



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 response. Maturation of dendritic cells is achieved by inflammatory cytokines (e.g. $\text{TNF}\alpha$) 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, and the nanoparticle effects on DC maturation in response to traditional inflammatory stimuli such as bacterial lipopolysaccharide.

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 differentiation into immature dendritic cells. On day 5-7 of culture, LPS and/or the nanoparticles are added to the cell cultures and incubation is continued for an additional 24-48 hr to stimulate maturation of DC. To test for the particles' potential ability to interfere with inflammation-induced DC maturation, LPS can be combined with nanoparticles prior to addition to the cells or after exposure to nanoparticle treatment. The choice of the treatment regimen depends on the project goal.

3. Reagents, Materials, and Equipment

Note: The NCL does not endorse any of the suppliers listed below; these reagents were used in the development of the protocol and their inclusion is for informational purposes only. Equivalent supplies from alternate vendors can be substituted. Please note that suppliers may undergo a name change due to a variety of factors. Brands and part numbers typically remain consistent but may also change over time.

3.1 Reagents

3.1.1 Human blood from normal donors

3.1.2 Ficoll-Paque Premium (GE Life Sciences, 17-5442-02)

- 3.1.3 Phosphate Buffered Saline (PBS) (GE Life Sciences, Hyclone SH30256.01)
 - 3.1.4 LPS O55:B5 (Sigma, L6529), LPS K12 E.coli (Invivogen, tlrl-pekllps) or equivalent
 - 3.1.5 Fetal Bovine Serum (FBS) (GE Life Sciences, Hyclone, SH30070.03)
 - 3.1.6 RPMI-1640 (GE Life Sciences, HyClone, SH30096.01)
 - 3.1.7 Penicillin streptomycin solution (GE Life Sciences, Hyclone, SV30010)
 - 3.1.8 L-glutamine (GE Life Sciences, Hyclone, SH30034.01)
 - 3.1.9 β -mercaptoethanol (Sigma-Aldrich, M7522)
 - 3.1.10 Trypan Blue solution (Gibco, 15250-061)
 - 3.1.11 HEPES (Gibco, 15630)
 - 3.1.12 Sodium Pyruvate, 100mM [100x] (Gibco, 11360-070)
 - 3.1.13 MEM NEAA, 100x (Gibco, 11140-050)
 - 3.1.14 Human AB serum (GeminiBio, GemCell, 100-512)
 - 3.1.15 DNase I, 10 units/ μ L (Sigma-Aldrich, Roche, 04 716 728 001)
 - 3.1.16 Human IL-4 (PeproTech, 200-04)
 - 3.1.17 Human GM-CSF (PeproTech 300-03)
 - 3.1.18 Simultest Isotype control IgG₁/IgG_{2a}-PE (BD 340041)
 - 3.1.19 HLA-ABC- FITC (BD Pharmingen, 555552)
 - 3.1.20 HLA-DR-PE (BD, 347367)
 - 3.1.21 CD86-FITC (BD Pharmingen, 555657)
 - 3.1.22 CD80-PE (BD Pharmingen, 557227)
 - 3.1.23 CD14- Alexa Fluor 488 (BD Pharmingen, 557700)
 - 3.1.24 CD83-PE (Beckman Coulter Life Sciences, PN IM2218U)
 - 3.1.25 Paraformaldehyde, 20% (Tousimis, 1008A)
- 3.2 Materials
- 3.2.1 Pipettes, 5 to 25 mL
 - 3.2.2 Pipettors, 2 to 1000 μ L
 - 3.2.3 Pipette tips, 2 to 1000 μ L
 - 3.2.4 6-, 24-, and 96-well TC plates
 - 3.2.5 96-well polypropylene plate

- 3.2.6 T100 TC flasks, low profile, CELLBIND surface (Corning, 3073)
- 3.2.7 15, 50 and 250 mL polypropylene tubes (Falcon)
- 3.2.8 Vacuum disposable filtration unit (Millipore)
- 3.3 Equipment
 - 3.3.1 Centrifuge
 - 3.3.2 Refrigerator
 - 3.3.3 Freezer, -20°C and -80°C
 - 3.3.4 Cell culture incubator with 5% CO₂ and 95% humidity
 - 3.3.5 Biohazard safety cabinet approved for level II handling of biological material
 - 3.3.6 Inverted microscope
 - 3.3.7 Vortex
 - 3.3.8 Hemacytometer
 - 3.3.10 Flow Cytometer
 - 3.3.11 Flow cytometry software for data analysis

4. Preparation of Reagents

4.1 Medium for cryopreservation of cells (if applicable)

RPMI-1640

20% FBS

10% DMSO

2 mM L-Glutamine

2.5 mM HEPES

Note: Fresh PBMC isolated from fresh whole blood are ideal. When fresh cells are not available, cryopreservation may be used. Various media in addition to that listed above are available and include among others a) ultrapure FBS+10% DMSO; b) IMDM + 20% FBS + 10% DMSO + trehalose 50 µg/mL [1]; c) CryoStor[®] CS10 (StemCell Technologies Catalog #07930). Labs are advised to select the cryopreservation medium empirically based on the cell viability and functionality analysis in comparison to freshly derived PBMC.

4.2 DC Culture Medium

RPMI-1640

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

50 µM 2-Mercaptoethanol

4.3 FACS blocking buffer

PBS

5% Human AB serum

4.4 FACS staining buffer

PBS

0.5% BSA

0.1% NaN₃ (optional)

4.5 Fixative

PBS

1% Paraformaldehyde

4.6 GM-CSF

Prepare stock solution by reconstituting commercial lyophilized cytokine Leukine Sargramostim in water to a concentration of 1.4×10^6 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 them 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, at a concentration 2X the highest tested concentration. Nanoparticles should be dissolved/resuspended in complete DC culture medium. The concentration is selected based on the 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 were reviewed elsewhere [2] 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:

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

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.

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

The assay will evaluate four concentrations: 10 X (or when feasible 100 X, 30 X or 5 X) 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, the highest final concentration is 1 mg/mL or the highest reasonably achievable concentration.

For example, if the final theoretical plasma concentration to be tested is 0.2 mg/mL, then a stock of 4 mg/mL will be prepared and diluted 10-fold (0.4 mg/mL), followed by two 1: 5 serial dilutions (0.08 and 0.016 mg/mL). When 500 μ L of each of these samples are combined in a culture plate well with 500 μ L of cells, the final concentrations of nanoparticles are 0.008, 0.04, 0.2 and 2 mg/mL. Each nanoparticle concentration is plated 3 times.

6. Isolation of Human PBMC from Leukopack or Whole Blood

- 6.1 Aliquot 15 mL of Ficoll-Paque into each 50 mL centrifuge tube.
- 6.2 Dilute the leukopack 1:2 with PBS; whole blood 1:3 with PBS.
- 6.3 Slowly layer 35 mL of diluted matrix from step 6.2 over Ficoll-Paque.
- 6.4 Centrifuge 15 min, 900xg at room temperature, slow acceleration and without brakes.
- 6.5 Remove the mononuclear cell layer at the interface and add it to a sterile 50 mL tube containing 25 mL of PBS. Depending on the total cell number, this layer varies from thin and translucent to thick and milky in appearance.
- 6.6 Centrifuge 15 min, 400xg
- 6.7 Discard the supernatants and resuspend the pellets in 50 mL PBS. Repeat centrifugation step as described in 6.6.
- 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 6.6. Discard PBS and resuspend cells in a small volume of DC culture medium (~3 mL).
- 6.9 Perform cell count and confirm cell viability, for example, by using Trypan Blue exclusion. Adjust cell concentration to 3×10^6 cells/mL in the DC culture medium.
- 6.10 Use cells from step 6.8 for further DC generation or freeze them in freeze/thaw medium.

7. Rapid Thaw Method of Cryopreserved PBMC

- 7.1 Retrieve cryovial containing PBMC from storage area (-70°C or liquid nitrogen).
- 7.2 Rapidly thaw cells in 37°C water bath while gently shaking.
- 7.3 Wipe off cryovials with 70% ethanol and carefully open cap.
- 7.4 Transfer cells to a labeled 15 mL polypropylene round bottom tube.
- 7.5 Immediately add 10 mL of freeze/thaw medium in a drop-wise manner, continuously swirling the tube contents.
- 7.6 Centrifuge the cell suspension at 400xg for 5 min.
- 7.7 Discard the supernatant and re-suspend the cell pellet in warm DC culture medium.
- 7.8 Perform cell count and determine viability, for example, by Trypan Blue exclusion.
- 7.9 Adjust the volume of cell suspension to 3×10^6 cells/mL using DC culture medium.

8. Generation of Immature Dendritic Cells

- 8.1 Isolate PBMC or thaw frozen PBMC using the rapid thaw method according to procedures described in sections 6 or 7, respectively.
- 8.2 Use 5 mL of cell suspension from step 6.9 or 7.9 (total number of cells is 15×10^6 cells).
- 8.3 Add 15 μ L of DNase (150 units). Incubate the cell suspension in a polypropylene tube with loosened cap at 37°C for 1-2 hr.
- 8.4 After incubation, spin the cells 5 min at 400xg, and resuspend the pellet in 5-10 mL of DC culture medium.
- 8.5 Perform a cell count and viability, then adjust cell concentration to 3×10^6 cells/mL in DC culture medium.
- 8.6 Add 3 mL of cell suspension to wells in 6-well TC plate. (T75 or T100 TC flask can be used. In this case, do not add more than 20 mL of cell suspension per flask.)
- 8.7 Incubate plate/flask for 1-2 hr at 37°C to allow the monocytes to adhere.

- 8.8 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 50mL for each T100 flask.) Do not use previously thawed IL-4 aliquots for initial set up.
- 8.9 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.
- 8.10 Add 4 mL of complete DC medium (prepared in step 8.8) to each well with adhered cells (20 mL for flask).
- 8.11 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.
- 8.12 Incubate plate (flask) for 5- 7 days at 37°C.
- 8.13 Feed DC culture on day 3-4 (can be late day 2). Mix cells gently and aspirate half of the volume of each well/flask. Centrifuge at 400xg for 5 min. Resuspend the cell pellet in the same volume of fresh DC media with 2X amount of IL-4 and GM-CSF. Add this cell suspension to initial wells or flasks.
- 8.14 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

- 9.1 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.
- 9.2 Centrifuge the tube at 400xg 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**.
- 9.3 Perform cell count and viability.

- 9.4 Adjust the cell concentration to 0.4×10^6 cells/mL.
- 9.5 Add 0.5 mL of cell suspension to each well of a 24-well TC plate (see example plate map 1 in section 13).
- 9.6 Prepare nanomaterial in DC culture medium at 2X of desired final concentration (see step 5 for details).
- 9.7 Add 0.5 mL of nanomaterial to appropriate wells. Add 0.5 mL of DC media to baseline control wells.
- 9.8 Add LPS at a final concentration of 20 ng/mL as positive control.

Notes:

- Generally, you will have wells with immature DC (in DC medium only) as baseline, wells with LPS only, as a standard for maturation, and wells with nanoparticle samples.
- When applicable, nanoparticle vehicle control can be included (e.g. when nanoparticle storage solution is not saline or PBS).
- 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 additional 24 hours. Use scientific judgement to adjust experimental design so to address the project need. See example plate map 1 in section 13.

- 9.9 Incubate DC for 24-48 hr at 37°C.
- 9.10 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

- 10.1 Centrifuge harvested DC at 400xg for 5 min. and resuspend the cell pellet in FACS Blocking buffer.
- 10.2 Transfer an equal amount of cell suspension (not more than 200 μ L) into each well of a 96-well polypropylene plate (see example of plate map 2 in section 13).

Ideally, you will have 100,000 – 200,000 cells /well. However, you can use as few as 10,000 cells/well.

- 10.3 Keep the plate at 4°C for 15-30 min.
- 10.4 Centrifuge the plate at 400xg 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.
- 10.5 Add 20 µL of appropriate antibody to each well. Add 150 µL of FACS staining buffer to each well.
- 10.6 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 section 13.
- 10.7 Keep the plate at 4°C for 15-30 min.
- 10.8 Wash the plate with FACS buffer 3 times, flicking the supernatant every time.
- 10.9 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.
- 10.10 For analysis: create FSC/SSC dot plot for each sample and make R1 gate (live cells) to 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 % of positive cells in M1, as well as Mean Channel Fluorescence (MCF) in M1.
- 10.11 Matured DC should have HLA-DR MCF increased; % of positive for CD86 and CD80 cells increased (although MCF may stay the same); % of CD83 positive cells should increase. 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. Abbreviations

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

12. References

1. Shinde P, Khan N, Melinkeri S, Kale V, Limaye L. Freezing of dendritic cells with trehalose as an additive in the conventional freezing medium results in improved recovery after cryopreservation. *Transfusion*. 2019;59(2):686-696. doi: 10.1111/trf.15028. PMID: 30456902.
2. 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.

13. Appendix

Example Plate Map 1

	1	2	3	4	5	6
A	Untreated Cells (baseline)	Untreated Cells (baseline)	PC	PC		
B	TS 1.0 mg/mL	TS 0.2 mg/mL	TS 0.04 mg/mL	TS 0.008 mg/mL		
C	TS 1.0 mg/mL	TS 0.2 mg/mL	TS 0.04 mg/mL	TS 0.008 mg/mL		
D	TS 1.0 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.

Example 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 1.0 mg/mL	TS 0.2 mg/mL	TS 0.04 mg/mL	TS 0.008 mg/mL	TS 1.0 mg/mL	TS 0.2 mg/mL	TS 0.04 mg/mL	TS 0.008 mg/mL	TS 1.0 mg/mL	TS 0.2 mg/mL	TS 0.04 mg/mL	TS 0.008 mg/mL
C	TS 1.0 mg/mL	TS 0.2 mg/mL	TS 0.04 mg/mL	TS 0.008 mg/mL	TS 1.0 mg/mL	TS 0.2 mg/mL	TS 0.04 mg/mL	TS 0.008 mg/mL	TS 1.0 mg/mL	TS 0.2 mg/mL	TS 0.04 mg/mL	TS 0.008 mg/mL
D	TS 1.0 mg/mL	TS 0.2 mg/mL	TS 0.04 mg/mL	TS 0.008 mg/mL	TS 1.0 mg/mL	TS 0.2 mg/mL	TS 0.04 mg/mL	TS 0.008 mg/mL	TS 1.0 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 1.0 mg/mL	TS 0.2 mg/mL	TS 0.04 mg/mL	TS 0.008 mg/mL								
G	TS 1.0 mg/mL	TS 0.2 mg/mL	TS 0.04 mg/mL	TS 0.008 mg/mL								
H	TS 1.0 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 nanoparticles 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.