

## Evaluation of Cross-presentation in Bone Marrow-derived Dendritic Cells *in vitro* and Splenic Dendritic Cells *ex vivo* Using Antigen-coated Beads

Andrés Alloatti<sup>1, \*</sup>, Fiorella Kotsias<sup>1, 2</sup>, Eik Hoffmann<sup>1, 3, 4</sup>, and Sebastian Amigorena<sup>1</sup>

<sup>1</sup>Institute Curie, PSL Research University, INSERM U932, Paris, France; <sup>2</sup>Department of Virology, University of Buenos Aires, Buenos Aires, Argentina; <sup>3</sup>VIB Inflammation Research Center, Unit of Molecular Signal Transduction in Inflammation, Ghent, Belgium; <sup>4</sup>Department of Biomedical Molecular Biology, Ghent University, Ghent, Belgium

\*For correspondence: [andres.alloatti@curie.fr](mailto:andres.alloatti@curie.fr)

**[Abstract]** Antigen presentation by MHC class I molecules, also referred to as cross-presentation, elicits cytotoxic immune responses. In particular, dendritic cells (DC) are the most proficient cross-presenting cells, since they have developed unique means to control phagocytic and degradative pathways.

This protocol allows the evaluation of antigen cross-presentation both *in vitro* (by using bone marrow-derived DC) and *ex vivo* (by purifying CD8<sup>+</sup> DC from spleen after incorporation of particulate antigen) using ovalbumin (OVA)-coupled particles. Cross-presentation efficiency is measured by three different readouts: the B3Z hybridoma T cell line (Karttunen *et al.*, 1992) and stimulation of antigen-specific CD8<sup>+</sup> T cells (OT-I) (Kurts *et al.*, 1996), either analyzing OT-I activation by CD69 expression or OT-I proliferation after labeling them with carboxyfluorescein succinimidyl ester (CFSE). By using this approach, we could show recently that DCs are able to increase cross-presentation efficiency transiently upon engagement of TLR4 (Alloatti *et al.*, 2015).

**[Background]** In mouse, antigen-presenting cells (APC) are able to take up exogenous antigens to process them and to load peptides derived from such exogenous antigens onto major histocompatibility complex (MHC) class I molecules. Peptide-MHC I complexes are subsequently transported to the plasma membrane, where they might be presented to CD8<sup>+</sup> T cells thereby promoting T cell activation, a process referred to as cross-presentation (Joffre *et al.*, 2012). Among the different APC, dendritic cells (DC) excel at cross-presentation and comprise of different subpopulations expressing the XCR1 marker, which have been shown to cross-present antigens very efficiently (*i.e.*, CD8<sup>+</sup> resident DC from spleen and CD103<sup>+</sup> migratory DC from skin and lung) (Dorner *et al.*, 2009; Crozat *et al.*, 2011). While the purification of DC residing in spleen or migratory DC is feasible, it is laborious and expensive. In order to study the cell biology of DC, primary cultures of bone marrow-derived DC (BMDC) can be easily differentiated from myeloid progenitors by culturing them with GM-CSF. Even though BMDC cannot be associated with any particular DC subtype (perhaps inflammatory DC), they constitute a valuable tool to study the main characteristics of DC cell biology. Herein, we introduce a detailed protocol to analyze cross-presentation of particulate antigen by BMDC, but also by CD8<sup>+</sup> splenic DC. Although previous protocols included different antigen forms and read-outs, the protocol described here aims to analyze cross-presentation in a comprehensive and concise way in different DC types.

## **Materials and Reagents**

1. 2 ml Eppendorf tubes
2. 15 ml centrifuge tubes
3. 50 ml centrifuge tubes
4. 14 ml tubes
5. Fisherbrand™ cell strainer (Thermo Fisher Scientific, Fisher Scientific, catalog number: 22-363-548)
6. Pre-separation filters (30 µm) (Miltenyi Biotec, catalog number: 130-041-407)
7. 1 ml insulin syringes (Terumo Medical, catalog number: SS+01H1)
8. 2.5 ml syringes
9. 25 G needles (Terumo, catalog number: AN\*2516R1)
10. Non-treated 96-well plates (Corning, Falcon®, round bottom, catalog number: 351177)
11. Non-treated 6-well plates (Sigma-Aldrich, catalog number: M8562-100EA)
12. Non-treated Petri dish, 145 x 20 mm (Greiner Bio One, catalog number: 639161)
13. Mice: C57BL/6 and C57BL/6 recombination activating gene 1-deficient OT-I TCR (Vα<sub>2</sub>, Vβ<sub>5</sub>) transgenic mice were obtained from Charles River Laboratories (CDTA, Orleans, France)
14. B3Z T cell line (a Kb-restricted, OVA-specific CD8<sup>+</sup> T cell hybridoma) (Kurts *et al.*, 1996)
15. Low endotoxin OVA (50 mg/ml stock) (Worthington Biochemical, catalog number: LS003062)
16. OVA peptide 257-264 (SIINFEKL) (Polypeptide, catalog number: SC1302)
17. Polybead® polystyrene 3.0 micron microspheres (Polysciences, catalog number: 17134)
18. Polybead® dyed blue 1.0 micron microspheres (Polysciences, catalog number: 15712)
19. PBS (1x, pH 7.4) (Thermo Fisher Scientific, Gibco™, catalog number: 10010-023)
20. Glycine (Sigma-Aldrich, catalog number: 50046)
21. Glutaraldehyde (25%) (Sigma-Aldrich, catalog number: G5882)
22. Iscove's modified Dulbecco's medium (IMDM) (Sigma-Aldrich, catalog number: I3390-500ML)
23. Penicillin-streptomycin (10,000 U/ml) (Thermo Fisher Scientific, Gibco™, catalog number: 15140122)
24. RPMI with GlutaMAX™ (Thermo Fisher Scientific, catalog number: 61870-010)
25. GlutaMAX™ supplement (100x) (Thermo Fisher Scientific, Gibco™, catalog number: 35050061)
26. β-mercaptoethanol (Thermo Fisher Scientific, Gibco™, catalog number: 21985-023)
27. MEM non-essential amino acids solution (100x) (Thermo Fisher Scientific, Gibco™, catalog number: 11140050)
28. Sodium pyruvate (100 mM) (Thermo Fisher Scientific, Gibco™, catalog number: 11360070)
29. CPRG (Roche Diagnostics, catalog number: 10884308001)
30. Fixable viability dye eFluor 780 (dilution 1/10,000) (Affymetrix, eBioscience, catalog number: 65-0865-14)
31. Low endotoxin fetal bovine serum (FBS, heat-inactivated for 20 min at 56 °C) (Biowest, catalog number: S1860)

32. Low endotoxin BSA (fraction V) (Euromedex, catalog number: UA1315)
33. CFSE (Thermo Fisher Scientific, Molecular Probes™, catalog number: C34554)
34. Liberase TM (Roche Diagnostics, catalog number: 05401119001)
35. DNase I (Roche Diagnostic, catalog number: 04536282001)
36. Red blood cell lysis buffer (Sigma-Aldrich, catalog number: R7757)
37. EasySep™ Mouse Pan-DC Enrichment Kit (STEMCELL Technologies, catalog number: 19763)
38. EasySep™ Mouse Naïve CD8<sup>+</sup> T Cell Isolation Kit (STEMCELL Technologies, catalog number: 19858)
39. FACS antibodies (all anti-mouse):
  - a. CD69-eFluor® 450 (clone H1.2F3, dilution 1/300) (Affymetrix, eBioscience, catalog number: 48-0691-82)
  - b. CD8a-PerCP-Cy5.5 (clone 53-6.7, dilution 1/300) (Affymetrix, eBioscience, catalog number: 45-0081-82)
  - c. TCR vβ 5.1-PE (clone MR9-4, dilution 1/500) (BD, Pharmingen™, catalog number: 553190)
  - d. CD25-FITC (clone 7D4, dilution 1/200) (BD, Pharmingen™, catalog number: 553072)
  - e. CD4-PE-Cy7 (clone RM4-5, dilution 1/300) (BD, Pharmingen™, catalog number: 552775)
  - f. CD25-APC (clone PC61.5, dilution 1/300) (Affymetrix, eBioscience, catalog number: 17-0251-81)
  - g. CD19 eFluor® 450 (clone 1D3, dilution 1/500) (Affymetrix, eBioscience, catalog number: 48-0193)
  - h. CD3 eFluor® 450 (clone 17A2, dilution 1/500) (Affymetrix, eBioscience, catalog number: 48-0032-80)
  - i. CD11c-FITC (clone HL3, dilution 1/500) (BD, Pharmingen™, catalog number: 553801)
  - j. CD11c-APC (clone N418, dilution 1/400) (Affymetrix, eBioscience, catalog number: 17-0114)
  - k. CD8-PE (clone 53-6.7, dilution 1/500) (BD, Pharmingen™, catalog number: 553032)
  - l. CD11b-PE (clone M1/70, dilution 1/300) (Affymetrix, eBioscience, catalog number: 12-0112)
  - m. CD40-PE (clone 3/23, dilution 1/150) (BD, Pharmingen™, catalog number: 553791)
  - n. CD86-PE (clone GL1, dilution 1/300) (Affymetrix, eBioscience, catalog number: 12-0862)
  - o. MHC Class II I-Ab-PE (clone AF6-120.1, dilution 1/300) (Affymetrix, eBioscience, catalog number: 12-5320)
40. BMDC culture medium (see Recipes)
41. B3Z and T cell culture (see Recipes)
42. Digestion medium for spleens (see Recipes)
43. MACS buffer (see Recipes)

## **Equipment**

1. Incubator (37 °C and 5% CO<sub>2</sub>)
2. Refrigerated centrifuge for tubes of 2 ml, 15 ml and 50 ml size as well as 96-well plates

3. Stuart test tube rotator wheel tolerating 4 °C (Bibby Scientific, model: SB3)
4. Multicolor flow cytometer (MAQSquant, Miltenyi; FACSverse, Becton Dickinson or similar)
5. Multicolor FACS sorter (for example, BD, model: FACSAria III)
6. Fluorometric plate reader (OD: 590 nm)

## **Software**

1. FlowJo 10 software (FlowJo, LLC.)
2. Prism 6 software (GraphPad Software, Inc.)

## **Procedure**

### Part I. Cross-presentation analysis of bead-bound OVA in BMDC *in vitro*

1. Preparation of beads (Day: -1, sterile environment)

The day before the cross-presentation experiment, particles are coupled to OVA (bead-bound OVA: bbOVA). Polystyrene beads of 3 µm diameter are coated with soluble OVA and soluble BSA ranging from 100% OVA (most concentrated) to 100% BSA (background). Since BSA is not cross-presented, the preparation of beads coated with 100% BSA is the accurate control to check whether beads without any antigen coating are able to induce any additional activation of DC.

- a. Estimate the amount of 3 µm beads that will be used during the cross-presentation experiment on day 0. Consider to prepare a minimum of at least four different conditions:
  - i. 100% OVA and 0% BSA: 10 mg/ml OVA
  - ii. 50% OVA and 50% BSA: 5 mg/ml OVA + 5 mg/ml BSA
  - iii. 25% OVA and 75% BSA: 2.5 mg/ml OVA + 7.5 mg/ml BSA
  - iv. 0% OVA and 100% BSA: 10 mg/ml BSA

- b. Dilute the beads differentially depending on the readout. For B3Z T cell hybridomas, beads are diluted 1/10 (which corresponds to a bead/cell ratio of approximately 200), while for OT-I T cells the dilution is 1/25 (which corresponds to a bead/cell ratio of 600).

The beads are added to BMDC in a final volume of 100 µl. In order to analyze cross-presentation in 10 samples with the 3 different read-outs of this protocol, you will need per condition:

10 samples for B3Z → 1 ml of culture medium → 100 µl of beads (1/10 dilution)

10 samples for OT-I CD69 expression → 40 µl of beads (1/25 dilution)

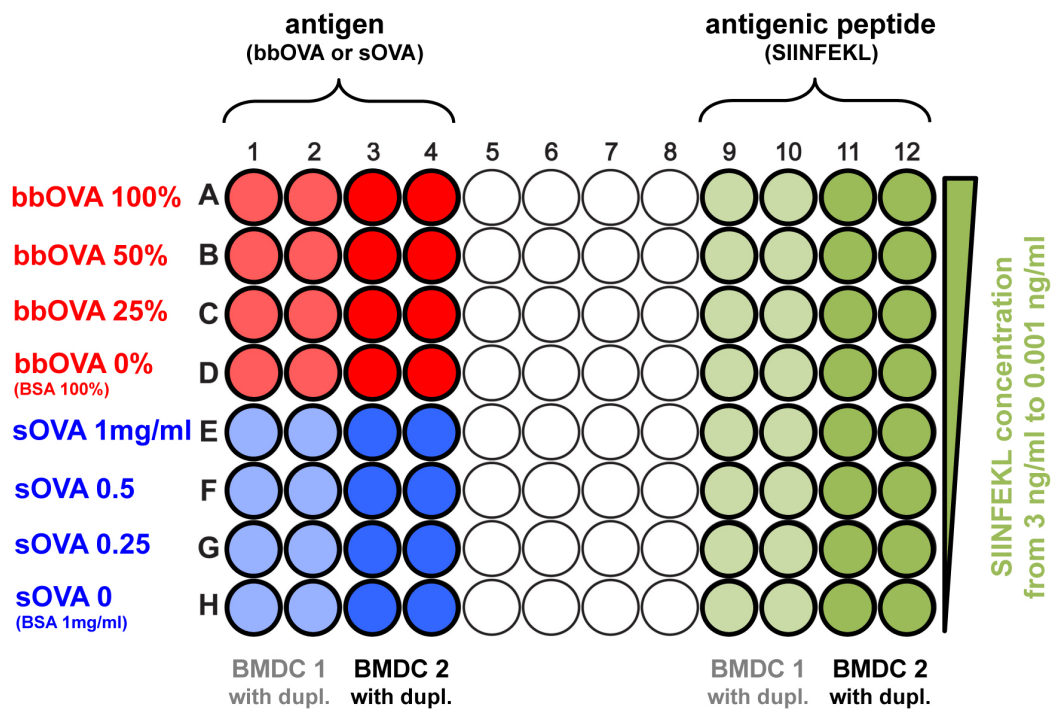
10 samples for OT-I proliferation → 40 µl of beads (1/25 dilution)

Therefore, a final bead volume of 200-250 µl is needed for 10 samples.

- c. Put a maximum of 250 µl of beads per 2 ml Eppendorf tube, and wash them with 1.5 ml of PBS. Centrifuge at 19,000 x g for 3 min at 4 °C. Repeat washing twice.

*Note: If you are assessing 10 samples with the 3 readouts, and doing 4 different conditions of bbOVA (100%, 50%, 25% and 0%) you should have at this point 4 tubes with 200-250  $\mu$ l of beads (1 for each condition).*

- d. Discard supernatant, and resuspend the beads in the different protein suspensions as shown in the following (250  $\mu$ l of beads per 750  $\mu$ l of soluble protein mix):  
Stock concentration of OVA: 50 mg/ml (Working concentration: 10 mg/ml)  
Stock concentration of BSA: 50 mg/ml (Working concentration: 10 mg/ml)  
Cond. 1) 100% OVA: 0% BSA  $\rightarrow$  150  $\mu$ l of OVA<sub>stock</sub> + 600  $\mu$ l PBS  
Cond. 2) 50% OVA: 50% BSA  $\rightarrow$  75  $\mu$ l of OVA<sub>stock</sub> + 75  $\mu$ l of BSA<sub>stock</sub> + 600  $\mu$ l PBS  
Cond. 3) 25% OVA: 75% BSA  $\rightarrow$  37.5  $\mu$ l of OVA<sub>stock</sub> + 112.5  $\mu$ l of BSA<sub>stock</sub> + 600  $\mu$ l PBS  
Cond. 4) 0% OVA: 100% BSA  $\rightarrow$  150  $\mu$ l of BSA<sub>stock</sub> + 600  $\mu$ l PBS
- e. Incubate overnight at 4 °C with permanent agitation on a rotator wheel.
2. Washing the beads (Day: 0, sterile environment)
  - a. Spin the bbOVA with 19,000 x g for 5 min at 4 °C. Subsequently, discard supernatant with a pipette trying not to touch the beads.
  - b. Resuspend the bbOVA in 1 ml of cold PBS by pipetting. Centrifuge at 19,000 x g for 5 min at 4 °C.
  - c. Repeat washing step twice.
  - d. Resuspend the bbOVA in the original volume (200-250  $\mu$ l in this example). The beads are ready to use. Keep bbOVA on ice.
3. Loading BMDC with bbOVA, Co-culture with T cells (Day: 0, sterile environment)  
Plate in 96-well round bottom plates: 100,000 BMDC per well for B3Z readout and 10,000 BMDC per well for both OT-I readouts (Figure 1).

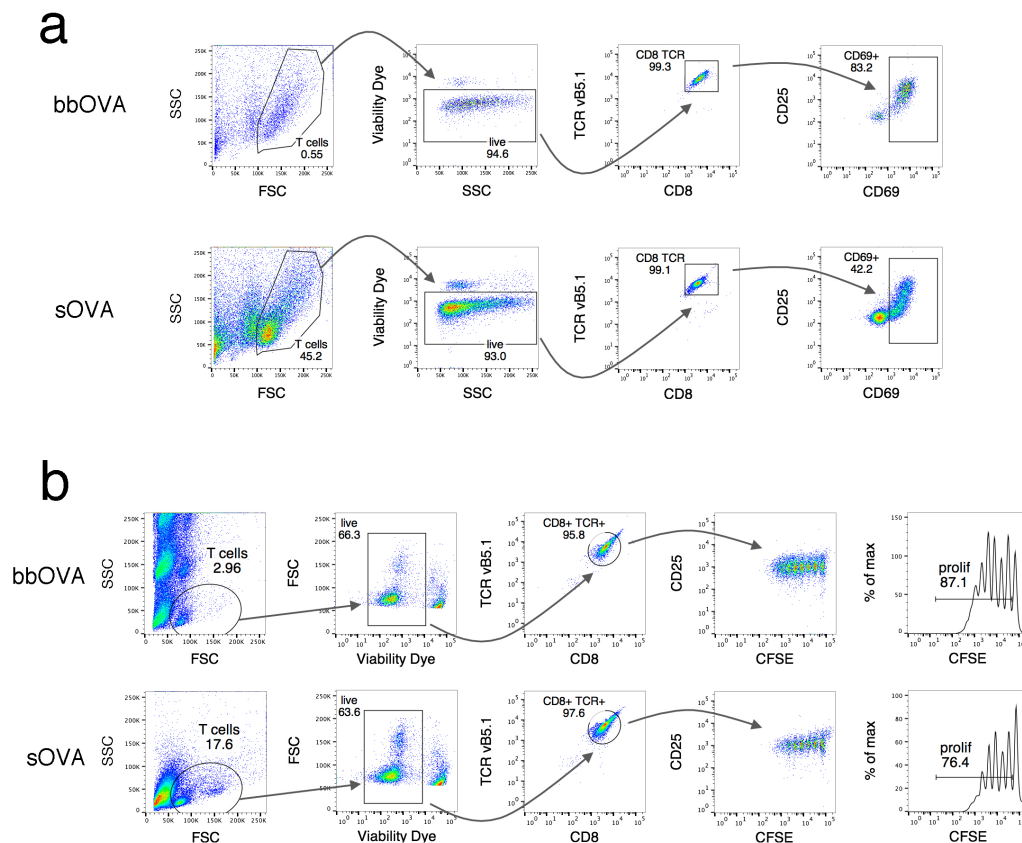


**Figure 1. Scheme of an average 96-well plate loaded with BMDCs.** Plate 100,000 BMDCs/well in 50  $\mu$ l of BMDC culture medium for B3Z readout, or 10,000 cells/well in 50  $\mu$ l of BMDC culture medium for OT-I readouts. Add the antigen to the right concentration in 50  $\mu$ l of BMDC culture medium for a final volume of 100  $\mu$ l. In the scheme, sOVA and SIINFEKL peptide are also analyzed (see Notes 2 and 3). In the scheme, a minimum setup is shown: two different BMDC samples are analyzed, which are plated in duplicates. Additional replicates are suggested to increase robustness of the obtained data.

- The correct amount of BMDC is plated in 50  $\mu$ l of BMDC culture medium per well.
- The final dilution of bbOVA in BMDC culture medium is, as mentioned before: 1/10 for B3Z and 1/25 for OT-I. Please consider that 50  $\mu$ l of bbOVA in BMDC culture medium will be added to 50  $\mu$ l of BMDC for a final volume of 100  $\mu$ l per well. Thereby, bbOVA concentration must be twice the final dilution (1/5 for B3Z, 1/12.5 for OT-I).
- For the OT-I T cell proliferation readout, incubate BMDC with bbOVA for 1 h at 37  $^{\circ}$ C. Consequently, cross-presenting BMDC are co-cultured with 60,000-100,000 CFSE-stained OT-I T cells and T cell proliferation is analyzed 72 h later (continues in step 5a).
- For B3Z and OT-I CD69 expression readouts, incubate BMDC with bbOVA for 4-5 h at 37  $^{\circ}$ C.
- Wash the cross-presenting BMDC with 100  $\mu$ l/well of 0.1% PBS-BSA (always keep reagents and solutions on ice) and centrifuge at 800  $\times$  g for 2 min at 4  $^{\circ}$ C. Flick the plate and repeat washing twice.
- Fix cross-presenting BMDC: add 50  $\mu$ l/well of freshly made PBS-glutaraldehyde (GTA) 0.008% (vol/vol) and incubate for 5 min on ice. Mix cells and PBS-GTA by pipetting.



- g. Add 50 µl of PBS-glycine 0.4 M to the PBS-GTA 0.008% solution, and centrifuge at 800 x g for 2 min at 4 °C. Flick the plate.
  - h. Add 100 µl of PBS-glycine 0.2 M and centrifuge at 800 x g for 2 min at 4 °C. Flick the plate.
  - i. Wash twice with 200 µl/well of B3Z and T cell culture medium (see Recipes). Centrifuge again and flick the plate.
  - j. Resuspend fixed, cross-presenting BMDC in 100 µl/well of cell culture medium.
  - k. Add 60,000-100,000 effector cells (B3Z or OT-I, see Notes 4 and 5) per well in 100 µl cell culture medium (to a final volume of 200 µl).
  - l. Incubate for 16 h at 37 °C for B3Z T cells or for 18-20 h at 37 °C for OT-I T cells.
4. Analysis of B3Z read-out and CD69 expression of OT-I cells (Day: 1)
- a. B3Z measurement:
    - i. Centrifuge at 800 x g for 2 min at 4 °C. Supernatant can be kept for subsequent cytokine analysis.
    - ii. Wash once with 100 µl/well of 0.1% PBS-BSA, spin down and flick.
    - iii. Add 120 µl/well of CPRG (Chlorophenol red-β-D-galactopyranoside). Mix properly to resuspend the B3Z cells.
    - iv. Incubate at 37 °C and check every 30 min until the solution changes its color to orange/red (usually, after 2 h).
    - v. Measure OD at 590 nm every 30-60 min until the reaction gets saturated (after 4-6 h).
  - b. Measurement of CD69 expression of OT-I cells:
    - i. Centrifuge at 800 x g for 2 min at 4 °C. Supernatant can be kept for subsequent cytokine analysis.
    - ii. Wash once with 100 µl/well of 0.1% PBS-BSA, spin down and flick the plate.
    - iii. Stain the cells for 15 min at 4 °C with 100 µl/well of fixable viability dye eFluor 780 + Fc block (1/100) in PBS. Centrifuge and flick the plate.
    - iv. Wash once with 0.1% PBS-BSA, centrifuge and flick the plate.
    - v. Stain the cells for 40 min at 4 °C with 70 µl/well of a combination of CD69-eFluor450, CD8a-PerCP-Cy5.5, TCR vβ 5.1-PE, CD25-FITC and CD4-PE-Cy7 diluted in 1% PBS-BSA (dilution factors are provided in Materials and Reagents). Centrifuge and flick the plate.
    - vi. Wash 3 times with 0.1% PBS-BSA, centrifuge and flick the plate.
    - vii. Resuspend in 100 µl/well of 0.1% PBS-BSA and analyze by flow cytometry (see Figure 2a).



**Figure 2. Gating strategy for cross-presentation analysis.** a. Activation of OT-I T cells by measuring CD69 expression at the cell surface. BMDC were incubated 5 h with bbOVA (upper panel) and sOVA (see Note 2) (lower panel), fixed and co-cultured with OT-I T cells. After 16 h, CD69 expression was measured. b. Proliferation of OT-I T cells. BMDC were incubated for 1 h in the presence of bbOVA (upper panel) and sOVA (lower panel) and co-cultured for 72 h with CFSE-labeled OT-I T cells. Proliferation was addressed by measuring loss of CFSE staining by flow cytometry and by calculating the proliferation index (right panel).

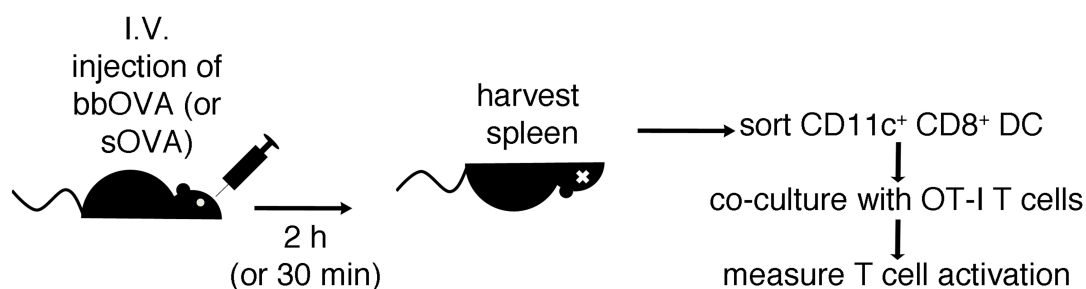
## 5. Analysis of OT-I T cell proliferation (Day: 3)

- Centrifuge at 800 x g for 2 min at 4 °C. Supernatant can be kept for subsequent cytokine analysis.
- Wash once with 100 µl/well of 0.1% PBS-BSA, spin down and flick the plate.
- Stain the cells for 15 min at 4 °C with 100 µl/well of fixable viability dye eFluor 780 + Fc block (1/100) in PBS. Centrifuge and flick the plate.
- Wash once with 0.1% PBS-BSA, centrifuge and flick the plate.
- Stain the cells for 40 min at 4 °C with 70 µl/well of a combination of CD8a-PerCP-Cy5.5, TCR vβ 5.1-PE, CD25-APC and CD4-PE-Cy7 diluted in 1% PBS-BSA (dilution factors are provided in Materials and Reagents). Centrifuge and flick the plate.
- Wash 3 times with 0.1% PBS-BSA, centrifuge and flick the plate.
- Resuspend in 100 µl/well of 0.1% PBS-BSA and analyze by flow cytometry (see Figure 2b).



## Part II. Cross-presentation of bbOVA by splenic CD8<sup>+</sup> dendritic cells, analyzed *ex vivo*

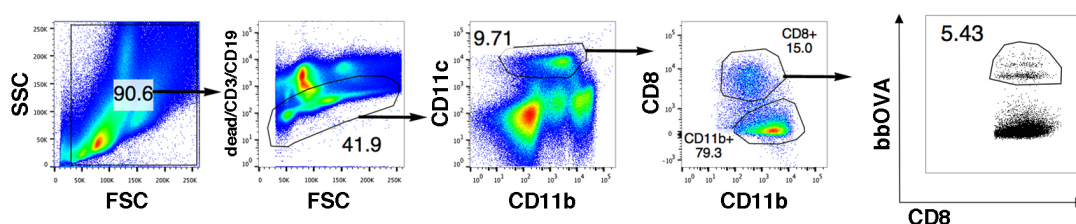
In this protocol, blue dyed beads coated with OVA are injected intravenously into mice. Subsequently, after DC have internalized the particulate antigen and cross-presented it *in vivo*, spleens are harvested and digested. CD11c<sup>+</sup> cells from whole digested spleens are then purified, and the enriched CD11c<sup>+</sup> fraction is stained. CD8<sup>+</sup> DC that have phagocytosed beads are sorted and co-cultured with OT-I T cells for further analysis (Figure 3).



**Figure 3. Workflow of the antigen cross-presentation approach *in vivo***

1. Preparation of beads (Day: -1)
  - a. Calculate the number of blue dyed 1.0  $\mu\text{m}$  beads that will be used.  $4.5 \times 10^9$  beads are injected per mouse (bead stock solution is around  $4.5 \times 10^{10}$  particles/ml). Therefore, 100  $\mu\text{l}$  per mouse are injected.
  - b. Wash the beads with 1.5 ml cold PBS (again 250  $\mu\text{l}$  of beads per 2 ml Eppendorf tube). Centrifuge at  $19,000 \times g$  for 5 min at  $4^\circ\text{C}$ . Repeat.
  - c. Resuspend each 250  $\mu\text{l}$  of beads in 500  $\mu\text{l}$  of OVA 50 mg/ml, and pool them (if needed) into a 15 ml centrifuge tube. Incubate overnight at  $4^\circ\text{C}$  with permanent agitation on a rotator wheel.
2. Washing the beads (Day: 0)
  - a. For washing purposes, divide the beads in 2 ml Eppendorf tubes (250  $\mu\text{l}$  per tube).
  - b. Spin the bbOVA at  $19,000 \times g$  at  $4^\circ\text{C}$  for 5 min. Subsequently, discard supernatant with a pipette, trying not to touch the beads.
  - c. Resuspend the bbOVA in 1.5 ml of cold PBS. Centrifuge at  $19,000 \times g$  at  $4^\circ\text{C}$  for 5 min.
  - d. Repeat washing step twice.
  - e. Resuspend the bbOVA in the original volume (250  $\mu\text{l}$  in this example) and pool them. The beads are ready to use. Store them on ice.
3. Injection of mice, purification of DC and co-culture with T cells (Day: 0) (see Figure 3)
  - a. Inject 100  $\mu\text{l}$  of beads per mouse intravenously (either in the eye vein or in the tail). Use Terumo 1 ml insulin syringes with 25 G needle. Wait 2 h (time for the bbOVA to be phagocytosed and cross-presented).
  - b. Sacrifice the mice and harvest spleens.

- c. Flush the spleens in non-treated 6-well plates with 2 ml of digestion medium (see Recipes) per spleen by using 25 G needles and 2.5 ml syringes. Incubate for 15 min at 37 °C.
- d. Mince the spleens with scalpel blades in the same well and incubate for another 15 min. Stop Liberase/DNase reaction by adding 2 ml of FBS.
- e. Smash and filter the digested spleens through the cell strainer with the flat part of a syringe plunger into 50 ml centrifuge tubes. Centrifuge and discard supernatant.
- f. Lyse red blood cells at RT (5 min of 2 ml of red blood cell lysis buffer per spleen is sufficient). Add 30 ml of PBS and centrifuge (350 x g, 5 min, 4 °C).
- g. Discard supernatant and resuspend each spleen in 600 µl of cold sorting medium (PBS + 0.5 % FBS). Pass the cells through the suggested Miltenyi pre-separation filters (or similar) and collect them in 14 ml tubes for magnetic purification.
- h. In order to isolate CD11c<sup>+</sup> cells, we use the EasySep Mouse Pan-DC Enrichment Kit, but any other negative selection-based enrichment procedure might be used. Follow the manufacturer's indications.
- i. Stain the CD11c<sup>+</sup>-enriched cell fraction for 40 min at 4 °C with 70 µl/well of a combination of CD19-eFluor 450, DAPI, CD3-eFluor 450, CD11c-FITC and CD8-PE (see Note 2).
- j. Sort the cells following the gating strategy shown in Figure 4.
- k. Plate phagocytic CD8<sup>+</sup> DC at different amounts to obtain three different, final DC/T cell ratios:  
30,000 DC/100,000 T cells  
3,000 DC/100,000 T cells  
300 DC/100,000 T cells
- l. Fix CD8<sup>+</sup> DC with 0.008% PBS-GTA as described before (Procedure of part I).
- m. Co-culture fixed DCs with 100,000 OT-I T cells to a final volume of 200 µl of B3Z and T cell culture medium.
- n. For T cell analysis, proceed as described before (T cell read-outs from Procedure of part I).



**Figure 4. Gating strategy to sort CD8<sup>+</sup> DC from spleen.** Total splenic cells were gated in order to exclude B, T and dead cells. Subsequently, gates were performed on CD11c<sup>+</sup> and CD8<sup>+</sup> cells. CD11c<sup>+</sup> CD8<sup>+</sup> cells that have phagocytosed at least one bead were sorted and co-cultured with OT-I T cells.

## **Data analysis**

Each single experiment needs to be performed with a sufficient number of technical replicates (at least in triplicates). Conclusions can be drawn from the analysis of an appropriate number of biological replicates. The results shown here were confirmed by a minimum of 3 independent experiments. Analysis of flow cytometry data was performed using FlowJo 10 software (FlowJo, LLC.). Statistical analysis was performed using Prism 6 software (GraphPad Software, Inc.).

## **Notes**

### Part I. Cross-presentation analysis of bead-bound OVA in BMDC *in vitro*

1. BMDC are generated by culture in GM-CSF-containing medium (see Recipes) for 10 days. Percentages of CD11c and CD11b are always higher than 85% in used cultures.
2. Cross-presentation of soluble OVA (sOVA) can be measured in parallel. In our lab, we generally use sOVA ranging from 2 mg/ml to 0 mg/ml (4 different concentrations are usually sufficient).
3. The best control for cross-presentation, to our knowledge, is the OVA peptide 257-264 (SIINFEKL). This peptide is incorporated into the cell and loaded onto MHC class I molecules without being processed. We strongly recommend using such a control in different dilutions (a range from 3 ng/ml to 0.001 ng/ml is sufficient) for all cross-presentation assays.
4. Culturing B3Z T cells: Grow B3Z T cells in B3Z and T cell culture medium (see Recipes), split cells every 2-3 days; detach the cells manually by hitting smoothly against the flask. The day before the cross-presentation experiment, seed them at a concentration of  $0.1 \times 10^6$  cells/ml. Co-culture them with cross-presenting BMDC. After 16 h, T cell activation is measured detecting  $\beta$ -galactosidase activity by optical density at 590 nm using CPRG as substrate for the reaction.
5. In order to get OT-I T cells for co-culture with BMDC, we use kits for mouse naïve CD8<sup>+</sup> T cell purification (obtained either from Stemcell or Miltenyi) of spleens and lymph nodes of OT-I mice. We use MACS buffer (recipes 4) for washing purposes.
6. To obtain CFSE-labeled OT-I T cells, resuspend  $1 \times 10^7$  cells/ml of CD8<sup>+</sup> T cells and label them with 10  $\mu$ M of CFSE proliferative dye for 10 min at room temperature in the dark. Wash cells once with 10 ml of PBS and resuspend in sterile PBS to a concentration of  $1 \times 10^7$  cells/ml.

### Part II. Cross-presentation of bbOVA by splenic CD8<sup>+</sup> dendritic cells, analyzed *ex vivo*

1. Cross-presentation of sOVA can be measured in parallel. Consider injecting 100  $\mu$ l of OVA (i.v.) at a concentration of 20 mg/ml. Wait for 30 min instead of 2 h and harvest spleens to purify cross-presenting DC.
2. With the labeling of CD19, CD3 and DAPI, it is possible to exclude B, T and dead cells, respectively, from the analysis. Be aware that blue dyed beads are visible in both, the APC and the APC-Cy7 channel. No fluorophore should be used in those channels.

3. Consider that the proportion of phagocytic CD8<sup>+</sup> DC in spleen is really low. You should get around 100,000-200,000 cells per spleen. While this amount is enough for OT-I T cells co-culture (to analyze both OT-I CD69 expression and CFSE OT-I proliferation), co-culture with B3Z T cells might require the sacrifice of many more mice as well as many hours of cell sorting. By the time you will get sufficient cells for B3Z analysis, they might be already dead. Therefore, we suggest to not address this readout for *ex vivo* analysis of cross-presentation.

## **Recipes**

1. BMDC culture medium  
IMDM containing 10% heat-inactivated FBS  
100 IU/ml penicillin  
100 µg/ml streptomycin  
2 mM glutamax  
50 µM β-mercaptoethanol  
1x MEM non-essential amino acids  
1x sodium pyruvate  
Supernatant from J558 plasmacytoma cells was used as GM-CSF source (Winzler *et al.*, 1997).
2. B3Z and T cell culture  
RPMI containing 10% heat-inactivated FBS  
100 IU/ml penicillin  
100 µg/ml streptomycin  
2 mM glutamax  
50 µM β-mercaptoethanol  
1x MEM non-essential amino acids  
1x sodium pyruvate
3. Digestion medium for spleens  
Liberase 0.2 mg/ml (resuspend one bottle of 5 mg Liberase in 3 ml of RPMI-1640, which is sufficient for 15 spleens; use 0.2 ml of this solution per spleen) and DNase I 0.1 mg/ml in RPMI-1640 (final volume: 1.5 ml per spleen).
4. MACS buffer  
1x PBS  
2% FBS (or 2% BSA)  
5 mM EDTA

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