Original Article

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Differentiation and Characterization of Natural Killer Cells Derived from Human Umbilical Cord Blood Mononuclear Cells

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Objective: Human umbilical cord blood (UCB) is widely used for the isolation of mononuclear cells (MNCs) and development of hematopoietic stem cells for clinical use. This study aimed at establishing an in vitro system for the induction of natural killer (NK) cell differentiation from human UCB-derived MNCs (UCB-MNCs) and characterizing their phenotypic changes and tumor-killing activity in vitro.

Methods: Previously cryopreserved UCB-MNCs were cultured with Flt3L and IL-15 for the induction of NK cell differentiation. Two other commercially available media were used to compare the potency in inducing NK cell differentiation and expansion. The additive effects of IL-21 on IL-15-initiated NK cell expansion and cytotoxic function were also examined by using direct cell counting and tumor-killing assay, respectively.

Results: Flow cytometry showed successful differentiation of UCB-MNCs into NK cells. We could expand NK cells up to 21 days with the cell number being increased up to 40 folds. Addition of IL-21 could significantly enhance the tumor-killing activity of induced NK cells.

Conclusions: The study provides a useful model for the establishment of NK cell expansion and differentiation from clinical specimens and for monitoring the phenotypic characteristics and cytotoxic function of induced NK cells. It is also a feasible platform for studying the mechanisms of NK differentiation processes or testing the efficacy of potential drugs for enhancing the differentiation or tumor-killing activity of NK cells.

Key words: nature killer cells, umbilical cord blood, interleukin-15, interleukin-21, tumor-killing activity

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Introduction

Human umbilical cord blood (UCB) has long been widely used for the isolation of mononuclear cells (MNCs) and further development of hematopoietic stem cells.[1] The UCB-derived MNCs (UCB-MNCs) contain adequate progenitor cells with characteristics different from those of adult bone marrow or peripheral blood stem cells. During the past two decades, human UCB has been donated worldwide and stored for use by unrelated patients with hematologic malignancies and/or bone marrow disorders who have no matched donors.[2] UCB has also been stored privately for autologous transplantation. Recent advances in stem cell research support the proposal that UCB cells are a more primitive population than adult marrow cells with several distinct advantages over other adult stem cell sources. Compared with bone marrow-derived stem cells, UCB-derived progenitor cells have longer telomeres, higher proliferation potential, reduced risks of viral contamination and graft-versus-host disease during allogeneic grafts as well as a better tolerance for human leukocyte antigen (HLA) mismatches.[3] The main advantages of UCB cells include the non-invasiveness of collection, easy characterization, and convenient storage.[4] Therefore, UCB is a clinically applicable and readily accessible cell source for allogeneic or autologous cell transplantation. Increasing evidence supports that the pluripotency of UCB-MNCs gives rise to adherent mesenchymal stem cells (MSCs) that have the capacity for multilineage differentiation,[5] including chondrocytes, osteocytes, adipocytes, myocytes, and neurons.[6] In particular, they can be harnessed for differentiation into different cells of hematopoietic lineages for therapeutic purposes.[7-9] The capability of UCB-MNCs suggests their therapeutic potential for various diseases.

Immune effector cells without major histocompatibility complex restriction, such as natural killer (NK) cells,[10] natural killer T (NKT) cells,[11] and gamma-delta (γδ) T cells,[12] are reported to have anti-tumor activity. For the past decade, cancer immunotherapy with these cells has been extensively studied, especially for hematological malignancies such as leukemia, lymphoma, and multiple myeloma.[13-15] In this regard, NK cells are essential in the innate immunity. Cytotoxic reactions of NK cells are restricted by HLA class I and several NK receptor repertories.[16] Earlier animal studies demonstrated that NK cells are deficient in IL-15-/- or IL-15Rα-/- mice,[17,18] while IL-15 promotes differentiation of NK cells from CD34+ hematopoietic progenitor cells both in vitro and in vivo.[19,20] These findings strongly support that IL-15 stimulation is important for NK cell development. Moreover, IL-15 enhances expression of functional molecules such as perforin, granzyme B, and interferon-γ (IFN-γ) in NK cells and hence increases antibody-dependent cellular cytotoxicity of NK cells.[21,22] Besides, Flt3 ligand (Flt3L) is one of critical factors for NK cell development, because Flt3L-deficient mice show phenotype of defective NK cells and lymphopoiesis.[23] Several reports have shown that the combination of IL-15 and Flt3L could synergistically expand and activate NK cells derived from UCB-MNCs.[24,25]

In our previous study, we have established a standard protocol for the isolation and cryopreservation of human UCB-MNCs at E-Da Hospital.[26] The adherent UCB-MNCs were well cryopreserved and, after ex vivo expansion, may serve as multipotent MSCs for clinical application. In this study, we further examined the NK cell differentiation potential of the non-adherent population in the previously cryopreserved UCB-MNCs. We sought for a standard in vitro differentiation protocol by monitoring the expansion folds of total UCB-MNCs, profiling NK marker expression of the expanded cells and evaluating their in vitro

tumor-killing function.

Materials and Methods

UCB cell culture

Human UCB samples were obtained from twenty mother donors less than 40 years of age in the Department of Obstetrics and Gynecology, E-Da Hospital, with informed consent (IRB approval no. EMRP07106N). Donor sera were screened for HBsAg, HIV, and VDRL to confirm negativity before using the UCB samples for subsequent experiments. The isolated UCB-MNCs without ex vivo expansion were stored in preservation solution (CellBanker II®, Nippon Zynyaku Kogyo Co., Japan) and cryopreserved in liquid nitrogen as previously described.[26]

NK cell induction

UCB-MNCs were resuspended at 1 × 10^6 cells/mL in RPMI 1640 medium containing 2 mM L-glutamine (GIBCO/BRL, Rockville, MD, USA) supplemented with 10% fetal bovine serum (FBS; GIBCO/BRL), 100 units/mL penicillin, 100 μg/mL streptomycin, 1 mM sodium pyruvate (GIBCO/BRL), and 1 mM nonessential amino acid (GIBCO/BRL). Alternatively, commercially available serum-free media, including stem cell growth medium (SCGM; CellGenix GmbH, Freiburg, Germany) and X-VIVO medium for hematopoietic cell culture (Lonza Bioscience, Basel, Switzerland), were used to compare the growth and differentiation rates of the expanded cells.

For differentiation experiments, UCB-MNCs were dispensed at a density of 1 × 10^6 cells/mL in 48-well plates in the presence of 50 ng/mL IL-15, either with or without 10 ng/mL of Flt3L or 50 ng/mL of IL-21 recombinant proteins (all purchased from PeproTech, Rocky Hill, NJ). The cells were incubated in 5% CO_2 at 37°C in humidified atmosphere for the duration of incubation period, and half volume of culture medium was changed with fresh medium containing cytokines every 4 days throughout the incubation period. Parts of the cells were harvested on days 7, 14, and 21 for cell count and surface maker staining. The stained cells were then analyzed by flow cytometry to determine the percentage of NK cells.

Table 1. A list of detecting antibodies used for immunofluorescent staining.

Target antigens	Clone names	Manufacturers	Cat. No.
CD3	UCHT1	Biolegend	300420
CD4	OKT4	Biolegend	317412
CD8	SK1	Biolegend	344703
CD34	561	Biolegend	343606
CD56	HCD56	Biolegend	318306
TCRα24	6B11	Biolegend	342906
TCRγδ	B1	Biolegend	331212
DNAM-1	11A8	Biolegend	338303
NKG2C	REA205	Miltenyi Biotec	130-103-700
NKG2D	1D11	Biolegend	320808
CD158	HP-MA4	Biolegend	339511
NKp30	P30-15	Biolegend	325207
NKp44	P44-8	Biolegend	325115
NKp46	9E2	Biolegend	331917
NKG2A	REA110	Miltenyi Biotec	130-098-817
CD158b	DX27	Biolegend	312603
CD158f	UP-R1	Biolegend	341303
CD158e1	DX9	Biolegend	312719
CD158d	mAb33	Biolegend	347007

Flow cytometry analysis

One million cells were collected and washed once with ice-cold staining buffer (PBS with 2% fetal calf serum; FCS). The cells were then stained with human-specific antibodies purchased from Biolegend (San Diego, CA) and Miltenyi Biotec (Norh Rhine-Westphalia, Germany) companies (details are listed in Table 1) and incubated for 30 minutes on ice in darkness, followed by two washes with ice-cold staining buffer. The fluorescent signals of stained cells were collected by a flow cytometer (Accuri C6, BD Bioscience, Franklin Lakes, NJ). The data were analyzed by FlowJo software (BD Bioscience). Total collected cells were first gated for living cells [Forward scatter (FSC) vs. Side scatter (SSC)]. The cells were then gated with indicated antibodies to identify specific immune cells.

Tumor-killing (cytotoxicity) assay

The cytotoxic function of expanded NK cells was examined by intracellular activated caspase-3 staining. The effector cells (E) and target cells (T) were mixed for 4 hours in 100 μL of RPMI 1640 supplemented with 5% FBS in 96-well plate to obtain different E:T ratios, namely, 10:1, 5:1, 2.5:1, and 1:1. Human K562 cells derived from chronic myelogenous leukemia were used as target cells, which were prelabeled with CellTrace™ far-red dye (Thermo/Fisher Scientific, Waltham, MA) and plated out at 5×10^4 cells/well. After a four-hour incubation, the mixed cells were harvested and stained with anti-activated caspase-3 antibody (Clone C92-605, catalog No. 559565, BD Bioscience). The death of far-red positive target cells was determined by measuring the caspase-3 activity through a flow cytometer. The cytotoxicity was calculated using the following formula [Percentage of caspase-3-specific target cell lysis = (No. of experimental caspase-3 positive target cells - No. of spontaneous caspase-3 positive target cells) / No. of

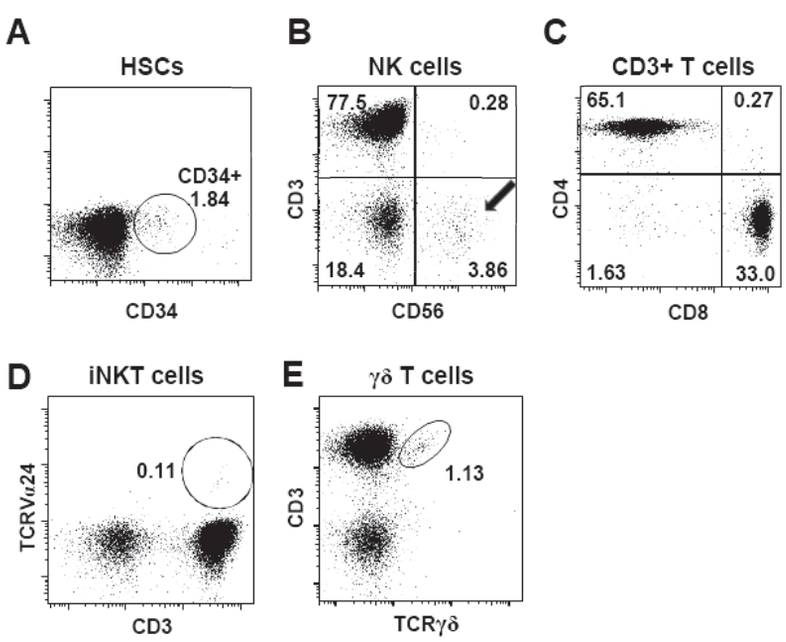

Fig. 1 Phenotypic characterization of UCB-MNCs before in vitro induction of natural killer (NK) cell differentiation. Previously isolated human UCB-MNCs were thawed from liquid nitrogen and immediately stained with indicated antibodies. The stained cells were then analyzed by flow cytometry using single- or two-parametric dot plot modes. (A) Hematopoietic stem cells (HSCs, CD34+); (B) NK cells (CD3-CD56+) (indicated by arrow); (C) T cells gated as CD3+ and then further divided into CD4+ and CD8+ (D) Invariant NKT (iNKT) cells (CD3+TCRVα24+); and (E) γδT cells (CD3+ TCRγδ+). The subpopulation percentages of circled areas and quadrants are shown in each panel.

total far-red positive target cell × 100 (%)].

Statistical analysis

All data are presented as mean ± SD. Comparisons between groups are done by using Student's t-test. Significance is declared when a *p* value is less than 0.05.

Results

Our previous study isolated human UCB-MNCs from 20 donor mothers, cryopreserved, and stored in liquid nitrogen for at least 3 years. These cryopreserved cells had been thawed and subjected to cell viability assays, which showed that, despite significant loss of viability, most clones of isolated UCB cells still had averaged viability of 85% after thawing. Earlier flow cytometry detection demonstrated that the adherent UCB-MNCs expressed MSC-specific surface antigens, including CD45, CD73, CD90 and CD105.[26] These results highly support the intact differentiation ability and multipotency of these isolated UCB-MNCs. We anticipated that the non-adherent population of these isolated UCB-MNCs might be suitable for ex vivo differentiation induction of NK cells.

To characterize the expression of surface CD markers in naive UCB-MNCs, the previously isolated and cryopreserved cells thawed from liquid nitrogen were immediately subjected to immunofluorescent staining for cell surface markers of CD34+ hematopoietic cells and those of immune cells, such as NK, invariant NKT (iNKT) and γδ T cells populations. The representative data of flow cytometry showed that there were 1 – 2% CD34+ hematopoietic cells among the isolated UCB-MNCs (Fig. 1A). Immune cell marker staining indicated that the UCB-MNCs contained nearly 80% of CD3+ T cells, while the prevalence of CD3-CD56+ NK cell population in UCB-MNCs was around 3.86% (Fig. 1B). Meanwhile, most of CD3+ T cells in UCB-MNCs expressed CD4 and/or CD8 markers (Fig. 1C). Besides, lower

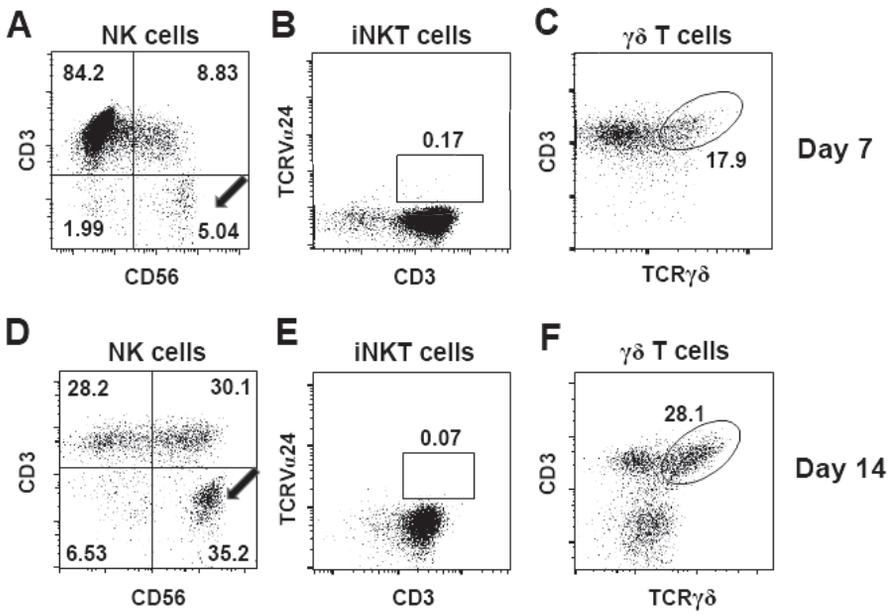

Fig. 2 Phenotypic characterization after in vitro induction of NK cell differentiation. Human UCB-MNCs cultured with Flt3L (10 ng/mL) and IL-15 (50 ng/mL) for 7 days (A-C) and 14 days (D-F) harvested for immunofluorescent staining, followed by flow cytometric identification. (A,D) NK subpopulation shown by two-parametric dot plot mode analyses. NK cells (CD3-CD56+) shown and indicated by arrow; (B,E) iNKT cells (CD3+TCRVα24+),and (C,F) γδT cells (CD3+TCRγδ+) . The subpopulation percentages of gated areas and quadrants are shown in each panel.

subpopulation of CD3+ T cells (around 0.11%) expressed TCRVα24, a marker for iNKT cells (Fig. 1D), while about 1.13% of γδ T cell subpopulation expressing TCRγδ was detectable in CD3+ UCB-MNCs (Fig. 1E).

Since a previous study showed that Flt3L and IL-15 can induce UCB-MNCs to become NK and T cells,[27] we next established the NK and T cell differentiation system by culturing the cells in RPMI medium supplemented with IL-15 and Flt3L recombinant proteins. After seven days of induction, the treated cells were again subjected to flow cytometry detection on CD marker expression. The analyzed data indicated that all population contained 5.04% of CD3-CD56+ NK cells, 84.2% of CD3+CD56- conventional T cells, and 8.83% of CD3+CD56+ cells (Fig. 2A). The frequency of iNKT cells, which is CD3+TCRVα24+ cells, was about 0.17% (Fig. 2B). Intriguingly, about 17.9% of cells were found to be γδT cells after seven days of induction (Fig. 2C). The same CD marker characterization further corroborated that, after 14-day induction, there were around 35.2% of CD3-CD56+ NK cells, 28.2% of CD3+CD56- conventional T cells, and 30.1% of CD3+CD56+ cells (Fig. 2D). The prevalence of iNKT cells (i.e., CD3+TCRVα24+ subset) remained at 0.07% level (Fig. 2E). The γδ T cell subpopulation remarkably increased from 17.9% to 28.1% after 14-day culture (Fig. 2F).

In parallel with CD marker characterization, the growth rate of all UCB-MNCs receiving NK cell induction was simultaneously monitored by direct cell counting following treatment with either IL-15 or Flt3L, or both. The results clearly showed an increase in cell number in all treatment groups in our culture system with the except of cells treated with Flt3L alone that demonstrated no significant expansion. More intriguingly, the total cell number in both IL-15 alone and IL-15 plus Flt3L treatment groups notably increased 25 folds after 14 days, and further elevated to 40 folds after 21 days of induction (Fig. 3A). However, there was no significant difference in cell number between the IL-15 alone group and IL-15 plus Flt3L group, even though the induction period was extended up to 30 days (data not shown). In fact, the overall cell number in all groups did not increase after 21 days.

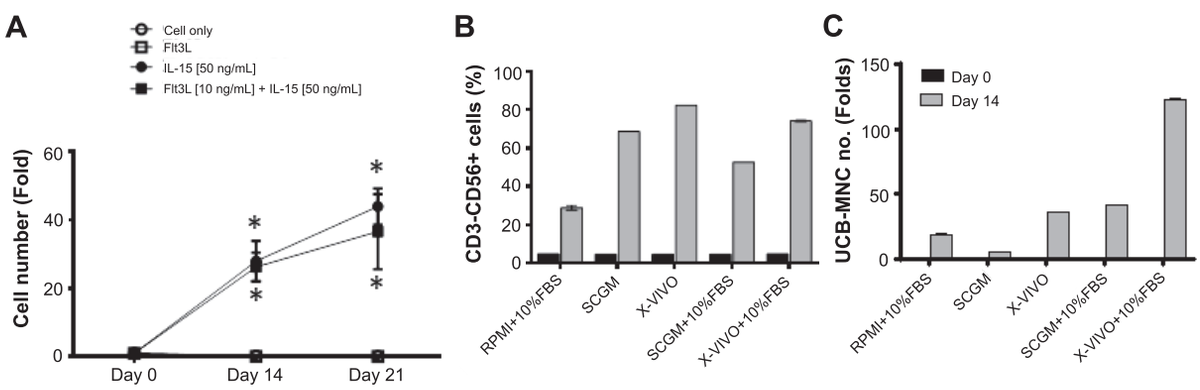

*Fig. 3 Expansion folds and differentiation rates of human UCB-MNCs after NK cell induction. (A) Effects of IL-15 and Flt3L on expansion of UCB-MNCs. UCB-MNCs were cultured in RPMI1640 medium supplemented with or without Flt3L (10 ng/mL) and IL-15 (50 ng/mL) for up to 21 days. Total cell numbers were counted at indicated time points and change in folds were calculated compared to the initial cell number. (B,C) Comparative observation on the growth and differentiation rates of UCB-MNCs grown with different media and supplements. (B) Before and after 14-day NK differentiation induction, the immunofluorescently stained UCB-MNCs were subjected to flow cytometry and the percentage of CD3-CD56+ cells were measured via flow cytometry. (C) Direct cell counting was performed to document the change in folds of total UCB-MNC number in different cell media. Induction folds were calculated relative to SCGM group. Data are expressed as mean ± SD. *p < 0.05, vs. compared with cell only group at the same time point.*

We then examined the effects of different culture media on NK cell differentiation and the expansion of UCB-MNCs. After being cultivated with RPMI, SCGM or X-VIVO media either with or without FBS for 14 days, the total cell number of UCB-MNCs was directly counted and the percentages of differentiated NK cells were measured using flow cytometry. Flow cytometric detection indicated that, compared with FBS-supplemented RPMI medium group containing about 30% of NK cells, serum-free SCGM or X-VIVO media exhibited better inducing effect on NK differentiation of UCB-MNCs. Treatments with these two media for 14 days could remarkably increase NK cell populations up to 60 – 80%, while additional 10% FBS supplementation in both media did not elevate the cell count but apparently suppressed NK cell differentiation from UCB-MNCs (Fig. 3B). However, after 14-day serum-free treatment, commercial X-VIVO medium exhibited the most prominent effect on the number of UCB-MNCs, while SCGM appeared not suitable for expansion of hematopoietic lineage cells (Fig. 3C). Moreover, FBS supplementation substantially increased the number of UCB-MNCs grown in SCGM by two folds as compared with that in RPMI medium, while the cells grown in FBS-supplemented X-VIVO medium had the highest increasing effect and raised the cell number by about five folds compared with RPMI groups.

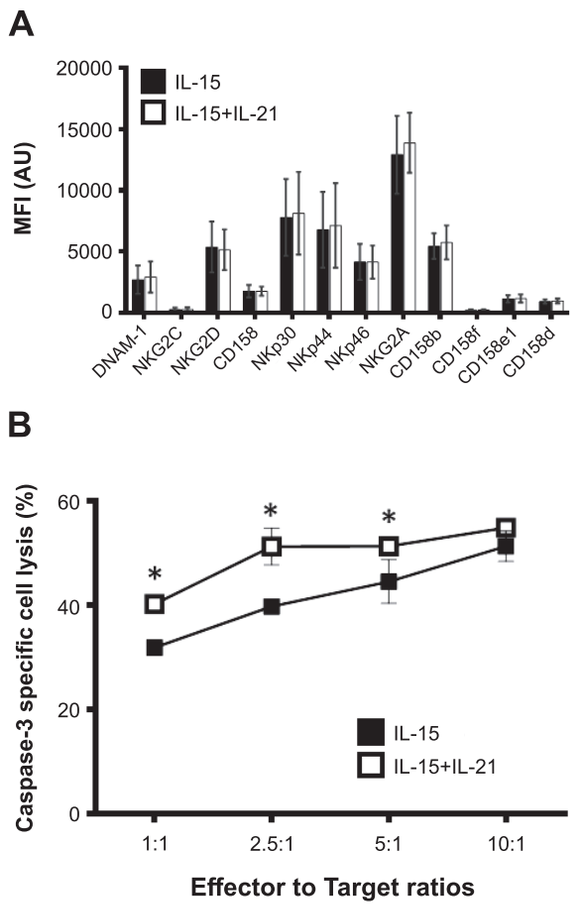

*Fig. 4 Function analysis of differentiated NK cells. After 14-day NK cell differentiation induced by IL-15 and Flt3L, the expanded human UCB-MNCs were further treated with either IL-15 alone or IL-15 and IL-21 for 3 days. (A) Flow cytometry was used to monitor expression of NK activation markers (DNAM-1, NKG2C, NKG2D and CD158), inhibition markers (NKG2A, CD158b, CD158f, CD158e1 and CD158d), and natural cytotoxicity receptors (NKp30, NKp44 and NKp46). Relative expression levels were shown as mean fluorescent intensity (MFI) in arbitrary unit (AU). (B) Tumor-killing activity of differentiated NK cells (effectors) was determined by co-culture with K562 cells (targets) at different effector-to-target cell ratios. Percentage of caspase-3-specific cell lysis of target cells was measured using flow cytometry. Data are expressed as mean ± SD. *p < 0.05, compared between groups in the same ratio.*

Because IL-21 has been shown to increase NK cell activity,[28] we then tested whether IL-21 treatment in our expanded NK cell could enhance NK cell functional activities. To this end, NK cell differentiation of UCB-MNCs was induced by IL-15 and Flt3L for 14 days, followed by 3-day stimulation with either IL-15 alone or IL-15 plus IL-21 cotreatment. Matured NK cells express different sets of surface markers that represent their functional status. Flow cytometry was used to monitor the expression levels of NK activation markers (DNAM-1, NKG2C, NKG2D and CD158), inhibition markers (NKG2A, CD158b, CD158f, CD158e1 and CD158d) and natural cytotoxicity receptors (NKp30, NKp44 and NKp46).[29] The results indicated that the presence of IL-21 did not alter the expressions of all receptors

tested (Fig. 4A). To examine the cytotoxic function of matured NK cells, the induced UCB-MNCs were subjected to tumor-killing assay by co-culture with K562 cells. Caspase-3-specific K562 cell lysis was detected by using flow cytometry. The results showed that cotreatment with IL-21 at lower E:T ratios (up to 5:1) significantly increased tumor-killing activity of induced NK cells, while no difference in cytotoxic activity was noted at a high E:T ratio, i.e., 10:1 (Fig. 4B), probably due to the reach of plateau of NK cell killing capacity. Overall, these data suggested IL-21 could enhance the tumor-killing activity of induced NK cells.

Discussion

In this study, we successfully established an in vitro differentiation system for the differentiation of human UCB-MNCs to NK cells. Our data strongly suggest that in vitro expansion of NK cells from UCB-MNCs can be attained by costimulation with IL-15 and Flt3L recombinant cytokines. Meanwhile, we examined the expanding effect of different culture media on UCB-MNCs during NK cell differentiation induction. Our data demonstrated that a commercially available medium originally designed for hematopoietic progenitor cell culture (i.e., X-VIVO) showed a higher potency in driving NK cell development and expansion under serum supplementation. However, the culture system still requires improvement before its application in clinical treatment. In particular, the number of induced NK cells need to be improved. Because 10^8 NK cells/kg are reportedly needed for a single infusion to a patient for therapeutic purpose, substantial expansion should be achieved when starting with one million cells for each infusion.

Our findings also demonstrated that IL-21 increased the tumor-killing activity of the differentiated NK cells. Several ways, such as co-culture with IL-21 feeder cell, can increase the NK cell expansion.[30] The effect of IL-21 feeder cells on NK cell number in this system remains to be elucidated. The other concern is that although supplementation with FBS in culture medium increased NK cell expansion (Fig. 3B), it may not be suitable for future clinical application. Other xeno-free additives (e.g., human serum or other serum-free media) may be needed to replace animal serum for NK cell expansion from UCB-MNCs.

In addition to NK cells, other cell populations like γδ T cells and NKT cells were identified in cryopreserved UCB-MNCs before NK cell induction, which may also be expanded for clinical use. In fact, similar to the function of NK cells, γδ T cells and NKT cells have been previously reported to have tumor-killing activity without a priming phase. More recently, several studies have found that both γδ T cells and NKT cells have therapeutic potential in cancer treatments.[31,32] In the present study, we intriguingly noted that about 18% of the expanded UCB-MNCs belonged to γδ T cells (Fig. 2C). Whether the phenotype and function of γδ T cells generated in this system are suitable for immunotherapy warrant further investigations. Our current system did not expand any NKT cells, as only 0.07% of the cells were TCRVα24+ after the 14-day culture (Fig. 2E). Because it have been previously reported that the number of iNKT cells can be expanded only under specific antigen stimulation,[33] it is not surprising that the expansion of iNKT cells was not observed in this culture system.

IL-21 promotes the maturation of NK cell progenitors and activates the anti-tumor effects of NK cells through the NKG2D pathway. IL-21 activates the cytotoxic machinery in NK cells, thereby providing potent cytotoxic effector arms against cancer cells. It may also reverse exhaustion and enhance the anti-tumor activity of NK cells.[28] Our data also demonstrated that IL-21 could significantly enhance

the tumor cytotoxic activity of induced NK cells via caspase-3-dependent activation (Fig. 4B). Similar to the finding of a previous study, our result showed that IL-21 did not enhance NKG2D expression.[34] We also found that the expression levels of several NK activation-related receptors remained unchanged following IL-21 treatment (Fig. 4A). Our data strongly suggested that IL-21-enhanced cytotoxic activity of NK cells may not be mediated through the activation/inhibition of receptor balance.

In conclusion, this study established practical protocols for the induction of differentiation and expansion of cryopreserved human UCB-MNCs into functional NK cells. The characterization of NK cell functions, including marker expression and tumor-killing activity, may enhance the efficacy of differentiated NK cells and facilitate their applications in preclinical animal studies and clinical cell therapy in the future.

Acknowledgements

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