NCL Method ITA-10

Preparation of Human Whole Blood and Peripheral Blood Mononuclear Cell Cultures to Analyze Nanoparticle Potential to Induce Inflammatory Cytokines, Chemokines and Interferons 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

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 six 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 with rats or 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). This warrants 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 to identify a nanoparticle’s 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 for the type of assay to use or between

singleplex and multiplex analysis. 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 hr 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 stabilities at room temperature (RT) and upon repeated freeze/thaw (FT) cycles. Please refer to individual NCL ELISA protocol or manufacturer's instruction of commercial kits for information about RT and FT stabilities. Section 12 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; their inclusion is for informational purposes only. Equivalent supplies from alternate vendors can be substituted.

3.1 Reagents for Whole Blood Cultures

1 Human blood anti-coagulated with Li-heparin and obtained from at least three healthy donors

2 PBS (GE Life Sciences, SH30256.01)

3 RPMI-1640 (Invitrogen, 11835-055)

4 Fetal bovine serum (GE Life Sciences, HyClone, SH30070.03)

5 Penicillin streptomycin solution (Invitrogen, 15140-148)

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

3.2 Reagents for PBMC Cultures

1. All materials listed in section 3.1
2. Ficoll Paque Premium (GE Healthcare, 17-5442-02)
3. Hank's balanced salt solution (HBSS) (Invitrogen, 24020-117)

Please refer to the table below for guidance in selecting whole blood or PBMC.

	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 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

1. Ultrapure LPS from K12 E.coli (Invivogen, tlrl-peklps)
2. ODN2216 is a CpG DNA oligonucleotide with a mixed backbone and the following sequence, 5'-ggGGGACGATCGTCGggggG-3, where lowercase letters show phosphorothioate linkages and capital letters refer to phosphodiester linkages between nucleotides. This can be custom ordered from IDT or equivalent supplier.
3. Phytohemagglutinin (PHA-M) (Sigma, L8902)

Please refer to the table below for guidance on concentrations and purpose of these 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
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 Materials

1. Pipettes, 0.05 to 10 mL
2. 24-well round bottom plates
3. Polypropylene tubes, 50 and 15 mL
4. Microcentrifuge tubes

3.5 Equipment

1. Centrifuge, 900x*g*, 2500x*g*, 18,000x*g*
2. Refrigerator, 2-8 ℃
3. Freezer, -20 ℃
4. Cell culture incubator with 5% CO_2 and 95% humidity.
5. Biohazard safety cabinet approved for level II handling of biological material
6. Inverted microscope
7. Vortex
8. Hemocytometer

Refer to manufacturer's instruction of commercial ELISA or multiplex kit, or to NCL assays ITA-22, ITA-23, ITA-24, or ITA-25 for additional reagent and material requirements for the selected study.

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 first 10 cc. For best results, whole blood should be used within 1 hr after collection. Prolonged storage (> 2 hr) of whole blood will lead to a decrease in cell function.

5. Preparation of PBMC

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

3. Centrifuge 30 min at 900x*g*, 18-20ºC, without brake.

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

4. Using a sterile pipet, remove the upper layer containing plasma and platelets and discard.
5. Using a fresh sterile pipet, transfer the mononuclear cell layer into a fresh centrifuge tube.
6. Wash cells by adding an excess of HBSS and centrifuging for 10 min at 400x*g*, 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.

7. Discard supernatant and repeat wash step once more.
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 Nanoparticles

When the experiment is done in 24 well plates, this assay requires 5 mL of nanoparticle solution dissolved/resuspended in complete culture medium, at a concentration 5X 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 [3] 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 10 mg/mL is prepared. This sample is then 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 sample dilutions are combined in a culture plate well with 800 µL of cells, the final nanoparticle concentrations tested in this assay are: 2.0, 0.2, 0.04 and 0.008 mg/mL. Each nanoparticle concentration is plated in triplicated.

An additional 600 µL 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 centrifuged for 10 minutes at 2,500x*g* and 800 µL of this cell-free supernatant is combined with 200 µL of test nanoparticles.

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:

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

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)

Add 1 mL of sterile water to 1 mg of LPS to the vial and vortex to mix. Aliquot 20 µL and store at a nominal temperature of -20°C. Avoid repeated freeze-thaw. On the day of the experiment, thaw one aliquotIts final concentration in PBMC or WB culture should be 20 ng/mL.

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 and gently rotate to mix. Store daily use aliquots at a nominal temperature of -20ºC. Avoid repeated freezing/thawing. On the day of the experiment dilute stock PHA-M solution in cell culture medium to a final concentration in the positive control sample of 10 µg/mL

7.5 ODN 2216 (1mg/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 the experiment thaw an aliquot at room temperature and dilute in culture media so that final concentration in the test sample is 5 µg/mL.

7.6 Negative Control

Use PBS as the negative control. Process this control the same way as the 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 the 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

1. Dilute whole blood 4X with complete culture media (e.g., 3 mL of whole blood and 9 mL of complete culture media).

2. Dispense 800 µL of diluted blood from step 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.
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 800µL of diluted blood from step 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 1 for 10 min at 2500x*g*. 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 controls for false-positive results. To test for potential false-negative result, supernatant from positive control can be spiked with nanoparticle at a final nanoparticle concentration identical to that in the test sample. Alternatively, a cell free supernatant containing nanoparticles can be spiked with cytokine standard in individual ELISA assays and analyzed against the 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 the 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.
4. Repeat steps 1-3 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.
5. Incubate 24 hr in a humidified 37°C, 5% CO_2 incubator.
6. Collect cultured blood in 1.5 mL centrifuge tubes and spin in a microcentrifuge at 18,000x*g* for 5 minutes.
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 best to prepare multiple aliquots(200 or 300 µL) for each supernatant. Refer to section 13 to see the acceptable number of

freeze/thaw cycles for the NCL ELISAs. Refer to the commercial plates manufacturer's instruction for any freeze/thaw limits relevant to those kits.

9. Experimental Procedure for PBMC

1. Adjust PBMC concentration to 1.3 x 10^6 viable cells/mL using complete RPMI medium.
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.
3. Dispense 800 µL of PBMC from step 1 per well in a 24 well plate containing 200µL of nanoparticles or complete culture medium to wells intended for cell free control. Refer to section 12 for plate layout. Gently shake plates to mix all components.

   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 a 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 the test sample. Alternatively, cell-free control supernatants can be spiked with relevant cytokine standards 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 the positive control or quality control, respectively. Additionally, to understand whether nanoparticles 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.
4. Repeat steps 1-3 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.
5. Incubate 24 hours in a humidified 37°C, 5% CO_2 incubator.

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

   Note: To avoid multiple freeze/thaw cycles, it is best to prepare multiple aliquots(200 or 300 µL) for each supernatant. Refer to section 12 to see the acceptable number of freeze/thaw cycles for various NCL ELISAs. Refer to the commercial plates manufacturer's instruction for any freeze/thaw limits relevant to those kits.

10. References

1. Suntharalingam GS et al. Cytokine storm in a phase 1 trial of the anti-CD28 monoclonal antibody TGN1412. New England Journal of Medicine, 2006, 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.

11. Abbreviations

FBS	fetal bovine serum
PBS	phosphate buffered saline
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

12. Appendix

Example 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; TS 1 and 2: test nanoparticle at two different test concentrations; VC1 and 2: vehicle control at same two test concentrations

Freeze/Thaw Limits for Culture Supernatants for NCL ELISA Assays

NCL Assay (Analyte)	Acceptable Number of F/T Cycles
ITA-22 (IL-8)	1
ITA-23 (IL-1β)	3
ITA-24 (TNFα)	3
ITA-25 (IFNγ)	2