NCL Method ITA-9.2

In Vitro Assay for Assessing Nanoparticle Effects on Monocyte/Macrophage Phagocytic Function

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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 evaluation of nanoparticle effects on the phagocytic function of immune cells. Phagocytosis is a receptor-mediated endocytosis peculiar to the phagocytic cells, e.g. cells of the mononuclear phagocytic system (MPS). Phagocytosis is an active process and requires actin polymerization. There are four primary receptors which mediate phagocytic uptake. Phagocytosis via three of these receptors (complement receptor (CR), FcγR receptor, and mannose receptor (MR)) is accompanied by inflammatory reactions (cytokine secretion). Phagocytosis via the fourth receptor (scavenger receptor (SR)) is not accompanied by inflammatory responses [1-5].

2. Principles and Limitation

HL-60 promyelocytic cells are used as the model phagocytic cell line, and Zymosan A is used as a model bioparticle. The phagocytic activity of HL-60 cells is visualized with luminol. Luminol is a dye which is not luminescent unless exposed to the low pH of the phagolysosome. Nanoparticles may either enhance or inhibit the cell phagocytic function. Such effects are monitored by comparing the Zymosan A uptake in control cells to that of cells exposed to test nanomaterials 24 hours prior to the addition of Zymosan A.

3. Reagents, Materials, Cell Lines, 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 Phosphate buffered saline (PBS) (GE Life Sciences, Hyclone, SH30256.01)
- 3.1.2 Zymosan A (Sigma-Aldrich, Z4250)
- 3.1.3 Fetal bovine serum (FBS) (GE Life Sciences, Hyclone, SH30070.03)

3.1.4 RPMI-1640 (GE Life Sciences, HyClone, SH30096.01)

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

3.1.6 Trypan Blue solution (Gibco, 15250-061)

3.1.7 Human AB serum or plasma pooled from at least three donors

3.1.8 Luminol (Sigma-Aldrich, 123072)

3.2 Materials

3.2.1 Pipettes covering the range 0.05 to 10 mL

3.2.2 Flat bottom 96-well white luminescence plates

3.2.3 Polypropylene tubes, 50 and 15 mL

3.3 Cell Lines

3.3.1 HL-60 promyelocytic cells (ATCC, CCL-240)

3.4 Equipment

3.4.1 Centrifuge capable of operating at 400xg and 2000xg

3.4.2 Refrigerator, 2-8ºC

3.4.3 Freezer, -20ºC

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

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

3.4.6 Inverted microscope

3.4.7 Vortex

3.4.8 Hemocytometer

3.4.9 Plate reader capable of working in luminescence mode

Note: The plates used for this assay have a solid white bottom; therefore, the plate should be read from the top. Depending on the type of the plate reader, one may need to use plate adaptor to provide optimal conditions for top read.

3.4.10 Warm gel-pack

Note: This material is optional and may be omitted. It is used to keep the plate warm for optimal phagocytosis. However, if it takes longer than 2 minutes to transfer the plate to the plate reader after addition of all

reagents, the phagocytosis process will begin before one starts to analyze the plate on the plate reader.

4. Reagent and Control Preparation

4.1 Complete RPMI-1640 Medium

The complete RPMI medium should contain the following reagents:

20% FBS (heat inactivated)

4 mM L-glutamine

100 U/mL penicillin

100 μg/mL streptomycin sulfate

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

4.2 Zymosan A Stock

Prepare Zymosan A stock at final concentration of 2 mg/mL in PBS. Use freshly prepared.

4.3 Opsonized Zymosan A

Combine Zymosan A stock and human AB serum or plasma. Use 1 mL of serum/plasma per each 0.5 mL of zymosan A stock. Incubate Zymosan A with serum/plasma for 30 minutes at 37°C. Wash Zymosan A particles with PBS (use 1 mL of PBS per each 0.5 mL of original Zymosan stock and a centrifuge setting of 2000xg for 2 min) and resuspend in PBS to a final concentration of 2 mg/mL.

4.4 Negative Control

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

4.5 Heat-Inactivated Fetal Bovine Serum

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

4.6 Luminol Stock (10 mM in DMSO)

Dissolve luminol in DMSO to a final concentration of 10 mM, e.g. dissolve 17.7 mg of luminal in 10 mL of DMSO. Prepare single use aliquots and store at -20°C; protect from light.

4.7 Luminol Working Solution (250 μM in PBS).

On the day of the experiment, thaw an aliquot of luminol stock solution and dilute with PBS to a final concentration of 250 μM, e.g. add 250 μL of 10 mM stock into 9.750 mL of PBS. Protect from light. Discard unused portion.

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.

5. Preparation of Study Samples

This assay requires 2 mL of nanoparticles at 5x the highest test concentration dissolved/resuspended in PBS. 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 [6] 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:

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

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.

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

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 serial 5-fold 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 cells, the final concentrations of nanoparticles are 0.008, 0.04, 0.2, 2 mg/mL. Each nanoparticle concentration is plated six times.

6. Cell Preparation

HL-60 is a non-adherent promyelocytic cell line derived by S.J. Collins, et al. from a patient with acute promyelocytic leukemia [5]. Cultures can be maintained by the addition of fresh medium or replacement of medium. Alternatively, cultures can be established by centrifugation with subsequent resuspension at $1x10^5$ viable cells/mL. **Do not allow cell concentration to exceed $1x10^6$ cells/mL.** Maintain cell density between $1x10^5$ and $1x10^6$ viable cells/mL. On the day of the experiment, count cells using trypan blue. If the cell viability is ≥90% proceed to the next step.

7. Experimental Procedure, Day 1

7.1 Adjust cell concentration to 1.25 x 10^6 cells per mL using complete medium.

7.2 Plate 800 µL of the cell suspension from step 7.1 per well on 24-well plate. Prepare 6 wells for each nanoparticle concentration, vehicle control, negative control and 8 wells for untreated cells. Refer to Appendix for example of a plate map.

7.3 Add 200 µL of test samples to corresponding wells. Cover the plates and incubate at 37°C overnight (18-24 hr).

8. Experimental Procedure, Day 2

8.1 Turn on plate reader, allowing it to warm up to 37°C. Place an empty white 96-well test plate inside the plate reader chamber, allowing it to warm to 37°C as well. Set up the instrumental parameters.

8.2 Harvest cells from step 7.3 into Eppendorf tubes and wash twice with PBS to remove nanoparticles. Do not pool the content of individual wells within the treatment group; each well serves as a separate replicate.

8.3 After the last wash reconstitute cell pellet in 240 µL of complete media. Use 20 µL of this suspension for determining cell count and viability by trypan blue staining or other relevant procedure.

8.4 Adjust cell concentration to 0.9-1 x 10^7 viable cells/mL using complete medium. Keep at room temperature.

8.5 Plate 100 µL of cell suspension per well on the 96 well white plate pre-warmed in step 8.1. Prepare 4 wells with 100 µL of PBS for no cells control and another 4 wells with 200 µL of PBS for Luminol only. Refer to Appendix for an example plate map.

Note: This step and steps 8.6 and 8.7 can be done at room temperature (20-22°C). However, when the room temperature is low, keep the plate on a warm gel pack during these steps.

8.6 Add 100 µL of Luminol working solution from step 4.7 to each well. Please refer to the note in step 8.5 for additional details about plate handling conditions.

8.7 Using multichannel pipette, quickly add 100 µL of opsonized Zymosan A from step 4.3 to all wells except blank wells. Please refer to the note in step 8.5 for additional details about plate handling conditions.
Note: This step can be performed on the bench close to the plate reader to minimize the time between sample addition and initiation of the kinetic reading.

8.8 Start kinetic reading on a luminescence plate reader immediately.
Note: Plate readers capable of both top and bottom reading may require a plate adaptor for top reads. Check user manuals before proceeding with the plate analysis on the plate reader.

9. Calculations

9.1 Using Excel or other relevant software, compare area under the curve (AUC) for all samples. An increase in the AUC at least 2-fold above the negative control (baseline) is considered a positive response. Use relevant statistical analysis to compare AUC values for test samples to that of the baseline.

9.2 A percent coefficient of variation is used to control precision and calculated for each control or test sample according to the following formula:

$$\%CV = \frac{standard\ deviation}{mean} * 100\%$$

10. Acceptance Criteria

10.1 %CV for each control and test sample should be $< 30\%$.

10.2 Samples demonstrating higher variability should be re-analyzed.

11. References

1. Antonini JM., van Dyke K., ye Z., DeMatteo M., Reasor MJ. Introduction of luminol-dependent chemiluminescence as a method to study silica inflammation in the tissue and phagocytic cells of rat lung. Environ. Health Perspect., 1994, 102(suppl10), 37-42.
2. Gref R., Luck M., Quellec P., et al. Stealth corona-core nanoparticles surface modified by PEG: influences of corona (PEG chain length and surface density)

and of the core composition on phagocytic uptake and plasma protein absorption. Colloids and surfaces B: Biointerfaces, 2000, 18: 301-313.

3. Leroux JC., Gravel P., Balant L., et al. Internalization of poly(D,L-lactic acid) nanoparticles by isolated human leukocytes and analysis of plasma proteins absorbed onto particles. J.Biomed.Materials Res., 1994, 28:471-481.
4. Mold C., Gresham HD., DuClos TW. Serum Amyloid P component and C-reactive protein mediate phagocytosis through murine FcγRs. J.Immunol., 2001, 166:1200-1205.
5. Collins SJ, Ruscetti FW, Gallagher RE, Gallo RC. Terminal differentiation of human promyelocytic leukemia cells induced by dimethyl sulfoxide and other polar compounds. Proc Natl Acad Sci U S A. 1978 May;75(5):2458-62.
6. 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

AUC	area under the curve
CV	coefficient of variation
DMSO	dimethyl sulfoxide
FBS	fetal bovine serum
PBS	phosphate buffered saline
RPMI	Roswell Park Memorial Institute
SD	standard deviation
VC	vehicle control

13. Appendix

Example Plate Map, Day 1

1	2	3	4	5	6
Untreated Cells	Untreated Cells	TS (1 mg/mL)	TS (1 mg/mL)	TS (0.2 mg/mL)	TS (0.2 mg/mL)
Untreated Cells	Untreated Cells	TS (1 mg/mL)	TS (1 mg/mL)	TS (0.2 mg/mL)	TS (0.2 mg/mL)
Untreated Cells	Untreated Cells	TS (1 mg/mL)	TS (1 mg/mL)	TS (0.2 mg/mL)	TS (0.2 mg/mL)
Untreated Cells	Untreated Cells				

1	2	3	4	5	6
TS (0.04 mg/mL)	TS (0.04 mg/mL)	TS (0.008 mg/mL)	TS (0.008 mg/mL)	NC	NC
TS (0.04 mg/mL)	TS (0.04 mg/mL)	TS (0.008 mg/mL)	TS (0.008 mg/mL)	NC	NC
TS (0.04 mg/mL)	TS (0.04 mg/mL)	TS (0.008 mg/mL)	TS (0.008 mg/mL)	NC	NC
VC	VC	VC	VC	VC	VC

NC: Negative Control; TS: Test Sample; VC: Vehicle Control

Example Plate Map, Day 2

	1	2	3	4	5	6	7	8	9	10	11	12
A	Untreated Cells	Untreated Cells	NC	NC	NC	VC	VC	VC	PBS No cells	PBS No cells		
B	Untreated Cells	Untreated Cells	NC	NC	NC	VC	VC	VC	PBS No cells	PBS No cells		
C	TS 0.008 mg/mL	TS 0.008 mg/mL	TS 0.008 mg/mL	TS 0.04 mg/mL	TS 0.04 mg/mL	TS 0.04 mg/mL	TS 0.2 mg/mL	TS 0.2 mg/mL	TS 0.2 mg/mL	TS 1.0 mg/mL	TS 1.0 mg/mL	TS 1.0 mg/mL
D	TS 0.008 mg/mL	TS 0.008 mg/mL	TS 0.008 mg/mL	TS 0.04 mg/mL	TS 0.04 mg/mL	TS 0.04 mg/mL	TS 0.2 mg/mL	TS 0.2 mg/mL	TS 0.2 mg/mL	TS 1.0 mg/mL	TS 1.0 mg/mL	TS 1.0 mg/mL
E												
F												
G	Untreated Cells	Untreated Cells	PBS	PBS								
H	Untreated Cells	Untreated Cells	PBS	PBS								

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

These wells receive both Luminol and Zymosan A (rows A-D)

These wells receive Luminol only; PBS is used instead of Zymosan A to adjust the volume (rows G and H)