A Comprehensive Experimental Guide to Studying Cross-Presentation in Dendritic Cells *in vitro*

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ABSTRACT

Cross-presentation was first observed serendipitously in the 1970s. The importance of it was quickly realized and subsequently attracted great attention from immunologists. Since then, our knowledge of the ability of certain antigen presenting cells to internalize, process, and load exogenous antigens onto MHC-I molecules to cross-prime $CD8^+$ T cells has increased significantly. Dendritic cells (DCs) are exceptional cross-presenters, thus making them a great tool to study cross-presentation, but the relative rarity of DCs in circulation and in tissues makes it challenging to isolate sufficient numbers of cells to study this process *in vitro*. In this paper, we describe in detail two methods to culture DCs from bone-marrow progenitors, and a

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method to expand the numbers of DCs present *in vivo* as a source of endogenous bona-fide cross-presenting DCs. We also describe methods to assess cross-presentation by DCs using the activation of primary $CD8^+$ T cells as a readout.

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Basic Protocol 8: $CD8^+$ T cell activation to assess cross-presentation

Support Protocol 7: Isolation and labeling of $CD8^+$ T cells with CFSE

KEYWORDS

Cross-presentation, Cross-priming, Dendritic Cells, DC, $CD8^+$ T Cell, MHC-I

Introduction

Antigen presentation is an essential step in the process of mounting an adaptive immune response against harmful non-self or altered-self entities, such as during viral infection or cancer. Antigen presenting cells (APCs) process and load antigenic peptides onto major histocompatibility complex (MHC) class I or class II molecules and display them on their surfaces to be recognized by $CD8^+$ and $CD4^+$ T cells, respectively (Blum et al., 2013; Cresswell, 2005). T cells recognize cognate peptides complexed with MHC molecules via T-cell receptors (TCRs). This interaction is complemented by T cell costimulatory signals induced by pattern recognition receptor (PRR) signaling, such as Toll-like receptors (TLRs), which detect microbial components, in order to specify the origin of the peptide as being non-self (Akira et al., 2006; Blander, 2018).

Virtually all nucleated cells can process intracellular antigens and load antigen-derived peptides onto MHC class I molecules to display on their surfaces, whereas only professional APCs, namely dendritic cells (DCs), macrophages, and B cells, can process endocytosed or phagocytosed extracellular antigens and load antigen-derived peptides onto MHC class II molecules (Blander, 2018). However, some professional APCs have been demonstrated to cross-present extracellular antigen-derived peptides onto MHC class I molecules to cross-prime and activate $CD8^+$ T cells. This process is termed cross-presentation, and it is critical for the instigation of an antigen-specific $CD8^+$ T cell response (Blander, 2018; Grotzke et al., 2017). *In vivo* and *in vitro* studies in both murine models and humans have shown that DCs are the best cross-presenting cell type (Embgenbroich & Burgdorf, 2018). DC cross-presentation provides a vital defense mechanism against many pathogens and malignancies that are able to evade or dampen the immune response of the infected/affected cell, thus rendering the process an

important therapeutic strategy in creating anti-cancer vaccines. Additionally, DC cross presentation is implicated in promoting central and peripheral immune tolerance (Joffre et al., 2012).

DC cross-presentation occurs via one of the two currently accepted pathways, pertaining mainly to the kinetics and processing of the exogenous antigens, and called the vacuolar pathway or the cytosolic pathway (Figure 1) (Blander, 2018). In the vacuolar pathway, antigens are taken up for processing in the endocytic compartment, and degraded primarily by the lysosomal protease Cathepsin S, whereas in the cytosolic pathway, antigens are internalized and translocated into the cytosolic space for processing and degradation by proteasomes (Shen et al., 2004). The resultant peptides, from the latter pathway, are subsequently transported by the protein transporter associated with antigen processing (TAP) either to transport the peptides into the endoplasmic reticulum or into the nascent endosome for loading onto MHC class I (MHC-I) molecules (Ackerman et al., 2006; Kovacsovics-Bankowski & Rock, 1995; Palmowski et al., 2006). A single mouse DC can cross-present exogenous peptides via both pathways simultaneously, though it has been demonstrated that a $CD8\alpha^+$ subpopulation of classical or conventional DCs (cDCs) known as class 1 DCs (cDC1) are only able to utilize the cytosolic pathway (Segura et al., 2009).

Over the years, we have extensively studied DC cross-presentation in mice. We have carefully optimized the conditions to study this process in murine cells, including the isolation and differentiation of murine DCs, the isolation of antigen-specific transgenic murine $CD8^+$ T cells, stimulating DCs for cross-presentation with soluble or particulate cargoes, and cross-priming $CD8^+$ T cells by DCs. We have optimized the necessary readouts, in order to delineate the cell biology of cross-presentation and the complex cross-talk between vesicular traffic and TLR signaling pathways (Blander, 2008, 2016, 2018; Nair-Gupta et al., 2014; Nair-Gupta &

Blander, 2013). This paper details such protocols with clear instructions to set-up cross-presentation assays *in vitro* along with the appropriate experimental readouts that need to be conducted.

[*Insert figure 1 near here]

Strategic Planning

An important consideration for *in vitro* cross-presentation assays is the method by which the cross-presenting DCs are prepared. In mice and in humans, cDCs that reside in lymphoid tissues can be broadly divided into two subsets, class 1 and class 2 cDCs. These subsets have distinct phenotypes, developmental requirements, and functions. $CD11b^+SIRP\alpha^+$ cDC2s present exogenous antigen to $CD4^+$ T cells, while $CD8\alpha^+CD103^+$ cDC1s are the most efficient at cross-priming $CD8^+$ T cells *in vivo* (Mildner & Jung, 2014). For decades, the standard method of culturing DCs *in vitro* has been to differentiate bone marrow progenitor cells in the presence of granulocyte-macrophage colony-stimulating factor (GM-CSF) for five days, which may require the addition of interleukin-4 (IL-4) (Inaba et al., 1992). Whilst these so-called GM-DCs have been used in cross-presentation assays for decades and have been indispensable in research elucidating the machinery required for cross-presentation, it has recently become apparent that the output of this culture system does not phenotypically resemble the bona fide cross-presenting cDC1s (Helft et al., 2015). This is not to say that all knowledge gained by work in GM-DCs is to no avail as our fundamental understanding of DC maturation and antigen presentation including cross-presentation comes from GM-CSF DCs. In the past couple of decades, a culture system which makes use of Fms-like tyrosine kinase 3 ligand (Flt3L) allows

for the production of DCs which more closely resemble cDC1s found *in vivo*, as well as their CD103$^+$ migratory counterparts (Brasel et al., 2000; Liu & Nussenzweig, 2010; Maraskovsky et al., 1996). Flt3L may give rise to some plasmacytoid DC (pDC)-like cells as well (Naik et al., 2005), and recently, Kirkling *et al.* described an *in vitro* culture system whereby feeder cells expressing Notch ligand Delta-like 1 drive optimal Flt3L-dependent development of murine cDC1s from bone marrow progenitor cells (Kirkling et al., 2018). These cDC1s express surface markers and exhibit migratory properties that closely resemble the surface markers and migratory phenotype of cDC1s seen *in vivo* (Kirkling et al., 2018). Despite these encouraging properties, the aforementioned Flt3L-dependent cDC1 culture system has mainly been utilized to study cDC1 development, with only a few groups choosing to use it for functional studies of cross-presentation (Beshara et al., 2018; Brawand et al., 2002; Lau et al., 2018; Masten et al., 2004; Ou et al., 2019; Pulendran et al., 1997; Waskow et al., 2008). Importantly, many of the fundamental principles of cross-presentation have been validated in Flt3L-dependent cDC1 as well. This includes the capacity of these cells to stimulate and cross-prime CD8$^+$ T cells (Masten et al., 2004).

In addition to the above described, *in vitro* differentiation of cDC1s from bone marrow-derived progenitor cells, cross-presenting cDC1s can also be directly obtained from the spleen of a mouse that has been implanted with a tumor of B16-melanoma cells secreting Flt-3L (B16-Flt3L) (Arora & Porcelli, 2016). These tumor cells provide a sustained systemic level of Flt3L *in vivo* which stimulates the propagation of all subsets of DCs in the mouse spleen. This generally results in mice developing splenomegaly, in which DCs comprise up to 60% of the total spleen cells after approximately 10 days. Commercial kits are subsequently used to isolate the cDC1 subtype using phenotypic markers. This method yields high numbers of immature cDC1s for use in downstream *in vitro* cross-presentation experiments.

Below, we have outlined the protocols most commonly used to isolate murine DC progenitor cells and differentiating them into GM-DCs and cDC1-like cells *in vitro*, in Basic Protocol 1, 2, and 3, respectively. In Basic Protocol 4, we outline a protocol to culture B16-Flt3L cells for subsequent tumor implantation in mice to expand cDC1s *in vivo* and isolation from the mouse spleen. The resultant non-activated and immature DCs will be used in downstream *in vitro* cross-presentation experiments using $CD8^+$ T cells as a readout. An overview of the workflow is schematized in Figure 2.

[*Insert figure 2 near here]

Basic Protocol 1: Isolation of bone marrow (BM) progenitor cells

Introduction

The study of cross-presentation using an *in vitro* system necessitates a large number of DCs. *In vivo*, DCs comprise a relatively rare immune cell subset, meaning that the isolation of enough immature DCs from an animal for use *in vitro* is impractical. In addition to the issue of rarity, the isolation of differentiated DCs from different tissues has the potential to induce activation and phenotypic changes which may preclude proper assessment of some of their functions (O'Connell et al., 2000). However, in the past decades, it has been possible to differentiate DCs from bone marrow progenitors *in vitro* (Inaba et al., 1992) and utilize them for controlled cross-presentation experiments. The protocol below outlines how to extract bone marrow from the femur and tibia of a mouse. The progenitor cells obtained from the bone marrow can then be differentiated into DCs *in vitro* for downstream experiments. Typically, the bone marrow recovered from the femur and tibia bones, of a single mouse, yield approximately 50 to 80 million DC progenitor cells.

Materials

Reagents, Solutions, Test Organisms, Cells

- 7-11 week old C57BL/6J mice (The Jackson Laboratory, cat. no. 000664), or mouse of desired genotype
- Phosphate Buffered Saline (PBS) pH 7.4 (Gibco, cat. no. 14190144)
- Endotoxin-free red blood cell (RBC) lysis buffer (Sigma, cat. no. R7757)
- Heat-inactivated fetal calf serum (FCS) with endotoxin levels of ≤0.06 EU/ml (R&D Systems. Formerly Atlanta Biologicals, cat. no. S11550H), *see Critical Parameters*
- Hank's Balanced Salt Solution (HBSS, VWR, cat. no. L0121-0500)
- RPMI 1640-supplemented media (*See Reagents and Solutions*)

Hardware

- Dissecting scissors
- Forceps
- 10 cm polystyrene petri dish
- 50 ml polypropylene conical tube
- 15 ml polypropylene conical tube
- Hemocytometer or a cell counter
- 27-gauge needle
- 10cc syringe

- 70 µm nylon mesh cell strainer

Protocol Steps

1. Obtain a 7-11 week-old C57BL/6J mouse with the correct genetic modification. *For instance, if the impact of Toll-like receptor signaling on cross-presentation is being investigated, then one might consider using mice deficient for the receptor or the signaling adaptors for the receptor.*
2. Sacrifice the mouse according to your institute's guidelines and minimize animal sufferings.
3. Place the mouse in supine position with its forelimbs and hindlimbs pinned onto a dissecting board and spray the mouse with ethanol before placing it into a tissue culture hood. *All steps from hereinafter are to be carried out under sterile conditions.*
4. Using forceps, gently lift the abdominal wall and use dissecting scissors to make a vertical cut from near the urethral orifice to the area just below the sternum. Take care not to cut or puncture any organs.
5. Make a diagonal cut alongside each of the hindlimbs to reveal the leg bones and tissues.
6. Using two forceps separate the leg tissue from the skin.
7. Using two forceps, carefully remove the femur and the tibia bones individually from the mouse.
8. Hold the tip of each bone with one set of forceps, whilst gently using another set to remove all associated muscle and tissue masses around the bones
9. Place the bones in a clean 10 cm petri dish and wash with PBS to remove any debris.

10. Transfer the bones to a clean 10 cm petri dish containing 2 ml HBSS medium + 2% FCS.

11. Carefully sever the epiphyses to expose the bone marrow cells, using sharp dissecting scissors.

12. Use a 27-gauge needle attached to a 10cc syringe containing HBSS medium with 2% FCS, flush the bone marrow cells carefully from each bone into a 10 cm petri dish.

13. Isolate and suspend the cells by gentle pipetting up and down several times, and transfer to a 15 cm conical tube.

14. Centrifuge the cells at 300 x g for 5 minutes at 4°C. Remove and discard the supernatant carefully.

15. Resuspend the cells in 1 ml of RBC lysis buffer and incubate for exactly 1 minute at room temperature.

16. Add 10 ml of HBSS medium + 2% FCS to neutralize the RBC lysis buffer

17. Centrifuge the cells at 300 x g for 5 minutes at 4°C. Remove and discard the supernatant carefully.

18. Resuspend the cells in 10 ml of HBSS medium + 2% FCS.

19. Pass the resuspended cells through a 70 μm cell strainer into a 50 ml conical tube.

20. Count the cells.

21. Centrifuge the cells at 300 x g for 5 minutes at 4°C. Remove and discard the supernatant carefully.

22. Resuspend the cells in supplemented RPMI 1640 media (*See Reagents and Solutions*) at a concentration of 1 x 10^6 per ml, and immediately continue to the differentiation protocol in Basic Protocol 2 section below.

Basic Protocol 2: *In vitro* differentiation of DCs with granulocyte-macrophage colony-stimulating factor (GM-CSF)

In vitro differentiation of bone marrow-derived DCs with GM-CSF, so called GM-DCs, begins with the isolation of bone marrow progenitor cells, as described in Basic Protocol 1. The bone marrow progenitor cells are then cultured in the presence of GM-CSF to activate the downstream signaling pathways that are necessary in driving DC differentiation (van de Laar et al., 2012). GM-CSF can either be obtained by filtering the conditioned media of cultured J558L cells transfected with murine GM-CSF cDNA, or by purchasing recombinant GM-CSF from commercial vendors. The bone marrow progenitor cells are cultured with GM-CSF supplemented media for five days, with an additional replenishment with the GM-CSF-containing media on day three. The DCs will appear in clusters that situate mainly around the edges of each well.

Materials

Reagents, Solutions, Test Organisms, Cells

- Bone marrow cells (*as isolated in Basic Protocol 1*)
- RPMI 1640-supplemented media (*See Reagents and Solutions*)
- Recombinant murine GM-CSF (R&D Systems, cat. no. ML-415), ***OR***

- Genetically engineered GM-CSF-expressing J558L cell conditioned media as a source of GM-CSF (*see Support Protocol 1*)

Hardware

- 24-well plates, polystyrene and tissue culture treated

Protocol Steps

1. Resuspend the bone marrow-derived progenitor cells at a concentration of 1 x 10^6 cells per ml in RPMI 1640-supplemented media.

2. Add 10 ng/ml recombinant murine GM-CSF or GM-CSF conditioned media *at the experimentally determined appropriate percentage (see Support Protocol 1)* to the resuspended cells.

3. Plate 1 ml of the cell suspension in each well of a 24-well plate, *i.e., 1 x 10^6 cells per well*.

4. Incubate the cells for 5 days (total) at 37°C in a humidified atmosphere containing 5% CO_2. Take extra care not to disturb the cells whilst they differentiate and grow so to avoid spontaneous maturation. *The cells do not need to be replated, and replating prior to experiments should especially be avoided.*

5. On day 3, add 1 ml of prewarmed fresh RPMI 1640-supplemented media containing 10 ng/ml recombinant murine GM-CSF or GM-CSF conditioned media to each well. *At the end of the incubation period, i.e., at day 5, clusters of DCs should be visible, particularly around the edges of the wells.*

- *J558L cell GM-CSF conditioned media can be substituted for recombinant GM-CSF.*

Support Protocol 1 Preparation of conditioned media from GM-CSF producing J558L cells

Whilst recombinant murine GM-CSF is readily available commercially, many groups choose to generate their own in an effort to cut down on costs. J558L cells that have been transduced with a GM-CSF expression vector are cultured for 3 days in RPMI 1640-supplemented media. The culture supernatant is then centrifuged and filter-sterilized. In order to determine the concentration of GM-CSF in the culture supernatant, an ELISA test is performed using the J558L media supernatant. Alternatively, a better evaluation would involve culturing and differentiating bone marrow progenitor cells with a serial dilution of the J558L conditioned media, then empirically calculating the best concentration guided by evaluating the resulting GM-DCs, from each J558L conditioned media dilution, and assessing their expression of DC markers, viability and maturation state.

Materials

Reagents, Solutions, Test Organisms, Cells

- J558L cells transfected with murine GM-CSF cDNA (Qin et al., 1997)
- IMDM-supplemented media (*See Reagents and Solutions*)
- Geneticin G418 (InvivoGen, cat. no. ant-gn-1)
- Murine GM-CSF ELISA Development Kit (PeproTech, cat. no. 900-K55), or another suitable ELISA kit

Hardware

- 15 ml polypropylene conical tube

- 50 ml polypropylene conical tube
- T25 tissue culture flask, polystyrene and tissue culture treated
- T175 tissue culture flask, polystyrene and tissue culture treated
- 500 ml 0.22 μm filter bottle, cellulose acetate membrane

Protocol Steps

1. Thaw a vial of GM-CSF producing J558L cells, containing 5 x 10^6 cells, slowly on ice and transfer the cells into a 15 ml conical tube containing 10 ml of prewarmed IMDM-supplemented media.
2. Centrifuge the cells at 300 x g for 5 minutes at room temperature. Remove and discard the supernatant carefully.
3. Resuspend the cells in 5 ml prewarmed IMDM-supplemented media without geneticin G418 and culture in a T25 flask and incubate overnight at 37°C in a humidified atmosphere containing 5% CO_2.
4. Transfer the cells to a T175 flask and add 175 ml prewarmed IMDM-supplemented media containing geneticin G418 at a concentration of 1mg/ml. Incubate as before.
5. After approximately 3 days, the cells should have expanded sufficiently as indicated by the color of the phenol red in the media turning orange. Phenol red is a pH sensitive dye which turns to orange and then yellow when the culture media becomes increasingly acidic as the cells proliferate and release waste products.
6. As soon as the medium becomes orange, the cells can now be propagated further by seeding into 2 x T175 flasks each with 175 ml of IMDM-supplemented media containing

geneticin G418 and cultured for an additional 3 days. *If a larger batch of GM-CSF is desired, this can be scaled up as appropriate.*

7. Carefully collect the culture supernatant in each flask at day 3 and transfer to 50 ml conical tubes.
8. Centrifuge the supernatant at 300 x g for 5 minutes at room temperature to pellet cellular debris. Collect the supernatant carefully, leaving approximately 1 ml of media at the bottom of the tube to avoid cellular contaminants from being collected.
9. Pool and filter the supernatant using a 500 ml 0.22 μm filter bottle. Aliquot into 50 ml conical tubes and store at -80ºC for later use.
10. Calculate the GM-CSF concentration in the supernatant by utilizing commercially available ELISA kits, or empirically by using serially diluted GM-CSF-containing media to differentiate bone marrow progenitor cells into GM-DCs, as described in *Basic Protocol 2*, then analyze the culture output as described in *Basic Protocol 5*.

- *GM-CSF producing J558L cells are J558 cells that have been transfected with murine GM-CSF cDNA. The J558 cell line (ATCC, cat no. TIB-6) does not endogenously express murine GM-CSF.*
- *J558L cells can be initially cultured in 20% FCS after thawing to aid establishing the cell culture (step 3) prior to the addition of geneticin G418 (step 4).*

Basic Protocol 3: *In vitro* differentiation of DCs with Flt3L

Introduction

As previously discussed, it has recently been argued that the GM-DC culture system (*see Basic Protocol 2*) does not phenotypically share many features with cDC1s, which have been

shown to be the *bona fide* cross-presenting subset of DCs *in vivo* (Helft et al., 2015). Subsequently, the protocol below describes the steps to obtain cDC1-like cells, and it starts with the bone marrow progenitor cells (*isolated in Basic Protocol 1*) and utilizes Flt3L in the culture media to drive the differentiation of cDC1-like cells over 7-11 days (Brasel et al., 2000; Naik et al., 2005; van de Laar et al., 2012).

Materials

Reagents, Solutions, Test Organisms, Cells

- Murine bone marrow progenitor cells (*see Basic Protocol 1*)
- RPMI 1640-supplemented media (*See Reagents and Solutions*)
- Recombinant Flt3L (PeproTech, cat. no. 250-31L), ***OR***
- B16-Flt3L cell Flt3L conditioned media (*see Support Protocol 2*)

Hardware

- 24-well plates, polystyrene and tissue culture treated

Protocol Steps

1. Resuspend the bone marrow-derived progenitor cells at a concentration of 1 x 10^6 cells/ml in RPMI 1640-supplemented media.
2. Add 300 ng/ml recombinant murine Flt3L or the equivalent in the form of B16-Flt3L conditioned media (% v/v) to the resuspended cells *(see Support Protocol 2)*.
3. Plate 2 ml of the cell suspension in each well of a 24-well plate, *i.e., 2 x 10^6 cells per well*.

4. Incubate the cells for 7 (total) days at 37°C in a humidified atmosphere containing 5% CO_2. Take extra care not to disturb the cells whilst they differentiate and grow so to avoid spontaneous maturation. *The cells do not need to be replated, and replating prior to experiments should especially be avoided.*

5. On day 3, carefully pipette 1 ml media out of each well, without disturbing the cells, and replace with 1 ml of prewarmed fresh RPMI 1640-supplemented media containing 300 ng/ml recombinant murine Flt3L or the equivalent in the form of B16-Flt3L conditioned media (% v/v) to each well.

- *B16-Flt3L cell Flt3L conditioned media can be substituted for recombinant Flt3L.*

Support Protocol 2 Preparation of Flt3L containing media from B16-Flt3L cells

Flt3L conditioned media, from cultured B16-Flt3L cells, can be used to differentiate bone marrow-derived progenitor cells to cDC1-like cells, in lieu of commercially available recombinant murine Flt3L, or as an economically viable alternative. The process is simple, and the protocol below describes in detail the steps involved to generate Flt3L containing media from B16-Flt3L cells. B16-Flt3L cells are a murine melanoma cell line that are transfected with the Flt3L cytokine gene and subsequently expresses high concentrations of Flt3L (Vargas et al., 2006). These adherent cells are cultured in DMEM-supplemented media until they reach approximately 85% confluency. The culture supernatant is then collected, centrifuged and filter-sterilized, and stored at -80ºC for later use. Prior to use in experiments, an ELISA test can be performed using a commercially available kit in order to determine the concentration of the Flt3L in the supernatant, which is to be diluted to a final working concentration of 300 ng/ml.

Materials

Reagents, Solutions, Test Organisms, Cells

- B16-Flt3L cells (RRID, CVCL_IJ12)
- DMEM-supplemented media (*See Reagents and Solutions*)
- PBS (Gibco, cat. no. 14190144)
- Trypsin-EDTA (0.25%), phenol red (Gibco, cat. no. 25200056)
- Murine Flt3L ELISA Development Kit (R&D Systems, cat. no. MFK00), or another suitable ELISA kit

Hardware

- T25 tissue culture flask, polystyrene and tissue culture treated
- T75 tissue culture flask, polystyrene and tissue culture treated
- T175 tissue culture flask, polystyrene and tissue culture treated
- 15 ml polypropylene conical tube
- 50 ml polypropylene conical tube
- 0.22 µm Steriflip filter system (Millipore Sigma, cat. no. SE1M003M00), or any other sterile 0.22 µm filter system.

Protocol Steps

1. Thaw a vial of B16-Flt3L cells quickly by dipping the vial halfway in a 37ºC water bath and agitating the vial, being careful not to allow the water to touch the area near the lid.

2. Transfer the cells into a 15 ml conical tube containing 10 ml of prewarmed DMEM-supplemented media.
3. Centrifuge the cells at 300 x g for 5 minutes at room temperature. Remove and discard the supernatant carefully.
4. Resuspend the cells in 5 ml prewarmed DMEM-supplemented media and culture in a T25 flask and incubate at 37°C in a humidified atmosphere containing 5% CO_2.
5. Once the culture reaches 80-90% confluency, aspirate the culture media, then wash once with PBS before incubating the cells with 3 ml trypsin for 5 minutes at 37°C.
6. Add 5 ml of DMEM-supplemented media to the flask to inactivate the trypsin and stop digestion. Collect the cell suspension in a 15 ml conical tube.
7. Centrifuge the cells at 300 x g for 5 minutes at room temperature. Remove and discard the supernatant carefully.
8. Resuspend the cells in 10 ml prewarmed DMEM-supplemented media and culture in a T75 flask and incubate at 37°C in a humidified atmosphere containing 5% CO_2.
9. Split the culture again once it reaches 80-90% confluency by trypsinizing the cells with 5 ml trypsin as before, and seed at 50% confluency in a T175 flask with 50 ml prewarmed DMEM-supplemented media. Incubate at 37°C in a humidified atmosphere containing 5% CO_2.
10. Allow the adherent cells to expand to a confluency of 85% or when the cell medium turns orange. At this point, the conditioned media can be collected, or the cells can be split further in order to make more conditioned media.
11. Carefully collect the culture supernatant and transfer to a 50 ml conical tube.

12. Centrifuge the supernatant at 300 x g for 10 minutes at 4°C to pellet cellular debris. Collect the supernatant carefully, leaving approximately 1 ml of media at the bottom of the tube to avoid cellular contaminants from being collected.

13. Pool, if there is more than one tube, and filter the supernatant. It is recommended to use a 0.22 µm steriflip 50 ml tube filter system, though other 0.22 µm filter systems may be used as well. Aliquot up to 50 ml of the conditioned media into 50 ml conical tubes and store at -80°Cfor later use.

14. Calculate the Flt3L concentration in the supernatant by utilizing commercially available ELISA kits. *However, many laboratories simply use the conditioned medium at a concentration of 10% (v/v) in the culture system in Basic Protocol 3.*

Basic Protocol 4: Expansion of cDC1s *in vivo* for use in *ex vivo* experiments

In order to expand the numbers of DCs which naturally occur in the spleen of mice, mice are injected subcutaneously with 1 x 10^7 B16-Flt3L cells. Within 7-11 days, the tumor will reach a size measuring approximately 2-10 mm in diameter, which makes it readily visible (Arora & Porcelli, 2016). The lymphoid organs, in particular the spleen, are then harvested and digested, followed by depleting RBC with a lysis buffer. The resulting single cell suspension consists mainly of the different subsets of DCs, which are labelled with microbeads for separation with a magnetic cell separation apparatus. The subsequent cDC1s that are isolated should then immediately be placed into culture, stimulated and given cross-presentation cargo. This is because removal and handling of the cDC1s from lymphoid organs causes them to spontaneously become activated and thus mature, which will render them useless for *in vitro* cross-presentation assays.

Materials

Reagents, Solutions, Test Organisms, Cells

- 7-11 week old C57BL/6J mice (The Jackson Laboratory, cat. no. 000664), or mouse of desired genotype
- B16-Flt3L cells (RRID, CVCL_IJ12)
- RPMI 1640-supplemented media (*See Reagents and Solutions*)
- RPMI 1640 media (Gibco, cat. no. 11875093)
- PBS (Gibco, cat. no. 14190144)
- Trypsin-EDTA (0.25%), phenol red (Gibco, cat. no. 25200056)
- Endotoxin-free red blood cell (RBC) lysis buffer (Sigma, cat. no. R7757)
- Heat-inactivated FCS (R&D Systems. Formerly Atlanta Biologicals, cat. no. S11550H)
- DNAse/collagenase solution
    - DNAse I (Roche, cat. no. 11 284 932 001)
    - Collagenase D (Roche, cat. no. 11 088 858 001)
- $CD8\alpha^+$ Dendritic Cell Isolation Kit, mouse (Miltenyi Biotec, cat. no. 130-091-169)

Hardware

- T175 flask, polystyrene and tissue culture treated

- 1.5 ml Eppendorf tube
- 1cc syringe
- 10cc syringe
- 25-gauge needle
- Dissecting scissors
- Forceps
- 70 µm nylon mesh cell strainer
- 6-well plate
- Pasteur pipette
- 15 ml polypropylene conical tube
- 10 cm polystyrene petri dish
- 50 ml polypropylene conical tube
- Hemocytometer or a cell counter
- LS magnetic separation column (Miltenyi Biotec, cat. no. 130-042-401)
- QuadroMACS™ Separator (Miltenyi Biotec, cat. no. 130-091-051)
- MACS MultiStand (Miltenyi Biotec, cat. no. 130-042-303)

Protocol Steps

1. Seed B16-Flt3L cells in a T175 flask and grow to 90% confluency, following the steps 1-8 outlined in Support Protocol 2.

2. Harvest the B16-Flt3L cells. Begin by aspirating the culture media, then wash once with ice-cold PBS before incubating the cells with 10 ml trypsin for 5 minutes at 37ºC.
3. Add 10 ml of DMEM-supplemented media to the flask to inactivate the trypsin and stop digestion. Collect the cell suspension in a 50 ml conical tube.
4. Centrifuge the cells at 300 x g for 5 minutes at 4ºC. Remove and discard the supernatant carefully. Wash once in ice-cold PBS and centrifuge as before.
5. Count the cells with a hemocytometer or a cell counter.
6. Resuspend the cells at a concentration of 1 x 10^8 cells per ml in PBS. Transfer the cells to a 1.5 ml Eppendorf tube and keep on ice. Immediately bring the cell suspension to the animal facility where the mice are kept.
7. Work in a biosafety hood and use a 1cc syringe fitted with a 25-gauge needle to draw 100 µl of the B16-Flt3L cells suspended in PBS for each mouse that is to be injected. *Do not pass the B16-Flt3L cells through the needle more than once as this may cause excessive cellular stress resulting in shearing the cells, hence reducing the total number of viable cells and in turn reducing the rate of successful implantation.*
8. Immobilize the mouse by holding it by the scruff of its neck, then turn it over so the abdomen faces you, and tuck its tail behind your pinky finger. Using your dominant hand, inject the mouse subcutaneously with the 100 µl B16-Flt3L cells contained in the syringe into the side flank. To minimize animal suffering and to successfully implant the tumor, place the needle in nearly parallel to the skin of the mouse and push the needle into the skin slowly. A small "bubble" should form just under the skin while injecting, which indicates correct application. *If immobilizing the mouse is proving difficult or direct*

injection not permitted by the institute, then the mouse can be anaesthetized using ketamine or isoflurane prior to the injection. Place the mouse back in its usual enclosure.

9. Wait for 7-11 days for the tumor to reach a noticeable size, at which point the various DC subsets, and in particular cDC1s in the spleen should have significantly increased in number. During the period following the tumor implantation, the animal's health must be assessed and monitored according to an accepted method, such as those published by Ullman-Culleré and Foltz (Ullman-Culleré & Foltz, 1999).

10. Sacrifice the mouse once the tumor has reached a diameter of 2-10 mm across and is visibly palpable. Follow your institute's guidelines on the humane criteria for determining at which point of tumor growth to sacrifice the animal and minimize its suffering.

11. Place the mouse in supine position with its forelimbs and hindlimbs pinned onto a dissecting board and spray it with ethanol before placing it into a tissue culture hood. *All steps from hereinafter are to be carried out under sterile conditions.*

12. Using a pair of forceps, gently lift the abdominal wall and use dissecting scissors to make a vertical cut from near the urethral orifice to the area just below the neck. Take care not to cut or puncture any organs.

13. Make two horizontal cuts on the left side of the mouse from top and bottom ends of the first cut to peel the abdominal wall back to reveal the internal abdominal organs. Using a pair of forceps, gently harvest the spleen.

14. Place the spleen inside of a 70 μm cell strainer that is sitting on a well of a 6-well plate containing RPMI 1640 media in the presence of DNAse/collagenase solution (100 μg/ml

DNAse I and 1 mg/ml collagenase D). Mash the spleen through the strainer using the plunger portion of a 10cc syringe.

15. Incubate the mashed-up spleen including the cell strainer in the DNAse/collagenase solution for 30 minutes at 37°C.
16. Mix the splenocyte suspension by gently pipetting up and down using a pasteur pipette, pipette through the cell strainer also, and transfer the cells to a 15 ml conical tube.
17. Centrifuge the cells at 300 x g for 5 minutes at room temperature. Remove and discard the supernatant carefully.
18. Resuspend the cells in 1 ml of RBC lysis buffer and incubate for exactly 1 minute at room temperature.
19. Add 10 ml of RPMI 1640-supplemented media to neutralize the RBC lysis buffer.
20. Centrifuge the cells at 300 x g for 5 minutes at 4°C. Remove and discard the supernatant carefully.
21. Wash the cells once in 10 ml PBS containing 5% FCS, then centrifuge as before.
22. Isolate the cDC1 subset using MACS mouse $CD8\alpha^+$ DC isolation kit, following the manufacturer's instructions (Miltenyi Biotec). All steps must be completed using pre-cooled buffers. All reagents and cell suspensions must be kept on ice to maintain a temperature of ~4°C. This is to maximize cell viability and minimize spontaneous maturation and activation of the DCs.
23. Estimate the number of DCs using a hemocytometer or a cell counter and immediately resuspend the cells at a concentration of 1 x 10^6 cells per ml in RPMI 1640-supplemented media.

24. Add 1 ml of cell suspension (*i.e.,* 1 x 10^6 cells) to each well of a 24-well plate, and immediately proceed to the addition of cross-presentation cargo and DC stimulation, *as described in Basic Protocol 6*.

Basic Protocol 5: Characterizing resting and activated DCs

Introduction

DCs can become activated with very little stimuli, which begins the maturation process. It is therefore imperative to ensure that all reagents used in DC culture are completely free of endotoxins. Additionally, it has been shown that the simple disruption of E-cadherin mediated contacts, that help form the clusters which appear in DC cultures, is enough to induce DC maturation (Jiang et al., 2007). Consequently, it is highly recommended to handle these cells with caution and minimize any disruptions whilst in culture, including excessive movement and handling of the wells or culture flasks, or excessive vibrations caused by opening and closing the incubator door, or by unbalanced centrifuge that is in close proximity to the incubator. The cross-presentation capability of DCs is negatively affected by their maturation, a state in which the phagocytic capability of DCs decrease (Blander, 2018). For this reason, when establishing an *in vitro* cross-presentation system, it is important to ensure the baseline 'resting' state of DCs prior to their use in cross-presentation experiments.

The method below describes a simple way to do this by staining the cultured DCs for activation markers prior to any stimulation and performing flow cytometric analyses. This should be done regularly, particularly when an already established reagent has been replaced with a new brand or new lot. In particular, close attention must be paid to the lot of serum, as serum is notorious for containing endotoxins at levels which many manufacturers deem to be an insignificant amount, but, in fact, is adequate to activate DCs *in vitro.* In addition to the

aforementioned flow cytometric analyses, ELISA tests for the pro-inflammatory cytokines TNFα and IL-6 are also utilized to assess DC activation by probing for the expression of these pro-inflammatory cytokines that are typically secreted as a result of TLR signaling, such as LPS. Stimulating the TLR signaling pathways in DCs will serve as a positive control for the phenotypic markers and cytokine expression of activated and matured DCs in comparison to resting and immature DCs.

Materials

Reagents, Solutions, Test Organisms, Cells

- GM-CSF or Flt3L *in vitro* differentiated DCs (*see Basic Protocol 2 and 3*)
- Lipopolysaccharides (LPS, Millipore Sigma, cat. no. L2880)
- RPMI 1640-supplemented media (*See Reagents and Solutions*)
- PBS (Gibco, cat. no. 14190144)
- Commercially available ELISA kits for detecting murine TNFα and IL-6 cytokines
- Trypsin-EDTA (0.25%), phenol red (Gibco, cat. no. 25200056)
- FACS buffer (*See Reagents and Solutions*)
- Aqua Live/Dead stain (Invitrogen, cat. no. L34957)
- Anti-mouse CD16/CD32 Fc-block antibody (eBioscience, clone 93, cat. no. 14-0161-85)
- Antibody cocktail & Isotype controls
    - Anti-mouse CD11b antibody (Invitrogen, clone M1/70, cat. no. 25-0112-81)
    - Anti-mouse CD11c antibody (BioLegend, clone N418, cat. no. 117320)

- Anti-mouse MHC-II antibody (BioLegend, clone M5/114.15.2, cat. no. 107631)
- Anti-mouse CD40 antibody (eBioscience, clone 1C10, cat. no. 12-0401-83)
- Anti-mouse CD80 antibody (BioLegend, clone 16-10A1, cat. no. 104706)
- Anti-mouse CD86 antibody (BioLegend, clone GL1, cat. no. 105028)

Hardware

- 1.5 ml Eppendorf tube
- 15 ml polypropylene conical tube
- Hemocytometer or a cell counter
- U-shaped 96-well plate, polystyrene and tissue culture treated
- Aluminum foil

Protocol Steps

1. Culture bone marrow derived DCs using either the GM-CSF protocol, or the Flt3L protocol (*see Basic Protocol 2 and 3, respectively*).
2. To evaluate the overall level of DC differentiation from the bone marrow-derived progenitor cells, as well as assessing the ability of these newly differentiated DCs to mature and become activated upon stimulation, add 100 ng/ml LPS directly to the wells housing the differentiated DCs in the 24-well plates and incubate for 6 hours, or overnight, at 37°C in a humidified atmosphere containing 5% CO_2. Make sure to include

unstimulated DC control wells, alongside the stimulated DC wells and conduct each condition in triplicates. Continue from step 7.

3. *Alternatively*, use only non-adherent immature DCs to assess the ability of these newly differentiated DCs to mature and become activated upon stimulation. To harvest non-adherent immature DCs, begin with flushing the media contained in the wells up and down several times using a p1000 pipette. Pipette carefully and do not let the pipetted contents touch the bottom of the pipette itself to avoid contamination. If not possible, use filtered pipette tips to avoid contamination. Ensure to flush around the edges of the wells, as this is where much of the DC clusters are formed. Then, collect the RPMI 1640 media-containing DCs in the wells and place into a 15 ml conical tube. Immediately add 1 ml of ice-cold PBS to the well, flush and collect the remainder of DCs.

4. Count the cells using a hemocytometer or a cell counter and centrifuge the cells at 300 x g for 5 minutes at 4°C. Remove and discard the supernatant carefully.

5. Resuspend the DCs at a concentration of 1 x 10^6 cells/ml in RPMI 1640-supplemted media.

6. Transfer 1 ml suspended DCs, i.e., 1 x 10^6, to a new 24-well plate well and immediately stimulate the DCs with 100 ng/ml LPS, and incubate for 6 hours, or overnight, at 37°C in a humidified atmosphere containing 5% CO_2. Make sure to include unstimulated DC control wells, alongside the stimulated DC wells and conduct each condition in triplicates.

7. Once the treatment incubation period has been completed, the DCs are now ready for the assessment of maturation markers via cytokine expression and secretion. Centrifuge the cells at 300 x g for 5 minutes at 4°C.

8. For cytokine secretion, carefully and slowly remove the supernatants with a pipette and transfer to labelled 1.5 ml Eppendorf tubes for ELISA tests. Do not disturb the DCs at the bottom of the wells. Continue with steps 9-10 below for ELISA tests, or for assessment of maturation markers on DCs by flow cytometry, continue from step 11 below.

9. Centrifuge the supernatants at 300 x g for 10 minutes at 4°C to pellet cellular debris. Carefully collect the supernatants without disturbing the area where a pellet may have formed, and aliquot into clean and labelled 1.5 ml Eppendorf tubes for immediate use or store in the refrigerator overnight for use the next day. *Avoid repeat freeze-thaw cycles. It is highly recommended to do the ELISA tests immediately after collecting the supernatant. This is because freezing and thawing the supernatant even once may alter the cytokine concentration. Plan ahead of the experiment, such as coating ELISA plates with the appropriate capture antibody overnight, if not using a kit or as and when required.*

10. Use commercially available ELISA kits to detect the levels of the pro-inflammatory cytokines TNFα and IL-6 in the LPS-stimulated DCs compared to unstimulated DCs as indicators of DC activation. Follow the ELISA manufacturers' guidelines.

11. Harvest the stimulated DCs (and negative control, unstimulated DCs) by adding 1 ml of PBS to each well and flushing the media/PBS contained in the well up and down several times using a p1000 pipette. Pipette carefully and do not let the pipetted contents touch the bottom of the pipette itself to avoid contamination. If not possible, use filtered pipette tips to avoid contamination. Ensure to flush around the edges of the wells, as this is where much of the DC clusters are formed. Collect the pipetted contents from all the wells and place into labeled 15 ml conical tubes and place it on ice. Immediately after the media is removed from the wells, add 1 ml of ice-cold PBS to each well and flush the

wells as before to collect remaining cells, then pool them with the other harvested cells in the corresponding 15 ml conical tube.

12. Add 500 µl trypsin to each well and incubate for no longer than 5 minutes at 37°C in a humidified atmosphere containing 5% CO_2. Lightly agitate the plate manually to dislodge adherent cells.

13. Add 3 ml of RPMI 1640-supplemted media to each well to neutralize the trypsin. Transfer contents to corresponding 15 ml conical tubes. Add 1 ml of ice-cold PBS to each well and flush the wells as before to collect remaining cells, then pool them with the other harvested cells in the corresponding 15 ml conical tube.

14. Count the cells suspended in the media/PBS using a hemocytometer or a cell counter.

15. Centrifuge the cells at 300 x g for 5 minutes at 4°C. Remove and discard the supernatant carefully. Wash once in ice-cold PBS and centrifuge as before.

16. Resuspend the DCs at a concentration of 2 x 10^6 cells/ml in PBS and add 100 µl (2 x 10^5 cells) of each treatment of interest into the wells of a 96-well plate.

17. Ensure adequate samples are included to satisfy all staining controls; including technical replicates, isotope controls, single stain controls, as well negative controls, i.e., unstained controls, for your experiment.

18. Wash again by adding another 100 µl of PBS to each of the wells and centrifuge as before.

19. Remove and discard the supernatant carefully using either an aspirator on a low setting, or manually using a pipette.

20. Resuspend all the DC in the wells in 100 µl PBS containing anti-CD16/CD32 (Fc-block) diluted 1/100 and Aqua Live/Dead stain diluted 1/1000. Control cells for these are resuspended in PBS only. Cover the 96-well plate with foil or another light impenetrable material, and incubate on ice or at 4°C for at least 30 minutes. *It is important to use PBS, not FACS buffer in this step, as the FCS in the FACS buffer will sequester the Aqua Live/Dead stain.*

21. Wash the cells by adding another 100 µl of FACS buffer to each of the wells and centrifuge as before.

22. Wash the cells once more using 100 µl FACS buffer, and centrifuge as before.

23. Add 100 µl of the appropriate antibody cocktail or corresponding isotype control antibodies diluted in the FACS buffer to the cells in each of the appropriate wells. Incubate for 30 minutes, on ice or at 4°C. The recommended surface marker controls are: CD11b, CD11c, MHC-II, CD80, CD86, and CD40*. For each individual laboratory, an antibody cocktail should be created based on available materials, being mindful of spectral overlap and the flow cytometer capabilities, with antibodies that have been titrated and validated.*

24. Wash the cells by adding 100 µl of FACS buffer to each of the wells and centrifuge as before. Wash twice more by adding 200 µl of FACS buffer to each of the wells and centrifuge as before.

25. Resuspend the cells in 200 µl of FACS and proceed to acquisition or fix the cells in a preferred fixative to store for later analyses. Use single stain controls for compensation. Sample data is shown below in Figure 3.

[*Insert Figure 3 near here]

- *Immature differentiated DCs should be positive for CD11b and CD11c and have moderate expression of MHC-II. In contrast, activated and matured DCs will upregulate surface MHC-II expression, as well as the induction of expression of the co-stimulatory markers CD80/CD86 and CD40, with the latter being considered as the clearest indicator of DC maturation.*
- *Compare and contrast the levels of expression of the aforementioned markers on cells that have not been stimulated with LPS to those that have been stimulated, to ensure that your DC culture system generates DCs that respond appropriately to TLR stimulation and most importantly have intact phagocytic activity. Cultured DCs that express low levels of DC maturation markers are indicative of DCs in the resting state. If the levels are similar to those on LPS-treated DCs, then the cultured DCs should not be used as they are already activated and importantly will have compromised phagocytic activity.*
- *It is vital to utilize this protocol every single time a new reagent is introduced, or an existing reagent is switched (e.g., new batch) in order to ensure they do not induce spontaneous maturation of DCs in culture.*
- *As a cost-effective measure, ELISA kits can be substituted for individually purchased ELISA components (such as the appropriate capture and detection antibodies, recombinant proteins used as standards, substrates, stop solution, blocking buffer etc.) to assay cytokine concentrations, and following protocols published previously by the Blander Lab and elsewhere (Nair-Gupta et al., 2014).*

Basic Protocol 6: DC stimulation, antigenic cargo, and fixation

DCs are able to internalize a wide range of microbial antigens or whole microbes for cross-presentation *in vivo*. It has been widely demonstrated that DCs engage different mechanisms to internalize cargo by utilizing different surface receptors, and process antigens from these cargoes for cross-presentation. The activation of various downstream signaling cascades during the process of internalization is in turn dictated by the engagement of different DC PRRs (Blander, 2018). Therefore, the choice of the model antigen is an important factor when designing *in vitro* cross-presentation assays, because this dictates the method by which the antigen will be internalized and processed.

Ovalbumin (OVA), for example, is a mannosylated soluble protein, and by engaging the mannose receptors on the DC surface, is internalized into $EEA^{+}Rab5^{+}$ endosomes (Gazi & Martinez-Pomares, 2009). Whilst soluble antigens of 0.5 μm in diameter or smaller in size, such as OVA and other proteins are internalized by endocytosis, particles larger than 0.5 μm in diameter (Aderem & Underhill, 1999), such as apoptotic cells, bacteria or beads coated by a model antigen are phagocytosed, and will engage a different set of receptors in the process. Such receptors include Clec9a, a C-type lectin-like receptor which engages filamentous actin on the surface of dying cells, and is essential for the cross-presentation of dead and dying cells (Ahrens et al., 2012). The protocol below outlines instructions to stimulate DCs, and to prepare and deliver cross-presentation cargoes to these cells for internalization via the endocytic or phagocytic pathways, as well instructions to prepare cargo that contains TLR ligands, namely LPS.

Materials

Reagents, Solutions, Test Organisms, Cells

- GM-CSF or Flt3L *in vitro* differentiated DCs (*see Basic Protocol 2 and 3*), or *in vivo* derived splenic DCs (*see Basic Protocol 4*)
- LPS (Millipore Sigma L2880)
- PBS (Gibco, cat. no. 14190144)
- Cross-presentation cargo (*see Support Protocols 3, 4, and 5*)
- 16% PFA (ThermoFisher, cat. no. 28906)
- Glycine (Fisher Scientific, cat no. BP381-5)
- RPMI 1640 media (Gibco, cat. no. 11875093)
- RPMI 1640-supplemented media (*See Reagents and Solutions*)

Hardware

- U-shaped 96-well plate, polystyrene and tissue culture treated
- 24-well plate, polystyrene and tissue culture treated
- Hemocytometer or a cell counter
- 15 ml polypropylene conical tube

Protocol Steps

1. Start with fully differentiated DC culture in 24-well plates, as prepared in Basic Protocol 2, 3, or 4.
2. Approximate the number of DCs in each well by counting the DCs in a representative well using a hemocytometer or a cell counter.

3. Calculate the number of DCs required for your experiments (*see table 1*) and harvest the required amount of DCs, accordingly. To harvest DCs, begin with flushing the media contained in the wells up and down several times using a p1000 pipette. Pipette carefully and do not let the pipetted contents touch the bottom of the pipette itself to avoid contamination. If not possible, use filtered pipette tips to avoid contamination. Ensure to flush around the edges of the wells, as this is where much of the DC clusters are formed. Then, collect the RPMI 1640 media-containing DCs in the wells and place into a 15 ml conical tube. Immediately add 1 ml of ice-cold PBS to the well, flush and collect the remainder of DCs.

4. Count the cells suspended in the media/PBS using a hemocytometer or a cell counter.

5. Centrifuge the cells at 300 x g for 5 minutes at 4°C. Remove and discard the supernatant carefully.

6. Resuspend the DCs at a concentration of 2 x 10^6 cells/ml in RPMI 1640-supplemted media.

7. Add 50 µl of the DC suspension (i.e., 1 x 10^5 DCs) to each well of a 96-well plate.

8. Stimulate the DCs with a cross-presentation cargo by the addition of 50 µl of a model antigen listed in Table 1, reconstituted in RPMI 1640-supplemted media, at the indicated concentrations (and for the time indicated). Add the antigen model or cargo slowly and gently to the center of the wells, try not to disturb the DC clusters to avoid spontaneous maturation. The concentrations of antigens and incubation times can be adjusted for the purpose and objective of the experiment conducted. An appropriate PRR agonist, such as LPS (100 ng per ml), must be included in the stimulation, if the model antigen lacks the ability to activate the appropriate PRR, in order to activate the signaling cascades

required for cross-presentation. Model antigens and cargoes must be reconstituted in the same media used to culture the DCs, i.e., RPMI 1640-supplemented media. Make sure the appropriate controls for your experiments are included in the plate, i.e., addition of model antigens in the presence or absence of a PRR agonist such as LPS. Make sure to include the appropriate controls for each condition tested.

Cross-presentation cargo	**Concentration**	**Incubation time**	**Notes**
Apoptotic cells	2 cells:1 DC	16 - 18 hours	Model infected apoptotic cells can be prepared, *see Support Protocol 4*
Antigen-coated microbeads	2 beads:1 DC	= or > 5 hours	LPS used as PRR agonist. *See Support Protocol 3*
Endocytic cargo, e.g., OVA	100ng per ml (a titration may be required for optimum concentration of each endocytic model antigen)	3 - 6 hours	An agonist such as LPS can be added alongside, depending on the experimental conditions
Bacteria	100 bacteria: 1 DC for cytokine measurements, OR 25 bacteria: 1 DC for T cell proliferation and activation readouts	1 - 12 hours	*See Support Protocol 5*

Table 1. **Different cross-presentation cargoes given to DCs.**

9. Centrifuge the 96-well plate at 300 x g for 2 minutes at room temperature and incubate at 37°C in a humidified atmosphere containing 5% CO_2, for the duration indicated in Table 1, or according to the objectives of your study. Generally, the larger the cargo, the more time it is required for the DCs to internalize and process the cargo.

10. At the end of the desired incubation period, centrifuge the stimulated DCs in the 96-well plate at 300 x g for 5 minutes at 4°C. Remove and discard the supernatant carefully using a p200 pipette. Avoid accidentally pipetting and discarding the DCs at the bottom of the wells.

11. Wash the DCs twice by adding 200 µl ice-cold PBS to each well and centrifuge and discard the supernatants, as before.

12. Fix the DCs by adding 100 µl of 0.05% PFA in PBS to each well and incubating the cells for 5 minutes at room temperature. Use fresh or recently thawed PFA. Frozen PFA must be thoroughly thawed in a water bath, and if a white precipitate forms after thawing then incubate at 60°C briefly.

13. Wash once by adding 100 µl PBS to each well containing PFA, centrifuge at room temperature and discard the supernatant carefully, as before. Wash again in 200 µl PBS, centrifuge and discard the supernatant, as before.

14. Quench the PFA fixation by adding 100 µl of 0.5 M glycine, dissolved in serum-free RPMI 1640 media, to each well and incubate for 5 minutes at room temperature. Centrifuge at room temperature and discard the supernatant, as before.

15. Wash four times in PBS, centrifuge and discard the supernatant, as before.

16. Add 200 µl RPMI 1640-supplemted media to each well and incubate for 20 minutes at 37°C in a humidified atmosphere containing 5% CO_2. This is to allow the release of

excess PFA from the DCs. Centrifuge and discard the supernatant as before, followed by two more washes in PBS. *It is absolutely crucial to wash away any residual PFA from the fixed DCs before adding and co-culturing CD8⁺ T cells (See Basic Protocol 8). This is because PFA is highly toxic to living cells, and as such, any residual PFA would kill the CD8⁺ T cells.*

17. For most downstream applications, the fixed DCs are now ready to use, such as co-culturing with CD8⁺ T cells to assess the cross-priming abilities of DCs as indicated by proliferation of CD8⁺ T cells *(See Basic Protocol 8).*

Support Protocol 3: Preparation of model antigen coated microbeads

Microbead-coated antigens are utilized to mimic the phagocytic internalization of microbes and insoluble antigens by DCs in cross-presentation. Streptavidin coated microbeads are normally utilized due to the extraordinarily high affinity of streptavidin tetramers for biotin, which makes the coupling of biotinylated model antigens to the microbeads extremely reliable, durable and convenient. Streptavidin coated microbeads are readily available to purchase from vendors. The protocol below outlines instructions to prepare microbeads coated with model antigens to use in DC cross-presentation experiments. GFP-OT is a biotinylated recombinant fusion protein which is comprised of GFP fused to OVA-derived SIINFEKL peptide. The SIINFEKL peptide is internalized, processed and loaded onto MHC-I molecules in DCs, which is then specifically recognized by CD8⁺ OT-I transgenic T cells. This recombinant GFP-OT protein can be custom ordered from GenScript.

Materials

Reagents, Solutions, Test Organisms, Cells

- ProMag® 3 Series Streptavidin Microspheres (Polysciences, cat. no. 86056)
- PBS (Gibco, cat. no. 14190144)
- Biotinylated model antigens, e.g.,
    - Biotinylated GFP-OT (Drutman & Trombetta, 2010) (custom order by GenScript or other commercial vendors)
    - Biotinylated LPS (Invivogen cat. no. tlrl-lpsbiot)
- Heat-inactivated FCS (R&D Systems, formerly Atlanta Biologicals, cat. no. S11550H)

Hardware

- 1.5 ml Eppendorf tube

Protocol

1. Wash the microbeads (Streptavidin Magnetic Microspheres) by resuspending the desired amount in PBS and centrifuging at 2,000 x g for 5 minutes at room temperature. Carefully aspirate and discard the PBS. This step is necessary to remove any EDTA, surfactant, or other reagents used in the preparation or storing of the microbeads but may interfere or contaminate with the coupling of biotinylated antigens to the microbeads and their use in cross-presentation experiments.
2. Incubate the microbeads in the presence of biotinylated GFP-OT for 30 minutes at room temperature in PBS, with constant shaking on a vortex. The GFP-OT protein should only occupy a quarter of the streptavidin molecules on the surface of the

microbeads in order to allow sufficient LPS binding as well. According to calculations, guided by the details provided by the manufacturer and the molecular weight of GFP-OT protein, 62 ng of GFP-OT protein would effectively saturate a quarter of 1 x 10^6 microbeads.

3. Wash the conjugated microbeads four times in PBS containing 0.5% FCS by centrifuging at 2,000 x g for 5 minutes at room temperature. *The FCS is important to allow proper pelleting of the beads with each centrifugation.*

4. Depending on your experimental design, incubate the conjugated microbeads in 100 µg/ml of biotinylated LPS for 30 minutes at room temperature with constant shaking on a vortex. It is highly recommended to compare the cross-presentation outcome of phagocytic cargo derived OVA in the presence or absence of LPS stimulation. Therefore, microbeads sequentially conjugated with GFP-OT then LPS would serve as TLR ligand positive antigenic cargo and those conjugated with GFP-OT alone would serve as TLR ligand negative antigenic cargo.

5. Wash the microbeads four times in PBS containing 0.5% FCS, as before.

6. The microbead-coated antigens can now be resuspended in the appropriate media for immediate use or stored at 4°C overnight for use in DC cross-presentation experiments the next day, as described in detail in Basic Protocol 6.

Support Protocol 4: Preparation of apoptotic cells

Similar to microbead-coated antigens, apoptotic cells are used as a source of cell-associated antigen that would be internalized by phagocytosis *in vivo*, and then processed and cross-presented by DCs. Typically, murine $CD19^+$ B cells are used as a model apoptotic cell,

which has been isolated from the lymphoid organs, specifically the spleen, of a transgenic mouse that endogenously expresses a model antigen of interest, such as OVA-expressing transgenic mice, which express the OVA antigen on the surfaces of all cells in the animal, including B cells. We have bred our OVA-transgenic mice onto a BALB/c background ($H2^d$) to avoid potential presentation of OVA by B cells to OVA-specific $H2^b$-restricted $CD8^+$ T cells. The $CD19^+$ $H2^d$ B cells are isolated and subjected to apoptosis *ex vivo* to be used as model of an uninfected apoptotic cell, or, alternatively, if blasting of B cells is required, then this is done by incubating the B cells with endotoxin free anti-mouse immunoglobulin (H+L), prior to induction of apoptosis (Blander & Medzhitov, 2006). Additionally, to prepare a model of infected apoptotic cells, the $CD19^+$ B cells are cultured *ex vivo* and stimulated with LPS to induce blasting of the B cells, prior to induction of apoptosis. Flow cytometric analysis is used to confirm the induction of apoptosis and absence of secondary necrosis. Apoptotic B cells are resuspended in RPMI to prepare them for addition to DC cultures. The protocol below outlines instructions to prepare such cells to use in DC cross-presentation experiments.

Materials

Reagents, Solutions, Test Organisms, Cells

- 7 - 11 week old ACT-mOVA mice transgenic for a membrane-bound form of ovalbumin (The Jackson Laboratory, cat. no. 005145)
- RPMI 1640-supplemented media with 2% heat-inactivated FCS (*See Reagents and Solutions*)
- RPMI 1640-supplemented media (*See Reagents and Solutions*)
- Endotoxin-free RBC lysis buffer (Sigma, cat. no. R7757)

- MACS buffer (*See Reagents and Solutions*)
- CD19 MicroBeads, mouse (Miltenyi Biotec, cat. no.130-121-301)
- Recombinant Murine IL-4 (PeproTech, cat. no. 214-14)
- Rabbit Anti-Mouse IgG (H+L)-UNLB (SouthernBiotech, cat. no. 6170-01)
- FITC Annexin-V Apoptosis detection kit with 7-AAD (BioLegend, cat. no. 640922)
- Heat-inactivated FCS (R&D Systems. Formerly Atlanta Biologicals, cat. no. S11550H)

Hardware

- Dissecting scissors
- Forceps
- 70 µm nylon-mesh cell strainer
- 6-well plate
- 10cc syringe
- Pasteur pipette
- 15 ml polypropylene conical tube
- 50 ml polypropylene conical tube
- LS magnetic separation column (Miltenyi Biotec, cat. no. 130-042-401)
- QuadroMACS™ Separator (Miltenyi Biotec, cat. no. 130-091-051)
- MACS MultiStand (Miltenyi Biotec, cat. no. 130-042-303)

- Hemocytometer or a cell counter
- UV-irradiator
- 15 cm polystyrene petri dish
- T175 flask, polystyrene and tissue culture treated

Protocol

Isolation and magnetic separation of antigen expressing splenic CD19$^+$ B cells

1. Four days prior to adding the apoptotic B cells to the DCs for cross-presentation experiments, sacrifice the mouse which expresses the required antigen and harvest its spleen. Follow your institute's sacrificing guidelines and minimize animal suffering.

2. Place the mouse in supine position with its forelimbs and hindlimbs pinned onto a dissecting board and spray it with ethanol before placing it into a tissue culture hood. *All steps from hereinafter are to be carried out under sterile conditions.*

3. Using a pair of forceps, gently lift the abdominal wall and use dissecting scissors to make a vertical cut from near the urethral orifice to the area just below the neck. Take care not to cut or puncture any organs.

4. Make two horizontal cuts on the left side of the mouse from top and bottom ends of the first cut to peel the abdominal wall back to reveal the internal abdominal organs. Using forceps, gently harvest the spleen.

5. Place the spleen inside of a 70 µm cell strainer that is sitting on a well of a 6-well plate containing RPMI 1640-supplemented media. Mash the spleen through the strainer using the plunger portion of a 10cc syringe.

6. Mix the splenocyte suspension by gently pipetting up and down using a pasteur pipette, pipette through the cell strainer as well, and transfer the cells to a 15 ml conical tube.

7. Centrifuge the cells at 300 x g for 5 minutes at room temperature. Remove and discard the supernatant carefully.

8. Resuspend the cells in 1 ml of RBC lysis buffer, and incubate for exactly 1 minute at room temperature.

9. Add 10 ml of MACS buffer to neutralize the RBC lysis buffer.

10. Centrifuge the cells at 300 x g for 5 minutes at 4°C. Remove and discard the supernatant carefully.

11. Resuspend cells in 176 µl of MACS buffer and 24 µl of CD19 MicroBeads. Incubate at 4°C for 25 minutes.

12. Sterilize the MACS separation apparatus (MACS™ Separator and MACS MultiStand) with ethanol prior to placing it into the biosafety cabinet. Carefully place a LS magnetic separation column into position in the MACS separation apparatus. Activate the column by adding 3 ml MACS buffer to it and allow it to completely pass through the column. Discard the eluate.

13. Add 800 µl of MACS buffer to the cells and add it to the column. Wash the 15 ml conical tube with 2 ml MACS buffer and add it to the column to recover the

majority of the $CD19^+$ B cells. Allow the volume to pass through the column and discard the eluate.

14. Wash the column three times by adding 3 ml MACS buffer and allowing it to pass through the column. Discard the eluate.

15. Remove the column from the MACS separation apparatus and add 5 ml of MACS buffer to the column. Use the column plunger to swiftly expel and elute the MACS buffer from the column into a clean 15 ml conical tube. Contained in this fraction are the $CD19^+$ OVA expressing B cells.

16. Count the $CD19^+$ B cells centrifuge at 300 x g for 5 minutes at 4°C. Remove and discard the supernatant carefully.

17. The $CD19^+$ B cells can now be subjected to apoptosis *ex vivo* to be used as model of uninfected apoptotic cell by following the steps in the next section of this protocol, *starting from step 20*. However, continue with the next steps if blasting of B cells is required to prepare model of either uninfected or infected apoptotic B cells.

18. Resuspend the cells at a concentration of 3 x 10^6 cells per ml in RPMI 1640-supplemented media, and plate 3 ml a well in a 6-well plate.

19. Induce blasting in the $CD19^+$ B cells by stimulating the cells either with; 100 µg/ml anti-mouse IgG (H+L) (and 50 ng/ml of recombinant murine IL-4), ***or*** add 25 µg/ml of LPS to each well. IL-4 can be added to aid B cell viability. Incubate the cells at 37°C in a humidified atmosphere containing 5% CO_2 for 3 days.

Inducing and assessing apoptosis in antigen expressing B cells

20. The night before the final day of culturing the $CD19^+$ B cells, add 10 ml FCS to a 20 cm petri dish and incubate at 37°C in a humidified atmosphere containing 5% CO_2 overnight.

21. Harvest the $CD19^+$ B cells from the 6-well plate. Count the cells, and resuspend in PBS at 1 x 10^6 cells per ml.

22. Remove the FCS in the petri dish and add up to 30 x 10^6 cells to the FCS-coated 15 cm petri dish. Induce the cells to undergo apoptosis by UV irradiation at 2.5 mJ/cm^2.

23. Pipette the cells up and down several times in the petri dish to dislodge any cells that may have adhered to the dish and transfer the cells to a 50 ml conical tube. Count the cells.

24. Centrifuge the cells at 300 x g for 5 minutes at room temperature. Remove and discard the supernatant carefully.

25. Resuspend the cells in RPMI 1640-supplemented media (containing serum). Incubate for different times (4, 6, 10 and 18 hours).

26. At each time point, take an aliquot of 1 x 10^6 of the cells to assess for apoptosis by flow cytometry. A time course must be conducted to determine the earliest time point at which Annexin-V positive cells appear.

27. Wash that portion of cells twice with 10 ml ice cold PBS, then resuspend in 1x Annexin-V binding buffer (contained in the kit) at a concentration of 1 x 10^6 cells

per ml. Transfer 100µl (i.e., 1 x 10^5) of the cell suspension to a new 15 ml conical tube for use as an unstained control.

28. Add 5 µl of the FITC Annexin-V dye and 5 µl of the 7-AAD dye (contained in the kit) to the remainder of the cells to stain for apoptosis and secondary necrosis. Gently vortex the cell suspension, and incubate for 15 minutes at room temperature in the dark.

29. Analyze the cells by flow cytometry immediately or within an hour. The Annexin-V binding buffer must be used to flush the flow cytometer prior to sample analysis. *Annexin-V positive but 7-AAD negative cells indicate the correct induction of apoptosis, since expression of 7-AAD is indicative of secondary necrosis, a more inflammatory form of cell death which could alter the results of downstream cross-presentation experiments.*

30. The apoptotic $CD19^+$ B cells can now be resuspended in the appropriate media (namely RPMI 1640-supplemented media) for immediate use in DC cross-presentation experiments, as described in detail in Basic Protocol 6.

- *It is recommended to add the apoptotic $CD19^+$ B cells as soon as a percentage of them begins to label with Annexin-V. This is because the apoptosis will continue in culture after the $CD19^+$ B cells are added to the DCs in culture. This is best practice because the DCs will internalize the apoptotic $CD19^+$ B cells as soon as the phosphatidylserine (target of the Annexin-V protein) is exposed on the outer leaflet of the plasma membrane. Waiting too long to increase the percentages of apoptotic cells comes at the cost of increasing the percentages of cells undergoing secondary necrosis which is inflammatory to the DCs and should be avoided when one is studying the non-inflammatory phagocytosis of apoptotic cells.*

Support Protocol 5: Preparation of recombinant bacteria

Bacteria are usually internalized by DCs through phagocytosis and the cargo processed for cross-presentation in these cells. This process involves DCs utilizing and engaging different surface receptors to capture and phagocytose bacteria. Subsequent processing of the microbe and the generation of peptides, as well as loading onto MHC-I molecules, may engage signaling cascades differing from those of endocytic cargoes or the phagocytosis of apoptotic cells. The protocol below outlines instructions for the preparation of recombinant bacteria for use in DC cross-presentation experiments.

Materials

Reagents, Solutions, Test Organisms, Cells

- Log-phase glycerol stock of recombinant OVA-expressing *E. coli* (*See previously published material* (Blander & Medzhitov, 2006))
- LB Broth Base (Invitrogen cat. no. 12780052)
- Ampicillin (Sigma cat. no. A5354)
- IPTG (Sigma cat. no. 367-93-1)
- Glycerol
- RPMI 1640-supplemented media (*See Reagents and Solutions*)

Hardware

- 250 ml Erlenmeyer (conical) flask
- Spectrophotometer capable of reading absorbance at 600nm
- 50 ml polypropylene conical tube
- 2 ml cryovials

Protocol Steps

1. Inoculate 1 ml of the log-phase of recombinant *E. coli*, which express your model antigen of interest, to 50 ml LB Broth in an Erlenmeyer flask and supplemented with 100 µg/ml of Ampicillin, *or other appropriate selective antibiotic*. *To prepare a log-phase E. coli working stock, grow a flask of E. coli to an* OD_{600} *reading of ~1, then aliquot to 1 ml cryovials containing 25% glycerol (diluted in* dH_2O*) and store at -80°C.*
2. Grow the bacteria in a shaker at 37°C to an OD_{600} reading of ~0.6 to ensure they are in log-phase. *The amount of time to reach 0.6 will vary depending on the batch and strain of bacteria, as well incubation temperature, and this will need to be determined in a separate experiment.*
3. Induce the expression of the model antigen of interest by incubating the bacteria with fresh 2 mM IPTG, and incubate in a shaker at 37°C for 5-6 hours. *It is important to always use freshly prepared/freshly thawed IPTG for induction of expression of the model antigen of interest.*
4. Heat-inactivate the bacteria in 60°C water for 45 minutes. To do this, dilute the induced culture to an OD_{600} reading of 0.6 in a 50 ml conical tube. Screw the tube cap very tightly, and wrap it with parafilm, then submerge and incubate the conical tube in a 60°C water

bath for 45 minutes. *The dilution to an OD_{600} reading of 0.6 is to ensure that the heat inactivation happens in the amount of time the bacteria are incubated at 60°C.*

5. Store the bacteria for up to 18 hours at 4°C prior to being added to DCs, or proceed immediately.
6. Count the number of heat-inactivated bacteria in the 50 ml conical tube with a spectrophotometer. Centrifuge the bacteria at 3,000 x g for 5 minutes. Remove and discard the supernatant carefully.
7. Wash the heat-inactivated bacteria once in RPMI 1640-supplemented media to get rid of any remaining salts from the LB Broth. Centrifuge and discard the supernatant as before.
8. Resuspend the heat-inactivated bacteria in RPMI 1640-supplemented media to the required concentration, for immediate use in DC cross-presentation experiments, as described in detail in Basic Protocol 6.

Basic Protocol 7: Immunocytochemistry immunofluorescence (ICC/IF)

Immunofluorescence microscopy, specifically confocal microscopy, is a powerful tool that has been utilized to gain an insight into the complex mechanisms which orchestrate cross-presentation in DCs. The protocol below has been optimized to be used to probe, visualize, and unravel the signaling molecules, including proteins, lipids, structures and organelles in DCs, that are responsible for the capturing, internalization and processing of endocytic and phagocytic cargoes, organelle fusion, the loading of peptides on MHC-I molecules, and cross-presenting them on the cell surface.

Materials

Reagents, Solutions, Test Organisms, Cells

- GM-CSF or Flt3L *in vitro* differentiated DCs (*see Basic Protocol 2 and 3*), or *in vivo* derived splenic DCs (*see Basic Protocol 4*)
- RPMI 1640 (Gibco, cat. no. 11875093)
- RPMI 1640-supplemented media (*See Reagents and Solutions*)
- PBS (Gibco, cat. no. 14190144)
- Heat-inactivated FCS (R&D Systems, formerly Atlanta Biologicals, cat. no. S11550H)
- 16% PFA (ThermoFisher, cat. no. 28906)
- Glycine (Fisher Scientific, cat no. BP381-5)
- BSA (Sigma cat. no. 9048-46-8)
- Saponin (Sigma, cat. no. S4521)
- VectaShield Hardset mounting medium (Vector Laboratories, cat. no H-1400)

Hardware

- Alcian blue-coated round 12mm glass coverslips (*see Support Protocol 6)*
- 24-well plate, polystyrene and tissue culture treated
- Hemocytometer or a cell counter
- 45° curved tapered fine point forceps
- Glass microscope slides

Protocol Steps

1. Place sterile Alcian Blue-treated 12mm glass coverslips into the wells of a sterile 24-well plate, using a set of curved fine point forceps.
2. Collect fully differentiated DCs, *as prepared in Basic Protocol 2, 3, or 4.* Harvest the DCs in the 24-well plate by flushing the media contained in the well up and down several times using a p1000 pipette. Pipette carefully and do not let the pipetted contents touch the bottom of the pipette itself to avoid contamination. If not possible, use filtered pipette tips to avoid contamination. Ensure to flush around the edges of the wells, as this is where much of the DC clusters are formed. Then, collect the media in the wells and place into a 15 ml conical tube. Immediately add 1 ml of ice-cold PBS to the well, flush and collect the remainder of DCs. If DCs were isolated as in *Basic Protocol 4*, skip this step and proceed to counting the cells, as described in step 3 below.
3. Count the cells suspended in the media/PBS using a hemocytometer or a cell counter.
4. Plate 2 x 10^5 DCs per well in 1 ml RPMI 1640-supplemented media into wells of a 24-well plate containing Alcian blue coated coverslips.
5. Centrifuge the cells at 300 x g for 2 minutes at room temperature to sediment the cells. Avoid excessive handling or shaking of the cells to prevent spontaneous DC maturation.
6. Incubate the cells for 30-60 minutes at 37°C in a humidified atmosphere containing 5% CO_2. *You should see that the cells are attached to the bottom of the plate and stretching out their dendrites, when viewed under a light microscope.*
7. Stimulate the DCs with the desired cross-presentation cargo and for the desired time, *as described in Basic Protocol 6*.

8. Aspirate the media from the wells and wash the coverslips once by adding 1 ml cold PBS to each well, then aspirate and discard the PBS. Fix the cells by adding 1 ml 1% PFA to each well and incubate for 20 minutes at room temperature.

9. Carefully aspirate the PFA and wash once with 2 ml PBS containing 2% BSA.

10. Quench the PFA fixation by adding 1 ml of serum-free RPMI 1640 media containing 0.5 M glycine to each well, and incubate for 20 minutes at room temperature.

11. Aspirate the PFA/media from each well and wash once with 2 ml PBS containing 2% BSA. *The fixed DCs can now be temporarily stored in PBS at 4°C or processed immediately.*

12. Permeabilize and block the DCs by adding 1 ml RPMI 1640 media containing 0.2% saponin and 10% FCS to each well. Incubate for 30 minutes at room temperature. Aspirate and discard the media.

13. Dilute primary antibodies in RPMI 1640 media containing 0.2% saponin and 10% FCS and incubate for 1 hour at room temperature, in the dark. Aspirate and discard the media containing the primary antibody. Minimize light exposure from this point forward.

14. Wash the coverslips four times with 2 ml RPMI 1640 media containing 0.2% saponin and 10% FCS. Leave coverslips for 1 minute in each wash. Aspirate and discard the wash buffer.

15. Dilute secondary antibody in RPMI 1640 media containing 0.2% saponin and 10% FCS and incubate for 30 minutes at room temperature, in the dark. Aspirate and discard the media containing the secondary antibody.

16. Wash the coverslips four times with 2 ml PBS. Leave coverslips for 1 minute in each wash. Aspirate and discard the wash buffer.
17. Mount the coverslips face down onto glass microscope slides using VectaShield or another suitable mounting media containing an appropriate nuclear dye, e.g., DAPI or Hoechst. Leave to air dry for one hour at room temperature or overnight at 4°C, in the dark. The mounted coverslips can now be imaged immediately or stored at 4°C, in the dark, for later use.
18. View the slides using a confocal microscope with sections spanning 0.4-0.9 μm on the z-axis. Representative data is shown in Figure 4.

[*Insert Figure 4 near here]

- *Labelling plasma membrane bound proteins or lipids, such as surface receptors or to compartmentalize the plasma membrane, must take place prior to the permeabilization step.*
- *The primary and secondary antibody concentrations and incubation times may need to be optimized empirically for best results or may need to be used according to the manufacturers' guidelines.*

Support Protocol 6: Preparation of Alcian blue coated coverslips

Introduction

Alcian blue is a polyvalent basic dye that is used to treat glass coverslips to form positively charged surfaces. This positively charged surface will allow the negatively charged plasma membrane of DCs to firmly adhere to the glass coverslip. This strong interaction is aided

by the binding of multiple localized charges and Van der Waals forces (Fadeel & Xue, 2009). Precationizing glass coverslips are essential to ensure the DCs remain adhered to the glass coverslips whilst subjected to the multiple wash and incubation steps during the sample preparation.

Materials

Reagents, Solutions, Test Organisms, Cells

- Alcian blue 8 GX dye (Sigma, cat. no. A9186)
- Distilled water

Hardware

- 12mm round glass coverslips, thickness 0.13-0.17 mm (Carolina, cat. no. 633029)
- 500 ml 0.22 µm filter bottle, cellulose acetate membrane
- Microwave
- Glass jar
- 45° curved tapered fine point forceps

Protocol Steps

1. Prepare 1% Alcian blue 8 GX dye in distilled water and filter using a 0.22 µm filter bottle. Store at 4°C.
2. Place coverslips in the Alcian blue solution in a glass jar. Ensure adequate coverage.

3. Heat by microwaving at High setting for 1-2 minutes. Take care not to boil the Alcian blue solution.
4. Let coverslips sit in the hot solution for 10 minutes. Ensure coverslips are well dispersed by occasionally swirling the glass jar every 2-3 minutes.
5. Wash coverslips with deionized distilled water to remove excess Alcian blue.
6. Dry coverslips by placing them individually on paper towels, using a pair forceps. Make sure the water droplets do not dry on the coverslips. This is best avoided by not having a large droplet on the coverslip but rather a thin film that should dry quickly.
7. Sterilize coverslips by autoclaving, and store in a sterile condition for later use.

- *Alcian blue is a strongly colored dye. Handle with care to avoid staining of skin and clothes*.

Basic Protocol 8: CD8⁺ T cell activation to assess cross-presentation

The *in vitro* co-culture system of transgenic CD8⁺ T cells with stimulated and activated DCs is a robust simulation of the physiological interactions between those two cells *in vivo.* Cross-priming of antigen-specific CD8⁺ T cells by activated DCs causes stimulation, activation and proliferation of the former, which in turn indicates successful cross-presentation of antigens in the latter. Therefore, assessing and monitoring the activation and proliferation of CD8⁺ T cells allows for several experimental opportunities to asses cross-presentation.

For instance, activated CD8⁺ T cells upregulate the expression of CD69 within several hours post stimulation that peaks at approximately 24 hours, which makes it ideal to be

detected by flow cytometric analysis and used as an early activation marker of $CD8^+$ T cells (Cibrián & Sánchez-Madrid, 2017; Simms & Ellis, 1996). Another flow cytometric method that is readily utilized to assess activated $CD8^+$ T cell proliferation is the dilution of carboxyfluorescein diacetate succinimidyl ester (CFSE). This method is a common, powerful, and sensitive technique to measure the cross-presentation capacity of APCs, including DCs. For instance, this detection technique is a $>10^4$-fold more sensitive than detection by a conformation dependent antibody such as the 25D1.16 antibody, which is one of the few available antibodies specific to formed peptide:MHC-I complexes (Nair-Gupta et al., 2014). T cells internalize the CFSE dye by diffusion, which is then cleaved by cellular esterases and subsequently reacts with amine and lysine resides in the cytosol of the cells. In order to induce activation and proliferation, a CFSE-labeled TCR-transgenic T cell only needs to encounter a very small number of cognate peptide:MHC-I complexes, and the subsequent cell division will result in two daughter cells where each carries half of the CFSE dye molecules as the parent cell. Further successive cell divisions will result in further halving of the stain in daughter cells, thus establishing an inverse correlation between the number of cell divisions and the level of detected CFSE dye. This change in fluorescence can be readily measured using a flow cytometer, and the resultant data provides a robust way to assay and evaluate the differences in cross-presentation under various conditions, including cross-priming abilities of APCs, the effect of antigens used, incubation time, amongst other factors and variables being studied (Quah & Parish, 2010).

The co-culture medium represents yet another opportunity to assess the activation and proliferation of the $CD8^+$ T cells. The co-culture medium can be used in ELISA tests for several secreted cytokines indicative of T cell activation and proliferation. Upon activation, $CD8^+$ T cells express cytokines for expression on the cell surface or to secrete into their environment either to directly engage in immune defense, as effector cytokines, or aid in their own proliferation (Charles A Janeway et al., 2001; Cox et al., 2013). Three of such cytokines, which are frequently

used to assay for $CD8^+$ T cell activation *in vitro,* are interferon gamma (IFN-γ), tumor necrosis factor alpha (TNF-α), and IL-2. IFN-γ works by directly interfering and inhibiting viral replication and replication of other pathogens, whilst at the same time causes infected cells to increase the production of the necessary molecules, including MHC-I molecules, to present antigens on their surface (Charles A Janeway et al., 2001; Wong & Pamer, 2003). TNF-α is a powerful pro-inflammatory cytokine and can synergize with IFN-γ to activate innate immune cells to kill infected or cancerous cells (Ye et al., 2018). TNF-α is secreted within hours of $CD8^+$ T cell stimulation (Brehm et al., 2005). IL-2 is a vital cytokine for the proliferation and development of many immune cells, including $CD8^+$ T cells (Boyman & Sprent, 2012; Zhang & Bevan, 2011).

$CD8^+$ T cells bearing a TCR which corresponds to the model antigen, whose cross-presentation by DCs is being monitored, are first harvested from the lymphoid organs, specifically the spleen, of TCR-transgenic mice, such as the OT-I mouse specific for OVA antigen. The cells are then labeled with the CFSE dye and co-cultured with fixed DCs, which had been previously stimulated with model antigen, or unstimulated for control experiments, at a ratio of 2 $CD8^+$ T cells for every DC. $CD8^+$ T cells require costimulation to remain viable, which is provided on the surface of DCs that have been stimulated with a PRR ligand such as LPS. However, $CD8^+$ T cells which are co-cultured with unstimulated DCs or DCs deficient for TLR or TLR signaling molecules require an exogenous source of CD28 costimulatory signals, such as anti-CD28 antibodies. The resulting CFSE-labeled $CD8^+$ T cells are analyzed with a flow cytometer. The protocols below outline instructions to prepare and assay such cells to use in DC cross-presentation experiments.

Materials

Reagents, Solutions, Test Organisms, Cells

- Fixed DCs, both antigen treated and untreated (*see Basic Protocol 6*)
- RPMI 1640-supplemented media (*See Reagents and Solutions*)
- CFSE-labelled TCR-transgenic $CD8^+$ T cells (*see Support Protocol 7*)
- Ultra-LEAF™ Purified anti-mouse CD3 Antibody (BioLegend, cat. no. 100238)
- Ultra-LEAF™ Purified anti-mouse CD28 Antibody (BioLegend, cat. no.102115)
- Commercially available ELISA kits for detecting murine IFN-γ, TNF-α, and IL-2 cytokines
- FACS buffer (*See Reagents and Solutions*)
- Alexa Fluor® 647 anti-mouse CD8β Antibody (Biolegend, clone YTS156.7.7, cat. no. 126612)
- Purified anti-mouse CD69 antibody (Biolegend, clone H1.2F3, cat. no. 104502)

Hardware

- Hemocytometer or a cell counter
- U-shaped 96-well plate, polystyrene and tissue culture treated
- 15 ml polypropylene conical tube
- 1.5 ml Eppendorf tube

Protocol Steps

1. Prepare fixed DCs stimulated with the desired cross-presentation antigens or cargoes, as described in detail in Basic Protocol 6, in three separate 96-well plates, containing identically treated DCs; with each plate designated for the following experiments; **A)** detecting $CD8^+$ T cell activation cytokine markers IFN-γ, TNF-α, and IL-2 using ELISA

tests; **B)** detecting the expression of the early activation marker CD69 on the surface of $CD8^+$ T cells by flow cytometric analysis; and **C)** assessing the proliferation of CFSE-labeled $CD8^+$ T cells (*See Support Protocol 7*) by flow cytometric analysis. Label each 96-well plate as "Experiment A, B, or C". Make sure the appropriate cross-presentation controls are included.

2. Resuspend freshly isolated and CFSE-labeled or unlabeled $CD8^+$ T cells, *as described in Support Protocol 7*, in RPMI 1640-supplemented media, at a concentration of 1×10^6 cells per ml. *Make sure the correct transgenic $CD8^+$ T cells are isolated which express the TCR specific for the model antigen used for cross-presentation by the DCs.*

3. Add 200 µl (*i.e.,* 2×10^5 $CD8^+$ T cells) of unlabeled $CD8^+$ T cells into the wells designated for experiments A and B, and CFSE-labeled $CD8^+$ T cells into the wells designated for experiment C, into the correct 96-well plates that already contain the fixed DCs. Make sure a positive control for $CD8^+$ T cell proliferation is included in each plate by adding 2 µg/ml anti-mouse CD3 and 0.5 µg/ml anti-mouse CD28 antibodies to $CD8^+$ T cells that are co-cultured with unstimulated fixed DCs, i.e., DCs that were not stimulated with antigens or cargoes, or exposed to PRR engaging agents.

4. Add 0.5 µg/ml anti-mouse CD28 antibodies to the wells which contain unstimulated control DCs, DCs deficient for TLR or TLR signaling adaptors, or DCs that were not stimulated in the presence of a PRR engaging agent, such as LPS, bacteria, or apoptotic cells. *Without PRR stimulation, the DCs will not express co-stimulatory ligands on their own, thus no cross-priming will occur even if the peptide:MHC-I complex is expressed on the DC surface.*

5. Incubate the cells at 37°C in a humidified atmosphere containing 5% CO_2. *In positive control wells, within 24-48 hours dark clusters of cells should be visible in the center of the wells to indicate that $CD8^+$ T cells have begun proliferating.*

6. At 20 hours post stimulation, collect the 96-well plate labeled **"Experiment A",** which had been designated for use in ELISA tests, carefully and slowly remove the supernatants with a p200 pipette and transfer to labelled 1.5 ml Eppendorf tubes for ELISA tests. Do not disturb the DCs at the bottom of the wells.

7. Centrifuge the supernatants at 300 x g for 10 minutes at 4°C to pellet cellular debris. Carefully aspirate the supernatants for immediate use in ELISA tests, or store at -80°C for later use. Measure the secretion of IFN-γ, TNF-α, and IL-2 cytokines as activation markers for $CD8^+$ T cells, (please *follow the manufacturers' guidelines for commercially available ELISA kits*).

8. At 24 hours post stimulation, collect the 96-well plate labeled **"Experiment B"** to use for flow cytometric analysis evaluating the expression of CD69 molecules on the surface of stimulated $CD8^+$ T cells as an early activation marker of these cells.

9. Centrifuge the cells at 300 x g for 5 minutes at 4°C and discard the supernatants carefully using a 200 pipette. Avoid accidentally pipetting and discarding the cells at the bottom of the wells.

10. Wash the cells twice by adding 200 µl of cold FACS buffer to each well of the 96-well plate, and centrifuge as before. Whilst washing the cells, prepare a staining cocktail by diluting the mouse anti-CD8β and anti-CD69 antibodies in FACS buffer. *The concentration of both of the mouse antibodies must be empirically calculated - guided by the manufacturer's instructions.*

11. Resuspend the cells in each well in 50µl of the staining cocktail, then incubate for 15 minutes at 4°C in the dark. *Remember to exclude some wells for use as negative controls for unstained and single stained cells for gating.*

12. Centrifuge the cells at 300 x g for 5 minutes at 4°C, and wash twice in FACS buffer, as before. Resuspend the cells in 200 µl of FACS buffer and analyze with a flow cytometer using the anti-CD8β antibody to gate for $CD8^+$ T cells and the anti-CD69 antibody to gate for activated $CD8^+$ T cells.

13. At 96 hours post stimulation, collect the final 96-well plate labeled **"Experiment C"** to use for flow cytometric analysis evaluating the dilution of the CFSE dye as a marker of proliferation of the cross-primed or stimulated $CD8^+$ T cells.

14. Prepare the samples for flow cytometric analysis by following steps 9-12 above with the following adjustments; omit the mouse anti-CD69 antibody from the staining cocktail prepared in step 10, use the CFSE dye to gate for CFSE-labeled $CD8^+$ T cells, and plot the data as a histogram. *On the flow cytometer, set the voltage such that the CFSE signal is positioned on the far right side of the histogram flow plot. This is important, as it will allow visualization of the separate peaks that correspond with each division of the CFSE-labeled $CD8^+$ T cells.* Sample data is shown below in Figure 5.

[*Insert Figure 5 near here]

- *As a cost-effective measure, ELISA kits can be substituted for individually purchased ELISA components (such as the appropriate capture and detection antibodies, recombinant proteins used as standards, substrates, stop solution, blocking buffer etc.) to assay cytokine concentrations, and following protocols published previously by the Blander Lab or elsewhere (Nair-Gupta et al., 2014).*

Support Protocol 7 - Isolation and labeling of CD8⁺ T cells with CFSE

Materials

Reagents, Solutions, Test Organisms, Cells

- 7 - 11 week old TCR-transgenic mice
    - OT-I mouse (Charles River Laboratories, C57BL/6-Tg (TcraTcrb) 1100Mjb/Crl)
    - CL4 mouse (The Jackson Laboratory, cat. no. #005308)
- PBS (Gibco, cat. no. 14190144)
- Endotoxin-free RBC lysis buffer (Sigma, cat. no. R7757)
- Heat-inactivated FCS (R&D Systems. Formerly Atlanta Biologicals, cat. no. S11550H)
- MACS buffer (*See Reagents and Solutions*)
- Mouse CD8α (Ly-2) MicroBeads (Miltenyi Biotec, cat. no. 130-117-044)
- RPMI 1640-supplemented media (*See Reagents and Solutions*)
- CellTrace™ CFSE Cell Proliferation Kit (Invitrogen, cat. no. C34554)

Hardware

- Dissecting scissors
- Forceps
- 70 µm nylon-mesh cell strainer

- 6-well plate
- 10cc syringe
- Pasteur pipette
- 15 ml polypropylene conical tube
- LS magnetic separation column (Miltenyi Biotec, cat. no. 130-042-401)
- QuadroMACS™ Separator (Miltenyi Biotec, cat. no. 130-091-051)
- MACS MultiStand (Miltenyi Biotec, cat. no. 130-042-303)
- Hemocytometer or a cell counter

Protocol Steps

Isolating transgenic CD8$^+$ T cells specific to a model antigen

1. Obtain a 7 - 11 week old transgenic mouse with the correct TCR specific for the model antigen of interest that is cross-presented by the DCs.
2. Sacrifice the mouse and harvest the spleen, brachial, and/or axillary lymph nodes. Follow your institute's sacrificing guidelines to minimize animal suffering.
3. Place the mouse in supine position with its forelimbs and hindlimbs pinned onto a dissecting board and spray it with ethanol before placing it into a tissue culture hood. *All steps from hereinafter are to be carried out under sterile conditions.*

4. Using a pair of forceps, gently lift the abdominal wall and use dissecting scissors to make a vertical cut from near the urethral orifice to the area just below the neck. Take care not to cut or puncture any organs.

5. Make two horizontal cuts on the left side of the mouse from top and bottom ends of the first cut to peel the abdominal wall back to reveal the internal abdominal organs. Using a pair of forceps, gently harvest the spleen.

6. Place the spleen inside of a 70 µm cell strainer that is sitting on a well of a 6-well plate containing RPMI 1640-supplemented media. Mash the spleen through the strainer using the plunger portion of a 10cc syringe.

7. Mix the splenocyte suspension by gently pipetting up and down using a pasteur pipette, pipette through the cell strainer as well, and transfer the cells to a 15 ml conical tube.

8. Centrifuge the cells at 300 x g for 5 minutes at room temperature. Remove and discard the supernatant carefully.

9. Resuspend the cells in 1 ml of RBC lysis buffer, and incubate for exactly 1 minute at room temperature.

10. Add 10 ml of MACS buffer to neutralize the RBC lysis buffer.

11. Count and centrifuge the cells at 300 x g for 5 minutes at 4°C. Remove and discard the supernatant carefully.

12. Resuspend the cells in 90 µl of MACS buffer and 10 µl of the mouse CD8α MicroBeads per 1 x 10^7 cells. Incubate at 4°C for 25 minutes.

13. Sterilize the MACS separation apparatus (MACS™ Separator and MACS MultiStand) with ethanol prior to placing it into the biosafety cabinet. Carefully place a LS magnetic

separation column into position in the MACS separation apparatus. Activate the column by adding 3 ml MACS buffer to it and allow it to completely pass through the column. Discard the eluate.

14. Add 800 µl of MACS buffer to the cells and add it to the column. Wash the 15 ml conical tube with 2 ml MACS buffer and add it to the column to recover the majority of the $CD8^+$ T cells. Allow the volume to pass through the column and discard the eluate.

15. Wash the column three times by adding 3 ml MACS buffer and allowing it to pass through the column. Discard the eluate.

16. Remove the column from the MACS separation apparatus and add 5 ml of MACS buffer to the column. Use the column plunger to swiftly expel and elute the MACS buffer from the column into a clean 15 ml conical tube. Contained in this fraction are the $CD8^+$ T cells.

17. Count the $CD8^+$ T cells, and centrifuge at 300 x g for 5 minutes at 4°C. Remove and discard the supernatant carefully.

18. Wash the $CD8^+$ T cells with10 ml of PBS and centrifuge as before.

19. Proceed to CFSE labeling in PBS, if the cells are to be used in a cross-presentation experiment where the readout is CFSE dilution. Otherwise, resuspend the $CD8^+$ T cells at a concentration of 1 x 10^6 cells per ml in RPMI 1640-supplemented media (and continue with the protocol in Basic Protocol 8).

Labeling $CD8^+$ T cells with CFSE dye

20. Resuspend the $CD8^+$ T cell fraction at a concentration of 2 x 10^7 cells per ml in PBS (without serum) in a 15 ml conical tube.

21. In a separate tube, dilute the CFSE dye which has been resuspended in DMSO in PBS (without serum) to a concentration of 10 μM.
22. Add equal volume of the CFSE diluted in PBS to the $CD8^+$ T cell suspension, to create a final concentration of 5 μM of CFSE dye in 1 x 10^7 cells per ml. Incubate the solution mixture for 8-10 minutes, at 37°C, in the dark. Lightly agitate the tube manually every 2 minutes.
23. Add 10ml PBS containing 2% FCS. (or at least 5 times the volume of CFSE and $CD8^+$ T cell mixture from the previous step). This step is important to remove any free dye remaining in the solution.
24. Centrifuge the cells at 300 x g for 5 minutes at room temperature. Remove and discard the supernatant carefully.
25. Count and wash the CFSE-labeled $CD8^+$ T cells once more in 10 ml PBS containing 2% FCS, and centrifuge as before.
26. Resuspend the CFSE-labeled $CD8^+$ T cells at a concentration of 1 x 10^6 cells per ml in RPMI 1640-supplemented media, and proceed with the protocol in Basic Protocol 8 to co-culture the cells with DCs for cross-priming and cross-presentation experiments.

- *Cervical, mesenteric and inguinal lymph nodes should not be collected, as these often contain a high proportion of activated T cells which could alter the results of the assay.*
- *The CFSE dye is toxic to cells, therefore, labelling $CD8^+$ T cells with the CFSE dye may result in losing some cells as a result.*

Reagents and Solutions: (this section details complex solutions that are used in protocols; thus, enabling them to be written as a single-line in your materials list).

1. RPMI 1640-supplemented media
    a. RPMI 1640 media (Gibco, cat. no. 11875093)
    b. 5% Heat-inactivated FCS (R&D Systems, formerly Atlanta Biologicals, cat. no. S11550H)
    c. 2mM L-Glutamine (Gibco, cat. no. 25030081)
    d. 100 Units/ml Penicillin-Streptomycin (Gibco, cat. no. 15140122)
    e. 1mM Sodium pyruvate (Gibco, cat. no. 11360070)
    f. 10mM HEPES buffer (Gibco, cat. no. 15630080)
    g. 1 X Non-essential Amino Acid solution (Gibco, cat. no. 11140050)
    h. 0.055mM 2-Mercaptoethanol (Gibco, cat. no. 21985023)

2. IMDM-supplemented media
    a. IMDM (Gibco, cat. no. 12440053)
    b. 5% Heat-inactivated FCS (R&D Systems, formerly Atlanta Biologicals, cat. no. S11550H)
    c. 2mM L-Glutamine (Gibco, cat. no. 25030081)
    d. 100 Units/ml Penicillin-Streptomycin (Gibco, cat. no. 15140122)
    e. 1mM Sodium pyruvate (Gibco, cat. no. 11360070)

f. 10mM HEPES buffer (Gibco, cat. no. 15630080)

g. 1 X Non-essential Amino Acid solution (Gibco, cat. no. 11140050)

h. 0.055mM 2-Mercaptoethanol (Gibco, cat. no. 21985023)

3. DMEM-supplemented media

a. DMEM (Gibco, cat. no.11965-092)

b. 5% Heat-inactivated FCS (R&D Systems, formerly Atlanta Biologicals, cat. no. S11550H)

c. 2mM L-Glutamine (Gibco, cat. no. 25030081)

d. 100 Units/ml Penicillin-Streptomycin (Gibco, cat. no. 15140122)

e. 1mM Sodium pyruvate (Gibco, cat. no. 11360070)

f. 10mM HEPES buffer (Gibco, cat. no. 15630080)

g. 1 X Non-essential Amino Acid solution (Gibco, cat. no. 11140050)

h. 0.055mM 2-Mercaptoethanol (Gibco, cat. no. 21985023)

4. FACS buffer

a. PBS (Gibco, cat. no. 14190144)

b. 5% heat-inactivated FCS (R&D Systems, formerly Atlanta Biologicals, cat. no. S11550H)

c. 0.1% Sodium Azide (NaN_3, Millipore Sigma, cat. No. S2002)

5. MACS buffer

a. PBS (Gibco, cat. no. 14190144)

b. 2% heat-inactivated FCS (R&D Systems, formerly Atlanta Biologicals, cat. no. S11550H)

c. 2mM EDTA (ThermoFisher, cat. no. 15575020)

COMMENTARY:

Understanding Results

BM derived and in vivo differentiated DCs should express the DC markers CD11b and CD11c on the cell surface. In resting and non-activated DCs, MHC-II molecules and the co-stimulatory molecules C40, CD80, and CD86 are expressed at low levels on the cell surface. The expression of the latter four molecules increase significantly upon DC stimulation and activation (see Basic Protocol 5 and Figure 3). Additionally, stimulated and activated DCs secrete pro-inflammatory cytokines such as TNFα and IL-6, which can be detected with ELISA tests (See Basic Protocol 5). Resting DCs or DCs which have been stimulated with an endocytic or a phagocytic cargo can be fixed and visualized with immunofluorescence microscopy and probed for different molecules and markers involved in cross-presentation (See Basic Protocol 7 and Figure 4).

Transgenic CD8+ T cells, which are specific for their cognate peptide cross-presented on the MHC-I molecule on the surface of DCs, are utilized to assess cross-presentation. These CD8+ T cells are co-cultured with fixed DCs (unstimulated or stimulated) and the proliferation of the

CD8+ T cells is measured by flow cytometry to assess if successful cross-priming and CD8+ T cell activation has taken place, and hence, a robust readout for cross-presentation of antigens by DCs (See Basic Protocol 8 and Figure 5). Co-cultured CD8+ T cells with unstimulated DCs or DCs deficient for TLR or TLR signaling molecules must be cultured with exogenous anti-CD28 costimulatory antibodies. Additionally, early CD8+ T cell activation markers can also be used to assess cross-presentation. The expression of CD69 molecules on the surface of CD8+ T cells increase significantly 24 hours post activation and can be measured by flow cytometry, whilst the secretion of cytokines such as IFN-γ, TNF-α, and IL-2 are also upregulated 20 hours post stimulation and can be detected in the supernatant as indicators of CD8+ T cell early activation markers with ELISA tests (See Basic Protocol 8). Add aCD28 sentence.

Critical Parameters

- Prior to starting any DC cross-presentation experiments, it is vitally important to test that all reagents are free from endotoxins. Particular attention must be paid to ensuring every batch of FCS, that is used to supplement any culture media or buffers at any stage of the DC cross-presentation experiments, is endotoxin free. This is because constituting components in the FCS solution, mainly endotoxins, may activate and mature DCs, hence making it difficult to plan controlled experiments thus resulting in irreproducible and invalid results. The steps outlined in Basic Protocol 5 are to be followed to test each new reagent, including new batches, that is introduced at any stage of the protocols described throughout, to ensure the said new reagent does not cause activation and maturation of DCs because of trace amounts of endotoxin that are not reported by manufacturers.

- When performing GM-CSF or Flt3L DC differentiation for the first time in your laboratory, the DC cultures should be analyzed by flow cytometry (*see Basic Protocol 5*) to determine the desired phenotypic expression profile of the DCs that are required for your experimental needs.
- Trypan blue exclusion test of cell viability can be incorporated into the protocol when counting cells to assess cell viability.

Troubleshooting

Problem	Possible cause	Solution
No cross-presentation	No PRR stimulation	Include a PRR agonist, e.g., LPS
	Spontaneous maturation of DCs	Take extra care whilst handling DCs and minimize vibrations
	Antigen (particularly if this is cell-associated antigen from apoptotic cells) was not delivered to the DC	*See Blander and Medzhitov, 2006* for a previously published protocol to verify phagocytosis of apoptotic cells by DCs (Blander & Medzhitov, 2006)
Cross-presentation in control/non-stimulated DCs	Non-endotoxin-free reagents used and/or contamination	Test all reagents for endotoxins prior to use
Apoptotic B cell blasts not staining for Annexin-V	Did not use the proper buffer	Ensure all staining and acquisition is done in the Annexin-V staining buffer included in the kit
Low yields of DCs	The incubation with RBC lysis buffer is too long	Be very careful to incubate for exactly one minute with the

		RBC lysis buffer
Cross-presentation is observed even in the absence of TLR ligands, for instance, when using the microbeads coated with model antigen	The concentration of model antigen is too high. In the presence of high amounts of model antigen, the need for TLR ligands can be bypassed. *See Blander and Medzhitov, 2006* (Blander & Medzhitov, 2006), where a titration with and without LPS was conducted	Carefully titrate the amount of model antigen given to the DCs by creating serial dilutions of the antigen, in whatever form it is being delivered, and evaluate cross-presentation
$CD8^+$ T cells dying after CFSE labeling	CFSE at high concentrations can be toxic to the $CD8^+$ T cells	Use less of the CFSE dye to label the $CD8^+$ T cells
$CD8^+$ T cells dying after co-culturing with PFA fixed DCs	Residual PFA has not been adequately washed away from fixed DCs. PFA is highly toxic to living cells, and as such, any residual PFA would kill the $CD8^+$ T cells	Follow the protocol to adequately wash away any residual PFA from the fixed DCs. If necessary, include extra washes and longer incubations before co-culture
No cross-presentation occurring in DCs stimulated with *E. coli* expressing the model antigen of interest	IPTG used to induce the expression of the model antigen of interest is old	Use freshly prepared, or freshly thawed, IPTG to induce the expression of the model antigen of interest

Table 2. **Troubleshooting**

Time Considerations

Protocol	**Time required**	**Hands-on time**
Basic Protocol 1	1 - 1.5 hours	45 minutes
Basic Protocol 2	5 days	1 hour

Support Protocol 1	7 days	1 hour
Basic Protocol 3	7 days	45 minutes
Support Protocol 2	7 - 10 days	1 - 2 hours
Basic Protocol 4	7 - 11 days	4 - 6 hours
Basic Protocol 5	8 - 10 hours	3 - 5 hours
Basic Protocol 6	3 - 24 hours	1 - 3 hours
Support Protocol 3	3 hours	2 hours
Support Protocol 4	4 days	3 hours
Support Protocol 5	1 day	2 hours
Basic Protocol 7	5 - 24 hours	3 - 4 hours
Support Protocol 6	1 - 2 hours	0.5 - 1 hours
Basic Protocol 8	4 days	4 - 6 hours
Support Protocol 7	1 day	2 - 4 hours

Table 3. **Time considerations.**

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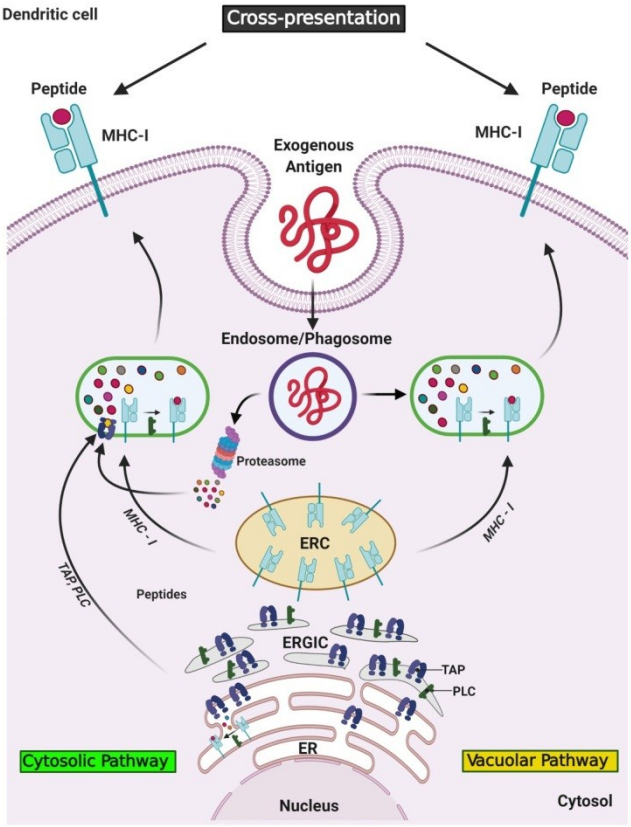

Figure 1. **An illustration of the two pathways by which cross-presentation is currently understood to occur in DCs.** Exogenous peptides are endocytosed or phagocytosed into the

cell. In the cytosolic pathway, the cargo is transported into the cytosolic space for processing via cellular proteasomes. The resultant peptides are either translocated back into a nascent endosome for loading of MHC-I molecules or conceivably, as less evidence exists in support of this, they are chaperoned to the ER for loading onto MHC-I molecules, and cross-presented on the surface of the cell. In the vacuolar pathway, the cargo is processed in the endosome/phagosome and the resultant peptides are loaded onto MHC-I molecules there, then presented on the surface of the cell. The endocytic recycling center (ERC) is an important source of MHC-I for cross-presentation of microbial antigens. The PLC and TAP molecules are provided by the ERGIC. ER: endoplasmic reticulum, ERGIC: ER-Golgi intermediate compartment, PLC: peptide-loading complex, TAP: transporter associated with antigen processing.

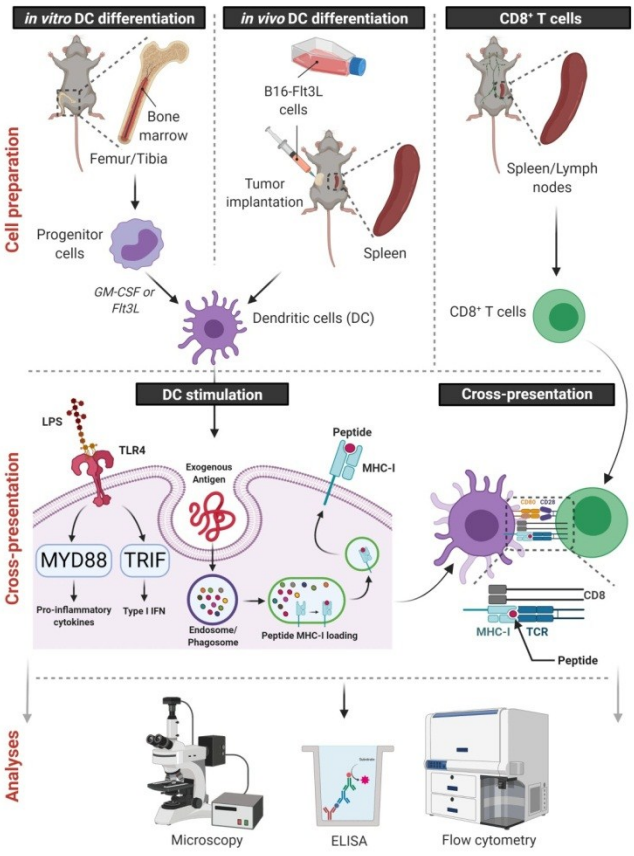

Figure 2. **A schematic workflow showing the outline for the preparation of DCs and CD8⁺ T cells, and the co-culture of the two to study cross-presentation i*n vitro*.** In the cell

preparation phase, DC progenitor cells are isolated from femur and tibia bone marrow of 7-11 week-old mice and are differentiated *in vitro* into GM-DCs or cDC1-like cells, by culturing them for 5 days or 7-10 days in the presence of GM-CSF, or Flt3L, respectively. *In vivo* differentiated cDC1 cells are isolated from the spleen of similar aged mice implanted with B16-Flt3L tumor cells for 7 days. Similar aged mice specific for the model antigen are used to obtain $CD8^+$ T cells from the spleen or lymph nodes. In the cross-presentation phase, DCs are given a model antigen cargo for cross-presentation (exogenous antigen) along with an appropriate stimulant, e.g., LPS *as shown here*. Isolated $CD8^+$ T cells are co-cultured with stimulated, fixed DCs to evaluate cross-presentation. In the analyses phase, DCs can be analyzed with immunofluorescence microscopy to visualize cargo internalization and subcellular localization and surface expression of MHC-I. ELISA tests can be used to detect the expression levels of appropriate cytokines which may indicate the activation of TLR signaling in DCs, whilst flow cytometry analysis can indicate the activation/maturation state of DCs by assessing surface markers. T cells that had been co-cultured with DCs are analyzed by flow cytometry to evaluate their activation state, and later to assess proliferation after cross-priming by DCs. ELISA tests can also be utilized to measure cytokine expression in activated $CD8^+$ T cells.

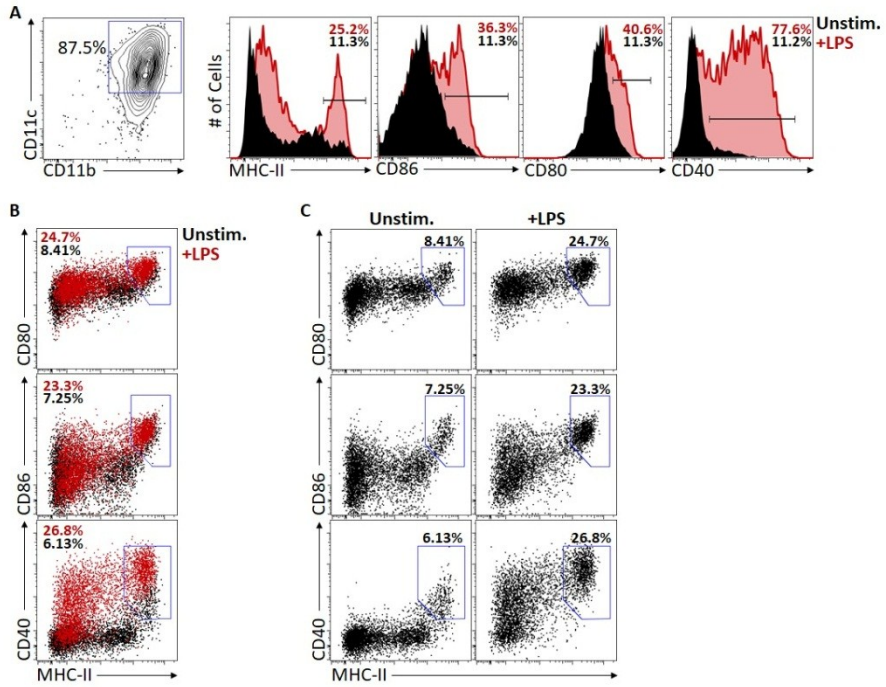

Figure 3. **Examples of DC maturation profiles as assessed by flow cytometry.** DCs were cultured as described in Basic Protocol 2. At the end of the culture period (18 hours), cells were analyzed with flow cytometry to assess the expression level of CD11b, CD11c, MHC-II, CD86, CD80, and CD40. **A.** CD11c and CD11b expression on the DC showing 87.5% of the cells are positive for these DC markers. Histogram plots gated on the $CD11b^{+}CD11c^{+}$ DCs show expression of MHC-II, CD86, CD80 and CD40 on unstimulated (black histograms) versus LPS stimulated (red histograms) DCs. It is expected that resting DC will have some level of expression of these markers, however, as the data show, their expression is markedly increased upon LPS stimulation. **B** and **C.** The same data as in (**A**) but plotted as dot plots rather than histograms and either as an overlap of the resting versus LPS-stimulated DCs (**B**) or each DC condition separately (**C**). This type of plotting of the data enables one to simultaneously assess the expression of each costimulatory molecule versus MHC-II. These are typical data that are consistent with GM-DCs being successfully cultured and activated in the presence of the TLR agonist LPS.

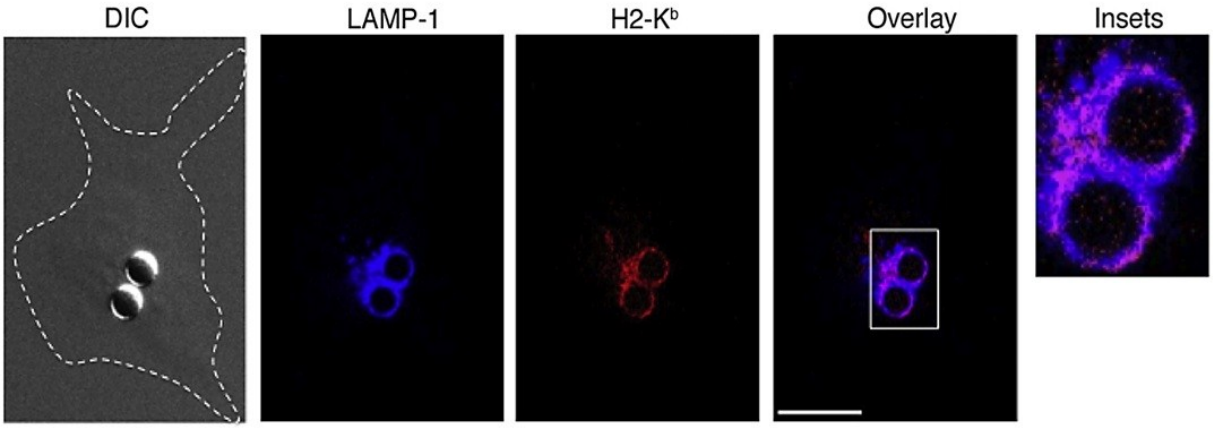

Figure 4. **Confocal image of GM-DCs showing the recruitment of MHC-I to phagosomes carrying LPS-conjugated beads.** DCs were cultured and given LPS-coated microbeads, *as described in Basic Protocol 6.* The DCs were then prepared as described in this protocol (*Basic Protocol 7*) and labelled with antibodies against: lysosomal-associated membrane protein (LAMP)-1 to probe the phagocytosed microbeads; and MHC-I (labelled with H2-K^b antibody) to probe the recruitment and colocalization of MHC-I molecules to the phagosome. Images are of a single z-stack plane acquired using a confocal microscope with a 63x/1.4 NA oil immersion objective. Scale bars represent 10 μm. Image adapted from previously published data by the Blander Lab (Nair-Gupta et al., 2014).

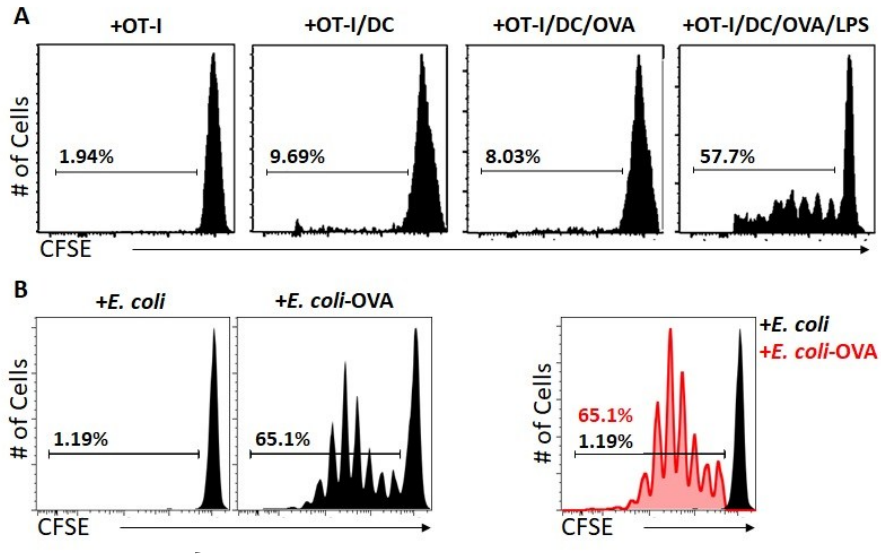

Figure 5. **The representative results of a CFSE dilution assay to measure the proliferation of OT-I T cells in response to cross-presented antigen.** $CD8^+$ T cells were co-cultured with DCs that had been given (**A**) soluble OVA protein as a model of endocytic antigen or (**B**) *E. coli* or recombinant OVA-expressing E. coli (*E. coli*-OVA). DC were incubated for a period of 4.5 hours then then fixed. **A.** Panels from left to right; OT-I T cells alone; OT-I T cells co-cultured with DCs in absence of a cross-presentation cargo; OT-I T cells co-cultured with DCs that had been given the endocytic cargo OVA to cross-present but in absence of LPS; OT-I T cells co-cultured with DCs that had been given the endocytic cargo OVA to cross-present in presence of LPS. **B.** The histogram plots show two different ways of depicting the CFSE dilution data, either as each histogram separately (left) or overlaid (right panel). All wells in (A) also contained anti-CD28 stimulating antibodies. As expected, TLR stimulation of DCs augments the cross-presentation of OVA but can only occur when the DCs are stimulated with LPS, thus allowing the DCs to cross-prime and activate the OT-I T cells causing them to proliferate, as seen by the dilution of the CFSE dye in panel 4 of (A). E. coli naturally contain LPS in their cell walls and stimulate robust costimulatory expression by DCs, thus no exogenous provision of anti-CD28 antibodies is necessary.