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Isolation and In Vitro Generation of Gene-Manipulated Human Plasmacytoid and Conventional Dendritic Cells

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Abstract

Our understanding of human lymphocyte development has increased significantly over the past 20 years. In particular, our insight into human T- and B-cell development has improved (1, 2). Nonetheless, there are many gaps in our understanding, particularly regarding the early stages of development of hematopoietic progenitor cells (HPCs) into downstream lineage-biased and lineage-restricted precursors and the molecular mechanisms underlying these activities. The same holds true for our knowledge of human dendritic cell (DC) development. While the amount of data on the different subsets of conventional DCs (cDCs) and plasmacytoid DCs (pDCs) rapidly increases in mice (3, 4), the developmental stages of different DC subsets in humans remain poorly defined (2). The relatively easy access to patient material and therefore human precursor cells that can be isolated from these tissues combined with the availability of in vitro and in vivo differentiation assays allows studies in the field of human hematopoietic development, including that of DCs. In addition, the opportunities to manipulate gene expression, by stable overexpression of a gene of interest or RNA interference-mediated knockdown, generate valuable information about the mechanisms underlying lineage commitment and differentiation.

Keywords

Human immune system; CD34⁺ hematopoietic stem cells; OP9; differentiation; plasmacytoid dendritic cells; conventional dendritic cells

1. Introduction

To date our knowledge of the mechanisms that regulate development and function of the hematopoietic system is largely derived from experiments performed in mouse models. While these studies are of the utmost importance, one has to take into account that considerable differences between mice and humans exist. This discrepancy invites for more extensive research on fundamental processes in human biology. Access to human tissues such as umbilical cord blood, buffy coats extracted from blood donations, and left-over samples from surgical procedures including postnatal thymus and fetal tissues has greatly facilitated research on development of the human hematopoietic system. These biological materials allow isolation of (subsets of) cell populations of the immune system and moreover they provide a source of human HPCs. Using in vitro assays we are able to

stimulate the differentiation of isolated human progenitor cells into most hematopoietic lineages (5–7). In addition we are able to modify the genetic profile of progenitor cells by means of retro- (8, 9) and lentiviral transduction (see chapter by van Lent et al. in this issue). Combined these techniques allowed the identification of factors that control the development and function of the cells that make up the human hematopoietic system.

The cytokine Flt3L (10) is vital for the development of human pDCs in vitro from CD34⁺ progenitor cells present in fetal liver, cord blood, and bone marrow (11). The subsequent stages of development of the type-I interferon (IFN)-producing pDCs in these compartments have been well characterized (11). The human postnatal thymus also contains CD34⁺ progenitor cells with pDC potential, however, the stages of development appear to be slightly different (11, 12). In recent years our lab has concentrated on gaining insight into the complex network of transcription factors that controls progenitor cell differentiation and the regulation of human pDC development. Using the methods described in this chapter we have identified the ETS transcription factor Spi-B as a key regulator in human pDC development (6, 13). Furthermore, we showed that the DeltaLike1/Notch1 signaling pathway controls the T/pDC lineage decision by controlling the expression of downstream lineage-specific transcription factors. Activation of the T-cell lineage-specific receptor Notch1 by its ligand DeltaLike1 negatively regulates Spi-B expression and thereby inhibits pDC development (7). In addition, we recently found that the E-protein TCF4/E2-2 is highly expressed in pDCs as compared to the CD34⁺ progenitor cells and that development of pDCs depends on E2-2 expression (14). This is in line with our previous observation that forced expression of Id2 and Id3, which are negative regulators of E-proteins, in CD34⁺ progenitor cells induces a profound block in pDC differentiation (15). This, however, left the development of conventional DCs unaffected (15). The Id2-induced block in pDC development can be released by co-transduction of E2-2 (14). Notably, Spi-B was unable to overcome this developmental block. Also, we observed that Spi-B downregulates Id2 expression likely allowing E2-2 to stimulate pDC development. Together a model is emerging in which Spi-B and E2-2 co-operate in the development of human pDCs.

The in vitro generated pDCs are fully functional as addition of the Toll-like receptor 9 (TLR-9)-specific ligand CpG or virus, such as Herpes simplex virus, Influenza virus, and HIV, induces production and secretion of type-I IFN (7, 16). In vitro generated pDCs stimulated with CpG or virus inhibit thymic T-cell development at an early stage (16). This suggests that the anatomical localization of T-cell progenitor cells in the cortex and pDCs in the medulla of the thymus (17, 18) is an important requirement to allow proper development of T cells.

Although in vitro systems have merit for investigations on development of particular lineage branches they never allow for studying the complexity of the entire hematopoietic system in one assay. Tremendous progress has been made in the generation of humanized mouse models allowing the simultaneous development of the different hematopoietic lineages and their function in an in vivo setting (6, 19, 20). These issues, however, are addressed in this issue by van Lent et al. Here we will focus on the techniques currently employed in our lab to isolate pDCs and cDCs from primary human material and to differentiate human HPCs into pDCs in vitro.

2. Materials

2.1. Isolation of Mononuclear Cells

1. A source of human hematopoietic cells (*see* Note 1).
 - a. Buffy coats isolated from human donor whole blood are obtained from the blood bank.
 - b. Umbilical cord blood is obtained from pregnant patients at delivery.
 - c. Postnatal thymus is obtained from surgical specimens removed from children up to 3 years of age undergoing open heart surgery. Tissue is processed the same day (*see* Note 2).
 - d. Fetal liver and fetal thymus are obtained from elective abortions with gestational age ranging from 13 to 17 weeks. The raw material can be maintained overnight in a cold room or fridge. Phosphate-buffered saline (PBS) buffer (Invitrogen, Grand Island, NY, USA).
2. RPMI-1640 (Invitrogen) supplemented with 2% fetal calf serum (FCS). Store at 4–8°C.
3. Penicillin and streptomycin (Invitrogen).
4. Stomacher-80 Biomaster lab system (Seward Ltd., Worthing, UK), Stomacher bags, and a bag-sealing device (e.g., Sealboy 236 Audion Elektro).
5. Ficoll-Paque Plus (GE Healthcare, Uppsala, Sweden). Store away from light at 4–8°C.
6. Fine-toothed tweezer (VWR, West Chester, PA, USA).
7. Regular tweezer (VWR).
8. Small spoon (optional) (VWR).
9. Scalpel (VWR).
10. Stainless steel mesh (VWR).

2.2. Enrichment and Sort of Hematopoietic Populations

1. MACS isolation kit depending on desired population (*see* Note 3).

¹Human fetal tissues are obtained from elective abortions. The use of fetal tissue was approved by the Medical Ethical Committee of the Academic Medical Center and was contingent on obtaining informed consent, in accordance with the Declaration of Helsinki. Gestational age was determined by ultrasonic measurement of the diameter of the skull and ranged from 13 to 17 weeks.

The use of postnatal thymus tissue, donor blood, and cord blood was approved by the Medical Ethical Committee of the Academic Medical Center and used with informed consent in accordance with the Declaration of Helsinki.

²Postnatal thymus samples are taken from children undergoing open-heart surgery ranging from 0 to 3 years of age. Although samples from older children can be obtained they typically contain severely reduced numbers of CD34⁺ progenitor cells as compared to children below the age of 3 years.

³According to the manufacturer direct and indirect CD34⁺ progenitor cell isolations have identical efficiencies. However, in our hands the direct isolation kit works significantly better for enrichment of CD34⁺ from postnatal thymus as compared to cells from fetal liver. It may be considered to use the indirect isolation kit for fetal liver cells due to the lower pricing compared to the direct kit. Of note, we use only about 25–50% of the amount of antibodies and beads that are recommended by the manufacturer.

- a. Direct CD34 human MicroBead Kit (Miltenyi Biotec, Bergisch Gladbach, Germany).
 - b. Indirect CD34 human MicroBead Kit (Miltenyi Biotec).
 - c. CD304 (BDCA4/neuropilin-1) MicroBead Kit (Miltenyi Biotec).
2. MACS pre-separation filters and recovery tubes (Miltenyi Biotec).
3. MACS buffer is prepared as follows: PBS pH 7.2, 0.5% bovine serum albumin, 2 mM ethylenediaminetetraacetic acid (EDTA). Store at 4–8°C.
4. MACS separator, Large Scale (LS) MACS separation columns (Miltenyi Biotec).
5. Anti-PE microbeads (Miltenyi Biotec).
6. 5-ml Polystyrene round-bottomed 12 mm × 75-mm tubes (Becton Dickinson (BD), Franklin Lakes, NJ, USA).
7. Fluorochrome-coupled monoclonal antibodies depending on intended population:
anti-human CD1a (BD), anti-human CD11c-PE (BD), anti-human CD3 (BD), anti-human CD34 (BD), anti-human CD38 (BD), anti-human CD45RA (BD), anti-human CD56 (BD), anti-human CD123 (BD), anti-human BDCA2 (Miltenyi Biotec), anti-human HLA-DR (BD).
8. Fluorescence-activated cell sorter for isolation of pure cell populations, e.g., FACS Aria (BD).

2.3. Virus Production

1. Retroviral construct, e.g., pMX, LZRS expressing the gene of interest.
2. Retroviral producer cells, e.g., Phoenix-ampho or Phoenix-GALV (*see* Note 9).
3. 70 µm Cell strainer (BD).
4. Iscove's Modified Dulbecco's Medium (IMDM) (Invitrogen) supplemented with 8% FCS.
5. Penicillin and streptomycin (Invitrogen).
6. Puromycin (Sigma-Aldrich, St. Louis, MO, USA) (when LZRS is used).

2.4. Progenitor Transduction

1. Iscove's Modified Dulbecco's Medium (IMDM) (Invitrogen).
2. Yssels supplement (21).
3. Pooled human serum (Invitrogen).
4. SCF, IL-7, and TPO (PeproTech EC, London, UK).
5. Retronectin (30 µg/ml; Takara Biomedicals, Otsu, Shiga, Japan).

⁹More detailed information on the Phoenix retroviral system can be obtained on <http://www.stanford.edu/group/nolan/>.

6. Multiwell 24-well plate non-tissue culture treated (BD).

2.5. In Vitro DC-Differentiation Assay

1. Complete MEM α : Minimum Essential Medium (MEM) α is prepared from MEM α medium powder without NaHCO₃ (Invitrogen). One vial powder medium is reconstituted in 1 l MilliQ water together with 2.2 g NaHCO₃ (Merck, Darmstadt, Germany) and filtered over a 500-ml 0.22- μ m Bottletop filter (Corning, Corning, NY, USA). Medium is supplemented with 20% Hyclone (Logan, UT, USA) serum and penicillin/streptomycin (Roche).
2. OP9 cell line (*see* Note 21).
3. Phosphate-buffered saline (PBS) (Invitrogen).
4. Trypsin (Invitrogen).
5. 6-Well tissue culture plates (Corning).
6. IL-7 and Flt3L (PeproTech EC, London, UK).
7. 70- μ m Nylon mesh (Spectrum Laboratories, Inc., CA, USA). Cut into 1 $\times$ 1 cm square pieces and autoclave before use.
8. Fluorescence-activated cell analyzer, e.g., FACS LSRII (BD).

2.6. Stimulation of In Vitro Generated pDCs

1. pDCs generated in vitro from CD34⁺ progenitors in OP9 cultures with IL-7 and Flt3L.
2. CpG oligodeoxynucleotide (CpG-ODN) 2216 ggGGGAC-GATCGTCgggggG and CpG oligodeoxynucleotide (CpG-ODN) 2243 ggGGGAGCATGCTCgggggG (Sigma-Aldrich, St. Louis, MO, USA).
3. Irradiated Herpes simplex virus, Influenza virus, or HIV.
4. Human IFN- α enzyme-linked immunosorbent assay (ELISA; Biosource International, Camarillo, CA, USA).
5. Brefeldin A (Sigma).
6. Fix and Perm Kit (Caltag, Burlingame, CA, USA).
7. FITC-labeled anti-IFN- α 2 mAb (Chromaprobe, Maryland Heights, MO, USA).

²¹OP9 is a bone marrow stromal cell line derived from M-CSF deficient mice (24). OP9 needs to be cultured at relatively high/semi-confluent density and preferably passaged 1:2–1:3 three times a week. When OP9 cells are cultured at low density they tend to change the morphology by stretching out (although we have no indication that this affects the functionality of these cells). For splitting, OP9 cells can be treated similar to other common adherent cell lines. However, during trypsinizing the OP9 cells tend to come off the plate in large sheets. Vigorous pipetting will break up the sheets after which the cells are passed through a 70- μ m cell strainer to remove the clumps.

3. Methods

3.1. Isolation of Nucleated Cells from Human Peripheral (or Umbilical Cord Blood)

1. Open the collection bag with sterile scissors.
2. Empty the bag in a T75-ml flask, the collected volume is around 25–50 ml.
3. The amount of collected umbilical cord blood is usually smaller, adjust all volumes accordingly.
4. Add PBS to a total of 200 ml PBS.
5. Divide the suspension over 50-ml Falcon tubes at 25 ml/tube. Four tubes in general generate enough cells for downstream applications.
6. Add 12 ml Ficoll-Paque and isolate nucleated cells from a regular density gradient by standard procedures.
7. Wash cells in RPMI-1640.

3.2. Isolation of Nucleated Cells from Human Postnatal Thymus

1. The collected tissue is processed the same day.
2. Put the thymus tissue in a sterile Petri dish and pull off the capsule layer with a set of fine-toothed tweezers. Subsequently, cut the tissue into small pieces using a sterile disposable scalpel.
3. Transfer the pieces into a sterile Stomacher bag with either tweezers or a small spoon, wash the Petri dish with a small amount of RPMI medium (with 8% FCS, penicillin, and streptomycin) and add the rinsing to the bag. The bag is sealed twice to avoid leakage and put into a Stomacher device for further disruption of the tissue.
4. Clean the bag with 70% ethanol and open the bag with sterile scissors. Empty the contents on an autoclaved stainless steel mesh and press the cells through the filter to obtain a single-cell suspension. Wash the filter and plate with RPMI 8% FCS, suspension is left overnight in a 50-ml tube at 4°C.
5. The following day thymocytes are isolated from a Ficoll-paque density gradient at a maximum of 600×10^6 cells/50-ml Falcon tube.
6. Wash the cell suspensions by centrifugation in RPMI-1640 2% FCS medium.

3.3. Isolation of Nucleated Cells from Human Fetal Liver

1. Transfer one fetal liver into one Stomacher bag, add 10–15 ml RPMI-1640 medium, and seal the bag twice to avoid leakage.
2. Disrupt the liver pieces mechanically in a Stomacher device for 1 min at high speed. The bag now contains a cell suspension mixed with non-dissociated liver stroma.

3. Wipe the bag with ethanol 70% and open it with sterile scissors. Transfer the contents of the bag into a 10-cm Petri dish.
4. Dilute the cell suspension with RPMI-1640 and recover the supernatant from the dish avoiding the pieces of stroma at the bottom of the dish. Pour new RPMI-1640 medium into the plate and repeat this procedure until the recovered medium is clear. Usually, pouring fresh medium three times is enough.
5. Add RPMI-1640 medium to the tubes until you reach approximately 20 ml. Wash the cell suspensions by centrifugation. Aspirate the fat-containing supernatant.
6. Isolate the cells from a Ficoll-paque density gradient.
7. Wash the cell suspensions by centrifugation and pool the cell pellets in RPMI-1640 2% FCS medium.

3.4. Isolation of CD123⁺CD45RA⁺ Plasmacytoid Dendritic Cells

1. Cells recovered from Ficoll-paque density gradient are enriched for BDCA4⁺ cells by immunomagnetic cell sorting, using a BDCA4⁺ cell separation kit (*see* Note 5).
2. Label the cells according to manufacturer's protocol (*see* Note 6).
3. Install the magnetic separation column in the MACS magnet and put a pre-separation filter on top of it. Prepare the collection tube for the column flow-through. Proceed to filter/column washing with MACS buffer, following the manufacturer's instructions.
4. After extensive washing of the column take it from the magnet. Add 5 ml MACS buffer to the column and flush out the BDCA4⁺ cells into a 15-ml tube using the plunger.
5. Add 5 ml of RPMI-1640 2% FCS medium to the tube and wash the cell suspension by centrifugation.
6. Resuspend the pellet in a volume of 1 ml of RPMI-1640 2% FCS medium and count the amount of nucleated cells in the suspension.
7. The BDCA4⁺ cell fraction is labeled with anti-CD123, anti-CD45RA, and lineage markers (*see* Note 7).
8. After washing-off excess antibodies in RPMI-1640 2% FCS medium cells are filtered over 70- μ m autoclave mesh to remove potential clumps.
9. On a flow cytometry sorter CD123^{hi}CD45RA⁺ cells are sorted to purity (Fig. 5.1, *see* Note 8).

⁵Human pDCs, as opposed to murine pDCs, do not express CD11c. Therefore, the described CD11c MACS protocol does not enrich for human pDCs.

⁶When using the BDCA4 cell separation kit it is advisable to pre-incubate the cells for several minutes with the FcR-blocker before adding the BDCA4-beads. This will increase the enrichment rate compared to simultaneous addition of FcR-blocker and BDCA4-beads to the cells.

⁷Although BDCA2 is the specific marker for human pDCs it is best not be used for cell sorting as it activates the pDCs (22).

⁸Typically, the tubes used for FACS sorting are polystyrene as cells are less likely to stick to the tube as compared to regular tubes.

3.5. Isolation of CD11c⁺HLA-DR⁺ Conventional DCs

1. Cells recovered from Ficoll-paque density gradient are enriched for CD11c⁺ cells by a two-step immunomagnetic cell protocol (*see* Note 5).
2. Cells are stained with anti-human CD11c-PE antibodies.
3. Incubate at 4°C for 15 min and wash.
4. Add anti-PE microbeads.
5. Incubate at 4°C for 15 min and wash.
6. Install the magnetic separation column in the MACS magnet and put a pre-separation filter on top of it. Prepare the collection tube for the column flow-through. Proceed to filter/column washing with MACS buffer, following the manufacturer's instructions.
7. After extensive washing of the column take it from the magnet. Add 5 ml MACS buffer to the column and flush out the CD11c⁺-enriched cells into a 15-ml tube using the plunger.
8. Resuspend the pellet in a volume of 1 ml of RPMI-1640 2% FCS medium and count the amount of nucleated cells in the suspension.
9. The CD11c⁺ cell fraction is labeled with CD3, CD19, CD56, CD11, and HLA-DR antibodies for 30 min at 4°C.
10. After washing-off excess antibodies in RPMI-1640 2% FCS medium cells are filtered over 70-µm autoclave mesh to remove potential clumps.
11. By flow cytometry Lin⁻CD11c⁺HLA-DR⁺ cDCs are sorted to purity (*see* Fig. 5.2, *see* Note 8).

3.6. Isolation of CD34⁺ Cells from Postnatal Thymus

1. Cells recovered from Ficoll-paque density gradient are enriched for CD34⁺ cells by immunomagnetic cell sorting, using a direct CD34⁺ cell separation kit (*see* Note 3).
2. Label the cells according to manufacturer's protocol (*see* Note 3).
3. Install the magnetic separation column in the MACS magnet and put a pre-separation filter on top of it. Prepare the collection tube for the column flow-through. Proceed to filter/column washing with MACS buffer, following the manufacturer's instructions.
4. After extensive washing of the column take it from the magnet. Add 5 ml MACS buffer to the column and flush out the CD34⁺ into a 15-ml tube using the plunger.
5. Add 5 ml of RPMI-1640 2% FCS medium to the tube and wash the cell suspension by centrifugation.
6. Resuspend the pellet in a volume of 1 ml of RPMI-1640 2% FCS medium and count the amount of nucleated cells in the suspension.

7. Stain the CD34⁺-enriched thymocytes with antibodies against CD34, CD1a, CD56, and BDCA2.
8. After washing-off excess antibodies in RPMI-1640 2% FCS medium cells are filtered over 70- μ m autoclave mesh to remove potential clumps.
9. Lin⁻CD34⁺CD1a⁻ cells with T/NK/pDC potential are sorted to purity by flow cytometry (Fig. 5.3) (*see* Notes 4 and 8).

3.7. Enrichment for CD34⁺ Hematopoietic Stem Cells (HSCs)

1. Cells recovered from Ficoll-paque density gradient are enriched for CD34⁺ cells by immunomagnetic cell sorting, using an indirect CD34⁺ cell separation kit (*see* Note 3) consisting of (1) anti-CD34 hapten-coupled antibody; (2) anti-hapten antibody conjugated with colloidal paramagnetic beads.
2. Label the cells according to manufacturer's protocol (*see* Note 3).
3. Install the magnetic separation column in the MACS magnet and put a pre-separation filter on top of it. Prepare the collection tube for the column flow-through. Proceed to filter/column washing with MACS buffer, following the manufacturer's instructions.
4. After extensive washing of the column take it from the magnet. Add 5 ml MACS buffer to the column and flush out the CD34⁺ into a 15-ml tube using the plunger.
5. Add 5 ml of RPMI-1640 2% FCS medium to the tube and wash the cell suspension by centrifugation.
6. Resuspend the pellet in a volume of 1 ml of RPMI-1640 2% FCS medium and count the amount of nucleated cells in the suspension.
7. Stain the CD34⁺-enriched fetal liver cells with antibodies against CD34, CD38, CD56, CD3, and BDCA2.
8. After washing-off excess antibodies in RPMI-1640 2% FCS medium cells are filtered over 70- μ m autoclave mesh to remove potential clumps.
9. Multipotent CD34⁺CD38⁻ HSCs are sorted to purity by flow cytometry (Fig. 5.4, *see* Note 8).

3.8. Production of High-Titer Amphotropic Retroviral Stocks (*see* Note 9)

- 1 Day 1: Near confluent Phoenix-ampho or Phoenix-GALV cells are split 1:5 to have the cells growing in log phase (*see* Note 10).
- 2 Day 2: Phoenix cells are plated at 1.5×10^6 cells/10-cm Petri dish in 8 ml IMDM 8% FCS.

⁴If required Lin⁻CD34⁺CD1a⁺ cells can simultaneously be sorted from the CD34⁺ MACS-enriched cells (Fig. 5.3). These cells are strongly biased to the T-cell lineage. Although they still have little NK-cell potential, they have completely lost their capacity to develop into pDCs (15).

¹⁰In case of low transfection efficiency it may be worthwhile to try using more or less plasmid DNA, as less DNA may also give higher transfection efficiency. If transfection efficiency stays low it is advised to redo the plasmid DNA isolation to obtain plasmid DNA of better quality.

- 3 Day 3: 25 µl FuGene is added to 400 µl RPMI without serum (the absence of serum is important). Under shaking or vortexing the RPMI/FuGene mixture is added dropwise to 10 µg retroviral plasmid DNA in a 2-ml eppendorf tube (*see* Note 11).
- 4 Incubate the DNA/FuGene mixture at RT for 20 min and divide it dropwise over the Phoenix cells.
- 5 If LZRS constructs were transfected continue from Step 9, otherwise continue from Step 6.

3.8.1. Retroviral Production from Non-LZRS Constructs

- 6 Day 5: For non-LZRS constructs the medium is overnight replaced with 4 ml IMDM 8% FCS medium.
- 7 Day 6: If desired repeat the same procedure as described on day 4 (*see* Note 12).
- 8 Continue from Step 14.

3.8.2. Retroviral Production from LZRS Constructs (see Note 13)

- 9 Day 4: Transfer the Phoenix cells from the 10-cm dish into a large T175 flask containing 20 ml IMDM 8% FCS.
- 10 Day 5: Add 40 µl puromycin 1 mg/ml to select for transfected-Phoenix cells (*see* Note 14).
- 11 Day 8: If the T175 flask is nearly confluent, remove half of the Phoenix cells by trypsinization from the flask and add fresh medium and puromycin.
- 12 Days 10–12: Around this time the puromycin selection is completed and over 90% of the Phoenix cells contain the LZRS plasmid. When the flask is around 70% confluent, the medium is replaced in the morning to wash away the puromycin. Handle the flask carefully, the Phoenix cells easily detach when grown at high density.
- 13 Put 16 ml IMDM 8% FCS overnight on the Phoenix cells.
- 14 Next day harvest the retroviral supernatant from the Phoenix cells. Spin the supernatant for 5 min at 1,250 rpm without brake to pellet detached cells and cell debris.

¹¹Amphotropic- and gibbon-ape-leukemia-virus (GALV)-pseudotyped retrovirus are much more effective in transducing human cells than the commonly used ecotropic-pseudotype virus to transduce murine cells. We never observed a clear advantage of either amphotropic- or GALV-typed virus for transduction of CD34⁺ progenitor cells, also not in double transduction experiments.

¹²The virus titers of the second harvest are generally somewhat increased from the first harvest. The second harvest typically gives increased transduction efficiencies.

¹³The LZRS retroviral plasmid contains a puromycin resistance and EBNA (for replication of the plasmid in human cells) sequences in the backbone. This combination allows positive selection and expansion of transfected and thus puromycin resistant Phoenix cells resulting in the easy generation of large amounts of high titer virus. The plasmid is thought to be present in the Phoenix cells up to 3 months.

¹⁴It takes about 2 days before the non-transfected Phoenix cells start to die due to the puromycin treatment.

- 15 A typical transduction of CD34⁺ progenitor cells requires 300 µl virus, therefore virus stocks are frozen in 400-µl aliquots. Snap-freeze the virus on dry ice and store at -80°C. The virus titer remains stable for years.

3.9. Retroviral Transduction of Human CD34⁺Progenitor Cells (see Note 15)

1. Sorted CD34⁺CD38⁻ fetal liver or CD34⁺CD1a⁻ post-natal thymocytes are cultured overnight at a density of 250,000–800,000 cells/1 ml in a standard 24-well tissue culture plate in IMDM with Yssels supplement, 5% human serum, and cytokines. Cytokines are 20 ng/ml SCF + 10 ng/ml IL-7 + 20 ng/ml TPO for fetal liver or 20 ng/ml SCF + 10 ng/ml IL-7 for postnatal thymocytes, respectively.
2. Cells are cultured overnight at 37°C to induce cell cycle progression.
3. Simultaneously, a non-tissue culture treated 24-well plate is coated with 0.5 ml/well retronectin overnight at 4°C (*see* Note 16).
4. The next morning the retronectin is collected from the plate (*see* Note 17). Retronectin-coated wells are incubated with 0.5 ml/well PBS 2% HSA for 20 min at room temperature to block non-specific sites. Just before use the plates are washed with PBS.
5. In the meantime the progenitor cells are collected from the overnight culture by resuspending the cells in the well and subsequent washing of the wells with IMDM with Yssels supplement. Progenitor cells are spun and taken up in the desired volume of IMDM with Yssels supplement and cytokines (no serum, *see* Note 18).
6. Virus is taken from the -80°C storage and quickly thawed in hot tap water.
7. Cells and virus are added onto the retronectin plate in a 1:1 ratio, 300 µl each with a final concentration of cytokines of 20 ng/ml SCF + 10 ng/ml IL-7 + 20 ng/ml TPO for fetal liver or 20 ng/ml SCF + 10 ng/ml IL-7 for postnatal thymocytes, respectively (similar to the concentrations used in Step 1) (*see* Note 19).
8. The plates are spun in a plate centrifuge for 90 min at 800 rpm and room temperature without brake.
9. After centrifugation the cells are incubated at 37°C for an additional 5–6 h.
10. Cells are collected from the wells, counted, and resuspended in the appropriate medium for subsequent differentiation assay.
11. A small amount of transduced cells can be cultured in a 96-well round-bottomed plate in IMDM Yssels 5% human serum with cytokines and analyzed subsequently

¹⁵Although CD34⁺CD38⁻ isolated from fetal liver can be efficiently transduced with lenti-viral constructs (see van Lent et al., this issue) this has been problematical for CD34⁺CD1a⁻ postnatal thymocytes. The reason for this is still unclear and may depend on both the lenti-viral construct (e.g., promoters used) or the virus titer.

¹⁶Instead of coating a 24-well plate with retronectin overnight at 4°C, 2 h at room temperature or in the incubator is also sufficient.

¹⁷Retronectin can be re-used up to three times without loss of transduction efficiency.

¹⁸The presence of high serum dose in the transduction cultures inhibits transduction efficiency (23).

¹⁹For transductions virus and cells are mixed at a 1:1 volume ratio. In case of double transduction with two different viral constructs 300 µl cells is mixed with 150 µl of each of the respective virus.

at day 3 after transduction for transduction efficiency. Transduction efficiencies up to 20% are anticipated (Fig. 5.5, *see* Note 20).

3.10. In Vitro pDC and cDC Differentiations

1. OP9 cells (or MS-5 or S17) are plated in a 6-well at 50,000 cells/well in 1 ml complete MEM α , preferably up to 24 h before starting co-cultures (*see* Note 21).
2. Sorted progenitor cells either CD34⁺CD38⁻ or CD34⁺CD1a⁻ (transduced or not-transduced) are counted, resuspended in 1 ml MEM α , and carefully added to the OP9 cells to a total culture volume of 2 ml (*see* Note 22).
3. The medium is supplemented with 5 ng/ml IL-7 and 5 ng/ml Flt3L (*see* Notes 23 and 24).
4. Fresh medium and cytokines (1 ml complete MEM α with 10 ng/ml IL-7 and 10 ng/ml Flt3L) are added every 2–3 days.
5. At the day of analysis the cultures are resuspended and filtered over a piece of autoclaved 70- μ m nylon mesh into a 15-ml tube. The peak in percentage and number of pDCs from CD34⁺CD38⁻ and CD34⁺CD1a⁻ is reached between day 5 and day 7, respectively (16).
6. Spin the cells, resuspend in 1 ml medium, and count the number of cells.
7. The cultures are analyzed by flow cytometry. pDCs stain double positive for CD123 (IL3R α) and BDCA2 antigens (Fig. 5.6). CD4, CD45RA, and HLA-DR staining may also be included. Although the percentage of OP9 falling into the lymphocyte gate of an FSC–SSC dotplot is usually neglectable, an antibody against human CD45 can be used to exclude contaminating OP9 cells.
8. Cells recovered from OP9 cultures started from CD34⁺CD38⁻ progenitor cells may be replated on fresh OP9 cells with cytokines as described above. Around day 11 CD10⁺CD19⁺ pro-B cells and CD11c⁺CD14⁺ cells of myeloid origin can be detected (6) (*see* Note 25).

3.11. Stimulation of In Vitro Generated pDCs

1. After 5–7 days of culturing CD34⁺ progenitors on OP9 cells with IL-7 and Flt3L the pDCs that have developed in the culture are stimulated by adding 10 μ g/ml

²⁰Typically, we observe lower transduction efficiencies when CD34⁺CD1a⁻ thymocyte precursors are used as compared to CD34⁺CD38⁻ fetal liver cells. In addition constructs with RNA interference or microRNA-encoding sequences negatively affect transduction of both cell types.

²²At the start of the differentiation assay the genes encoded by the viral constructs are not expressed at detectable levels yet. Therefore, both transduced and non-transduced progenitor cells are cultured concomitantly, which requires the use of a fluorescent marker, such as GFP, expressed from the retroviral construct in order to be able to discriminate transduced and non-transduced cells by flow cytometric analysis.

²³Although it is clear that Flt3L is the cytokine driving DC development we typically use the combination of IL-7 and Flt3L in the differentiation assays to allow a better comparison between OP9 DC and OP9-DeltaLike1 T-cell differentiation cultures (7). Also increased expansion and cell survival of progenitor cells is observed in the presence of IL-7.

²⁴In addition to Flt3L and IL-7 thrombopoietin (TPO) can be added to the cultures to increase the number of pDCs derived from CD34⁺ fetal liver or cord blood progenitor cells (25).

²⁵Under the conditions described here CD34⁺CD38⁻ HSC will give rise to a small percentage of pro-B cells. Lowering or completely leaving out cytokines will increase the percentage (but decrease the absolute cell number) of proB cells in the culture. Sorted CD19⁺ from the OP9 co-cultures can be further differentiated into BCR⁺ B-cells with CD40L + IL-2/IL-4 (26).

(CpG-ODN) 2216 or the negative control (CpG-ODN) 2243 or virus at 10 PFU/cell to bulk cultures. Alternatively, CD123⁺BDCA2⁺ pDCs are sorted from the bulk cultures before stimulation.

2. After 24–48 h of stimulation IFN- α production is measured by analyzing the supernatant by enzyme-linked immunosorbent assay (ELISA) according to manufacturer's instruction.
3. Intracellular IFN- α can be detected by FACS after 8–12 h of stimulation.

To optimize the detection of intracellular cytokines Brefeldin A (10 μ g/ml) may be added during the last 2 h of stimulation. After stimulation, pDCs are, if required, labeled with CD123 and BDCA2 and subsequently fixed and permeabilized using the Fix and Perm Kit according to the manufacturer's instructions and are labeled with anti-IFN- α 2 mono-clonal antibody.

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Fig. 5.1. Sorting of CD123⁺CD45RA⁺ pDCs from BDCA4⁺ MACS-enriched cells. Dotplot “Before sorting” is gated lineage negative and doublets are excluded.

Fig. 5.2. Sorting of CD11c⁺HLA-DR⁺ cDCs from CD11c⁺ MACS-enriched cells. Dotplot “Before sorting” is gated lineage negative and doublets are excluded.

Fig. 5.3.
Sorting of CD34⁺CD1a⁻ and CD34⁺CD1a⁺ subsets from CD34⁺ MACS-enriched cells.
Dotplot “Before sorting” is gated lineage negative and doublets are excluded.

Fig. 5.4. Sorting of CD34⁺CD38⁻ from CD34⁺ MACS-enriched fetal liver cells. Dotplot “Before sorting” is gated lineage negative and doublets are excluded.

Fig. 5.5.
Transduction efficiency measured 3 days after transduction.

Fig. 5.6. BDCA2⁺CD123⁺ pDCs derived from CD34⁺CD38⁻ fetal liver cells or CD34⁺CD1a⁻ postnatal thymocytes on OP9 in the presence of Flt3L and IL-7.