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**Novel Characteristics of Murine Bone Marrow-Derived Macrophages
and Human Macrophage-Like Cells**

**A thesis submitted in partial fulfillment of the requirements for the degree of
Master of Science at Virginia Commonwealth University**

By

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B.S. Biology

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2004

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Table of Contents

Acknowledgment.....	ii
List of Figures.....	iv
List of Abbreviations.....	v
Abstract.....	vi
Study 1.....	1
Introduction.....	2
Methods and Materials.....	5
Results.....	9
Discussion.....	11
Figures.....	12
Study 2.....	17
Introduction.....	18
Methods and Materials.....	20
Results.....	24
Discussion.....	26
Figures.....	27
List of References.....	35
Vita.....	39

List of Figures

Study 1

Figure 1.	Catecholamine synthesis pathway.....	12
Figure 2.	BMM staining for F4/80.....	13
Figure 3.	Catecholamine synthesis inhibition in mouse BMM with α -mpt.....	14
Figure 4.	Catecholamine synthesis inhibition in mouse BMM with fusaric acid.....	15
Figure 5.	Adrenergic regulation of IL-1 β in mouse BMM.....	16

Study 2

Figure 1.	MAC-1 staining of human bone marrow-derived cells.....	27
Figure 2.	F4/80 staining of human bone marrow-derived cells.....	28
Figure 3.	AS02 staining of human bone marrow-derived cells.....	29
Figure 4.	Phagocytosis assay.....	30
Figure 5.	Phagocytosis of FITC-Bacteria in RAW264.7 cells.....	31
Figure 6.	Phagocytosis of FITC-Bacteria in human bone marrow-derived cells.....	32
Figure 7.	TNF- α production in human bone marrow-derived cells.....	33
Figure 8.	IL-1 β production in human bone marrow-derived cells.....	34

List of Abbreviations

α -mpt.....	α -methyl- <i>para</i> -tyrosine
BMM.....	bone marrow-derived macrophages
cRPMI.....	complete RPMI medium
DMEM.....	Dulbecco's Modified Eagle's Medium
FBS.....	Fetal Bovine Serum
FITC.....	Fluoresceine
GM-CSF.....	granulocyte macrophage-colony stimulating factor
HEPG2.....	human hepatoma cell line
IL-1 β	Interleukin-1 β
IL-6.....	Interleukin-6
L929.....	mouse fibroblast cell line
LPS.....	lipopolysaccharide
M-CSF.....	macrophage-colony stimulating factor
PBS.....	phosphate buffered saline
PE.....	phycoerythrine
RAW264.7.....	mouse macrophage cell line
RT-PCR.....	reverse transcription-polymerase chain reaction
TNF- α	tumor necrosis factor- α

Abstract

NOVEL CHARACTERISTICS OF MURINE BONE MARROW-DERIVED MACROPHAGES AND HUMAN MACROPHAGE-LIKE CELLS

George T. Georges, B.S.

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University.

Virginia Commonwealth University, 2006

Major Director: Jennifer K Stewart, Ph.D., Associate Professor, Department of Biology

These studies provide evidence for novel properties of macrophages derived from bone marrow stem cells. In study I, treatment of activated mouse bone marrow-derived macrophages (BMM) with either catecholamine synthesis inhibitors (α -methyl-*para*-tyrosine and fusaric acid) or the β_2 adrenergic receptor antagonist ICI 118,551 demonstrated that BMM produce catecholamines. The catecholamines modulated macrophage cytokine production through autocrine actions on adrenergic receptors. In study II, undifferentiated human bone marrow cells were incubated in 30% mouse L929 fibroblast conditioned medium and generated adherent cells within three days. The cells were clearly identifiable as macrophages based on surface proteins and phagocytic activity but produced only low levels of the cytokines tumor necrosis factor- α and interleukin-1 β . Cytokine production did not increase in response to the bacterial

endotoxin lipopolysaccharide (LPS). Generation of these macrophage-like cells was not repeatable with other samples of human bone marrow, but the cells continue to proliferate in cell culture and will be investigated further in future studies.

Study 1

**Synthesis and Autocrine Actions of Catecholamines in Mouse
Bone Marrow-Derived Macrophages**

Introduction

Macrophages are a critical part of the innate immune response of host defense. Upon entering the body, bacteria and other foreign antigens are identified by the cells of the immune system, which act quickly to eliminate them. Macrophages are characterized by their ability to become activated in the presence of the foreign antigen. Activation stimulates macrophages to engulf particles by phagocytosis and to produce cytokines that are important immune regulators.

Macrophages are derived from the pluripotent hematopoietic bone marrow stem cells. The process of differentiation requires chemical signals that allow the cells to develop along a specific cell lineage. In the case of macrophages, macrophage-colony stimulating factor (M-CSF) and granulocyte macrophage-colony stimulating factor (GM-CSF) are required to commit the cells to the myeloid cell lineage (17). The stem cells first develop into monocytes in the blood, whereby further development through continued stimulation results in the formation of localized, tissue-specific macrophages.

Macrophages are known to express α_2 and β_2 adrenergic receptors that enable the catecholamines, norepinephrine and epinephrine, to modulate cytokine production and influence immune function. Previous studies have demonstrated that binding of catecholamines or other adrenergic agonists to β_2 adrenergic receptors inhibits macrophage production of tumor necrosis factor- α (TNF- α) and interleukin-1 β (IL-1 β)

(3; 11; 12; 16; 21; 22). Binding to α_2 adrenergic receptors stimulates production of these cytokines (5; 6; 22; 23).

Although catecholamines are produced primarily by neurons and the adrenal medulla, macrophages have been reported to take up catecholamines from extracellular fluids (1; 13), and catecholamines have been measured in peritoneal macrophages by two laboratories (15; 22). Scott Brown in our laboratory detected tyrosine hydroxylase (TH) mRNA, the rate limiting enzyme of the catecholamine synthesis pathway, in the RAW264.7 macrophage cell line following treatment with the bacterial endotoxin, lipopolysaccharide (LPS) (2). TH mRNA increased three fold from 24 h to 48 h after LPS treatment. In the same study, both intracellular and extracellular norepinephrine levels decreased in the presence of α -methyl-*para*-tyrosine (α -mpt), which prevents the activity of TH. These findings suggest that catecholamines are synthesized in RAW264.7 macrophages.

It is unclear whether catecholamines in primary macrophages are synthesized by these cells or taken up and stored for subsequent release. Several studies suggest that macrophages release norepinephrine. For example, our laboratory demonstrated that the RAW264.7 macrophage cell line releases norepinephrine (2), and Miller and colleagues found that human synovial macrophages release norepinephrine (19). Spengler and colleagues reported that intracellular levels of norepinephrine in peritoneal macrophages are reduced 30 minutes after the cells are stimulated with LPS (22), suggesting that intracellular catecholamines are released from macrophages into the extracellular environment upon stimulation. Additionally, there is evidence that macrophage

production of cytokines is modulated by autocrine actions of macrophage-derived catecholamines binding adrenergic receptors. For example, treatment of peritoneal macrophages with adrenergic receptor antagonists (in the absence of exogenous catecholamines) modulates LPS stimulated production of the cytokines tumor necrosis factor-alpha (TNF- α) (22) and interleukin-1 β (IL-1 β) (7).

Although it is now clear that mouse peritoneal macrophages release catecholamines that exert autocrine effects on cytokine production, it is not known if this regulatory mechanism is present in newly differentiated murine bone marrow-derived macrophages. This question is important because bone marrow stem cells from experimental animals and human subjects are important precursors of macrophages, and these cells are often the major source of macrophages used for research. The focus of this study is to investigate whether mouse BMM synthesize catecholamines and whether catecholamines released by these cells display the same autocrine regulation of cytokines through the adrenergic receptors as is seen in mouse peritoneal macrophages.

Methods and Materials

Cells

Female CBA/J mice (6-8 weeks of age) were obtained from Harlan (Indianapolis, IN), and 6 month old female C57BL6J/129 mice were obtained from Taconic (Germantown, NY). Mice were euthanized with CO₂. Cells were collected from tibia and femurs (4) and washed with complete RPMI (cRPMI) medium consisting of RPMI 1640 (Invitrogen) supplemented with 10% heat inactivated-FBS, 1% L-glutamine, 1% non-essential amino acids, 1% minimal essential medium vitamins, 100 units/ml penicillin and 100 µg/ml streptomycin. The cells were seeded in tissue culture plates in fresh cRPMI with 30% L929-cell conditioned medium (a source of macrophage colony stimulating factor) (8; 18). Cells were incubated at 37 °C in a humidified 5% CO₂ atmosphere. After 24 hours, non-adherent cells were transferred to new 6 well tissue culture-treated plates to eliminate any early adherent cells such as fibroblasts. After 1 week numerous adherent cells were evident, and the medium was replaced with fresh 30% L-cell/70% cRPMI. The adherent cells were identified with a macrophage surface antigen and used in experiments the same week.

Two cell lines, L929 mouse fibroblasts, and RAW264.7 macrophages were obtained from American Type Cell Culture (Manassas, VA). The cells were maintained in cRPMI in 75 cm² tissue culture flasks. Stock cultures of L929 cells were sub-cultured weekly, and RAW264.7 cells were sub-cultured twice weekly. For collection of L929 conditioned medium, approximately 200,000 L929 cells were seeded in 75 cm² flasks in 25 ml of cRPMI. Medium was removed after one week, and fresh cRPMI was added. The cells were maintained for a second week; then medium was again harvested, and cells were discarded. The media samples were centrifuged at 500 x g and 4 °C for 10 minutes, and filtered with either 0.22 µm PVDF-syringe filters (Fisher Scientific) or Steriflip vacuums filters with 0.22 µm PVDF membranes (Millipore Corporation, Billerica, MA), and stored frozen at -20 °C in polypropylene tubes.

Bone marrow-derived mast cells were obtained from Mohit Kashyap in the laboratory of Dr. John Ryan, Virginia Commonwealth University. Mast cells were differentiated and maintained as previously described (9).

Identification of Macrophages with F4/80

F4/80 is a surface antigen expressed by mature macrophages. Compared with other commonly used markers that are found on both fibroblasts and macrophages, F4/80 is more specific for macrophages (14). For F4/80 detection, cells were washed with fresh cRPMI medium and adjusted to 0.5×10^6 cells/ml. An aliquot (180 µl) of the cells was added to a 96 well v-bottom plate, washed with phosphate buffered saline (PBS), and incubated with 10 µl of ascites 2.4G2 for 10 min to prevent non-specific binding to Fc-II and Fc-III receptors. Samples were incubated with phycoerythrine (PE)-labeled anti-

mouse F4/80 antibody (eBioscience, San Diego, CA) at 2 $\mu\text{g/ml}$ final concentration, while control samples were incubated with PE Rat IgG2a isotype control (eBioscience, San Diego, CA) at 20 $\mu\text{l/well}$. Following a 30 minute incubation, the wells were washed, and cells binding the labeled antibody were detected with a BD FACS Calibur flow cytometer. Results were analyzed with the BD CellQuest Pro software. Mouse mast cells and RAW264.7 macrophages were assayed as negative and positive controls, respectively.

Effects of catecholamine synthesis inhibitors on LPS-stimulated IL-6 production

Adherent cells in a 6 well plate were rinsed and removed with 3 ml of 0.25% (w/v) Trypsin + 0.53 mM EDTA. Fresh cRPMI was added to the cells, and 1.5×10^6 cells per well were plated in a 24 well tissue culture-treated plate. The cells were incubated for 4 h at 37°C in 5% CO₂ to allow for adherence. BMM were then washed twice with warm (37°C) phosphate buffered saline, pH 7.4, and treated with 30 ng/ml LPS (*E. coli* – serotype 055:B5, Sigma) and catecholamine synthesis inhibitors (either 2 mM α -mpt or 500 μM fusaric acid) in a final volume of 0.5 ml serum-free RPMI. α -mpt inhibits the activity of tyrosine hydroxylase, which converts L-tyrosine to L-dopa, and fusaric acid inhibits the activity of dopamine- β -hydroxylase, which converts dopamine to norepinephrine (20) (Figure 1). The macrophage monolayer in each well was washed with PBS, lysed in 150 μl of lysis buffer (0.05 M Tris (pH 7.5), 0.3 M NaCl, 2 mM EDTA, 0.5% Triton X-100, 2.0 $\mu\text{g/ml}$ leupeptin, 1 $\mu\text{g/ml}$ aprotinin and 0.2 mM phenylmethylsulfonylfluoride), and then frozen at -20°C for future determination of

total cellular protein with the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA) and read at 595 nm in the microplate spectrophotometer.

Effects of the β_2 adrenergic antagonists on LPS stimulated IL-1 β production

Bone marrow cells were treated with 30 ng/ml LPS (*E. coli* –serotype 055:B5, Sigma, St. Louis, MO) and 10 μ M ICI 118,551 (β_2 antagonist, Tocris, Ellisville, MO) in a final volume of 0.5 ml serum-free RPMI. Following a 24 h incubation, extracellular fluid was removed, centrifuged at 500 x g for 10 min at 4°C and then frozen at -20°C for future IL-1 β assay. The macrophage monolayer in the wells was washed with PBS, lysed in 150 μ l lysis buffer as described above, and then frozen at -20 °C for future determination of total cellular protein with the Bio-Rad protein assay.

Measurement of IL-6 and IL-1 β

Extracellular cytokine concentrations were assayed with an OptEIA Multi Component Elisa Set for Mouse IL-6 and a set for IL-1 β (BD Biosciences, San Diego, CA) in accordance with the manufacturer's instructions. The plate was read at 450 and 570 nm with a μ Quant Universal Microplate Spectrophotometer (Bio-Tek Instruments, Winooski, VT). Concentrations were calculated with the manufacturer's KC4 software. Statistical analysis of all data was performed with GraphPad Prism, version 4.02.

Results

Verification of macrophage phenotype

Approximately 50% of the bone marrow-derived cells began to show adherence on day two of incubation following marrow extraction. By day 4, nearly 80% of the cells in each flask were healthy differentiated adherent cells with defined pseudopodia. The cells displayed a high fluorescence intensity for the mature macrophage marker F4/80, and the expression of F4/80 in the BMM was similar to that of the positive control (RAW 264.7 macrophage cells) (Figure 2).

Effects of catecholamine synthesis inhibitors on IL-6 release

Catecholamine synthesis inhibitors significantly reduced extracellular levels of IL-6. When treated with LPS + the tyrosine hydroxylase inhibitor α -mpt, extracellular IL-6 decreased three fold when compared with LPS treatment alone (Figure 3). When the BMM were treated with LPS + fusaric acid, an inhibitor of dopamine- β -hydroxylase, similar results were observed (Figure 4). These findings suggests that mouse BMM synthesize catecholamines that exert autocrine effects on the release of IL-6. Furthermore, because fusaric acid inhibits synthesis of norepinephrine and epinephrine, but not dopamine (Figure 2), the results suggest that IL-6 release is stimulated by BMM-derived norepinephrine or epinephrine.

Autocrine actions of macrophage-derived catecholamines on IL-1 β release

When the BMM were treated with 30 ng/ml LPS and the β_2 -adrenergic receptor antagonist ICI, the extracellular IL-1 β levels increased two fold over those observed with LPS alone (Figure 5) suggesting that β_2 adrenergic agonists released by BMM inhibit LPS-stimulated production of IL-1 β . These results parallel those previously observed with peritoneal macrophages (7) and suggest that BMM-derived catecholamines display an autocrine effect on IL-1 β mediated through the β_2 -adrenergic receptor.

Discussion

This study is the first to demonstrate that newly differentiated bone marrow-derived macrophages (BMM) produce catecholamines, which modulate extracellular levels of IL-1 β and IL-6 produced by the macrophages. The autocrine actions of catecholamines on the β_2 -adrenergic receptor in BMM are consistent with the results of previous studies on peritoneal macrophages (7). Further investigation is needed to determine whether BMM express the α_2 -adrenergic receptor and to determine the receptor mediating autocrine effects of BMM-derived catecholamines on release of IL-6. These findings are important for our understanding of regulatory mechanisms in BMM and because BMM are frequently used to study macrophage functions.

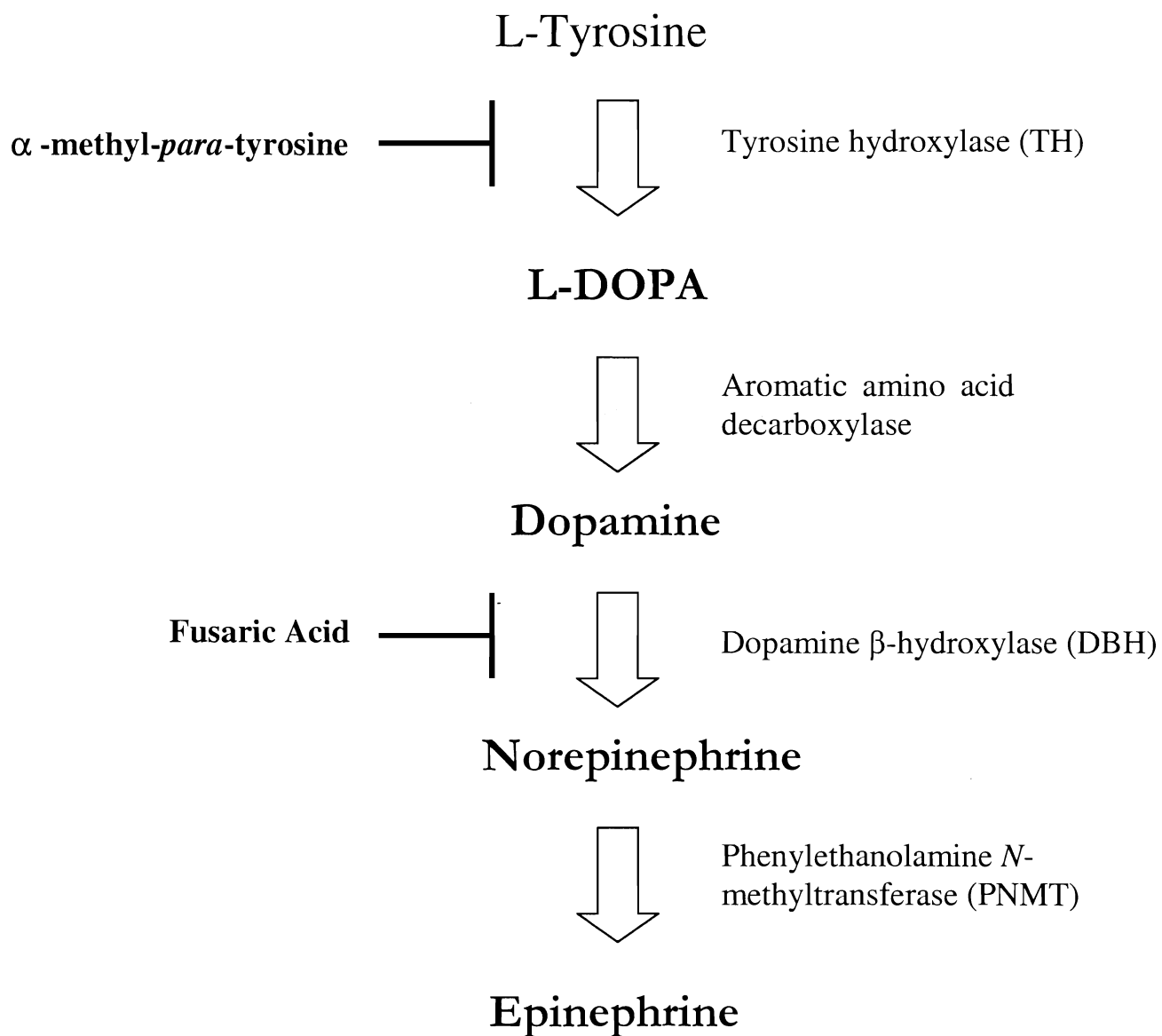


Figure 1. Catecholamine synthesis pathway. α -methyl-*para*-tryosine inhibits the actions of tyrosine hydroxylase (TH), preventing the synthesis of L-DOPA, dopamine, norepinephrine, and epinephrine. Fusaric acid inhibits the actions of Dopamine β -hydroxylase (DBH), preventing the synthesis of norepinephrine and epinephrine only.

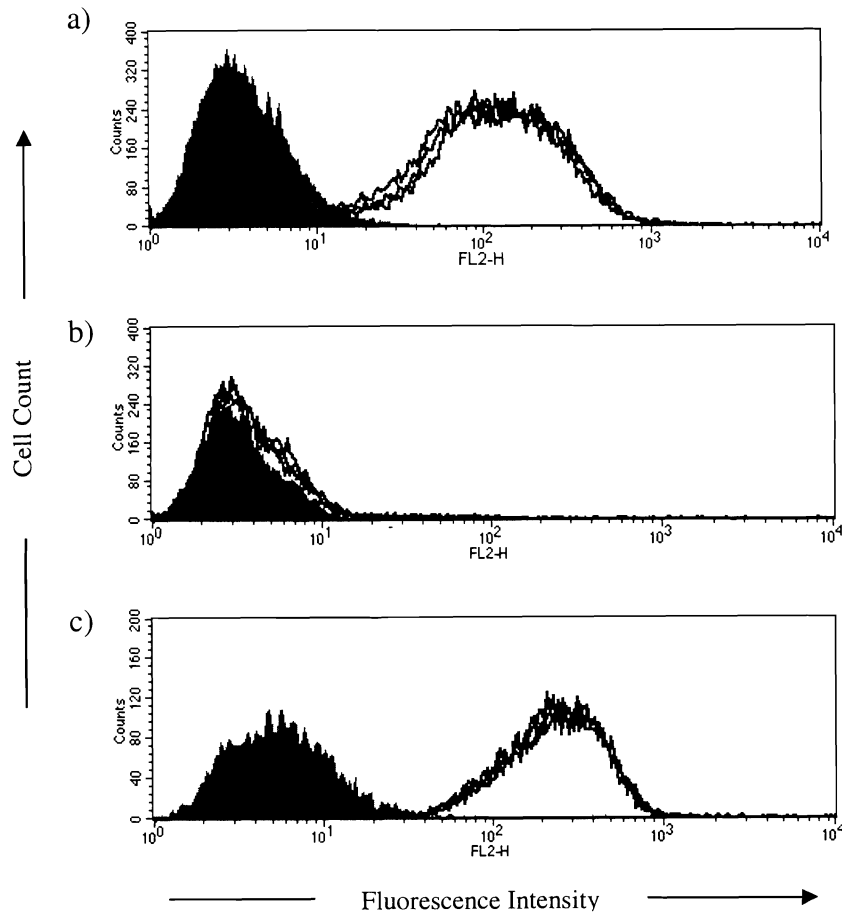


Figure 1: Expression of F4/80 in mouse BMM. The shaded regions indicate non-specific background staining, and lines represent triplicate samples with F4/80 staining. (a) The RAW 264.7 macrophage cells were a positive control. (b) Mast cells, a negative control, showed no significant expression of F4/80. (c) The F4/80 staining of bone marrow-derived cells was comparable to that of the positive controls cells.

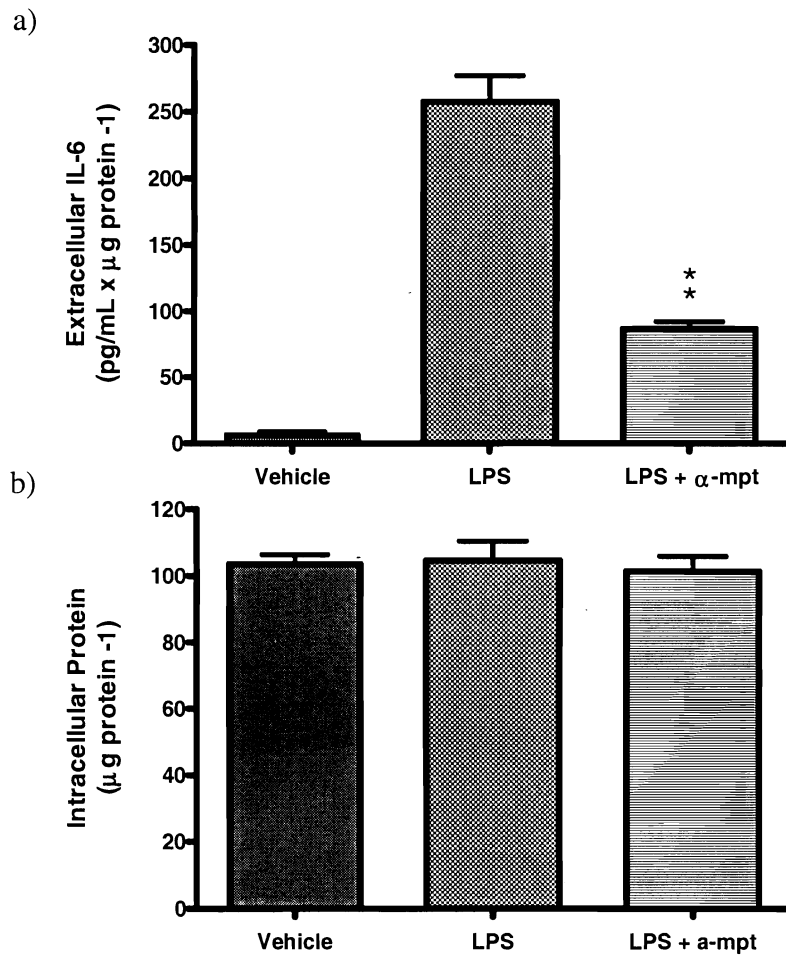


Figure 3: Bone marrow derived macrophages were treated with LPS and the catecholamine synthesis inhibitor α -methyl-*para*-tyrosine (α -mpt). a) Extracellular IL-6 was measured to determine the effects of catecholamine synthesis on mouse BMM cytokine release. b) Protein levels were not different among treatment groups. * P < 0.001 compared to LPS alone.

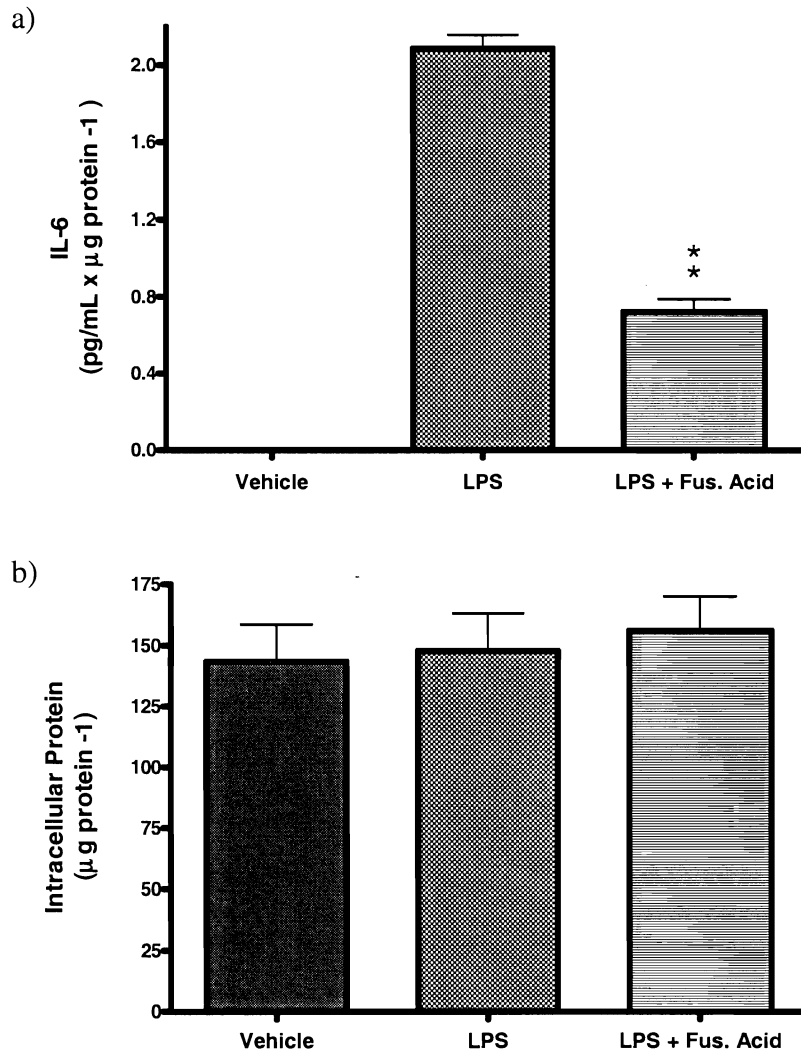


Figure 4: Bone marrow derived macrophages were treated with LPS and the catecholamine synthesis inhibitor fusaric acid. (a) Extracellular IL-6 was measured to determine the effects of norepinephrine and epinephrine synthesis on mouse BMM cytokine release. (b) The concentration of protein in each treatment group did not differ significantly. * $P < 0.001$ compared to LPS alone.

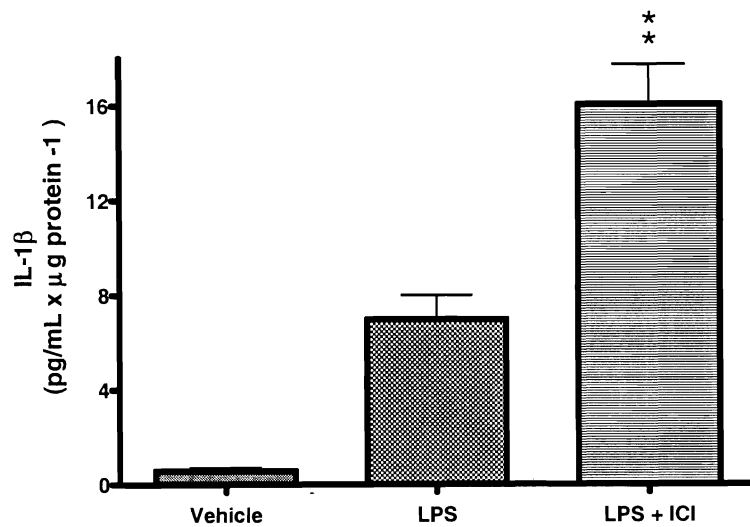


Figure 5: Adrenergic receptor antagonists modulate LPS-stimulated release of IL-1 β in BMM. Murine BMM were treated with LPS and 10 μ M ICI 118,551 (ICI) (β_2 adrenergic receptor antagonist) for 24 hrs. IL-1 β was measured with a BD ELISA Kit. LPS significantly stimulated the production of IL-1 β , while the addition of ICI further increased extracellular levels of IL-1 β . * $P < 0.01$ compared to LPS alone.

Study 2

**Differentiation and Characterization of Human Bone Marrow-Derived
Macrophage-Like Cells**

Introduction

Hematopoiesis is the critical process of bone marrow stem cell development. Undifferentiated cells derived from the bone marrow have the potential to become one of many mature specialized cell components of blood. The process of cell differentiation is mediated through a cell signaling mechanism involving the actions of specific cytokines and growth factors. In the case of macrophages, hematopoietic stem cells respond to macrophage-colony stimulating factor (M-CSF) and granulocyte macrophage-colony stimulating factor (GM-CSF)(8; 17). M-CSF is a survival, growth, differentiation, and activating factor for macrophages and their progenitor cells. In the presence of these cytokines, the cells commit to the myeloid cell lineage. These premature cells known as monocytes are directed into the blood circulation where they continue their development into macrophages under the continued influence of these cytokines. Interleukin-6 (IL-6) is a cytokine produced by several types of cells including fibroblasts and is also important in macrophage development (10).

L929 cells are a mouse fibroblast cell line known for continuous production of M-CSF and IL-6. Studies have shown that macrophages differentiate from mouse femur-derived stem cells when grown in L929 conditioned medium (4). These mouse bone-marrow derived macrophages have been the subject of several ongoing experiments within our laboratory, and we were interested in continuing this research with human

bone marrow-derived macrophages. Although investigators recently discovered a human cell line that produces factors needed for human macrophage differentiation (10), this cell line is not available. Treating human stem cells with L929-conditioned medium did not seem feasible because mouse derived M-CSF and IL-6 are documented as not displaying cross-reactivity with human cytokine receptors (8) Nevertheless, we could find no evidence that mouse L929 medium has ever been tested on human cells. It is known that native cytokine proteins found in conditioned medium may differ in biological activity from the recombinant proteins often used to test for cross reactivity (18). Also, conditioned medium may contain unidentified factors that affect growth and differentiation.

In this study, the effects of mouse L929 fibroblast conditioned medium on development of undifferentiated human bone marrow cells was investigated.

Methods and Materials

Cells

Human bone marrow cells (cat. # ABM001-1) were obtained from Stem Cell Technologies (Berkeley, CA). Cells were washed with complete RPMI (cRPMI) medium consisting of RPMI 1640 (Invitrogen) supplemented with 10% heat inactivated-FBS, 1% L-glutamine, 1% non-essential amino acids, 1% minimal essential medium vitamins, 100 units/ml penicillin and 100 µg/ml streptomycin. The cells were seeded into tissue culture plates in fresh cRPMI with 30% L929-cell conditioned medium (a source of macrophage colony stimulating factor)(4; 18). Cells were incubated at 37 °C in a humidified 5% CO₂ atmosphere for 1 week. Adherent cells were visible within 4 days.

Mouse L929 fibroblasts and RAW264.7 macrophages were maintained as described in Study 1. Human dermal fibroblasts were obtained through Dr. Dorne Yager from the Plastic Surgery Department, Medical College of Virginia at Virginia Commonwealth University. These cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, cat. # 30-2002) from ATCC (Manassas, VA) supplemented with 10% heat inactivated-FBS and 100 µg/ml streptomycin. HEPG2 cells were obtained through Dr. Qibing Zhou, Department of Chemistry at Virginia Commonwealth University, and were maintained by Lin Zhang. All cells were incubated at 37°C in a humidified 5% CO₂ atmosphere.

Identification of Surface Markers with Flow Cytometry

Fibroblasts are a major contaminant in adherent cell cultures. They adhere to the surface of flasks and resemble macrophages in shape and size. To address this concern, the adherent bone marrow-derived cells were assayed with flow cytometry to detect cell specific surface antigens for both macrophages and fibroblasts. Cells were washed and adjusted to 1×10^6 cells/ml cRPMI. In a v-bottom tissue culture-treated plate, 180 μ l of bone marrow cells were added to each well, washed, and blocked with 10 μ l of 2.4G2 rat ascites for 10 minutes at 4°C. Samples included positive and negative control cells. Cells were incubated with antibodies to surface markers for 30 minute at 4 °C, then analyzed by flow cytometry (BD, Facs Calibur) with BD CellQuest Pro software. Macrophage cell surface markers included Mac-1 (identified with 2 μ g/ml (phycoerythrin) PE-anti-Mac-1, eBiosciences) and F4/80 (identified with 2 μ g/ml PE-anti-F4/80, eBiosciences). The fibroblast marker ASO2 (identified with 2 μ g/ml 1° mouse anti-human ASO2 and 10 μ g/ml 2° PE-goat anti-mouse ASO2, eBiosciences) was used to identify any contaminating fibroblasts. For macrophage identification with Mac-1, cells were compared with the mouse macrophage cell line RAW264.7, which were the positive control cells, and with the mouse fibroblast L929 cell line that was used as a negative control. For macrophage identification with F4/80, RAW264.7 cells were positive controls, and human dermal fibroblasts were negative control cells. For fibroblast identification, human dermal fibroblasts and HEPG2 hepatocytes were used as positive and negative controls respectively.

Phagocytosis Assay

Phagocytosis of bacteria was measured with the Vybrant Phagocytosis Kit (Invitrogen V-6694). All cells, including adherent human bone marrow-derived cells, positive controls (RAW264.7) and negative controls (human dermal fibroblasts) were adjusted to 1×10^6 cells/ml in

cRPMI medium, and 150 μ l/well were added to a 96 well plate. Cells were incubated for 1 h to allow for adherence. Following incubation, the medium was replaced with *E. coli* fluorescein (FITC)-labeled BioParticle suspension, and the cells were then incubated for 2 h. The cells were then washed, and any remaining extracellular fluorescence was immediately quenched with trypan blue. Samples were read in a Wallac Victor² multilabel counter (Perkin Elmer) at settings of 480 nm excitation and 520 nm emission.

Cells were also incubated separately with the FITC-labeled BioParticle suspension on microscope slides for 2 h and mounted with Vectasheild mounting media. The cells were viewed with an Opelco IX-70 fluorescent inverted phase contrast microscope (OPELCO, Dullas, VA). Images were saved with the QCapture software (OPELCO) and adjusted for size with Arcsoft Photoimpression software.

Effects of LPS and adrenergic antagonists on TNF- α & IL-1 β

Bone marrow derived cells were scraped gently from tissue culture-treated flasks (Corning brand, Fisher Scientific)(4), and for each treatment, 1.5×10^6 cells were added to each well in a 24 well tissue culture-treated plate (Costar brand, Fisher Scientific) in 0.5 ml serum-free cRPMI. The cells were incubated for 4 h at 37 °C in 5% CO₂ to allow cell adherence. The cells were then washed twice with warm (37 °C) phosphate buffered saline, pH 7.4, treated with 30 ng/ml LPS (*E. coli* –serotype 055:B5, Sigma) and either 10 μ M ICI (β_2 antagonist, ICI-118,551, Tocris) or 5 μ M Yohimbine (α_2 antagonist, Sigma), all in a final volume of 0.5 ml serum-free RPMI. Following a 24 h incubation, extracellular fluid was removed, centrifuged at 500 x g for 10 min at 4 °C and then frozen at -20 °C for future IL-1 β / TNF- α assays. The cell monolayers in the wells were washed with PBS, lysed in 150 μ l lysis buffer (0.05 M Tris (pH 7.5), 0.3 M NaCl, 2 mM EDTA, 0.5% Triton X, 2.0 μ g/ml leupeptin, 1 μ g/ml aprotinin and 0.2

mM phenylmethylsulfonylfluoride), and then frozen at -20 °C for future determination of total cellular protein.

Measurement of IL-1 β , TNF- α , and total cellular protein

Extracellular concentrations of IL-1 β were assayed with the OptEIA Multi Component Elisa Set for Mouse IL-1 β (BD Biosciences, San Diego, CA). The assay was performed in accordance with the manufacturer's instructions. The plate was read at 450 and 570 nm with a μ Quant Universal Microplate Spectrophotometer (Bio-Tek Instruments, Winooski, VT), and concentrations were calculated with the manufacturer's KC4 software. Cell lysates were assayed for total protein with the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA) and read at 595 nm in the microplate reader.

Results

Identification of Cells

Identifying surface antigens that are specific for macrophages was an important initial step in characterizing the human bone marrow-derived cells. The first of these surface markers identified was MAC-1. When analyzed with flow cytometry, the MAC-1 surface antigen was identified on all of the cells, including the negative control cells (L929 fibroblasts), indicating that it was not a specific indicator of macrophages (Figure 1). The F4/80 surface antigen showed a higher degree of specificity for macrophages (Figure 2) (14). Compared with the positive and negative controls, human bone marrow-derived cells exhibited a high fluorescence intensity for F4/80, indicating that these cells were similar to mature macrophages. When the cells were examined for ASO2, a fibroblast specific surface marker, the bone marrow-derived cells exhibited low fluorescence intensity equaling the nonspecific fluorescence (Figure 3). Human dermal fibroblasts (positive control) exhibited a high fluorescence intensity for ASO2 as expected. This result suggests that the bone marrow-derived cells are not fibroblasts.

Phagocytosis

The phagocytosis assay revealed that the human bone marrow-derived cells are phagocytic (Figure 4). They cells engulfed the bacteria with a little over half the efficiency of the RAW macrophage cell line (positive control). Human dermal fibroblasts did not show any phagocytic activity. This finding indicates that the cells not only express macrophage specific surface antigens but also are phagocytic.

RAW macrophages that were incubated on slides with the fluorescent bioparticle suspension (FITC-Bacteria) were viewed with fluorescence microscopy (Figure 5). Cells not receiving the suspension were examined for background fluorescence. The RAW macrophages did not show any significant background fluorescence, and phagocytosis of particles was evident in cells treated with the FITC-Bacteria. Human bone marrow derived cells exhibited phagocytic activity comparable to that exhibited with the RAW macrophage cell line and exhibited no background fluorescence in the absence of the fluorescent bioparticle incubation (Figure 6). A bright-field view of the slide without the fluorescent incubation confirmed the presence of the cells when no fluorescence was evident.

Cytokine Production

The human bone marrow-derived cells did not produce cytokines in a manner typical of macrophages. Low concentrations of TNF- α were produced continuously but did not increase in response to LPS stimulation. The constitutive release of TNF- α did not appear to be altered by LPS in either a time or dose dependent pattern (Figure 7). Furthermore, the cells produced only low concentrations of IL-1 β (Figure 8). LPS and adrenergic antagonists known to modulate IL-1 β release (7; 22) also had no significant effect on extracellular levels of this cytokine.

Discussion

The human bone marrow-derived cells investigated in this study can be described as macrophage-like cells, as they have been found to express macrophage specific surface antigens and exhibit phagocytic activity. The cells do not, however, produce cytokines in response to LPS as would be expected of macrophages. This new cell line proliferates in culture in cRPMI (without L929-conditioned medium) and reaches confluency within several days of seeding. Further studies are needed to investigate the mechanisms regulating cytokine production in this new cell line.

Newly differentiated monocytes require specific signals for continued development to macrophages. It is unclear whether L929-conditioned medium provided the signals for the human bone marrow cells to differentiate into macrophage-like cells or whether the cells were transformed *in vivo*. Cytokines such as M-CSF and IL-6 have been identified for their key roles in macrophage differentiation. For example, IL-6 is reported to up-regulate M-CSF receptors on the surface of macrophages (10). L929 fibroblasts produce mouse IL-6 and M-CSF, but it is unclear whether these mouse cytokines bound to receptors on the human cells. Also, it is possible that other unidentified growth factors needed for normal mouse bone marrow cell differentiation and development may not have cross reacted with receptors on human cells. Because we were not able to repeat this study with human bone marrow cells incubated in L929-conditioned medium, it is conceivable that the cells in this study were transformed to a continuously proliferating cell line *in vivo*. The cells are easily maintained in culture and will be used for future studies of macrophage-like functions.

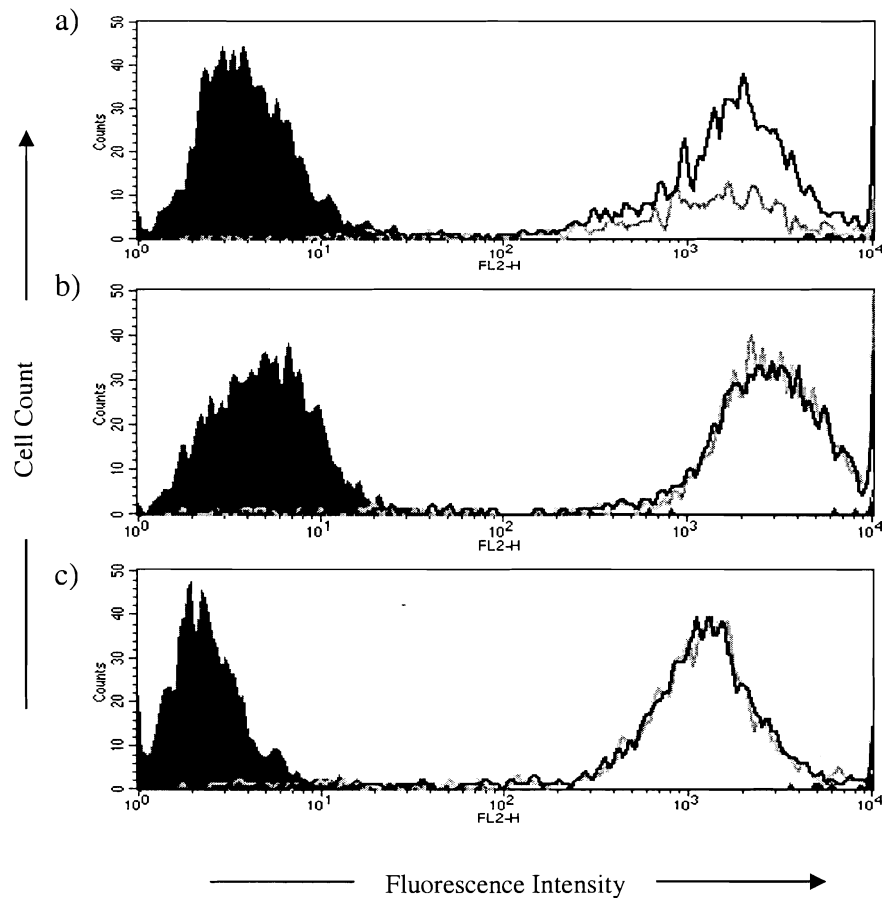


Figure 1: MAC-1 staining for macrophages. Shaded areas indicate background non-specific staining. Lines represent duplicate samples. (a) RAW 264.7 macrophages stained for MAC-1 (positive control). (b) Mouse L929 fibroblasts, a negative control, also showed high fluorescence for MAC-1 indicating that MAC-1 is not a macrophage-specific marker. (c) Human bone marrow-derived cells also stained for MAC-1.

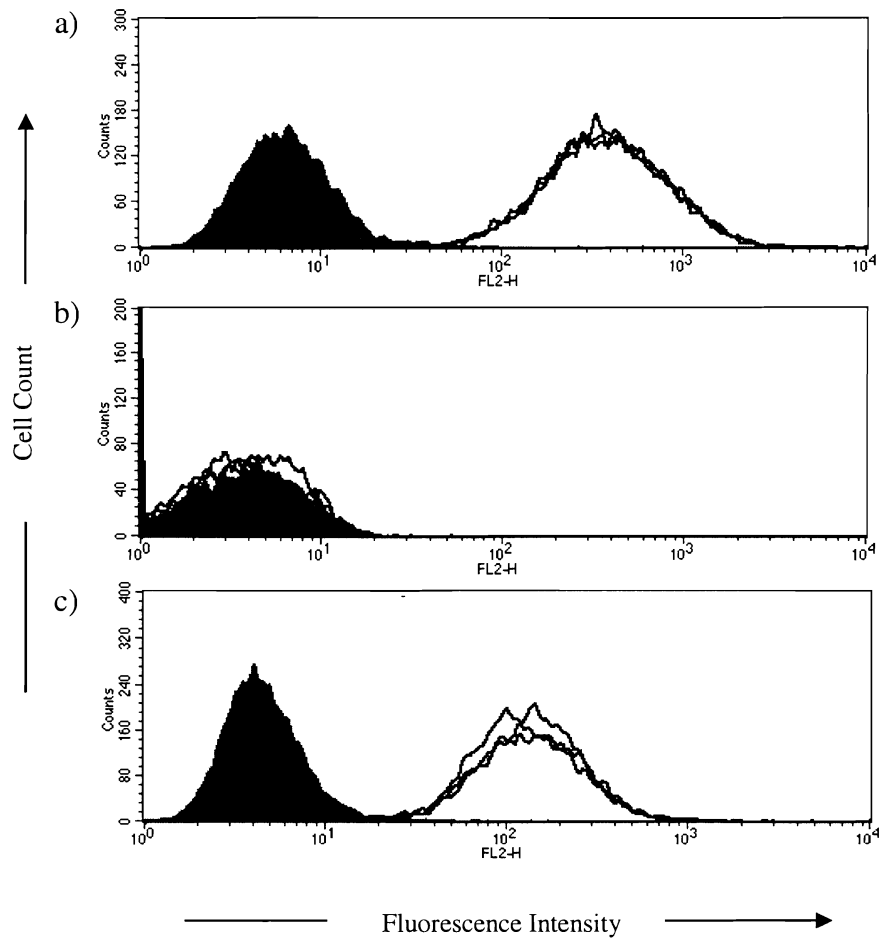


Figure 2: F4/80 (mature macrophage marker) staining. Shaded areas indicate background non-specific staining. Lines represent triplicate samples. (a) The RAW 264.7 macrophage cell line was stained as a positive control for this marker. (b) Human dermal fibroblasts showed no specific staining. (c) Human bone marrow-derived cells were positive for F4/80.

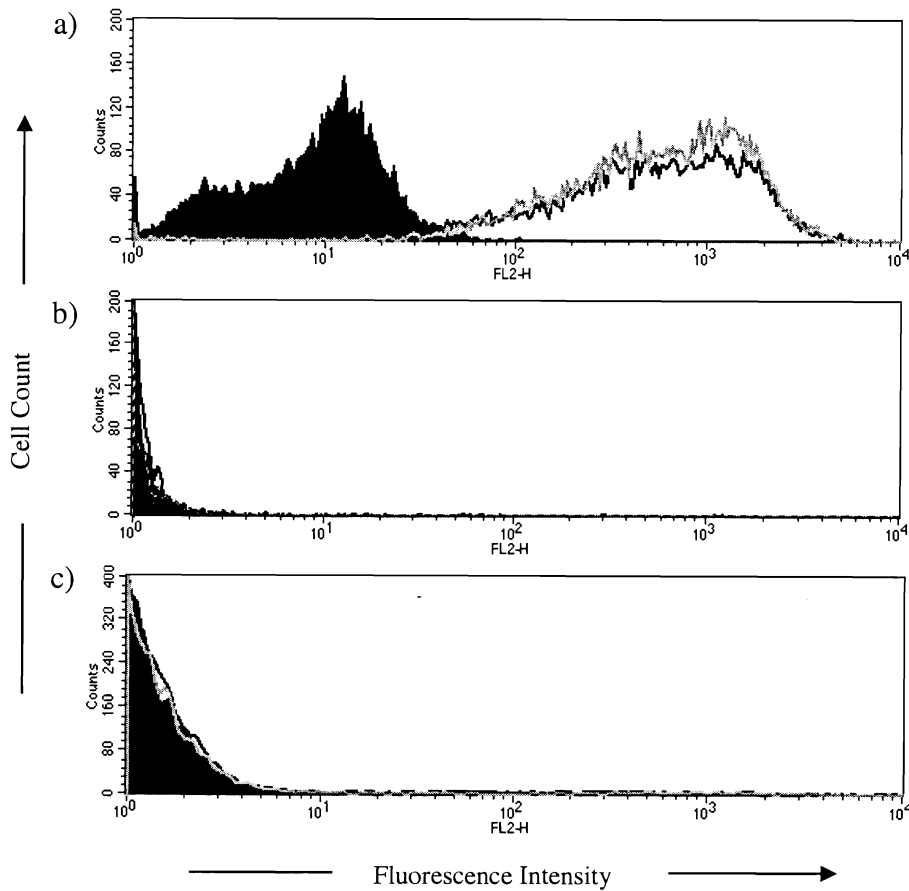


Figure 3: ASO2 (fibroblast-specific surface marker) staining. Shaded areas indicate background non-specific staining. Lines represent triplicate samples. (a) Human dermal fibroblasts, positive control cells, stained for ASO2. (b) A hepatocyte carcinoma cell line HEP-G2, negative control cells, showed no staining for the fibroblast-specific protein. (c) The human bone marrow-derived cells showed no staining for ASO2.

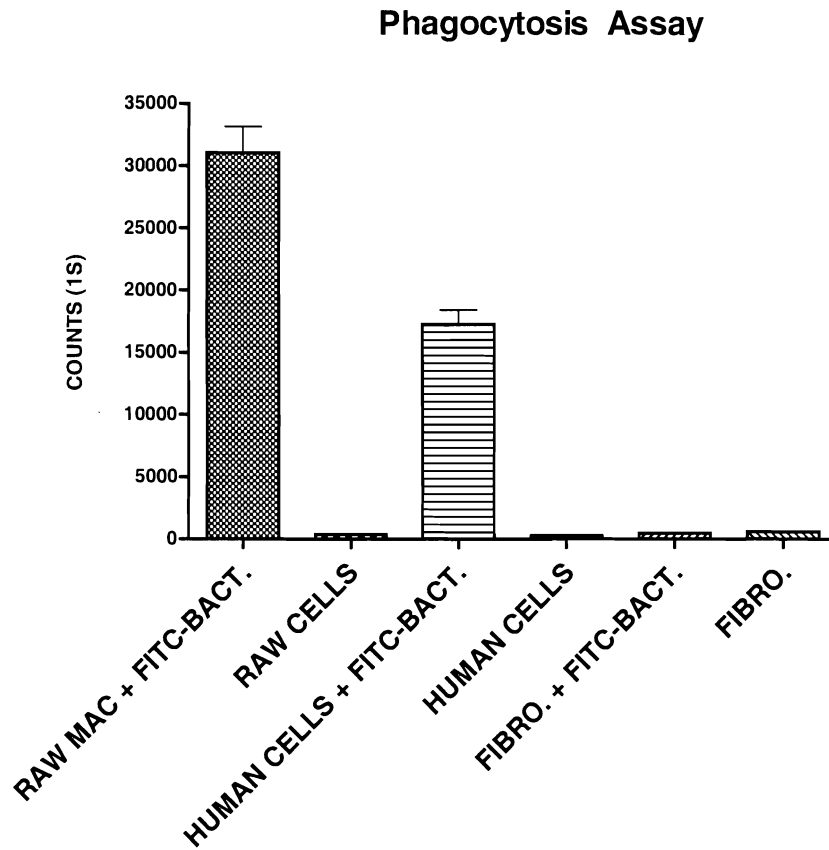
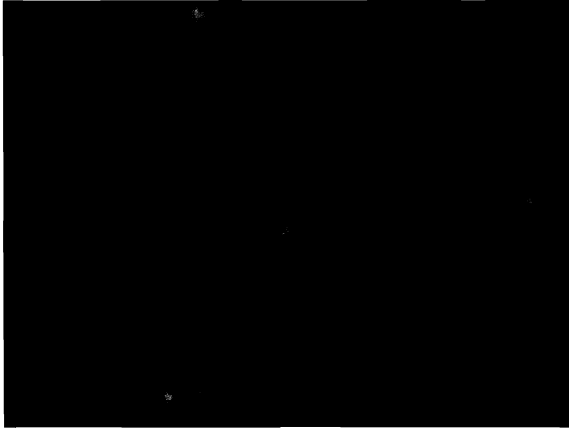


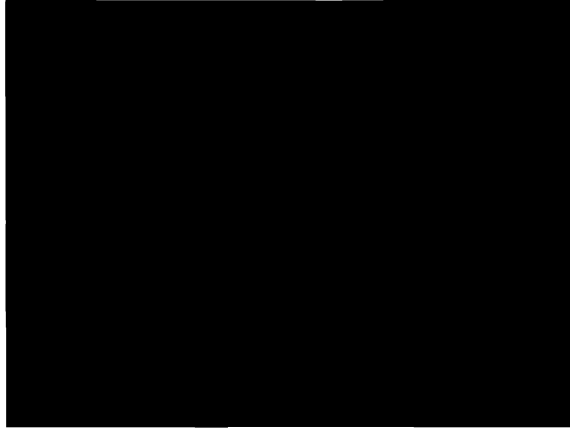
Figure 4: Phagocytic activity of human bone marrow-derived cells and control cells. Phagocytic cells (RAW), non-phagocytic (human dermal fibroblasts) (FIBRO), and human bone-derived cells were assayed in the absence of bacteria to account for background fluorescence or incubated with fluorescein-labeled bacteria (FITC-BACT).

a)



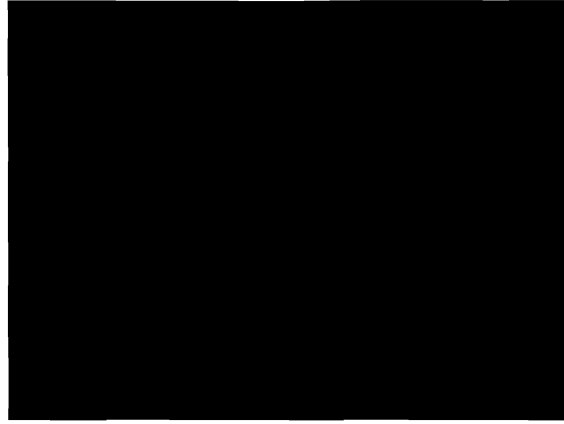
RAW macrophages
treated w/ FITC-Bact.

b)



Raw macrophages w/out
FITC-Bact. treatment

Figure 5: RAW 264.7 macrophages incubated with the fluorescent bioparticle suspension. (a) High fluorescence of intracellular bacteria was observed with an inverted phase contrast microscope. (b) Cells that were not incubated with fluorescent bacteria showed minimal to no fluorescence, indicating that endogenous macrophage fluorescence is negligible. Magnification = 400X



HUMAN CELLS

Top Left: Human Cells Treated w/ FITC-Bact.

Top Right: Human Cells w/out FITC-Bact.

Bottom: Bright field view of human cells w/out FITC-Bact.



Figure 6: Cells treated with the fluorescent bacteria revealed a high level of phagocytosis activity (top left). Cells that were incubated without the bacteria showed only minimal background fluorescence as expected (top right). A bright field view (bottom right) of the cells (top right) confirmed the presence of the bone marrow-derived cells on the slide. Magnification = 400X

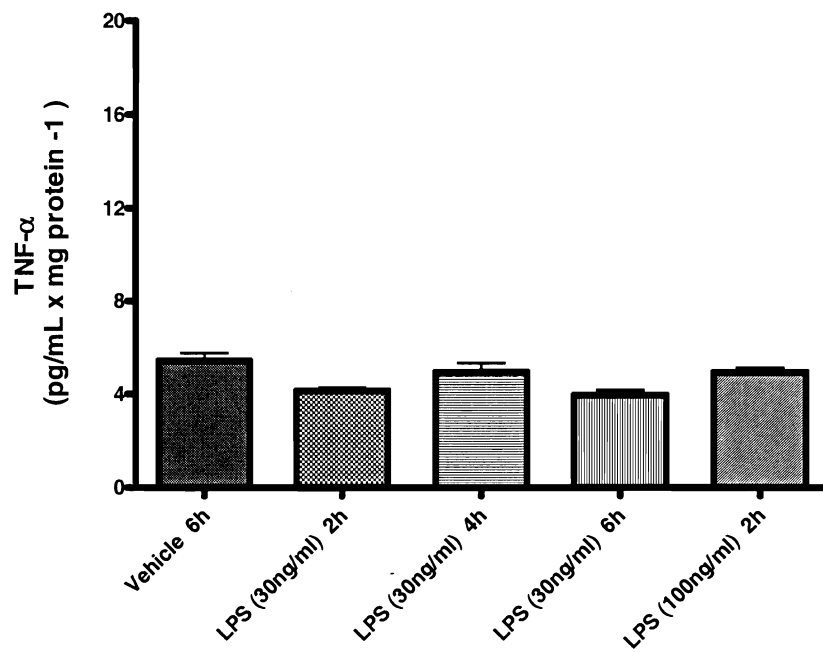


Figure 7: TNF- α production by the human macrophage-like cells following a 2,4, and 6 hour incubation with 30 ng/ml LPS. Additionally, cells were treated for a 2 h incubation with 100 ng/ml LPS.

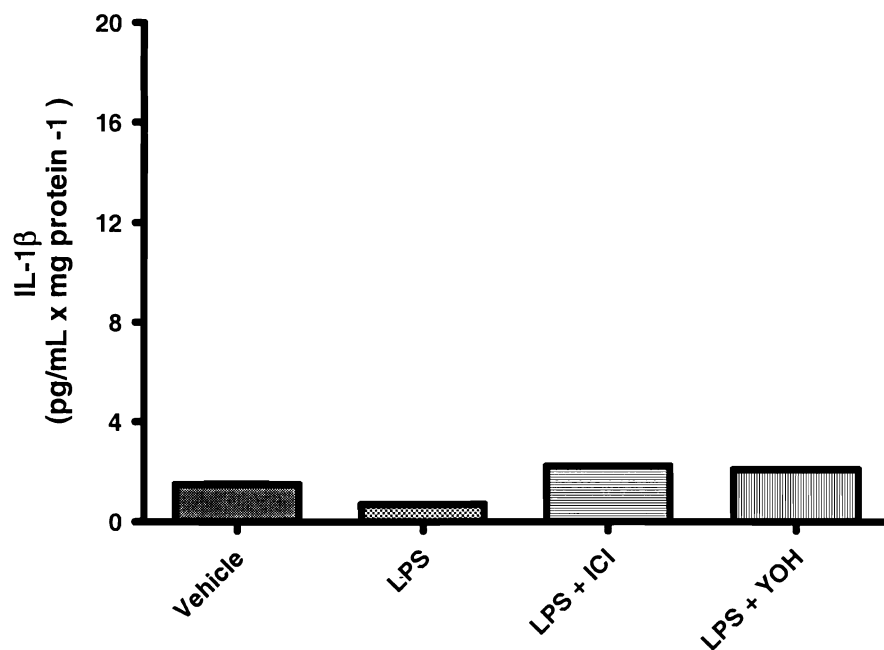


Figure 8: IL1- β production by the human macrophage-like cells assayed following a 24 h incubation with LPS, LPS+ICI-118,551 (ICI), or LPS+Yohimbine (YOH). These bone marrow-derived cells did not respond to LPS stimulation and produced little IL-1 β .

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Vita

George Tharwat Georges was born on November 4, 1981 in Egypt. He moved to the United States of America at the age of three. He grew up in Hampton, Virginia where he attended Phoebus High School and began developing a love for science. George continued his education at Virginia Commonwealth University where he majored in Biology and earned his Bachelor of Science degree. Having been involved with undergraduate research in genetics and molecular biology, he enrolled into the graduate program in biology at Virginia Commonwealth University where he has continued to develop his research skills pursuing a thesis in neuroimmunology and earned his Master of Science degree. George foresees a future in education and the medical sciences.