Differentiation of Peripheral Blood Monocytes into Dendritic Cells

BASIC PROTOCOL

Dendritic cells (DCs) are potent antigen-presenting cells that are important in the initiation and control of cellular immune responses. Commonly used in T cell–stimulation experiments, DCs are typically "matured" in vitro with microbial products or proinflammatory cytokines, and are then loaded with antigens from any number of sources, including peptides, whole proteins, cell lysates, RNA, microbes, or killed tumor cells. A detailed protocol is presented here that uses the simplest and most common method for the generation of mature human dendritic cells—i.e., differentiating them from peripheral blood monocytes. It should be noted that *UNIT 7.32* contains protocols for isolating and purifying DCs from peripheral blood, as well as a method for depleting DCs from mononuclear cell suspensions.

The 7-day procedure begins with the plating of peripheral blood mononuclear cells (PBMCs) onto 10-cm tissue culture dishes. Monocytes adhere to the plastic dishes during a 1-hr incubation step, and lymphocytes, which do not adhere to plastic, are removed by washing. The monocytes are then induced to differentiate into immature DCs by culturing for 5 days in the presence of IL-4 and GM-CSF. On Day 5, the immature DCs are harvested and transferred to 6-well plates. After an overnight culture step, the immature DCs are stimulated to mature by culturing for an additional 18 to 24 hr in the presence of a cocktail of three proinflammatory cytokines (IL-1β, IL-6, and TNFα) and prostaglandin E_2 (PGE_2). This cocktail is referred to as monocyte conditioned medium mimic (MCM mimic). This method routinely results in Day 7 cell yields of 5% to 10% relative to the number of PBMCs plated on Day 0, with 80% to 90% of the cells having morphologic, phenotypic, and functional characteristics of mature DCs.

Materials

Peripheral blood mononuclear cells (PBMC; *UNIT 7.1*)
RPMI-10 (see recipe)
RPMI 1640 medium (e.g., Invitrogen; no serum or other additives), prewarmed to 37°C
PBS/5% albumin (see recipe)
400 IU/μl IL-4 (see recipe)
100 IU/μl GM-CSF (see recipe)
100× monocyte conditioned medium (MCM) mimic (see recipe)
Heat-inactivated human AB serum containing 10% (v/v) DMSO
Isopropanol
Liquid nitrogen

10-cm tissue culture dishes (BD Falcon)
Inverted microscope
15- and 50-ml conical polypropylene centrifuge tubes
Tabletop centrifuge
6-well tissue culture plates (BD Falcon)
1.0 or 1.8-ml cryovials
"Mr. Frosty" Cryo 1°C freezing container (Nalgene)
Liquid nitrogen freezer or equivalent

Additional reagents and equipment for determining cell viability by trypan blue exclusion (*APPENDIX 3B*)

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NOTE: All solutions and equipment coming into contact with live cells must be sterile, and aseptic technique should be used accordingly.

NOTE: All culture incubations should be performed in a humidified 37°C, 5% CO_2 incubator unless otherwise specified.

Initiate PMBC culture (Day 0)

1. Resuspend PBMC at a concentration of 5×10^6 viable cells/ml in RPMI-10. Plate 10 ml of this cell suspension onto each 10-cm tissue culture dish.

   Plating 50×10^7 cells per 10-cm tissue culture dish ensures reproducible saturation of the dish with monocytes. A saturated dish should yield 5–6 $\times 10^6$ immature DCs after 5 days in culture. There may be donor-to-donor variability in the number of cells to plate, and as few as 35×10^6 PBMCs may be sufficient.

2. Incubate 1 to 2 hr. Do not stack the dishes for this step, so as to allow them to rapidly equilibrate to 37°C.

   During this incubation, monocytes will adhere to the plastic surface of the tissue culture dish, whereas lymphocytes will remain unattached (see also UNIT 7.6).

3. For every tissue culture dish to be used, prepare 10 ml of RPMI-10 with 1× cytokines by adding 50 µl of 400 IU/µl IL-4 and 100 µl of 100 IU/µl GM-CSF per 10 ml of RPMI-10. Mix thoroughly but gently.

   This is a 1× working solution with a concentration of 200 IU/ml IL-4 and 100 IU/ml GM-CSF. Prepare fresh; do not store.

   IL-4 and GM-CSF induce the differentiation of monocytes (large $CD14^+$ cells) into immature DCs (which are large, nonadherent, $CD14^-$ $CD83^-$ cells). It is a good idea to carry out titration experiments to determine the optimal amount of these cytokines needed to achieve the desired effect. GM-CSF is required for cell survival and to promote monocyte differentiation into DCs, whereas IL-4 prevents monocyte differentiation into macrophages. Macrophages are easily identified as large, adherent cells with a "fried egg" appearance. In the authors' experience, 100 IU/ml GM-CSF and 200 IU/ml IL-4 have been sufficient to induce monocyte differentiation into immature DCs. Increasing the concentration of these cytokines has no adverse effects, but tends not to improve the yield of immature DCs on Day 5.

4. When incubation is complete, remove dish from incubator and wash by pipetting the medium already there up and down several times while gently rocking the dish, then removing the medium. Add 10 ml prewarmed RPMI 1640 medium and repeat the wash.

   When pipetting up, one should tilt the plate toward oneself while aspirating up the medium. When pipetting down, direct the stream of medium over the entire surface of the dish by moving the pipet. Do not scrape the surface of the dish with the pipet. Pipet out all of the medium after each wash, but work quickly, so that the cells do not dry out between washes.

5. After the second wash, add 10 ml RPMI-10 with 1× cytokines (see step 3).

   Check the dish under the inverted microscope after adding the medium with cytokines. There should be very few freely floating cells (these can be identified by tapping the plate and looking for moving cells). It may be necessary to wash again if many cells are still free-floating, but it is best not to overmanipulate the dish.

6. Return the dish to the incubator on a new shelf.

   Using a new shelf helps one keep track of which plates have received fresh cytokines.

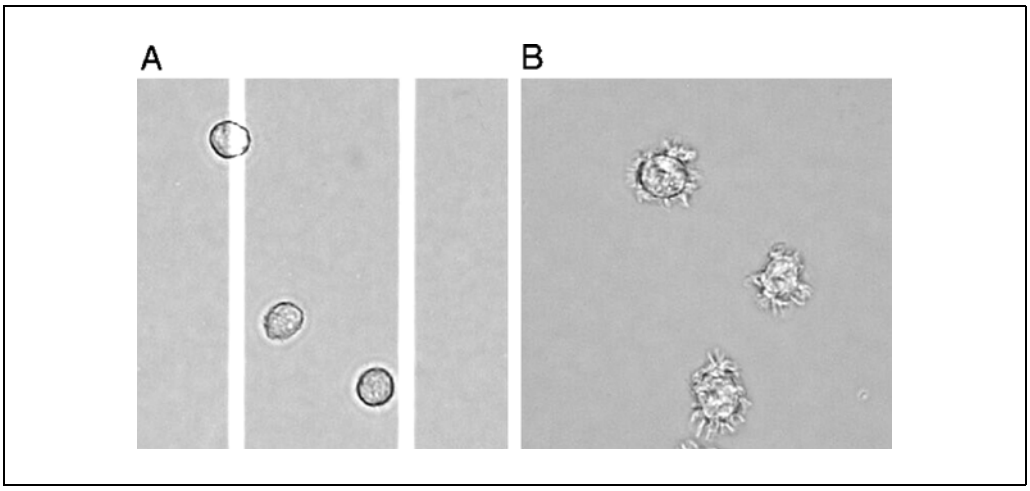

Figure 22F.4.1 Immature DCs (**A**) and MCM mimic-matured DCs (**B**) as viewed in a hemacytometer at 400× magnification under an Olympus BX51 microscope with an Olympus U Plan Fluorite 40×/0.75 NA objective. Images were captured using an Optronics MicroFire digital camera and MicroFire image acquisition software, and processed with Adobe Photoshop.

7. Repeat steps 4, 5, and 6 (i.e, washing twice, adding medium with cytokines, and returning to the incubator, respectively) for all of the remaining tissue culture dishes.

8. Incubate 5 days. Refresh the IL-4 and GM-CSF on Day 2 or 3 of incubation (see below).

   It does not matter whether the cytokines are added on Day 2 or Day 3. This is left up to the investigator's discretion.

Refresh cytokines (Day 2 or 3)

9. For every tissue culture dish, prepare 1 ml RPMI-10 with 10× cytokines by adding 5 µl of 400 IU/µl IL-4 and 10 µl of 100 IU/µl GM-CSF per 1 ml of RPMI-10. Mix thoroughly but gently.

   This is a 10× solution with a concentration of 2000 IU/ml IL-4 and 1000 IU/ml GM-CSF. It is used to refresh the cytokines in dendritic cell cultures on Day 2 or 3. Use this solution immediately (do not prepare and store in advance).

10. View the cells under an inverted microscope and assess for cell density, cell viability, dendritic or veiled appearance (Fig. 22F.4.1), amount of cell debris, relative number of nonadherent cells, and relative number of lymphocytes.

    It is important to understand the morphological changes to expect in the cell culture. After washing out most of the lymphocytes on Day 0, the remaining monocytes can be seen adhering tightly to the plastic surface of the tissue culture dish, many of them with a flattened appearance. A fair number of adherent platelets may be seen, which are much smaller than monocytes or lymphocytes. Most of the platelets will not survive the week-long culture period. During the first 5 days of culture, the monocytes will detach from the plastic as they differentiate into immature DCs. There will be a fair amount of cell death and debris visible, but by Day 5 it is reasonable to expect a viable cell yield of 10% to 15% relative to the number of PBMCs plated on Day 0. Immature DCs are large, relatively round cells with few or no cytoplasmic projections (see Fig. 22F.4.1). They are 2 to 3 times the size of lymphocytes. If the expected morphology is not observed, it may be an indication that the IL-4 or GM-CSF stocks have lost activity (see Troubleshooting).

11. Add 1 ml of RPMI-10 with 10× cytokines to each dish. Mix each culture thoroughly by swirling. Return the dishes to the incubator.

Harvest immature DCs (Day 5)

12. View the cells under an inverted microscope and assess their morphology as described in step 10.

    Over 90% of the cells should be loosely adherent or nonadherent by Day 5, with the vast majority having the morphological characteristics of immature DCs. If the expected morphology is not observed, it may indicate a problem with the IL-4 or GM-CSF (see Troubleshooting).

13. Harvest the cultures by pipetting up and down with a 10-ml pipet to resuspend all loosely adherent or nonadherent cells. Transfer and pool the harvested nonadherent cells in a labeled 50-ml conical centrifuge tubes or other sterile container. Discard the empty tissue culture dishes.

14. Record the total volume of harvested cells. Mix the cells thoroughly, take a 10-µl aliquot fo cell suspension, and determine the total number of viable cells (*APPENDIX 3B*).

    The Day 5 yield (ie, the number of viable cells harvested on Day 5 divided by the number of cells plated on Day 0) should be 10% to 15%.

15. Prepare fresh RPMI-10 with 1× cytokines as described in step 3. Centrifuge the harvested cell suspension 6 min at 500 × *g*, room temperature. Resuspend the cells at a concentration of 5×10^5 cells/ml in RPMI-10 with 1× cytokines.

16. Transfer 3 ml of cell suspension per well into labeled 6-well tissue culture plates. Incubate overnight.

    Much less cell debris should be visible when the cultures are viewed under an inverted microscope once the cells are washed and replated on Day 5.

Add maturation stimulus (Day 6)

17. View the cells under an inverted microscope and assess their morphology as described above.

18. Add 30 µl of 100× MCM mimic to each well. Mix thoroughly by pipetting up and down and by swirling the plates. Return the plates to the incubator and continue incubating overnight.

    If the cells are to be loaded with antigens, many investigators prefer to add the antigen source just prior to adding the maturation stimulus (see Commentary).

    MCM mimic is a potent cocktail of proinflammatory cytokines and prostaglandin E_2 that stimulates dendritic cell maturation during overnight (18 to 24 hr) culture. The final (1×) concentrations of the four components in tissue culture medium are: 5 ng/ml TNF-α, 5 ng/ml IL-1β, 150 ng/ml IL-6, and 1 µg/ml prostaglandin E_2 (PGE_2). Many other compounds may also be used to mature DCs (see Commentary).

Harvest mature DCs (Day 7)

19. View the cells under an inverted microscope and assess their morphology as described above.

    About two-thirds of the cells should survive from Day 5 to Day 7. By Day 5, some of the DCs may begin to show early changes associated with DC maturation—the characteristic cytoplasmic projections (dendrites) or veils (Fig. 22F.4.1). These changes are much more evident on Day 7 following the overnight maturation stimulus. By Day 7, over 80% of the cells in the culture should be large, nonadherent cells with numerous cytoplasmic projections and/or veils. There will be some cell debris and evidence of cell death, but this should not be a predominant feature. It is reasonable to expect a 5% to 10% yield of mature DCs on Day 7 relative to the number of PBMCs plated on Day 0.

20. Harvest cells as on Day 5 (see step 13). Count viable cells (*APPENDIX 3B*). Either use cells in in experiments, or freeze as in step 21.

Cells kept in the incubator in RPMI-10 without cytokines for up to 3 days will maintain their mature phenotype. Mature DCs can be successfully loaded with a number of different antigen sources for T cell stimulation experiments (see Commentary).

21. Centrifuge the cells 6 min at 500 × g, room temperature. Resuspend cells at the desired concentration (usually 1×10^6 cells/ml) in heat-inactivated human AB serum containing 10% DMSO. Divide suspension into aliquots in 1.0 or 1.8-ml cryovials, then place the vials in a "Mr. Frosty" 1°C freezing container filled with isopropyl alcohol to promote controlled-rate freezing (also see *APPENDIX 3G*). Place the "Mr. Frosty" in a −70°C freezer.

DCs that have been loaded with antigens and then frozen and thawed retain the ability to stimulate antigen-specific T lymphocytes.

22. After the controlled-rate freezing step is complete, transfer the DCs to a liquid nitrogen freezer or equivalent for long-term storage.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***APPENDIX 5****.*

GM-CSF, 100 IU/μl

Stock solution (0.5 mg/ml): Using aseptic technique, reconstitute lyophilized GM-CSF (R&D Systems) in PBS/5% albumin (see recipe) at a concentration of 0.5 mg/ml. Store frozen at −20° to −80°C in 10-μl aliquots.

Working solution (100 IU/μl): Check the manufacturer's specifications for the specific activity of the lot from which the stock solution was prepared (should be ∼20,000 IU/μg). Thaw a frozen aliquot of GM-CSF stock solution and dilute in PBS/5% albumin (see recipe) to a concentration of 100 IU/ml. Mix thoroughly but gently. Do not vortex excessively. Store up to 8 to 14 days at 2° to 8°C.

IL-4, 400 IU/μl

Stock solution (1 mg/ml): Using aseptic technique, reconstitute lyophilized IL-4 (R&D Systems) in PBS/5% albumin (see recipe) at a concentration of 1 mg/ml. Store frozen at −20° to −80°C in 10- to 20-μl aliquots.

Working solution (400 IU/μl): Check the manufacturer's specifications for the specific activity of the lot from which the stock solution was prepared (should be ∼10,000 IU/μg). Thaw a frozen aliquot of IL-4 stock solution and dilute in PBS/5% albumin (see recipe) to a concentration of 400 IU/ml. Mix thoroughly but gently. Do not vortex excessively. Store up to 8 to 14 days at 2° to 8°C.

Monocyte conditioned medium (MCM) mimic, 100×

Prepare stock solutions as follows using aseptic technique:

50 μg/ml IL-1β: Reconstitute lyophilized IL-1β (R&D Systems) in PBS/5% albumin (see recipe) at a concentration of 50 μg/ml. Store frozen at −20° to −80°C in 20-μl aliquots.

150 μg/ml IL-6: Reconstitute lyophilized IL-6 (R&D Systems) in PBS/5% albumin (see recipe) to a concentration of 150 μg/ml. Store frozen at −20° to −80°C in 200-μl aliquots.

1 mg/ml PGE_2: Dissolve PGE_2 powder (Sigma, cat. no. P-0409) at a concentration of 1 mg/ml in 100% ethanol. Store in aliquots up to 2 to 3 years at 4°C, protected from light.

continued

50 μg/ml TNF-α: Reconstitute lyophilized TNF-α (R&D Systems) in PBS/5% albumin (see recipe) at a concentration of 50 μg/ml. Store frozen at −20° to −80°C in 20-μl aliquots.

Prepare 100× MCM mimic as follows:

Combine 200 μl of 1 mg/ml PGE_2 stock solution with 1560 μl of PBS/5% albumin (see recipe) in a 1.8-ml microcentrifuge tube. Mix thoroughly (the ethanol in the PGE_2 solution will cause some of the albumin to precipitate, but this goes back into solution after thorough mixing). Use this solution to rinse out a tube containing a thawed 20-μl aliquot of 50 μg/ml TNF-α stock solution, then use the resulting solution to rinse out a tube containing a thawed 20-μl aliquot of 50 μg/ml IL-1β, and then use this solution to rinse out a tube containing a 200-μl aliquot of 150 μg/ml IL-6 stock solution, finally pooling the solution back into the original 1.8-ml microcentrifuge tube.

The final volume should be 2 ml. 100× MCM mimic contains 0.5 μg/ml TNF-α, 0.5 μg/ml IL-1β, 15 μg/ml IL-6 and 100 μg/ml PGE_2. It can be prepared ahead of time and frozen in aliquots at −20° to −80°C if desired.

PBS/5% albumin

Mix 40 ml phosphate-buffered saline (PBS; *APPENDIX 2A*) with 10 ml of 25% (w/v) human (or bovine) albumin. Filter sterilize and store up to 1 month at 4°C.

RPMI-10

450 ml RPMI 1640 medium with L-glutamine (e.g., BioWhittaker)
50 ml heat-inactivated human AB serum
5 ml 1 M HEPES buffered saline (e.g., BioWhittaker)
1 ml 10 mg/ml gentamicin
Filter sterilize
Store up to 1 month at 4°C.

COMMENTARY

Background Information

Dendritic cells (DCs) are a heterogeneous population of lineage-negative, HLA-DR^+ mononuclear cells that play a central role in the induction and control of immune responses (Banchereau et al., 2000; Shortman and Liu, 2002). Depending on how they are manipulated, DCs can be used either to stimulate T cell–mediated immunity or to induce immune tolerance, both in vitro and in vivo (Steinman et al., 2003; Ardavin et al., 2004; O'Neill et al., 2004).

In the peripheral blood, DCs can be classified into two major populations by staining with antibodies to CD11c and CD123 (the IL-3 receptor α chain): $CD11c^+CD123^{lo}$ "myeloid" DCs and $CD11c^-CD123^{hi}$ "plasmacytoid" DCs. Three general methods are used to prepare DCs from human blood. First, as described in *UNIT 7.32*, both myeloid DCs and plasmacytoid DCs may be directly purified from the blood by density gradient centrifugation (Hsu et al., 1996), fluorescence activated cell sorting (Fonteneau et al., 2003), or immunomagnetic beads (Penna et al., 2001). The major drawback of these approaches is that it is difficult and often expensive to obtain the DCs in large numbers, since DCs as a whole represent only about 0.1% of peripheral blood mononuclear cells.

Myeloid DCs may be generated in larger numbers by differentiating them from peripheral blood precursors—either $CD34^+$ hematopoietic progenitor cells (Caux et al., 1992; Banchereau et al., 2001) or $CD14^+$ monocytes (Bender et al., 1996). $CD34^+$ hematopoietic progenitor cells are difficult to obtain in adequate numbers without stimulating blood donors with GM-CSF, whereas monocytes are plentiful in the peripheral blood and can be easily differentiated into large numbers of immature myeloid DCs by culturing in the presence of IL-4 and GM-CSF, as outlined here (also see *UNIT 7.32*, Alternate Protocol).

It is often desirable to mature the DCs prior to use. Maturation is a terminal differentiation process that transforms DCs from cells specialized for antigen capture into cells

specialized for lymphocyte stimulation. DC maturation is induced by components of pathogens or by host molecules associated with inflammation or tissue injury—stimuli often collectively referred to as "danger signals" (Matzinger, 2002). Mature DCs develop characteristic cytoplasmic extensions or "veils" (Fig. 22F.4.1), up-regulate adhesion and costimulatory molecules involved in the formation of the immunological synapse, and produce cytokines and chemokines that promote lymphocyte activation. The up-regulation of CD83, a molecule thought to be involved in DC-DC interactions and $CD4^+$ T cell development, is often used as a marker of DC maturation (Lechmann et al., 2002).

In the protocol described here, the DCs are matured with a commonly used "cocktail" of three proinflammatory cytokines (IL-1β, IL-6, and TNF-α) and prostaglandin E_2 (Jonuleit et al., 1997; Lee et al., 2002). There are many other potential stimuli that may be used, however (Banchereau et al., 2000; O'Neill et al., 2004), including monocyte conditioned medium, bacterial lipopolysaccharide, CD40 ligand, Toll-like receptor agonists such as bacterial CpG motif DNA (which matures plasmacytoid DCs), and synthetic antiviral compounds like Imiquimod (which matures both plasmacytoid and myeloid DCs). It is important to note that different maturation stimuli can produce DCs with significant phenotypic and functional differences (Lanzavecchia and Sallusto, 2001).

For lymphocyte stimulation experiments, DCs may be loaded with many different sources of antigens. Examples include MHC-restricted peptides (Dhodapkar et al., 1999), purified or recombinant proteins (Timmerman et al., 2002), immune complexes (Rafiq et al., 2002), killed cells or cell lysates (Albert et al., 1998), nonreplicating recombinant viral vectors (Engelmayer et al., 2001), plasmid DNA (Larregina et al., 2004), or in vitro transcribed RNA (Nair et al., 2000). In general, antigens or their precursors are loaded prior to maturation, although some antigen sources such as peptides and RNA seem to work as well or better when loaded onto mature DCs.

Critical Parameters

The in vitro differentiation of monocytes into DCs is entirely driven by cytokines, so it is important that the cytokine preparations used be kept potent. In this protocol, this is done by using cytokine solutions that contain 5% albumin, which acts to stabilize proteins in solution. Stock solutions of cytokines are frozen in aliquots and kept at −80°C, and working solutions (which are stored refrigerated) are kept as concentrated as possible and used for only 1 to 2 weeks.

There are no other specific requirements for the culture medium or its supplements—tissue culture media other than RPMI have been used successfully by many laboratories, and the media can be supplemented with serum or plasma at concentrations ranging from 1% to 10%. Serum-free media such as AIM-V (Invitrogen) or X-VIVO 15 (Bio-Whittaker) may also be used. PBMCs may be obtained from whole blood, buffy coats, or leukapheresis products (also see *UNIT 7.1*). PBMCs derived from leukapheresis products may be frozen and thawed before use.

Troubleshooting

If lower than expected cell yields are obtained, the potency of the IL-4 and GM-CSF should be tested, or new aliquots of these cytokines should be used. Control cultures that lack one of the cytokines can be used to demonstrate the effect of loss of activity on cell yield, morphology, and phenotype. In the absence of GM-CSF, essentially all of the cells will die by Day 5, whereas in the absence of IL-4 almost all of the Day 5 cells will be firmly adherent, with a "fried egg" appearance. In the case of inadequate maturation, the potency of the maturation cytokines and PGE_2 can also be tested by maturing the DCs in the presence of any of these components individually (each on its own should induce at least some of the cells to express CD83).

Anticipated Results

On Day 5, one should expect a viable cell yield of 10% to 15% relative to the number of PBMCs plated on Day 0 (each 10-cm tissue culture dish should yield $5–6 \times 10^6$ cells). About a third of the cells will die between Day 5 and Day 7, so on Day 7 there should be $\sim 1 \times 10^6$ cells per well in each 6-well plate. The overall yield of mature DCs for the procedure should be between 5% and 10% relative to the number of PBMCs plated on Day 0. By flow cytometry, the DCs should have high-level expression of HLA-DR, CD80, CD86, and CD83, and should stain negatively for CD14. The cells should stain positively for CCR7 and should migrate in a CCL19 chemokine gradient in transwell migration assays (Lee et al., 2002; Scandella et al., 2002). Less than 10% of the cells should be lymphocytes, which are about one-third the size of a DC.

Time Considerations

The protocol spans 8 days from start to finish, although most of the time is for cell incubation. The amount of hands-on time will vary depending on the number of cells prepared. The following estimates are for preparations using up to 500×10^6 PBMCs (10 tissue culture dishes). On Day 0, allow 2 to 3 hr to plate the PBMCs, incubate, wash, and prepare and add medium with cytokines. On Day 2 or 3, allow at least 30 min to assess cell morphology and to prepare and add 10× medium with cytokines. On Day 5, allow 1 to 2 hr to harvest the nonadherent cells and to replate into 6-well plates in medium with cytokines. On Day 6, allow at least 1 hr to prepare and add 100× MCM mimic (allow more time if loading the cells with antigen). On Day 7, allow at least 1 hr to harvest, count, and freeze the mature DCs (as for Day 6, allow more time if loading with antigen).

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