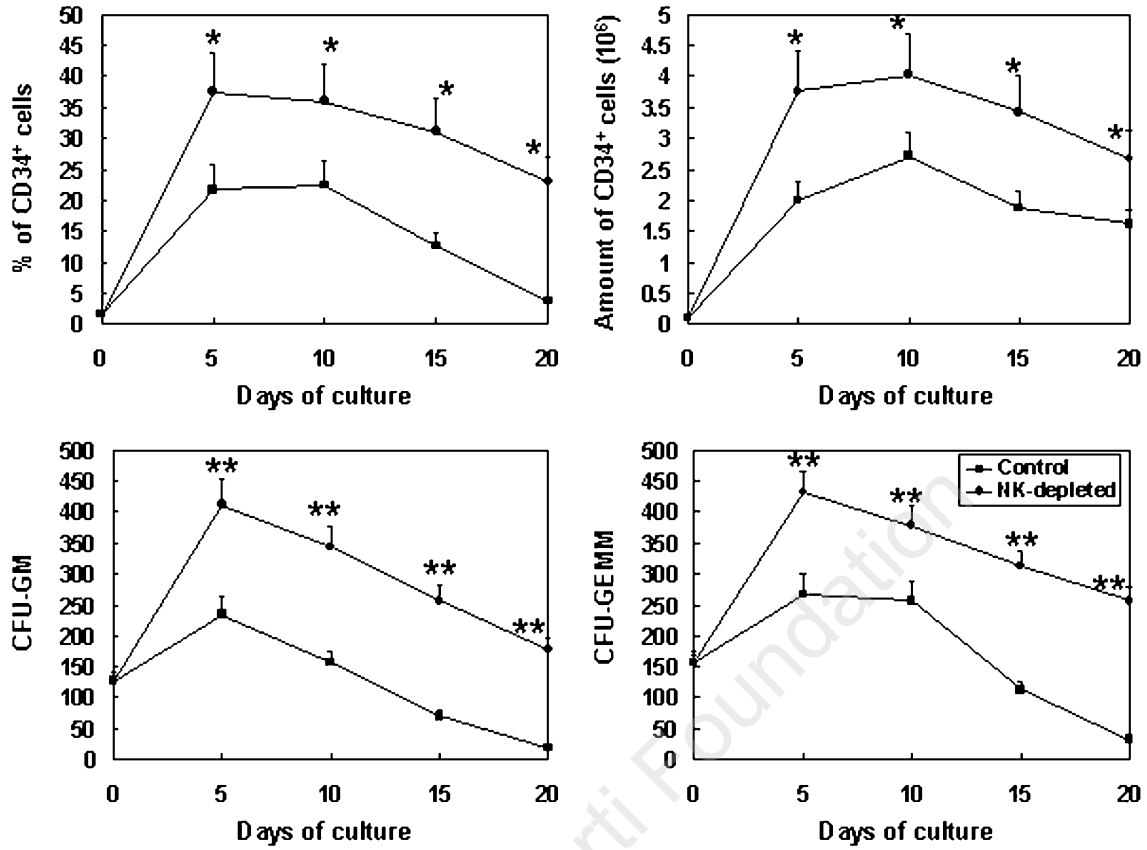

Figure 2. Effects of NK-cell depletion on expansion of hematopoietic progenitor cells from cord blood. NK-cell-depleted and -undepleted CB-MNC cells (around 2×10^6/mL/well containing 2×10^4 CD34+ cells) were incubated in 24-well plastic plates in liquid culture containing IL-1, 3, 6, SCF, GM-CSF and G-CSF for a total of 20 days. The HPC contents were analyzed at day 5, 10, 15 and 20 by flow cytometry to detect CD34+ cells [(A) percentage by flow cytometry and (B) absolute cumulative amount of CD34+ cells by multiplying the percentage by the total MNC cell number], and colony assays to detect contents (per 2,000 MNC cells) of CFU-GM (C) and CFU-GEMM (D) as described in Design and Methods. The data were from one representative experiment with three separate cord blood samples.

NK cells inhibit expansion of hematopoietic progenitor cells partly by producing IFNγ and TNFα

We then investigated the underlying mechanisms of how NK cells inhibited hematopoiesis of CB-MNC in our *ex vivo* expansion system. Though NK cells produce a variety of soluble factors to improve or inhibit hematopoiesis and lymphopoieis, the best recognized cytokines are IFNγ and TNFα, which are produced in relatively large amounts by NK cells. Interestingly, as shown in Figure 3, intracellular IFNγ and TNFα were almost not expressed by NK cells in freshly isolated CB-MNC (day 0), but their percentage increased more than 10-fold in whole MNC after 4 or 8 days of incubation (day 4, day 8) in the liquid culture system containing 6 cytokines which might activate NK cells. If calculating the percentage of NK cells themselves, around 50% of NK cells were involved in the production of both cytokines.

We speculate that the decline of HPC production in long-term cultures is correlated to products of activated NK cells, thus anti-IFNγ or anti-TNFα or a combination of both antibodies was added to the liquid culture. The antibodies eliminate almost all their corresponding soluble antigens (i.e. IFNγ and TNFα) in the supernatant by antigen-antibody reaction. As shown in Table 1, neutralization of IFNγ or TNFα significantly increased CFU-GM formation ($p<0.05$) and CD34+ cell content ($p<0.05$), the increased magnitudes at day 10 being about 50% for both anti-IFNγ and anti-TNFα groups in five separate experiments each with 3-6 samples. The combined neutralization of IFNγ and TNFα most significantly increased CFU-GM formation ($p<0.01$) and CD34+ cell contents ($p<0.05$), the increase in both CFU-GM and CD34+ cell content being around 75.7% to 100% in this case. The results indicate that IFNγ and TNFα, at least partly produced by acti-

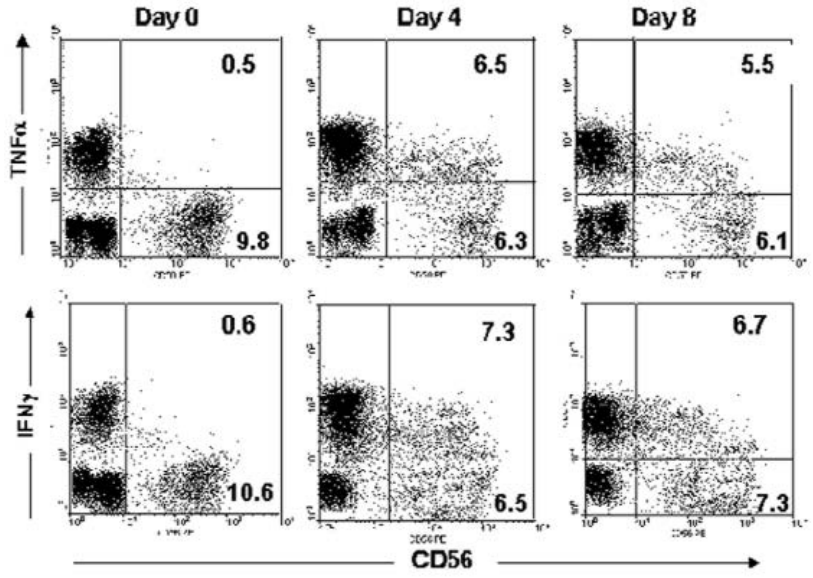

Figure 3. Intracellular IFNγ and TNFα of NK cells in liquid culture. CB-MNC cells were incubated as in Figure 2. Intracellular staining for IFN and TNFα expression was performed on day 0, day 4 and day 8 of liquid culture using the Pharmingen Intracellular Staining Kit. Dead cells were gated out with a forward vs side scatter window. The surface staining was first performed with phycoerythrin (PE)-conjugated anti-CD56, and then cells were permeabilized by a saponin-based method (Pharmingen) and stained with CyChrome-anti-IFN- or -TNFα mAbs.

vated NK cells, are responsible for inhibiting HPC amplification in CB-MNC cultures and that neutralization of both cytokines may improve production of HPC from CB.

Comparison of NK-cell depletion with depletions of other immunocompetent cells

Since there are a variety of MNC cells in CB, including $CD3^+$ T cells, $CD19^+$ B cells and $CD14^+$ monocytes (Mo), and it was reported that $CD3^+CD8^+$ Tc cells might inhibit hematopoiesis in leukemia and normal marrow as reported previously,[27-29] we compared NK-cell depletion with depletion of other MNC cells in amplification of HPC from CB-MNC. As shown in Table 2, $CD3^+$ T-cell depletion exerted similar results as NK-cell depletion in CFU-GM formation when examined at day 10 of co-culture. Interestingly, combining depletion of NK cells and T cells did not improve HPC production but, in contrast, inhibited CFU-GM formation. $CD19^+$ B-cell or $CD14^+$ Mo depletion did not change CFU-GM formation, and as speculated, simultaneous depletion of NK cells, T cells, B cells and Mo cells further reduced HPC production compared with simultaneous depletion of NK cells and T cells. The results indicate that MNC play an important role in hematopoiesis in that NK cells or T cells may exert inhibitory effects on *ex vivo* expansion of CB-MNC and that depletion of NK cells or T cells, but not their combined depletion, may benefit the expansion of HPC from CB.

Discussion

The development of methods to expand HPC *in vitro* would have a profound impact on stem cell transplantation. Therefore, *ex vivo* expansion of HPC cells is currently a major research area in clinical settings. In the last few years a number of studies have been performed to identify culture conditions that are able to expand primitive hematopoietic progenitors, especially transplantable stem cells. CB transplantation is an increasingly encouraging alternative to BM transplantation because of the easier accessibility of this source of stem cells and the possible allowance of more HLA mismatches. Despite the positive findings so far, the clinical use of such protocols requires additional consideration and work. The expansion procedures must be scaled-up to fit the body size requirements of human recipients, since the outcome of CB transplantation is significantly influenced by the number of cells infused into the recipient.[7,32] The current evidence demonstrates that the clinical requirement for CB transplant is more than 37×10^6 nucleated cells per kg body weight.[7,33] To overcome this limitation, a number of protocols for *ex vivo* expansion of CB-HPC have been developed.[9-12] The clinical efficacy of CB expansion has been recently evaluated by Shpall *et al.*[34] and McNiece *et al.*[35] Their results suggest that expanded CB cells may be a promising alternative source of HPC for cancer patients receiving high-dose chemotherapy. Dick's group demonstrated a 2- to 4-fold expansion of human CB stem cells in 4 days' culture supplemented with SCF, FL, G-CSF, IL-3 and IL-6.[15] Aglietta's group showed extensive and sustained (more than 6 months) amplification of human CB primitive progenitors using suspension cultures supplemented with TPO and FL,[16] and subsequently demonstrated that transplantable stem cells were also greatly expanded under the same culture conditions.[17] In contrast to these

Table 1. Anti-cytokine treatment for expansion of CFU-GM and CD34+ cells from cord blood.

Exp	Control		Anti-IFNγ		Anti-TNFα		Anti-IFNγ+ Anti-TNFα	
	CFU-GM	CD34 %	CFU-GM	CD34 %	CFU-GM	CD34 %	CFU-GM	CD34 %
1 (n=3)	220±16	21.33±4.43	331±21*	33.65±6.73*	326±17*	31.13±5.33*	387±25°	38.74±6.43*
2 (n=3)	21±13	20.33±3.40	348±20*	35.12±6.41*	316±18*	32.33±5.03*	432±27°	39.13±5.83*
3 (n=3)	205±12	21.33±4.42	312±18*	37.55±5.93*	327±23*	33.83±5.12*	412±24°	39.15±5.77*
4 (n=5)	215±11	19.33±3.21	335±16*	36.01±5.22*	305±22*	34.33±5.49*	405±22°	39.77±4.43
5 (n=6)	223±12	18.39±3.12	365±19*	38.31±4.44*	325±24*	35.33±5.11*	415±24°	41.89±6.43*

*Anti-IFNγ mAb (10 μg/mL) and anti-TNFα mAB (10 μg/mL) were added to liquid culture containing IL-1, IL-3, IL-6, SCF, GM-CSF and G-CSF at the beginning of the experiment and again when half the medium was replaced each week to maintain the concentration of antibodies. The contents of CFU-GM and CD34± cells in HPC were assayed on day 10. The data are from five representative experiments, each with three to six separate cord blood samples. The CFU-GM assays and flow cytometry for CD34± cells were performed as described in Design and Methods. *p< 0.05; °p< 0.01 when compared with control group.*

recent studies using stroma-free culture, however, the importance of cell-cell interactions between stromal cells and HPC in early stages of hematopoiesis has been repeatedly reported. Various stromal cell lines have been established that support long-term hematopoiesis in culture. Some of these have been shown to be capable of supporting maintenance or expansion of transplantable stem cells.[18-21] Protocols including stromal-free liquid culture and stromal-dependent culture for preclinical studies will further increase the understanding of hematopoietic processes and ultimately improve the expansion of HSC from CB and thus clinical applications.

When NK cell-depleted or –undepleted (control) CB-MNC were cultured in a liquid culture system containing hematopoietic cytokines, the content of CD34+ stem cells was increased at each time point (day 5, 10, 15 and 20) (Figure 2). There were two possible effects of NK-cell depletion: first, NK-cell depletion might improve the renewal of CD34+ stem cells by increasing the percentage and absolute number of CD34+ cells at each time point; secondly, NK-cell depletion might retard the apoptosis or cell death of CD34+ cells by decreasing the decline of CD34+ cell content in late stages of culture. We speculate that NK cells in a liquid culture system can induce apoptosis or cell death or inhibit renewal of CD34+ cells through soluble factors (for example IFNγ and TNFα) and/or cell-to-cell contact after responding to hematopoietic cytokine stimuli. Although a decrease in the number of CD34+ cells in the liquid culture system might result from a extremely complicated process including modulations to both the cell cycling of CD34+ cells and regulatory factors such as cellular or soluble factors, we for the first time found that NK cells play a critical role in negatively regulating hematopoiesis of CB. Regarding inhibitory soluble factors, it was noted that after co-culture with hematopoietic cytokines, NK cells produced large amounts of cytokines such as IFNγ and TNFα (Figure 3), which have been convincingly shown to inhibit hematopoiesis by several laboratories[22-26] and us in this study (Table 1). It is well known that NK cells are major producers of IFNγ and TNFα after *in vivo* and *in vitro* stimuli,[36-40] so an activation-induced hematopoiesis-inhibiting function (including expression of IFNγ and TNFα) of NK cells by hematopoietic cytokine stimuli should be considered when liquid culture of CB is used as an *ex vivo* expansion system. Because NK cells might continuously produce several kinds of inhibitory cytokines, such as IFNγ and TNFα, several antibodies against each cytokines must be continuously added to the liquid culture. Thus, in this situation depletion of NK cells, which needs to be performed only once *in vitro*, would be a better choice than neutralization of inhibitory cytokines. Regarding cell-to-cell contacts, our observations corroborate reports from several other laboratories that murine NK cells may inhibit dendritic cell maturation by cell-to-cell contact[41] and that a murine NK-cell subset may inhibit hematopoiesis by contact between NK cells and CD34+ cells.[42] So, it is possible that NK cells play an inhibitory role on a variety of blood cells by mechanisms of cell-to-cell contact through membrane molecules.[43] Whether NK cells inhibit hematopoiesis of CB in liquid culture by cell-to-cell contact, and, if so, which membrane molecule (s) and their ligand (s) are involved in these processes are currently under investigation in our laboratory.

In view of the potential clinical applications, an additional objective of our *in vitro* study was to evaluate whether other lymphocytes or monocytes also inhibit hematopoiesis of CB. Since CB contains a variety of MNC, including T cells, B cells and monocytes, in addition to NK cells, we compared NK-cell depletion with depletion of other MNC

Table 2. Comparison of NK-cell depletion with depletion of other cells for expansion of CFU-GM from cord blood.

Exp	*Control*	*-CD56*	*-CD3*	*-CD19*	*-CD14*	*-CD3,56*	*-CD3,14,19,56*
1 (n=3)	249±12	436±25°	386±19°	218±13	257±15	137±11	67±5
2 (n=3	231±11	417±27°	412±21°	198±14	262±19	146±11	51±7
3 (n=3)	282 ±14	416±23°	405±23°	25212	201±15	157± 12	34±5

*Cell depletion was performed in two steps: the first step was to add a specific mAb together with rabbit complement into CB-MNC and incubate for one hour (CDCC-treated); the second step was to load the CDCC-treated cells onto a column containing MACS specific Ab-coated magnetic beads according to the instructions in the manipulation manual. The pass-through cells were specific Ab-free cells examined by flow cytometry. The specific Ab against T cells is anti-CD3 mAb, that for NK cells is anti-CD56 mAb, for B cells it is anti-CD19 mAb and for monocytes it is anti-CD14 mAb. The control group cells were treated with normal murine serum plus rabbit complement, and then passed through a column containing normal murine serum-coated magnetic beads. CFU-GM of HPC in liquid culture was assayed on day 10. The data were from three representative experiments, each with three separated cord blood samples. *p< 0.05; °p< 0.01 when compared with control group.*

subsets in amplification of HPC from CB MNC. $CD3^+$ T-cell depletion exerted similar results as NK-cell depletion on CFU-GM when examined at day 10 (Table 2). The underlying mechanisms of the inhibitory effects of T cells on hematopoiesis of CB are probably similar to those of NK cells, since T cells are also major producers of IFNγ and TNFα. Interestingly, combined depletion of NK cells and T cells did not improve HPC production but, on the contrary, inhibited CFU-GM formation and $CD34^+$ cell content. In this regard, it is possible that the decreased hematopoiesis of CB is due to an absolute reduction of the supply of hematopoietic cytokines or membrane molecules in a total amount supplied by NK cells and T cells together. This indicates that NK cells and/or T cells probably supply supportive factors for hematopoiesis such as hematopoietic cytokines (IL-3, GM-CSF, etc.) and membrane molecules (CD40L, ECM, VCAM, CXCR4, etc.),[43] which are essential for growth or renewal of HPC of CB, at the same time as producing inhibitory factors. We speculate that NK cells supply a similar pattern of cytokines, both inhibitors and activators, as do T cells. The second possibility is that the inhibition of HPC could be due to a cytotoxic depletion mechanism whereby CDCC-induced cytolysis of a larger number of NK cells and T cells causes the release of cytotoxic factors in culture, hence reducing the number of primitive cells available for expansion. We added the supernatant from CDCC-induced cytolysis to a similar CB-MNC culture system, and observed that the exchange of culture medium did not influence HPC expansion, indicating that the possibly released inhibitory soluble factors do not interfere with the *ex vivo* expansion of HPC from CB (*data not shown*). In addition, B-cell or monocyte depletion did not change CFU-GM formation, indicating that CDCC itself did not affect the *ex vivo* expansion. Simultaneous depletion of NK cells, T cells, B cells and monocytes resulted in a larger reduction in HPC than did simultaneous depletion of NK cells and T cells. The results indicate that MNC in CB play a complicated role in hematopoiesis, and that NK cells (or a subset) or T cells may exert inhibitory effects on *ex vivo* expansion of CB-HPC.

In this study, we examined $CD34^+$ cells, CFU-GM and CFU-GEMM to calculate the amounts of hematopoietic progenitor cells (HPC), but we cannot estimate the number of hematopoietic *stem cells* exactly by the *in vitro* colony assay method. The number of hematopoietic stem cells could be assessed by specific *in vivo* experiments such as the long-term NOD/SCID-repopulating cell assay.[44-46] The next step is to elucidate the effect of NK-cell depletion on *ex vivo* expansion of hematopoietic *stem cells*, which is more important in hematopoiesis research and has implications for clinical stem cell transplantation, by using NOD/SCID-repopulating cell assays. The effect of NK-cell depletion might also differ depending on the source of the HPC. It would also be very interesting to know whether NK-cell depletion affects the *ex vivo* expansion of hematopoietic progenitor cells (or even NOD/SCID-repopulating *stem cells*) from adult peripheral blood $CD34^+$cells or bone marrow, which are more extensively used in clinical practice. The effect of NK-cell depletion might also be different depending on the donor situations. If NK-cell depletion affects expansion of HPC for autologous BM or peripheral blood stem cells of patients suffering from various specific diseases, the possible utilization in clinical stem cell transplantation will be obviously widened.

In conclusion, cord blood mononuclear cells were co-cultured in a liquid culture system for a total of 20 days with or without depletion of NK cells. The percentage and absolute amounts of $CD34^+$ cells increased significantly in the NK-cell-depleted cultures. This finding was further confirmed by examination of functional hematopoietic progenitor cells including CFU-GM and CFU-GEMM. Intracellular IFNγ and TNFα in $CD56^+$ NK cells were increased more than 10-fold after incubation with

hematopoietic cytokines; neutralization of IFNγ or TNFα present in the liquid culture using specific antibodies also significantly increased CFU-GM formation and CD34+ cell content. The results indicate that depletion of NK cells may be beneficial to produce hematopoietic progenitor cells from cord blood in an *ex vivo* expansion system for clinical stem cell transplantation. The inhibitory effect of NK cells on hematopoiesis is at least partly the result of these cells' soluble products, IFNγ and TNFα. The effect of NK-cell-depletion on hematopoietic stem cells should be further assessed by using an *in vivo* repopulating cell assay in NOD/SCID mice.

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Pre-publication Report & Outcomes of Peer Review

Contributions

ZT hypothesized the inhibitory effects of NK cells on hematopoiesis, designed the experiments to compare each group, performed the cellular experiments and flow cytometric analysis and wrote the paper. RS contributed to the novel ideas on inhibitory effects of NK cells on hematopoiesis, co-designed and performed most experiments including the colony assay and flow cytometry, and gave substantial contributions to data interpretation and critical review of the draft. HW and JZ participated in the discussion of the experimental design and most of the experiments, especially the *ex vivo* expansion of cord blood cells. All authors approved the version for publication and are listed according to a criterion of decreasing individual contribution to the work, with the exception of the last author (ZT) who had the most important role in the whole research and writing the paper.

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Disclosures

Conflict of interest: none.

Redundant publications: no substantial overlapping with previous papers.

Manuscript processing

This manuscript was peer-reviewed by two external referees and by Professor Mario Cazzola, Editor-in-Chief. The final decision to accept this paper for publication was taken jointly by Professor Cazzola and the Editors. Manuscript received December 4, 2002; accepted March 27, 2003.

In the following paragraphs, the Editor-in-Chief summarizes the peer-review process and its outcomes.

What is already known on this topic

Although a huge number of investigations have been done on *ex vivo* expansion of hematopoietic cells from umbilical cord blood, few studies have examined the effect of removal of inhibitory factors.

What this study adds

This study shows that depletion of NK cells may be beneficial in producing hematopoietic progenitor cells from cord blood in *ex vivo* expansion systems.