ARTICLES

Investigation of Macrophage Differentiation and Cytokine Production in an Undergraduate Immunology Laboratory

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Abstract: We have developed a semester-long laboratory project for an undergraduate immunology course in which students study multiple aspects of macrophage biology including differentiation from progenitors in the bone marrow, activation upon stimulation with microbial ligands, expression of cell surface markers, and modulation of cytokine production. In the first part of the semester, students differentiate macrophages from mouse bone marrow stem cells and perform immunophenotyping on their macrophages using myeloid markers that are either constitutively expressed or expressed upon activation with microbial ligands. Students use a low-cost image cytometer to both visualize and quantify cellular expression of myeloid markers. Students then perform literature research, design, and execute a series of experiments aimed at investigating the role of natural anti-inflammatory compounds on TNF-α production in these macrophages. The soup-to-nuts investigative approach in which students generate "their own" macrophages and study their functions over the course of the semester fostered a sense of ownership and accomplishment

Keywords: Immunology, image cytometry, macrophage, ELISA

INTRODUCTION

Macrophages are myeloid lineage professional phagocytes that play a key role as a first line of defense against invading pathogens. In addition to their immediate role in phagocytosis of microbes, macrophages express toll-like receptors (TLRs) and other pattern recognition receptors that enable a powerful and exquisitely tailored cytokine response to infection. Macrophage cytokines coordinate an immune response that includes stimulation of vascular permeability and recruitment of neutrophils and other immune cells to the infection site. Along with dendritic cells, activated macrophages play an important role in antigen processing and presentation to T cells, placing them at a critical juncture between innate and adaptive immunity (Murray & Wynn, 2011).

Macrophage biology can be modeled using a variety of cell culture approaches ranging from macrophage-like cell lines such as RAW 264.7 to primary resident tissue macrophages. Macrophage-like cells lines such as RAW 264.7 are robust and fairly easy to culture with minimal expertise, and in this respect, offer an attractive model for studying macrophage function. However, exclusive use of cell lines presents two major drawbacks: 1) the immortalized nature of the cells and their associated genetic abnormalities may limit conclusions that can be drawn from their use, and 2) in an undergraduate teaching laboratory, a cell line-only approach makes it all too easy for students to overlook the fact that myeloid lineage cells such as macrophages are born, differentiate, and carry out their functions in the context of a complex network of immune cells and organs *in vivo*. However, for the undergraduate laboratory, isolation of primary macrophages such as thioglycollate-elicited peritoneal macrophages, splenic macrophages, and alveolar macrophages is often not realistic, especially in institutions that lack mouse facilities. Here, we propose that the generation and study of bone marrow-derived macrophages (BMDM) in the undergraduate laboratory represents a fertile middle ground between cell lines and primary cells that offers the ease of a cell line together with a higher degree of authenticity. BMDM are commonly used in research labs and are a widely accepted monocyte/macrophage model as they closely approximate the attributes of primary macrophages and do not display immortalized growth, but can be isolated in large quantities using well-established protocols and can withstand cryopreservation (Franke et al., 2011; Manzanero, 2012; Weischenfeldt & Porse, 2008; Zanzoni et al., 2009). The process of BMDM differentiation starting with isolation of mouse femurs allows students to observe and think about hematopoiesis from the starting point of a whole organism. The harvesting of bones from sacrificed mice and subsequent BMDM differentiation protocol involves a single visit to an animal facility. Instructors at small colleges lacking animal facilities can generally obtain extra mice no longer needed for research from investigators at nearby R1 universities.

Here, we describe a semester-long laboratory project for an undergraduate immunology laboratory course that reinforces multiple principles of cellular immunology, including hematopoiesis, TLR signaling, cytokine production, and co-activation. The course is designed for students with no prior experience in cell culture, but can be easily adapted depending on the level of the students. The goal of this paper is to present our course structure accompanied by detailed methods. We will also present a brief assessment of student engagement and discuss ways in which our methods can be adapted to other courses.

COURSE STRUCTURE

Immunology (BIO3012) at Merrimack College is an elective course typically taken by juniors and seniors who have previously completed Introductory Biology and Genetics. Cell Biology is not a prerequisite, therefore, no prior knowledge was assumed of the students regarding mechanisms of cellular differentiation and signal transduction. Like most science courses taught at Merrimack College, the course is four credits and includes a lecture and laboratory which are taught by the same instructor in order to maximize connections between the theoretical and practical aspects of the subject. This laboratory course is divided into three parts: 1) Introduction to Mammalian Cell Culture, 2) BMDM Differentiation and Analysis, and 3) Independent Investigation as described below and as shown in Table 1.

Introduction to Mammalian Cell Culture.

In the first week of lab, students were given a small flask of RAW 264.7 cells and were asked to seed several wells of a 24-well plate. The purpose of this exercise was to provide an opportunity to learn and practice aseptic technique, proper use of the Class II Biosafety cabinet, and use of the hemocytometer. Following cell seeding, students stimulated production of the pro-inflammatory cytokine TNF-α by treatment of select wells with whole microbes (*E. coli*) or purified lipopolysaccharide (LPS). LPS is a molecule that is present in the cell wall of all Gram-negative bacteria and is a strong stimulator of cytokine production via toll-like receptor 4 (TLR4)-mediated signaling (Poltorak et al., 1998). In the lecture portion of the course, the molecular aspects of innate immunity were discussed at length, including TLR signaling as well as signaling induced by other families of pattern recognition receptors. Culture supernatants from stimulated and unstimulated RAW 264.7 cells were collected and saved for use as positive controls for subsequent TNF-α ELISAs to be performed later in the course. (If time allows, the samples could be immediately analyzed via ELISA).

BMDM Differentiation and Analysis.

To introduce students to the techniques and concepts that will be used throughout the semester, we dedicated the second week of lab to the analysis of a current primary research article focusing on macrophage toll-like receptor signaling (Thanawastien et al., 2009). For this, students presented figures in a round-table format. In the third week of the lab the instructor travelled to the University of Massachusetts Medical School to sacrifice donated mice and harvest femurs under their Institutional Animal Care and Use Committees IACUC protocol. Although students did not directly participate in the handling or dissection of mice, we spent time in class discussing the importance and

Table 1. Semester laboratory schedule. The semester consisted of thirteen 2.5 hour laboratory periods; activities in each laboratory period are shown. In addition to the regularly scheduled lab times, students were occasionally asked to come in to the lab for short periods of time to treat or fix cells.

Week #	Activity
1	Introduction to Cell Culture: Students are given a 25 mm flask of RAW cells –students harvest cells, determine concentration via hemacytometer, seed in 24 well dish, treat wells +/- Lipopolysaccharide, harvest supernatants
2	Journal Club: Thanawastien et al., 2009.
3	Crush mouse femurs and stimulate myeloid differentiation via treatment with MCSF
4	BMDM harvest and creation of BMDM frozen stocks
5	Seed BMDMs and culture +/- LPS to stimulate activation, fix and save cells
6	Image cytometric immunophenotyping of BMDM with CD11b and CD80 fluorescent antibodies
7	Image cytometry data analysis
8	Grant revew panel
9	BMDM seeding and anti-inflammatory treatment, collect supernatant
10	TNFα ELISA
11	Cytotoxicity Assay
12	Extra time to repeat experiments, plan final presentations
13	Anti-Inflammatory "Lunch" and Student Presentations

function of IACUC in animal research, which is critical in the field of immunology. Additionally, a video was viewed in class to expose students to the entire procedure including femur harvest (Trouplin et al., 2013). Working in pairs, students liberated marrow cells from femurs and tibias, seeded cells, and cultured cells in the presence of macrophage colony stimulating factor (M-CSF) to induce monocyte/macrophage differentiation (Klappacher et al., 2002; Stanley et al., 1997).

Students were asked to perform their own literature searches to determine which cell surface markers will identify myeloid lineage cells, as well as markers of myeloid activation. Students came up with several potential markers, and reviewing the students' answers together as a class served as an excellent introduction to a general discussion of immunophenotyping. As part of the discussion, we stressed the fact that no single cell surface marker can positively identify the myeloid lineage, monocyotes, or macrophages. Through classroom discussions, we decided to analyze expression of CD11b and CD80. CD11b (complement receptor) is a marker commonly used to identify myeloid lineage cells, and CD80 (B7-1) is a macrophage co-stimulation signal for T cell activation whose expression is induced upon activation with microbial ligands (Linsley & Ledbetter, 1993).

Others have implemented creative teaching modules that incorporate flow cytometry in the setting of undergraduate teaching laboratories (Boothby et al., 2004; Fuller-Espie, 2010; Ott & Carson, 2014; Szeberenyl, 2007). As an alternative to flow cytometry, students used image cytometry to immunophenotype our BMDM with respect to CD11b (constitutive myeloid) and CD80 (activation-associated). Image cytometric analysis resulted in vivid images that correlated well with scatter plots representing cell population data.

Independent Investigation.

Following analysis of myeloid cell surface markers by image cytometry, students designed and executed a series of experiments aimed at investigating the anti-inflammatory properties of natural plant-based compounds. In lecture, the inflammatory response in health and disease was discussed at length. Students were introduced to literature on natural anti-inflammatory compounds. Students were asked to write a grant proposal detailing experiments they would conduct using their isolated BMDM in order to determine whether a chosen compound counteracts inflammation at the cellular level. For example, one student group proposed to test the hypothesis that epigallocatechin gallate (EGCG), a component of green tea, inhibits LPS-stimulated TNF-α production in BMDM. Compounds studied by all student groups are shown in Table 2.

To encourage students to deeply think about their hypothesis and the details of their experiments, they were asked to draft a miniature version of an NIH-style grant proposal; the instructor reviewed and commented on each student's draft, giving feedback for improvement in the final draft. Additionally, the class was divided into peer review groups in which each group (3-4 students) was given an equal number of draft proposals to review. By participating in the review process, students were able to see examples of others writing, and reported that the process was extremely helpful in helping them improve their own writing. We believe that the drafting process made a daunting assignment more approachable and allowed the students the chance to improve their writing skills.

The remainder of the semester was dedicated to performing experiments designed by the students to test their hypothesis that compound "X" modulated production of TNF-α by BMDM. We limited our analysis to TNF-α for financial reasons, however, a panel of two or more cytokines could be analyzed if desired. In addition to cytokine analysis, students examined cytotoxicity to ensure that variations in cytokine levels were not due to varying levels of toxicity in response to cell treatment.

Table 2: Compounds tested by students for anti-inflammatory effects in BMDM. The qualitative effect of each compound on inhibition of LPS-induced TNF-α production in BMDM is also indicated, with ++ indicating strong inhibition of TNF-α production, + indicating weak inhibition, and – indicating no inhibition or inconclusive data.

Compound	Source in Nature	Inhibition of TNFα Production observed?
Epigallocatechin-3-gallate	Green tea	++
Fucoidan	Brown algae, seaweed	+
Resveratrol	Grape skins	++
Neem	Neem leaf	+
Harpagophytum procumbens extract	Devil's Claw	-
Quercetin	Flavenoid with wide distribution	++
Parthenolide	Feverfew leaves	-
Ocimum basilicum (eugenol)	Eugenol	-
Curcumin	Turmeric	++
γ-aminobutyric acid (GABA)	Animals and a variety of plants	++

MATERIALS AND METHODS

The complete student lab manual and an instructor's preparation guide, including reagent ordering information, is available within the ACUBE Resources site.

RAW 264.7 Culture

RAW 264.7 cells were obtained from the American-Type Culture Collection and maintained in Dulbecco's Modified Eagle Medium (DMEM, Sigma) supplemented with 10% fetal bovine serum (Atlanta Biologicals) and penicillin/streptomycin (Life Technologies). For student experiments, RAW 264.7 cells were seeded in 24 well plates with 1.0 x 10^5 cells/well. Seeding was carried out during the regularly scheduled lab period, where students were also instructed on aseptic technique, proper use of the Class II Biosafety Cabinet, and enumeration of live/dead cells by trypan blue staining followed by hemocytometer counting. The following day, students came in briefly to treat cells with 10 μL overnight *E. coli* (DH5α) culture or 10 ng/mL LPS (Sigma). Cells were treated for 4 hours and supernatants collected and saved at -20°C.

Bone Harvest

Five six week-old male C57/BL6 mice were sacrificed by CO_2 euthanasia followed by cervical dislocation as approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Massachusetts Medical Center. C57/BL6 mice are commonly used in immunology studies, but many other strains would make a suitable substitute. Either male or female mice may be used. In C57/BL6 mice, the optimal age range for BMDM production is 6 weeks-3 months. Older mice (up to one year) may be used, although they typically produce lower BMDM yields. Femurs and tibias were separated from tissue as described previously (Zanzoni et al., 2009). Bones were harvested 20 hours prior to the scheduled laboratory period and were immersed in DMEM and stored at 4°C or on ice during transport.

BMDM Differentiation

BMDM media consisted of phenol red-free high glucose DMEM (Sigma) supplemented with 20% fetal calf serum (Atlanta Biologicals), 1 mM sodium pyruvate (Sigma), 2 mM glutamine (Sigma), 100 Units/mL penicillin/streptomycin (Life Technologies) and 10% L929 cell supernatant, prepared as described previously (Yamamoto et al., 1981). Phenol red-free DMEM was used throughout our experiments as the presence of phenol red in the media interferes with the colorimetric cytotoxicity assay used later in the semester.

Students worked in pairs to extract bone marrow from a single femur and tibia. Bones were crushed mechanically using an ethanol-sterilized mortar and pestle inside the Biosafety cabinet. The mechanical crushing method produces less pure cultures than flushing of bones with fine-gauge needles, however, we have found this method to be preferable in the hands of undergraduates as it avoids the unacceptable risk of needle-stick injuries. Crushed bones were submerged in 10 ml BMDM media and further dissociated by repeated pipetting. Samples were transferred to sterile 50-mL conical tubes and large debris (bone fragments and connective tissue) was allowed to settle by leaving tubes undisturbed for 2 minutes on ice. The supernatant was transferred to a new 50 mL conical tube, then centrifuged at 1200 rpm for 5 minutes to pellet cells. Cells were resuspended in 5 ml BMDM, counted and seeded at 1.0 x 10^7 cells per plate in 25 cm petri dishes (not cell culture-treated) in a total of 25 mL BMDM media. Typically, the femurs and tibias of a single mouse will produce enough material for 5-10 plates. Following three days of incubation at 37°C and 5% CO_2, cells were fed with 20 ml additional BMDM media and returned to the incubator for an additional four days.

Following seven days total incubation, media was removed and BMDM were washed three times in cold magnesium/calcium-free PBS and incubated in 10 ml cold PBS for 30 minutes on ice. The ice incubation causes BMDM to round up and loosen their attachment to the plates. To collect BMDM, cells were pipetted up and down several times in PBS. This process requires quite a bit of patience and "elbow grease", as BMDM are highly adherent. Students must be encouraged to keep pipetting up and down (typically at least 20 times on each plate), using the stream of fluid to force cells off the plates until no "cloudy" areas are observed. To check that most cells have been removed, plates may be checked in the microscope. Cells were transferred to a sterile 50 mL conical tube on ice and each plate was rinsed with 10 mL fresh PBS. Cells were then concentrated by centrifugation at 1200 rpm for 5 minutes and resuspended in the equivalent of 1 mL BMDM media per plate. Cells were counted and resuspended in freezing media (BMDM media supplemented with 10% DMSO) to a final concentration of 1.0 x 10^7 cells/mL and 1 mL aliquots stored in cryovials at -80°C (if available, liquid nitrogen is preferable, although cells will remain stable at -80°C for the remainder of the semester).

BMDM Culture and Activation

To recover frozen BMDMs, cryovials were thawed in a 37°C water bath only until just thawed, then placed on ice. The contents were diluted in 9 mL BMDM media in a 15 mL sterile conical tube. To remove DMSO, cells were centrifuged at 1200 rpm for 5 minutes, resuspended in 1 mL fresh BMDM media, and an aliquot was stained with trypan blue and counted in order to determine cell viability and concentration.

To generate samples of resting and activated BMDM samples for subsequent immunophenotyping, cells were seeded at 5 x 10^6 cells/plate in 10 cm

plates. The following day, two plates were treated with 10 ng/mL LPS (Sigma) in order to activate macrophages, and the other two were treated with the equivalent volume of media only (inactivated). Following 24 hours in LPS, cells were washed with 10 mL PBS and fixed in 4% formaldehyde/PBS, removed from wells by scraping, and stored at 4°C until the next laboratory period.

For investigation of natural compounds, students seeded BMDMs in 24 well plates at a density of 7.0 x 10^5 cells/well. Following seeding and overnight incubation, students treated their cells with a variety of concentrations of their test compound (typically, 1 μM, 10 μM, and 100 μM, although a variety of concentrations were used based on their literature research). Following 12-16 hour "pre-treatment" with putative anti-inflammatory compounds, a subset of wells was treated for 2 hours with 10 ng/mL LPS. It is important here to include controls that are exposed to putative anti-inflammatory but not LPS, as well as an LPS-only control and a no-treatment control.

Image cytometric immunophenotyping

Conical tubes containing fixed cells were centrifuged on high speed for one minute to pellet cells. Cells were then resuspended in PBS/10% Fetal calf serum to a final concentration of ~ 3 x 10^6 cells/ml. To reduce background staining caused by binding of the Fc region of fluorescent antibodies to macrophage Fc receptors, 5 μL Fc block reagent (BD Pharmingen) was added to each sample and incubated with agitation at 4°C for 5 minutes. 100 μL cells were transferred to new tubes and incubated in the presence of 1.25 μL phycoerythrin(PE)-anti-mouse-CD11b (Biolegend) AND 1.25 μL Alexa-fluor-488-anti-mouse-CD80 (Biolegend). Following 30 min incubation in the dark at 4°C to avoid photobleaching of the fluorophores, cells were washed 5X in PBS/10% Fetal calf serum, then resuspended in 100 μL PBS/10% Fetal calf serum.

The Cellometer Vision instrument (Nexcelom Biosciences) has been described in previous publications (Chan et al., 2011a, 2011b). The system utilizes bright-field (BR) and dual-fluorescent imaging modes to quantitatively analyze and measure the concentration and fluorescent intensity of target cells. The fluorescence optics modules (FOMs) utilize filters with Excitation/Emission of 470 nm/535 nm and 525 nm/595 nm for FITC and PE detection, respectively. The cell samples (20 μL) were pipetted into the Nexcelom disposable counting chambers, inserted into the instrument, and then focused using the BR imaging. Following image capture, data was exported to a proprietary .NXDAT file format, which was then analyzed in the FCS Express Software (De Novo, Los Angeles, CA).

Natural Compounds

Compounds were purchased from Sigma and resuspended in DMSO unless otherwise directed. Solutions were sterilized by filtration through 0.22 μm filters. Final concentrations used were typically 1, 10, and 100 mM.

ELISA

TNF-α ELISA (eBioscience) was carried out according to manufacturer's instructions using 100 μL cell supernatants. If desired, the RAW cell supernatants generated earlier in the course may also be tested here (RAW cells typically produce higher levels of TNF-α, requiring dilution of supernatants prior to adding to ELISA wells (1:10) to avoid saturation. Absorbance at 450 nm was quantified using a BioTek ELx800 plate reader.

Cytotoxicity Assay

To rule out the possibility that TNF-α levels are modulated due solely to cytotoxicity, we used the Cytotox Assay (Promega) to measure culture supernatants for the presence of lactate dehydrogenase, a cytosolic enzyme. Assays were carried out in 96-well plates and A_{680} measured using the BioTek ELx800 plate reader.

Institutional Review Board Approval

Student surveys were approved by the Merrimack College IRB.

RESULTS AND DISCUSSION

Representative student-generated data of image cytometric analysis of CD11b/CD80 surface expression is shown in Figure 1. As expected, scatterplot analysis of the non-activated BMDM shows that 93.3% of are $CD11b^+/CD80^-$ and only 4.9% are $CD11b^+/CD80^+$. We observed a shift in the subset of CD80 positive cells following LPS-mediated activation, with 36.6% of cells $CD11b^+/CD80^+$. Previously published values typically show higher levels of CD80 induction upon LPS treatment of BMDM (Franke et al., 2011), however, we suspect that the mechanical crushing method used to extract marrow from bones led to decreased purity in our sample. One extremely powerful aspect of image cytometry is that students can observe fluorescence in the captured images, which provides a visual framework to help them better understand abstract scatterplots and histograms.

A list of all compounds tested and their qualitative effects on TNF-α production in BMDM is shown in Table 2. One of the most potent inhibitors of LPS-inducted TNF-α production was the green tea compound epigallocatechin gallate (EGCG). TNF-α ELISA results show a dose-dependent decrease in TNF-α levels upon pre-treatment with EGCG (Figure 2). To determine whether variations in TNF-α levels were due to variations in cell viability, students also tested cellular supernatants for release

of lactate dehydrogenase (LDH), an abundant cytosolic enzyme released from cells upon death. LDH levels remained constant regardless of EGCG concentration (data not shown).

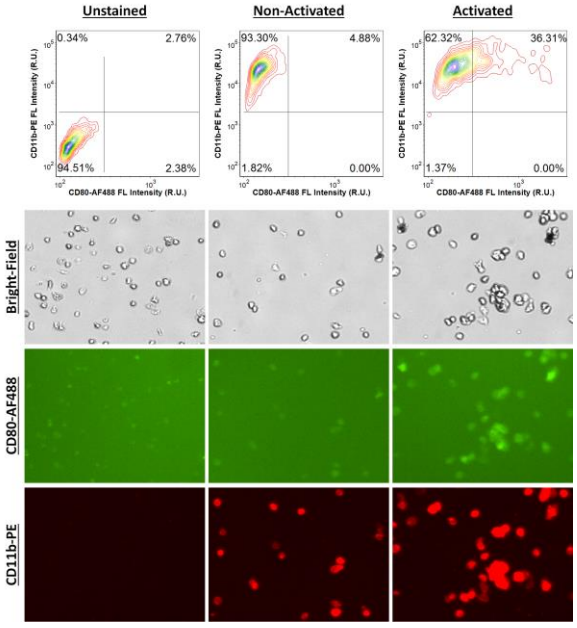

Figure 1. Image cytometric analysis of bone marrow-derived macrophages. BMDM were cultured in either DME/20% FBS/M-CSF (Non-activated) or DME/20% FBS/M-CSF + 10 ng/ml LPS for 24 hours (Activated). Cells were fixed with 4% formaldehyde/PBS and incubated phycoerythrin (PE)-anti-CD11b and Alexfluor-488-anti-CD80. Representative bright-field, CD80 positive (green pseudo color) and CD11b positive (red pseudo color) images resulting from Cellometer Vision analysis are shown. Scatterplots show that the majority (93.3%) of non-activated BMDM are $CD11b^{+}/CD80^{-}$. Activation of BMDM by treatment with LPS induces a shift in CD80 expression, with 36.61% of cells $CD11b^{+}/CD80^{+}$. Fluorescent images also show activation with LPS induces an increase in the intensity of CD11b staining. In addition, the cell morphology in bright-field shows increase in cell size that can be observed by the students. To assess background levels of autofluorescence, a sample of unstained BMDM is shown (left panel).

We have found that image cytometry is ideal for use in the undergraduate laboratory for both practical and pedagogical reasons. Observation of raw images shows a clear difference in the proportion of cells expressing CD11b and CD80 when comparing activated and resting BMDM. Also, direct observation of bright-field and fluorescent images shows that LPS treatment induces an increase cell size and in the *intensity* of CD11b staining. Direct observation of these images enabled students to quickly understand and explain the population-based scatter plots generated based on multiple images.

Exam questions showed that all students were able to apply their understanding of cytometric analysis to other situations (sample questions are available within the ACUBE member resources site).

Practically speaking, image cytometry instrumentation has a smaller footprint, lower price, and minimal maintenance, making it an ideal tool for teaching and research at smaller colleges and departments. All of the kits and reagents needed for this laboratory can be purchased for approximately $1500. In addition to its use in immunophenotyping, image cytometry can be used to visually and quantitatively assess multiple cellular phenotypes, including viability, apoptosis, necrosis, autophagy, cell cycle progression, and mitochondrial potential (Chan et al., 2011a, 2011b; Robey et al., 2011).

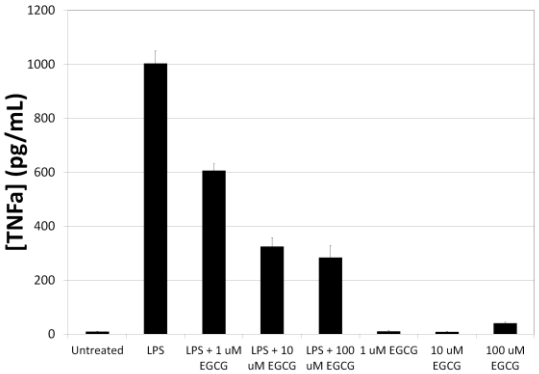

Figure 2. EGCG inhibits production of TNF-α. BMDM were seeded at 7.0 x 10^5 cells/well in 24 well dishes and pre-treated overnight with shown concentrations of EGCG or DMSO (carrier) prior to treatment with 100 ng/ml LPS. Supernatants were collected after 2 hours of treatment +/- LPS and subjected to TNF-α□□□□□□. This experiment was performed in triplicate; similar results were found when repeated.

Student attitudes and engagement were assessed by administering questionnaires in which students ranked their level of agreement/disagreement with several statements on a 5-point Likert scale (Figure 3). Large gains in student confidence were observed for discipline-specific skills. More modest gains were observed in the more general skills including designing and executing experiments. We suspect that this is due in part to the fact that most science labs at Merrimack College include experimental design and hypothesis testing into the laboratory curriculum. Since Immunology is populated by juniors and seniors, it is likely that they had all gained some level of competency in this realm through previous courses. This is supported by relatively high "pre" scores in response to the statement: "I am comfortable designing testable hypotheses and designing/executing my own

experiments." To assess student understanding directly, we administered an in-lab quiz based on analysis of data from the primary literature (this is available on the ACUBE Resources site). Over 90% of students performed well (score of 85% or higher)

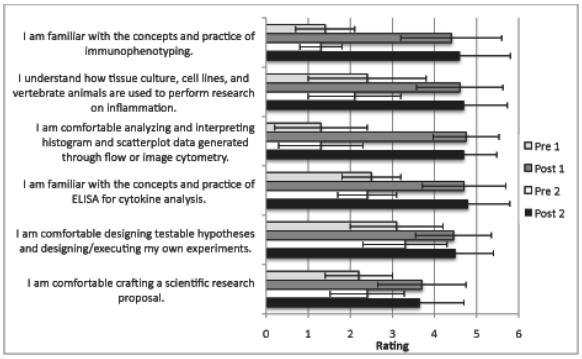

Figure 3. Survey of student learning and attitudes. Survey questions were administered to students prior to (pre) and following (post) the laboratory activities. Students were asked to rate their level of agreement with each statement on a 5-point Likert scale, with 1 indicating "strongly disagree" and 5 indicating "strongly agree". These surveys were given to students taking the course in two separate semesters (semester 1, n=14 students; semester 2, n=13 students).

on assessments that challenged them to analyze immunophenotyping and ELISA data.

Student surveys indicated a high degree of interest, a sense of ownership of their project, and a sense of accomplishment. A sampling of student comments supports this idea: "I felt independent to the point of being expert and accomplished."; "I was grossed out by the mouse bones at first, but once I successfully got my own macrophages, I felt really invested in the project."; "Although writing the proposal was hard, it helped me to organize my thoughts."; "I learned so much from this – great lab design! I really wanted to see what happened and didn't even mind coming in after-hours." Another sign of student engagement in the course was that students came up with the idea to hold an "anti-inflammatory lunch" at the same time as the final presentations. This idea had its genesis in an observation made by a student: that many of the compounds tested by students are found in edible items. Our lunch menu included sushi (containing fucoidan in sea weed), green tea (containing ECGC), grapes (containing resveratrol) and Neem leaf tea.

There are multiple opportunities to either expand upon or modify this laboratory module. In the absence of either image or flow cytometric analysis, expression of myeloid markers may be performed using standard immunofluorescence, western blotting, and/or quantitative real-time PCR. Our lab used BMDM, however, the above activities (minus the differentiation procedure itself) can easily be performed using a macrophage-like cell line such as RAW 264.7 or J77A.4. Instructors wishing to use real murine bone marrow may learn the technique on their own with the recent advent of video-based resources that focus on scientific methods (Trouplin et al., 2013). Instructors may also expand upon the immunophenotyping exercise to include examination of additional markers such as F4/80, CD115 (M-CSF receptor), and others. Additionally, there are plentiful opportunities to use image cytometry to experimentally examine factors that affect the bone marrow differentiation process itself, for example, the concentration of M-CSF added and/or the presence of additional growth factors and cytokines.

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