

In Vitro and In Vivo Methods for Analysis of Nanoparticle Potential to Induce Delayed-Type Hypersensitivity Reactions

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Abstract

Delayed-type hypersensitivity (DTH) reactions are among the common reasons for drug withdrawal from clinical use during the post-marketing stage. Several in vivo methods have been developed to test DTH responses in animal models. They include the local lymph node assay (LLNA) and local lymph node proliferation assay (LLNP). While LLNA is instrumental in testing topically administered formulations (e.g., creams), the LLNP was proven to be predictive of drug-mediated DTH in response to small molecule pharmaceuticals. Global efforts in reducing the use of research animals lead to the development of in vitro models to predict test-material-mediated DTH. Two such models include analysis of surface marker expression in human cell lines THP-1 and U-937. These tests are known as the human cell line activation test (hCLAT) and myeloid U937 skin sensitization test (MUSST or U-SENS), respectively. Here we describe experimental procedures for all these methods, discuss their in vitro–in vivo correlation, and suggest a strategy for applying these tests to analyze engineered nanomaterials and nanotechnology-formulated drug products.

Key words Nanoparticles, Hypersensitivity, Leukocyte proliferation, Sensitizer, Allergen, Irritant, LLNA, LLNP, hCLAT, MUSST

1 Introduction

According to several reports from industry, academia, and regulatory agencies, many drugs are withdrawn from clinical use due to their adverse effects on immune system function [1–4]. Although nanotechnology-formulated drugs represent a relatively new category of pharmaceutical products, their safety assessment is conducted using the regulatory framework established for other products, such as small molecules, therapeutic nucleic acids, and biologics; and, among other tests, it includes analysis of immunotoxicity. Acute immunotoxicity commonly observed in response to nanomaterials is related to hemolysis, complement activation, opsonization, phagocytosis, and cytokine secretion, and can be accurately tested using in vitro assays [1]. Identifying changes in

the immune system function is more complex and requires more sophisticated approaches. Since many low molecular weight compounds can induce allergic contact dermatitis, evaluation of skin sensitization is one of the key safety endpoints for materials used in cosmetics [2]. Delayed-type hypersensitivity (DTH) reactions to systemically administered drugs are also a safety concern which cannot be revealed by standard toxicity studies [3]. Estimation of a material's potential to allergic contact dermatitis in human was historically done using Guinea Pig Maximization Test, Buehler's Test, and more recently by Local Lymph Node Assay (LLNA). Local Lymph Node Proliferation Assay (LLNP) is a modification of LLNA which was shown to accurately predict DTH reaction to systemically administered pharmaceuticals [3]. The change in LLNP involves subcutaneous (s.c.) injection of a test material instead of topical application onto a skin surface like in LLNA. Both LLNA and LLNP require administration of a test material to mice once a day for three consecutive days, followed by 2 days of rest and then intravenous (i.v.) administration of 3H-thymidine. Animals are sacrificed 5 h after thymidine injections and draining lymph nodes are analyzed by scintillation counting. 3H-thymidine incorporation into DNA of lymph node resident leukocytes is used as an indication of T-cell activation involved in allergic sensitization. Several years ago, the European Cosmetics Association (COLIPA), through a number of inter-laboratory studies, validated two in vitro assays that are proposed to decrease and possibly eliminate animal use in analysis of DTH reactions. These tests are the human cell line activation test (hCLAT) and myeloid U937 skin sensitization test (MUSST or U-SENS) [2, 4–8]. For the purpose of this chapter, we will refer to the MUSST/U-SENS assay as MUSST. They were originally developed by several researchers in Japan [2, 9–13], and subsequently adopted by several European labs [14–17]. The principle of these methods is based on the fact that allergic sensitization requires formation of T-cell memory and the essential step in the process is the activation of antigen-presenting cells such as dendritic cells. Due to the complexity of isolation and high inter-donor variability between primary human dendritic cell cultures, the researchers have selected monocyte-macrophage cell lines THP-1 and U-937. They found that these cells possess one of the properties of activated primary dendritic cells, i.e., they express CD86 and/or CD54 in response to allergens. Despite original publications describing the expression of both CD54 and CD86 by THP-1 cells, both our laboratory and BD Biosciences found that THP-1 lack CD86 (NCL unpublished data and reference 18). The hCLAT and MUSST tests described in this chapter are adaptations of previously published methods and evaluate the expression of surface markers CD54 and CD86 in THP-1 and U937 cell lines, respectively. Both in vitro and in vivo DTH tests are schematically depicted in Fig. 1. These assays apply to engineered nanomaterials

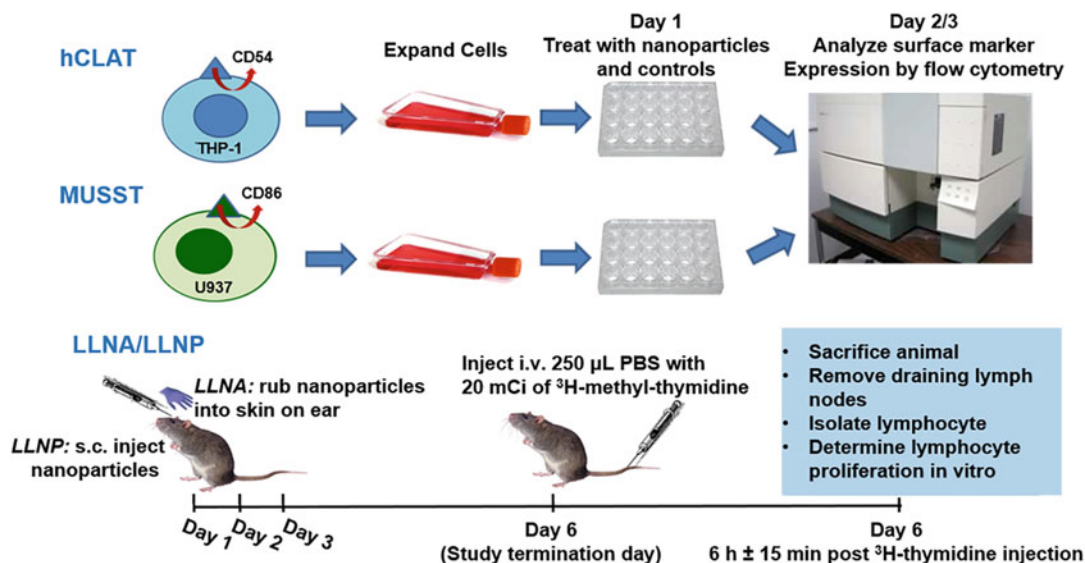


Fig. 1 Schematic of LLNA, LLNP, MUSST, and hCLAT methods. This figure depicts the major experimental steps of in vitro MUSST and hCLAT, and in vivo LLNA and LLNP methods

and nanotechnology-formulated drug products. However, due to the complexity of these novel materials, the in vitro–in vivo correlation between LLNA, LLNP, and hCLAT, MUSST assays may be observed for some but not all nanoparticles (Fig. 2). In vitro methods have a greater rate of positive response than in vivo tests; moreover, among in vitro assays MUSST is more sensitive in identifying positive responses than hCLAT (Fig. 3). Therefore, the in vitro assays can be used for rapid screening of multiple nanoformulations. However, positive responders established in these in vitro tests require verification using a relevant in vivo method.

2 Materials

2.1 hCLAT and MUSST

1. Phosphate buffered saline (PBS).
2. Heat-inactivated fetal bovine serum (FBS). Thaw a bottle of FBS at room temperature or overnight at 2–8 °C and allow to equilibrate to room temperature. Incubate for 30 min at 56 °C in a water bath mixing every 5 min to heat-inactivate it. Single-use aliquots may be stored at 2–8 °C for up to 1 month or at a nominal temperature of –20 °C for a longer duration.
3. 10 mg/mL stock glycerol. Prepare 10 mg/mL stock by adding 80 μ L of glycerol into 9.92 mL sterile water and filter through 0.2- μ m filter. Make sure to use pyrogen-free water.
4. Reagent to distinguish between life and dead cells, such as trypan blue solution or arcindine orange/propidium iodide (AO/PI) staining solution.

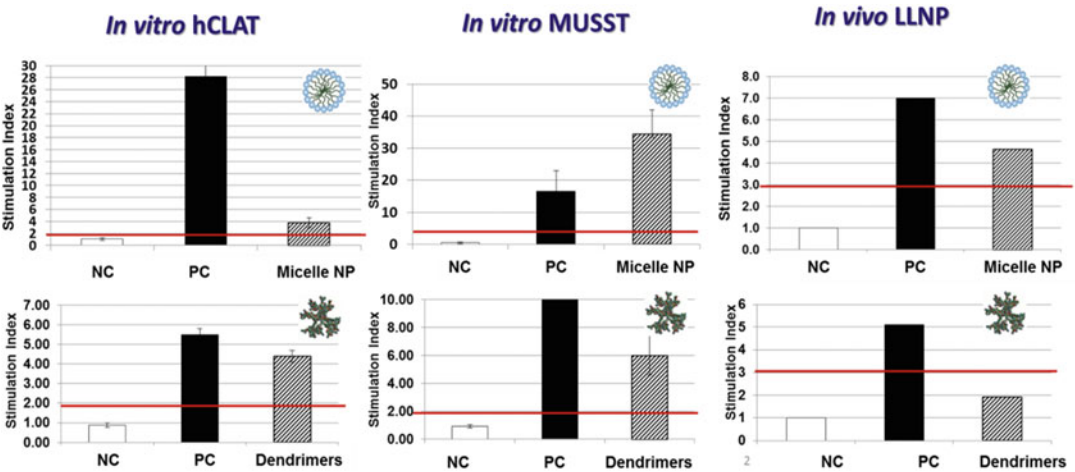


Fig. 2 Correlation between hCLAT, MUSST, and LLNP assays is observed for some but not all nanomaterials. Micelle nanoparticle (Cremophor EL) and dendrimers (generation 6 carboxy-terminated PAMAM dendrimers) were tested in vitro in hCLAT and MUSST, and in vivo in LLNP assay. The concentration of dendrimers tested in vitro is 100 $\mu\text{g/mL}$. That of Cremophor is 8.5 mg/mL and is equivalent to the cremophor concentration present in Taxol when the API (paclitaxel) concentration is 25 μM . Taxol is the cremophor-based formulation of paclitaxel used in the clinic. It is associated with both acute and delayed-type hypersensitivity. Animals were s.c. injected with 100 μL of nanoparticle stock solution. The concentration of the stock was 8.5 mg/mL and 0.1 mg/mL for cremophor and dendrimers, respectively. NC: negative control (1 mg/mL glycerol in hCLAT and MUSST and PBS in LLNP), PC: positive control (250 $\mu\text{g/mL}$ Nickel sulfate, 250 μM TNBS, and 50 mg/kg Streptozotocin in hCLAT, MUSST, and LLNP, respectively). Shown are mean $\pm$ SD ($N = 3$). The red line depicts the minimum positive stimulation index of 3

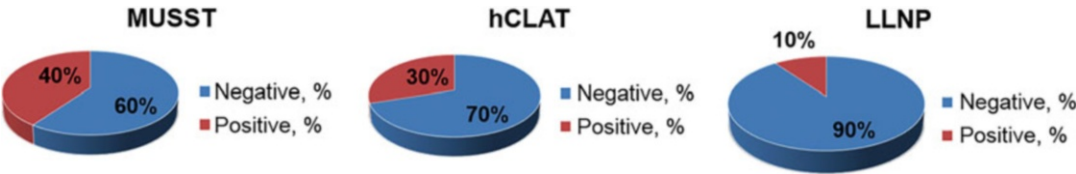


Fig. 3 Comparison of positive response rates between in vitro and in vivo tests. Twenty formulations including colloidal gold and colloidal silver nanoparticles with various sizes and surface coatings, generations 3, 4, 5, and 6 PAMAM dendrimers with different surface functionalities, iron oxide nanoparticles, liposomes, and emulsions were analyzed in vitro using hCLAT and MUSST and in vivo using LLNP assay. Particles producing a stimulation index ≥ 3 were considered positive, while those with a stimulation index of less than 3 were considered negative. Percentage of positive and negative responders in each test is presented in the charts. Not all particles that were positive in vitro were positive in vivo, indicating that results correlated between in vitro and in vivo conditions for some but not all nanoparticles (Fig. 2, for example). The data show that in vitro assays have a greater rate of positive responses

5. Albumin from bovine serum (BSA).
6. Bovine Serum Albumin (BSA) blocking buffer. BSA reagent is provided as lyophilized powder. Dissolve 50 mg of the reagent and in 50 mL ice-cold PBS. Mix well and place on ice. Prepare fresh before each use. Discard any leftover after the experiment is complete.

7. Globulins Cohn fraction II blocking buffer. Globulins Cohn fraction II, III human is provided as lyophilized powder. Dissolve 5 mg of the reagent in 50 mL of ice cold PBS. Mix well and place on ice. Prepare fresh before each use. Discard any leftover after the experiment is complete.
8. 7-Amino-Actinomycin D (7-AAD).
9. Cellometer (NexCelome) or Hemacytometer to perform cell count.
10. Flow Cytometer (e.g., FACSCalibur) (*see* **Notes 1** and **2**).

2.2 hCLAT Only

1. Monocytic leukemia cell line THP-1 (ATCC).
2. R-phycoerythrin (PE) mouse IgG1 isotype control (BD Pharmingen, cat# 34076) (*see* **Note 2**).
3. PE mouse anti-human CD54 (BD Pharmingen, cat# 555511).
4. 10 mg/mL nickel(II) sulfate hexahydrate stock solution. Weigh 100 mg of nickel(II) sulfate and dissolve in 10 mL of sterile water to obtain a 10 mg/mL stock solution. Filter this stock through 0.2- μ m filter.
5. Complete RPMI solution: 20% heat-inactivated FBS, 4 mM L-glutamine, 2-mercaptoethanol to a final concentration of 0.05 mM, and 100 U/mL penicillin. Store at 2–8 °C protected from light for no longer than 1 month. Before use warm the medium in a water bath to 37 °C.

2.3 MUSST Only

1. Monocytic leukemia cell line U937 (ATCC).
2. Fluorescein isothiocyanate (FITC) mouse IgG1 isotype control (BD Pharmingen, cat# 555748).
3. FITC mouse anti-human CD86 (BD Pharmingen, cat# 555657).
4. 170 mM 2,4,6-Trinitrobenzenesulfonic acid solution (TNBS) stock.
5. Complete RPMI medium: 20% FBS (heat-inactivated), 4 mM L-glutamine, 100 U/mL penicillin. Store at 2–8 °C protected from light for no longer than 1 month. Before use warm the medium in a water bath to 37 °C.

2.4 LLNP and LLNA

1. 7–12 weeks old male and female mice of the following strains: CBA/Ca, CBA/j, or Balb/c.
2. Streptozotocin.
3. PBS.
4. 1 mCi ³H-thymidine.
5. 5% trichloroacetic acid (TCA) solution. Prepare 5% solution by dissolving 5 g of TCA in 100 mL of distilled water.

6. Tuberculin 1 mL syringe.
7. 12×75 mm tube with cell strainer cap.
8. Scintillation liquid.
9. Scintillation counter.
10. 20 mL scintillation vials.

3 Methods

3.1 Preparation of Controls and Cell Lines

1. For *bCLAT* and *MUSST*, prepare glycerol as negative control. Use 100 μ L of 10 mg/mL glycerol stock per 900 μ L of cell suspension to achieve a final concentration of 1 mg/mL.
2. For *bCLAT*, prepare THP-I cell line. Cultures can be maintained by the addition of fresh medium or replacement of culture medium. Alternatively, cultures can be established by centrifugation with subsequent resuspension at $2\text{--}4 \times 10^5$ viable cells/mL. Subculture is needed when cell concentration reaches 8×10^5 cells/mL. It is important not to allow cell concentration to exceed 1×10^6 cells/mL. Maintain cell density between 1×10^5 and 1×10^6 viable cells/mL. Do not use cells exceeding 22 passages.
3. For *bCLAT*, prepare nickel(II) sulfate hexahydrate as a positive control. Dilute the 10 mg/mL stock solution by adding 1 mL of the stock to 3 mL of media to obtain a 2.5 mg/mL intermediate solution. Add 100 μ L of the 2.5 mg/mL intermediate solution to 900 μ L of cell suspension to obtain a final concentration of 250 μ g/mL.
4. For *MUSST*, prepare U937 cell line. Cultures can be maintained by the addition of fresh medium or replacement of culture medium. Alternatively, cultures can be established by centrifugation with subsequent resuspension at $1\text{--}2 \times 10^5$ viable cells/mL. Subculture is needed when cell concentration reaches 8×10^5 cells/mL. It is important not to allow cell concentration to exceed 1×10^6 cells/mL. Maintain cell density between 1×10^5 and 2×10^6 viable cells/mL. Do not use cells exceeding 22 passages.
5. For *MUSST*, prepare TNBS solution as a positive control. Dilute the 170 mM TNBS stock solution by adding 18 μ L of the stock to 982 μ L of the media to obtain a 3 mM working stock. Add 100 μ L of the 3 mM working stock solution to 900 μ L of the cell suspension to obtain a final concentration of 300 μ M.
6. For *LLNP*, prepare ^3H -thymidine dosing solution. Add 11.5 mL of PBS to a dosing vial. Remove stock of the radiolabeled material from storage and add 1 mL of this stock to

the dosing vial containing PBS. Mix well to prepare a dosing solution. To confirm the concentration of this dosing solution, remove two 8 μL aliquots of the solution and mix with 20 mL of water, then use 1 mL aliquots of these diluted dosing solutions and mix each with 10 mL of the scintillation liquid. These aliquots are used to confirm that each milliliter of the dosing solution contains $\sim 80 \mu\text{Ci}$ of ^3H -thymidine. Dispose of all radioactive material and perform post work contamination survey according to the protocol approved by your institutional committee.

7. Prepare streptozotocin by dissolving lyophilized powder in sterile PBS or saline at concentration 1.5–2 mg/mL. The final dose of streptozotocin is 50 mg/kg and the injection volume is 50–100 μL per animal.

3.2 Preparation of Study Samples

1. The *bCLAT* and *MUSST* require 0.4 mL of nanoparticles dissolved/resuspended in complete culture medium to a concentration of $10\times$ the highest tested 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 [1] and are summarized in Box 1. The assay will evaluate four concentrations: $10\times$ (or when feasible $100\times$, $30\times$, or $5\times$) of theoretical plasma concentration, theoretical plasma concentration,

Box 1. Example Calculation of Nanoparticle Concentration for In Vitro Test

In this example, assume the mouse dose is known to be 123 mg/kg.

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

Blood volume constitutes approximately 8% of body weight (e.g. a 70 kg human has approximately 5.6 L (8% of 70) of blood), allowing for a very rough estimation of the maximum blood concentration.

$$\begin{aligned} \text{in vitro concentration}_{\text{human matrix}} &= \frac{\text{human dose}}{\text{human blood volume}} = \frac{70 \text{ kg} \times 10 \frac{\text{mg}}{\text{kg}}}{5.6 \text{ L}} \\ &= \frac{700 \text{ mg}}{5.6 \text{ L}} = 0.125 \text{ mg/mL} \end{aligned}$$

Box 1 Estimation of nanomaterial concentration for in vitro assay. The box shows an example of calculating nanoparticle concentrations for the in vitro assays. Reproduced with permission from ref. 1

and two 5-fold 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 0.1 mg/mL, then a stock of 10 mg/mL will be prepared and diluted 10-fold (1 mg/mL), followed by two 5-fold serial dilutions (0.2 and 0.04 mg/mL, respectively). When 0.1 mL of each sample is added to the plate and mixed with 0.9 mL of cell suspension, the final nanoparticle concentrations tested in the assay are 1.0, 0.1, 0.02, and 0.004 mg/mL.

2. For *LLNP* and *LLNA*, prepare test nanoparticles in an appropriate vehicle which may be peculiar to each formulation. Nanomaterials are tested at three doses, selected based on the existing information about the test nanomaterial from previous toxicological studies: (1) at the maximum tolerated dose (MTD) (e.g., a dose which demonstrated a moderate decrease in leukocyte count and was not accompanied by life-threatening abnormalities), (2) half of MTD, and (3) quarter of MTD.

3.3 *hCLAT* Experimental Procedure

1. Adjust THP-1 cell number to 1×10^6 viable cells/mL and aliquot 900 μ L of the cell suspension to each well on the 24-well plate.
2. Add 100 μ L of test nanoparticles or controls to appropriate wells. Prepare duplicate wells for each sample. Incubate for 24 h at 37 °C and 5% CO₂.
3. After 24 h of incubation with test materials and controls, harvest the cells and determine cell viability by trypan blue or another relevant method.
4. Harvest cells into round bottom 12 $\times$ 75 mm² tubes and centrifuge at $400 \times g$ for 5 min.
5. Aspirate supernatant and wash cells with 2 mL of ice-cold PBS.
6. Resuspend cells in 400 μ L of ice-cold Cohn fraction blocking buffer and incubate on ice for 10 min.
7. Wash cells two times with 1 mL of BSA blocking buffer using centrifugation at $400 \times g$ at 4 °C for 5 min to pellet the cells. Discard wash supernatant.
8. After final wash, resuspend cells in 100 μ L of ice-cold BSA blocking buffer.
9. Add 20 μ L of monoclonal antibody against CD54 and isotype control antibody to appropriate tubes (final concentration of antibody is 5 μ g/mL).
10. Incubate at 4 °C for 30 min.

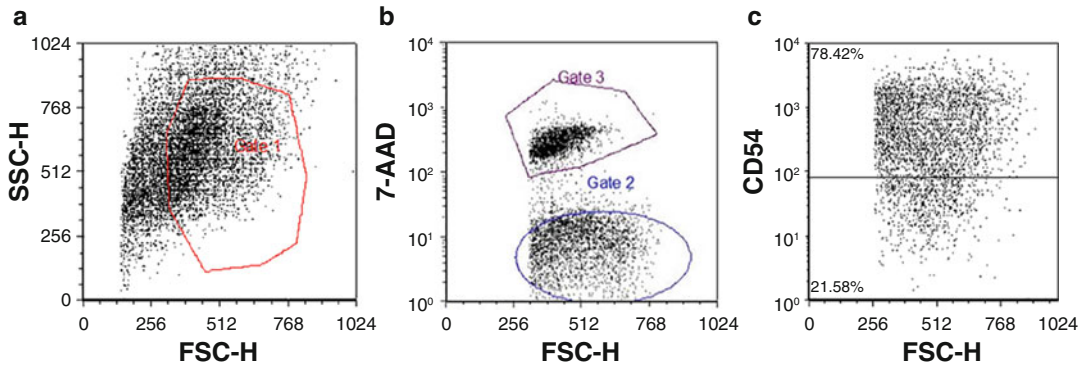


Fig. 4 An example of flow cytometry analysis of CD54 expression in THP-1 cells. **(a)** Forward scatter (FSC) vs. side scatter (SSC) dot plot gated on cells of interest. **(b)** FSC vs. FL3 cells stained with 7AAD dot plot showing 7AAD negative cells (gate 2). Gate 3 shows dead cells. **(c)** FSC vs. FL2 profile of cells stained with mouse anti-human CD54-PE dot plot gated to show only cells from gate 2

11. Wash cells twice with 1 mL of BSA blocking buffer and centrifugation at $400 \times g$ for 5 min to pellet the cells and discard the wash supernatant.
12. After final wash resuspend the cells in 400 μ L of BSA blocking buffer.
13. Add 5 μ L of 7AAD to each sample.
14. Analyze the samples using flow cytometer (*see* **Notes 1** and **2**; Fig. 4).

3.4 MUSST Experimental Procedure

1. Adjust U937 cell count to 1.25×10^5 cells/mL and aliquot 900 μ L of the cell suspension to each well on the 24-well plate.
2. Add 100 μ L of test nanoparticles or controls to appropriate wells. Prepare duplicate wells for each sample.
3. After 48 h of incubation with test materials and controls, harvest the cells and determine cell viability using trypan blue or another similar approach.
4. Harvest cells into polystyrene round bottom 12×75 mm² tubes and centrifuge at $400 \times g$ for 5 min.
5. Aspirate supernatant and wash cells with 2 mL of ice-cold PBS.
6. Resuspend cells in 400 μ L of ice-cold Cohn fraction blocking buffer and incubate on ice for 10 min.
7. Wash cells two times with 1 mL of BSA blocking buffer using centrifugation at $400 \times g$ at 4 °C for 5 min to pellet the cells and discard wash supernatant.
8. After final wash, resuspend cells in 100 μ L of ice-cold BSA blocking buffer.
9. Add 20 μ L of monoclonal antibody against CD86 and isotype control antibody to appropriate tubes (final concentration of the antibody is 5 μ g/mL).

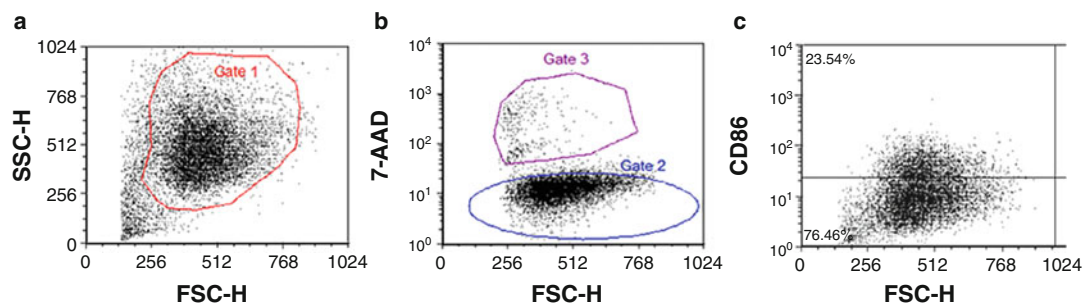


Fig. 5 An example of flow cytometry analysis of CD86 expression in U937 cells. **(a)** Forward scatter (FSC) vs. side scatter (SSC) dot plot gated on cells of interest. **(b)** FSC vs. FL3 cells stained with 7AAD dot plot showing 7AAD negative cells (gate 2). Gate 3 shows dead cells. **(c)** FSC vs. FL1 profile of cells stained with mouse anti-human CD86 FITC dot plot gated to show only cells from gate 2

10. Incubate at 4 °C for 30 min.
11. Wash cells twice with 1 mL of BSA blocking buffer and centrifuge at $400 \times g$ for 5 min to pellet the cells. Discard the wash supernatant.
12. After final wash, resuspend the cells in 400 μ L of BSA blocking buffer.
13. Add 5 μ L of 7AAD to each sample.
14. Analyze the samples using flow cytometer (*see* **Notes 1** and **2**; **Fig. 5**).

3.5 LLNA and LLNP Experimental Procedure

1. On Day 1, weight each mouse before applying treatment (*see* **Note 3**). If using LLNA, apply 25 μ L of the test solution (either negative control [PBS], positive control [10% neutral buffered formalin], or nanoparticles) topically to the dorsal surface of both ears (*see* **Note 4**). Just before application of the treatment, wipe the dorsal surface of each ear with acetone to aid in absorption. If using LLNP, inject 100 μ L of the test solution (either negative control [PBS], positive control [streptozotocin], or nanoparticles) s.c. in the midline of the head (*see* **Notes 5** and **6**).
2. Repeat Day 1 treatment on two subsequent days (Days 2 and 3). Observe each animal for signs of local irritation.
3. On Days 4, 5, and 6 of the study, observe each animal for signs of local irritation and record animal weights.
4. On Day 6 (72 ± 3 h) after the first treatment, inject each animal intravenously (i.v.) with 250 μ L of sterile PBS containing 20 μ Ci of ³H-thymidine via the lateral tail vein. Use a 1 mL syringe with a needle size no larger than 25 gauge (*see* **Note 7**).
5. Sacrifice the animals 5 h $\pm$ 45 min after injection of the thymidine using carbon dioxide asphyxiation or another technique approved by your institute's animal care committee. Isolate the

draining lymph nodes of each ear and collect them into vials containing sterile PBS (*see* **Note 8**).

6. Place lymph nodes into the top compartment of the cell strainer cap. Using the plunger from the 1 mL syringe, press the lymph node into the 5 mL round bottom tube. Prepare one tube per individual animal.
7. Rinse the cap with 5 mL of PBS to move all single cells into the tube. Discard the strainer cap and remaining tissue debris.
8. Centrifuge for 10 min at $190 \times g$ and 4°C . Remove supernatant and leave 1 mL of solution above the pellet.
9. Repeat **step 8** two more times.
10. After the final wash, remove all PBS and gently agitate to break up the pellet.
11. Add 3 mL of 5% TCA and incubate at 4°C overnight.
12. Centrifuge for 10 min at $190 \times g$ and 4°C . Remove supernatant and resuspend the pellet in 1 mL of 5% TCA.
13. Transfer each sample to a scintillation vial and add 10–15 mL of scintillation fluid.
14. Shake well. Dark adapt the samples for 30 min.
15. Analyze the samples using a scintillation counter.

3.6 Calculations and Data Interpretation—*hCLAT and MUSST*

1. A percent coefficient of variation should be calculated for each control or test according to the following formula:

$$\%CV = \frac{\text{Standard deviation}}{\text{Mean}} \times 100\%$$

2. Measure the stimulation index (SI) using the following formula:

$$SI = \frac{\text{Mean \% positive cells}_{\text{test sample}}}{\text{Mean \% positive cells}_{\text{negative control}}}$$

An $SI \geq 3$ is considered a positive response.

3. %CV for each control and test sample should be less than 30%.
4. If a positive control or negative control fails to meet acceptance criterion described above, the assay should be repeated.
5. Within the acceptable assay, if two of three replicates of unknown sample fail to meet acceptance criterion described in **step 3** above, this unknown sample should be re-analyzed.

3.7 Calculations and Data Interpretation—LLNA and LLNP

1. The proliferative response (PR) in each sample is determined by the number of radioactive disintegrations per minute per lymph node, using the following formula:

$$PR = \frac{(DPM_{\text{test sample}} - DPM_{\text{background}})}{LN}$$

where DPM is the result reported by the scintillation counter and LN is the number of the lymph nodes in the sample. The background DPM ($DPM_{\text{background}}$) is determined by analyzing 10 mL of the scintillation liquid without any sample.

2. Measure SI according to the formula:

$$SI = \frac{PR_{\text{test sample}}}{PR_{\text{negative control}}}$$

An SI of ≥ 3 is considered a positive response.

4 Notes

1. To set up the instrument for data acquisition and analysis, please refer to the operation manual of your flow cytometer. The protocol described in this chapter was developed using a FACSCalibur. With this instrument, we first optimize the instrument settings to bring the cells in focus using forward and side scatter. Next, we create a gate around the cell population and analyze cells from this gate on the fluorescent channel 3 (FL-3) to distinguish between dead and live cells. We create a gate around the live cell population in FL-3. Cells negative for 7-AAD staining detected in this channel are the live cells, while cells positive in 7-AAD staining are dead cells and they are excluded from the analysis. We acquire CD54+ cells and CD86+ cells from the 7-AAD negative population using fluorescent channels 2 and 1 (FL-2 and FL-1), respectively. The FL-1 and FL-2 on the FACSCalibur instrument detect the FITC and PE labels of the antibodies, respectively. Each test sample is also stained using the isotype control antibody. The percent positive cells in each sample stained with CD54 or CD86 antibody is determined against respective isotype controls of the same sample. If antibodies with different fluorophores are used, one would need to select the appropriate isotype control and adjust both the antibody concentration and the acquisition channel.
2. NCL does not endorse suppliers. However, we found that a new user benefits from knowing the instruments and catalog information of reagents used in our assays. Therefore, ideas of

reagents used at the NCL are included in this chapter in parentheses. When using reagents and instruments from other sources, the assay performance may change. We recommend performing assay qualification to verify the assay functionality and validity before proceeding with test samples.

3. This procedure requires an animal study protocol and radiation program approved by relevant institutional committees at your organization.
4. Consider using a collar to prevent animals from ingesting the treatments during the grooming process.
5. The point of injection is very important. It should be in the midline, between the eyes on the top of the head. Point the needle toward the back of the animal so that the end of the needle is just forward of the point between the ears. Injection should be performed slowly so that the bulk of the liquid is placed either between or just forward of the ears. Injecting too far to the rear will result in reduced response in the draining lymph nodes.
6. Typical injection volume is 100 μL , but it may be lower and vary between 50 and 100 μL . The volume should not exceed 5 mL/kg, and the needle size should be 25 G or smaller.
7. Placing animals on a warm plate may be needed to dilate veins for easier i.v. injections.
8. The lymph nodes should be processed as soon as possible within 2 h after isolation from the animals.

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