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# Differential activation of cord blood and peripheral blood natural killer cells by cytokines

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**Abstract**

*Background aims.* Natural killer (NK) cells play important roles in the clearance of infection and transformed cells. Cord blood (CB) is currently used as a source of hematopoietic stem cells for transplantation and is a potential source of NK cells for immunotherapy. We previously showed that CB NK cells are immature and less cytotoxic as compared with peripheral blood (PB) NK cells. We aimed to identify which cytokines, among interleukin (IL)-2, IL-12, IL-15 and IL-18 and their combinations, could fully activate CB NK cells as compared with PB NK cells. *Methods.* We performed a comprehensive analysis of phenotype and functionality of cytokine-activated NK cells. *Results.* Our results show that the lower responsiveness of CB NK cells to IL-2 is associated with lower levels of expression of IL-2 receptors and decreased phosphorylation of STAT5 as compared with PB NK cells. Activation of CB NK cells with IL-15+18 led to the most robust proliferative response and higher interferon-γ and tumor necrosis factor-α secretion, whereas activation with IL-15+2 promoted enhanced cytotoxicity. PB NK cells responded significantly better to IL-2 than to CB NK cells but were also fully activated with other cytokine treatments including IL-15, IL-15+2 or IL-15+18. It was also possible to use cytokines to generate memory-like NK cells, with sustained ability to produce interferon-γ, from both CB and PB. *Conclusions.* CB NK cells are fully functional on activation with IL-15+2 or IL-15+18 rather than IL-2 alone as observed for PB NK cells. These cytokines should be considered in the future to activate CB NK cells for therapeutic purposes.

**Key Words:** *cord blood, cytokine activation, memory-like NK cells, natural killer cells*

## Introduction

Natural killer (NK) cells play significant roles in eradicating tumor and virus-infected cells [1,2]. To discriminate between healthy and transformed cells, NK cells express a variety of activating and inhibitory receptors that allow them to recognize targets that have lost major histocompatibility complex expression or upregulated stress ligands, resulting in triggering of their cytotoxic function [3].

Currently, NK cell–based immunotherapy approaches aim to exploit their cytotoxic functions. Interleukin (IL)-2 has been shown to enhance NK cell–mediated killing of different tumor cell lines *in vitro*. However, the use of *ex vivo*–expanded autologous NK cells together with the systemic administration of IL-2 to treat patients with hematologic malignancies and solid tumors showed limited success [4,5]. In contrast, allogeneic NK cells offer greater antitumor activity potentially as the result of killer immunoglobulin–like receptors (KIR) mismatch [6,7]. Moreover, the transfer of IL-2 or IL-15 expanded allogeneic NK cells alone or together with cytokine infusion has been shown to be a safe and effective approach to treat different types of cancer [8–10].

Umbilical cord blood (CB) has been increasingly used as an alternative source of hematopoietic stem cells (HSCs) for transplantation. CB has clinical advantages over other HSC sources including off-the-shelf availability, less stringent human leukocyte antigen matching and reduced incidence and severity of graft-versus-host disease while preserving the graft-versus-leukemia effect [11–14]. After CB transplantation, NK cells show early reconstitution, which is associated with a positive graft-versus-leukemia effect [15,16]. Hence, NK cells are believed to be key effectors in the clearance of residual cancerous cells after transplant. Furthermore, CB contains a high percentage of NK cells and altogether these properties suggest CB as a promising source of NK cells for cellular immunotherapy [17,18]. However, CB NK

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cells have been shown to be distinct from PB NK cells. Gaddy *et al.* [19] reported the existence of immature $CD56^{-}CD16^{+}$ NK cells in CB, a population previously described in transplant recipients [20] and patients infected with hepatitis C or with HIV [21]. Furthermore, CB NK cells have a lower capacity to form conjugates with target cells because of a lower expression of adhesion molecules [22,23]. It has also been shown that CB NK cells express high levels of lectin-like inhibitory receptors (CD94/NKG2A) and low levels of KIR, which indicates that CB NK cells have an immature phenotype [18,22,24].

Separate studies have established that CB NK cells can respond to cytokines [19,22,25,26]. However, those studies only addressed some aspects of NK cell activation and functions, whereas some features such as activation of signal transduction pathways or migration capacity of NK cells after cytokine treatment remain to be investigated. Moreover, it has been shown that higher doses of IL-2 are required to achieve significant activation of CB NK cells as compared with PB NK cells [18,25], which suggests that CB NK cells may have distinct activation mechanisms. To date, no systematic study has been performed to assess which cytokine treatment can generate fully functional CB NK cells. Therefore, the aim of this study was to fully characterize the activation of CB NK cells, in comparison to PB NK cells, through the use of IL-2, IL-12, IL-15 and IL-18 and their combinations because these cytokines are involved in NK cell stimulation and proliferation. Other cytokines such as IL-21 have been reported to increase NK cell cytotoxicity but also exhibit regulatory functions and can induce apoptosis, IL-10 secretion, or NKG2D downregulation on NK cells and were therefore not included in the present study [27–29]. We analyzed the phenotype and function of cytokine-activated PB and CB NK cells by comparing proliferation, cytotoxicity toward K562 cells, cytokine secretion and NK cell migration. Through the use of a published protocol that was based on cytokines [30,31], memory-like NK cells were generated from CB for the first time. Altogether, this study provides the basis for the design of optimized strategies for adoptive transfer of NK cells.

## Methods

### *Samples*

Peripheral blood mononuclear cells were isolated from the blood of healthy volunteers after written informed consent was given. CB samples were obtained with prior written consent and ethical committee approval from the Anthony Nolan Cord Blood bank (NREC 10/H0405/27), Nottingham, United Kingdom. Only fresh peripheral blood mononuclear cells and CB samples were used. The study had full ethical approval from the local research ethics committee.

### *NK cell purification and culture conditions*

NK cells were isolated from PB or CB mononuclear cells with the use of the NK Cell Isolation Kit II (Miltenyi Biotec, Bergisch Gladbach, Germany), following the manufacturer's recommendations. The purities of NK cell isolation were 92.27% ± 4.5% for CB and 90% ± 2.2% for PB. Approximately $1.5–2.5 \times 10^6$ NK cells were obtained from $1 \times 10^7$ CB mononuclear cells, and $1.0–1.5 \times 10^6$ NK cells were obtained from $1 \times 10^7$ PB mononuclear cells. NK cells were resuspended at $1 \times 10^6$ cells/mL in media containing cytokines (as described in Table I), 10% fetal bovine serum and 5 µmol/L $\beta$-mercaptoethanol and were cultured at 37°C with 5% $CO_2$ [25,32–34]. IL-2 was purchased from Prospec (Ness-Ziona, Israel), IL-15 and IL-12 were purchased from Peprotech (London, United Kingdom) and IL-18 from MBL International Co (Buckingham, United Kingdom). For cultures longer than 40 h, the medium was changed by hemi-depletion every 48 h.

### *Flow cytometry*

Flow cytometric analysis was performed with the use of a BD FACSCalibur (BD Biosciences, San Jose, CA, USA), and data analysis was performed with the use of FlowJo version 6.2.4. The following monoclonal antibodies from BD Biosciences (Oxford, United Kingdom) were used: 7AAD, annexin V, CCR7 (3D12), CD3 (SK7), CD16 (NKP15), CD56 (B159), CD62L (Dreg56), CD69 (L78), CD94 (HP-3D9), CD158a (HP-3EA), CD158b (CH-L), CXCR3 (1C6/CXCR3), IL-2R$\beta$ (Mik-B3), IL-12R$\beta$1 (2.4 E6), IL-12R$\beta$2 (2B6/12B2) and p-STAT5 (pY694). NKp44 (P44-8) was from Biolegend (London, United Kingdom), NKG2A (Z199) was from Beckman Coulter (High Wycombe, United Kingdom) and

Table I. Cytokine conditions used to activate PB and CB NK cells.

| Cytokine | PB NK cells | CB NK cells | Incubation time |
|---|---|---|---|
| IL-2 | 200 IU/mL [33] | 1000 IU/mL [25] | 5 days |
| IL-12 | 10 ng/mL [34] | 10 or 50 ng/mL | 40 h |
| IL-15 | 20 ng/mL [33] | 20 or 100 ng/mL | 5 days |
| IL-18 | 100 ng/mL [32] | 100 ng/mL | 40 h |
| IL-15+2 | 20 ng/mL and 200 IU/mL, respectively | 20 ng/mL and 1000 IU/mL, respectively | 5 days |
| IL-15+18 | 20 ng/mL and 100 ng/mL, respectively | 20 ng/mL and 100 ng/mL, respectively | 5 days |