NCL Method ITA-26

Detection of Intracellular Complement Activation in Human T Lymphocytes

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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

The complement present in plasma is a group of approximately 30 proteins which are produced by the liver. Plasma complement plays an important role in innate immunity and promotes adaptive immunity against pathogens. Activation of plasma complement may occur in response to a drug product. Such undesirable activation results in immediate-type hypersensitivity reactions independent of immunoglobulin E. These reactions are often referred to as Complement Activation Related Pseudoallergy (CARPA). Although CARPA has been observed in the clinic with various types of drug products, nanoformulations, and especially PEGylated liposomes, are frequent causes of CARPA in patients [1, 2].

Activation of plasma complement is initiated via three main pathways, the classical, the alternative and the lectin pathway, all of which converge at the complement component 3 (C3) to form a common, terminal pathway leading to the formation of the membrane attack complex [3]. Cleavage of C3, C4 and C5 components of the plasma complement results in generation of several split products, C3a, C4a, and C5a, which are also known as anaphylatoxins. The anaphylatoxins are the main triggers of the symptoms observed in CARPA. The larger split products (C3b, C4b, and C5b) are opsonins which bind to and accelerate the clearance of pathogens.

Recently, the concept of complement has been expanded to include so-called intracellular complement [4]. The complement protein C3 has been found in many cells including lymphocytes, monocytes, endothelial and even cancer cells. The activation of intracellular complement generates split products which are exported from the cell onto the membrane. Since the activation of intracellular complement in T-lymphocytes was found to correlate with autoimmune disorders [4], and growing evidence is available for the involvement of T-lymphocytes in the development of drug-induced hypersensitivity reactions [5], understanding the ability of nanomaterials to activate intracellular complement may aid in establishing long-term safety profiles for these materials.

2. Principles

This assay has been adapted from the original study described by Liszewski, et al., [4] and optimized to support nanoparticle screening. Human peripheral blood mononuclear cells are treated with nanoparticles and controls, and the presence of the complement split products on the

cell surface is then monitored by flow cytometry. Although the gating strategy used in this protocol is specific to CD3-positive lymphocytes (T-cells), this protocol can be adjusted to detect intracellular complement on the surface of other cells. The assay requires 400 µL of the test nanomaterial at a concentration determined through previous cytotoxicity testing of the nanomaterial. The details of nanoparticle concentration selection are provided in section 4.2 of the protocol.

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

1. Human blood coagulated with Li-heparin
2. PBS (HyClone, AQB 22934)
3. Ficoll-Paque Premium (GE HealthCare ,17-5442-03)
4. Fetal bovine serum (Hyclone, SH30070.03)
5. RPMI1640 (Invitrogen, 11875-119)
6. Pen/Strep solution (Invitrogen, 15140-148)
7. Cellometer ViaStain AOPI Staining Solution in PBS (Nexcelom Biosciences, CS2-0106; or other reagent for distinguishing between live and dead cells)
8. Albumin, from bovine serum (BSA) (Sigma, A4503)
9. Purified Mouse Anti-Human CD3 IgG2a (Abcam ab86883; use for positive control)
10. Purified Mouse Anti-Human CD3 IgG2a (eBioscience 16-0037; use flow cytometry to gate CD3 positive cells)
11. Purified Mouse Anti-Human C3adesArg, clone 2991, (NEO-Epitope) IgG1 (Hycult Biotech, HM2074)
12. Purified Mouse IgG1 Isotype control (BD Pharmingen, 554121)
13. Purified Mouse IgG2a Isotype control (BD Pharmigen, 554126)
14. APC Rat Anti-Mouse IgG2a (eBioscience, 17-4210)
15. PE Rat Anti-Mouse IgG1 (eBioscience, 12-4015)

3.2 Materials

1. Polystyrene round bottom 12 x 75 mm^2 Falcon tubes (Corning, 352058)
2. Pipettes, 0.05 to 10 mL
3. 24-well cell culture plates (Corning, 3524)
4. Polypropylene tubes, 5 mL

3.3 Equipment

1. Centrifuge, 400xg (or ~1440 rpm)
2. Refrigerator, 2-8°C
3. Freezer, -20°C
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. Cellometer or hemocytometer
9. Flow Cytometer (e.g., FACSCalibur)

4. Reagent and Control Preparation

4.1 Complete RPMI

The complete RPMI medium should contain 10% FBS (heat-inactivated), 4 mM L-glutamine, and 100 U/mL penicillin and 100 µg/mL streptomycin sulphate. Store at 2-8°C protected from light for no longer than 1 month. Before use, warm the medium in a 37°C water bath.

4.2 Preparation of test nanomaterials

The important aspect of this test is to assess nanoparticles at non-cytotoxic concentrations, i.e., those not affecting cell viability. Therefore, there is no standard concentration for this test. Each nanomaterial should be evaluated for cytotoxicity before analyzing in this assay. To achieve this, titrate nanoparticles in peripheral blood mononuclear cells (PBMC) starting with the highest reasonable/achievable concentration to select the highest concentration which results in cell viability no less than 80% upon 1 hr incubation with the nanomaterial. Trypan blue exclusion test, MTT, 7-ADD, Cellometer or other relevant cell viability assays can be used for this

purpose. Since the dilution factor for this assay is 5, the stock concentration of nanoparticle required for the test should be 5 times higher than this concentration. For example, if the cell viability test determined that the highest non-toxic concentration of nanoparticles in PBMC is 0.1 mg/mL, then you will need 400 µL of a nanoparticle stock at a concentration 0.5 mg/mL. Two hundred (200) µL of this stock will be used directly to treat the cells, and the rest of the material will be used to prepare three serial 1:5 dilutions of this stock. In this example, the final concentrations tested in the assay will be 0.1, 0.02, 0.004 and 0.0008 mg/mL. If a test-nanomaterial is loaded with a cytotoxic drug, the concentrations should reflect the drug and the carrier concentrations. It is also important to include a carrier control.

4.3 Heat-inactivated fetal bovine serum

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

4.4 Staining buffer (0.1% bovine serum albumin (BSA) in PBS)

Weigh 50 mg of the reagent and add into 50 mL ice-cold 1X PBS. Mix well and place on ice. Prepare fresh before each use. Discard any leftover after experiment is complete.

4.5 Positive control

Cells treated for 1 hr with 5 µg/test of Mouse Anti-Human CD3 IgG2a (Abcam cat#ab86883).

4.6 Negative control

Use cells without treatment as a negative control.

Note: For flow cytometry analysis include a tube with unlabeled cells, which is processed as other tubes except no antibodies added.

5. Isolation of Human 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, 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 the tube at a 45° angle.

5.3 Centrifuge 30 minutes at 900xg, 18-20°C without brake.

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

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

5.6 Wash cells by adding an excess of HBSS and centrifuging for 10 min at 400xg, 18-20°C. The HBSS volume should be ~3 times the volume of the mononuclear layer.
Note: Typically, 4 mL of blood/PBS mixture results in ~2 mL of the mononuclear layer and requires at least 6 mL of HBSS for wash step. We use 10 mL of HBSS per each 2 mL of cells.

5.7 Discard supernatant and repeat wash step one more time.

5.8 Resuspend cells in complete RPMI1640 medium. Count cells and determine viability using AOPI staining solution. If viability is at least 90%, proceed to the next step.

6. Treatment with Nanoparticles and Controls

6.1 Adjust cell count to$1.25x10^6$ cells/mL.

6.2 Aliquot 800 µL of cell suspension prepared in step 5.8 per each well of a 24 well-plate.
Note: plan additional wells for isotype controls.

6.3 Add 200 µL of test-nanoparticles or control to appropriate wells.

6.4 After 1 hour of incubation with test-materials and controls, harvest the cells and measure cell viability. If viability is ≥80% proceed to the next step. If not, stop the experiment.

7. Preparation of Cells for Flow Cytometry

7.1 Harvest cells into polystyrene round bottom 12 x 75 mm^2 Falcon tubes and centrifuge at 400xg for 5 minutes.

7.2 Aspirate supernatant and wash cells with 2 mL ice-cold PBS.

7.3 Resuspend cells with 100 µL of staining buffer.

7.4 Add 1 µg/test of Purified Mouse Anti-Human C3adesArg (clone 2991)—except for tubes with isotype controls and a tube with unlabeled cells..

7.5 Add 1 µg/test of Purified Mouse IgG1 and 0.25µg/test of Purified Mouse IgG2a in isotype control #1 and only 1 µg/test of Purified Mouse IgG1 in isotype control #2.
Note: It is convenient to prepare an appropriate amount of working solutions of antibodies in staining buffer and add 100 µL of antibodies containing staining solution to the cells.

7.6 Add 0.25 µg/test of CD3 (eBioscience) to tubes except PC and isotype control #1 and unlabeled cells.
Note: Isotype controls should be tested with the highest concentration of each tested nanoparticle.

7.7 Incubate on ice for 20 minutes.

7.8 Wash cells two more times with 1 µL of PBS by centrifugation at 400xg at 4°C for 5 minutes and resuspend cells with 100 µL of ice-cold staining buffer.

7.9 Add 0.25 µg/test of PE Rat Anti-Mouse IgG1 and 0.5 µg/test APC Rat Anti-Mouse IgG2a to tubes.

7.10 Incubate at 4°C for 20 minutes.

7.11 Wash cells twice with 1 mL of PBS by centrifugation at 400xg for 5 minutes.

7.12 Resuspend with 400 µL of PBS.

7.13 Test samples using flow cytometer.
Note: All staining procedures should be performed on ice, and centrifuge temperature must be adjusted to 4°C.

8. Flow Cytometry

At the NCL, we use FACSCalibur. The procedure below is based on our experience with this instrument. If you are using another cytometer, please follow the procedure specific to that instrument.

8.1 Switch on the instrument. Make sure the sheath tank is full and the waste tank is empty.

8.2 To get consistent results, run BD FACSComp software using BD CaliBRITE beads.

8.3 Adjust FSC vs. SSC dot plot using unlabeled cells to get the population of cells in the plot. See Figure 1A below for example.

8.4 Set the threshold to remove most of the debris.

8.5 Gate lymphocyte population (gate 1). See Figure 1A below for example.

8.6 Create an FL2 vs. FL4 dot plot. Limit this dot plot to the cell population from gate 1.

8.7 Adjust FL2 and FL4 settings to get cells in the left low corner of FL2 vs. FL4 dot plot using unlabeled/untreated cells and isotype control #1.

8.8 Set up quadrant regions based on isotype control #2. See Figure 1B below for example.

8.9 Acquire and save the data.

8.10 Follow the instrument closing procedure.

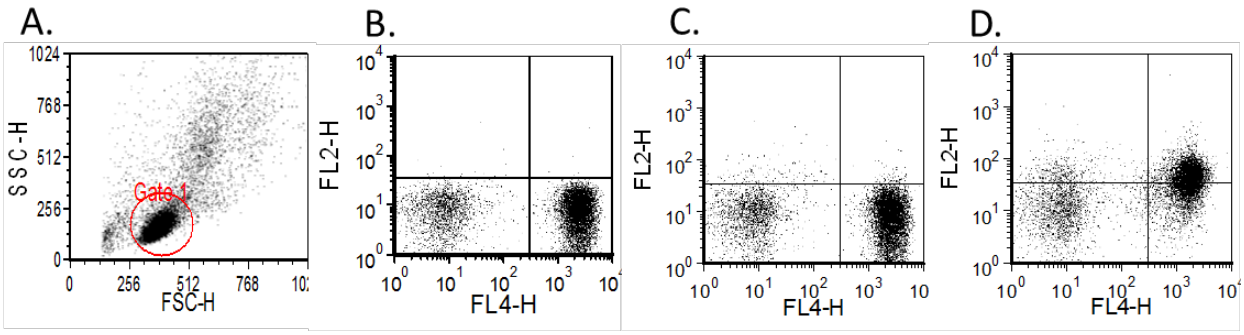

Figure 1. Example of Flow Cytometry Analysis of C3a Expression. (A) FSC vs. SSC dot plot and gate to select the cell population of interest. (B) Isotype control #2 (FL2 vs. FL4) (C) Negative control (FL2 vs. FL4) (D) Positive control (FL2 vs. FL4).

9. Data Analysis and Report

Use appropriate software to analyze the data acquired in step 8.9. CellQuest or other flow cytometry software can be used. Here, FCSExpress from De Novo Software Solutions, Inc. was used. Report the data as percent double positive cells, which is determined by comparing the number of double positive cells against that in the negative control.

10. Acceptance Criteria

10.1 The run is acceptable if no nonspecific binding is detected in isotype control #1.

10.2 The run is acceptable if the positive control is positive, i.e., more than 10% of cells are positive in C3adesArg staining.

11. References

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