An improved protocol for ex vivo expansion of highly lytic NK cells from human umbilical cord blood CD34$^+$ cells

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Abstract

A number of different cytokine combinations have been tested for their ability to support the proliferation and differentiation of human $CD34^+$ hematopoietic stem cells \(HSC) into mature NK cells. Here, we describe an improved protocol based on multiple cytokines \(SCF, Flt3-L, IL-15 and IGF-1) culture for obtaining a high number of lytic NK cells from human umbilical cord blood $CD34^+$ cells.More than 6 x 10^7 highly lytic NK cells can be obtained from 1 x 10^5 fresh $CD34^+$ HSC using this protocol. This facilitates the use of the above culture system as a tool for researchers to study the development, properties and functions of NK cells in vitro, and this procedure also facilitates cellular adoptive immunotherapy by providing sufficient numbers of highly enriched $CD3^-$ $CD56^+$ effector cells with highly cytotoxic potential.

Introduction

NK cells are important effectors of the innate immune system, and the cytotoxic function of NK cells is crucial to many processes such as defending against pathogens and tumors[1, 2]. In vitro, human NK cells can differentiate from bone marrow- or cord blood-derived hematopoietic progenitor cells in appropriate culture conditions. In early studies, NK-cell development from purified HSC was shown to be stromal-cell dependent [3]. It has later been demonstrated that the stromal-cell requirements may be replaced by the provision of combined cytokines such as SCF, Flt3-L and IL-15 to the cultures[4]. In particular, SCF and Flt3-L directly induce the expression of IL-2 receptor-βchain on HSC, thereby rendering them susceptible to the NK-cell commitment induced by IL-15[5]. Recently, a number of different cytokine combinations have been tested for their ability to support the proliferation and differentiation of human $CD34^+$ hematopoietic stem cells \(HSCs) into mature NK cells. However, a cytokine that has not been extensively studied in this regard is insulin-like growth factor \(IGF)-1. IGF-1, also known as somatomedin C, is one of the main endocrine mediators of growth and development under physiological conditions[6]. In addition to this important function, IGF-1 also regulates the hematopoiesis and direct effector functions of diverse aspects of cells of the innate and acquired immune systems. In this study, we found that IGF-1 may foster the IL-15-induced NK differentiation of human umbilical cord blood \(UCB) $CD34^+$ cells. Here, we described an improved protocol based on combined cytokines \(SCF, Flt3-L, IL-15 and IGF-1) culture for ex vivo expansion of highly lytic NK cells from human UCB/$CD34^+$ cells.

Reagents

Human CD34 Microbead Kit \(Miltenyi Biotec, 130-046-702) Fluorescently labelled anti-CD34, anti-CD3, anti-CD56, anti-perforin and anti-CD107a \(BD Pharmingen). GMP Serum-free Stem Cell Growth Medium \(SCGM, CellGro®/ CellGenix™, 20802-0500) Fetal Bovine Serum \(FBS) heat inactivated \(Gibco, 10100-147) Recombinant Human SCF \(PeproTech, AF-300-07) Recombinant Human Flt3-Ligand \(PeproTech, AF-300-19) Recombinant Human IL-15\(PeproTech, 200-15) Recombinant human IGF-1 \(PeproTech, AF-100-11) Fixation/Permeabilization Solution Kit \(BD Pharmingen, 554714): including both the Fixation

and Permeabilization Solution \(BD Pharmingen, 554722) and the Perm/Wash™ buffer \(BD Pharmingen, 554723)

Equipment

Cell culture disposables: 96, 24 and 6 well plates, 50 ml and15 ml centrifuge tubes, 1.7 ml microcentrifuge tubes, pipettes, pipette tips miniMACS separator \(Miltenyi Biotec) MACS columns \(Miltenyi Biotec, 130-042-201) Flow cytometer \(FACS Calibur, BD Pharmingen) FACS tubes round bottom CO2 incubator \(Forma, 3111) Centrifuge suitable for 15 ml and 50 ml tubes \(Hettich) Microcentrifuge suitable for 1.7 ml tubes \(Sigma, 1-15K)

Procedure

1. Collect fresh cord blood \(typically 60–80 ml samples) from the umbilical cords of the placentas of normal, full-term, non-stressed newborns of consenting mothers. 2. Cord blood samples were diluted 1:2 in PBS. Cord blood mononuclear cells \(CBMC) were isolated by Ficoll-Hypaque centrifugation at 400 g for 30 minutes using standard procedures and washed extensively in PBS. ▲ CRITICAL STEP Ensure that the cells were processed within 4 h of collection. 3. Isolate $CD34^+$ cells from CBMCs with the Miltenyi human CD34 Microbead Kit according to the manufacturer's instructions \(Miltenyi Biotec). ▲ CRITICAL STEP Cell purity analysis by FACS, purity should be routinely higher than 90%. 4. Determine the number of the $CD34^+$ cells by gently mixing 10 µl of the above isolated cells with 80 µl of PBS and 10 µl of Trypan Blue. Use a Neubauer chamber to count the number of viable \(not stained) $CD34^+$ cells. 5. The isolated $CD34^+$ cells were cultured in warm SCGM supplemented with 100 U/ml Penicillin/Streptomycin and 10% FBS in the presence of recombinant human SCF \(30 ng/ml), Flt3-L \(50 ng/ml), IL-15 \(50 ng/ml) and IGF-1 \(100 ng/ml). ▲ CRITICAL STEP Depending on the cell number of the starting population \(5×10^4 cells or 1×10^5 cells per culture), cultures were grown in 0.1 or 1.0 ml medium in 96- or 24-well culture plates, respectively. 6. The cultures were incubated at 37°C in a humidified atmosphere with 5% CO2 for 4 weeks. ▲ CRITICAL STEP Every 3–4 days, half of the medium volume was replaced with fresh medium containing the same concentration of freshly added cytokines. Cell density was thereby adjusted to 1×10^6 cells/ ml in 6-well culture plates. 7Total number of viable cells differentiated from $CD34^+$ cells by various cytokine combinations, as counted for up to 4 weeks. 8. At day 28, collect cells, the percentage of $CD3^-$ $CD56^+$ cells was measured by FACS analysis. 9. NK cell function was evaluated through flow cytometry monitoring of perforin and CD107a expression. \(1) For perforin assay: Intracellular staining for perforin was performed following surface staining for $CD56^+$ $CD3^{neg}$ cells \(to identify NK cells). Stained cells were fixed and permeabilized with the Fixation/Permeabilization Solution Kit \(BD Pharmingen), as detailed in manufacturers' instructions: a. Thoroughly resuspend cells with 250 µl of BD Cytofix/Cytoperm solution in the 1.5 ml Eppendorf tube and incubate for 20 min. at 4°C. b. Wash cells two times in 1 ml BD Perm/Wash buffer. c. Thoroughly resuspend fixed/permeabilized cells in 50 µl of BD Perm/Wash buffer containing a predetermined optimal concentration of a PE-conjugated anti-perforin Ab or appropriate negative control. Incubate at 4°C for 30 minutes in the dark. d. Wash cells 2 times with BD

Perm/Wash buffer and resuspend in staining buffer and then analyzed by flow cytometry. \(2) For CD107a assay, the cytokine-differentiated NK cells were isolated, then 2×10^5 NK cells and 2×10^5 cells of the erythroleukemia cell line K562 \(American Type Culture Collection) were mixed by gentle pipetting, spun down for 1 min at 200 g, and incubated for 2 h at 37°C in 200 µl of SCGM media. NK cells were then stained with an Alexa-647-labeled anti-CD56-Ab, PE-Cy5-labeled anti-CD3-Ab, and degranulation was detected with a PE-labeled anti-CD107a-Ab \(all Abs from BD Biosciences).

Anticipated Results

Following this protocol, purified $CD34^+$ HSCs from human umbilical cord blood \(UCB) were maintained with Flt3-L and SCF, IL-15 and IGF-1 for up to 4 weeks. To monitor cell expansion in response to cytokine stimulation, cells were counted weekly. Our data showed that proliferation was increased substantially in $CD34^+$ cell cultures containing SCF, Flt3-L, IL-15 and IGF-1 \(Fig. 1). Moreover, a significant increase was observed in the percentages and absolute cell numbers of $CD3^-$ $CD56^+$ NK cells when cells were cultured in the above multiple cytokines with IGF-1 \(Fig. 2). NK cell function was evaluated through flow cytometry monitoring of perforin and CD107a expression. We found that SCF, Flt3-L and IL-15 with IGF-1 significantly increased perforin expression and CD107a release in human $CD34^+$-derived $CD56^+$ NK cells compared with that of $CD56^+$ effectors derived from the same $CD34^+$ population in the presence of SCF, Flt3-L and IL-15 alone \(Fig.3).

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