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Title: In Vitro Generation of Human NK cells Expressing Chimeric Antigen Receptor through Differentiation of Gene-Modified Hematopoietic Stem Cells

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Running Head: CAR-NK cells from HSC

Summary

NK cells represent a very promising source for adoptive cellular approaches for cancer immunotherapy, and extensive research has been conducted, including clinical trials. Gene modification of NK cells can direct their specificity and enhance their function, but the efficiency of gene transfer techniques is very limited. Here we describe two protocols designed to generate mature human NK cells from gene-modified hematopoietic stem cells. These protocols use chimeric antigen receptor as the transgene, but could potentially be modified for the expression any particular transgene in human NK cells.

Key words: hematopoietic stem cells, gene transfer, lentiviral vector, OP9 cell line, NK cells

1. Introduction

Natural Killer (NK) cells are innate immune cells that mediate spontaneous cytotoxicity against tumor and virus-infected cells, and many clinical trials have attempted to harness their properties for cellular therapies (*1–6*). Gene transfer technology can enhance the efficacy of such efforts by improving NK cell survival or function, or engineering antigen specificity (*7–9*).

Chimeric antigen receptors (CAR) are engineered fusion proteins that combine the antigen specificity of antigen-binding moieties of monoclonal antibodies and intracellular activation motifs capable to activate immune cells. Preliminary evidence suggests that NK cells with specificity directed by chimeric antigen receptors may have enhanced cytotoxicity (*10–12*).

Generation of mature NK cells from hematopoietic stem cells provides the opportunity of generation of younger NK cells and expansion of specific gene-modified clones starting from a smaller number of previously isolated and cryopreserved initial cells, with the added advantage of generation of multiple batches from the same donor (*13–16*). In this chapter we describe a protocol for NK cell differentiation from human hematopoietic stem cells (HSC) modified to express chimeric antigen receptors using co-culture with a feeder stroma of murine OP9-DL1 cells in presence of human recombinant cytokines (*15*). Alternatively, we describe a feeder-free protocol for generation of gene-modified NK cells from human hematopoietic stem cells using insulin-like growth factor 1 (IGF-1) (*17*). The transgene used in this protocol is a CD19-specific CAR, but the HSC could potentially receive any type of persistent gene modification for expression in differentiated NK cells.

2. Materials

2.1. *Isolation and cryopreservation of umbilical cord blood HSC*

1. Human primary cells from umbilical cord blood collected within 48 hours from the CD34 isolation procedure (*see Note 1*).
2. Ficoll-Paque PLUS (GE Healthcare Life Sciences).
3. CD34 MicroBead Kit UltraPure (Miltenyi Biotec).
4. MidiMACS Starting Kit with LS columns (Miltenyi Biotec).
5. MACS BSA Stock Solution (Miltenyi Biotec).
6. autoMACS Rinsing Solution (Miltenyi Biotec).
7. Dulbecco's PBS (DPBS).
8. Freezing medium: 10% DMSO in heat-inactivated fetal bovine serum (Omega).

2.2. *Lentiviral transduction of HSC*

1. Cryopreserved primary human CD34-positive hematopoietic stem cells (HSC) isolated from umbilical cord blood (*see Note 1*).
2. Transduction medium: Freshly prepared X-Vivo15 medium (Lonza), 50 ng/mL of recombinant human Stem Cell Factor (SCF) (R&D Systems), 50 ng/mL of recombinant human Flt-3 ligand (R&D Systems) and 50 ng/mL of recombinant human thrombopoietin (R&D Systems).
3. Fibronectin fragment CH-296 RetroNectin (Takara Shuzo Co.).
4. 48 well non-tissue culture-treated plate, sterile.
5. Third-generation lentiviral vector carrying second-generation chimeric antigen receptor construct (**Fig. 1a**) (*see Note 2*).

2.3. Differentiation of HSC to NK cell lineage

2.3.1. Co-culture with OP9-DL1 stromal cells

1. OP9 stromal cell line (ATCC) gene-modified to express Delta-like 1 (OP-DL1) (see **Note 3**).
2. Alpha-20 medium: alpha-Minimum Essential Medium (alpha-MEM) enriched with 20% of heat-inactivated fetal bovine serum (Omega), 2 mM L-glutamine, 50 U/mL penicillin and 50 µg/mL streptomycin.
3. NK differentiation medium: alpha-20 medium, enriched with 5ng/mL of recombinant human SCF (R&D Systems), 5ng/mL of recombinant human Flt-3 ligand (R&D Systems), 5ng/mL of IL-7 (R&D Systems) and 10ng/mL of IL-15 (R&D Systems).
4. 12 well tissue culture-treated plates, sterile.
5. T-75 and T-150 culture flasks.
6. 0.05% trypsin EDTA 1x.
7. Dulbecco's PBS (DPBS).
8. 70 µm cell strainer

2.3.2. Feeder-free differentiation protocol

1. AIM V CC medium: AIM V Complete medium (Life Technologies), 5% human AB serum (Corning) and 2 mM GlutaMAX (Life Technologies), freshly supplemented with 30 ng/mL of recombinant human cytokines SCF (R&D Systems), 50 ng/mL of recombinant human Flt-3 ligand (R&D Systems), 50 ng/mL of recombinant human IL-15 (R&D Systems) and 100 ng/mL of recombinant human IGF-1 (R&D Systems).

2. Falcon 48, 12 and 6 well tissue culture-treated microplates, sterile.

3. Methods

3.1. Isolation and cryopreservation of umbilical cord blood HSC (see Note 1)

Isolation of CD34-positive cells from umbilical cord blood collected within 48 hours will increase cell recovery. The cord blood unit can be kept at room temperature until isolation procedure. The isolation procedure should be conducted in a biosafety cabinet to ensure sterility and personal protection.

1. Dilute umbilical cord blood with equal volume of PBS.
2. Pipette 15 mL of Ficoll into a 50 mL conical tube.
3. Layer 35 mL of cord blood onto Ficoll.
4. Centrifuge at 400 g for 30 minutes at 20°C without brake.
5. Prepare isolation buffer by diluting MACS BSA Stock Solution into autoMACS Rinsing Solution according to the manufacturer's instructions, and keep it ice-cold.
6. Collect the buffy coat of all post-centrifugation Ficoll tubes with a sterile Pasteur pipette or a serological pipette into a new 50 mL conical tube.
7. Dilute such collected buffy coat with equal volume of PBS.
8. Centrifuge at 300 g for 15 minutes at 4°C, with brake on.
9. Remove supernatant.
10. Collect all cell pellets into one 50 mL conical tube, and complete to 50 mL with ice-cold isolation buffer.
11. Count cells.
12. Centrifuge at 250 g for 5 minutes at 4°C, with brake on.

13. Remove supernatant and resuspend up to 1×10^8 cells in 300 μL of ice-cold isolation buffer.
14. Follow the manufacturer's instructions to use the reagents of the Miltenyi Biotec CD34 MicroBead Kit UltraPure and the MidiMACS Kit to perform immunomagnetic positive selection of CD34 HSC using the LS columns.
15. After completion of the isolation protocol, count the CD34-positive HSC and use immediately for transduction, or cryopreserve for posterior use. HSC cells kept in culture with human cytokines will undergo mostly onto myeloid lineage differentiation and lose expression of CD34.

3.2. *Lentiviral transduction of HSC*

3.2.1. *High-titer lentivirus for modification of HSC*

Our protocol for packaging and titer analysis of high-titer lentiviral vectors for gene modification of primary human cells is very extensive and has been published elsewhere (*15, 18*). In brief, such vectors should have titer of at least 5×10^7 – 1×10^8 TU/mL to ensure very efficient transduction, what requires post-packaging procedures to obtain 100-1000-fold concentration. The vector used in this protocol is a third-generation replication-incompetent HIV-based lentivirus carrying a CD19-specific CAR and enhanced green fluorescent protein (EGFP) (**Fig. 1**) (see **Note 2**).

3.2.2. *Lentiviral transduction of HSC*

Before a NK differentiation protocol is initiated, the investigators should evaluate in preliminary experiments the optimal transduction conditions using the available preparations of lentiviral vectors in human HSC (*18*) (see **Note 4**). Gene-modified HSC should be placed in NK

differentiation culture conditions immediately after lentiviral transduction, and full expression of integrated vector copies will take place around ten days after transduction. We routinely use overnight cytokine pre-stimulation of HSC in plates coated with fibronectin fragment CH-296 RetroNectin before treatment with the lentiviral vector (*15, 18*).

1. RetroNectin coating of transduction plate: fill the wells of a non-tissue culture-treated 48 well plate with 500 μL /well of RetroNectin 20 $\mu\text{g}/\text{mL}$ in PBS and incubate for 2h in room temperature; after aspirating the RetroNectin solution, fill the wells with 1 mL/well of blocking solution with 2% fetal bovine serum (FBS) in PBS and incubate for 30 minutes in room temperature; remove the blocking solution and wash twice with 1mL/well of wash buffer with 0.025M HEPES in PBS (*see Note 5*).
2. Resuspend human CD34-positive cells at 1×10^6 cells/mL in transduction medium.
3. Plate 400 μL /well in the RetroNectin coated 48 well transduction plate (from step 1).
4. Incubate the plate for 14h in 5% CO_2 at 37°C for pre-stimulation; the cells will attach to the bottom coated with RetroNectin.
5. After pre-stimulation, add the necessary volume of lentiviral vector to ensure transduction efficiency at a viral vector copy number of 1-3 copies/cell (*15, 18*) as determined in preliminary experiments using available vector preparations (*see Note 4*).
6. Incubate transduction plate with added lentiviral vectors for 24h in 5% CO_2 at 37°C before transferring HSC to differentiation cultures.

3.3. Culture and passaging of OP9-DL1 stromal cells:

It is fundamental to keep healthy, low-passage, stromal cells for co-culture with the primary human cells (*see Notes 3 and 6*).

1. Start OP9-DL1 stromal cell culture with 2×10^6 - 5×10^6 cells in a T-75 flask using alpha-20 culture medium without any cytokines. (*see Note 7*)
2. To passage OP9-DL1: first carefully aspirate and remove the culture medium; rinse with 20 mL of PBS at room temperature, and completely remove PBS.
3. Add 12 mL of trypsin for a T-75 flask (or 30 mL for a T-150 flask) and incubate at 37°C for 5 to 15 minutes until cell layer is dispersed.
4. Add at least the same volume of alpha-20 medium to resuspend cells in order to inactivate the trypsin.
5. Count the cells and add 2×10^6 - 5×10^6 cells to a T-75 flask, or 5×10^6 - 10×10^6 cells to a T-150 flask, for continued culture (*see Note 8*).
6. For the co-culture with human cells, plate 2×10^5 - 5×10^5 per well in a tissue culture-treated 12 well plate, in alpha-20 medium (*see Note 9*).

3.4. Differentiation of HSC to NK cell lineage

3.4.1. Differentiation by co-culture with OP9-DL1 stromal cells:

All co-cultures should be performed using freshly prepared NK differentiation medium from the first day.

1. Vigorously pipette the HSC in the transduction plate after adding 0.5-1mL of NK differentiation medium per well, in order to detach all cells adherent to the bottom.
2. Count cells and adjust concentration to 5×10^5 cells/mL in NK differentiation medium.
3. Carefully remove the alpha-20 medium from the confluent OP-DL1 stroma cultured in the 12 well plates (*see Note 9*).

4. Seed the transduced HSC cells suspended in the NK differentiation medium by slow and gentle pipetting against the wall, at 3×10^5 - 5×10^5 cells per well.
5. Gently add freshly prepared NK differentiation medium by slow pipetting against the wall for a total volume of 2 mL per well.
6. Split HSC every 3-4 days; vigorously pipette using P-1000 to resuspend cells and detach stroma; remove stromal cells using a $70 \mu\text{m}$ cell strainer into a 50mL conical tube, then repeat steps 2-5 in this section (*see Note 10*).
7. Maintain co-culture of transduced HSC with stromal cells for 35-40 days to obtain functional NK cells (*see Notes 11 and 12*) (**Fig. 2a**). CD56⁺ NK cells can be sorted via fluorescence activated cell sorting (FACS) or immunomagnetic bead selection for continued culture or functional assays such as cytotoxicity assessment.

3.4.2. Feeder-free differentiation culture (alternate protocol):

All media changes and splits should be performed using AIM V CC from the first day (*see Note 13*).

1. Vigorously pipette the HSC in the transduction plate after adding 0.5-1mL of AIM V CC per well, in order to detach all cells adherent to the bottom.
2. Transfer cells to an appropriately sized conical tube. Centrifuge at 300g for 10 minutes. Remove supernatant and resuspend cell pellet by flicking the tube (*see Note 14*). Add 1 mL (or appropriate volume) of AIM V CC.
3. Count cells using a viability dye (e.g., trypan blue) and adjust viable cell concentration to 0.5×10^6 cells/mL in AIM V CC.

4. Plate cells into appropriately sized wells of tissue-culture treated microplates. For example, plate as many as 0.5×10^6 cells in 1 mL of AIM V CC into one well of a 24 well-plate, or up to 3×10^6 cells in 6 mL of AIM V CC into the well of a 6 well-plate.
5. Cells are incubated at 37°C in a humidified atmosphere with 5% CO_2 .
6. Differentiating cells will need to be split up to twice a week. Pipet to detach gently adherent cells and transfer half the media and cells to a neighboring well that has not been used or to a new equally sized plate. Replenish the media to the original volume in both wells with fresh AIM V CC (*see Note 15*).
7. In order to remove cellular debris, every 10-12 days the cells should be washed and completely replenished with fresh media and cytokines. Pipet to detach the slightly adherent cells, ignoring the strongly adherent cell populations. Transfer the non-adherent cells to conical tubes and centrifuge at $300g$ for 10 minutes. Remove supernatant and resuspend cell pellet in an appropriate volume of AIM V CC for counting cells. Count cells using a viability dye and adjust viable cell concentration to 1×10^6 cells/mL in AIM V CC. Plate cells into appropriately sized wells of Falcon tissue culture treated microplates. For example, plate as many as 5×10^6 cells in 5 mL AIM V CC into one well of a 6 well-plate. (*see Note 15*).
8. CD56+ NK cells will begin to appear around Day 14, peak around Day 28 and begin to decline thereafter (**Fig. 2b**).
9. Between days 14 and 28, CD56+ NK cells can be sorted via fluorescence activated cell sorting (FACS) or immunomagnetic bead selection for continued culture or functional assays such as cytotoxicity assessment. Sorted NK cells can be cultured at 1×10^6 cells/mL, but will no longer appreciably expand, in AIM V medium (Life Technologies)

supplemented with 5% human AB serum (Corning) and 2 mM GlutaMAX (Life Technologies) and 20 ng/mL of recombinant human IL-15 (R&D Systems). In our hands, sorted NK cells in this media remain cytotoxic for approximately two weeks longer.

4. Notes

1. The presented protocols can be applied to any source of human CD34-positive cells (*14*). However, the final yield of differentiated NK cells is higher using fetal liver or umbilical cord blood, as opposed to bone marrow or mobilized peripheral stem cells (*19*).
2. The presence of a co-delivered marker, such as enhanced green fluorescent protein (EGFP) or a selection component, will allow enrichment for gene-modified NK cell precursors at any step of the differentiation protocol.
3. It is not clear if DL1 expression is necessary for successful generation of mature and functional NK cells. Preliminary evidence suggests that OP9-DL1 co-culture promotes faster differentiation and higher cell proliferation (*13, 15, 20*).
4. In clinically-relevant experiments, the viral vector copy number per cell in HSC should be kept within 1-3 copies/cell, in order to avoid genotoxicity.
5. A plate coated with RetroNectin can be preserved at 4°C for up to 7 days with every well filled with 1mL of wash buffer.
6. In the co-culture protocol have used non-irradiated OP9-DL1 stromal cells.
7. Start OP9-DL1 stromal cell culture 1-2 weeks before HSC transduction, in order to have at least a full T-75 flask with confluent stroma before starting the protocol.

8. Passage OP9-DL1 cells cultured in flasks every 3-5 days and when no more confluent than 90% split cells at 1:5-1:10, i.e., using one-fifth to one-tenth of the volume of cell suspension from one flask to re-seed a new flask. Fully confluent stroma decays in about 10-14 days. Timing of the passage of OP9-DL1 cells with the seeding of plates for the co-culture with HSC will optimize the protocol and avoid waste.
9. OP9-DL1 cells should be at 90% confluence before seeding the human cells, what usually takes 2-4 days after seeding on a tissue culture-treated 12 well plate.
10. Seed the HSC over fresh confluent OP9-DL1 stromal cells each time HSC are passaged. During the first two weeks the human cells will proliferate faster and may require passaging more often.
11. In the OP9-DL1 co-culture protocol, differentiated NK cells will develop cytotoxicity against the stromal cells around day 35 of culture, as a surrogate of successful differentiation.
12. The use of a NK cell expansion protocol will increase the final yield of the cell generation when used in sequence to the protocols described in this chapter. Cells should be transferred to the expansion protocol at day 30 or later, when cells have higher expression of CD16 and other mature NK cell-specific markers.
13. In preparation of AIM V CC, human AB serum must be heat inactivated at 56°C for 30 min and sterile filtered to remove excess lipids. Alternatively, the AIM V Complete medium can be sterile filtered prior to the addition of cytokines.

14. In order to resuspend the cell pellet, it is important to flick the tube to disperse the cells rather than to directly pipet the pellet, which can cause shearing and decrease viability of cells at this point.
15. As HSC differentiate, adherent cells such as endothelial cells and macrophages will become observable. NK and NK progenitors, however, are not strongly adherent; therefore, during splits, pipet to detach cells that are easily detachable and do not attempt to remove strongly adherent cells. If the well becomes overgrown with adherent cells, do not continue to use it for NK differentiation and expansion.

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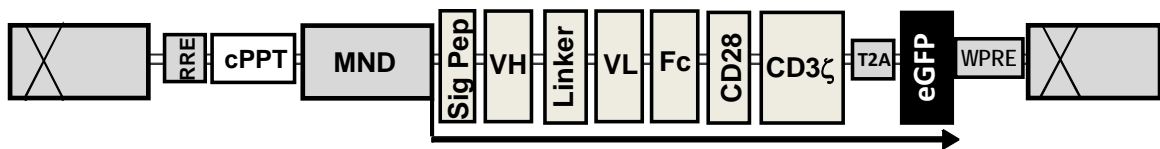
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Figure Legends

Figure 1. Lentiviral vector for transduction of human HSC. (a) Diagram of third generation lentiviral vector carrying anti-CD19 CAR and EGFP. (b) Co-expression of CAR and EGFP detected by flow cytometry in NK cells differentiated in vitro from human HSC, day 40 of culture. CAR expression was detected by using PE-Texas Red-conjugated polyclonal F(ab')₂ fragment goat antihuman IgG1 Fc γ (Jackson ImmunoResearch Laboratories)(15).

Figure 2. Expression of NK-specific surface markers along progression of in vitro differentiation culture from gene-modified human HSC. (a) Co-culture with OP9-DL1 stromal cells. (b) Feeder-free culture. Representative plots were taken at days 14, 29 and 40 of both NK differentiation culture protocols. Gated human cells positive for EGFP were evaluated for the CD56, CD16, CD94, CD57, CD158 and CD335 surface markers.

A.



MND-CD19RCD28-EGFP

B.

