Video Article

In Vitro Generation of Murine Plasmacytoid Dendritic Cells from Common Lymphoid Progenitors using the AC-6 Feeder System

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Abstract

Plasmacytoid dendritic cells (pDCs) are powerful type I interferon (IFN-I) producing cells that are activated in response to infection or during inflammatory responses. Unfortunately, study of pDC function is hindered by their low frequency in lymphoid organs, and existing methods for *in vitro* DC generation predominantly favor the production of cDCs over pDCs. Here we present a unique approach to efficiently generate pDCs from common lymphoid progenitors (CLPs) *in vitro*. Specifically, the protocol described details how to purify CLPs from bone marrow and generate pDCs by coculturing with γ-irradiated AC-6 feeder cells in the presence of Flt3 ligand. A unique characteristic of this culture system is that the CLPs migrate underneath the AC-6 cells and become cobblestone area-forming cells, a critical step for expanding pDCs. Morphologically distinct DCs, namely pDCs and cDCs, were generated after approximately 2 weeks with a composition of 70-90% pDCs under optimal conditions. Typically, the number of pDCs generated by this method is roughly 100-fold of the number of CLPs seeded. Therefore, this is a novel system with which to robustly generate the large numbers of pDCs required to facilitate further studies into the development and function of these cells.

Video Link

The video component of this article can be found at https://www.jove.com/video/53211/

Introduction

Dendritic cells (DCs) are professional antigen-presenting cells that play an important role in controlling immune responses[1]. While DCs are heterogeneous, they can be broadly classified into two populations, conventional DCs (cDCs) and plasmacytoid DCs (pDCs)[2,3]. In addition to lymphoid organs, cDCs and pDCs are also found in tissues including lung, intestine, and skin[2]. The morphology of cDCs and pDCs differs, with cDCs exhibiting dendrite-like projections and the shape of pDCs being more plasma cell-like. In addition, the common mouse DC marker, CD11c, is more highly expressed on cDCs than on pDCs. Moreover, cDCs can be further divided into $CD11b^{+}CD24^{-}$ cDCs and $CD11b^{-}CD24^{+}$ cDCs, both of which express higher levels of MHC class II and costimulatory molecules than do pDCs[2]. Mature pDCs, on the other hand, have been shown to selectively express Siglec-H and BST2[4]. Functionally, cDCs are better antigen presenting cells than are pDCs; however, pDCs can produce a vast amount of type I interferon upon virus infection or inflammatory stimulation[5].

Both cDCs and pDCs are short-lived, and therefore, must be constantly replenished from progenitors within the bone marrow (BM)[6,7]. Adoptive transfer of common myeloid progenitors (CMPs) and common lymphoid progenitors (CLPs) into lethally-irradiated mice demonstrates that cDCs and pDCs can be generated from both lineages[8-10]. However, there is a subset of pDCs that express RAG1/2 and possess rearranged D-J segments at the IgH locus[11,12]. These cells also share molecular similarities with B lymphocytes, including expression of the B220 marker, nucleic acid-sensing receptors (TLR7/TLR9), and transcription factors (*Spib* and *Bcl11a*)[13], features all believed to be signatures of the lymphoid lineage. Therefore, CLPs may be good choices for *in vitro* generation of pDCs because of lineage similarity.

While the frequency of both cDCs and pDCs in humans and mice is very low[6], DCs, particularly cDCs, can be generated *in vitro* from BM or progenitors in the presence of cytokines, such as GM-CSF[11,14] or Flt3 ligand (FL) using feeder-free systems [11,15,16]. Unfortunately, however, it is not possible to produce large numbers of pDCs *in vitro* using FL[11,15,16]. Previously we demonstrated that pDCs can be efficiently generated *in vitro* from CLPs using the AC-6 feeder system[17]. The advantage of using the AC-6 stromal cell line in the culture system is that it provides cell-cell contact and secretion of cytokines that support the generation of large numbers of pDCs from CLPs. Although production with this system is quite robust, careful replication of the procedures described below is required in order to ensure efficient generation of pDCs.

Protocol

C57BL/6 wild-type mice were purchased from the National Laboratory Animal Center (NLAC), Taiwan. All mice were bred and kept under specific pathogen-free conditions. Protocols and animal use procedures were reviewed and approved by the Institutional Animal Care and Use Committee of National Taiwan University College of Medicine (Permit Number: 20120075). In addition, researchers made every effort to reduce the potential for pain, suffering, or distress in the animals while performing experiments. All procedures described were carried out at RT while wearing gloves.

1. Preparation of AC-6 Feeder cells

Note: The AC-6.21 (AC-6 in short) stromal cell line[18] (provided by I. Weissman, Stanford University) should be maintained in RPMI supplemented with 15% heat-inactivated fetal bovine serum (FBS). To serve as feeder cells, AC-6 cells are γ-irradiated with 3,000 rad (30Gy) one day before the co-culture to prevent their proliferation. Note that AC-6 cells tend to lose their differentiation-supporting ability if left overcrowded during maintenance, or if their passage number is over 20.

1. Wash AC-6 cells once with 1 ml Dulbecco's Phosphate-Buffered Saline (DPBS) and remove DPBS by aspiration. Treat AC-6 cells with 0.8 ml trypsin solution (0.05 % trypsin and 0.5 mM EDTA in DPBS) for 3-5 min at 37^{o}C, 5% CO_2 and then stop the reaction by diluting it with 10 volumes of culture medium to make single cell suspension.
2. Seed AC-6 cells at 5.9x10^4 cells/well into 12-well plates and incubate at 37^{o}C, 5% CO_2 O/N.
   Note: The density of AC-6 cells is very important as a lower density will favor cDC generation. Seeding at a density of 5.9x10^4 cells/well will allow AC-6 to reach confluency by the next day, which is optimal for derivation of pDCs from CLPs.
3. Irradiate AC-6 cells at 3,000 rad (30 Gy) using γ-irradiator.
   Note: The dose used to irradiate AC-6 is optimized to prevent the cells from proliferating and yet keep them viable long enough to provide the cytokines and cell-cell contact required for differentiation of DCs from CLPs.
4. Aspirate medium and replace with 1 ml complete RPMI (RPMI with 10 % heat-inactivated FBS, 50 µg/ml gentamycin and 50 µM β-mercaptoethanol).

2. Isolate BM Cells from Mice

1. Sacrifice mice by CO_2 asphyxiation and cervical dislocation. Place the mice on a dissection tray and sterilize with 70% ethanol. Make an incision at the mid-abdomen and remove the skin from the distal part of the mouse including the skin covering the lower extremities.
2. Dissect mice in a semi-sterile hood. To release the femur and tibiae, clip the femur above and tibiae below the knee joint. Detach the muscles from the bones using scissors and place the bones in a 6 cm petri dish containing 5 ml complete RPMI.
3. Move to a sterile culture hood. Fill a 3 ml syringe connected to a 27G needle with complete RPMI. Cut off both ends of the bones using scissors, and insert the 27G needle to flush the marrow out of the bones by gently injecting complete RPMI once from each end.
4. Prepare a single cell suspension by gentle pipetting the cells up and down 3-5 times in the culture dish using the syringe without the needle.
5. Centrifuge the cells for 5 min at 500 x g and decant the supernatant.
6. Lyse red blood cells with 1 ml ACK buffer (150 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA) incubating for 1 min and stopping the reaction by diluting with 10 volumes of complete RPMI. Allow cells to stand for 5 min in order to pellet down dead cell clumps and tissue debris by gravity.
   Note: Do not stand more than 5 min as this will result in cell loss due to settling of viable cells.
7. Slowly decant cell-containing supernatant to a new tube leaving debris behind in the original tube.
8. Centrifuge for 5 min at 500 x g to pellet the cells, then remove and discard the supernatant.
9. Resuspend total BM cells (typically 4-6 x 10^7 BM cells are obtained from one mouse) in 100 µl FACS buffer (1x PBS+ 2% FBS+ 1mM EDTA).

3. FACS Analysis and Sorting of CLPs

1. Add anti-CD16/32 (hybridoma supernant clone 2.4G2, 50 µl/reaction or 1-2 µg/reaction) into cells in FACS buffer (step 2.9) for 1-2 min in order to block Fc receptors.
   Note: Anti-CD16/32 is also called Fc block, which is added to prevent non-specific binding of antibodies to cells expressing Fc receptors including granulocytes, monocytes, and B lymphocytes.
2. Simultaneously stain cells with the following fluorescent dye-labeled antibodies (for 4x10^7 cells) for 15 min on ice, avoiding ambient light.
   Antibodies: PE-conjugated lineage markers (0.2 µg each) including anti-CD3 (17A2), anti-CD8 (53-6.7), anti-B220 (RA3-6B2), anti-CD19 (eBio1D3), anti-CD11b (M1/70), anti-Gr-1 (RB6-8C5), anti-Thy1.1 (HIS51), anti-NK1.1 (PK136), anti-TER119 (TER-119), and anti-MHC-II (NIMR-4), 0.2 µg anti-c-Kit-PerCP-Cy5.5 (2B8), 1 µg anti-Sca-1-FITC (D7), 0.4 µg anti-M-CSFR-APC (AFS98), and 0.2 µg anti-IL-7Rα-PE-Cy7 (A7R34).
3. Wash cells with 3 ml FACS buffer, and centrifuge for 5 min at 500 x g.
4. Resuspend the cells in 300 µl FACS buffer and filter cells through a 40 µM cell strainer.
5. Wash the tube with an additional 100 µl FACS buffer to recover any remaining cells and filter into a sterile FACS sorting tube using the same cell strainer.
6. Perform flow cytometric analysis immediately after the staining using appropriate filters and voltages for signal detection. Use 488 nm laser to detect the FITC-, PerCP-Cy5.5-labeled antibodies, 561 nm laser to detect the PE- and PE-Cy7-labelded antibodies and 633 nm laser to detect the APC-labeled antibody.
7. Sort out CLPs according to the following markers lin$^-$c-kit^{int}Sca-1^{int}M-CSFR$^-$IL-7Rα$^+$ (as stained in step 3.2) using a cell sorter. Collect the sorted cells in a 15 ml tube containing 8 ml of complete RPMI as a cushion.
8. Record the absolute number of sorted cells as shown by the cell sorter at the end of the run. Typically, 5x10^4 CLPs can be obtained from the BM of one mouse.

4. Coculture CLPs and AC-6

1. Centrifuge the sorted cells from step 3.7 for 10 min at 500 x g, remove the supernatant and resuspend the cell pellet with enough complete RPMI to obtain a cell density around $5x10^4$ cells/ml. Seed 500 cells/well into the 12-well plate containing feeder cells prepared in step 1.4.
2. Add 100 ng/ml FL[19] (hu-Flt3L-Ig generated in-house using an expression system provided by M. Manz, University Hospital Zürich, Switzerland) and incubate at 37°C, 5% CO_2 with periodic visual monitoring of DC development under a microscope.
   Note: Commercially available recombinant human FL or mouse FL can be used as a substitute for hu-Flt3L-Ig to support DC development.
3. Collect the cells in the supernatant at day 12 and wash the wells once with 0.5 ml complete RPMI medium combining the resulting supernatants. Add 0.5 ml fresh medium and scrap the adherent cells with a cell scraper.
4. Combine the cell-containing medium from both parts and centrifuge for 5 min at 500 x g.
5. Decant supernatant and resuspend cells in 50 µl FACS buffer. Add 50 µl anti-CD16/32 hybridoma supernatant and incubate for 1-2 min to block Fc receptors.
6. Enumerate and stain all the cells with the following antibodies (0.05 µg each) anti-CD11c-APC (N418), anti-CD11b-FITC, and anti-B220-PE.
7. Wash and centrifuge the cells as described in step 3.3.
8. Resuspend the cells in 100 µl FACS buffer, gate on $CD11c^+$ and analyze for cDCs ($CD11c^+CD11b^+B220^-$) and pDCs ($CD11c^+CD11b^-B220^+$)[17].

Representative Results

A total of $4\text{-}6x10^7$ BM cells are typically isolated from femurs and tibiae of one 6-8 wk-old, wild-type C57BL/6 mouse. To sort out CLPs, total BM cells are stained with PE-conjugated antibodies against lineage markers (CD3, CD8, B220, CD19, CD11b, Gr-1, Thy1.1, NK1.1, TER119, and MHC-II), anti-c-Kit-PerCP/Cy5.5, anti-Sca-1-FITC, anti-M-CSFR-APC and anti-IL-7Rα-PE/Cy7, analyzed and sorted with a cell sorter. The sorting strategy for CLP is shown in **Figure 1**. Typically, $5x10^4$ CLPs are obtained from one C57BL/6 mouse. To obtain more CLPs, BM cells from 3-4 mice may be combined and protocol adjusted accordingly before sorting.

Following the coculture of 500 CLPs with $5.9x10^6$ AC-6 feeder cells, cobblestone area-forming cells (CAFC) start to appear by day 3-4. By day 8, round-shaped pDCs appear and suspend on top of the CAFC (**Figure 2A**). The number of pDCs peaks around day 12-14 at which point pDCs start to undergo apoptosis following their full development, and the numbers begin to gradually decrease. cDC colonies appear later than pDC colonies, at day 6-8, and the morphology of cDCs are larger, spindle-like, and adherent (**Figure 2B**).

Typically, $5\text{-}6x10^4$ cells can be generated from 500 CLPs after 2 weeks of coculture. After staining the cells with anti-CD11c-APC, anti-CD11b-FITC and anti-B220-PE, analysis by flow cytometry shows that the percentage of pDCs ($CD11c^+CD11b^-B220^+$) and cDCs ($CD11c^+CD11b^+B220^-$) is 91% and 6%, respectively (**Figure 3**). Therefore, with this method pDCs can be expanded as much as 100-fold.

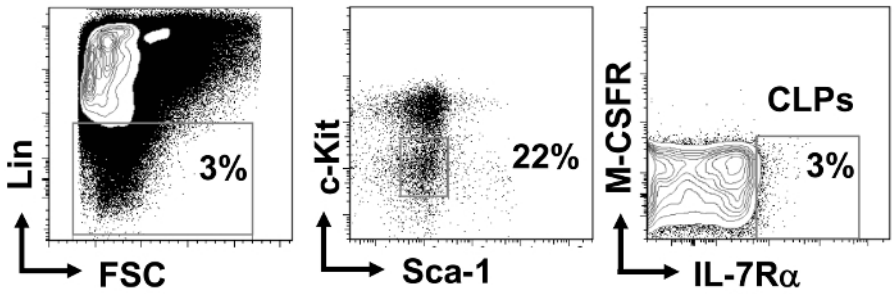

Figure 1. Gating strategies for CLPs. BM cells isolated from one mouse were stained with fluorescently-labeled antibodies and analyzed for CLPs, which were defined as $lin^-c\text{-}Kit^{int}$ $Sca\text{-}1^{int}M\text{-}CSFR^-IL\text{-}7R\alpha^+$. Please click here to view a larger version of this figure.

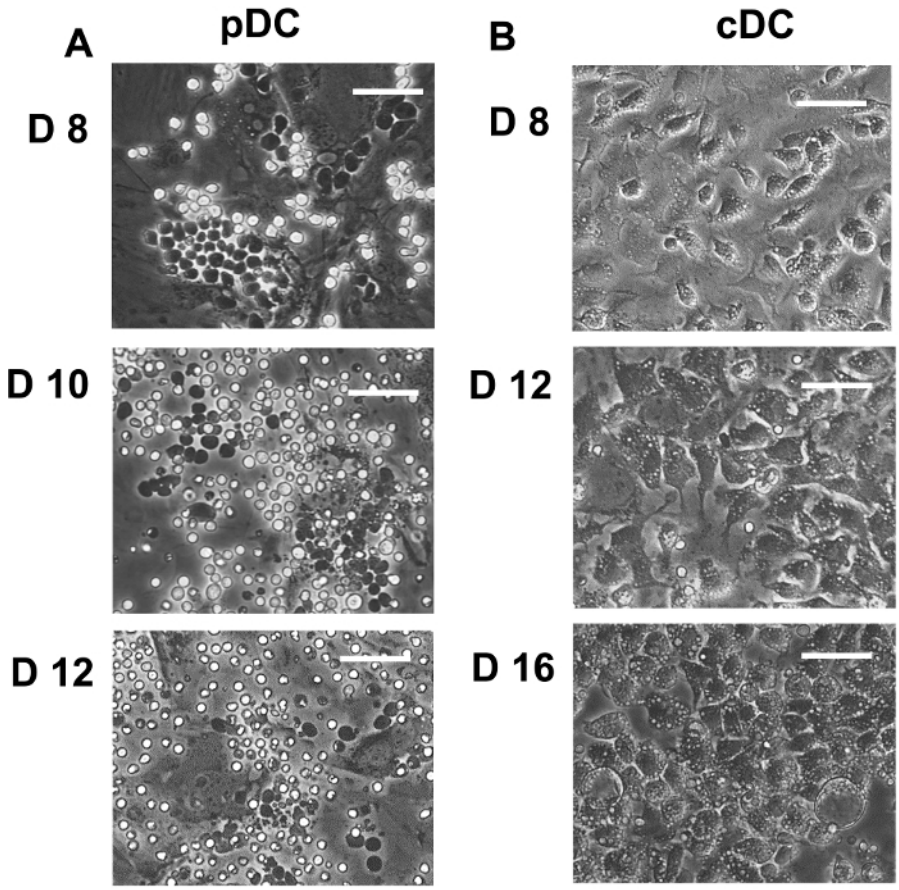

Figure 2. Morphology of cDC and pDC colonies in CLP-AC-6 cocultures. 500 CLPs were cocultured with γ-irradiated AC-6 feeder cells in the presence of 100 ng/ml FL. **(A)** pDC colonies at day 8, 10, and 12, and **(B)** cDC colonies at day 8, 12, and 16 were photographed under light microscopy at 200 x (scale bar 50 µm). Please click here to view a larger version of this figure.

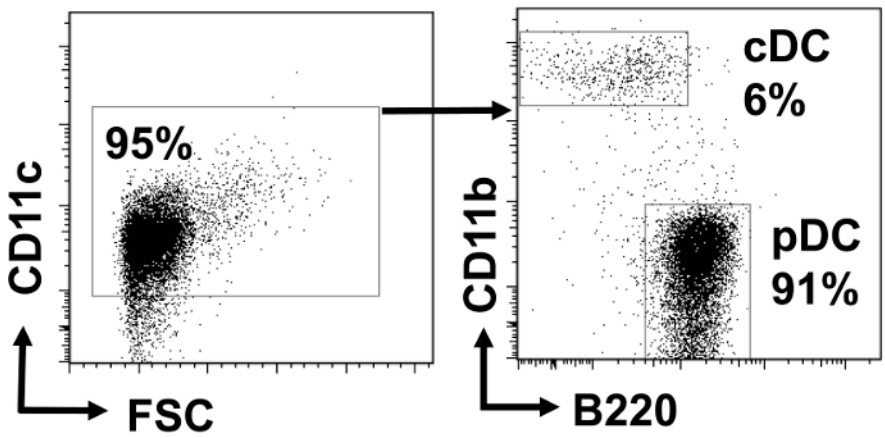

Figure 3. Flow cytometric analysis of CLP-derived pDCs and cDCs. Five hundred CLPs and γ-irradiated AC-6 feeder cells ($5.9x10^4$/ml) were cocultured *in vitro* for 12 days in the presence of FL (100 ng/ml). The cells were stained with anti-CD11c-APC, anti-CD11b-FITC, and anti-B220-PE and analyzed by flow cytometry. Cells were first gated on $CD11c^+$ and then analyzed for cDCs ($CD11c^+CD11b^+B220^-$) and pDCs ($CD11c^+CD11b^-B220^+$). Please click here to view a larger version of this figure.

Discussion

Here we describe an *in vitro* culture system for the robust generation of DCs, and pDCs in particular, from a small number of CLPs. The uniqueness of this culture system is due to the use of AC-6 cells, a stromal cell line, as feeders. This approach has been shown to provide not only the cytokines, such as IL-7, SCF, M-CSF and FL[20], but also the cell-cell contact[21] necessary to support DC development. AC-6 cells have been used previously to facilitate the study of *in vitro* DC development from several progenitors[8,16,22]. We found that it is critical to seed an optimal density of AC-6 feeder cells to support the efficient differentiation of pDCs from CLPs. Specifically, seeding $5.9x10^4$ γ-irradiated AC-6

cells in 12-well plates results in confluency the next day, which is suitable for pDC development. A lower AC-6 cell density ($3.9x10^4$/well in 12-well plate) tends to support cDC development[24]. Therefore, it is recommended to first seed several concentrations of AC-6 cells in order to optimize conditions when scaling up the culture system[23]. Moreover, it is also important that the AC-6 cells be scrupulously maintained, including careful attention to passage number, and never allowed to reaching confluency. If left overcrowded or if their passage number is greater than 20, these cells tend to lose the ability to support hematopoiesis *in vitro*. Additionally, the concentration of FL also affects DC development. Specifically, at low doses (25-50 ng/ml) cDCs are preferentially developed, while at high doses (100-200 ng/ml) pDCs are predominantly generated[24]. In addition to AC-6 cell density and FL dosage, careful selection of a batch of FBS that efficiently supports DC development in this culture system is central to the success of the procedure.

While *in vitro*-derived cDCs and pDCs can be distinguished by their surface markers, namely $CD11c^+CD11b^+B220^-$ and $CD11c^+CD11b^-B220^+$, respectively, their morphologies are also discernible by microscopy. cDCs are larger, spindle-shaped and are adherent, while pDCs are smaller, round-shaped and grow in suspension (**Figure 2**). The kinetics of DC differentiation also differs, with pDCs appearing earlier and cDCs later. Additionally, the process of pDC development is significantly influenced by AC-6 cell density with higher AC-6 density speeding up the developmental process. It should be noted that pDCs start to die following complete development and thus, the ratio of cDCs versus pDCs in this culture system gradually increases at later time points. Therefore, periodic monitoring of DC development by microscopy and flow cytometry is highly recommended in order to determine when pDCs start to undergo apoptosis.

Of note, the described method can also be applied to other DC progenitors, such as CDPs; however, the main DC population generated from these cells are cDCs[17], even at high AC-6 densities or FL doses. The low pDC potential of CDPs is consistent with reports from different groups using either CDP-feeder[11] or feeder-free[16] systems, two different *in vitro* culture systems. These results suggest that the AC-6 feeder system can faithfully reflect the differentiation potential of different progenitors.

The pDCs generated from this culture system are $CD11c^+CD11b^-B220^+$. Moreover, they express relatively high levels of *Tcf4* (encodes E2-2) and *Rag1*, two pDC-specific genes, but lower levels of Id2, a cDC-specific gene, than do cDCs[17]. Even though these pDCs lack Siglec-H and BST2, markers considered characteristic of murine pDCs, they are still able to produce higher levels of IFN-I than do cDCs and upregulate CD86 when infected with vesicular stomatitis virus (VSV)[24]. This suggests that these pDCs, although incompletely differentiated, are still functional. Moreover, we have already successfully used this method to delineate a synergistic role of IFN-I and FL in pDC development from CLPs[17], confirming that this technique is valuable for the study of DC development.

In conclusion, here we present a simple, but efficient, method to generate pDCs from a small number of CLPs using the AC-6 feeder coculture system, which can facilitate developmental or functional studies of pDCs.

Disclosures

Authors have nothing to disclose.

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