NCL Method ITA-10
Version 1.2

Cytokine Induction

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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

This document describes a protocol for assessing an effect of nanoparticle formulation on cytokine production by peripheral blood mononuclear cells. Lymphocytes are isolated from human blood anti-coagulated with Li-heparin using Ficoll-Paque Plus solution. The cells then are incubated with or without lipopolysaccharide in the presence or absence of nanoparticles for 24 hours. After this incubation step cell culture supernatants are collected and analyzed by cytometry beads array for the presence of IL-1β, TNFα, IL-12, IL-10, IL8 and IL-6. The assay therefore allows for measurement of nanoparticles ability to induce cytokines or to suppress cytokines induced by LPS. The assay requires 600 μL of a test-nanomaterial at two concentrations determined by toxicity assays GTA-1 and GTA-2.

2. Reagents

2.1. Human blood pooled from at least 3 donors anti-coagulated with Li-heparin

2.2. Ficoll-Paque Plus, Amersham Biosciences, cat# 17-1440-02

2.3. PBS, HyClone, cat# AQB 22934

2.4. LPS O55:B5 or equivalent, Sigma, cat# L6529

2.5. Fetal bovine serum, Hyclone.

2.6. RPMI-1640, Invitrogen, cat#11835-055

2.7. Hanks balanced salt solution (HBSS), Invitrogen, cat#24020-117

2.8. Pen/Strep solution, Invitrogen, cat#15140-148

2.9. β-mercaptoethanol, Sigma cat#M7522

2.10. Trypan Blue solution, Invitrogen, cat#15250-061

2.11. Human Inflammation kit, BD Biosciences, cat#551811

Note: The NCL does not endorse any of the material/instrument suppliers listed. Their inclusion is for informational purposes only.

3. Equipment

3.1. Pipettes covering range from 0.05 to 10 mL

3.2. 96-well plates

3.3. Polypropylene tubes 5 and 15 mL

3.4. Centrifuge

3.5. Refrigerator, 2-8 °C

3.6. Freezer, -20°C

3.7. Cell culture incubator with 5% CO_2 and 95% humidity.

3.8. Biohazard safety cabinet approved for level II handling of biological material

3.9. Inverted microscope

3.10. Vortex

3.11. Hemacytometer

3.12. Flow Cytometer, FACS Calibur or equivalent

3.13. CBA software for data analysis

4. Reagent and Control Preparation.

5.1. Complete RPMI-1640 medium

The complete RPMI medium should contain the following reagents:

10% FBS (heat inactivated)

2 mM L-glutamine

50 μM β-mercaptoethanol

100U/mL penicillin

100 μg/mL streptomycin sulfate

Store at 2-8 °C protected from light for no longer than 1 month. Before use warm in a water bath.

5.2. Lipopolysaccharide 1mg/mL (LPS, Stock)

Add 1 mL of sterile PBS or cell culture medium per 1 mg of LPS to the vial and vortex to mix. Store daily use aliquots at a nominal temperature of -20 °C.

5.3. Positive control

Dilute stock LPS solution in cell culture medium to a final concentration of 1 μg/mL. Store at room temperature. Discard unused portion after experiment is set-up.

5.4. Negative Control

Use PBS as a negative control.

5.5. Heat-inactivated fetal bovine serum

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

5. Test-nanomaterial preparation.

This assay requires 1800 μL of nanoparticles dissolved/resuspended in complete culture medium, i.e three 100 μL replicates per sample analyzed in duplicate, 600 μL per set using cells derived from one donor. For the original screen we recommend use as high concentration of nanoparticles in the sample as possible. The following issues have to be considered when selecting the concentration: i) solubility of nanoparticles in a biocompatible buffer; ii) pH within physiological range; iii) availability of nanomaterial, and iv) stability.

6. Isolation of human lymphocytes.

6.1. Place freshly drawn blood into 15 or 50 mL conical centrifuge tube, add equal volume of room-temperature PBS and mix well.

6.2. Slowly layer the Ficoll-Paque solution underneath the blood/PBS mixture by placing the tip of the pipet containing Ficoll-Paque at the bottom of the blood sample tube. Alternatively blood/PBS mixture may be slowly layered over Ficoll-Paque solution. Use 3 mL of Ficoll-Paque solution per 4 mL of blood/PBS mixture.

Note: to maintain Ficoll-blood interface it is helpful to hold tube at 45° angle.

6.3. Centrifuge 30 minutes at 900g, 18-20 °C without brake.

6.4. Using sterile pipet, remove upper layer containing plasma and platelets and discard it.

6.5. Using fresh sterile pipet transfer mononuclear cell layer into another centrifuge tube.

6.6. Wash cells by adding excess of HBSS and centrifuging for 10 min at 400g, 18-20 °C. The HBSS volume should be ~3 times the volume of mononuclear layer.

Note: usually 4mL of blood/PBS mixture results in ~2mL of mononuclear layer and requires 6mL of HBSS for wash step.

6.7. Discard supernatant and repeat wash step one more time.

6.8. Resuspend cells in complete RPMI1640 medium. Count an aliquot of cells and determine viability using trypan blue exclusion.

7. Experimental Procedure.

7.1. Adjust cell concentration to 2 x 10^6 cells/mL using complete RPMI medium

7.2. Dispense 100 μL of blank media (baseline), negative control, positive control and test samples per well on 96 well plate. Prepare duplicate wells for each sample.

7.3. Prepare "LPS+nanoparticles" wells by combining 50 μL of 2μg/mL LPS and 50μL of 2x concentrated nanoparticles.

7.4. Dispense 100 μL of cell suspension per well. Gently shake the plate to allow all components to mix.

Repeat steps 7.1-7.4 for cells obtained from each individual donor. There is no limit to the number of donors used in this test. It is advised to test each nanoparticle formulation using cells derived from at least three donors.

7.5. Incubate 24 hours in a humidified 37C, 5% CO2 incubator.

7.6. Collect supernatants into 0.5mL centrifuge tubes and spin in a microcentrifuge at a maximum speed for 5 minutes. Transfer supernatants into fresh tubes and either analyze fresh or store at -80C for future analysis.

7.7. On the day of analysis, thaw supernatants at room temperature, then place them on ice.

7.8. Dilute culture supernatants 1:5 with assay buffer provided with human inflammation kit.

7.9. Follow BD Biosciences kit instructions to prepare assay standard curve, cytokine detection beads and cytometer calibration beads.

7.10. In a falcon 5mL tube combine 50μL of cytokine detection beads with 50 μL of PE-detection reagent and one of the following: 50μL of assay buffer (reagent blank), 50μL of calibration standard or 50 μL of culture supernatant. Cover tubes and incubate in dark for 3 hours. During this incubation step perform calibration of flow cytometer.

7.11. At the end of incubation add 1mL of wash buffer provided with the kit to each tube, and centrifuge 5 minutes at 200g, then collect and discard supernatants.

7.12. Add 300 μL of wash buffer provided with the kit to each tube, vortex and analyze on flow cytometer.

8. Calculations

Data are analyzed using CBA software (BD Biosciences). The software calculates mean fluorescent intensity (MFI) for each sample, builds standard curve using 4-parameter regression model and calculates concentration of each cytokine based on sample MFI response extrapolated from corresponding standard curve.

9. Acceptance Criteria

9.1. The experiment should be performed at least twice using two separate sets of donor's blood. Statistical analysis is then performed to compare cytokine response of baseline (culture supernatant from untreated cells) with that of controls (culture supernatant from cells treated with either PBS or LPS, negative and positive controls, respectively) or study samples (culture supernatants from cells treated with test-nanomaterials).

9.2. Negative control is considered acceptable if cytokine levels in this sample do not exceed that of baseline levels more than 2 times.

9.3. Positive control is acceptable if cytokine levels in this sample are at least 3 times greater than that of baseline.

9.4. Study sample is considered positive in terms of cytokine induction if cytokine level(s) in the study sample is(are) higher that that of the baseline level(s) ($p<0.05$).

9.5. A study sample is considered capable of suppressing cytokine induction by inflammatory stimuli if cytokine level(s) in the sample "LPS+nanoparticles" is lower than that of positive control ($p<0.05$).

9.6. Positive and negative controls should be monitored for inter-assay variability and should be consistent. Test results for study sample from both experiments should be reported.

9.7. If significant variability is observed in results obtained using leukocytes from three initial donors, the experiment need to be repeated with additional donor cells