NCL Method ITA-34

Detection of Antigen Presentation by Murine Bone Marrow-Derived Dendritic Cells

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This protocol assumes an intermediate level of scientific competency with regard to techniques, instrumentation, and safety procedures. Rudimentary assay details have been omitted for the sake of brevity.

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1. Introduction

Nanoparticles have been extensively tested as therapeutic vaccine delivery vehicles for the treatment of cancer. A key effort in this area is the delivery of antigens to antigen-presenting cells (APCs), such that tumor-specific peptides are presented in the context of class I major histocompatibility complex (MHC) molecules. The purpose of this protocol is to determine the ability of nanoparticles to deliver antigen to APCs, such that an antigenic class I peptide sequence is properly presented in the context of MHC. For the purposes of this assay, the model antigen ovalbumin (OVA) is used in the context of murine cells. The protocol requires the synthesis of nanoparticles that deliver SIINFEKL ($OVA_{257-264}$), the immunodominant class I peptide derived from OVA, either in its peptide form or as part of a larger molecule (such as the whole OVA protein). The 25-D1.16 antibody, which binds to SIINFEKL presented in the context of the mouse H2-K^b MHC molecule [1], is used to detect antigen presentation on APCs after nanoparticle delivery. The results of this protocol may be used to infer the ability of the nanoparticle system to deliver other similar antigens.

2. Principles

Bone marrow-derived dendritic cells (BMDCs) are used for the antigen presentation assay. Bone marrow cells are collected from 8-12 week-old C57BL6 mice and cultured in medium supplemented with murine granulocyte-macrophage-colony-stimulating factor (GM-CSF) for differentiation into BMDCs. After 7 days of culture, differentiated BMDCs are treated with nanoparticles and relevant controls for 24 hours. After incubation, cells are then stained with 25-D1.16 or an isotype control and analyzed for fluorescence by flow cytometry. Although cell lines can be used instead of primary BMDC, care must be taken to select cell lines that express the H2-K^b MHC molecule, since SIINFEKL is an H2-K^b-restricted antigen. The DC2.4 cell line, which is derived from C57BL6 mice and is capable of presenting OVA-derived antigens [2], is one example of an appropriate cell line.

3. Reagents, Materials, and Equipment

Note: The NCL does not endorse any of suppliers listed below; their inclusion is for informational purpose only. Equivalent supplies from alternative vendors can be substituted.

3.1 Reagents

1. Phosphate buffered saline (PBS) (HyClone, SH30256.01)
2. Cell culture grade water, endotoxin-free (HyClone, SH30529.02)
3. Ficoll-Paque Premium (GE HealthCare, 17-5442-03)
4. Fetal bovine serum (Hyclone, SH30070.03)
5. RPMI1640 (Invitrogen, 11875-119)
6. Pen/Strep solution (Invitrogen, 15140-148)
7. L-glutamine (Hyclone, SH30034.01)
8. Cellometer ViaStain AOPI Staining Solution in PBS (Nexcelom Biosciences, CS2-0106; or other reagent for distinguishing between live and dead cells)
9. Albumin, from bovine serum (BSA) (Sigma, A4503)
10. Formaldehyde, 20% (Tousimis, 1008A)
11. Recombinant Murine GM-CSF (Peprotech, 315-03)
12. SIINFEKL positive control: OVA 257-264 class I peptide (Invivogen, vac-sin)
13. 25-D1.16 PE antibody: Anti-Mouse OVA257-264 (SIINFEKL) peptide bound to H-2Kb PE (eBioscience, 12-5743-82)
14. Isotype PE antibody: Mouse IgG1 k Isotype PE (eBioscience, 12-4724-41)
15. ACK Lysis buffer (Lonza, 10-548E)
16. 70% Ethanol

3.2 Materials

1. Pipettes, 0.05 to 10 mL
2. Polypropylene tubes, 50 and 15 mL
3. Cell culture dish, 10 cm
4. 6-well culture plate (Thermo Fisher Scientific, 140685)
5. 24-well cell culture plate (Corning, 3524)
6. Polystyrene round bottom 12 x 75 mm^2 (Falcon tubes, 352058)
7. Single edge razor blades (VWR, 10040-386)
8. 5 mL sterile, individually wrapped syringes
9. 25G needles
10. Cell strainer, 40 μm (Corning, 352340)

3.3 Equipment

1. Refrigerator, 2-8°C

2. Freezer, -20°C
3. Cell culture incubator with 5% CO_2 and 95% humidity
4. Biohazard safety cabinet approved for level II handling of biological material
5. Vortex
6. Cellometer, Source or Hemocytometer to perform cell count
7. Flow Cytometer (e.g., FACSCalibur)
8. Centrifuge, 400xg (or ~1440 rpm)

4. Animals

This protocol utilizes 8-12 week-old C57BL/6 males or females. Each set of mouse femurs and tibiae generates a large number of BMDCs ($>50x10^6$ cells). Therefore, there is no need to pool cells. For work conducted outside NCI at Frederick facilities, ensure that animal work is supported by Animal Care and Use Committee (ACUC) approved protocols.

NCI at Frederick is accredited by AAALAC International and follows the Public Health Service *Policy for the Care and Use of Laboratory Animals* (Health Research Extension Act of 1985, Public Law 99-158, 1986). Animal care is provided in accordance with the procedures outlined in the *Guide for Care and Use of Laboratory Animals* (National Research Council, 1996; National Academy Press, Washington, D.C.). All animal protocols are approved by the NCI at Fredrick institutional Animal Care and Use Committee.

5. Reagent and Control Preparation

5.1 Complete RPMI

The complete RPMI medium should contain 10% FBS (heat-inactivated), 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin sulphate. Store at 2-8°C protected from light for no longer than 1 month. Before use, warm the medium in a 37°C water bath.

5.2 Heat-inactivated fetal bovine serum

Thaw a bottle of FBS at room temperature or overnight at 2-8°C and allow to equilibrate to room temperature. Incubate 30 minutes in a 56°C water bath, mixing every 5 minutes to heat-inactivate it. Single-use aliquots may be stored at 2-8°C for up to one month or at a nominal temperature of -20°C indefinitely.

5.3 GM-CSF

Prepare stock solution by reconstituting commercial lyophilized recombinant murine GM-CSF in endotoxin-free cell culture grade water to a concentration of 0.5 mg/mL. Prepare small aliquots at 5 µL each and store them at -80ºC for up to 12 months. On the day of experiment, add freshly thawed GM-CSF to complete RPMI to a final concentration of 20 ng/mL.

5.4 FACS Staining Buffer

FACS staining buffer should be prepared by diluting neat FBS in 1X sterile Ca^{2+}/Mg^{2+}-free PBS to a final concentration of 1% FBS (i.e. by adding 0.5 mL FBS to 49.5 mL 1X PBS). Store this solution at 2-8°C.

5.5 Fixative

The flow cytometry fixative should be prepared on the day of the experiment by diluting 20% formaldehyde in 1X sterile Ca^{2+}/Mg^{2+}-free PBS to a final concentration of 2% formaldehyde (i.e. by adding 1 mL 20% formaldehyde to 9 mL PBS). Store this solution at 2-8°C.

5.6 Positive Control

The positive control is the SIINFEKL peptide (OVA257-264). Reconstitute a peptide stock solution by adding 1 mL of endotoxin-free water to 1 mg of peptide. Aliquot this solution and store at -20°C for up to 6 months. On the day of experiment, add freshly thawed SIINFEKL peptide to complete RPMI to a final concentration of 5 µg/mL.

5.7 Negative Control

Use PBS as a negative control.

5.8 Vehicle Control

Vehicle control is the buffer or media used to formulate test nanomaterials. Common excipients used are trehalose, sucrose, and albumin. However, other reagents and materials are also used alone or in combination. Vehicle control should match formulation buffer of the test-nanomaterial by both composition and concentration. This control can be skipped if nanoparticles are stored in PBS.

6. Preparation of Nanoparticles

When the experiment is conducted in 24-well plates, the assay requires 1.5 mL of nanoparticles dissolved/re-suspended in complete culture medium at a concentration 5X higher than the highest final test concentration. Test concentrations are based on the calculated plasma concentration of the nanoparticle at the intended therapeutic dose. For the purpose of this protocol this concentration is called "theoretical plasma concentration". Considerations for estimating theoretical plasma concentration have been reviewed elsewhere [3] and are summarized in Box 1 below.

Box 1. Example Calculation to Determine Nanoparticle Concentration for In Vitro Tests

In this example, we assume a mouse dose of 123 mg/kg. Therefore, the scaled equivalent human dose would be:

$$Human\ dose = \frac{mouse\ dose}{12.3} = \frac{123\ mg/kg}{12.3} = 10\ mg/kg$$

Blood volume constitutes approximately 8% of the body weight. Therefore, an average human of 70 kg body weight has approximately 5.6 L of blood. Assuming all the nanoparticle injected goes into the systemic circulation, this provides a rough approximation of the potential maximum nanoparticle concentration in blood, which is used as the in vitro test concentration.

$$in\ vitro\ concentration_{human\ matrix} = \frac{human\ dose}{human\ blood\ volume} = \frac{70\ kg * 10\ mg/kg}{5.6\ L} = 0.125\ mg/mL$$

This assay will evaluate four concentrations: 10X (5X if 10X cannot be achieved, or 100X or 30X when feasible) of the theoretical plasma concentration, the theoretical plasma concentration, and two 1:5 serial dilutions of the theoretical plasma concentration. When the intended therapeutic concentration is unknown, 1 mg/mL is used as the highest concentration. Alternatively, the highest reasonably achieved concentration can be used if 1 mg/mL is unattainable.

For example, if the theoretical plasma concentration to be tested is 0.2 mg/mL, a stock of 10 mg/mL is prepared. This sample is then diluted 10-fold (1.0 mg/mL), followed by two 1:5 serial dilutions (0.2 and 0.04 mg/mL). When 0.2 mL of each of these sample dilutions are combined in

a culture plate with 800 µL cells, the final concentrations of nanoparticles are 0.008, 0.04, 0.2, and 2 mg/mL. Each nanoparticle concentration is plated two times.

7. Isolation and Counting of Bone Marrow Cells

7.1 Position euthanized mouse on its back and rinse fur thoroughly with 70% alcohol. (Euthanize animals according to the protocol approved by your institution.)

7.2 Cut a slit in the fur just below the rib cage without cutting the peritoneal membrane.

7.3 Firmly grasp skin and peel back to expose hind limbs. Trim away skin after the limbs are fully exposed.

7.4 Using sterile sharp dissecting scissors, cut around the hip joint of each limb to free it from the pelvis. Cut through ligaments and excess tissue, being careful not to break the limb bones in the process.

7.5 After freeing the limbs, cut away as much of the muscle tissue from the bone as possible. Leave the footpads on the limb at this stage. Use kimwipes to aid in this process by gently massaging the bone to clean away residual tissue.

7.6 Sterilize the cleaned bones and attached footpads by complete immersion in 70% ethanol for 1 minute.

7.7 Transfer cleaned and sterilized mouse legs to a cell culture dish with 20 mL media. From this point onwards, only manipulate the legs with sterile forceps and a razor. Thoroughly sterilize gloves, and do not touch the media in the dish. To sterilize tools, immerse in 70% ethanol for at least 5 minutes.

7.8 Using a razor blade, cut away the footpad at the ankle joint. Discard the footpad.

7.9 Using the same razor blade, cut the femurs near the hip joint and knee joint to expose the interior marrow shaft. Cut the tibia near the other end of the knee joint to expose the marrow of the tibia. Keep all parts of the limbs (hip joint, knee joint, femurs, tibiae) submerged in the media.

7.10 Using a 5cc syringe with a 25G needle, draw up 5 mL of complete media.

7.11 For each limb section, use forceps to keep the bone steady and insert bevel of needle into joint opening / marrow shaft. Gently flush out the marrow into the culture dish, and continue flushing until bones are white instead of red. For the joint sections, a brief media flush in the joint openings will suffice to dislodge available cells.

7.12 Insert a 40 μm cell strainer into the opening of a 50 mL conical tube. Using a 10 mL pipette, collect all the media in the culture dish and pipette it through the cell strainer to collect the filtered cells into the conical tube.

7.13 Centrifuge collected cells at 400xg for 5 minutes. Resuspend the cells in 5 mL of ACK lysis buffer for the hypotonic lysis of red blood cells. Immediately centrifuge the cells at 400xg for another 5 minutes, then resuspend cells in 10 mL of complete medium.

7.14 Perform cell count and viability assay. For the Cellometer, dilute 20 μL of collected cells with 20 μL of AOPI staining solution, then pipette 20 μL of mixed cells into a counting slide and run cell counts on the automated cell counter. Anticipated yield is approximately $50x10^6$ bone marrow cells per hindlimb.

8. Generation of Immature Dendritic Cells

8.1 Resuspend BMDCs at $1x10^6$ cells/mL in complete medium supplemented with 20 ng/mL GM-CSF. For this example, cells isolated from two hindlimbs of a single animal are resuspended in 100 mL of complete medium, supplemented with 4 μL of recombinant mouse GM-CSF at a stock concentration of 0.5 mg/mL.

8.2 Distribute all media containing bone marrow cells into 6-well plates, dispensing 5-6 mL per well.

8.3 Culture bone marrow cells in an incubator maintained at 37ºC, 5% CO_2 and 95% humidity for 5 days.

8.4 On day 5, media in the 6-well plates should be yellow or orange-yellow in color. Dislodge non-adherent and semi-adherent cells by pipetting the media in each well up and down using a 1 mL pipette. Collect all non-adherent and semi-adherent cells into 50 mL conical tubes and discard the plates with adherent cells.

8.5 Centrifuge collected cells at 400xg for 15 minutes.

8.6 Aspirate the supernatant and resuspend cells in 100 mL of complete medium, freshly supplemented with 20 ng/mL GM-CSF.

8.7 Distribute cells into new 6-well plates, dispensing 5-6 mL per well.

8.8 Continue culturing cells in the incubator for 2 more days.

8.9 After the final 2-day culture, cell media should once again be yellow in color. Collect all non-adherent and semi-adherent cells as in step 8.4, and wash cells as in step 8.5.

8.10 Resuspend collected BMDCs in 20 mL of complete medium and determine cell count and viability.

9. Treatment with Nanoparticles and Controls

9.1 Adjust BMDC cell count to $1.25x10^6$ cells/mL.

9.2 Aliquot 800 µL of BMDC suspension per designated well of a 24-well plate.

9.3 Add 200 µL PBS to the negative control well. Add 200 µL of an appropriate vehicle control to wells marked as "VC". Add 200 µL of complete media to wells marked as "unlabeled cells".

9.4 Add 200 µL of test nanoparticles and control to designated wells and incubate for 24 hours at 37°C, 5% CO_2, and 95% humidity.

9.5 Two hours before the end of step 9.4 (test nanoparticle incubation), add 200 µL of media with 5 µg/mL of SIINFEKL to each well marked as "PC".

9.6 At the end of the incubation period, collect all cells from the plate and transfer samples into pre-labeled polystyrene round-bottomed 12 x 75 mm^2 Falcon tubes.

9.7 Prepare 25-D1.16 PE staining solution by adding 2 µL of antibody stock solution to 798 µL of FACS staining buffer.

9.8 Prepare Isotype PE staining solution by adding 2 µL of antibody stock solution to 798 µL of FACS staining buffer.

9.9 Wash cells with 2 mL PBS and aspirate / decant supernatant.

9.10 Resuspend cells in 100 µL of appropriate staining solution.

9.11 Incubate cells at room temperature, protected from light, for 30 minutes.

9.12 Wash cells with 1 mL PBS and aspirate / decant supernatant.

9.13 Fix cells with Fixative (2% formaldehyde) for 15 minutes at room temperature. Samples are now ready to be analyzed by flow cytometry.

10. Flow Cytometry

At the NCL, we use FACSCalibur. The procedure below is based on our experience with this instrument. If you are using another cytometer, please follow the procedure specific to that instrument.

10.1 Switch on the instrument. Make sure the sheath tank is full, and the waste tank is empty.

10.2 To get consistent results, run BD FACSComp software using BD CaliBRITE beads.

10.3 Adjust FSC vs. SSC dot plot using unlabeled cells to get the population of cells in the plot. See Figure 1 below for example.

10.4 Set the threshold to remove most of the debris.

10.5 Gate on the major cell population as BMDCs (gate1). See Figure 1 for example.

10.6 Create an FL2 histogram for the BMDC cell population. Adjust the FL2 voltage such that the majority of unlabeled or untreated cells have an intensity below 10^1. See Figure 2 for an example. Note that some treatments may activate BMDCs and increase their FSC and SSC; this may require adjustment as necessary.

10.7 Create FL2 histogram for each analyzed live cell population. See Figure 2 for an example.

10.8 Analyze shift of FL2 fluorescence in treated samples comparing to NC. Compare test sample data against corresponding isotype control to ensure that FL2 fluorescence is due to specific antibody binding.

10.9 Acquire and save data.

10.10 Follow the instrument closing procedure.

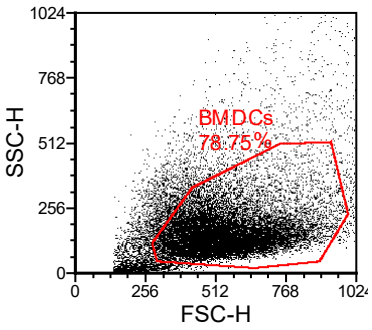

Figure 1. Gating example for BMDC cell populations.

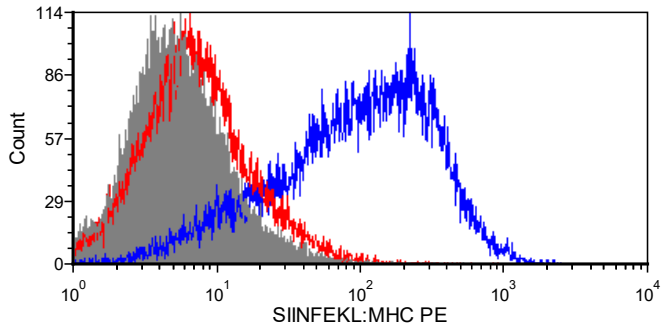

Figure 2. Detection of SIINFEKL presentation on APCs after pulsing with 1 µg/mL SIINFEKL peptide. Grey–NC with 25-D1.16 staining, blue–PC with 25-D1.16 staining, red–PC with isotype control staining.

11. Data Analysis and Report

Use appropriate software to analyze the data acquired in step 10.9. CellQuest or other flow cytometry software can be used. Here, FCSExpress from De Novo Software Solutions, Inc. was used.

Set up a gate excluding NC cells but including most PC cells. Cells in this gate may be designated 25-D1.16 positive; see Figure 3 for an example. Report the data as GeoMean of samples, % 25-D1.16 positive cells, and flow cytometry plots where appropriate.

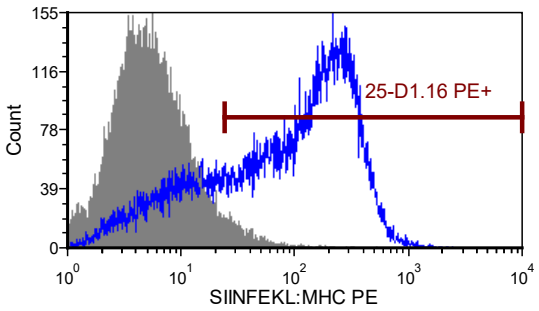

Figure 3. Gating example for 25-D1.16 positive cells. Grey–NC with 25-D1.16 staining, blue–PC with 25-D1.16 staining.

12. References

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13. Abbreviations

APC – antigen presenting cell

MHC – major histocompatibility complex

OVA – ovalbumin, or chicken egg albumin

GM-CSF – granulocyte-macrophage colony-stimulating factor

BMDC – bone marrow derived dendritic cell

FL – fluorescence

NC – negative control

PBS – phosphate buffered saline

PC – positive control

RPMI – Roswell Park Memorial Institute

TS – test samples

VC – vehicle control

14. Appendix

Example Plate Map

	1	2	3	4	5	6
A	NC	VC	PC	PC (isotype)		
B	TS (0.008 mg/mL)	TS (0.04 mg/mL)	TS (0.2 mg/mL)	TS (2 mg/mL)		
C	TS + Isotype (0.008 mg/mL)	TS + Isotype (0.04 mg/mL)	TS + Isotype (0.2 mg/mL)	TS + Isotype (2 mg/mL)		
D	Unlabeled Cells					

NC – negative control, VC – vehicle control, PC – positive control, TS – test sample. Shaded cells – samples to be stained with isotype control antibody