NCL Method ITA-10

Preparation of Human Whole Blood and Peripheral Blood Mononuclear Cell Cultures to Analyze Nanoparticle Potential to Induce Cytokines In Vitro

Nanotechnology Characterization Laboratory
Frederick National Laboratory for Cancer Research
Leidos Biomedical Research, Inc.
Frederick, MD 21702
(301) 846-6939
ncl@mail.nih.gov
http://www.ncl.cancer.gov

https://ncl.cancer.gov

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.

Method written by:

Timothy M. Potter[1]

Edward Cedrone[1]

Barry W. Neun[1]

Marina A. Dobrovolskaia[1,*]

1 - Nanotechnology Characterization Lab, Cancer Research Technology Program, Frederick National Laboratory for Cancer Research sponsored by the National Cancer Institute, Frederick, MD 21702

*- address correspondence to: marina@mail.nih.gov

Please cite this protocol as:

Potter TM, Cedrone E, Neun BW, Dobrovolskaia MA, NCL Method-10: Preparation of Human Whole Blood and Peripheral Blood Mononuclear Cell Cultures to Analyze Nanoparticle Potential to Induce Cytokines In Vitro.

https://ncl.cancer.gov/resources/assay-cascade-protocols DOI: 10.17917/GBQR-BN14

1. Introduction

Cytokine storm is a condition characterized by high plasma levels of inflammatory cytokines, chemokines and interferons which can be commonly induced by pathogens or their components (endotoxin, lipoproteins, DNA, RNA etc.). Cytokine storm can also be induced in response to certain drugs (e.g., recombinant proteins, therapeutic antibodies, macromolecular nucleic acid-based therapeutics). It is accompanied by fever, hypo- or hypertension and may progress to a more severe life-threatening condition called systemic inflammatory response syndrome (SIRS). For example, cytokine storm was a severe side effect in the phase I clinical trial of the experimental monoclonal antibody therapeutic TGN1412, which resulted in 6 healthy donor volunteers becoming critically ill and requiring intense care [1]. All patients had high serum levels of TNFα, IFNγ and other pro-inflammatory messengers [1]. Cytokine storm to this drug was not observed in preclinical studies involving rats and cynomolgus monkeys [1], but was easily detectable in vitro using a cytokine release assay in human primary blood cells [2].

Nanoparticles can be used for delivery of therapeutic proteins, antibodies and nucleic acids, or contain biologicals (antibodies, proteins or nucleic acids) as targeting agents. In addition, some nanoparticles can be made of biological molecules (e.g., self-assembling peptides or siRNAs). These details warrant studying both nanotechnology platforms and their macromolecular payload and targeting agents for the ability to induce inflammatory cytokines. Human whole blood and peripheral blood mononuclear cells (PBMC) are considered reliable and predictive models for this purpose. The data obtained from such in vitro studies is intended to supplement other preclinical data to create a nanoparticle safety profile towards its clinical development.

2. Principle

Whole blood or peripheral blood mononuclear cells derived from healthy donor volunteers are cultured in the presence of controls and nanoparticles in order to identify nanoparticle potential to induce cytokine storm. The culture supernatants prepared according to this protocol can be analyzed by commercial assays specific to human cytokines, chemokines and interferons, or by NCL protocols ITA-22 (IL-8), ITA-23 (IL-1β), ITA-24 (TNFα) and ITA-25

(IFNγ). NCL uses commercial PBL ELISA kits to test for the presence of type I interferons as well as multiplex kits. There is no harmonized approach as to which type of assay to use or choice between singleplex or multiplex analysis. NCL uses protocol ITA-27 to analyze supernatants by multiplex ELISAs (4-plex, 14-plex and 15-plex). Scientific judgement and the critical path of the project should determine the type of the cytokines and method for analysis of supernatants.

It takes 24 hours to culture whole blood or PBMC to collect supernatants, and an additional 5-6 hours to complete the ELISA or multiplex. If ELISA or multiplex analysis cannot be conducted immediately after incubation of whole blood or PBMC with nanoparticles, the culture supernatants can be frozen at -20°C. Different cytokines have different stability at room temperature (RT) and upon repeated freeze/thaw (FT) cycles. Please refer to individual NCL ELISA protocols or manufacturer's instruction for commercial kits for the information about RT and FT stabilities. Section 13 contains information about FT limits for NCL ELISA assays. When such information is not available, as in the case of some commercial kits, analyze supernatants immediately and prepare multiple aliquots for repeat analysis in order to avoid multiple FT cycles.

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 for Whole Blood Cultures

- 3.1.1 Human blood anti-coagulated with Li-heparin and obtained from at least three healthy donors
- 3.1.2 Phosphate Buffered Saline (PBS) (GE Life Sciences, Hyclone SH30256.01)
- 3.1.3 RPMI-1640 (GE Life Sciences, HyClone, SH30096.01)
- 3.1.4 Fetal bovine serum (GE Life Sciences, HyClone, SH30070.03)

3.1.5 Penicillin streptomycin solution (GE Life Sciences, Hyclone, SV30010)

3.1.6 L-glutamine (GE Life Sciences, Hyclone, SH30034.01)

3.2 Reagents for PBMC cultures

3.2.1 All reagents listed in section 3.1

3.2.2 Ficoll Paque Premium (GE Healthcare, 17-5442-02)

3.2.3 Hank's balanced salt solution (HBSS) (Gibco, 14175-095)

3.2.4 Trypan Blue solution (Gibco, 15250-061)

Table 1. Guidance for selecting whole blood vs. PBMC culture

	Whole Blood	PBMC
Primary Analysis	Inflammatory cytokines and type II interferon	Type I interferons
Nanoparticles	Any payload except nucleic acid-based API or targeting ligand	Payload or targeting ligand composed of therapeutic nucleic acid (e.g., siRNA, ASN, aptamer)

Note: Pro-inflammatory cytokines and type II interferon can also be detected in PBMC cultures. In addition to the cells present in PBMC, type II interferon is also produced by neutrophils, which are present only in whole blood cultures. Therefore, the latter is the preferable model to study type II interferon.

3.3 Controls

3.3.1 Ultrapure LPS from K12 E.coli (Invivogen, tlrl-peklps)

3.3.2 ODN2216 is CpG DNA oligonucleotide with a mixed backbone and the following sequence, 5'-ggGGGACGATCGTCGggggG-3, where lowercase letters show phosphorothioate linkage and capital letters refer to phosphodiester linkage between nucleotides. This can be custom ordered from IDT or equivalent supplier.

3.3.3 Phytohemagglutinin (PHA-M) (Sigma, L8902)

Table 2. Guidance on concentrations and purpose of positive controls

	LPS	ODN2216	PHA-M
Primary Analysis	Positive control for inflammatory cytokines (TNFα, IL1β, IL-6, IL-8, IL-10, IL-12)	Positive control for type I interferons IFNα and IFNβ	Positive control for type II interferon, IFNγ
Final Assay Concentration	20 ng/mL	5 µg/mL	10 µg/mL

Note: Other agents can be used as the assay positive control. Use scientific judgement for selecting other controls.

3.4 Equipment and Materials

3.4.1 Pipettes covering a range from 0.05 to 10 mL

3.4.2 24-well round bottom plates

3.4.3 96-well U-bottom plates

3.4.4 Polypropylene tubes, 15 and 50 mL

3.4.5 Microcentrifuge tubes

3.4.6 Centrifuge

3.4.7 Refrigerator, 2-8ºC

3.4.8 Freezer, -20ºC

3.4.9 Cell culture incubator with 5% CO_2 and 95% humidity

3.4.10 Biohazard safety cabinet approved for level II handling of biological material

3.4.11 Inverted microscope

3.4.12 Vortex

3.4.13 Hemocytometer

Refer to manufacturer's instruction of commercial ELISA or multiplex kit or to NCL assays ITA-22 (IL-8), ITA-23 (IL-1β), ITA-24 (TNFα), ITA-25 (IFNɣ) or ITA-27 (multiplex) for additional reagent and material requirements for the selected study. Table 3 provides additional details regarding acceptable freeze/thaw limits for ELISA reagents used in the

noted NCL assays. NCL uses reagents and procedure from PBL Assay Science for VeriKine human IFNα ELISA (cat # 41100) and VeriKine human IFNβ ELISA (cat #41410).

Table 3. Freeze/Thaw Limits of Culture Supernatants for NCL ELISA/Multiplex Protocols

NCL Assay	Analyte	Acceptable number of Freeze/Thaw Cycles
ITA-22	IL-8	1
ITA-23	IL1β	3
ITA-24	TNFα	3
ITA-25	IFNɣ	2
ITA-27 (4-plex)	IFNα, IFNβ, IFNω, IFNλ	1
ITA-27 (14-plex)	IFNα, IFNβ, IFNω, IFNλ, IL-2, IL-4, IL-5, IL-7, IL-13, IL-15, IL-17, IL-22, IL-23, IL-27	1
ITA-27 (15-plex)	IFNy, IL-1α, IL-1β, IL6, IL8, IL-10, IL-12, IL-21, IP-10, MCP-1, MCP-2, MIP-1α, MIP-1β, RANTES, TNFα	1

4. Collection and Handling of Whole Blood for Culture

Collect whole blood from healthy donor volunteers who have not been on medication and who are clear from infection for at least two weeks prior to blood donation. Use Li-heparin tubes and discard the first 10 cc. For the best results whole blood should be used within 1 hour after collection. Prolonged storage (> 2 hr) of whole blood will lead to a decrease in cell function.

5. Preparation of PBMC

5.1 Place freshly drawn blood into 15 or 50 mL conical centrifuge tubes. Add an equal volume of room-temperature PBS and mix well.

5.2 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, the blood/PBS mixture may be slowly layered over the Ficoll-Paque solution. Use 3 mL of Ficoll-Paque solution per 4 mL of blood/PBS mixture. For example, use 15 mL Ficoll-Paque per 20 mL of diluted blood in a 50 mL tube. To maintain Ficoll-blood interface it is helpful to hold the tube at a 45º angle.

5.3 Centrifuge 30 min at 900xg, 18-20ºC, without brake.

Note: For certain types of centrifuges it may be advisable to set acceleration speed to minimum as well.

5.4 Using a sterile pipet, remove the upper layer containing plasma and platelets and discard.

5.5 Using a fresh sterile pipet, transfer the mononuclear cell layer into a fresh centrifuge tube.

5.6 Wash cells by adding an excess of HBSS and centrifuging for 10 min at 400xg, 18-20 ºC. The HBSS volume should be approximately three times the volume of mononuclear layer.

Note: Typically 4 mL of blood/PBS mixture results in about 2 mL of mononuclear layer and requires at least 6 mL of HBSS for the wash step. We use 10 mL of HBSS per each 2 mL of cells.

5.7 Discard supernatant and repeat wash step once more.

5.8 Resuspend cells in complete RPMI-1640 medium. Dilute cells 1:5 or 1:10 with trypan blue, count cells and determine viability using trypan blue exclusion. If viability is at least 90%, proceed to step 9.

6. Preparation of Study Samples

When experiment is done in 24-well plates, the assay requires 5 mL of nanoparticles dissolved/re-suspended in complete culture medium at a concentration 5X the highest final test concentration. When experiment is done in 96-well plates, the assay requires 1 mL of nanoparticles dissolved/re-suspended in complete culture medium at a concentration 5X the highest final test concentration. 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 [3] and are summarized in Box 1 below.

The assay will evaluate 4 concentrations: 10X (or when feasible 100X, 30X or 5X) of the theoretical plasma concentration, 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 10 mg/mL will be prepared and diluted 10-fold (1 mg/mL), followed by two 1:5 serial dilutions (0.2 and 0.04 mg/mL). When 200 µL of each of these samples are combined in a culture plate well with 800 µL of whole blood or cells, the final concentrations of nanoparticles are 0.008, 0.04, 0.2, and 2 mg/mL. Each nanoparticle concentration is plated 3 times. An additional 600 µL per dose is required for a cell-free control. When the cell-free control is prepared for the whole blood plate, an aliquot of the blood diluted in PBS from step 8.1 is spun down for 10 minutes at 2,500xg, and 800 µL of this cell free supernatant is combined with 200 µL of test nanoparticles.

For 96-well plate experiments, 40 µL of sample is combined with 160 µL of whole blood or cells per well to achieve the same concentrations as above. An additional 150 µL per dose is required for cell-free controls. Cell-free controls require 160 µL of blood supernatant or PBMC growth medium to be combined with 40 µL of test nanomaterials per well.

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.

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

7. Reagent and Control Preparation

7.1 Heat Inactivated Fetal Bovine Serum (FBS)

Thaw a 50 mL aliquot of FBS and equilibrate to room temperature. Place the tube in a 56°C water bath and incubate with mixing for 35 min. The heat inactivation takes 30 min; the initial 5 min is used to bring the entire content of the vial to 56°C. Allow the serum to equilibrate to room temperature and use to prepare complete culture media.

7.2 Complete RPMI-1640 Medium

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

7.3 Lipopolysaccharide (LPS, 1 mg/mL stock)

E.coli K12 LPS is supplied as a lyophilized powder. Reconstitute by adding 1 mL of sterile water per 1 mg of LPS to the vial and vortex to mix. Stocks with higher concentration (5-10 mg/mL) can also be prepared. Aliquot 20 µL and store at a nominal temperature of -20°C. Avoid repeated freeze-thaw cycles.

On the day of experiment, thaw one aliquot and use at a final concentration of 20 ng/mL in PBMC or WB culture.

7.4 Phytohemagglutinin (PHA-M, 1 mg/mL stock)

Add 1 mL of sterile PBS or cell culture medium per 1 mg of PHA-M to the vial and gently rotate to mix. Store daily use aliquots at a nominal temperature of -20ºC. Avoid repeated freezing/thawing. On the day of experiment dilute stock PHA-M solution in cell culture medium so that its final concentration in the positive control sample is 10 µg/mL.

7.5 ODN 2216 (1 mg/mL stock)

This oligonucleotide is supplied as lyophilized powder. Reconstitute in pyrogen free, nuclease free water to a final concentration of 1 mg/mL. Prepare single use 5 µL aliquots and store at -20°C. On the day of experiment thaw an aliquot at room temperature and dilute in culture media so that the final concentration in the test sample is 5 µg/mL.

7.6 Negative Control

Use PBS as a negative control. Process this control the same way as test samples.

7.7 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.

8. Experimental Procedure for Whole Blood Culture (24-well)

8.1 Dilute whole blood 4-fold with complete culture media (e.g., 3 mL of whole blood and 9 mL of complete culture media).

8.2 Dispense 800 µL of diluted blood from step 8.1. per well in a 24-well plate. Refer to Section 12 for plate layout.

Note: If positive control supernatants are used to prepare inhibition enhancement controls, do not forget to add extra replicates of the positive control sample.

8.3 Dispense 200 µL of blank media (baseline), negative control, positive control, vehicle control and test samples into corresponding wells on 24-well plate containing 800 µL of diluted blood from step 8.1. Prepare triplicate wells for each sample. Prepare cell-free control by dispending 200 µL of nanoparticles into 800 µL of cell-free supernatant prepared by spinning an aliquot of whole blood from step 8.1 for 10 min at 2500xg. Gently shake plates to allow all components to mix.

Note: The cell-free samples will be processed the same way as the whole blood samples and will serve as controls for false-positive results. To test for potential false-negative results, supernatant from positive control can be spiked with nanoparticle at a final nanoparticle concentration identical to that in test sample. Alternatively, a cell free supernatant containing nanoparticles can be spiked with cytokine standard in individual ELISA assay and analyzed against relevant quality control. If nanoparticle inhibits detection of cytokine, a decrease in the cytokine level will be seen when compared to the level of cytokine in positive control or in quality control samples. Additionally, to understand whether nanoparticle may potentiate or inhibit cellular response to the assay positive control (LPS, PHA-M or ODN2216), the positive control should be co-cultured with nanoparticles in the presence of cells; such testing would increase the required amount of nanomaterial detailed in section 6.

8.4 Repeat steps 8.1-8.3 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 blood derived from at least three donors.

8.5 Incubate 24 hours in a humidified 37C, 5% CO_2 incubator.

8.6 Collect cultured blood into 1.5 mL centrifuge tubes and spin in a microcentrifuge at a 18,000xg for 5 minutes.

8.7 Transfer supernatants into fresh tubes and either proceed with ELISA analysis or aliquot and store at -20°C.

Note: To avoid multiple freeze/thaw cycles, it is better to prepare multiple (200 or 300 µL) aliquots for each supernatant. Please refer to Table 3 to see acceptable number of freeze/thaw cycles for various NCL ELISAs. Refer to the commercial plate manufacturer's instruction for any freeze/thaw limits relevant to those kits.

9. Experimental Procedure for Whole Blood Culture (96-well)

9.1 Dilute whole blood 4-fold with complete culture media (e.g., 3 mL of whole blood and 9 mL of complete culture media).

9.2 Dispense 160 µL of diluted blood from step 9.1. per well in 96-well plate. Refer to section 12 for plate layout.

Note: If positive control supernatants are used to prepare inhibition enhancement controls, do not forget to add extra replicates of the positive control sample.

9.3 Dispense 40 µL of blank media (baseline), negative control, positive control, vehicle control and test samples into corresponding wells on 96-well plate containing 160 µL of diluted blood from step 9.1. Prepare triplicate wells for each sample. Prepare cell-free control by dispending 40 µL of nanoparticles into 160 µL of cell-free supernatant prepared by spinning an aliquot of whole blood from step 9.1 for 10 min at 2500xg. Gently shake plates to allow all components to mix.

Note: The cell-free sample will be processed the same way as the whole blood samples and will serve as control for false-positive results. To test for potential false-negative results, supernatant from positive control can be spiked with nanoparticle at a final nanoparticle concentration identical to that in test sample. Alternatively, a cell-free supernatant containing nanoparticles can be spiked with cytokine standard in individual ELISA assay and analyzed against relevant quality control. If nanoparticle inhibits detection of cytokine, a decrease in the cytokine level will be seen when compared to the level of cytokine in positive control or in quality control samples. Additionally, to understand whether nanoparticle may potentiate or inhibit cellular response to

the assay positive control (LPS, PHA-M or ODN2216), the positive control should be co-cultured with nanoparticles in the presence of cells; such testing would increase the required amount of nanomaterial detailed in section 6.

9.4 Repeat steps 9.1-9.3 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 blood derived from at least three donors.

9.5 Incubate 24 hours in a humidified 37C, 5% CO_2 incubator.

9.6 Collect cultured blood into 0.5 mL centrifuge tubes and spin in a microcentrifuge at a 18,000xg for 5 minutes.

9.7 Transfer supernatants into fresh tubes and either proceed with ELISA analysis or aliquot and store at -20°C.

Note: To avoid multiple freeze/thaw cycles, it is better to prepare multiple (60-80 µL) aliquots for each supernatant. Please refer to Table 3 to see acceptable number of freeze/thaw cycles for various NCL ELISAs. Refer to the commercial plate manufacturer's instruction for any freeze/thaw limits relevant to those kits.

10. Experimental Procedure for PBMC (24-well)

10.1 Adjust PBMC concentration to 1.3 x 10^6 viable cells/mL using complete RPMI medium.

10.2 Dispense 200 µL of blank media (baseline), negative control, vehicle control, positive control and test samples into corresponding wells on 24-well plate. Refer to section 12 for plate layout.

Note: If positive control supernatants are used to prepare inhibition enhancement controls, do not forget to add extra replicates of the positive control sample.

10.3 Dispense 800 µL of PBMC from step 10.1 per well in 24-well plate containing 200 µL of nanoparticles or complete culture medium to wells intended for cell free control. Refer to section 12 for plate layout. Gently shake plates to allow all components to mix.

Note: For each nanoparticle concentration prepare cell-free control by plating 800 µL of complete culture medium and 200µL of nanoparticle sample. The resulting sample will be processed the same way as PBMC samples and will serve as control for false-positive results. To test for potential false-negative results, supernatant from positive control can be spiked with nanoparticle at the final nanoparticle concentration identical to that in the test sample. Alternatively, cell-free control supernatants can be spiked with relevant cytokine standard used in ELISA or multiplex. If nanoparticle inhibits detection of cytokine a decrease in the cytokine level will be seen when compared to the level of cytokine in positive control or quality control, respectively. Additionally, to understand whether nanoparticle may potentiate or inhibit cellular response to the assay positive control (LPS, PHA-M or ODN2216), the positive control should be co-cultured with nanoparticles in the presence of cells; such testing would increase the required amount of nanomaterial detailed in section 6.

10.4 Repeat steps 10.1-10.3 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 blood derived from at least three donors.

10.5 Incubate 24 hours in a humidified 37C, 5% CO_2 incubator.

10.6 Collect cultured blood into 1.5 mL centrifuge tubes and spin in a microcentrifuge at a maximum speed for 5 minutes. Transfer supernatants into fresh tubes and either proceed with ELISA analysis or aliquot and store at -20°C.

Note: To avoid multiple freeze/thaw cycles, it is better to prepare multiple (200 or 300 µL) aliquots for each supernatant. Please refer to Table 3 to see acceptable number of freeze/thaw cycles for various NCL ELISAs. Refer to the commercial plate manufacturer's instruction for any freeze/thaw limits relevant to those kits.

11. Experimental procedure for PBMC (96-well)

11.1 Adjust PBMC concentration to 1.3 x 10^6 viable cells/mL using complete RPMI medium.

11.2 Dispense 40uL of blank media (baseline), negative control, vehicle control, positive control and test samples into corresponding wells on a 96-well U-bottom plate. Refer to section 12 for plate layout.

Note: If positive control supernatants are used to prepare inhibition enhancement controls, do not forget to add extra replicates of the positive control sample.

11.3 Dispense 160 µL of PBMC from step 11.1 per well in 96-well plate containing 40 µL of nanoparticles or complete culture medium to wells intended for cell free control. Refer to section 12 for plate layout. Gently shake plates to allow all components to mix.

Note: For each nanoparticle concentration prepare cell-free control by plating 160 µL of complete culture medium and 40µL of nanoparticle sample. The resulting sample will be processed the same way as PBMC samples and will serve as control for false-positive results. To test for potential false-negative results, supernatant from positive control can be spiked with nanoparticle at the final nanoparticle concentration identical to that in the test sample. Alternatively, cell-free control supernatants can be spiked with relevant cytokine standard used in ELISA or multiplex. If nanoparticle inhibits detection of cytokine a decrease in the cytokine level will be seen when compared to the level of cytokine in positive control or quality control, respectively. Additionally, to understand whether nanoparticle may potentiate or inhibit cellular response to the assay positive control (LPS, PHA-M or ODN2216), the positive control should be co-cultured with nanoparticles in the presence of cells; such testing would increase the required amount of nanomaterial detailed in section 6.

11.4 Repeat steps 11.1-11.3 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 blood derived from at least three donors.

11.5 Incubate 24 hours in a humidified 37C, 5% CO_2 incubator.

11.6 Spin the 96-well test plate in a centrifuge at 700xg for 10 minutes. Transfer supernatants into fresh plates and either proceed with ELISA analysis or aliquot and store at -20°C.

Note: To avoid multiple freeze/thaw cycles, it is better to prepare multiple (60-80 µL) aliquot plates for each supernatant. Please refer to Table 3 to see acceptable number of freeze/thaw cycles for various NCL ELISAs. Refer to the commercial plate manufacturer's instruction for any freeze/thaw limits relevant to those kits.

12. Example Plate Maps

Example of 24-well Plate Template for Culturing Whole Blood or PBMC.

	1	2	3	4	5	6
A	**NC (PBS)**	**PC (20 ng/mL LPS)**	**TS 1**	**TS 2**	**VC 1**	**VC 2**
B	**NC (PBS)**	**PC (20 ng/mL LPS)**	**TS 1**	**TS 2**	**VC 1**	**VC 2**
C	**NC (PBS)**	**PC (20 ng/mL LPS)**	**TS 1**	**TS 2**	**VC 1**	**VC 2**
D	**TS 1 Cell-free**	**TS 2 Cell-free**	**TS 1 Cell-free**	**TS 2 Cell-free**	**VC 1 Cell-free**	**VC 2 Cell-free**

Row D does not contain cells.

NC: negative control; PC: positive control (see Table 2); TS 1 and 2: test nanoparticle at two different test concentrations; VC1 and 2: vehicle control at same two test concentrations, respectively.

Example of 96-well Plate Template for Culturing Whole Blood or PBMC

	1	2	3	4	5	6	7	8	9	10	11	12
A	Donor 1 NC	Donor 1 NC	Donor 1 NC	Donor 1 PC1 (LPS)	Donor 1 PC1 (LPS)	Donor 1 PC1 (LPS)	Donor 1 PC2 (PHA-M)	Donor 1 PC2 (PHA-M)	Donor 1 PC2 (PHA-M)	Donor 1 PC3 (ODN2216)	Donor 1 PC3 (ODN2216)	Donor 1 PC3 (ODN2216)
B	Donor 1 TS1	Donor 1 TS1	Donor 1 TS1	Donor 1 TS2	Donor 1 TS2	Donor 1 TS2	Donor 1 TS3	Donor 1 TS3	Donor 1 TS3	Donor 1 TS4	Donor 1 TS4	Donor 1 TS4
C	Donor 1 VC1	Donor 1 VC1	Donor 1 VC1	Donor 1 VC2	Donor 1 VC2	Donor 1 VC2	Donor 1 VC3	Donor 1 VC3	Donor 1 VC3	Donor 1 VC4	Donor 1 VC4	Donor 1 VC4
D	Donor 1 TS1 Cell-Free	Donor 1 TS1 Cell-Free	Donor 1 TS1 Cell-Free	Donor 1 TS2 Cell-Free	Donor 1 TS2 Cell-Free	Donor 1 TS2 Cell-Free	Donor 1 TS3 Cell-Free	Donor 1 TS3 Cell-Free	Donor 1 TS3 Cell-Free	Donor 1 TS4 Cell-Free	Donor 1 TS4 Cell-Free	Donor 1 TS4 Cell-Free
E	Donor 2 NC	Donor 2 NC	Donor 2 NC	Donor 2 PC1 (LPS)	Donor 2 PC1 (LPS)	Donor 2 PC1 (LPS)	Donor 2 PC2 (PHA-M)	Donor 2 PC2 (PHA-M)	Donor 2 PC2 (PHA-M)	Donor 2 PC3 (ODN2216)	Donor 2 PC3 (ODN2216)	Donor 2 PC3 (ODN2216)
F	Donor 2 TS1	Donor 2 TS1	Donor 2 TS1	Donor 2 TS2	Donor 2 TS2	Donor 2 TS2	Donor 2 TS3	Donor 2 TS3	Donor 2 TS3	Donor 2 TS4	Donor 2 TS4	Donor 2 TS4
G	Donor 2 VC1	Donor 2 VC1	Donor 2 VC1	Donor 2 VC2	Donor 2 VC2	Donor 2 VC2	Donor 2 VC3	Donor 2 VC3	Donor 2 VC3	Donor 2 VC4	Donor 2 VC4	Donor 2 VC4
H	Donor 2 TS1 Cell-Free	Donor 2 TS1 Cell-Free	Donor 2 TS1 Cell-Free	Donor 2 TS2 Cell-Free	Donor 2 TS2 Cell-Free	Donor 2 TS2 Cell-Free	Donor 2 TS3 Cell-Free	Donor 2 TS3 Cell-Free	Donor 2 TS3 Cell-Free	Donor 2 TS4 Cell-Free	Donor 2 TS4 Cell-Free	Donor 2 TS4 Cell-Free

Rows D & H do not contain cells.

NC: negative control; PC: positive control (see Table 2); TS 1-4: test nanoparticle at four different test concentrations; VC 1-4: vehicle control at same four test concentrations, respectively.

13. References

1. Suntharalingam GS et al. Cytokine storm in a phase 1 trial of the anti-CD28 monoclonal antibody TGN1412. New England Journal of Medicine2006, 355: 1018-1028.
2. Stebbings R et al. Cytokine storm in the phase I trial of the monoclonal antibody TGN1412: better understanding the cause to improve the preclinical testing of immunotheraputics. J Immunology, 2007, 179: 3325-3331.
3. 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.

14. Abbreviations

FBS	fetal bovine serum
PBS	phosphate buffered saline
RPMI	Roswell Park Memorial Institute
VC	vehicle control
PBMC	peripheral blood mononuclear cells
FT	freeze/thaw
IL	interleukin
LPS	lipopolysaccharide
ODN	oligodeoxyribonucleotide
PHA-M	phytohemagglutinin
TNF	tumor necrosis factor
IFN	interferon