# NCL Method ITA-14

# Analysis of Nanoparticle Effects on Maturation of Monocyte Derived Dendritic Cells In Vitro

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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

Dendritic cells (DC) are antigen-presenting cells. They play an important role in initiation and regulation of the cellular immune responses. Maturation of dendritic cells is achieved by inflammatory cytokines (e.g., TNF-α), or pathogen associated molecular patterns (e.g., bacterial lipopolysaccharide, LPS). This protocol can be used to study the in vitro efficacy of nanoparticle-based vaccine formulations. In addition, it can also be used to assess the potential cytotoxicity of nanoparticle formulations to dendritic cells, as well as nanoparticle effects on DC maturation in response to traditional inflammatory stimuli such as bacterial lipopolysacharide.

## 2. Principles

This document describes a protocol for assessing the effect of nanoparticles on the maturation of dendritic cells derived from peripheral blood monocytes. Monocytes are cultured for 5-7 days in the presence of interleukin 4 (IL-4) and granulocyte macrophage-colony stimulating factor (GM-CSF) to allow for differentiation into immature dendritic cells. On day 5-7 of culture, LPS and/or the test nanoparticles are added to the cell culture and incubation is continued for an additional 24–48 hr to stimulate maturation of DC. To test for the particles' potential to interfere with inflammation-induced DC maturation, LPS can be combined with the nanoparticles prior to addition to the cells or after exposure to nanoparticle treatment. The choice of the treatment regimen depends on the goal of the project.

## 3. Reagents, Materials, and Equipment

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

### 3.1 Reagents

1. Human blood from normal donors
2. Ficoll-Paque Plus (GE-Life Sciences, 17-1440-03)
3. PBS (Gibco, 14190)
4. LPS O55:B5 or equivalent (Sigma, L6529)
5. Fetal bovine serum (heat inactivated, low endotoxin) (Lonza, 14-507F)
6. RPMI-1640 (Gibco, 21870)

7. Pen-Strep-L-Glutamine solution (Gibco, 10378-016)
8. β-mercaptoethanol (Sigma, M7522)
9. Trypan Blue solution (Gibco, 15250-061)
10. HEPES (Gibco, 15630)
11. Sodium pyruvate (Gibco, 11360)
12. MEM NEAA (Gibco, 11140)
13. Human AB serum (GemCell, 100-512)
14. DNAse I, 10 units/μL (Roche, G8101782)
15. Human IL-4 (Peprotech, 200-04)
16. Human GM-CSF (Leukine Sargramostim) (Immunex-Berlex, 58406-33)
17. Simultest Isotype control (BD, 340041)
18. HLA-ABC-FITC (BD Pharmingen, 555552)
19. HLA-DR-PE (BD, 347367)
20. CD86-FITC (BD Pharmingen, 555657)
21. CD80-PE (BD Pharmingen, 557227)
22. CD14-FITC (BD Pharmingen, 55397)
23. CD83-PE (Beckman Coulter, PN IM2218U)
24. Paraformaldehyde (Sigma, P6148 )

3.2 Materials

1. Pipettes, 5 to 25 mL (Corning)
2. Pipetters, 2 to 1000 μL (Ranin)
3. 6-, 24-, and 96-well TC plates (Corning)
4. 96-well polypropylene plate (Corning)
5. T100 TC flasks, low profile, CELLBIND surface (Corning, 3073)
6. Polypropylene tubes, 15, 50 and 250 mL (Falcon)
7. Disposable vacuum filtration unit (Millipore)

3.3 Equipment

1. Centrifuge, 400x*g*, 900x*g*
2. Refrigerator, 4°C
3. Freezer, -20°C and -80°C
4. Cell culture incubator, 5% $CO_2$ and 95% humidity

5. Biohazard safety cabinet approved for level II handling of biological material
6. Inverted microscope
7. Vortex
8. Hemacytometer
9. Coulter particle counter
10. Flow Cytometer (FACSCalibur)
11. CellQuest software for data analysis

## 4. Preparation of Reagents

4.1 Freeze/Thaw Medium

The freeze/thaw medium consists of RPMI-1640 with 20% FBS, 2 mM L-glutamine, 2.5 mM HEPES, 100 U/mL penicillin, and 100 μg/mL streptomycin.

4.2 DC Culture Medium

DC culture medium consists of RPMI-1640 with 10% FBS (all new lots must be screened for good DC generation), 2 mM L-glutamine, 25 mM HEPES, 100 U/mL penicillin, 100 μg/mL streptomycin, 0.1 mM MEM NEAA, 1 mM sodium pyruvate, and 50 μM β-mercaptoethanol.

4.3 FACS blocking buffer

FACS blocking buffer consists of PBS with 5% Human AB serum.

4.4 FACS staining buffer

FACS staining buffer consists of PBS with 5% BSA. Addition of 0.1% $NaN_3$ is optional.

4.5 Fixative

Fixative is PBS containing 1% paraformaldehyde.

4.6 GM-CSF

Prepare stock solution by reconstituting the commercial lyophilized cytokine Leukine Sargramostim in water to a concentration of $1.4 \times 10^6$ IU/mL (250 μg/mL). Store in glass vials at 4°C. Do not freeze. On the day of experiment add to DC media to a final concentration of 100 IU/mL.

4.7 IL-4

Prepare stock solution by reconstituting commercial lyophilized cytokine to a concentration of 100 μg/mL in PBS. Prepare small aliquots and store at -80°C. On the day of experiment add to DC media to a final concentration of 50 ng/mL.

4.8 Vehicle Control

Vehicle control is the buffer or media used to formulate test nanomaterials. Common excipients used in nanoformulations 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 or saline.

## 5. Preparation of Test Nanoparticles

This assay requires 2.5 mL of nanoparticle solution dissolved/resuspended in complete DC culture medium, at a concentration 2X 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 [1] and are summarized in Box 1 below.

This assay evaluates 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 serial 1:5 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 4 mg/mL is prepared. This sample is then diluted 10 fold (0.4 mg/mL), followed by two 1:5 serial dilutions (0.08 and 0.016 mg/mL). When 0.5 mL of each of these sample dilutions is added to the plate and mixed with 0.5 mL of cell suspension, the final nanoparticle concentrations tested in this assay are: 2.0, 0.2, 0.04 and 0.008 mg/mL.

***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:

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

## 6. Isolation of Human Peripheral Blood Mononuclear Cells (PBMC) from Leukopack or Whole Blood

1. Aliquot 15 mL of Ficoll-Paque into each 50 mL centrifuge tube.
2. Dilute the leukopack 1:2 with PBS; Dilute whole blood 1:3 with PBS.
3. Slowly layer 35 mL of diluted matrix from step 2 over Ficoll-Paque.
4. Centrifuge 15 min, 900x*g* at room temperature, without brake.
5. Remove the mononuclear cell layer at the interface and add to a sterile 50 mL tube containing 25 mL PBS. Depending on the total cell number, this layer can vary from thin and translucent to thick and milky in appearance.
6. Centrifuge 15 min, 400x*g*
7. Discard the supernatants and resuspend the pellets in 50 mL PBS. Repeat centrifugation step as described in step 6.
8. Combine all washed cells into one tube and adjust the volume to 50 mL with PBS. Repeat centrifugation step as described in step 6. Discard PBS and resuspend cells in a small volume of DC culture medium (~3 mL).
9. Perform cell count and confirm cell viability, for example, by using trypan blue exclusion. Adjust cell concentration to $3x10^6$ cells/mL in DC culture medium.
10. Use cells from step 8 for further DC generation or freeze them in freeze/thaw medium.

## 7. Rapid Thaw Method of Cryopreserved PBMC

1. Retrieve cryovial containing PBMC from storage area (-70°C or liquid nitrogen).
2. Rapidly thaw cells in a 37°C water bath while gently shaking.
3. Wipe cryovials with 70% ethanol and carefully open cap.
4. Transfer cells to a labeled 15 mL polypropylene round bottom tube.
5. Immediately add 10 mL of freeze/thaw medium in a drop-wise manner, continuously swirling the tube contents.
6. Centrifuge the cell suspension at 400x*g* for 5 min.
7. Discard the supernatant and resuspend the cell pellet in warm DC culture medium.
8. Perform the cell count and determine viability, for example, by trypan blue exclusion.
9. Adjust the volume of cell suspension to $3x10^6$ cells/mL using DC culture medium.

## 8. Generation of Immature Dendritic Cells

1. Isolate PBMC or thaw frozen PBMC using the rapid thaw method according to the procedures described in Sections 6 or 7, respectively.
2. Use cells from step 6.9 or 7.9 in 5 mL of DC media (total number of cells is $15x10^6$ cells).
3. Add 15 µL of DNase (150 units). Incubate the cell suspension in a polypropylene tube with loosened cap at 37°C for 1–2 hr.
4. After incubation, spin the cells 5 min at 400x*g*, and resuspend the pellet in 5-10 mL of DC culture medium.
5. Perform the cell count and viability, and adjust cell concentration to $3x10^6$ cells/mL in DC culture medium.
6. Add 3 mL of cell suspension to wells in a 6-well TC plate. (T75 or T100 TC flask can also be used; In this case, do not add more than 20 mL of cell suspension per flask.)
7. Incubate plate/flask for 1–2 hr at 37°C to allow the monocytes to adhere.
8. For each well of the 6-well plate utilized, prepare 5 mL of **DC medium containing IL-4 (50 ng/mL) and GM-CSF (100 IU/mL)**. (Prepare 25 mL of complete DC medium for each T75, or 50 mL for each T100 flask.) Do not use previously thawed IL-4 aliquots for initial set up.

9. After incubation, gently pipette up and down, aspirate and collect the media containing non-adherent cells. Wash each well (flask) three times with sterile PBS. Combine all washes with the initial supernatant.
10. Add 4 mL of complete DC medium (prepared in step 8) to each well with adhered cells (20 mL for flask).
11. Perform cell count on total non-adherent cells collected. Approximate the number of adherent monocytes by subtracting the number of non-adherent cells from the total number of plated PBMC.
12. Incubate plate (flask) for 5–7 days at 37°C.
13. Feed DC culture on day 3–4 (can be late day 2). Mix cells gently and aspirate ½ of the volume of each well/flask. Centrifuge at 400x*g* for 5 min. Resuspend the cell pellet in the same volume of fresh DC media with 2X the amount of IL-4 and GM-CSF. Add this cell suspension to initial wells or flasks.
14. At the end of day 5–7, monocytes (large, round CD14+ cells) will be used to differentiate to immature dendritic cells (large, irregular shaped, CD14-).
    **Note**: Cells should detach from the plate as they keep differentiating toward immature DC phenotype.

## 9. Maturation of Dendritic Cells with or without Nanomaterial

1. On day 5-7, harvest immature DC into a 50 mL polypropylene tube. Wash each well or flask with PBS two times and collect all washes in one tube. Some DC may be attached to the plate; in this case add 2 mL of 5 mM sterile EDTA to each well and incubate the plate for 10–15 min at room temperature. Collect the floating cells and add them to the previous washes.
2. Centrifuge the tube at 400x*g* for 5 min. Discard the supernatant and resuspend the cell pellet in 1 mL of fresh DC culture media **without IL-4 and GM-CSF.**
3. Perform cell count and viability.
4. Adjust the cell concentration to $0.4x10^6$ cells/mL.
5. Add 0.5 mL of cell suspension to each well of a 24-well TC plate (see example plate map 1 in Appendix).

6. Prepare nanomaterial in DC culture medium at 2X the desired final concentration (see Section 5 for details).
7. Add 0.5 mL of nanomaterial to appropriate wells. Add 0.5 mL of DC media to baseline control wells.
8. Add LPS at a final concentration of 20 ng/mL as positive control.

   **Notes**:

   a. Generally you will have wells with immature DC (in DC medium only) as a baseline, wells with LPS only, as a standard for maturation, and wells with nanoparticle samples.
   b. When applicable, nanoparticle vehicle control can be included (e.g., when nanoparticle storage solution is not saline or PBS).
   c. When analysis of nanoparticle effect on LPS-induced DC maturation is needed, prepare experimental wells with nanomaterial and LPS. Alternatively, cells can be treated with nanoparticles for 24 hours, washed, and then treated with LPS for an additional 24 hours. Use scientific judgement to adjust experimental design so as to address the project need. See example plate map 1 in Appendix.
9. Incubate DC for 24–48 hr at 37°C.
10. Harvest DC and perform flow analysis of DC phenotype, staining for HLA-ABC, HLA-DR, CD86, CD80, CD83, and CD14.

## 10. Analysis of DC Maturation by Flow Cytometry

1. Centrifuge harvested DC at 400x*g* for 5 min, and resuspend the cell pellet in FACS blocking buffer.
2. Transfer an equal amount of cell suspension (not more than 200 μL) into each well of a 96-well polypropylene plate (see example of plate map 2 in Appendix). Ideally, you will have 100,000–200,000 cells/well. However, as few as 10,000 cells/well can be used.
3. Keep the plate at 4°C for 15–30 min.
4. Centrifuge the plate at 400x*g* for 5 min. Decant the supernatant from each well by flicking the plate. Wash the cells once with FACS staining buffer. Decant the supernatant again.

5. Add 20 μL of appropriate antibody to each well. Add 150 μL of FACS staining buffer to each well.
6. You will have four wells total for each DC sample: Isotype control; HLA-ABC/HLA-DR; CD86/CD80;CD14/CD83. See example plate map 2 in Appendix.
7. Keep the plate at 4°C for 15–30 min.
8. Wash the plate with FACS buffer three times, flicking the supernatant every time.
9. After the last wash, add 100 μL of 1% paraformaldehyde in PBS to each well. Wrap the plate in foil and store at 4°C. Analyze the samples within one week. Before flow cytometry, transfer cell suspension from each well to a FACS tube and add 200 μL of PBS.
10. The samples can be analyzed "fresh" (without fixation), if time permits.
11. For analysis: create FSC/SSC dot plot for each sample and make R1 gate (live cells) exclude cell debris. Create histograms for each staining in R1 gate. Set up M1 marker on Isotype control histograms and transfer this marker to all other histograms. Perform histogram statistics, looking for % positive cells in M1, as well as Mean Channel Fluorescence (MCF) in M1.
12. Mature DC should have increased HLA-DR MCF, increased % positive for CD86 and CD80 cells (although MCF may stay the same), and increased % positive CD83 cells. Some immature DC may still be CD14+, but this percentage should decrease after maturation with LPS. Use scientific judgement for interpretation of the data generated using nanoparticles or, when needed, nanoparticle combination with LPS.

## 11. References

1. Dobrovolskaia MA, McNeil SE. Understanding the correlation between in vitro and in vivo immunotoxicity tests for nanomedicines. J Control Release. 2013;172(2):456-66.

## 12. Abbreviations

| | |
|---|---|
| API | active pharmaceutical ingredient |
| BSA | bovine serum albumin |
| DC | dendritic cells |
| DMSO | dimethyl sulfoxide |
| EDTA | ethylenediaminetetraacetic acid |
| FACS | fluorescence activated cell sorting |
| FBS | fetal bovine serum |
| FITC | Fluorescein isothiocyanate |
| FSC | forward scatter |
| GM-CSF | granulocyte macrophage colony stimulating factor |
| HEPES | 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid |
| HLA ABC | human leukocyte antigen, class I (A, B, and C) |
| HLA-DR | human leukocyte antigen, class II (DR) |
| IL | interleukin |
| IU | international units |
| LPS | lipopolysaccharide |
| MCF | mean channel fluorescence |
| MEM NEAA | minimal essential medium - non-essential amino acids |
| PBMC | peripheral blood mononuclear cells |
| PE | phycoerythrin |
| SSC | side scatter |
| TC | tissue culture |

## 13. Appendix

### Example Plate Map 1

| | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|
| **A** | **Untreated Cells (baseline)** | **Untreated Cells (baseline)** | **PC** | **PC** | | |
| **B** | **TS 2.0 mg/mL** | **TS 0.2 mg/mL** | **TS 0.04 mg/mL** | **TS 0.008 mg/mL** | | |
| **C** | **TS 2.0 mg/mL** | **TS 0.2 mg/mL** | **TS 0.04 mg/mL** | **TS 0.008 mg/mL** | | |
| **D** | **TS 2.0 mg/mL** | **TS 0.2 mg/mL** | **TS 0.04 mg/mL** | **TS 0.008 mg/mL** | | |

PC: positive control, LPS; TS: test sample.

Also add a vehicle control if nanoparticles are formulated in buffers other than PBS or saline.

Prepare second plate in which nanoparticles are combined with LPS if analysis of nanoparticle effects on LPS-induced maturation is needed. Certain experiments treat cells with nanoparticles for 24 hours prior to exposure to LPS; in this case all wells receive LPS except for the baseline.

**Plate Map 2**

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **A** | Cells Only | Cells Only | PC | PC | Cells Only | Cells Only | PC | PC | Cells Only | Cells Only | PC | PC |
| **B** | TS 2.0 mg/mL | TS 0.2 mg/mL | TS 0.04 mg/mL | TS 0.008 mg/mL | TS 2.0 mg/mL | TS 0.2 mg/mL | TS 0.04 mg/mL | TS 0.008 mg/mL | TS 2.0 mg/mL | TS 0.2 mg/mL | TS 0.04 mg/mL | TS 0.008 mg/mL |
| **C** | TS 2.0 mg/mL | TS 0.2 mg/mL | TS 0.04 mg/mL | TS 0.008 mg/mL | TS 2.0 mg/mL | TS 0.2 mg/mL | TS 0.04 mg/mL | TS 0.008 mg/mL | TS 2.0 mg/mL | TS 0.2 mg/mL | TS 0.04 mg/mL | TS 0.008 mg/mL |
| **D** | TS 2.0 mg/mL | TS 0.2 mg/mL | TS 0.04 mg/mL | TS 0.008 mg/mL | TS 2.0 mg/mL | TS 0.2 mg/mL | TS 0.04 mg/mL | TS 0.008 mg/mL | TS 2.0 mg/mL | TS 0.2 mg/mL | TS 0.04 mg/mL | TS 0.008 mg/mL |
| **E** | Cells Only | Cells Only | PC | PC | | | | | | | | |
| **F** | TS 2.0 mg/mL | TS 0.2 mg/mL | TS 0.04 mg/mL | TS 0.008 mg/mL | | | | | | | | |
| **G** | TS 2.0 mg/mL | TS 0.2 mg/mL | TS 0.04 mg/mL | TS 0.008 mg/mL | | | | | | | | |
| **H** | TS 2.0 mg/mL | TS 0.2 mg/mL | TS 0.04 mg/mL | TS 0.008 mg/mL | | | | | | | | |

PC: Positive Control; NC: Negative Control; TS: Test Sample

Prepare second plate in which nanoparticles are combined with LPS if analysis of nanoparticle effects on LPS-induced maturation is needed.

Each set of samples, shaded red, blue, green and yellow, will be used to test one of the following groups: Isotype control; HLA-ABC/HLA-DR; CD86/CD80; and CD14/CD83.