NANOTECHNOLOGY CHARACTERIZATION LABORATORY

NCL Method ITA-3
Version 1.0

Mouse Granulocyte-Macrophage Colony-Forming Unit Assay

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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.

Method is written by: _____ 16 Dec 2005.

Marina A. Dobrovolskaia, Ph.D. Date

_____ 12/16/05.

Timothy M. Potter, B.S. Date

Method validation was conducted on: 7/11/2005 – 7/26/2005.

Date

Testing facility: NCI-Frederick, Bldg. 469, Room 253.

1. Introduction

This document describes a protocol for quantitative analysis of granulocyte-macrophage colony-forming units. The assay employs murine bone marrow (BM). Hematopoietic stem cells of BM proliferate and differentiate to form discrete cell clusters or colonies. The BM cells are isolated from 8-12 weeks old mice and cultured in methylcellulose-based medium supplemented with cytokines (mSCF, mIL-3 and hIL-6) either untreated (baseline) or treated with nanoparticles (test). These cytokines promote formation of granulocyte and macrophage (CFU-GM) colonies. After twelve days of incubation at 37 °C in the presence of 5% CO2 and 95% humidity number of colonies is quantified in baseline and test samples. A percent of CFU inhibition is then calculated for each test sample. The basic protocol for BM isolation and culture was adopted from technical manual # 28405 developed by StemCell Technologies Inc. The assay requires 450 μL of a test-nanomaterial.

2. Reagents

2.1. MethoCult medium, StemCell Technologies Inc cat. # 03534

2.2. Fetal Bovine Serum prescreened for hematopoietic stem cells, StemCell Technologies Inc. cat. # 06200

2.3. Iscove's MDM with 2% FBS, StemCell Technologies Inc cat. # 07700

2.4. Sterile distilled water

2.5. Blunt-end 16 gauge needles, StemCell Technologies Inc cat. # 03534

2.6. Cisplatin (positive control), Sigma cat#P4394

2.7. Sterile Ca^{2+}/Mg^{2+}-free DPBS, Sigma D8537 (negative control)

Note: Equivalent reagents from other vendors can be used

3. Equipment

3.1. Pipettes covering range from 0.05 to 10 mL

3.2. Prescreened 35mm culture dishes, StemCell Technologies Inc cat. # 27100

3.3. Blunt-end 16 gauge needles, StemCell Technologies Inc cat. # 03534

3.4. 100mm petri dishes

3.5. Plastic beakers

3.6. Polypropylene tubes 5 and 15 mL

3.7. Centrifuge

3.8. Refrigerator, 2-8 °C

3.9. Freezer, -20°C

3.10. Cell culture incubator with 5% CO_2 and 95% humidity.

3.11. CO_2 euthanasia box, or appropriate equipment approved by institution

3.12. Scissors for tissue dissection

3.13. Forceps

3.14. Biohazard safety cabinet approved for level II handling of biological material

3.15. Inverted microscope

3.16. Vortex

3.17. Hemacytometer

4. Animals

6-12 weeks old C56BL6 males or females. Use of the pooled cells derived from at least two (2) animals is highly recommended.

5. Reagent and Control Preparation

5.1. MethoCult medium

The MethoCult medium is supplied in 100mL size batches. It is recommended by manufacturer that the medium to be thawed at room temperature or in a refrigerator overnight, vortexed to mix well ingredients, then left at a room temperature for approximately 5 minutes to allow air bubbles to dissipate. Use a 16 gauge blunt-end needle to dispense 3 mL of the MethoCult medium into sterile 15mL tube. Store the aliquoted medium at a nominal temperature of -20 °C. Before the test thaw the required number of aliquots at room temperature for approximately 20 minutes and keep on ice prior to use. Repeated freezing and thawing should be avoided.

5.2. 50mM Cisplatin (Positive Control)

Cisplatin is supplied in a lyophilized form. Reconstitute the lyophilized powder by adding appropriate amount of DMSO to make a stock solution with nominal concentration of 50mM. Prepare small aliquots and store at a nominal temperature of -80 °C. Prior to use in the assay thaw an aliquot of the stock solution at room temperature and dilute in IMEM supplemented with

2% FBS to bring the concentration to 2mM. One hundred fifty (150) μL of this intermediate solution is then added to 3mL of culture medium. Final concentration of cisplatin in the positive control sample is 50 μM.

6. Preparation of Study Samples

This assay requires 450 μL of nanoparticles, i.e. three 150 μL samples, each of which is analyzed in duplicate. The following questions have to be considered when selecting the concentration: i) solubility of nanoparticles in a biocompatible buffer; ii) pH within physiological range; iii) availability of nanomaterial, and iv) stability. For the initial screen the test concentration is selected based on results from general toxicity assays. A nanomaterial, which revealed toxicity in general toxicity assays, is tested at two concentrations selected at the low and the high end of the dose response curve. A nanomaterial, which did not reveal toxicity in a general toxicity assays is tested at one concentration equal to highest dose tested in general toxicity assay.

7. Isolation and Counting of Bone Marrow Cells

7.1. Position euthanized mouse on its back and rinse fur thoroughly with 70% alcohol (Euthanize animals according to the protocol approved by your institution).

7.2. Cut a slit in the fur just below the rib cage without cutting the peritoneal membrane.

7.3. Firmly grasp skin and per back to expose hind limbs.

7.4. Using sterile sharp dissecting scissors cut the knee joint in the center. Cut through ligaments and excess tissue.

7.5. Grasp femur with forceps and cut femur near hip joint.

7.6. Free tibia by cutting near the ankle joint.

7.7. Trim the ends of the long bones to expose the interior marrow shaft. Put bones in sterile petri dish or in sterile culture medium and place on ice. Bones can be collected from multiple animals.

7.8. Using a 3cc syringe with 21 or 22 gauge needle, draw up to 1-3 mL of cold Iscove's MDM supplemented with 2% FBS.

7.9. Insert bevel of needle into marrow shaft and flush marrow into 15 mL tube. Repeat this procedure for all bones. The same medium can be used to isolate marrow from 1-3 animals. The bone shallow should appear white once all the marrow has been expelled.

7.10. Keeping needle below medium surface, gently draw medium with cells up and down with 3cc syringe and 21 gauge needle 3-4 times to make a single cell suspension.

7.11. Keep cells in medium on ice until use.

7.12. Perform a nucleated cell count. To do so, first dilute the cells with 3% acetic acid with methylene blue 1: 100 (e.g.10μL cells + 990 μL 3% acetic acid/methylene blue). Then use either hemocytometer or automatic cell counter. An average cell count is expected to be 1-2 x 10^7 for femur and 0.6-1 x 10^7 for tibia.

7.13. If cell viability (at least 90%) and count are acceptable proceed to the next step.

8. Experimental Procedure

8.1. Label lids of 35mm culture dishes at the edge using a permanent fine felt marker.

8.2. Thaw MethoCult medium at room temperature or in refrigerator overnight.

8.3. Vortex tubes to ensure all components are thoroughly mixed.

8.4. Dilute cells isolated according to the procedure described in section 6 with Iscove's medium supplemented with 2% FBS to 4 x 10^5 cells per mL.

8.5. Add 150μL of cell suspension and 150 μL of either Iscove's medium with 2% FBS (baseline), PBS (negative control), Cisplatin (positive control), or nanoparticles (test sample) to 3 mL of MethoCult medium.

8.6. Vortex tubes to ensure all cells and medium components are mixed thoroughly.

8.7. Let tube stand for 5 minutes to allow bubbles to dissipate.

8.8. Attach a 16 gauge blunt-ended needle to a 3 cc syringe, place the needle below the surface of solution and draw up approximately 1 mL. Gently depress the plunger and expel medium completely. Repeat until no air space is visible.

8.9. Draw up MethoCult medium with cells into syringe and dispense 1.1 mL per 35 mm dish. All samples are tested in duplicate (N=2). Two duplicates are analyzed for each nanoparticle.

8.10. Distribute the medium evenly by gently tilting and rotating each dish.

8.11. Place two (2) covered dishes with cells and one (1) uncovered dish filled with 3mL of sterile water into 150 mm Petri dish.

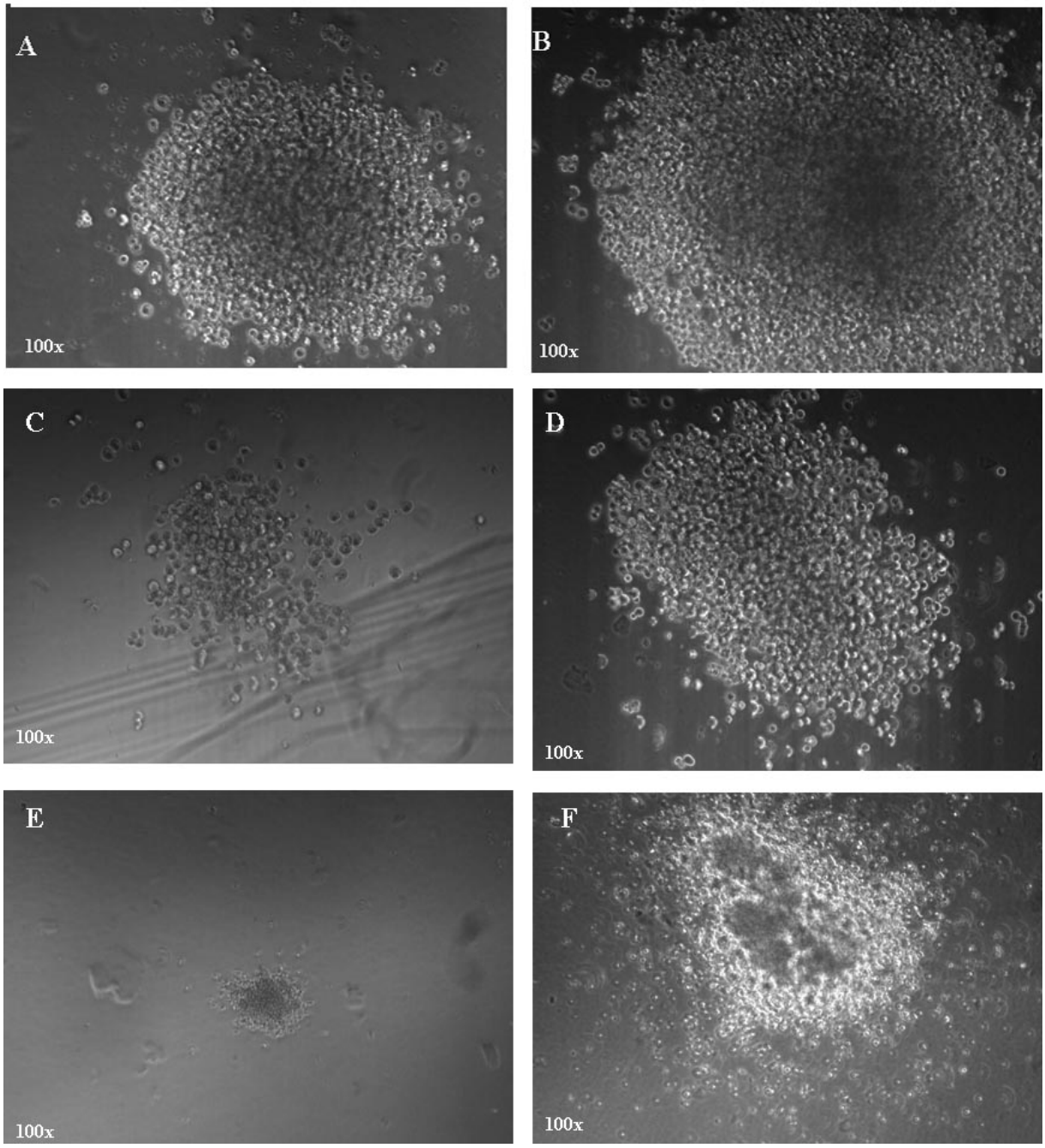
8.12. Place cultures in an incubator maintained at 37 °C, 5% CO2 and 95% humidity.

8.13. Incubate for 12 days. On the 12th day remove dishes from incubator, identify and count colonies as described below. Representative values of CFU-GM for C57BL6 mice at 8-12 weeks of age is 64±16.

9. Description of CFU-GM

This classification includes CFU-granulocyte (CFU-G), CFU-macrophage (CFU-M) and CFU-granulocyte macrophage (CFU-GM). The colonies contain 30 to thousands of CFU-G, CFU-M or both cell types (CFU-GM). Made up of at least 30 cells per colony. CFU-GM colonies often contain multiple clusters and appear as dense core surrounded by cells. The monocytic lineage cells are large cells with an oval to round shape and appear to have a drainy or grey center. The granulocytic lineage cells are round, bright, and are much smaller and more uniform in size than macrophages. It is easy to see individual cells of a CFU-GM colony, especially in the periphery of the colony.

Colonies seen on Figures A and B below are CFU-GM. Colonies on Figures C and D are CFU-M. Figure E demonstrates an example of single CFU-G colony. Few CFU-G colonies growing together are shown on Figure F.


A
100x
B
100x
C
100x
D
100x
E
100x
F
100x

10. Calculations

A Percent Coefficient of Variation should be calculated for each control or test according to the following formula**:** %CV=SD/Mean x 100%

A Percent CFU Inhibition is calculated as follows:

$$\% \text{ CFU-Inhibition} = \frac{(\text{Baseline CFU-GM} - \text{Test CFU-GM})}{\text{Baseline CFU-GM}} \times 100\ \%$$

Baseline refers to the assay negative control

11. Acceptance Criteria

11.1. %CV for each control and test sample should be less than 30%

11.2. If positive control or negative control fail to meet acceptance criterion described in 11.1 the assay should be repeated.

11.3. Within the acceptable assay if two of three replicates of unknown sample fail to meet acceptance criterion described in 11.1 this unknown sample should be re-analyzed.

11.4. If two duplicates of the same study sample demonstrated results different more then 30%, this sample should be reanalyzed.

12. References

12.1. Mouse Colony-Forming cell Assays Using MethoCult. Technical manual. StemCell Technologies Inc., cat # 28405.

12.2. Dominique Pifat. Assay Validation. www.fda.gov/cber/summaries/120600bio10.ppt - 05-24-2003.