Video Article

Generation of Bone Marrow Derived Murine Dendritic Cells for Use in 2-photon Imaging

Melanie P. Matheu[1], Debasish Sen[1], Michael D Cahalan[1], Ian Parker[2]

[1]Department of Physiology and Biophysics, University of California, Irvine (UCI)

[2]Department of Neurobiology and Behaviour, University of California, Irvine (UCI)

URL: https://www.jove.com/video/773
DOI: doi:10.3791/773

Keywords: Immunology, Issue 17, dendritic cells, mouse, bone marrow, 2-photon imaging, cell culture

Date Published: 7/9/2008

Citation: P. Matheu, M., Sen, D., Cahalan, M.D., Parker, I. Generation of Bone Marrow Derived Murine Dendritic Cells for Use in 2-photon Imaging. *J. Vis. Exp.* (17), e773, doi:10.3791/773 (2008).

Abstract

Several methods for the preparation of murine dendritic cells can be found in the literature. Here, we present a method that produces greater than 85% CD11c high dendritic cells in culture that home to the draining lymph node after subcutaneous injection and present antigen to antigen specific T cells (see video). Additionally, we use Essen Instruments Incucyte to track dendritic cell maturation, where, at day 10, the morphology of the cultured cells is typical of a mature dendritic cell and <85% of cells are CD11chigh. The study of antigen presentation in peripheral lymph nodes by 2-photon imaging revealed that there are three distinct phases of dendritic cell and T cell interaction[1,2]. Phase I consists of brief serial contacts between highly motile antigen specific T cells and antigen carrying dendritic cells[1,2]. Phase two is marked by prolonged contacts between antigen-specific T cell and antigen bearing dendritic cells[1,2]. Finally, phase III is characterized by T cells detaching from dendritic cells, regaining motility and beginning to divide[1,2]. This is one example of the type of antigen-specific interactions that can be analyzed by two-photon imaging of antigen-loaded cell tracker dye-labeled dendritic cells.

Video Link

The video component of this article can be found at https://www.jove.com/video/773/

Protocol

1) Remove both femur bones from one mouse

1. Use dissection scissors to cut away muscle and expose the femur bone above and below the joints (knee and hip). Grasp the center of the femur with dissection tweezers and cut above and below the joints to leave as much of the epiphysis intact as possible.
2. Clean off as much muscle as possible using small dissection scissors. Transfer the femur into a dish of RPMI.
3. All procedures should be carried out in the hood from this point on, using only sterile media, instruments, pipette tips and culture dishes.

2) Sterilize Femur bones:

1. In the hood, transfer bones from the RPMI to a small culture dish filled with 70% ethanol on ice.
2. Allow bones to soak in ethanol for 5 minutes on ice.

3) Collect Bone marrow under sterile conditions:

1. Transfer bones to a small culture dish containing sterile filtered Primary DC culture media.
2. With sterile tweezers, hold the bone over a discard dish or tube, and with sterile scissors, cut each epiphesis (ends of the bone) off. The cut should expose bone marrow which is bright red in the center of the bone.
3. With a sterile syringe (26-28 gauge needle), suck up about 100-200 mL of sterile media.
4. Hold the bone with the sterile tweezers over a fresh dish of sterile media and insert the needle into one side of the bone. Position the tip of the needle near the top of the bone and slowly wash the bone out with the sterile media. If you are in the center of the bone, the needle should slide in easily.
5. The bone marrow washes out, either in small pieces or as a single piece. It should be flushed out of the bone and into the dish of sterile media.
6. Repeat this step as needed to completely wash the marrow out of the bone.
7. When the bone is clean, it will be white and translucent.
8. Repeat this procedure with the other femur bone. Discard each femur bone once it is clean.

4) Re-suspend and Spin down Bone Marrow cells:

1. Transfer cells to a sterile falcon tube.
2. If marrow is still intact, very gently pipette media up and down in the dish, to break up the marrow into a single cell suspension. This may take several minutes and should be done slowly to avoid killing cells by sheer force.

3. Centrifuge cells as you would any other mammalian cells.

5) Cell Lysis:

1. Remove the tube from the centrifuge after the spin is done and pour media off (decant) into a small waste (50 mL falcon tube) in the hood.
2. Resuspend pellet by gently flicking the bottom of the tube.
3. Using sterile filtered water and 10x DPBS or 10x PBS, carry out a water lysis to remove the red blood cells (RBCs) using the following volumes:
   - 900 mL sterile filtered water
   - 100 mL 10x DPBS or 10x PBS
4. Add 100 mL of 10x PBS 5-10 seconds after adding the water. It is very important do this immediately so that only the RBCs are lysed. It is a good idea to have both pipettes filled and ready.
5. Add 5 mL – 10 mL of sterile DC basic media.

6) Count Cells:

1. In the hood, mix the 6 to 11 mL of bone marrow cells by gently swirling the tube with a slight inversion, a 45 degree tilt (not so that the media touches the top of the tube)
2. Remove 10 mL of media into a sterile tube for counting.
3. Re-cap the cells and centrifuge again.

7) Plating Dendritic cells:

1. Cells should be plated at a density of 1 x 106/mL.
2. Remove cells from centrifuge, decant media and re-suspend cells in the appropriate amount of Primary DC Media for plating.
3. Place in tissue culture incubator.

8) Culture Care and Maturation:

Always check cultures before adding more media or using in any experiments. Check the general health of cells and look for potential contamination.

DAY 0: Dendritic cells plated.

DAY 3: Remove 75% of the media and non-adherant cells and add back Primary DC media.

DAY 6: After Initial Cell Plating: Re-plate the cells using the following procedure:

1. Remove media, which should contain some non-adherant DCs at this point, and place in a sterile 50 mL falcon tube, leaving plates very slightly wet (you don't want the adherent cells to dry out)
2. Add 3 mM EDTA in PBS to each plate and allow to sit for 5 minutes.
3. Hold the plate at a 45 degree angle and gently pipette media up and down against the bottom of the plate to gently dislodge non-adherant cells.
4. EDTA/PBS should become thicker indicating cells are being collected from the bottom of the plate.
5. After several minutes of this, cell mixture can be transferred to the 50 mL falcon tube.
6. Spin all cells after performing this procedure for each plate.
7. Count and plate the cells at a density of 1 x 10^6 per plate in Secondary DC Media in new sterile 10 cm culture dishes. Return cells to tissue culture incubator.

DAY 10-11

Mature DCs ready for stimulation/antigen loading.

9) Dendritic Cell Stimulation:

1. Use LPS (lipopolyscaccharide) in combination with desired peptide for activation and peptide loading. Depending on your source of LPS, different stimulation conditions may give better results. Additionally, the desired amount of peptide for DC presentation should be optimized. Stimulation conditions in this protocol were 100 ng/mL LPS and 100 ug/mL OVA (ovalbumin).
2. Load antigen/treat with LPS for 18-24 hours prior to harvest/use.

Dendritic Cell Media: Sterile filter after everything has been added

Please see the discussion section for a review of different dendritic cell culture conditions and for the resulting dendritic cell phenotype. These culture conditions are designed to produce mature dendritic cells that rapidly home to draining lymph nodes, 18-24 hours after subcutaneous injection and present antigen efficiently.

Primary DC Media: Sterile filter after everything has been added

- 500 mL IMDM (remove 55-60 mL for 500mL final volume)
- 50 mL heat inactivated FBS
- 5 mL of 200 mM L-Gln, final concentration of 2 mM
- 100 IU/mL Penicillin and 100 mg/mL streptomycin (5 mL of 10,000 IU/mL Pen and 10,000 mg/mL Strep stock)

- 50 uM B-Me (14.3 M B-Me stock, dilute 1:100 in chemical hood, use 35 ul per 100 mL media for 50 micromolar concentration)
- 20-30 ng/mL GM-CSF
- 100-400 IU/mL IL-4 roughly 10- 40 ng/mL

SecondaryDC media for maturation*

100 ng/mL TNF-alpha (add to Primary DC media)
* *Note*: GM-CSF + IL-4 should produce immature dendritic cells. Using GM-CSF + IL4 (400 IU/ul) + TNF-alpha (100 ug/mL) will create cells that resemble mature monocyte derived dendritic cells. Mature dendritic cells are known not to take up antigen as efficiently as immature dendritic cells and cultures may have endogenous TNF-a released from stromal cells[4].

Discussion

Dendritic cells are key mediators of the adaptive immune response and the most efficient antigen presenting cell characterized to date. Methods for human and mouse dendritic cell culture in the literature differ in the type of cytokines used to influence the development of different dendritic cell types. Notably, GM-CSF, flt3L, IL4, IL13, TNF-alpha and IFN-gamma are used in different combinations to produce mature, immature, inflammatory and steady-state like murine bone marrow derived dendritic cells in vitro[3-7]. Here, we present a simple method for the production of mature murine dendritic cells that are capable of homing to draining lymph nodes after subcutaneous injection, presenting antigen and activating na ve T cells. The resulting dendritic cell population is typically>85% CD11chigh with inducible expression of CD80/86 upon LPS activation. The addition of TNF-alpha to the culture on day 7 is optional and produces a more mature phenotype8. The maintenance of *in vitro* Langerhans dendritic cell cultures, TNF-alpha is an important survival factor but does not stimulate the cells to mature[9]. It should be noted that TNF-alpha from endogenous sources (likely stromal cells) has been reported to be present in the culture media[7]. Other successful methods for imaging subcutaneously injected dendritic cells include positive selection for CD11c+ cells from the spleen as well as concurrent labeling and activation of the endogenous dendritic cell population1, [2, 10]. Production of CD11chigh dendritic cells from bone marrow is a robust method that consistently produces large numbers of dendritic cells capable of antigen presentation *in vivo*, important in imaging the process of adaptive immune system activation[11, 12].

Acknowledgements

National Institutes of Health Kirchstein Fellowship predoctoral fellowship AI-64128 (MPM), GM-41514 (MDC), GM-48071 (I.P.)

References

1. Miller, M. J., Safrina, O., Parker, I. & Cahalan, M. D. Imaging the single cell dynamics of CD4+ T cell activation by dendritic cells in lymph nodes. J Exp Med 200, 847-56 (2004).

2. Henrickson, S. E. et al. T cell sensing of antigen dose governs interactive behavior with dendritic cells and sets a threshold for T cell activation. Nat Immunol 9, 282-91 (2008).

3. Jefford, M. et al. Functional comparison of DCs generated in vivo with Flt3 ligand or in vitro from blood monocytes: differential regulation of function by specific classes of physiologic stimuli. Blood 102, 1753-63 (2003).

4. Inaba, K. et al. Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor. J Exp Med 176, 1693-702 (1992).

5. Lutz, M. B. et al. Differential functions of IL-4 receptor types I and II for dendritic cell maturation and IL-12 production and their dependency on GM-CSF. J Immunol 169, 3574-80 (2002).

6. Xu, Y., Zhan, Y., Lew, A. M., Naik, S. H. & Kershaw, M. H. Differential development of murine dendritic cells by GM-CSF versus Flt3 ligand has implications for inflammation and trafficking. J Immunol 179, 7577-84 (2007).

7. Inaba, K., Swiggard, W. J., Steinman, R. M., Romani, N. & Schuler, G. Isolation of dendritic cells. Curr Protoc Immunol Chapter 3, Unit 3 7 (2001).

8. Winzler, C. et al. Maturation stages of mouse dendritic cells in growth factor-dependent long-term cultures. J Exp Med 185, 317-28 (1997).

9. Koch, F. et al. Tumor necrosis factor alpha maintains the viability of murine epidermal Langerhans cells in culture, but in contrast to granulocyte/macrophage colony-stimulating factor, without inducing their functional maturation. J Exp Med 171, 159-71 (1990).

10. Miller, M. J., Hejazi, A. S., Wei, S. H., Cahalan, M. D. & Parker, I. T cell repertoire scanning is promoted by dynamic dendritic cell behavior and random T cell motility in the lymph node. Proc Natl Acad Sci U S A 101, 998-1003 (2004).

11. Wei, S. H. et al. Ca2+ signals in CD4+ T cells during early contacts with antigen-bearing dendritic cells in lymph node. J Immunol 179, 1586-94 (2007).

12. Castellino, F. et al. Chemokines enhance immunity by guiding naive CD8+ T cells to sites of CD4+ T cell-dendritic cell interaction. Nature 440, 890-5 (2006).

