



## **NCL Method ITA-35**

### **Antigen-Specific Stimulation of CD8+ T-cells 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

The stimulation of antigen-specific T-cell responses is critical for therapeutic cancer vaccines. Extensive efforts have been made to develop nanoparticles as carriers of therapeutic cancer vaccines to antigen presenting cells (APCs) of the immune system. The purpose of this protocol is to use nanoparticles to deliver vaccines to APCs and measure the antigen-specific T-cell responses that are instigated. 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<sub>257-264</sub>), 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). CD8<sup>+</sup> T-cells that recognize SIINFEKL are purified from the transgenic OT-I mouse model [1], and activated using nanoparticle-treated APCs. The resulting T-cell proliferation, as well as secretion of the cytokines interferon gamma (IFN- $\gamma$ ) and interleukin-2 (IL-2), are used to measure antigen-specific T-cell activation. The results of this protocol may be used to infer the ability of the nanoparticle system to deliver other similar antigens.

## 2. Principles

This experiment is performed by co-culturing nanoparticle-treated APCs with antigen-specific CD8<sup>+</sup> T-cells. Bone marrow-derived dendritic cells (BMDCs) are used to process and present antigen delivered by test nanoparticles. 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. To determine the impact of nanoparticle treatment in the presence of adjuvant, LPS may be added during the treatment step. Treated BMDCs are then washed and co-incubated with purified SIINFEKL-specific CD8<sup>+</sup> T-cells, which are pre-labeled with carboxyfluorescein succinimidyl ester (CFSE) to track cell proliferation. After a further 3-day culture, supernatants are collected, and cells are fixed and analyzed by flow cytometry. The readouts from this experiment are measures of cell proliferation as determined by cell count and CFSE dilution, as well as the determination of IFN- $\gamma$  and IL-2 concentrations in cell supernatants by enzyme-linked immunosorbent assay (ELISA).

### 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. MEM Non-Essential Amino Acids Solution, 100X (Thermo Fisher Scientific, 11140-050)
9. Sodium pyruvate, 100 mM (Thermo Fisher Scientific, 11360-070)
10. MEM Vitamin Solution, 100X (Thermo Fisher Scientific, 11120-052)
11. 2-Mercaptoethanol, 55 mM (Thermo Fisher Scientific, 21985-023)
12. Cellometer ViaStain AOPI Staining Solution in PBS (Nexcelom Biosciences, CS2-0106; or other reagent for distinguishing between live and dead cells)
13. Albumin, from bovine serum (BSA) (Sigma, A4503)
14. Formaldehyde, 20% (Tousimis, 1008A)
15. Recombinant Murine GM-CSF (Peprotech, 315-03)
16. SIINFEKL positive control: OVA 257-264 class I peptide (Invivogen, vac-sin)
17. Lipopolysaccharide from E.coli K12 (Invivogen, LPS-EK)
18. CellTrace CFSE Cell Proliferation Kit (Thermo Fisher Scientific, C34570)
19. CD8a<sup>+</sup> T-Cell Isolation Kit, mouse (Miltenyi Biotec, 130-104-075)
20. Anti-mouse CD8a FITC (eBioscience, 11-0081-81)
21. Anti-mouse V alpha 2 TCR PE (eBioscience, 12-5812-80)
22. Rat IgG2A k isotype control FITC (eBioscience, 11-4321-80)
23. Rat IgG2A k isotype control PE (eBioscience, 12-4321-41)
24. ACK Lysis buffer (Lonza, 10-548E)
25. 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<sup>2</sup> (Falcon tubes, 352058)
7. Single edge razor blades (VWR, 10040-386)
8. 1 mL and 5 mL sterile, individually wrapped syringes
9. 25G needles
10. Cell strainer, 40 µm (Corning, 352340)
11. 1.7 mL polypropylene tubes
12. LS Columns for separation (Miltenyi Biotec, 130-042-401)
13. 6 µm polystyrene beads (Polysciences Inc, 07312-5)
14. 96-well polypropylene cluster tubes, 8-tube strip format (Corning®, 4408)

### 3.3 Equipment

1. Refrigerator, 2-8°C
2. Freezer, -20°C
3. Cell culture incubator with 5% CO<sub>2</sub> 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)
9. Separation magnet capable of handling LS Columns, i.e. QuadroMACS (Miltenyi Biotec, 130-090-976)

## 4. Animals

This protocol utilizes two types of mice: 8-12 week-old C57BL/6 male or female mice for BMDC culture, and 8-12 week-old OT-I TCR transgenic female mice (C57BL/6-Tg(TcraTcrb)1100Mjb/J, The Jackson Laboratory).

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 (ACUC). For work conducted outside NCI at Frederick facilities, ensure that animal work is supported by ACUC approved protocols.

## 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 T-Cell Medium

The complete T-cell medium should contain 10% FBS (heat inactivated), 2 mM L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, 1X MEM non-essential amino acids, 1 mM sodium pyruvate, 0.4X MEM vitamin solution, and 50 µM 2-mercaptoethanol. 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.

### 5.4 Isolation Buffer

The CD8<sup>+</sup> T-cell isolation medium should contain 1X PBS, 0.5% bovine serum albumin, and 2 mM EDTA. Filter the isolation buffer before use and store at 2-8°C.

### 5.5 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  $\mu$ L each and store them at -80°C 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.

#### 5.6 FACS Staining Buffer

FACS staining buffer should be prepared by diluting neat FBS in 1X sterile Ca<sup>2+</sup>/Mg<sup>2+</sup>-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.

#### 5.7 Fixative

The flow cytometry fixative should be prepared on the day of the experiment by diluting 20% formaldehyde in 1X sterile Ca<sup>2+</sup>/Mg<sup>2+</sup>-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.

#### 5.8 LPS

Reconstitute 1 mg LPS in 1 mL of endotoxin-free water. Aliquot and store at -20°C for up to 6 months. On the day of experiment, dilute freshly thawed LPS in complete RPMI medium to a final concentration of 200 ng/mL.

#### 5.9 CFSE

Reconstitute a 5 mM working solution of CFSE by adding 18  $\mu$ L of DMSO (provided in kit) to a vial of CFSE. Store this vial at -20°C, protected from light.

#### 5.10 Positive Control

The positive control is the SIINF EKL peptide (OVA<sub>257-264</sub>). 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, dilute freshly thawed SIINF EKL peptide in PBS to a final concentration of 4  $\mu$ g/mL.

#### 5.11 Negative Control

Use PBS as a negative control.

#### 5.12 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 [2] and are summarized in Box 1 below.

### ***Box 1. Example Calculation to Determine Nanoparticle Theoretical Plasma Concentration***

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

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

The blood volume of a human is 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 a human. The theoretical plasma concentration, i.e. in vitro test concentration, is calculated by:

$$\text{theoretical plasma concentration} = \frac{\text{human dose}}{\text{human blood volume}} = \frac{70 \text{ kg} \times 10 \text{ mg/kg}}{5.6 \text{ L}} = 0.125 \text{ mg/mL}$$

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  $\mu$ L cells, the final concentrations of nanoparticles are 0.008, 0.04, 0.2, and 2 mg/mL.

## **7. Isolation and Counting of Bone Marrow Cells**

- 7.1 Position euthanized C57BL/6 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 complete RPMI medium. From this point onwards, only manipulate the legs with sterile forceps and a razor. Thoroughly sterilize gloves, and do not touch the medium 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  $\mu\text{m}$  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 400 x g 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  $\mu\text{L}$  of collected cells with 20  $\mu\text{L}$  of AOPI staining solution, then pipette 20  $\mu\text{L}$  of mixed cells into a counting slide and run cell counts on the automated cell counter. Anticipated yield is approximately  $50 \times 10^6$  bone marrow cells per hindlimb.

## **8. Generation of Immature Dendritic Cells**

- 8.1 Resuspend BMDCs at  $1 \times 10^6$  cells/mL in complete RPMI medium supplemented with 20 ng/mL GM-CSF. For this example, cells isolated from two hindlimbs of a single animal may be resuspended in 100 mL of complete medium, supplemented with 4  $\mu\text{L}$  of recombinant mouse GM-CSF at the 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 37°C, 5% CO<sub>2</sub> 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 RPMI medium and determine cell count and viability.

## 9. Treatment with Nanoparticles and Controls

- 9.1 Adjust BMDC cell count to 250,000 cells/mL in complete RPMI medium.
- 9.2 Seed 100  $\mu$ L BMDCs (25,000 cells) in each designated well of a 96-well U-bottomed plate. Set up all experimental groups in triplicate.
- 9.3 Add 50  $\mu$ L PBS to 2 sets of negative control (NC) wells.
- 9.4 Add 50  $\mu$ L of 4  $\mu$ g/mL SIINFEKL to 2 sets of positive control (PC) wells.
- 9.5 For one set of negative control wells, add 50  $\mu$ L of LPS (200 ng/mL in complete RPMI medium). (NC + LPS)
- 9.6 For one set of positive control wells, add 50  $\mu$ L of LPS. (PC + LPS)
- 9.7 Add 50  $\mu$ L of test nanoparticles at 4X the desired final concentration to designated wells.
- 9.8 In treatment conditions where the addition of LPS is desired, add 50  $\mu$ L of 200 ng/mL LPS.
- 9.9 In all samples where LPS is *not* added, add 50  $\mu$ L of complete RPMI medium to make up final volume to 200  $\mu$ L.
- 9.10 Incubate BMDCs for 24 hours at 37°C, 5% CO<sub>2</sub>, and 95% humidity.
- 9.11 After 24 hr incubation, centrifuge plate at 400xg for 5 minutes.
- 9.12 Using a multichannel pipette, without disturbing the cell pellet, very carefully remove 150  $\mu$ L of supernatant and discard. Add 200  $\mu$ L of fresh complete RPMI medium.  
*Note: Do not use a vacuum-driven aspirator or manifold to wash the cells. BMDCs are loosely adherent and will be removed under vacuum.*

- 9.13 Centrifuge plate at 400xg for 5 minutes.
- 9.14 Using a multichannel pipette, without disturbing the cell pellet, carefully remove 100  $\mu$ L of supernatant and discard. Final volume in the BMDC wells should be 100  $\mu$ L of complete RPMI medium.

## 10. Extraction and Homogenization of OT-I Spleen

- 10.1 Position euthanized OT-I mouse on its right side (with left side facing up) and rinse fur thoroughly with 70% alcohol. (Euthanize animals according to the protocol approved by your institution.)
- 10.2 Using a pair of sterilized forceps, lift up the skin on the left flank. Cut away the fur just below the rib cage without cutting the peritoneal membrane.
- 10.3 The spleen is visible just under the peritoneal membrane. Cut a slit in the peritoneal membrane without damaging the spleen. Gently lift out the spleen using the forceps, cutting away supporting tissue. Transfer the spleen to a 15 mL tube containing cold T-cell medium.
- 10.4 In a 6-well plate, fill one well with 5 mL of T-cell medium. Place a 40  $\mu$ m cell strainer into the well such that the bottom of the cell strainer is submerged in the medium. Place the spleen into the cell strainer.
- 10.5 Remove the plunger of a 1 mL syringe. Do so by extending the syringe within the wrapper, carefully peeling back the wrapper, and removing the plunger by its stem without touching the flat end of the plunger.
- 10.6 Using the flat end of the plunger, homogenize the spleen by crushing it against the cell strainer. Move the plunger against the strainer in a circular motion until no red chunks remain.
- 10.7 Remove and discard the cell strainer and plunger. Transfer the splenic homogenate to a 15 mL tube.

## 11. CD8<sup>+</sup> T-Cell Labeling and Purification

*Note: CD8<sup>+</sup> T-cell separation is performed according to the manufacturer's protocol. From step 11.8 onwards, an example of  $50 \times 10^6$  nucleated spleen cells is used. Exact cell counts used during the experiment are determined based on cell yield and experiment requirements.*

- 11.1 Centrifuge homogenate from a single spleen at 400xg for 5 minutes. Resuspend cells in 500  $\mu$ L PBS. Process spleens separately if working with several samples.
- 11.2 Add 1  $\mu$ L of 5 mM CFSE to a separate volume of 500  $\mu$ L PBS. Vortex to mix well.
- 11.3 Add the 500  $\mu$ L of diluted CFSE to the 500  $\mu$ L volume of splenic homogenate and mix by pipetting up and down 5 times.
- 11.4 Incubate at 37°C for 10 minutes. Protect cells from light at all stages after this step.
- 11.5 Quench the CFSE labeling reaction by adding 5 mL of ice-cold T-cell media.
- 11.6 Centrifuge cells at 400xg for 5 minutes. Wash cells with 5 mL of PBS and centrifuge again with the same settings.
- 11.7 Resuspend labeled cells in 5 mL of T-cell media. Stain cells with AOPI and determine nucleated cell count on a Cellometer or other instrument.
- 11.8 Resuspend  $50 \times 10^6$  cells in 200  $\mu$ L isolation buffer in a 1.7 mL tube. Add 50  $\mu$ L of Biotin-Antibody Cocktail, mix well, and incubate for 5 minutes at 2-8°C.
- 11.9 Add 150  $\mu$ L isolation buffer to the cells. Vortex the vial of Anti-Biotin MicroBeads for 10 seconds. Add 100  $\mu$ L of Anti-Biotin MicroBeads to the cells, mix well and incubate for 10 min at 2-8°C.
- 11.10 Place LS Column in the magnetic field of the QuadroMACS magnet. Prepare column by adding 3 mL of isolation buffer and discarding the flow through.
- 11.11 Apply cell suspension onto the column. Collect flow through containing unlabeled cells, representing the enriched CD8<sup>+</sup> T-cells.
- 11.12 Wash column with another 3 mL of isolation buffer, collecting this fraction as well.
- 11.13 Resuspend purified CD8<sup>+</sup> T-cells in 5 mL of T-cell medium. Determine cell count and viability.

## 12. Cell Stimulation Experiment

- 12.1 Adjust purified CD8<sup>+</sup> T-cell count to  $5 \times 10^5$  cells/mL using T-cell medium (50,000 cells per 100  $\mu$ L).
- 12.2 Add 100  $\mu$ L of purified CD8<sup>+</sup> T-cells to the plate of treated BMDCs.
- 12.3 Centrifuge plate for 15 seconds at 400xg to pellet cells.
- 12.4 Incubate T-cell and BMDC co-culture for 66–72 hours at 37°C, 5% CO<sub>2</sub>, 95% humidity.

*Note: Monitor the cell culture periodically. As cells are activated, the media will change color from pink to yellow. Excessive yellow color may indicate overstimulation and may warrant early termination of the experiment.*

### **13. Assessing T-Cell Purity**

*Note: Purity of isolated OT-I CD8<sup>+</sup> T-cells may be verified by flow cytometry immediately after purification. This procedure generally produces CD8<sup>+</sup> populations with over 95% purity. Nevertheless, it is important for investigators to verify that the isolation procedure works when setting it up for the first time.*

- 13.1 Prepare staining solutions. To 400  $\mu$ L of FACS staining buffer, add 1  $\mu$ L each of anti-mouse CD8a FITC and anti-mouse V alpha 2 TCR PE antibodies (CD8 staining mix). To 400  $\mu$ L of FACS staining buffer, add 1  $\mu$ L each of isotype control FITC and isotype control PE antibodies (isotype staining mix).
- 13.2 Add  $0.5 \times 10^6$  purified T-cells to each of two 5 mL round-bottomed FACS tubes.
- 13.3 Add 1 mL PBS to each tube and centrifuge at 400xg for 5 minutes.
- 13.4 Aspirate or decant supernatant. Resuspend cells in 100  $\mu$ L of either CD8 or isotype staining mix prepared in step 13.1.
- 13.5 Stain cells for 30 minutes at room temperature. Protect from light.
- 13.6 Add 1 mL PBS to each tube and centrifuge at 400xg for 5 minutes. Aspirate or decant supernatant. Resuspend cells in 200  $\mu$ L of fixative (2% formaldehyde) and store at 2-8°C until analysis by flow cytometry.

### **14. Ending Experiment**

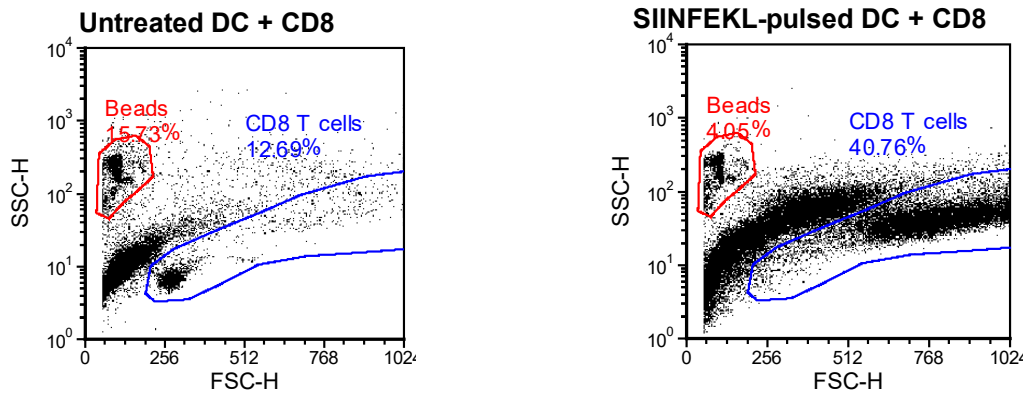
- 14.1 After co-culture is complete, centrifuge plate at 400xg for 5 minutes.
- 14.2 Carefully remove 150  $\mu$ L of supernatant per well and transfer to another 96-well plate for storage. Freeze supernatants at -80°C for storage.
- 14.3 Add 100  $\mu$ L of 3% formaldehyde to cells and mix well to fix the cells. This treatment gives a final formaldehyde concentration of 2% in a volume of 150  $\mu$ L. Incubate at room temperature for 15 minutes to fix, protected from light.
- 14.4 Transfer all samples to racked cluster tubes. Samples are now ready to be analyzed by flow cytometry; run them within 2-4 days after experiment.

14.5 (Perform this step just before flow cytometry analysis, if cell count using internal bead control is desired) Vortex the vial of 6  $\mu\text{m}$  beads to mix well. To a tube of 10 mL PBS, add 3 drops of beads and vortex to mix. Determine bead concentration using a cell counter. Add 50  $\mu\text{L}$  of pre-counted beads to each tube.

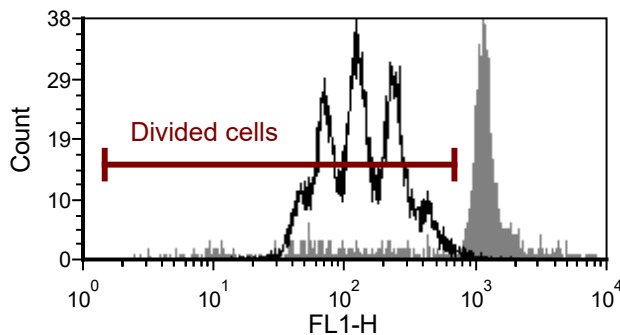
## 15. Flow Cytometry

*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.*

- 15.1 Switch on the instrument. Make sure the sheath tank is full, and the waste tank is empty.
- 15.2 To get consistent results, run BD FACSCComp software using BD CaliBRITE beads.
- 15.3 Adjust FSC vs. SSC dot plot using unlabeled cells to get the population of cells in the plot. See Figure 1 below for example.
- 15.4 Set the threshold to remove most of the debris.
- 15.5 Gate on the CD8<sup>+</sup> T-cell population and on the population of 6  $\mu\text{m}$  beads (Figure 1). Each population is distinct and easily distinguished; T-cells are low in FSC and SSC, while beads are low in FSC but high in SSC. Note that the T-cell population will change in FSC and SSC as T-cells are activated and expand (compare Figure 1A and Figure 1B). Adjust FSC and SSC settings to account for this.
- 15.6 Create an FL1 histogram for the T-cell population. Adjust the FL1 voltage such that the untreated, CFSE-labeled cells have an intensity above  $10^3$ . See Figure 2 for an example.
- 15.7 Acquire and save data.
- 15.8 Follow the instrument closing procedure.



**Figure 1. Gating example for T-cell and bead populations.** (Left) CD8<sup>+</sup> T-cells co-cultured with untreated BMDCs. (Right) CD8<sup>+</sup> T-cells co-cultured with SIINFEKL-treated BMDCs. Note that the T-cell population increases in both size (FSC) and granularity (SSC). The bead population remains in the same position. They have high SSC and are separate from the rest of the cells / debris. Also note that SSC is set here to a logarithmic scale. Specific settings may vary between users and instruments.



**Figure 2. Detection of CFSE dilution in dividing CD8<sup>+</sup> T-cells.** Grey – NC incubated with untreated BMDCs. Black – PC incubated with SIINFEKL-treated BMDCs.



## 16. Data Analysis and Report

Use appropriate software to analyze the data acquired in step 15.7. CellQuest or other flow cytometry software can be used. Here, FCSExpress from De Novo Software Solutions, Inc. was used.

- 16.1 Draw a gate on all cells with a lower fluorescence intensity than untreated cells for the “divided cells” gate. See Figure 2 for example.
- 16.2 Collect the following data from all samples: Bead gate event count, T-cell gate event count, FL1 gMFI of T-cells, and % of divided T-cells/total T-cells.
- 16.3 Determine the true cell count for each well using the following formula: True cell count = True bead concentration per well / Bead event count x T-cell event count.
- 16.4 Analyze changes in treated samples and controls comparing to NC. Report and analyze the following three metrics: True T-cell count, CFSE gMFI, and % divided T-cells.

## 17. References

1. Clarke, S.R., et al., *Characterization of the ovalbumin-specific TCR transgenic line OT-I: MHC elements for positive and negative selection*. Immunology and Cell Biology, 2000. **78**: p. 110-117.
2. Dobrovolskaia, M.A. and S.E. McNeil, *Understanding the correlation between in vitro and in vivo immunotoxicity tests for nanomedicines*. J Control Release, 2013. **172**(2): p. 456-66.

## 18. Abbreviations

APC – antigen presenting cell

OVA – ovalbumin, or chicken egg albumin

BMDC – bone marrow derived dendritic cell

GM-CSF – granulocyte-macrophage colony-stimulating factor

CFSE – carboxyfluorescein succinimidyl ester

ELISA – enzyme-linked immunosorbent assay

FL – fluorescence

NC – negative control

PBS – phosphate buffered saline

PC – positive control

RPMI – Roswell Park Memorial Institute

TS – test samples

VC – vehicle control

## 19. Appendix

### Example Plate Map

	1	2	3	4	5	6	7	8	9	10	11	12
A	NC	PC	VC	TS (0.008 mg/mL)	TS (0.04 mg/mL)	TS (0.2 mg/mL)	TS (2 mg/mL)					
B	NC	PC	VC	TS (0.008 mg/mL)	TS (0.04 mg/mL)	TS (0.2 mg/mL)	TS (2 mg/mL)					
C	NC	PC	VC	TS (0.008 mg/mL)	TS (0.04 mg/mL)	TS (0.2 mg/mL)	TS (2 mg/mL)					
D	NC	PC	VC	TS (0.008 mg/mL)	TS (0.04 mg/mL)	TS (0.2 mg/mL)	TS (2 mg/mL)					
E	NC	PC	VC	TS (0.008 mg/mL)	TS (0.04 mg/mL)	TS (0.2 mg/mL)	TS (2 mg/mL)					
F	NC	PC	VC	TS (0.008 mg/mL)	TS (0.04 mg/mL)	TS (0.2 mg/mL)	TS (2 mg/mL)					
G												
H												

NC – negative control, VC – vehicle control, PC – positive control, TS – test sample. Shaded cells – samples with 50 ng/mL LPS added.