

***In vitro* Ag Cross-presentation and *in vivo* Ag Cross-presentation
by Dendritic Cells in the Mouse**

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[Abstract] Antigen cross presentation is important for effective immune responses to tumors and viral infections. Dendritic cells are professional antigen presenting cells and are unique in their ability to cross-present exogenous antigens on MHC class I molecules and activate antigen specific cytotoxic T cells. This protocol describes antigen cross presentation by dendritic cells (DCs) (bone marrow derived DCs and splenic DCs) in an *in vitro* and in an *in vivo* assay system using soluble ovalbumin protein.

Materials and Reagents

1. Anti-Mouse CD45.1-APC (eBiosciences, catalog number: 17-0453-81)
 2. Rat anti-mouse CD8-PerCpCy5.5 (Biolegend, catalog number: 100733)
 3. Anti-mouse CD3-PE (Biolegend, catalog number: 100307)
 4. Anti-mouse CD19-PE (Biolegend, catalog number: 115507)
- Note: The above antibodies have been tested by the authors but may be substituted with antibodies conjugated with other fluorochromes obtained from other manufacturers.*
5. B3Z CD8⁺ T cell hybridoma is a kind gift from Dr. PK Srivastava (University of Connecticut Health Center)
 6. RPMI Medium 1640 + L-Glutamine (Life Technologies, catalog number: 11875-093)
 7. Phosphate buffered saline (PBS) (Life Technologies, Gibco®, catalog number: 10010-023)
 8. Fetal bovine serum (FBS) (Hyclone, catalog number: SH303397.03)
 9. RBC lysis buffer (Sigma-Aldrich, catalog number: R7757)
 10. β-mercaptoethanol (Sigma-Aldrich, catalog number: M7522)
 11. Antibiotic-Pen-Strep (Life Technologies, Gibco®, catalog number: 15140-122)
 12. Nonidet-P40 (NP40) (US Biological, catalog number: N3500)
 13. CPRG Chlorophenol red-D-galactopyranoside (Roche Diagnostics, catalog number: 10884308001)
 14. Endograde ovalbumin (Biovendor, catalog number: 321000)
 15. CFSEcarboxyfluorescein diacetate, succinimidyl ester (Life Technologies, Molecular Probes®, catalog number: C34554)

16. CD8a (Ly-2) microbeads (Miltenyi Biotec, catalog number: 130-049-401)
17. Anti-PE MicroBeads (Miltenyi Biotec, catalog number: 130-048-801)
18. CD11c MicroBeads, mouse (Miltenyi Biotec, catalog number: 130-052-001)
19. Recombinant GM-CSF (Pierce Biotechnology, catalog number: RMGMCSF20)
20. 16% paraformaldehyde (Electron Microscopy Sciences, catalog number: 15710)
21. L-glutamine
22. BMDC medium (see Recipes)
23. Soluble ovalbumin protein solution (see Recipes)
24. FACS buffer (see Recipes)
25. MACS buffer (see Recipes)
26. CPRG solution (see Recipes)
27. CPRG lysis solution (see Recipes)
28. CFSE dye solution (see Recipes)

Equipment

1. LSR II flow cytometer (Becton-Dickinson)
 2. MACS Multi Stand (Miltenyi Biotec, catalog number: 005126)
 3. Microplate reader (Bio-Rad Laboratories)
 4. FlowJo software (Tree Star)
 5. 96-well plates round bottom (Corning Incorporated, catalog number: 3799)
 6. 96-well plates flat bottom (Cell Star, catalog number: 655180)
 7. 100 mm TC-Treated culture dishes (Corning Incorporated, catalog number: 430293)
 8. 40 μ m cell strainer (BD Biosciences, Falcon[®], catalog number: 352340)
 9. FACS tube, polystyrene (BD Biosciences, Falcon[®], catalog number: 352054)
- Note: Perform the entire method with sterile pyrogen free dishes or plates, pipettes, tips, microfuge and conical tubes. Autoclave and filter all the buffers through 0.22 μ m filter.*
10. Incubator
 11. 10 mm culture dish
 12. 5 ml conical tube

Procedure

A. *In vitro* Ag cross-presentation

1. Isolate bone marrow derived dendritic cells (BMDCs) in RPMI Medium 1640 + L-Glutamine + 10% heat inactivated FBS + 50 μ M β -mercaptoethanol +1% Pen-Strep + 20 ng/ml GM-CSF and grow for 5 days at 37 °C in a 5% (v/v) CO₂ incubator. Briefly,

- sacrifice mouse, remove the skin and clean the tissue from the femurs and tibia of freshly prepared mouse hind legs using forceps and scissors. Flush the bone marrow from both ends of the bone with 25 g needle and a 10 cc syringe filled with 1x PBS (pH 7.4) into a 50 ml tube. Centrifuge cells at 300 x g for 5 min at room temperature (RT). Lyse RBCs with 3 ml of RBC lysis buffer for 5 min at RT. Wash cell suspension twice in RPMI Medium 1640 + L-glutamine + 10% heat inactivated FBS + 50 μ M β -mercaptoethanol +1% Pen-Strep by centrifuging at 300 x g for 5 min at RT. Pass cell suspension through 40 μ m cell strainer. Determine the number of cells/ml. Seed 2 x 10⁶ cells in 10 ml BMDC medium (RPMI Medium 1640 + L-Glutamine + 10% heat inactivated FBS + 50 μ M β -mercaptoethanol +1% Pen-Strep) containing 20 ng/ml GM-CSF in a 10 mm tissue culture treated culture dish. Grow the cells at 37 °C under a 5% (v/v) CO₂ for 5 days. Feed the cells at day 3 by adding 10 ml BMDC medium. Extract the DCs by pipetting the non-adherent or loosely adherent cells and transferring to a 50ml tube and centrifuging at 300 x g for 5 min at RT.
2. Isolate CD8⁺ splenic DCs as described (Ghosh *et al.*, 2012). Briefly, isolate total splenocytes by mechanical shear using the frosted surface of glass slides in 10 ml 1xPBS (pH 7.4). Perform RBC lysis with 3 ml of RBC lysis buffer for 5 min at RT. Wash twice with 10 ml 1x PBS (pH 7.4) by centrifuging at 300 x g for 5 min at RT. Pass the cell suspension through 40 μ m cell strainer. Incubate the cell suspension (1 x 10⁷ cells in 0.1 ml MACS buffer) with anti-mouse CD3-PE and CD19-PE Abs (1:100) in MACS buffer (1x PBS, pH 7.2 + 0.5% FBS + 2 mM EDTA) for 30 min at 4 °C in the dark. After washing twice with 3 ml MACS buffer by centrifuging at 300 x g for 10 min at 4 °C, resuspend cells (1 x 10⁷) in 90 μ l MACS buffer. Magnetically label the R-Phycoerythrin (PE)-conjugated CD3⁺ and CD19⁺ cells with anti-PE microbeads (according to manufacturer's instruction using a MACS Multi Stand. Add 10 μ l of anti-PE microbeads and incubate for 15 min at 4 °C in the dark.
 3. Wash cell suspension twice with 3 ml MACS buffer by centrifuging at 300 x g for 10 min at 4 °C.
 4. Resuspend cells in 500 μ l of MACS buffer.
 5. Load the magnetically labeled cells onto a MACS column in the magnetic field of a MACS separator. The magnetically labeled CD3-PE and CD19-PE cells will be retained within the column while the unlabeled cells that are depleted of CD3⁺ and CD19⁺ cells will be in the flow through fraction. Pass the cell suspension through a MACS column twice to completely deplete CD3⁺ T cells and CD19⁺ B cells.
 6. Next, magnetically label the flow-through fraction depleted of CD3⁺ and CD19⁺ cells with CD11c and CD8 microbeads according to manufacturer's instruction, by magnetic separation using a MACS Multi Stand. Resuspend cells in 80 μ l MACS buffer. Add 10 μ l each of CD11c and CD8 microbeads and incubate for 15 min at 4 °C in the dark.

7. Wash cell suspension twice with 3 ml MACS buffer by centrifuging at 300 x g for 10 min at 4 °C.
8. Resuspend cells in 500 µl of MACS buffer.
9. Load the magnetically labeled cells onto a MACS column in the magnetic field of a MACS separator. The magnetically labeled CD11c⁺ CD8⁺ cells will be retained within the column while the unlabeled cells will be in the flow through fraction. Remove the column from the magnetic field. Add 5 ml MACS buffer onto the column and place in a 5 ml conical tube. Elute the magnetically labeled CD8⁺ CD11c⁺ cells as positively selected fraction by flushing the column with a plunger. Increase the purity of the cell population by passing the cells over a second MACS column. Verify the purity of the cell population by flow cytometry.
10. Seed 4 x 10⁴ BMDCs or CD8⁺ CD11c⁺ splenic DCs in RPMI + 10% FBS on round-bottomed 96-well plates - 50 µl/well.
11. Add 1 x 10⁵ B3Z cells in RPMI + 10% FBS - 50 µl/well.
 B3Z is a CD8⁺ T-cell hybridoma that specifically recognizes OVA residues 257–264 (SIINFEKL) presented on the murine Kb MHC class I molecule (Karttunen *et al.*, 1992) containing the LacZ reporter gene driven by transcriptional control of the nuclear factor of activated T cells (NFAT) element of the human IL2 enhancer. The presentation of the SIINFEKL epitope (SL8) to B3Z cells activates NFAT and results in the induction of β-gal synthesis by B3Z. The amount of β-gal produced can be measured by the hydrolysis of the chromogenic substrate CPRG and is an indication of the amount of SL8/Kb complexes presented on the surface of antigen presenting cells. The advantage of using the B3Z is that the LacZ assay is sensitive enough to detect antigen, expressing APCs at a low frequency and these cells are also easy to maintain in cultures. Culture these cells at 37 °C (5% CO₂) in DMEM with 10% heat-inactivated fetal calf serum and supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin sulfate, 0.05 mM MEM nonessential amino acids, and 1 mM sodium pyruvate. Add varying concentrations of endofree ovalbumin protein (0.625-1 mg/ml) in 1x PBS (pH 7.4).
12. Incubate at 37 °C for 18-24 h in a 5% (v/v) CO₂ incubator.
13. Next day, spin the plate at 500 x g for 2 min at RT, gently aspirate the medium and wash the cell cultures twice with 100 µl of 1x PBS. The cells are non-adherent or loosely adherent so centrifuging the plate is necessary to avoid loss of cells during the washing step.
14. Add 100 µl of 0.15 mM of CPRG solution in CPRG lysis buffer (0.5% NP-40 in 1x PBS).
15. Incubate at RT in the dark for 6-24 h.
16. Transfer the contents to flat bottom 96-well plate by pipetting. In this step centrifuging the plate is not necessary as the cells are already lysed.

17. Avoid the bubbles present in the wells while transferring the contents.
18. Read the absorption at 570 nm, with 650 nm as the reference wavelength in a microplate reader. BMDCs incubated with B3Z but without the antigen serve as background control.

B. *In vivo* Ag cross-presentation

1. Harvest the spleen and lymph nodes (inguinal and mesenteric) from Rag^{-/-} CD45.1 OT-1 mice and isolate single cell suspension by mechanical shear using the frosted surface of glass slides in MACS buffer (1x PBS + 0.5% FBS + 2 mM EDTA).
2. Lyse RBCs (for spleen only as processing the lymph node does not require lysis of RBCs) with 3 ml of RBC lysis buffer for 5 min at RT.
3. Wash cell suspension twice in 1x PBS by centrifuging at 300 x g for 5 min at RT.
4. Pass cell suspension through 40 µm cell strainer and wash twice with 3 ml MACS buffer by centrifuging at 300 x g for 5 min at RT.
5. Determine cell number.
6. Resuspend 1 x 10⁷ cells in 90 µl MACS buffer.
7. Add 10 µl of CD8a microbeads per 1 x 10⁷ cells in 90 µl MACS buffer and incubate for 15 min at 4 °C in the dark. Magnetically label the CD8⁺ cells with CD8 microbeads (the microbeads are not conjugated to any fluorophore).
8. Wash cell suspension twice with 3 ml MACS buffer by centrifuging at 300 x g for 10 min at 4 °C.
9. Resuspend cells in 500 µl of MACS buffer.
10. Subject to magnetic separation using MACS column according to the manufacturer's instruction using a MACS Multi Stand. Load the magnetically labeled CD8⁺ cells onto a MACS column in the magnetic field of a MACS separator. The magnetically labeled CD8⁺ cells will be retained within the column while the unlabeled cells will be in the flow through fraction. Remove the column from the magnetic field. Add 5 ml MACS buffer onto the column and place in a 5 ml conical tube. Elute the magnetically labeled CD8⁺ cells as positively selected fraction by flushing the column with a plunger. Increase the purity of the CD8⁺ cell population by passing the cells over a second MACS column.
11. Resuspend 1 x 10⁷ cells/ml of CD8⁺ T lymphocytes and label with 10 µM of CFSE proliferative dye for 10 min at room temperature in the dark (add 2 µl of 5 mM CFSE dye to 10⁷ cells in 1 ml 1x PBS).
12. Wash cells once with 10 ml 1x PBS and resuspend in sterile 1x PBS to a concentration of 1 x 10⁷ cells/ml.
13. On day 0, adoptively transfer 1 x 10⁶ CFSE labeled CD8⁺ T lymphocytes to mice (0.1 ml of 1 x 10⁷ cells/ml) intravenously.

14. On day 1, after 24 h, challenge mice with 100 µg of endofree soluble ovalbumin protein/mice in 0.1 ml of 1x PBS intradermally by injecting into the right flank of the mouse.
15. After 3 days, harvest spleen and lymph nodes (inguinal and mesenteric) by mechanical shear as in step 1. Briefly, isolate total splenocytes by mechanical shear using the frosted surface of glass slides in 10 ml 1x PBS (pH 7.4). Perform RBC lysis with 3 ml of RBC lysis buffer for 5 min at RT. Wash twice with 10 ml 1x PBS (pH 7.5) by centrifuging at 300 x g for 5 min at RT.

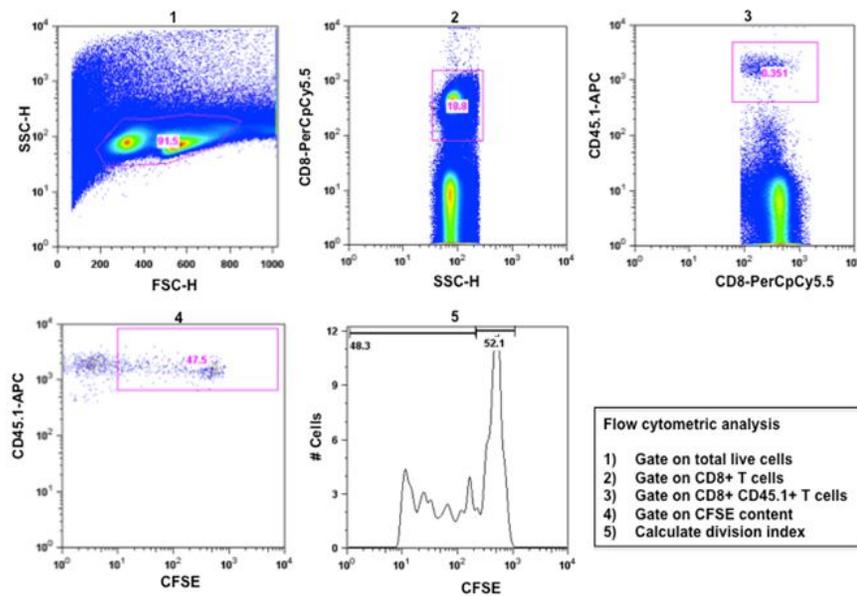


Figure 1. Activation of CD8⁺ T lymphocyte activation by *in vivo* cross presentation of soluble OVA antigen. CFSE-labeled CD45.1⁺ OT-I cells were intravenously injected into mice, followed by intradermal administration of soluble OVA after 24 h. 3 days later splenic lymphocytes were isolated and stained with anti-CD8-PerCpCy5.5 and anti-CD45.1-APC antibodies and OT-I cell proliferation was analyzed by flow cytometry. 1. Total splenocytes were gated on live cell population. 2. Total live cell population was gated on CD8⁺ T- cells. 3. Total CD8⁺ T-cell population was gated on CD8⁺ CD45.1⁺ T-cells. 4. CFSE content of CD8⁺ CD45.1⁺ OT-1 cells was determined as a measure of T-cell proliferation. 5. Number of cell division in the CD8⁺ CD45.1⁺ T-cell population indicated by the division index was determined by FlowJo software.

16. Lyse RBCs (for spleen only as processing the lymph node does not require lysis of RBCs) as in step 2 and isolate single cell suspension in 5 ml FACS buffer (1x PBS+ 2% heat inactivated FBS).
17. Pass cells suspension through 40 µm filter.
18. Determine cell number.
19. Immunostain 1 x 10⁶ cells with anti-CD45.1-APC (1:100) and anti-CD8-PerCpCy5.5 (1:100) antibodies in 0.1 ml FACS buffer for 30 min at 4 °C in the dark in FACS tubes.
20. Wash cells thrice with 3ml FACS buffer by centrifuging at 300 x g for 5 min at RT.
21. Fix cells with 0.5 ml 2% Paraformaldehyde in 1x PBS (pH 7.4) for 30 min at 4 °C.
22. Wash cells thrice with 3 ml FACS buffer by centrifuging at 300 x g for 5 min at RT.
23. Resuspend cells in 0.5 ml of FACS buffer.
24. Analyze cells by flow cytometry in LSRII for CFSE content (488 nm wavelength) in CD8⁺ CD45.1+ T-lymphocytes.
25. Calculate the division index as the average number of divisions for all the cells in the original population using FlowJo software.
26. Flow cytometric analysis.

Recipes

1. BMDC medium (500 ml)
 - 445 ml RPMI Medium 1640 + L-Glutamine
 - 50 ml heat inactivated FBS
 - 2 µl of 14 M β-mercaptoethanol
 - 5 ml of 100% Pen-Strep
 - 20 µl of 10 µg/ml GM-CSF [Reconstitute 10 µg of GM-CSF in 1 ml 1x PBS (pH7.4) Store at -80 °C in aliquots]
2. Soluble ovalbumin protein solution (1 ml)
 - 10 mg of ovalbumin
 - 1 ml 1x PBS (pH 7.4)
3. FACS buffer (500 ml)
 - 490 ml 1x PBS (pH 7.4)
 - 10 ml 100% Heat-inactivated FBS
4. MACS buffer (500 ml)
 - 495.5 ml 1x PBS (pH 7.2)
 - 2.5 ml 100% Heat-inactivated FBS
 - 2 ml 500 mM EDTA (pH 8.0)

(FBS can be replaced with 0.5% BSA. Addition of EDTA prevents cell clumping)

5. CPRG solution (500 ml)
 - 45.5 mg CPRG powder
 - 500 ml CPRG lysis solution
6. CPRG lysis solution (500 ml)
 - 2.5 ml 100% NP-40
 - 498 ml 1x PBS
7. CFSE dye solution (18 μ l of 5 mM stock solution) (prepare according to manufacturer's instruction)
 - 50 μ g of CFSE
 - 18 μ l DMSO (supplied by the manufacturer)

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References

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