

Article

High Expression of NKG2A/CD94 and Low Expression of Granzyme B Are Associated with Reduced Cord Blood NK Cell Activity

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Human umbilical cord blood (CB) has recently been used as a source of stem cells in transplantation. NK cells derived from CB are the key effector cells involved in graft-versus-host disease (GVHD) and graft-versus-leukemia (GVL). It was reported that the activity of CB NK cells was lower than that of adult peripheral blood (PB) NK cells. In this study, we analyzed the expression of some NK cell receptors and cytotoxicity-related molecules in CB and PB NK cells. The expressions of activating NK receptors, CD16, NKG2D and NKp46, did not show significant difference between CB and PB NK cells. But the expression of inhibitory receptor NKG2A/CD94 was significantly higher on CB NK cells. As to the effector function molecules, granzyme B was expressed significantly lower in CB NK cells, but the expressions of intracellular perforin, IFN- γ , TNF- α and cell surface FasL and TRAIL did not show difference between CB and PB NK cells. The results indicated that the high expression of NKG2A/CD94 and low expression of granzyme B may be related with the reduced activity of CB NK cells. *Cellular & Molecular Immunology*. 2007;4(5):377-382.

Key Words: NK, cord blood, NK cell receptor, NKG2A, granzyme B, cytotoxicity

Introduction

Natural killer (NK) cells are a population of large granular lymphocytes with a CD56⁺/CD3⁻ phenotype (1, 2). They are distinguishable from B and T lymphocytes by lack of antigen receptors. NK cells kill a variety of tumor cells, virus-infected cells and allogeneic cells in a non-major histocompatibility complex restricted manner and provide the first line of immune defense (3, 4).

Although NK cells lack the antigen-specific receptors, they distinguish between normal cells and abnormal cells by their cell surface receptors (5). After binding to potential target cell, NK cell activating and inhibitory receptors interacted with ligands and transmitted signals, and then all

the signals were integrated to determine whether NK cell stays and responds (6, 7). The effector function of NK cells is regulated by a balance between the inhibitory signals delivered by the MHC class I-specific inhibitory receptors and the activating signals transmitted by activating receptors (2, 8). NK cell effector function is mainly mediated through: 1) releasing cytoplasmic cytotoxic granules (granzyme and perforin) by exocytosis; 2) secreting cytokines (IFN- γ , TNF- α , etc.); and 3) the engagement of death receptors on target cells by their cognate ligands (e.g., FasL and TRAIL) on NK cells (9-12).

Human umbilical cord blood (CB) has recently been used as a source of stem cells in partially HLA-mismatched transplantation (13-16). NK cells derived from CB are the key effector cells involved in graft-versus-host disease (GVHD) and graft-versus-leukemia (GVL) (17-19). So much interest was focused on the characteristics of CB. It was reported that the activity of CB NK cells was lower than that of adult peripheral blood (PB) NK cells (20-22). Analysis of adhesion molecules on NK cells showed that low expression level of adhesion molecules on CB CD56^{dim} NK cells may contribute to the low NK cell activity (21). In this study, we investigated the expressions of some NK cell-related receptors and intracellular cytokines in CB and PB NK cells. We sought to determine whether the expression pattern of the NK cell receptors and cytokines between PB and CB was related to the low activity of CB NK cells. The results showed that CB NK cells expressed high level of

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NKG2A/CD94, one of NK cell inhibitory receptors; and the expression of cytotoxic granzyme B was significantly lower in CB NK cells than that in PB NK cells. So this may suggest that high expression of NKG2A/CD94 and low expression of granzyme B are associated with the reduced CB NK cell activity.

Materials and Methods

Blood samples

CB samples were collected from the umbilical cords of the placentas of normal, full-term, non-stressed newborns of consenting mothers at the Obstetrics and Gynecology of Anhui Provincial Hospital. Adult PB samples were obtained from healthy donors at Hefei Blood Bank. All blood samples were collected following approval of the University of Science and Technology of China Institutional Review Board and obtaining informed consent.

Cell preparation

Blood samples were processed within 8 hours of collection. CB or adult PB samples were diluted 1:2 in PBS. Mononuclear cells were isolated by Ficoll-Hypaque centrifugation using standard procedures.

Antibodies

Anti-CD16, CD8, CD94, CD158a, IFN- γ , granzyme B conjugated with FITC, anti-CD56, CD161, CD158b, NKG2D, NKp46, TRAIL, perforin conjugated with PE, anti-TNF- α conjugated with Alexa-488, anti-CD56 conjugated with Alexa-647, anti-CD3 conjugated with APC-Cy7 was purchased from BD PharMingen. Anti-FasL conjugated with PE was from eBioscience and anti-NKG2A conjugated with PE was purchased from R&D systems.

Cell staining and flow cytometry

Mononuclear cells were washed twice and blocked with mouse IgG (1 μ g/ 10^6 cells) for 30 min at 4°C. Then the cells were incubated with saturating concentrations of the appropriate mAbs for 30 min at 4°C. Thereafter, cells were washed twice and analyzed by using FACSaria (Becton Dickinson).

Intracellular granzyme B and perforin detection

Freshly isolated mononuclear cells (5×10^6 /ml) were stained with anti-CD56 and -CD3 antibodies for 30 min at 4°C, followed by fixed and permeabilized using the Fix/Perm cell permeabilization kit (BD Biosciences) for 20 min at room temperature. Then the cells were stained with anti-granzyme B or perforin for 1 hour at room temperature, washed twice with permeabilisation buffer, and analyzed by flow cytometry.

Intracellular cytokine detection

Freshly isolated mononuclear cells (5×10^6 /ml), cultured with RPMI 1640 supplemented with 10% fetal bovine serum (FBS, both from GIBCO), were stimulated with PMA (30

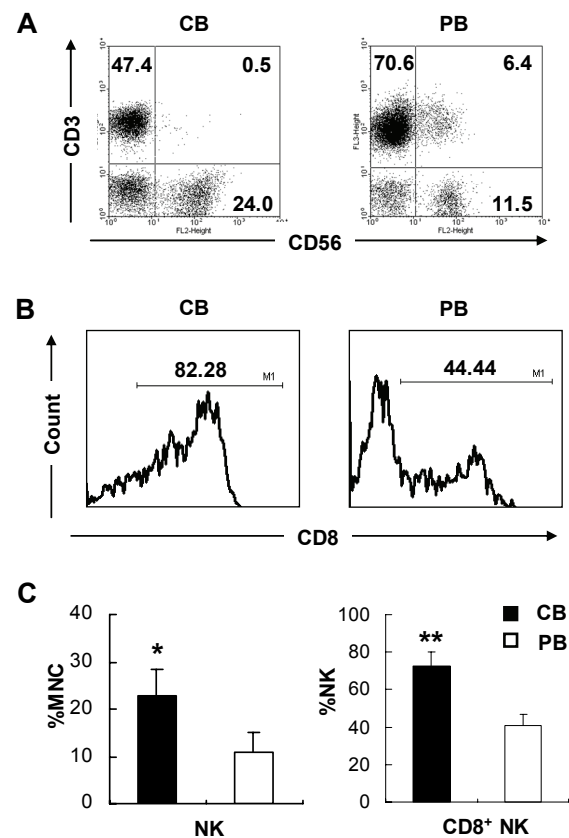


Figure 1. High percentages of NK cells and CD8⁺ NK cells in CB. Freshly isolated CBMCs or PBMCs were stained with anti-CD3, CD56 and CD8 mAbs, and analyzed by flow cytometry. (A) The relative percentage of CD56⁺CD3⁻ NK cells in CBMCs or PBMCs was analyzed. (B) The CD56⁺CD3⁻ cells were gated and the percentage of CD8⁺ NK cells in NK cells was analyzed. For (A) and (B), one representative example was shown from the 6 independent experiments. (C) The percentage of NK cells in CBMCs or PBMCs and CD8⁺ NK cells in NK cells were calculated. Data were collected from 6 independent experiments and analyzed by Student's *t* test. **p* < 0.05, ***p* < 0.01.

ng/ml, Sigma) and ionomycin (1 μ g/ml, Sigma). One hour later, monensin (10 μ g/ml, Sigma) was added to prevent the secretion of the induced cytokines into the supernatant. After 4 hours of culture at 37°C and 5% CO₂, the cells were harvested and labeled with anti-CD56 and -CD3 antibodies for 30 min at 4°C. Cells were fixed and permeabilized using the Fix/Perm cell permeabilization kit for 20 min at room temperature and stained with anti-IFN- γ or TNF- α for 1 hour at room temperature, washed twice with permeabilisation buffer, and analyzed by flow cytometry.

Cytotoxicity assay

Cytotoxic activity was assessed by using a ⁵¹Cr release assay as previously described (23). Briefly, 10⁶ K562 target cells were labeled with 100 μ Ci of Na⁵¹CrO₄ for 1 h at 37°C. Mononuclear cells were washed twice and resuspended in complete RPMI 1640 culture medium. Effector cells were

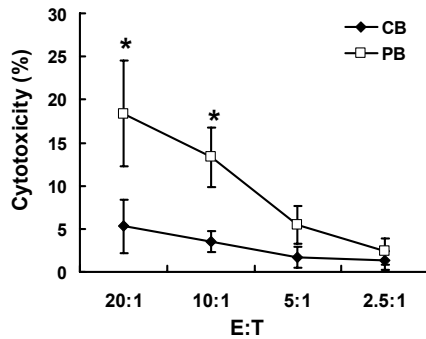


Figure 2. Reduced NK cell activity in CB. The cytotoxic activity of CBMCs and PBMCs against K562 cells was evaluated with 4-hour ^{51}Cr -release assay as described in Materials and Methods. Values represent the mean from 5 independent experiments. Data were analyzed by Student's *t* test. *, $p < 0.05$.

incubated with K562 in triplicate at 96-well round bottom plates for 4 hours at 37°C in 5% CO_2 . Supernatants were assessed for ^{51}Cr release using a γ -counter. Culture medium or 1% Triton X-100 was added to incubate with target cells for calculation of spontaneous or maximum release, respectively. The percentage of specific ^{51}Cr release was calculated as follows: $(\text{cpm experimental release} - \text{cpm spontaneous release}) / (\text{cpm maximal release} - \text{cpm spontaneous release}) \times 100\%$. Spontaneous release did not exceed 10% of the maximum release.

Statistical analysis

Results are expressed as mean \pm SD. The statistical analysis was performed by the Student's *t* test or the Mann-Whitney test. $p < 0.05$ was considered statistically significant.

Results

High percentage and low activity of CB NK cells

Freshly isolated cord blood mononuclear cells (CBMCs) or peripheral blood mononuclear cells (PBMCs) were stained with CD3 and CD56 antibodies, and analyzed by flow cytometry. The percentage of NK cells in CB was $22.63 \pm 5.95\%$, higher than that of PB $10.95 \pm 3.99\%$ ($p < 0.05$) (Figure 1A). It was reported that CD8^+ NK cells showed higher cytotoxicity than that of CD8^- NK cells (24, 25). In other words, the higher percentage of CD8^+ NK cells, the higher NK cell cytotoxicity. Our results indicated that the percentage of CD8^+ NK cells in CB NK cells was higher than that in PB NK cells ($72.78 \pm 7.27\%$ vs $42.26 \pm 3.97\%$, $p < 0.01$) (Figure 1B). However, cytotoxicity assay of NK cells against K562 cells showed that CB NK cell activity was significantly lower than that of PB ($p < 0.05$) (Figure 2), which was similar with previous report (20-22).

NK cell receptor expressions on CB and PB NK cells

Although the percentage of NK cells in CB was higher and CB had more CD8^+ NK cells, the cytotoxicity of CB NK

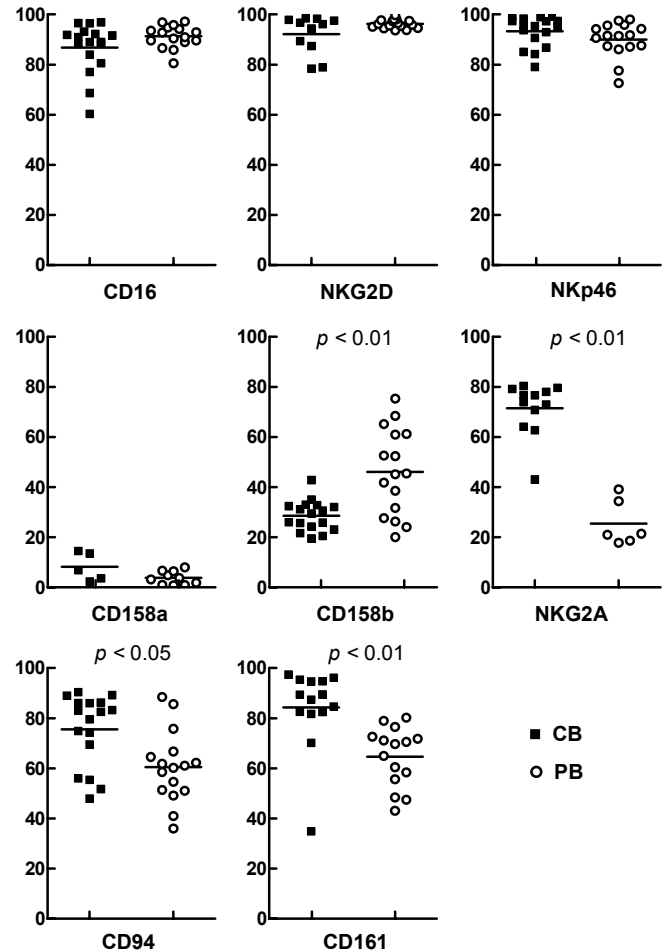


Figure 3. The expressions of NK cell receptors on CB and PB NK cells. Freshly isolated CBMCs or PBMCs were stained with anti-CD3, CD56 and indicated antibodies (CD16, NKG2D, NKp46, CD158a, CD158b, NKG2A, CD94 and CD161) and analyzed by flow cytometry. The $\text{CD56}^+\text{CD3}^-$ cells were gated and the expression percentage of each NK cell receptor on NK cells was shown. The data were analyzed by Mann-Whitney test.

cells was low. NK cell recognition through NK cell receptors is necessary for NK cell cytotoxicity target cells. We presumed that the expressions of NK cell activating or inhibitory receptors in CB were different from those in PB, so the expressions of NK cell activating and inhibitory receptors on CB NK cells were examined.

The PBMCs or CBMCs were stained with CD3, CD56 and another antibody, $\text{CD56}^+\text{CD3}^-$ NK cells were gated, and the expressions of NK cell receptors were analyzed. The results showed that CD16 expression on CB NK cells was similar to PB NK cells ($86.76 \pm 10.04\%$ vs $91.31 \pm 4.42\%$, respectively). The expressions of NKG2D on CB and PB NK cells were $92.13 \pm 7.59\%$ and $96.22 \pm 1.72\%$, and those of NKp46 were $93.26 \pm 6.06\%$ and $89.98 \pm 6.94\%$, respectively (Figure 3). The results showed that the expressions of activating receptors, CD16, NKG2D and NKp46 did not show significant difference between CB and PB NK cells.

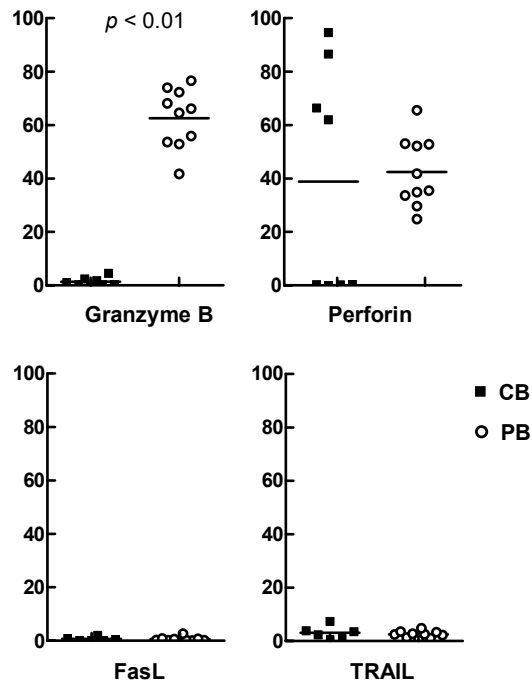


Figure 4. The expressions of cytotoxicity-related molecules in CB and PB NK cells. Freshly isolated CBMCs or PBMCs were stained with anti-CD56 and anti-CD3 mAbs, as well as FasL or TRAIL. The CD56⁺CD3⁺ NK cells were gated and the expression percentage of FasL or TRAIL on NK cells was shown. For granzyme B and perforin detection, the cells were stained with anti-CD56 and anti-CD3 mAbs, fixed and permeabilized, then stained with granzyme B and perforin and analyzed by flow cytometry. Data were analyzed by Mann-Whitney test.

As to the inhibitory receptors, the expressions of CD158a on CB and PB NK cells were $9.57 \pm 7.08\%$ and $4.67 \pm 16.78\%$, respectively. Whereas CD158b had a lower expression on CB NK cells ($28.61 \pm 6.07\%$), than that on PB NK cells ($46.10 \pm 17.67\%$), $p < 0.01$. NKG2A was expressed on $72.20 \pm 12.49\%$ CB NK cells, but only on $23.82 \pm 7.83\%$ PB NK cells ($p < 0.01$) (Figure 3).

In addition, the expressions of CD94 and CD161, which were the markers of immature NK cells, were also analyzed by flow cytometry. The expressions of CD94 on CB and PB NK cells were $76.90 \pm 13.55\%$ and $60.53 \pm 12.16\%$, respectively ($p < 0.05$). CD161 was expressed on $84.32 \pm 15.15\%$ CB NK cells and $66.00 \pm 12.83\%$ PB NK cells ($p < 0.01$) (Figure 3).

The detection of effector function molecules

NK cells performed the cytotoxicity function mainly through producing cytotoxic granules. The expression of granzyme B showed significant difference between CB and PB NK cells, $1.52 \pm 1.74\%$ vs $62.62 \pm 11.16\%$, $p < 0.01$. Perforin was expressed at comparable levels between CB ($39.92 \pm 43.74\%$) and PB NK cells ($42.39 \pm 12.92\%$) (Figure 4).

In addition, NK cells induced target cell apoptosis through the interaction of FasL or TRAIL with reciprocal

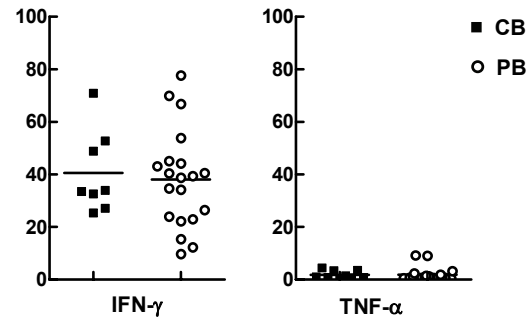


Figure 5. The expressions of IFN- γ and TNF- α in CB and PB NK cells. Freshly isolated CBMCs or PBMCs were stimulated with PMA/ionomycin for 4 hours and stained with anti-CD56 and anti-CD3 mAbs. Then cells were fixed, permeabilized, stained with anti-IFN- γ or TNF- α mAbs and analyzed by flow cytometry. The CD56⁺CD3⁺ NK cells were gated and the expression percentage of IFN- γ or TNF- α in NK cells was shown. Data were analyzed by Mann-Whitney test.

ligand on target cells. The results showed that FasL and TRAIL were expressed at very low levels in resting CB and PB NK cells, $0.79 \pm 0.75\%$ vs $0.77 \pm 0.72\%$, $3.82 \pm 1.84\%$ vs $2.73 \pm 1.09\%$, respectively, and did not show difference between them (Figure 4).

The expressions of IFN- γ and TNF- α in CB and PB NK cells

Besides cytotoxic granules, cytokines secreted by NK cells also played important role in NK cell cytotoxicity. The expressions of IFN- γ in CB and PB NK cells were $40.62 \pm 15.62\%$ and $33.07 \pm 18.65\%$, respectively. TNF- α expressions in both kinds of NK cells were very low, $1.83 \pm 1.57\%$ vs $1.84 \pm 1.65\%$, and did not show significant difference (Figure 5).

Discussion

Umbilical CB has become a valuable alternative source of haematopoietic stem cells for allogeneic transplantation (14-16, 26). NK cells derived from CB are the key effector cells involved in GVHD and GVL (17-19). CB had higher NK percentage and more CD8⁺ NK cells than PB NK cells. And it was reported that CD8⁺ NK cells were more cytotoxic than their CD8⁺ counterparts (24, 25). But in our and others results, CB NK cells showed reduced cytotoxicity compared with PB NK cells (20-22).

NK cell recognition target cells and response by releasing cytotoxic granules and secreting cytokines were important steps for NK effector functions (2, 6, 12). As to our results, it did not show significant difference for the expressions of activating receptors, NKG2D and Nkp46 on CB and PB NK cells. However, the expressions of inhibitory receptors CD158b and NKG2A were significantly different between CB and PB NK cells. Though CD158b was expressed lower in CB NK cells, NKG2A was significantly higher in CB NK cells, in accordance with the high level of CD94 on CB NK

cells as previously reported (27).

As mentioned above, it is more complex for NK cell recognition than for T lymphocytes, for which T cell receptor triggering is the key event in effector function deployment (28). NK cells have dozens of surface molecules and receptors that are involved in the cell decision (6, 7). The cooperation of activating NK receptors and inhibitory NK receptors regulated NK cell functions. NK cell response was dependent on the amount of activating and inhibitory NK receptors and their counterpart ligands on the target cells, and the signal strength they transmitted. When the strength of activating signals is powerful over the sum of inhibitory signals, NK cells will respond and kill the target cells. Conversely, NK cells detach the target cells (6-8). So the higher expressions of NKG2A/CD94 on CB NK cells may be an important factor for the low activity of CB. In addition, the expression level of CD161, a marker of immature NK cells, was also higher on CB NK cells. This might indicate that CB NK cells were immature and the ability to respond the foreign antigens is slower and weaker than adult PB NK cells.

Once NK cells decide to respond and clear target cells, they will reorganize and release pre-reserved granzyme, perforin, and transcribe and secrete cytokines, as well as through interaction of FasL and TRAIL with their reciprocal ligand on target cells to perform effector cytotoxicity (1, 2, 10-12). The expressions of intracellular perforin, IFN- γ (29), TNF- α , and cell surface FasL (29) and TRAIL (29) did not show significant difference between CB and PB NK cells. PB NK cells expressed high levels of granzyme B, whereas almost no granzyme B was expressed in CB NK cells. Therefore, the deficiency of granzyme B in CB NK cells was another reason for reduced CB NK cell activity.

Acknowledgements

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