Chapter 2

In Vitro Generation of Human XCR1$^+$ Dendritic Cells from CD34$^+$ Hematopoietic Progenitors

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Abstract

Dendritic cells (DCs) are a heterogeneous population of professional antigen-presenting cells which play a key role in orchestrating immune defenses. Most of the information gained on human DC biology was derived from studies conducted with DCs generated in vitro from peripheral blood CD14$^+$ monocytes (MoDCs) or from CD34$^+$ hematopoietic progenitors. Recent advances in the field revealed that these types of in vitro-derived DCs strikingly differ from the DC subsets that are naturally present in human lymphoid organs, in terms of global gene expression, of specialization in the sensing of different types of danger signals, and of the ability to polarize T lymphocytes toward different functions. Major efforts are being made to better characterize the biology and the functions of lymphoid organ-resident DC subsets in humans, as an essential step for designing innovative DC-based vaccines against infections or cancers. However, this line of research is hampered by the low frequency of certain DC subsets in most tissues, their fragility, and the complexity of the procedures necessary for their purification. Hence, there is a need for robust procedures allowing large-scale in vitro generation of human DC subsets, under conditions allowing their genetic or pharmacological manipulation, to decipher their functions and their molecular regulation. Human CD141$^+$CLEC9A$^+$XCR1$^+$ DCs constitute a very interesting DC subset for the design of immunotherapeutic treatments against infections by intracellular pathogens or against cancer, because these cells resemble mouse professional cross-presenting CD8α$^+$Clec9a$^+$Xcr1$^+$ DCs. Human XCR1$^+$ DCs have indeed been reported by several teams to be more efficient than other human DC subsets for cross-presentation, in particular of cell-associated antigens but also of soluble antigens especially when delivered into late endosomes or lysosomes. However, human XCR1$^+$ DCs are the rarest and perhaps the most fragile of the human DC subsets and hence the most difficult to study ex vivo. Here, we describe a protocol allowing simultaneous in vitro generation of human MoDCs and XCR1$^+$ DCs, which will undoubtedly be extremely useful to better characterize the functional specialization of human XCR1$^+$ DCs and to identify its molecular bases.

Key words Human immune system, CD34$^+$ hematopoietic stem cells, Differentiation, XCR1$^+$ dendritic cells, Monocyte-derived dendritic cells, Cross-presentation

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1 Introduction

The development of better vaccines or immunotherapies against cancer or intracellular pathogens is one of the major current challenges of immunological research. Innovative approaches are needed to induce efficient and long-lasting memory cytotoxic CD8 T cell responses. Conversely, in the case of inflammatory or autoimmune diseases, better treatments are needed to selectively dampen deleterious inflammatory reactions or to specifically inactivate autoimmune lymphocytes, without compromising overall immune responses of the patients to avoid increasing sensitivity to infections or cancers. Dendritic cells (DCs) are professional antigen-presenting cells which are exquisitely efficient for the activation of naïve T lymphocytes upon their primary encounter with their cognate antigen [1]. During this interaction, DCs instruct the functional polarization of T lymphocytes toward different activities depending on the physiopathological context, including promoting either immunity or tolerance. DCs exert this function by delivering to T cells the three types of output signals necessary for their priming: the triggering of the T cell receptor by peptide-MHC-I complexes (signal 1), the triggering of co-stimulation receptors by the co-stimulation molecules induced on mature DCs (signal 2), and cytokines which can contribute to promote the proliferation or survival of T cells and which instruct their differentiation toward specific functions (signal 3). The nature of the output signals delivered to T lymphocytes by DCs is determined by the integration by DCs of a variety of input signals that they can detect in their environment. Indeed, DCs are equipped with a variety of innate immune recognition receptors (I2R2s) allowing them on the one hand to detect pathogens, infections, transformed cells, and cytokines and on the other hand to engulf molecules, microorganisms, or cellular debris [2]. The combinations of I2R2s which are engaged on DCs during their activation control the type of maturation that DCs undergo, either tolerogenic or immunogenic, with the expression of specific combinations of positive or negative co-stimulation molecules and the production of particular patterns of immunoactivating or immunosuppressive cytokines. Hence, DC functions are highly plastic, allowing to induce the type of immune responses needed depending upon the physiopathological context [3]. The existence of distinct DC subsets specialized in different functions is another key feature contributing to promote a diversity of DC responses to match the diversity of the threats which the immune system has to face [4]. Indeed, several DC subsets exist which express different arrays of I2R2s [2] and have different potentials for exerting distinct functions such as the production of various cytokines or the activation of CD8 T cells [5].

A particular subset of mouse DCs excels at inducing protective CD8 T cell responses, in particular through uptake and processing of exogenous antigens for their presentation in association with major histocompatibility class-I (MHC-I) molecules, a process called cross-presentation [6]. Cross-presentation is critical for the induction of CD8 T cell responses against cancer, since most tumors are not derived from APCs and are inefficient for priming. Cross-presentation is also critical for the induction of CD8 T cell responses against intracellular pathogens that do not infect DCs or that escape direct antigen presentation in infected DCs, for example, by downregulating the expression of MHC or activating co-stimulation molecules or by enhancing the expression of inhibitory co-stimulation molecules [7]. Besides selectively expressing CD8α or CD103, the professional cross-presenting mouse DC subset is characterized by its unique expression of the endocytic receptor Clec9a, of the Toll-like receptor Tlr3, and of the chemokine receptor Xcr1 [8]. These three molecules have been shown or are thought to play a major role in endowing mouse Xcr1⁺ DCs with their high efficiency for CD8 T cell activation. Clec9a promotes the cross-presentation of dead cell-associated antigens, by allowing recognition of filamentous actin on dying cells for uptake by Xcr1⁺ DCs and intracellular routing into proper endosomes [9–11]. Tlr3 allows XCR1⁺ DCs to sense the abnormal presence of double-stranded RNA in the materials that they have engulfed in their endosomes, which triggers their production of interferons-β (IFN-β) and IFN-λ, which, in turn, at least for IFN-β, can enhance antigen cross-presentation by, and boost immunogenic maturation of, Xcr1⁺ DCs [12]. The ligand for Xcr1, Xcl1, is selectively produced by activated natural killer (NK) and CD8 T cells and might promote their physical encounters with Xcr1⁺ DCs as a mechanism amplifying the activation of cytotoxic effector lymphocytes by Xcr1⁺ DCs [13–15]. In vivo, targeting of mouse Xcr1⁺ DCs for vaccination, by co-administration of adjuvants and antigen-fused specific antibodies, yielded very encouraging results [16–19]. However, the efficacy of the immune responses induced and the generation of protective long-term memory require improvement. Moreover, how to translate this vaccination strategy for human health was not obvious.

In 2008, we discovered that the previously identified human CD141/BDCA3⁺ DC subset is equivalent to mouse Xcr1⁺ DCs, based on the similarities of their gene expression programs including unique expression of XCR1, CLEC9A, and TLR3 [20, 21]. Since 2010, we and several other teams have confirmed that human XCR1⁺ DCs are more efficient than other human DC subsets for the cross-presentation of cell-associated antigens or even of soluble antigens in specific experimental settings, in particular when delivered to late endosomes or lysosomes [13, 22–25]. However, in

other experimental settings, all human DC subsets from the blood, spleen, or tonsils were found to be equally efficient for cross-presentation of soluble or particulate antigens [23, 26, 27]. Human XCR1$^+$ DCs represent in average only 0.02 % of human peripheral blood mononuclear cells, thus constituting the rarest human DC subset. They also seem to be the most fragile human DC subset upon ex vivo isolation. Hence, human XCR1$^+$ DCs are the most difficult human DC subset to study ex vivo. Thus, alternative approaches are needed to study human XCR1$^+$ DCs. This is all the more important as most of the information gained on human DC biology was derived from studies conducted with DCs generated in vitro from peripheral blood CD14$^+$ monocytes (MoDCs) or from CD34$^+$ hematopoietic progenitors [21]. MoDCs are the most frequently used human DC subset for immunotherapeutic treatments in cancer patients or in individuals infected with human immunodeficiency virus type 1, but only with moderately encouraging results [28, 29] at least until recently [30].

Based on the combination and optimization of procedures previously reported by others [31, 32], we developed a protocol allowing simultaneous in vitro generation of high numbers of human MoDCs and XCR1$^+$ DCs from CD34$^+$ hematopoietic stem cells [33]. This system uniquely enables rigorous side-by-side functional comparison of XCR1$^+$ DCs and MoDCs from the same culture [33], which will undoubtedly be extremely useful to better characterize their respective functional specializations and to identify how they are molecularly regulated.

2 Materials

2.1 Enrichment of CD34$^+$ Hematopoietic Progenitor Stem Cells

1. A source of human CD34$^+$ hematopoietic cells (*see* **Note 1**).
2. CD34$^+$ cell selection kit (*see* **Note 2**).
3. Ficoll-Paque PLUS (GE healthcare). Store away from light at around +4 °C.
4. Dilution buffer (DB): Phosphate-buffered saline (PBS), 2 % fetal calf serum (FCS), 1 mM EDTA.
5. T 75 mL flask.
6. 50 mL Falcon tubes.
7. Sterile scissors.
8. Serological pipettes 5, 10, and 25 mL.

2.2 Expansion of Hematopoietic Precursors

1. StemSpan (StemSpan™ SFEM, serum-free medium for expansion of hematopoietic cells, Stem cell technology). This medium can be stored at −20 °C in aliquots.
2. FCS (*see* **Note 3**).

3. Recombinant human cytokines: FLT3-L, SCF, IL-3, TPO (Peprotech).
4. Amplification medium: StemSpan, FCS 10 %, FLT3-L (100 ng/mL), SCF (100 ng/mL), IL-3 (20 ng/mL), and TPO (50 ng/mL), to be prepared extemporaneously.
5. U-bottom 96-well tissue culture-treated plates.
6. Roswell Park Memorial Institute medium (RPMI).
7. 15 or 50 mL polypropylene tissue culture Falcon tubes.

2.3 Cryopreservation and Revival of Expanded Hematopoietic Precursors

1. Iscove's Modified Dulbecco's Medium (IMDM).
2. DMSO.
3. Deoxyribonuclease I from bovine pancreas (Nalgene, Sigma Aldrich).
4. FCS.
5. Cryotubes, e.g., Nunc® CryoTubes®, cryogenic vial, 1.8 mL, internal thread, round-bottomed, starfoot, free standing (Sigma).
6. Isopropanol.
7. Freezing container (e.g., Mr. Frosty, Nalgene).
8. Freezing medium#1 (FM1): IMDM, 30 % FCS.
9. Freezing medium#2 (FM2): IMDM, 30 % FCS, 20 % DMSO, to be prepared extemporaneously.
10. 15 or 50 mL polypropylene tissue culture Falcon tubes.
11. Water bath adjustable to 37 °C.

2.4 Differentiation of DCs from Expanded Hematopoietic Precursors

1. RPMI.
2. FCS.
3. Recombinant human cytokines: FLT3-L, SCF, GM-CSF, IL-4 (Peprotech).
4. Medium#2: RPMI, 10 % FCS, 10 mM HEPES, 1 mM sodium pyruvate, penicillin, streptomycin, 2 mM L-glutamine, 50 μM β mercaptoethanol.
5. Differentiation medium#1: medium#2, 100 ng/mL FLT3-L, 20 ng/mL SCF, 2.5 ng/mL IL-4, 2.5 ng/mL GM-CSF, to be prepared extemporaneously.
6. Differentiation medium#2: medium#2, 200 ng/mL FLT3-L, 40 ng/mL SCF, 5 ng/mL IL-4, 5 ng/mL GM-CSF, to be prepared extemporaneously (*see* **Note 4**).
7. U-bottom 96-well tissue culture-treated plates.
8. 15 or 50 mL polypropylene tissue culture Falcon tubes.

2.5 Staining for Flow Cytometry Analysis

Staining for flow cytometry can be used to phenotypically identify the different cell populations at the end of the culture, for sorting DC subsets, or for evaluation of DC subset maturation following their stimulation.

1. Fluorochrome-coupled monoclonal antibodies depending on the intended cell populations or biological process to study. The manufacturers, molecular targets, fluorochrome conjugation, hybridoma clones, and dilutions of use in our experimental settings (vol:vol) are given in Table 1 for the different antihuman antibodies used.

Table 1
List of the different fluorochrome-conjugated antihuman antibodies used for phenotyping

Antigen	Fluorochrome	Clone	Suppliers	Final dilution (v/v)
CD141	APC/FITC	AD5-14H12	Miltenyi Biotec	1:30
CD141	PE	VI E013	BD Bioscience	1:30
CLEC9A	APC	683409	R&D Systems	1:30
CLEC9A	APC/PE	8F9	Miltenyi Biotec	1:30
CADM1	Purified	3E1	MBL	1:1000
CD11C	V450	B-ly6	BD Biosciences	1:100
CD11B	FITC	ICRF44	eBioscience	1:100
CD23	PE	EBVCS2	eBioscience	1:50
CD32	APC	6C4	eBioscience	1:50
CD206	PE-Cy7	19.2	eBioscience	1:100
CD209	APC	eB-h209	eBioscience	1:100
CD86	AF700	2331 (FUN-1)	BD Biosciences	1:50
CD83	APC	HB15e	BD Biosciences	1:30
HLA-DR	AF700	LN3	eBioscience	1:100
CD40	PE		Beckman Coulter	1:10
TLR3	PE	34A3	Innate Pharma	1:30
TLR4	PE	HTA125	eBioscience	1:100
CD1B	APC	SN13 K5-1B8	eBioscience	1:50
CD103	PE-Cy7	B-ly7	eBioscience	1:100
CD14	FITC	M5E2	BD Biosciences	1:30
IgG (H + L)	FITC	Chicken	Southern Biotech	1:400
CLEC9A	PE	8F9	BioLegend	1:30

2. U-bottom 96-well tissue culture-treated plates.
3. FACS buffer: PBS, 1 mM EDTA, 10 mM HEPES.
4. Staining buffer (SB): FACS buffer, 2 % FCS.
5. Human TruStain FcX™ (Fc Receptor Blocking Solution, BioLegend).
6. Blocking buffer (BB): SB complemented 1:20, vol:vol, with Human TruStain FcX™ (e.g., 50 µL TruStain FcX™ for 1 mL SB).
7. LIVE/DEAD® Fixable Aqua Dead Cell Stain Kit (Invitrogen).
8. 0.5 % paraformaldehyde working solution: prepare 4 % (w/v) stock solution in PBS, adjusted to pH 7, according to manufacturer's instructions. Stock solution should be aliquoted in 10 mL volumes in 15 mL polypropylene tubes and frozen at −20 °C. Extemporaneously prepare 0.5 % working solution by diluting stock solution 1/8 in PBS.
9. OneComp eBeads (eBioscience) for compensation control.
10. Fluorescence-activated cell sorter for analysis of cells.

2.6 Sort of DC Subsets at the End of the Culture

1. Antibodies, FACS buffer, SB, and BB (*see* Subheading 2.5 above), all sterile.
2. 15 or 50 mL polypropylene tissue culture Falcon tubes.
3. FACS tubes (5 mL polystyrene round-bottomed 12 mm × 75 mm snap-cap tissue culture tubes).
4. Fluorescence-activated cell sorter for isolation of cells.
5. PBS containing 2 % BSA for coating the FACS tubes, sterile.
6. Collection medium: RPMI, 10 % FCS, sterile.
7. 70 µM cell strainers.
8. SYTOX® Blue Dead Cell Stain Kit (Invitrogen).
9. OneComp eBeads (eBioscience) for compensation control.

2.7 Stimulation of Sorted DC Subsets with Different Adjuvants

1. Adjuvants: Poly(I:C) (high molecular weight), R848 (imidazoquinoline compound), and LPS (ultrapure LPS from *Salmonella minnesota*).
2. 15 mL polypropylene Falcon tissue culture tubes.
3. U-bottom 96-well tissue culture-treated plates.
4. Medium#2.
5. Differentiation medium#2.
6. Round-bottomed 96-well tissue culture-treated plates.

3 Methods

The in vitro generation of XCR1$^+$ DCs consists in a two-step culture system (*see* Fig. 1). The first phase is a 7-day expansion of enriched CD34$^+$ cells. During this step, the CD34$^+$ cells proliferate under the instruction of a combination of cytokines (FLT3-L, SCF, TPO, and IL-3). This step allows amplifying the cells up to 100-fold. The expanded cells can be directly differentiated or cryopreserved for future use. The second phase is the differentiation of

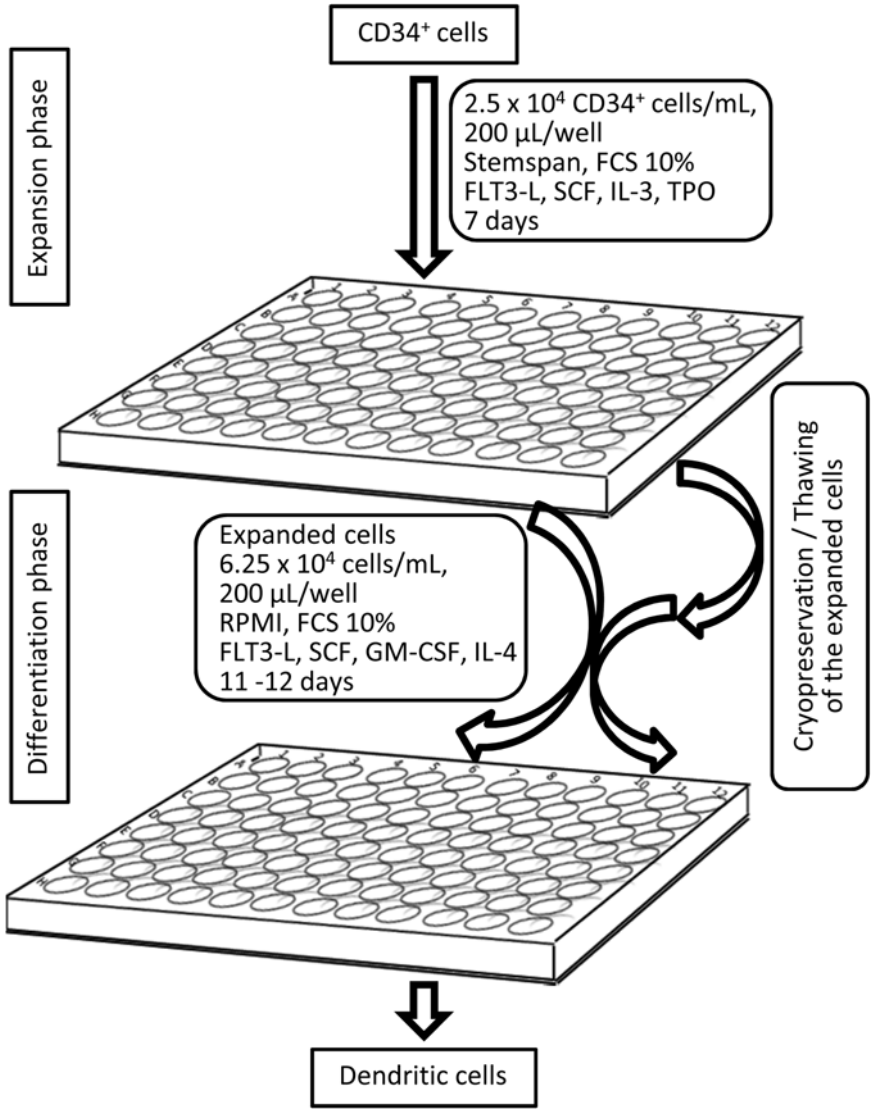

Fig. 1 General overview of the procedure for in vitro generation of XCR1$^+$ DCs from CD34$^+$ hematopoietic stem cell progenitors

the expanded cells, for 11–12 days, under the instruction of a different combination of cytokines (FLT3-L, SCF, IL-4, and GM-CSF). At the end of the culture, the different cell populations can be identified phenotypically by flow cytometry and eventually sorted for functional characterization as exemplified here by characterization of their responses to the stimulation by adjuvants.

All the experimental procedure should be performed under sterile cell culture conditions and with the appropriate safety measures required for handling human samples.

3.1 Isolation of the Mononuclear Cells (MNCs) from Cord Blood, Bone Marrow Aspirate, or G-CSF-Mobilized Peripheral Blood Apheresis

1. Bring the Ficoll-Paque from +4 °C to room temperature (RT).
2. Open the cord blood/bone marrow aspirate/peripheral blood apheresis collection (with sterile scissors in the case of collection bags), and transfer content to a T 75 mL tissue culture flask (for bags) or to 50 mL tissue culture tubes. Measure the volume.
3. Dilute the cord blood at 1:1 (v/v) with DB or dilute the bone marrow aspirate or peripheral blood apheresis at 1:2 (v/v).
4. Mix well.
5. Prepare one 50 mL tube for each 20–30 mL of the diluted cord blood or bone marrow aspirate or peripheral blood apheresis.
6. Dispense 15 mL of Ficoll-Paque to each of the 50 mL tube.
7. Carefully overlay 20–30 mL of blood on 15 mL of Ficoll-Paque (*see* **Note 5**).
8. Centrifuge the tubes at 800×*g* for 25 min at 20 °C without break.
9. Collect and pool the mononuclear cells at the interphase in new 50 mL tubes.
10. Centrifuge at 800×*g* for 5 min.
11. Resuspend the pellets in 10 mL of DB.
12. Centrifuge at 450×*g* for 6 min.
13. Resuspend the pellets in 5 mL of DB.
14. Determine the viable cell count with trypan blue. The cells are ready to use for CD34+ cell enrichment.

3.2 Enrichment of CD34+ Hematopoietic Progenitor Stem Cells

Enrich CD34+ cells using commercially available CD34+ enrichment kits according to the manufacturer's protocols. Enriched CD34+ cells can be directly used for the culture or cryopreserved for future use.

3.3 Expansion of Hematopoietic Precursors

1. Prepare the amplification medium as described in Subheading 2.
2. Wash the CD34+cells and resuspend them in the amplification medium at a cell density of 2.5×10^4 CD34+ cells/mL.

3. Plate 200 μL/well of the cell suspension, in U-bottom 96-well tissue culture-treated plates.
4. Harvest the cells on the seventh day: transfer the cells into 15 or 50 mL tubes and centrifuge at 450 × *g* for 5 min.
5. Resuspend the cells in RPMI, 10 % FCS, and determine the viable cell count using trypan blue. Expanded progenitors can be directly used for the culture or cryopreserved for future use (*see* **Note 6**).

3.4 Cryopreservation of Expanded Hematopoietic Precursors

1. The day before, prepare the freezing container by replenishing with fresh isopropanol according to the manufacturer's instructions. Precool it overnight at around +4 °C (*see* **Note 7**).
2. Prepare FM1 and FM2 and incubate them on ice for a time long enough to allow them to cool to +4 °C (for ≥10 min, depending on the volume) (*see* **Note 7**).
3. Label the appropriate number of cryotubes with sample name, cell number, date, etc.
4. Cool the cryotubes in ice for >10 min.
5. Harvest the cell culture and determine the viable count.
6. Resuspend the cells in FM1, in half of the final volume of cell suspension to be frozen (*see* **Note 8**).
7. Keep the cell suspension in ice for a time long enough to allow it to cool to +4 °C.
8. Add drop by drop to the cell suspension an identical volume of FM2, to achieve a 1:1 mixture of cell suspension and FM2, with continuous gentle agitation of the cell suspension tube. The tubes must be kept cold, on ice, during the entire procedure.
9. Transfer the cells to cryotubes, on ice.
10. Transfer the vials to the precooled freezing container.
11. Cool the freezing container at −80 °C overnight.
12. The day after, transfer the vials to liquid nitrogen for long-term storage.

3.5 Revival of Frozen Expanded Hematopoietic Precursors

1. Set the water bath at 37 °C.
2. Transfer the vials to the water and thaw the cells rapidly until only a small piece of ice is left in the tube (*see* **Note 9**).
3. Transfer the cells to a 15 mL polypropylene tissue culture tube.
4. Dilute the cell suspension fivefold in cold IMDM, 5 % FCS, 20 U/mL DNase I.
5. Gently mix the cell suspension, on ice.
6. Centrifuge the cell at 450 × *g* for 5 min at low break.
7. Resuspend the cells in medium#2.

3.6 Differentiation of DCs from Expanded Hematopoietic Precursors

1. Count the cells and resuspend them in differentiation medium#1 at a cell density of 6.25×10^4 cells/mL.
2. Plate 200 µL/well of the cell suspension in U-bottom 96-well tissue culture-treated plates.
3. On day 6: remove half of the medium from each well (100 µL) and replenish with differentiation medium#2.
4. The cells can be harvested on day 11 or 12 (*see* **Note 10**).

3.7 Phenotypic Identification of the Different Cell Populations at the End of the Culture

The in vitro culture consists in three different populations based on the expression of CD11c and CD141 (*see* **Note 11**) (*see* Fig. 2). The CD11c^{high}CD141^{+} (XCR1^{neg}) fraction specifically expresses CD11b, CD206, CD209, and TLR4. These cells are the equivalents of MoDCs as classically derived in cultures of monocytes with GM-CSF and IL-4. The CD11c^{low}CD141^{high} (XCR1^{+}) fraction specifically expresses CLEC9A, CADM1, TLR3, and XCR1. These cells are equivalent to the bona fide blood XCR1^{+} DC (*see* **Note 12**). The third fraction is low or negative for CD141 and CD11c (DN cells).

1. Prepare the staining buffer and blocking buffer as detailed in Subheading 2.
2. Resuspend the cells in blocking buffer ($2–10 \times 10^5$ cells/50 µL) (*see* **Note 13**).
3. Incubate at +4 °C for 5–10 min.
4. Prepare the desired antibody cocktails in blocking buffer, at twice the final dilution of use (*see* **Note 14**).

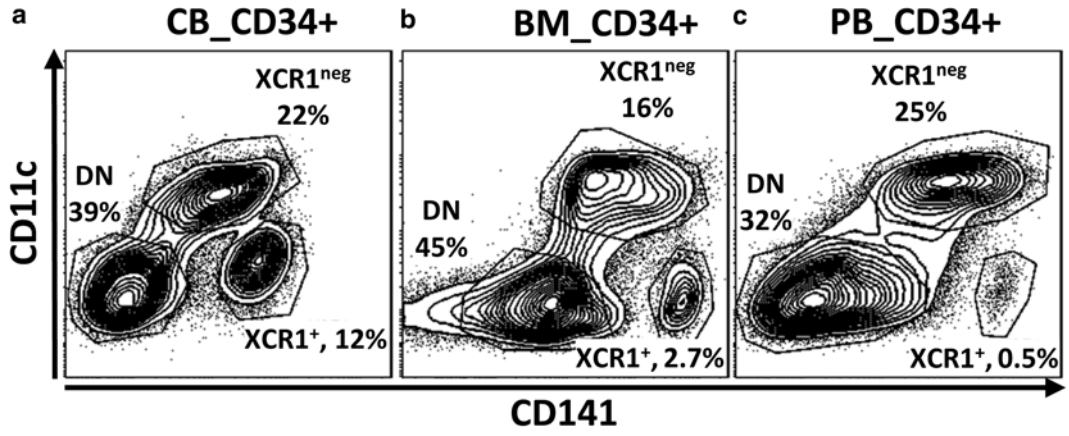

Fig. 2 Phenotypic identification of cell subsets in the cultures. The dot plot shows the different cell populations in the in vitro culture of CD34^{+} cells from cord blood (**a**), bone marrow (**b**), and G-CSF-mobilized peripheral blood (**c**). All culture generates three different populations based on the expression of CD11c and CD141. The XCR1^{+} cells correspond to the bona fide CD141^{+}CLEC9A^{+} cells present in human blood. The XCR1^{neg} cells are equivalent to MoDCs

5. Accordingly to the staining design, add to each cell suspension well 50 µL of the adequate antibody combination and aqua dead cell stain (*see* **Note 15**).
6. Incubate for 30 min at +4 °C.
7. Add 100 µL of SB to each well and centrifuge at 450 × *g* for 4 min.
8. Wash the cells pellets with 200 µL of SB and centrifuge at 450 × *g* for 4 min.
9. Resuspend the cell pellets in 100 µL of 0.5 % paraformaldehyde and incubate for 10–15 min for fixing the cells.
10. Spin the cells at 450 × *g* for 4 min.
11. Resuspend cell pellets in 200 µL of SB. The cells are ready for the FACS analysis.
12. Prepare single staining tubes for compensation with adequate compensation beads according to the manufacturer recommendations (*see* **Note 16**).

3.8 Sorting of the DC Subsets at the End of the Differentiation Culture

Purifying the different cell subsets in the culture is essential for their functional characterization and transcriptomic analysis (*see* **Note 17**). DCs can be sorted in two major populations, $CD11c^{high}CD141^{+}$ cells ($XCR1^{neg}$ fraction) and $CD11c^{low}CD141^{high}CLEC9A^{+}$ cells ($XCR1^{+}$ fraction) (*see* Fig. 2).

1. Coat the 5 mL snap-cap collection tubes overnight with PBS, 2 % BSA.
2. Extemporaneously fill the collection tubes with 250 µL/tube of collection medium.
3. Harvest the culture by collecting the cells in 15 or 50 mL polypropylene tissue culture tubes.
4. Centrifuge the cells at 450 × *g* for 5 min.
5. Resuspend the cell pellet in 5 mL of sterile SB and determine the viable cell number.
6. Prepare the single staining tubes with individual markers for compensation setup.
7. Prepare the antibody cocktails for phenotypic identification of subsets as described in Subheading 3.7.
8. Suspend ten million cells/200 µL of SB, and incubate the desired combination of antibodies for 30 min on ice in 15 mL tubes or 5 mL polystyrene round-bottomed snap-cap FACS tubes.
9. Wash the cells with 500 µL of SB.
10. Resuspend the cells in 1 mL of staining buffer containing SYTOX® Blue Dead Cell Stain.

11. Filter the cell suspension through a 70 μM cell strainer into 5 mL polystyrene round-bottomed snap-cap FACS tubes.
12. The live single cells should be plotted in CD11c vs. CD141 dot plot (*see* Fig. 2). Check that CLEC9A is specifically expressed on all $CD11c^{low}CD141^{high}$ cells and that CD209, CD206, and CD11b are specifically expressed on $CD11c^{high}CD141^{+}$ cells.
13. Sort the $XCR1^{+}$ DC as $CD11c^{low}CD141^{high}$ cells and the $XCR1^{neg}$ DCs as $CD11c^{high}CD141^{+}$ cells and if required the DN cells as $CD11c^{neg/low}CD141^{neg}$ cells (*see* **Note 18**).
14. Collect the sorted cells into the collection tubes.
15. Transfer a small fraction of sorted cell populations (~1:20 of the total volume) each into a properly labeled FACS tube, and complete it with SB to reach a total volume of 150 μL. Analyze these tubes by flow cytometry for measuring the purity achieved by the sorting procedure (*see* **Note 19**).

3.9 Stimulation of Sorted DC Subsets with Different Adjuvants and Evaluation of Their Activation

1. Transfer the sorted cells to 15 mL Falcon tubes and spin down at 450 × *g* for 5 min.
2. Resuspend the cells in a volume of medium#2 that should theoretically yield a cell concentration of 3×10^6 cells/mL based on the number of cells sorted for each population.
3. Determine the viable cell count.
4. Adjust the cell density to 10^6 cells/mL in medium#2.
5. Transfer 100 μL/well in U-bottom 96-well plate.
6. Add to each well 100 μL of differentiation medium#2 alone or supplemented with the selected TLR ligands, poly(I:C) at 5 μg/mL final, R848 at 10 μg/mL, or LPS at 1 μg/mL (*see* **Note 20**).
7. Incubate the cells for 16 h in a cell culture incubator.
8. Spin the culture plates at 450 × *g* for 5 min.
9. Transfer 150 μL of cell supernatant into a round-bottomed tissue culture-treated polypropylene 96-well plate, and freeze it at −20 °C for eventual later measurement of cytokine or chemokine titers by ELISA, Luminex, or any suitable method of your choice.
10. Resuspend cell pellets in 180 μL of staining buffer, centrifuge plates at 450 × *g* for 5 min, and discard supernatant.
11. Stain cells for flow cytometry as explained in Subheading 3.7, for the evaluation of their maturation as assessed by their enhanced expression of CD83, CD86, or HLA-DR under the different stimulation conditions.

4 Notes

1. The protocol works best with cord blood $CD34^+$ cells. This protocol also allows generating $XCR1^+$ DCs by using $CD34^+$ cells from bone marrow or from G-CSF-mobilized peripheral blood. Alternatively, $CD34^+$ cells can be purchased from different commercial suppliers. The frequency of $XCR1^+$ DCs varies from donor to donor but is generally higher (3–10 %) when using $CD34^+$ cells from cord blood as compared to bone marrow or to G-CSF-mobilized peripheral blood.
2. $CD34^+$ cells selection kits are available from different manufacturers (EasySep™ Human Cord Blood CD34 Positive Selection Kit, Stem cell technology; Dynal® CD34 Progenitor Cell Selection System, Dynal, Invitrogen; CD34 MicroBead Kit, human, Miltenyi Biotec). All the kits are efficient for the enrichment of $CD34^+$ cells. In our studies, we mainly utilized the Dynal or EasySep kits.
3. The selection of FCS is one of the most critical factors to ensure obtaining the highest possible frequencies of $XCR1^+$ DCs in the cultures, which can vary for the same $CD34^+$ cells from 0 to >10 % depending on the FCS. The frequency of the other cell subsets does not seem to be strongly affected by the FCS batches. Hence, a preliminary but very important step for establishing the culture system is to screen for a proper batch of FCS. Practically, small-scale cultures from two to three different donors must be seeded in parallel with different batches of FCS and compared for the frequency and absolute yields of $XCR1^+$ DCs obtained at the end of the culture.
4. This medium is used for feeding the cultures by replacement of half of their volume with fresh medium on the sixth day of the differentiation culture. Accordingly, it contains twice the concentrations of cytokines as compared to differentiation medium#1.
5. Preparation of the overlay of diluted samples on Ficoll-Paque is a critical step for the recovery of MNCs. To prepare the separate layers of Ficoll-Paque and diluted samples, it is crucial to hold the Ficoll-Paque containing tubes in a slanting position and to add the samples very gently in a very slow but continuous flow on the walls of the tubes by handling the pipette at a vertical angle with the tube walls. The loaded tubes should be handled very gently to avoid any mixing of Ficoll-Paque and samples which may reduce the recovery of MNCs.
6. Cryopreservation of expanded cells prior to their differentiation does not affect their propensity at generating $XCR1^+$ DCs. This step provides flexibility, including by allowing screening

CD34+ samples in small-scale cultures to select those yielding a higher frequency of XCR1⁺ DCs for later experiments requiring large-scale cultures.

7. All the items used in this step (FM1, FM2, cryotubes, freezing container, cell suspension, etc.) must be precooled by keeping them on ice, or at about +4 °C, for a minimum of 10–15 min or more depending upon the total volume of the cell suspension to be cryopreserved.
8. Cells are frozen in FM2. $1–5 \times 10^6$ cells can be cryopreserved in 1 mL per vial. The cells should be suspended at a 2× concentration in FM1 and diluted with FM2 at 1:1 ratio (i.e., if planning to cryopreserve 2×10^6 cells in 1 mL, suspend the cells in 500 μL of freezing medium 1, in one 50 mL polypropylene tissue culture tube, and add 500 μL of precooled freezing medium 2, drop by drop, under gentle mixing, on ice).
9. This is to ensure that the cell suspension does not warm above +4 °C.
10. The maximum yield of XCR1⁺ DCs is at days 11–12. Culturing cells longer does not improve the frequency or yield of XCR1⁺ DCs.
11. The combination of CD11c and CD141 allows discrimination between XCR1⁺ DCs ($CD141^{high}CD11c^{low}$) and MoDCs ($CD141^{+}CD11c^{high}$) versus other cell types ($CD11c^{-}CD141^{-}$) present in the culture. However, to ensure rigorous identification of cell types, it is highly recommended to examine their expression of additional markers, in particular CLEC9A or CADM1 which are specifically expressed on XCR1⁺ DCs versus CD206 and CD209 which are specifically expressed on MoDC subsets [33].
12. Other studies recently reported different protocols for in vitro generation of human XCR1⁺ DCs subsets [34, 35]. These protocols always use FLT3-L but with different combinations of cytokines including SCF, IL-3, IL-6, TPO, GM-CSF, or IL-4 and in one case using an antagonist of the aryl hydrocarbon receptor (StemRegenin 1) [35]. These protocols are especially interesting as they were reported to also simultaneously yield cells equivalent to the two other human blood DC subsets, pDCs and CD1c⁺ DCs. However, further characterization of the cell populations obtained with these protocols is required to ensure that each of them as identified corresponds to a single, homogeneous, cell type. The extent to which these in vitro-generated cell subsets are similar to human blood DC subsets also needs to be investigated in greater details.
13. Cells can be stained in U-bottom 96-well plate or in 5 mL round-bottomed FACS tubes.

Table 2
Differential expression of selected markers between the XCR1$^+$ and XCR1$^-$ subsets of DCs generated from CD34$^+$ cells

Marker	XCR1$^+$ DCs	XCR1^{neg} DCs
CD11c	Low	High
CD141	High	+
CLEC9A	+	–
XCR1	+	–
CADM1	+	–
TLR3	+	–
TLR4	–	+
CD209	–	+
CD206	–	+
CD11b	–	+
CD14	–	+
CD1b	+	++
CD23	–	+
CD32	–/+	++
CD103	–/+	++

14. For characterization of the cell populations by multiparameter flow cytometry analysis, a combination of CD11c and CD141 staining is required in each tube, in combination with the staining of other markers such as listed in Table 2 to ensure the identity of the cell populations.
15. Dead cell discrimination dyes must be used, according to the recommendations from the manufacturer, to ensure proper discrimination of cell subsets without the confounding factors of nonspecific binding of antibodies to dying cells or autofluorescence of these cells.
16. For compensation of the spectral overlaps between the different fluorochromes selected for the phenotyping, it is necessary to prepare single staining tubes, one for each of the individual antibodies used. Single stainings for compensation can be achieved with adequate compensation beads according to the manufacturer's recommendations (OneComp eBeads, eBioscience). However, it is highly recommended to additionally prepare and acquire single stainings performed with the cell suspension, in order to be able to replay compensation after data acquisition using this set of single stainings if needed.

17. Sorted cells at the end of Subheadings 3.8 and 3.9 can be processed for gene expression profiling as described previously [33] and detailed in another chapter of this book (Chapter 16).
18. The cells should be sorted with 100 μm nozzle and under low pressure (and hence low speed) to limit mechanical stress, using sterile FACS buffer instead of sheath fluid and keeping the collection tubes at 4 °C to preserve the best cell viability possible.
19. It is necessary to measure the purity achieved after each sort, by flow cytometry analysis of a small fraction of each sorted cell population.
20. XCR1⁺ DCs specifically and strongly respond to poly(I:C) and are also activated by R848 but not by LPS. MoDCs in these cultures exhibit a strong response to LPS and are also activated R848 but not strongly by poly(I:C) [33].

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