

Chapter 2

In Vitro Development of Human Killer–Immunoglobulin Receptor-Positive NK Cells

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Abstract

The in vitro culture system outlined in this chapter allows for the delineation of events that occur during the development of CD34⁺ hematopoietic precursor cells into mature KIR⁺ human NK cells. This system can also be utilized to study the effects of gene overexpression or knockdown on the process of NK cell differentiation through retroviral transduction and long-term culture. The necessary soluble factors and contact-dependent conditions for in vitro human NK cell development have been worked out in our laboratory over the past 16 years.

Key words: Human NK cell development, lymphocyte differentiation from hematopoietic precursors

1. Introduction

Both mouse and human natural killer cells recognize transformed and virally infected cells and influence the direction of the adaptive immune response in infectious settings (1). However, there are several notable differences between mouse and human NK cells with respect to differentiation markers, making developmental comparisons between the species difficult (2–4). To evaluate and manipulate human natural killer cell differentiation, a robust ex vivo cell culture system is necessary. To this end, our laboratory has developed a long-term culture system for studying human NK cell development from primitive progenitor cells (5–9).

In 1992, we were the first to show that primitive progenitors from adult bone marrow can give rise to functional NK cells

when cultured in contact with human bone marrow stroma (5). In 1994, we found that in the absence of factors known to support NK cell differentiation, direct contact with human allogeneic stroma is critical for NK cell differentiation (6). In 1998, after an explosion of new data describing novel NK cell receptors recognizing class I MHC, we found that our *in vitro* model of NK cell differentiation supported the acquisition of KIR (specifically KIR3DL1 in this initial description) (7).

Limitations of these initial studies included the heterogeneity and variability of primary human stroma, low cloning frequency when plating primitive stem cell populations, and the inability to support differentiation at the single cell level. These issues raise the possibility of starting progenitor contamination. Therefore, definitive study of NK cell precursors was technically difficult.

In 1999, we pioneered the use of a novel murine stromal cell line, called AFT024, which was derived from day 14 gestational fetal liver cells immortalized by a retrovirus containing a temperature-sensitive SV40 T antigen. The most important finding for the success of NK cell development cultures is that AFT024 could officially support NK cell differentiation of human cells at the single cell level. Using this system, we have shown that KIR and NKG2/CD94 receptors are acquired late in NK cell development (8). In 2008, we further improved this system by comparing NK cell development on a novel murine cell line called EL08-1D2 cloned from a culture of embryonic liver at day 11 of gestation. EL08-1D2 was chosen for these studies because of its ability to support generation of human hematopoietic progenitors from CD34⁺ umbilical cord blood cells without the addition of any cytokines. Use of EL08-1D2 identified several novel properties of this stromal feeder.

First, when IL-15 was eliminated from the culture medium, there was an accumulation of CD56⁺ NK cell precursors defined as CD34⁺/CD7⁻, CD34⁺/CD7⁺, and CD34⁻/CD7⁺. The role of IL-3 and IL-3 plus Flt3 ligand was established, and c-kit ligand and IL-7 appear to add efficiency to the system but are not absolutely required for NK cell differentiation. EL08-1D2 was superior to AFT024 for supporting differentiation of NK cell precursors, NK cell commitment, the acquisition of KIR, and overall proliferation (9). EL08-1D2 has been the stromal feeder of choice in our laboratory based on its ability to recapitulate the acquisition of class I recognizing receptors and developmental intermediates which may be important in NK cell maturation (10).

There has been support in the literature for NK cell differentiation cultures in the absence of stroma. In review of this literature, our stromal-based cultures seemed to better allow the acquisition of NK cell receptors where NK cell differentiation in the absence of stroma but in the presence of high concentrations of human cytokines allows NK cell commitment with poor expression of

KIR (11). Our data support the notion that unique signals from stroma are important in the acquisition of NK cell receptors. Further proof is the finding that CD56^{bright} KIR[−] cells can transition into CD56^{dim} KIR⁺ cells on EL08-1D2 and human IL-15 (12).

In summary, methods for NK cell differentiation cultures using EL08-1D2 will be described here. Other stromal cell lines, such as OP9 and MS5, have been used in the literature for similar purposes and could possibly be substituted here but direct comparisons with EL08-1D2 have not been performed in our laboratory (13, 14). CD34⁺ hematopoietic progenitors from umbilical cord blood are highly efficient in NK cell development in terms of both cloning efficiency and proliferation. Other hematopoietic stem cell sources such as bone marrow, peripheral blood progenitors, and fetal liver have been tested in our laboratory and can be substituted here if desired, but their efficiency may differ from the bulk of our work using human umbilical cord blood progenitors.

2. Materials

This protocol includes the experimental procedures that our laboratory has developed to retrovirally transduce CD34⁺ cells prior to long-term culture in order to study the effects of gene overexpression or knockdown. If the user does not desire to carry out retroviral transduction, **Section 2.3** and steps 24–42 in **Section 3.3** can be excluded from the protocol.

2.1. EL08-1D2 Cell Culture and Irradiation

1. EL08-1D2 cells: mouse embryonic liver cells. These cells were obtained from E. Dzierzak at Erasmus University MC, Rotterdam, the Netherlands.
2. *EL08-1D2 medium*: 202.5 ml α -MEM medium (Gibco, Carlsbad, CA), 250 ml Myelocult[®] M5300 medium (Stem-Cell Technologies Inc., Vancouver, BC, Canada), 37.5 ml fetal calf serum (*see Note 1*), 5 ml Penicillin + Streptomycin, 5 ml 100 \times Glutamax (Gibco), 35.0 μ l β -mercaptoethanol (0.143 M stock: 1:100 in H₂O), and hydrocortisone at a final concentration of 10^{−6} M. Hydrocortisone must be added fresh at the time of use. This medium (without hydrocortisone) can be stored at 4°C for up to 1 month. Note that these additives should be included on top of what is already present in the Myelocult[®] M5300 medium.
3. Ultrapure water with 0.1% gelatin (Chemicon Intl., Billerica, MA).
4. Trypsin/EDTA (Gibco).

5. Flat-bottom 96-well cell culture plates (Becton Dickinson, Franklin Lakes, NJ).
6. Multi-channel pipetter.
7. Cesium irradiator.

2.2. CD34⁺ Cell Isolation from Umbilical Cord Blood

1. Histopaque[®]-1077 (Sigma-Aldrich, St. Louis, MO).
2. Ammonium chloride solution (StemCell Technologies Inc.).
3. Coulter Particle Counter[®] (Beckman Coulter, Fullerton, CA).
4. PBS/0.3% BSA.
5. Direct CD34 Progenitor Cell Isolation Kit, human (Miltenyi Biotech, Oberlin, CA).
6. MACS[®] LS separation columns (Miltenyi Biotech).
7. MACS[®] pre-separation filters (Miltenyi Biotech).
8. MACS[®] magnetic stand and magnets (Miltenyi Biotech).
9. *Cycling Medium*: Iscove's Modified Dulbecco's Medium (with L-glutamine), 20% fetal bovine serum, 1% Penicillin + Streptomycin with 20 ng/ml IL-7, 20 ng/ml c-kit ligand, 20 ng/ml Flt3 ligand, and 20 ng/ml thrombopoietin. This medium can be stored at 4°C for up to 1 month.
10. 24-well cell culture plates (Becton Dickinson).

2.3. Retroviral Transduction and Cell Sorting of CD34⁺ Cells

1. Four separate 3 ml aliquots of retroviral supernatant (*see Note 2*).
2. Transwells with sterile 6-well culture plates — 0.4 µm PTFE membrane, 24 mm insert (Corning Incorporated, Corning, NY).
3. Tweezers.
4. RetroNectin (r-Fibronectin) (Otsu, Shiga, Japan).
5. PBS/2% BSA.
6. Iscove's Modified Dulbecco's Medium (with L-glutamine) without fetal bovine serum (Gibco).
7. Cycling medium (*see Section 2.2*).
8. APC-conjugated anti-CD34 monoclonal antibody (clone 8G12, mouse IgG₁; BD Biosciences)
9. APC-conjugated IgG₁ isotype control monoclonal antibody (BD Bioscience).
10. 5 ml polystyrene round-bottom tubes with cell-strainer cap (BD Biosciences).
11. A fluorescence-activated cell sorter.

2.4. Long-Term Culture of Sorted CD34⁺ Cells on the EL08-D12 Stromal Line

1. *Basal Culture Medium*: A 2:1 (vol:vol) mix of Dulbecco's Modification of Eagle's Medium (DMEM) with 4.5 g/l glucose, L-glutamine, and sodium pyruvate/Ham's F12 Medium. This medium mixture is then supplemented with 24 μ M 2-mercaptoethanol, 50 μ M ethanolamine, 20 mg/l ascorbic acid, 50 μ g/l sodium selenite, 1% penicillin + streptomycin and 20% heat-inactivated human AB serum (Valley Biomedical, Inc., Winchester, VA). The following cytokines must also be added: 10 ng/ml IL-15, 5 ng/ml IL-3, 20 ng/ml IL-7, 20 ng/ml c-kit ligand, and 10 ng/ml Flt3 ligand. This medium can be stored at 4°C for up to 1 month.
2. Multi-channel pipetter.

3. Methods

3.1. General Culture Conditions for EL08-1D2 Cells

1. Coat sterile cell culture flasks (75 cm² or 150 cm²) with enough sterile ultrapure water with 0.1% gelatin to cover the bottom of the flask. Let the flask sit in the culture hood at room temperature for 10 min. The gelatin water is necessary to promote adherence of the EL08-1D2 cells.
2. After 10 min, remove the ultrapure water with 0.1% gelatin and gently rinse the bottom of the culture flask with PBS. Remove the PBS.
3. Plate cells at approximately 4000 cells/cm² on gelatin-coated flasks in an appropriate volume of EL08-1D2 medium (12 ml for a 75 cm² flask or 24 ml for a 150 cm² flask) containing 20% conditioned EL08-1D2 medium (*see Note 3*).
4. Culture cells in an incubator set to 32°C 5% CO₂. This is important, as the cells grow in a temperature-sensitive manner.
5. Once the cells are 95–99% confluent, they can be split into new flasks.
6. To split cells, remove the spent medium, filter, and store at –20°C. Add enough 1× trypsin/EDTA to cover the bottom of the flask and incubate at room temperature until the cells start to lift off the bottom of the flask. Add 5 ml of 0.2 μ m-filtered EL08-1D2 conditioned supernatant to neutralize the trypsin and collect cells in a 15 ml centrifuge tube.
7. Count cells using a hemocytometer. Remove the desired number of cells (approximately 4000 cells/cm²) to seed a new flask(s).

8. Centrifuge at $550 \times g$ for 4 min and decant supernatant.
9. Resuspend cells in freshly prepared EL medium and gently transfer to a flask(s) freshly coated with gelatin water.
10. Return flask(s) to a 32°C 5% CO_2 incubator.

3.2. Irradiation of EL08-1D2 Cells

1. Use a multi-channel pipette to add 50 μl of ultrapure water with 0.1% gelatin per well into the desired number of flat-bottom 96-well cell culture plates. Let plates incubate for 10 min at room temperature.
2. After 10 min, remove the ultrapure water with 0.1% gelatin and gently rinse the bottom of the culture plates with PBS. Remove the PBS.
3. Resuspend EL08-1D2 cells in fresh EL08-1D2 medium (with 20% conditioned medium and hydrocortisone) at a concentration of 10^4 cells/ml.
4. Add 10^3 cells per well (100 μl) to the gelatin-coated and rinsed plates. Incubate plates at 32°C 5% CO_2 for 3–5 days until the plates are 95–99% confluent.
5. Irradiate confluent plates with 30 Gy of radiation and let the plates incubate at 32°C 5% CO_2 for at least 2 h before use. Irradiated plates can be kept for up to 1 week before use in an NK cell developmental assay.

3.3. CD34⁺ Cell Isolation and Retroviral Transduction

Day 1

1. Warm Histopaque[®]-1077 and PBS to room temperature.
2. Transfer umbilical cord blood into a sterile container and add 50 ml PBS to dilute the blood.
3. Add 20 ml Histopaque[®]-1077 to the necessary number of 50 ml centrifuge tubes.
4. Slowly layer 30 ml diluted blood over the Histopaque[®]-1077.
5. Centrifuge at $550 \times g$ for 30 min at room temperature with the brake off.
6. Slowly aspirate the plasma layer leaving approximately $\frac{1}{2}$ inch of liquid above the lymphocyte/Histopaque[®]-1077 interface.
7. Harvest the lymphocytes from the lymphocyte interface and transfer to a new 50 ml centrifuge tube. Fill up to 50 ml with PBS and centrifuge at $850 \times g$ for 5 min.
8. Gently decant the supernatant and resuspend cells in 10 ml ice-cold ammonium chloride solution. Incubate the cells on ice for 10 min.
9. Centrifuge for 5 min at $850 \times g$. Gently decant supernatant and resuspend cells in 20 ml PBS.

10. Determine total cell number using a Coulter Particle Counter[®] (*see Note 4*).
11. Wash the cells by adding another 30 ml of PBS to the cell suspension. Centrifuge for 5 min at $850 \times g$.
12. Gently decant the supernatant and resuspend cells in 300 μ l PBS/0.3% BSA per 10^8 cells. Keep the PBS/0.3% BSA cold throughout the isolation procedure.
13. Label cells for isolation by adding 100 μ l FcR blocking reagent for every 10^8 cells. Next, add 100 μ l CD34 Microbeads for every 10^8 cells. Incubate for 30 min on ice in the dark.
14. Add 30 ml of PBS/0.3% BSA and centrifuge for 5 min at $850 \times g$.
15. Gently decant the supernatant and resuspend the cells in 0.5 ml of PBS/0.3% BSA.
16. Set up one MACS[®] LS column per cord blood donor on a MACS[®] Magnetic Stand. Place a MACS[®] pre-separation filter on top of each column and equilibrate the column with 3 ml of PBS/0.3% BSA.
17. Pipette the labeled cells into the MACS[®] pre-separation filter and let the cells pass through the column. There is no need to save the effluent, as the CD34⁺ cells will be retained within the column.
18. Wash the column three times with PBS/0.3% BSA.
19. Remove the MACS[®] pre-separation filter, pull the column off the magnet, and transfer the column to a 15 ml centrifuge tube. Apply 5 ml of PBS/0.3% BSA and forcefully plunge the liquid through the column to purge the labeled cells.
20. Repeat steps 16–19 with a new MACS[®] LS column. It is necessary to double-column the labeled cells in order to obtain a CD34⁺ population with a high level of purity.
21. Count the total number of cells in the CD34⁺ fraction using a hemocytometer.
22. Centrifuge cells for 4 min at $550 \times g$ at 4°C (*see Note 5*).
23. Gently decant the supernatant and resuspend the cell pellet in Cycling Medium at a concentration of 5×10^5 cells/ml. Transfer the cells in Cycling Medium to a 6-well or 24-well cell culture plate and place in an incubator set to 37°C 5% CO₂. If cells are resuspended in less than 2 ml of medium (1×10^6 cells or less), they should be added to a well of a 24-well plate. Larger volumes should be added to a 6-well plate. The number of cells isolated will vary widely between

umbilical cord blood donors. Let the cells proliferate in the Cycling Medium for 3 days (*see* **Note 6**).

Day 4

24. Three days after the CD34⁺ cell isolation, reconstitute one vial of RetroNectin at a concentration of 0.05 mg/ml according to the manufacturer's instructions.
25. Transfer two transwells per umbilical cord donor to a 6-well culture plate using a pair of sterilized tweezers.
26. Coat transwells in the 6-well plate with 0.1 mg of RetroNectin (2 ml of 0.05 mg/ml RetroNectin per well). Incubate plate for 2 h at room temperature.
27. Remove RetroNectin from both the top and the bottom of the transwells.
28. Wash by adding 2 ml of PBS/2% BSA to the transwell. Let the transwells sit for $\frac{1}{2}$ h at room temperature (*see* **Note 7**).
29. Remove PBS/2% BSA from both the top and the bottom of the transwells.
30. Add 2 ml of Iscove's Medium (without FBS) to each transwell and let the transwell sit for $\frac{1}{2}$ h at room temperature.
31. Remove all Iscove's Medium from the top and the bottom of the transwells.
32. Add 2×10^5 CD34⁺ cells (at a concentration of 4×10^5 cells per ml) to each transwell along with 3 ml of the desired viral supernatant. Do not let the virus sit at room temperature longer than necessary before adding it to the transwell.
33. Place plates in an incubator set to 37°C 5% CO₂ for 6 h.
34. Remove the viral supernatant from the bottom of the transwells, taking care to disturb the cells on the top of the transwell as little as possible.
35. Place the transwell plate back into the incubator for 5 min to allow the rest of the viral supernatant to pass through the transwell.
36. Remove the remaining viral supernatant from the bottom of the transwells.
37. Slowly add 3 ml Cycling Medium to the top of each transwell and place the transwell plate back in the incubator overnight.

Day 5

38. Remove the Cycling Medium from the bottom of the transwells and allow all of the medium to pass through. Remove the remaining medium from the bottom of the transwells.
39. Add 3 ml of the same type of viral supernatant used during the previous day for transduction to each transwell.

40. Place plates in an incubator set to 37°C 5% CO₂ for 6 h.

41. Repeat steps 34–37 in this section.

Day 7

42. Harvest the cells from each transwell by pipetting up and down a few times and washing the top of the transwells with 1 ml of medium taken from the bottom of the transwells. Place the cells into a 15 ml centrifuge tube.

43. Remove 100 µl from each GFP control sample and put aside on ice for an isotype control.

44. Count cells using a hemocytometer. Generally, you should expect to observe a two- to threefold increase in cell number over the course of the retroviral transduction due to expansion in the Cycling Medium.

45. Centrifuge cells at $550 \times g$ for 4 min.

46. Gently decant the supernatant and add 10 µl of anti-CD34 APC-conjugated monoclonal antibody per 1×10^6 cells.

47. Add 1.5 µl of APC-conjugated mouse IgG₁ antibody to the 100 µl of cell set aside for the isotype control.

48. Incubate samples in the dark on ice for 30 min.

49. Wash off excess antibody by adding 5 ml of PBS/0.3% BSA and centrifuging at $550 \times g$ for 4 min.

50. Gently decant supernatant and resuspend stained cells in approximately 0.3 ml of PBS/0.3% BSA.

51. Pass the labeled cells through the strainer cap and into a 5 ml FACS tube.

52. Collect the double-positive GFP⁺/CD34⁺ population using a fluorescence-activated cell sorter (*see* **Note 8** and **Fig. 2.1A**).

53. Resuspend sorted GFP⁺CD34⁺ cells at a concentration of 500 cells per ml in Basal Culture Medium.

54. Remove the 96-well plates containing irradiated EL08-D12 cells from the incubator and gently remove the EL08-D12 medium using a multi-channel pipette.

55. Add 100 µl of sorted cells in Basal Culture Medium to each well containing irradiated EL08-D12 cells. This will result in a plating of 50 transduced CD34⁺ cells per well.

56. Place plates in an incubator set to 37°C and 5% CO₂.

**3.4. Long-Term
Culture of Sorted
CD34⁺ Cells on the
EL08-1D2 Stromal
Line**

1. After 7 days have passed, add 100 µl of Basal Culture Medium to each well and place the plates back into the incubator.

2. After 14 days have passed, carefully remove 150 µl of medium from each well without disturbing the cells on the bottom of the wells. Add 150 µl of Basal Culture Medium to

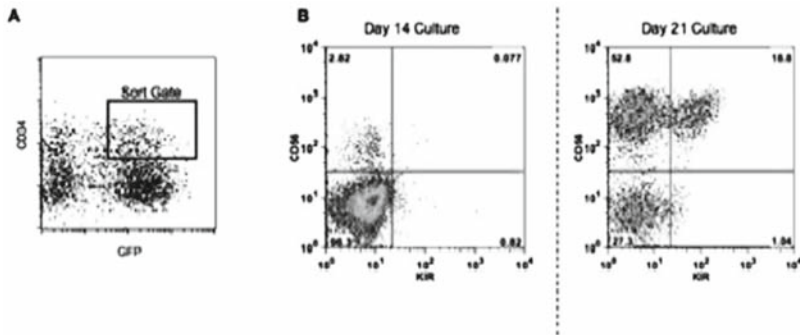


Fig. 2.1. **(A)** Representative phenotypes of retrovirally transduced CD34⁺ cells harvested at the time of sorting. A fairly conservative CD34⁺/GFP⁺ gate should be selected to collect this population for long-term culture. The CD34⁺ cells in this plot represent cells that have already begun to differentiate during the cycling phase of the protocol. These cells should not be collected. **(B)** Representative phenotypes of long-term cultures of CD34⁺ cells differentiated on EL08-1D2 stroma in the presence of 10 ng/ml IL-15, 5 ng/ml IL-3, 20 ng/ml IL-7, 20 ng/ml c-kit ligand, and 10 ng/ml Flt3 ligand. CD56 and KIR (mixture of DX9, EB6, GL183, and FES172 monoclonal anti-KIR antibodies conjugated with a common fluorochrome) expressions on cells harvested from day 14 and day 21 cultures are shown. As this figure illustrates, the major transition of hematopoietic cells into mature NK cells occurs between 2 and 3 weeks after plating.

each well. At this time point, a proportion of the cells in culture begin to express CD56, but do not express significant levels of KIR as determined by FACS analysis.

3. After 21 days, change the medium again by removing 150 μ l of medium from each well and adding back 150 μ l of Basal Culture Medium. At this time point, a significant number of cells in culture express both CD56 and KIR as determined by FACS analysis (*see* **Note 9** and **Fig. 2.1B**).
4. Full NK cell maturation will be observed after 21 or 28 days in culture, and this is usually used as an endpoint for in vitro NK cell development experiments (*see* **Note 10**).

4. Notes

1. The fetal calf serum used for EL08-D12 cell culture is specially designed for the maintenance of stromal cell lines from mouse embryonic tissues and is distributed by Stem-Cell Technologies, Inc. Other types of serum should not be substituted without comparative testing.
2. For this protocol, it is necessary to prepare ahead of time two 3 ml aliquots of retroviral supernatant prepared using a control GFP construct for each umbilical cord blood donor. You will also need two 3 ml aliquots of retroviral supernatant prepared using a construct containing GFP and your gene-of-interest for each umbilical cord donor.

We have consistently used Murine Stem Cell Virus (MSCV) promoter constructs for gene overexpression in CD34⁺ cells. We package MSCV retroviral particles in the 293kj cell line using the PCL packaging plasmid as previously described (15).

3. The “conditioned” EL08-1D2 medium refers to the supernatant from EL08-1D2 culture flasks that are at or near confluency. This medium should be harvested, 0.2 μ m-filtered, and frozen at -20°C for storage up to 6 months. Conditioned medium should be added to the EL Medium immediately before use at a 20% concentration.
4. Because of the high cell counts per unit of umbilical cord blood, counting with a hemacytometer will be less accurate. We also recommend that the user add 5–6 drops of Zap-oglobinTM II Lytic Reagent (Beckman Coulter) to the Isotone reagent before counting to obtain a more accurate lymphocyte count.
5. There is considerable variability in CD34⁺ cell yields between umbilical cord donors. This may be because of age from procurement to use or due to individual unit variability. Final cell counts generally fall between 3×10^5 cells and 1.5×10^6 cells. It is our experience that fresh umbilical cord blood units are more efficient in NK cell differentiation cultures than cryopreserved cells which work but with slightly less efficiency.
6. If gene modification with an EGFP marker is considered, it is important to setup the experiment so that you can sort the cells using a fluorescence-activated cell sorter 6 days after the CD34⁺ cell isolation.
7. Because of variability in the manufacturing of the transwells, the RetroNectin solution may or may not pass through individual transwells. This will not affect the experiment. However, if the RetroNectin does not pass through, we recommend adding 1 ml of PBS/0.3% BSA to the bottom of the transwell and 1 ml of PBS/0.3% BSA to the top of the transwell during the washing step to soak the transwell so that viral supernatant can pass through.
8. The percentage of GFP⁺/CD34⁺ cells in culture depends on the size of the retroviral construct and the potency of the virus. We routinely observe that, when using conservative gating, 1–5% of cells are GFP⁺/CD34⁺ at the time of the sort.
9. A reduction in the number of EL08-D12 cells will usually be observed after 21 days in culture due to their lysis by NK cells. The EL08-D12 stromal layer will disappear completely at later time points.

10. There is considerable variability between umbilical cord donors with respect to the rates of differentiation and proliferation in culture, which may be due to the intrinsic sensitivity of individual donors to the cytokines used in culture. Therefore, we recommend that at least four to six donors are analyzed and compared for each experiment.

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