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The unique profile of cord blood natural killer cells balances incomplete maturation and effective killing function upon activation

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ABSTRACT

Cord blood (CB) is increasingly used as a source of stem cells for hematopoietic stem cell transplantation, and natural killer (NK) cells may be the effectors of the antileukemic response observed after CB transplantation. Here, we analyzed the phenotype and functions of CB NK cell subsets. We determined that the percentage of NK cells was higher in CB compared with peripheral blood (PB). Furthermore, there was a higher percentage of the $CD56^{bright}$ subset in CB. CB NK cells reached a late stage of differentiation, but exhibited higher expression of NKG2A and expressed fewer killer-cell immunoglobulin-like receptors, suggesting an incomplete maturation. CB NK cells highly expressed CXCR4, but did not express L-selectin, highlighting unique homing properties of CB NK cells. CB NK cells proliferated in response to interleukin-2 and degranulated in response to stimulation with tumor cells, but failed to lyse K562 cells in ^{51}Cr-release assay. CB NK cells exhibited a lower interferon-γ production in comparison with PB NK cells. Culture with IL-2 increased CB NK cell functions. Our study sheds light on CB NK cell properties and highlights the potential of CB as a source of NK cells for immunotherapy.

1. Introduction

Hematopoietic stem cell transplantation is most commonly indicated for leukemia, but is also used to treat different hematological and nonhematological malignancies, immunodeficiency, and autoimmunity [1]. Cord blood (CB) has been increasingly used as an alternative source of stem cells for hematopoietic stem cell transplantation in adults because it presents several advantages including faster availability, lower human leukocyte antigen matching requirement, lower incidence and severity of graft-versus-host disease [2–4], and preservation of the graft versus leukemia effect described after allogeneic bone marrow transplantation.

The use of intense immunosuppression after CB transplantation (CBT) in conjunction with the naivety of the T cells infused with the graft and the slow T cell reconstitution generate a long period of immunodeficiency after CBT. Natural killer (NK) cells constitute between 15 and 30% of CB lymphocytes [5,6], and after CBT, NK cell reconstitution occurs very early on, so NK cells constitute most of the lymphocytes in circulation. The observation that they are capable of killing leukemia cells *ex vivo* [7] supports the hypothesis that NK cells are most likely to be a main effector of the graft-versus-leukemia effect observed after CBT.

It has been reported that the phenotype of CB NK cells demonstrates some similarities to peripheral blood (PB) NK cells [8]. CB $CD56^{+}$ cells have been demonstrated to express CD94, some killer-cell immunoglobulin-like receptors (KIR), and activating receptors such as NKG2D and NKp46 [6,9]. Nonetheless, the current literature relating to the phenotype and functions of CB NK cells indicated some inconsistencies [8]. CB NK cells have been reported to be mature by some investigators [6,8,10] and immature by others [11]. In some circumstances, they have been demonstrated to be as cytolytic as PB NK cells [6,10] or deficient in killing target cells, potentially because of an immature phenotype [11], a high expression of NKG2A [9], or a low expression of adhesion molecules [12]. However, incubation with interleukin-2 (IL-2), IL-12, or IL-15 can increase their cytolytic activity [6,11].

In addition to the $CD56^{dim}$ and $CD56^{bright}$ subsets, CB has been demonstrated to be characterized by the presence of $CD16^{+}CD56^{-}$ cells, which are not typically present in healthy PB. Previous studies have reported that these cells are a unique cell subset present in CB [10,11,13] that exhibited reduced lytic activity and might be the precursor of mature NK cells. Tanaka et al. reported an increased

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number of $CD16^+CD56^-$ NK cells in PB after CBT [12]. This NK cell subset was also reported in immunocompromised hosts with viral infections such as human immunodeficiency virus and hepatitis [14–16].

All these studies focused on $CD56^+$ or $CD56^-$ cells, and the characteristics of CB $CD56^{dim}$ and $CD56^{bright}$ NK cells especially before infusion into patients must still be explored. Although a few studies focused on the functions and phenotype of CB $CD56^+$ cells, none has addressed the question of their developmental stages and homing properties. In this study, we investigated the characteristics of CB $CD56^{dim}$ and $CD56^{bright}$ NK cells in term of expression of adhesion molecules, inhibitory and activating receptors, maturation markers and chemokine receptors, cytotoxic capacity, and interferon-γ (IFN-γ) production in comparison with PB NK cells. Overall, our data indicate that CB NK cells are differentiated, but not fully mature, and have different homing properties than PB NK cells; our data also indicate that IL-2 treatment can complete the maturation of CB NK cells and therefore increase their effector functions.

2. Subjects and methods

2.1. Collection of blood samples

Peripheral blood mononuclear cells were isolated from the blood of healthy volunteers upon informed consent. CB samples were obtained from the Anthony Nolan Cord Blood Bank (Nottingham, UK) or from the Programa Concordia Banc de Sang i Teixits (Barcelona, Spain). Samples were collected using routine banking procedures into a cord blood donation bag containing a citrate–phosphate–dextrose anticoagulant buffer and processed within 24 hours of collection.

2.2. Purification of CB and PB NK cells

Mononuclear cells were isolated by density-gradient centrifugation using Ficoll–Paque Premium (GE Healthcare Bio-sciences, Uppsala, Sweden). Purified NK cells were obtained by negative selection using the NK cell isolation kit II (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions.

2.3. Flow cytometry analysis

The following monoclonal antibodies were purchased from BD Pharmingen (San Diego, CA): anti-CD3 (HIT3a and SK7), anti-CD14 (M5E2), anti-CD16 (NKp15), anti-CD19 (ID3), anti-CD34 (581), anti-CD45 (HI30), anti-CD48 (BCM1), anti-CD56 (clone B159), anti-CD62L (Dreg 56), CD94 (HP-3D9), CD107a (H4A3), anti-CD117 (104D2), anti-CD122 (Mik-B3), anti-CD127 (L112), anti-CD158a (HP-3E4), anti-CD158b (CH-L), anti-CD226 (DX11), anti-FAS-L (NOK-1), anti-LFA-1 (HI111), anti-NKp30 (p30-15), anti-NKp46 (9E2/Nkp46), and anti-TRAIL (RIK-2). Anti-CCR6 (53103), anti-CXCR1 (42705), anti-CXCR4 (12G5), anti-CXCR7 (358426), and anti-NKG2C (34591) were purchased from R&D Systems (Minneapolis, MN); anti-NKG2A (z199) from Beckman Coulter (Marseille, France); and anti-NKG2D (BAR 221) from Miltenyi Biotec. Anti-CD25 (BC96), anti-CD49d (9F10), and anti-integrin β7 (FIB504) were purchased from eBiosciences (San Diego, CA). For intracellular staining, 10^6 mononuclear cells were incubated at 37°C for 5 hours in medium with or without phorbol myristate acetate (PMA; 100 ng/mL) and ionomycin Ca^{2+} ionophore (1 μg/mL; all reagents were from Sigma Chemical Co.–Aldrich (St. Louis, MO). Two microliters of GolgiStop (BD Biosciences) was added during the last 4 hours of incubation. Thereafter, cells were surface stained with anti-CD3, anti-CD16, and anti-CD56 antibodies permeabilized using the Fix/Perm cell permeabilization kit (BD Biosciences) for 5 minutes at room temperature and then stained for IFN-γ (BD Biosciences). The cells were then washed and analyzed on a FACSCalibur instrument (BD Biosciences). When expression of perforin (dG9, BD Biosciences) and granzyme B (GB11, BD Biosciences) was analyzed, the intracellular staining was performed directly without prior stimulation.

2.4. Proliferation analysis

Purified NK cells were resuspended in phosphate-buffered saline (PBS) at 10^7 cells/mL and labeled with 2 μM carboxyfluorescein diacetate succinimidyl ester (Molecular Probes, Invitrogen, Carlsbad, CA) for 10 minutes in the dark at 37°C. Cells were then washed twice with complete culture medium and cultured for 7 days in the presence of 200 or 1,000 UI IL-2/mL.

2.5. Degranulation assay

NK cells (2×10^5) were washed twice in PBS and added to 2×10^5 target cells in 200 μL complete medium and incubated for 2 hours at 37C°C. The cells were spun down and stained for CD56, CD3, and CD16 in PBS supplemented with 2% fetal bovine serum and 2 mM EDTA for 45 minutes on ice. The cells were then washed, resuspended in PBS containing 2% fetal bovine serum and 2 mM EDTA, and analyzed by flow cytometry.

2.6. Cytotoxicity assay

For cytotoxicity assay, ^{51}Cr-release assay was performed. K562 were used as target cells and pulsed with 100 μCi $Na_2[^{51}Cr]O_4$ for 45 minutes (PerkinElmer, Cambridge, UK). Freshly isolated NK cells or NK cells cultured for 7 days with 1,000 UI IL-2/mL were added to the target cells at effector-to-target ratios of 1:1, 5:1, and 10:1 in triplicate. ^{51}Cr-release was assessed in the supernatant of each culture after 4 hours. The maximum release was determined by lysis of target cells with 2% Triton X-100. The percentage of specific lysis was calculated as (experimental release – spontaneous release)/(maximum release – spontaneous release) $\times$ 100.

2.7. Statistics

Statistical comparisons were performed with GraphPad Prism software (GraphPad Software, San Diego, CA) using the nonparametric Mann–Whitney test or, when indicated, the unpaired *t* test or the *F* test to compare variances. Results are presented as means $\pm$ SD. *p* values <0.05 were considered statistically significant.

3. Results

3.1. CB NK cells are characterized by a heterogeneous CD56 expression

The analysis of the percentage of $CD56^+CD3^-$ cells in CB and PB lymphocytes indicated that, as previously reported [5], CB contained more NK cells (18.21 $\pm$ 1.146%) compared with adult PB (12.69 $\pm$ 1.637%; $p = 0.0161$; Fig. 1A). The markers CD56 and CD16 define distinct PB NK cell subsets [17,18]; $CD56^{bright}$ NK cells are predominantly cytokine producers, whereas $CD56^{dim}$ NK cells mediate natural and antibody-dependent cellular cytotoxicity (ADCC). Expression of CD16 and CD56 was analyzed by flow cytometry in purified NK cells. The percentage of $CD56^{bright}$ NK cells was slightly higher in CB than in PB ($p = 0.0135$; Fig. 1B). CD56 expression was more heterogeneous in CB NK cells, and the mean fluorescence intensity (MFI) for CD56 was lower in CB NK cells than in PB NK cells ($p < 0.0001$; Fig. 1C).

3.2. CB NK cells are differentiated NK cells

The stages of human NK cell development have recently been defined by Freud et al. [19,20], who proposed a developmental pathway from a $CD34^+$ NK cell progenitor to a functionally mature NK cell defined by a different expression level of CD34, CD117, and CD94. To determine the developmental stage of CB NK cells, CB and PB NK cells were stained for CD16, CD56, CD94, and CD117. Based