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Methods and reagents

Ex vivo expansion of CD3^{depleted} cord blood-MNCs in the presence of bone marrow stromal cells; an appropriate strategy to provide functional NK cells applicable for cellular therapy

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ABSTRACT

Considering umbilical cord blood (UCB) as a rich source of hematopoietic stem cells, we introduced a cost-effective approach to expand CD3^{depleted} UCB-MNCs into functional NK cells. CD3^{depleted} UCB-MNCs were expanded in the presence or absence of a feeder [bone marrow stem cells (BMSCs) or osteoblasts], with or without cytokines and their differentiation into NK cells was determined by flow cytometry. NK cell function was quantified by LAMP-1/CD107a expression, TNF-α/IFN-γ release, and LDH release/PI staining in targets. Higher expansion of NK cells was observed after two weeks in the presence of BMSCs and cytokines (104 ± 15) compared to osteoblasts and cytokines (84 ± 29, $p < 0.05$). On day 14, CD3^{depleted} UCB-MNCs in the presence of BMSCs and cytokines showed lower expression of CD3, CD19, CD14, CD15 and CD69 as well as higher expression of CD2 and CD7, which were suggestive of cell differentiation into mature NK cell lineage. Strong cytotoxicity of expanded cells was also identified with higher LDH release and PI% in targets. Significant upregulation of LAMP-1 with decreased release of IFN-γ and TNF-α from effectors were observed. We demonstrate an effective expansion of UCB-NK cells that maintained their functional capabilities applicable for cellular therapies.

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1. Introduction

Allogenic hematopoietic stem cell transplantation (HSCT) is usually complicated with graft-versus-host disease (GvHD) mediated by the donor's alloreactive T cells. The immunological responses of these cells against the recipient's tissues may lead to damage, primarily in the gastrointestinal tract, liver and skin. Contrary to T cells, NK cells are not involved in GvHD induction and they can even reduce it via the inhibition and lysis of autologous alloreactive GvHD-inducing T cells (Olson et al., 2010; Cerboni et al., 2007). This regulatory role of NK cells may affect T cell activation and proliferation, leading to reduced severity and delayed progression of GvHD following transplant (Alter et al., 2004). Therefore, oppose to GvHD, the graft-versus-leukemia (GvL) effect, mainly supported by the cooperation of cytotoxic T cells and NK cells, benefits transplant recipients by the eradication of residual malignant cells leading to relapse attenuation (Hosseini et al., 2012, 2013, 2015). The potential roles of NK cells in the reduction of post-transplant complications have drawn a significant interest for the use of these cells in cancer immunotherapy. So far, adoptive transfer of NK cells has proven to be an efficient approach for cancer treatment in various animal models (Alici et al., 2007; Basse et al., 2002; Siegler et al., 2005). These findings encourage scientists to develop similar protocols for cancer treatment in the clinical setting using adoptive transfer of either autologous or allogeneic NK cells. However, the isolation of clinical grade NK cells for adoptive immunotherapy is one of the most important obstacles that challenge the safety, feasibility and effectiveness of this therapeutic approach (Sutlu and Alici, 2009). NK cells comprise only a minor fraction of lymphocytes that are identified by their CD3^{-}/CD56^{+} phenotype. These cells are account for 5% to 15% of circulating lymphocytes and their low number in peripheral blood mononuclear cells (PBMCs) is an obstacle for adoptive transfer of NK cells. To overcome this problem, different methods of isolation and expansion of NK cells have been introduced to obtain a sufficient and effective source of cells for clinical trials (Childs and Berg, 2013). Prior to any clinical setting, some key points about the source of NK cells are the number, purity and state of proliferation and activation of cells. The purity of NK cells can be usually obtained by the depletion of contaminating T cells and NKT cells from PBMCs (Sutlu and Alici, 2009). Typically, simple purification of NK cells with a single-step or two-step procedure is applicable through the isolation of NK cell population from PBMC or other apheresis products that have undergone CD3^{+} T cell depletion with or without CD56^{+} selection to increase NK cell purity (Childs and Berg, 2013).

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To provide optimal sources of NK cells for adaptive transfer protocol, different methods for cell culture have been developed to expand large numbers of NK cells ex vivo. Cell expansion in cytokine-containing medium with or without stromal feeder cells has been extensively studied by different groups (Childs and Berg, 2013; Sutlu and Alici, 2009). Studies showed that, even after a short overnight incubation, cytokine-containing medium alone is capable of quick activation of NK cells, leading to enhanced cytotoxic and functional potential of these cells ex vivo. However, in the absence of feeder cells, either short- or long-term cell cultures containing cytokines, such as IL-15 and IL-2, given alone or in combination with other growth factors, have not been shown to provide significant ex vivo expansion of NK cells (Klingemann and Martinson, 2004; McKenna et al., 2007, 2012). These studies showed that sufficient and effective NK cell expansion is better achieved in the presence of feeder cells. So far, irradiated PBMCs, Epstein-Barr virus-transformed lymphoblastoid cell lines, gene-modified K562 cells expressing NK cell-stimulatory molecules such as 4-1BB ligand and membrane bound IL-15, and other irradiated tumor cell lines have been frequently used as feeder layers for the expansion of NK cells (Srivastava et al., 2008; Luhm et al., 2002; Berg et al., 2009; Shook and Campana, 2011; Imai et al., 2005).

Primitive stem cell populations including hematopoietic progenitor cells derived either from bone marrow (BM) or UCB and human embryonic stem cells can be used for NK cell expansion after being differentiated to relevant committed cells (Luevano et al., 2012; Woll et al., 2005). Studies showed the critical role of BM stromal cell system in the differentiation and formation of blood cells by providing extracellular matrix and required cytokines (Taichman and Emerson, 1998). These feeder cell systems include the stromal cells derived from BM that support hematopoiesis (bone marrow stromal cells, BMSCs) and their progeny including chondrocytes, osteoblasts and marrow adipocytes (Balakumaran et al., 2015). The use of stromal cell cultures to support hematopoietic cell expansion ex vivo has been shown by different groups (Childs and Berg, 2013). As a commonly used stromal layer, expanded bone marrow stromal cell cultures express many factors associated with hematopoietic cell differentiation and expansion including interleukin (IL)-1, IL-1β, IL-6, IL-8, IL-11, IL-12, IL-14, stem cell factor (SCF), FMS-like tyrosine kinase 3-ligand (Flt3-L), leukemia inhibitory factor (LIF) and M-colony stimulating factor (CSF) (Rafii et al., 1997). Osteoblasts are also part of the stromal cell system in the BM, which can support development of hematopoietic cells with the production of hematopoietic growth-promoting cytokines including G-CSF, M-CSF, GM-CSF, IL-1β, IL-7, tumor necrosis factor (TNF)-α, LIF, vascular endothelial growth factor (VEGF), stromal cell-derived factor (SDF)-1, osteoprotegerin (OPG) and IL-6 (Taichman et al., 2000; Neve et al., 2011; Shiozawa et al., 2008). Consequently, to find an optimized condition of NK cell expansion suitable for cell therapy, we co-cultured CD3^{depleted} CB-MNCs with either bone marrow stromal cells (BMSCs) or osteoblastic cells as feeder layer in the presence or absence of cytokines relevant to NK cell proliferation and differentiation.

2. Materials and methods

2.1. Collection and preparation of CB and BMSCs

See Supplementary methods.

2.2. Differentiation of BMSCs into osteoblast cells

See Supplementary methods.

2.3. Preparation of feeder cells

See Supplementary methods.

2.4. NK cell expansion cultures

CD3^{depleted} UCB-MNCs were plated with or without the feeder cell layer (BMSCs or osteoblastic cells), in the presence or absence of a combination of cytokines including IL-2 (250 IU/mL), IL-15, IL-3 and FLT-3L (10 ng/mL). All cytokines were obtained from Miltenyi Biotec (USA). We performed the expansions with different ratios of CD3^{depleted} UCB-MNCs:BMSCs and CD3^{depleted} UCB-MNCs:osteoblast cells to determine optimum condition. Given the insignificant difference between the ratio of 8:1 and 4:1, we chose to use the former ratio for our experiments (Fig. 1A).

Cells harvested at day 14 of culture were firstly counted with trypan blue exclusion and subsequently assessed by flow cytometry. Fold expansion of NK cells was calculated as follows.

$$\text{Fold expansion} = [(\text{final expanded MNCs number}/\text{initial MNCs number}) \times (\text{final \% of NK cells}/\text{initial \% of NK cells})]$$

2.5. Flow cytometric analyses of surface antigens

See Supplementary methods.

2.6. Cytotoxicity assay

Cytolytic potential of effectors [either fresh, non-activated CD3-^{depleted} UCB-MNCs with no feeder and cytokine or 2 week-expanded CD3^{depleted} UCB-MNCs in the presence of BMSCs feeder and cytokines including IL-2 (250 IU/mL), IL-15, IL-3 and FLT-3L (10 ng/mL)] were assessed against target (tumor cell lines) using a flow cytometry-based assay. The cell lines used in this study were the NK cell cytotoxicity-sensitive cell line, K562 (human erythroleukemia), and the NK cell cytotoxicity-resistant cell line, Daudi (human Burkitt lymphoma).

To perform this assay, target cells were labeled with the green fluorescent membrane dye, DIOC18 (3,3′-dioctadecyloxacarbocyanine perchlorate; 0.03 mM; SIGMA-Aldrich, USA), at 37° C for 15 min. These cells were washed three times with PBS and re-suspended in complete medium. The target cells were added to effector cells at various effector-to-target (E:T) ratios of 10:1, 5:1 and 1:1 then incubated for 4 h at 37 °C (Fig. 1B). After incubation, the cell mixtures were stained with Propidium Iodide (PI; 50 μg/mL; BioVision, Switzerland), which can only cross the plasma membrane of nonviable cells and binds to double-stranded DNA. Dead target cells were recognized as DIOC18 and PI double positives. To evaluate spontaneous lysis, targets were individually stained with PI. The percentage of target cell lysis was calculated as follows:

$$\text{Cytotoxicity \%} = (\text{total lysis of target \%}) - (\text{spontaneous lysis of target \%})$$

We also examined cytotoxicity activity of mentioned effectors against targets by a non-radioactive cytotoxic assay (LDH release; DGKC methods, Parsazmun, Iran). Briefly, the mixture of effector and target were resuspended in phenol red free RPMI 1640 to obtain E:T ratios of 10:1, 5:1 and 1:1. After incubation at 5% CO2 and 37 °C in humidified atmosphere for 4 h, the cell mixture was centrifuged at 200g for 5 min and then 10 μL of its supernatant was applied for LDH measurement (Experimental) according to the kit procedure. The targets or effectors were separately applied for LDH measurement to assess effector spontaneous or target spontaneous LDH release. For target maximum LDH release, targets were stimulated with calcium ionophore (10 μM; sigma, USA) for 20 min at 37 °C.

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