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Differentiation of U-937 Monocytes to Macrophage-Like Cells Polarized into M1 or M2 Phenotypes According to Their Specific Environment: A Study of Morphology, Cell Viability, and Cd Markers of an In Vitro Model of Human Macrophages

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DIFFERENTIATION OF U-937 MONOCYTES TO MACROPHAGE-LIKE CELLS

POLARIZED INTO M1 OR M2 PHENOTYPES ACCORDING TO THEIR SPECIFIC

ENVIRONMENT: A STUDY OF MORPHOLOGY, CELL VIABILITY, AND CD

MARKERS OF AN IN VITRO MODEL OF HUMAN MACROPHAGES

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I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY <u>Fatma Abdulhadi </u>ENTITLED <u>Differentiation of U-937 Monocytes to Macrophage- like Cells Polarized to M1 and M2 Phenotypes According to Their Specific Environment: A Study of Morphology, Cell Viability, and CD Markers of An In Vitro Model of Human Macrophages </u>BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF <u>Master of Science</u>.

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ABSTRACT

Abdulhadi, Fatma. M.S. Microbiology and Immunology Graduate Program, Wright State University, 2014. Differentiation of U-937 Monocytes to Macrophage- like Cells Polarized to M1or M2 Phenotypes According to Their Specific Environment: A Study of Morphology, Viability, and CD Markers of An In Vitro Model of Human Macrophages.

In this study, the human leukemic monocyte lymphoma cell line U-937 was used as an in vitro model for monitoring monocyte/macrophage differentiation. Phorbol 12-myristate (13) (PMA) was used to activate U-937 cells into macrophage-like cells (M0). After 24 hours of PMA treatment, non- adherent U-937 cells became tightly adherent to the culture plates forming M0 cells. M0 cells were then polarized into the M1 macrophage phenotype by treatment with LPS and IFN-γ for another 24 hours. Each of the cytokines IL-4, IL-13, or IL-10 was applied separately to three M0 cultures for 24 hours to induce the M2 macrophage phenotype. M1 and M2 phenotypes displayed distinct morphological characteristics. M1 cells appeared large, with cellular processes (pseudopodia), and intracellular vacuoles while the M2 cells large aggregated into large masses. The undifferentiated U--937 cells expressed less CD206 and CD86 but greater amounts of CD163, CD80, and CD200R than did the differentiated U937 cells (M0 macrophages). These observations suggest that the differentiated M0 cells would be better at antigen presentation since they expressed a 6-fold increase the CD86 costimulatory molecule and half the amount of the CD80 costimulatory molecule as did undifferentiated U937 cells. M1 polarized macrophages expressed lesser amounts of CD14, CD86, CD80, CD163, CD206, and CD200R than M0 cells which may reflect the production of toxic substances such as reactive oxygen molecules, nitric oxide and TNF-α. Both the differentiation and polarization processes caused decreases in cell viability no difference seen between M0 and M1 populations at the 24 hour observation time.

Marked differences in expression of these CD markers were obvious in the M2 subpopulations with the IL-4-polarized M2 cell showing marked elevations in expression of CD206 and CD86 and the IL-13-polarized M2 cells showing marked increase in expression of CD14. These differences highlight the plasticity of the macrophage in different microenvironments and may be useful in interpreting experimental results in other systems.

Table of Contents

List of Figures

List of Tables

List of Abbreviations

BSA = Bovine serum albumin

CD14 = Cluster of differentiation 14

CD86 = Cluster of differentiation 86

CD206 = Cluster of differentiation 206

CD163= Cluster of differentiation 163

CD200R= Cluster of differentiation 200R

CD80= Cluster of differentiation80

IL-4 = Interleukin-4

IL- IL-10 = Interleukin-10

M2 (IL-4)= M2 phenotype induced by interleukine-4

M2 (IL-10)= M2 phenotype induced by interleukine-10

M2 (IL-13)= M2 phenotype induced by interleukin 13

FCS = Fetal calf serum

IFN-γ = Interferon-gamma

IL-13 = Interleukin-13

iNOS = inducible nitric oxide synthase

LPS = Lipopolysaccharide

PBS = Phosphate buffered saline

TNF-α = Tumor necrosis factor-alpha

TCGF =T cell growth factor

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Dedication

I would like to dedicate my thesis project to my family,

For unconditioned love, patient, and support

Introduction

Macrophages are distributed in tissues throughout the body; these resident macrophages can act as "professional" phagocytic cells. They express numerous cell receptors that enable them to detect any abnormal signal within the host. They engulf and clear foreign materials, apoptotic cells and debris and also help in recruitment of additional macrophages to the site of the inflammation. Macrophages play an important role in antimicrobial defense, anti cancer immune responses, and contribute to other pathologies: metabolism and obesity, bronchial asthma and allergy, tumorigenesis, autoimmune diseases, atherosclerosis, wound healing and fibrosis. Local microenvironmental signals influence the heterogeneity and function of macrophage [1]. Macrophages are classified into two types: classically activated macrophages (M1) cells and alternatively activated macrophages (M2). The M2 category includes other tissue macrophages. The process of specialization into M1 and M2 is called macrophage polarization. M1 and M2 cells possess opposite functions. M1macrophages are induced by interferon-gamma (IFN-γ) and lipopolysaccharide (LPS) and produce pro-inflammatory cytokines such as inducible nitric oxide synthases (iNOS) and tumor necrosis factor-α (TNF-α). M2 macrophages, anti-inflammatory cells, are stimulated by interleukin-4 (IL-4) or (IL-13) or interleukin-10 (IL-10) to secrete anti-inflammatory cytokines such as interleukin-10 (IL-10), which contribute in wound healing and angiogenesis [2] [3].

Two human macrophage cell lines (2 MAC and K1m) have been established from the peripheral blood of healthy persons and both necessitate culture in a conditioned media containing T cell growth factor (TCGF). The propagation of these cell lines requires contact with an irradiated peripheral blood leukocyte-feeder layer. Depending on the source of the blood leucocyte, K1m cells exhibited variation in propagation that have not been explained [4] [5]. The difficulties in isolation of primary macrophages either by blood donation or using invasive procedures such as bronchoscopy or tissue biopsy limits the use of primary macrophages in the laboratory very limited. The main obstacle still is to obtain adequate cell number for experimentation. Since self- replicating macrophage cell lines have not been established yet, researchers tend to use monocytic cell lines such as U-937 and TH-P as a macrophage- like cell lines. Using macrophage-like cell lines is more convenient in terms of obtaining large cell numbers [6].

In this study, human leukemic monocyte lymphoma cell line U-937 cells were used as an in vitro model for monitoring monocyte/macrophage differentiation. The aim of this project was to study the expression of cell surface markers protein of U-937 cells after treating them with polarizing agents. Results from this study may offer a prediction of the behavior of differentiated tissue macrophages within the body. Phorbol 12-myristate (13) (PMA) is used to activate U-937 cells and change them into adherent macrophages. After 24 hours of treatment with PMA, both LPS and IFN-γ were used to induce polarization to the M1 phenotype, while treatment with IL-4, IL-13, orIL-10 was used to polarize cells to the M2 phenotype. Morphological features, cell viability, and cluster differentiation markers: CD14, CD86, CD80, CD206, CD163, and CD200R have been identified using immunofluorescent staining and flow cytometry.

Hypothesis: U-937 monocytes can be differentiated into macrophages that, according to their specific microenvironments, polarize into M1 or M2 phenotypes, each of which demonstrates differences in morphology, viability, and expression of CD markers.

Aims of the current Study:

- ❖ Differentiate U-937 monocytes into macrophages (M0) using phorbol 12-myristate (13) (PMA);

- ❖ Polarize M0 into M1 and M2 phenotypes using LPS, IFN-γ for M1 and each of IL-4, IL10, or IL-13 for M2;

- ❖ Examine the effect of polarization on morphology, cell viability, and expression levels of CD14, CD86, CD206, CD163, CD200R, and CD80 of the polarized cells and compare them to the control.

Literature Review

Macrophage Origin and function

Monocytes originate in bone marrow where common myeloid progenitor cells give rise to monocytes and neutrophils. From the bone marrow, monocytes are released into the blood stream, travel in the peripheral blood for several days and eventually replenish tissue macrophages. Circulating monocytes end up either as resident macrophages that occupy different tissues or specialized dendritic cells and osteoclasts. In 1939, Ebert and Flog reported their observation regarding extravascular migration of the monocytes that become tissue macrophages. Multiple stimuli such as immune, metabolic or pro-inflammatory factors help monocyte recruitment from blood into the tissues to become tissue macrophages. These macrophages contribute to host defense, tissue homeostasis, repair and modeling of the tissues and clearance of apoptotic cells [7].

Macrophages are considered one of the important phagocytic cells, they carry a diversity of surface receptors to recognize a variety of abnormal signals. All the subpopulations of the macrophages in the body respond to environmental signals. Thus, they act in either a pro-inflammatory or anti-inflammatory manner. Upon treatment with IFN-γ and LPS, macrophages respond by producing pro-inflammatory molecules such as iNOS and TNF-α, these are called M1or classical activated macrophages. After treatments with each IL-4 or IL-13, the macrophages respond by releasing anti-inflammatory molecules, which are important in tissue remodeling and angiogenesis.

These anti-inflammatory macrophages are called M2 or alternative activated macrophage subpopulation [2] [3]. In addition, each of the TGF-ß receptors, IL-10 receptors, or FC gamma receptors in the presence of LPS, lead to multiple M2 like subpopulations [8] [9] . Clinically, The equilibrium between pro-inflammatory and anti-inflammatory macrophage populations is vital since each macrophage subtypes can exacerbate certain diseases [10].

Macrophages bridge immune systems

Classically activated macrophages (M1) are effector cells that exert broad-spectrum microbicidal capacity appear during cell mediated immune responses (adaptive immunity). However, innate immune responses also produce IFN-γ, which can prime macrophages to produce inflammatory cytokines, oxygen and nitrogen radicals, to increase their microbicidal and tumerocidal capacity. Although macrophages play a fundamental role in the innate immune system during inflammation, innate and adaptive immune systems are able to control macrophage activation to be aggressive to a variety of pathogens and to produce immunoregulatory cytokines. Classically activated macrophages induce T helper 17 (Th17) to secrete IL-17, which is an important cytokine to recruit polymorphonuclear leucocyte and contribute to inflammatory autoimmune diseases. Early after trauma or infection, endogenous stimuli (innate responses) are generated. These rapid stimuli usually have transient effects on the macrophages physiology. For instance, innate immunity can temporarily induce the microbicidal capacity, while adaptive immune immunity can sustain the environment for classically activated macrophages to kill intracellular pathogens. Also, antigen specific immune cell (adaptive responses signals) produce radical changes on macrophages that can persist [7].

M1 Macrophage polarization with IFN-γ and LPS

IFN-γ, TNF, and toll like receptor ligands such as (**LPS**) stimulate macrophage populations that have antimicrobial and anti cancer ability by producing pro-inflammatory cytokines and mediators. Natural killer cells (NK) are important early innate immune cells that initiate production of IFN-γ. T helper-1 (Th-1), antigen specific cells, secretes a constant amount of IFN-γ. IFN-γ knock out mice are more prone to viral, bacterial, and protozoal infections. Also, people with mutations in IFN-γ signaling pathways suffer from a variety of microbial infections. However, stimulation with IFN-γ alone without TNF makes M1 cells less able to clear Leishmania infection. Leishmania spp, an intracellular organism, lacks TLR ligands, which is important for release of TNF. Exogenous supply with TNF or TLR ligand such as **LPS** eliminate the parasite. These findings proved the importance of TNF or TLR ligands (LPS**)** in M1 development. This cross talk between the host and the pathogen makes classically activated macrophages efficiently able to kill intracellular organisms [9].

Stimulation of M1 macrophages starts by binding IFN-γ to its receptor and activation of Jak/Stat pathways. Stat, which is a signal transducer, activates nuclear factor kappa B (NF-κB). NF-κB, an ubiquitous transcription factor, controls the expression of many inflammatory and immune genes. Mitogen activated protein kinases (MAPKs), signaling pathways, are activated following TLR (LPS) or TNF receptor activation. MAPKs lead eventually to cellular proliferation, differentiation, and activation of the macrophages. Some intracellular organisms such as Leishmania spp and Mycobacterium tuberculosis prevent IFN-γ production by interfering with these signaling pathways [9].

M2 polarization with IL-4, Il-13, and IL-10

Alternatively activated macrophages are induced by innate and adaptive immune responses. Granulocytes may respond and produce cytokine IL-4; however, basophils and mast cells are the main early innate cells that secrete IL-4. These cells can produce IL-4 in response to chitin present in some fungi and parasites. The role of IL-4 is to prime the resident macrophages to develop into programmed cells inducing wound healing. IL-4 activates the arginase enzyme, which is responsible for converting arginine into ornithine (amino acids), precursor of collagen and polyamines. Thus, IL-4 enhances production of extracellular matrix, which is important in tissue repair and also cancers [9].

Adaptive immune responses also contribute to IL-4 production. T helper 2 cells (TH2) are the first adaptive response cells to be induced, especially in the mucosal surface of the lungs and intestine. TH2 cells secrete IL-4 and IL-13. In-vitro treatments of macrophages with IL-4 and or IL-13 lead to reduction of pro-inflammatory cytokines, oxygen, and nitrogen radicals. Additionally, these cells fail to present antigens to T cells and cannot kill intracellular parasites. Alternative activated macrophages can be dis-regulated and cause tissue fibrosis as in chronic Schistosomiasis. Interestingly, macrophages that lack IL-4 receptors failed to cause this pathology, and treatment with IL-4 antibody decreases the fibrosis. M2 cells have been found in the lungs of mice with experimentally induced asthma, reflects the important function of M2 cells in airway remodeling activity, which is similar to the role of these cells in tissue repair and wound healing [9].

IL-10 is considered a crucial anti-inflammatory cytokine and inhibits production of IL-1 and TNF. IL-1 and TNF have synergistic actions on inflammatory pathways and

processes by amplifying the signals through induction of secondary mediators such as chemokines, prostaglandins, and PAF. IL-10 effectively prevent production of IL-1α, IL-1β, IL-6, IL-10 itself, IL-12, IL-18, GM-CSF, G-CSF, M-CSF, TNF, LIF and PAF by activated monocytes/macrophages (10, 11, 154–156). IL10 exerts an important role in regulating the conversion of macrophages into CD163^{+} M2 phenotype in regenerative muscle [10,11, 12].

Figure 1. Polarization of macrophages into M1 and M2 phenotypes via classical activation pathway and alternative pathway respectively. (a) M1-polarization by LPS and IFN-γ, which secreted by TH1 and NK cells. M1 produces inflammatory cytokines such as IL-12, I L-23 in high amount, while secrets IL-10 in low amount. (b) TH2 cells, basophils and innate lymphoid cells secrete IL-4, IL-13 or IL-33 in to polarize macrophages into M2 phenotype. M2 phenotype secretes high amount of IL-10 (anti-inflammatory cytokine) while secrets other inflammatory cytokines in low amount (adapted from S. K. Biswas and A. Mantovani, 2010).

MACROPHAGE PLASTICITY

One of the major obstacles that researchers face in studying macrophage polarization is macrophage plasticity. Macrophages have a difficult time in maintaining a discrete phenotype because they can switch phenotype depending on the type, concentration, and longevity of the stimulating factors present in the microenvironment. M2 cells can express M1 genes products if they are stimulated by IFN-γ. The plasticity of the macrophage genes explains the plasticity of the macrophage response to different stimuli such as cytokines, chemotactic factors, microorganisms, cell debris and dead cells, and interaction with different types of T cells. This scenario is completely opposite to that of other cells such as lymphocytes, which can display only a fixed phenotype after polarization because chromatin modification is fixed after exposure to the polarizing cytokines. Therefore, in response to microenvironment, macrophages and their monocytic precursors can alter their function and exhibit a marked heterogeneity. Currently, the distinction of the expression pattern of surface markers, which are absolute to macrophage subsets, is still in doubt, especially in human macrophages [9].

U-937 and PMA

U-937 cell line was obtained from a 37-year- old man with hystocytic lymphoma. These cells are non-adherent and autonomously proliferating cells oval in shape with minimum to no variation in morphology and large irregular nuclei [13]. Many researches used U-937 cells as an in vitro model to study tumor cell behavior in hematopoietic patients [14]. These cells have monoblastic characteristics, which mean that they can be differentiated into macrophage-like cells by using several inducers. Phorbol-12-myristate-13 acetate (PMA) exerts pleotropic effects on U-937 and can convert them into macrophage- like

9

cells. Upon addition of the PMA reagent, U-937 transforms from non-adherent to tightly adherent cells through up regulation of the adhesion molecules [15]. PMA alters the gene expression via activation of protein kinase c (PKC), which leads to maturation U-937 into macrophage like morphology [16]. Several transcriptional factors that bind to Cis element are also influenced by PMA such as NFkB, Ap1, and Ap2. The differentiation process also alters the B2 integrin family such as CD11a, CD11b, CD11c, and CD18, which are adhesion receptors [17].

Cluster of Differentiation (CD markers).

Cluster of differentiation (CD) markers are used to differentiate between M1 and M2 cells. The first CD marker is CD14, present at the cell surface; CD14 binds to LPS and CD11/18. These interactions lead to activation of toll like receptor 4 (TLR4), which initiates the signal transduction cascade(s), and ultimately activates the transcription factors [18]. CD86 (B7-2) is a co-stimulatory molecule at the cell membrane of the antigen presenting cells. CD86 co-stimulates the naive T cells, which become activated T cells [19]. The third CD marker is CD206, a mannose receptor, which is an integral protein that is expressed in the cell membrane of the macrophages and other cells. Following binding of mannose-rich glycoconjugates or pathogens on the surface of the macrophages, this receptor undergoes endocytosis and phagocytosis of the bound ligands . The fourth is CD80, another co-stimulatory molecule, which activates T cells. In-fact, CD86 and CD80 belong to single-family, B7 co-stimulatory molecules, where B7-1 (CD80) and B7-2 (CD86) interact with CD28 and CTLA of T cells. The interaction of B7:CD28 is crucial to activate T cells, and the absence of this interaction leads to T cell anergy [20]. The fifth CD marker is CD200R, which is a novel inhibitory receptor that

regulates the threshold activation in myeloid cells such as macrophages and mast cells [21]. CD80 was found to be a specific marker to human M1 polarized macrophages and CD 200R was a specific marker to polarized human M2 [22]. Hemoglobin Scavenger receptor CD163 is widely expressed in lymphoid tissue macrophages such as spleen, bone marrow, and liver; however, it also can be expressed in inflammation sites. The main role of this receptor is metabolizing hemoglobin and transforming it from pro-inflammatory to anti-inflammatory metabolites, which are carbon monoxide and bilirubin. It has endocytic activity to eliminate the hemoglobin [23]. Thus, this receptor is considered one of the anti-inflammatory receptors.

Materials and Methods

Cell culture

U-937 are monocytic pleural effusion cells isolated from a 37 year-old male with histiocytic lymphoma (ATCCD CRL-2593.2). The cells were cultured in 44.5 ml RPMI supplemented with 2mM(.0146grams) glutamine, one mM (0.5ml) sodium pyruvate, and 10% (5 ml) fetal calf serum (FCS) (Fisher Scientific). Cells were grown in $25cm^2$ vented culture flasks untill a concentration of $3x10^6$ cells/ ml. Incubation conditions were set at 37°C and 5% CO_2. Cells were collected into 15 ml centrifuge tubes and centrifuged at 2500 RPM and 4 degree Celsius for 5 minutes (Sorvall RT7, Wright State University). Supernatant was withdrawn and cells were suspended in one ml 10% RPMI. Cell concentrations of $1x10^6$ were added to 60mm culture dishes for differentiation.

Differentiation

Monocytes were induced to differentiate via phorbol 12- myristate13-acetate (PMA) 20ng/ml [15]. After the PMA addition, cells were incubated at 37°C and 5% CO_2

for 24 hours. Differentiation was identified by tight adherence of the cells to the bottom of the dish. Cell stripper was used to collect the cells for experiments.

Polarization

Culture plates were treated with 100ng/ml LPS and 20ng/ml IFN-γ to induce the M1 phenotype and 20ng/ml IL-4, 20ng/ ml IL-13, or 50ng/ml IL-10 to induce the M2 phenotype (Kigler et al., 2009). Each cytokine used to induce M2 was used individually. Control cells consisted of U-937 cells without treatment and U-937 cells treated with PMA (M0) are used over the same time frame. At 24 hours plates were analyzed for morphological differences and for cluster differentiation marker expression by flow cytometry and immunohistochemistry.

Cell Viability

U-937macrophages were grown to approximately 50% confluency, and then PMA was added to change them into macrophages (M0) and make them adhere to the bottom of the dish. IFN-γ/LPS was used to obtain the (M1) phenotype and each of IL-4, IL-10, and IL-13 was used to obtain (M2) phenotype. Untreated U-937 cells were used as a control. After 24 hours, cells were removed from culture dishes using a cell scraper and cell stripper. Cells were then centrifuged at 1500 revolutions per minute (4˚C) for 5 minutes. The supernatant was aspirated following centrifugation and the cell pellet was re-suspended in 1 mL of 10% RPMI. Trypan blue (Fisher Scientific) was used to determine viability, and a hemocytometer was used to obtain cell counts.

Immunofluorescence staining

 The U-937 cells were differentiated on glass coverslips added to 60mm culture dishes. After 24 hours of polarization into M1 or M2 phenotypes, supernatant was withdrawn

and the coverslip was rinsed briefly with 1% BSA. Next the cells were fixed with 5ml 4%

paraformaldehyde for 15 minutes at room temperature. After fixation, cells were rinsed

three times with 1 % BSA in PBS three minutes each and then permeabilized by using

ice-cold acetone (5ml for 10 minutes at -20℃). Then cells were rinsed three times with

1% BSA, suspended in PBS for three minutes each. 5ml of blocking puffer (3% BSA,

suspended in PBS and 100 ul 0,2% Triton-X-100) was added to the cells for one hour at

room temperature. Cells were rinsed with 1% BSA, suspended in PBS three times for

three minutes each. Primary antibodies (diluted in the blocking buffer 3% BSA) were

added to the coverslip in a dark room at room temperature. CD-86 antibodies conjugated

to PE and CD-14 conjugated to FTC, and CD-206 conjugated to APC, CD80 antibodies

conjugated to FTC, CD163 antibody conjugated to PE, CD200R conjugated to PE were

used to show cluster differentiation of the polarized macrophages. The cells were kept in

dark containers overnight in a refrigerator at 4°C. The following day, the cells were

rinsed with 1% BSA, suspended in PBS three times for three minutes each, and a hard set

mounting medium (VectaShield from Vector Laboratories) was used to mount the

coverslips on the microscope slides. All the antibodies were purchased from Biolegend.

Flow Cytometry

Cells were grown and polarized into M1 and M2 phenotypes. The cells were

grown in 200mm petri dishes in order to obtain enough polarized adherent cells for flow

cytometry. Untreated U-937 cells and U-937 treated with just PMA (M0) were used as

controls. After 24 hours following the treatment, the media were aspirated and the cells

harvested using cell stripper. Upon collection, the cells were centrifuged at 1500

revolutions per minute at 4 degree Celsius for five minutes (Sorvall RT7, Wright State University). The supernatant fluid was aspirated, and the pellet was mixed with 1 ml 10% RPMI and placed in ice. A heamocytomter was used to count the cells manually; one million cells were taken from the suspension of each sample. The cells were rinsed four times with 1% BSA, suspended in PBS and centrifuged at 1500 revolutions per minute at 4 degree Celsius for five minutes. After the final washing the pellet was suspended in a blocking puffer (3% BSA, suspended in PBS) for about 30 minutes at room temperature. Next, the cells were rinsed four times with 1% BSA, suspended in PBS and centrifuged at 1500 revolutions per minute at 4 degree Celsius for five minutes. Immunofluorescence antibodies (CD14, CD86, 206, CD80, CD200R, CD163) were incubated with each sample for 45 minutes. Negative isotype antibodies for each CD marker antibody were used. Following the staining, the cells were rinsed four times using 1% BSA, suspended in PBS and centrifuged at 1500 revolutions per minute at 4 degree Celsius for five minutes. The final pellets were suspended in 500 μL of ice cold PBS with 0.5% sodium azide. Accuri C6 flow cytometer was used to analyze the samples directly.

Table 1

 Summary of Antibody Concentration for Flow Cytometry and Immunofluorescence Staining

Antibody	FITC anti-human CD-14 Antibody (5μl/million cells) (Biolegend)	PE anti-human CD86 Antibody (5μl/million cells) (Biolegend)	FITC anti-human CD80 Antibody (Biolegend)
Isotype Control	FITC Mouse IgG1, κ Isotype Ctrl (FC) Antibody (5μl/million cells) (Biolegend)	PE Mouse IgG2b, κ Isotype Ctrl Antibody (5μl/million cells) (Biolegend)	FITC Mouse IgG1, κ Isotype Ctrl Antibody (5μl/million cells) (Biolegend)

Antibody	APC anti-human CD206 (MMR) Antibody (5μl/million cells) (Biolegend)	PE anti-human CD163 Antibody (5μl/million cells) (Biolegend)	PE anti-human CD200 Receptor Antibody (5μl/million cells) (Biolegend)
Isotype Control	APC Mouse IgG1, κ Isotype Ctrl Antibody (5μl/million cells) (Biolegend)	PE Mouse IgG1, κ Isotype Ctrl Antibody (5μl/million cells) (Biolegend)	PE Mouse IgG1, κ Isotype Ctrl Antibody (5μl/million cells) (Biolegend)

Statistics:

Each experiment in this study was performed at least three times, and the data were

analyzed by Sigma Plot.12 software and represented as mean ± SEM.

Results

PMA treatment causes an increase in the cell size and adherence but a decrease in confluency of U-937 cells.

Control cultures of U-937 consisted of replicating non- adherent round cells generally lacking processes with minimum changes in the morphology (Figure 2 a). In contrast, 50% of the cells that had been treated with 20 ng/ml PMA for 24 hours to differentiate the monocytic cells to M0 macrophages appeared flat, larger in size, and were tightly adherent to the culture dishes with a tendency to aggregate to the plastic surfaces of the dishes (Figure 2 b). The PMA treated cells (M0, M1, and M2) were adherent and were difficult to detach from the plastic surface in contrast to the untreated U-937 cells which remained in suspension

M1 and M2 phenotypes displayed distinct morphological characteristics

After treatment of U-937 cells with PMA for 24 hours, IFN-γ and LPS were added to the cells for another 24 hours to polarize M0 cells to the M1 macrophage phenotype. M1 cells appeared large, with cellular processes (pseudopodia), and visible intracellular vacuoles (Figure 2: c, d). Each of IL-4, IL-10, and IL-13 cytokines was applied separately to three culture plates at 24 hours following PMA treatment to polarize the M0 cells to M2 phenotypes. M2 cells appeared large and noticeably aggregated in large masses (Figure 3: a, b, c) in contrast to M1 phenotype (Figure 2A: c, d).

Treatment with PMA and the polarization agents exerts a significant impact on cell viability

Cell viability experiments were performed in triplicate for U-937, M0, M1, M2 (IL-4), M2 (IL-10), and M2 (IL-13). In contrast to untreated U-937 cells, the number of the viable cells were decreased after treating the U-937 cells with PMA and the other polarizing agents to 26% M0, 29% M1, 25% M2 (IL-4), 22% M2 (IL-10), 20% M2 (IL-13). One-way ANOVA showed these reductions compared to undifferentiated unpolarized U-937 cells to be significant ($p = 0.001$) (Figure 4). It is well documented that each of these substances (PMA and IL-4 and IL-10, LPS and INF-γ) is cytotoxic to the cells affecting cell viability [25-28].

When U-937 cells were treated with PMA and polarized to M1 phenotype following treatment with LPS& INF-γ, significantly less CD14 was expressed in M1 cells and also M0 cells.

Immunohistochemistry staining was performed for U-937, M0, and M1, few differences in expression of CD14 were seen when U-937 cells were compared to M0 and M1 cells (Figure 5). However, in a representative experiment, flow cytometry showed a decrease in expression of CD14 to 23% in M1 cells and 37% in M0 cells compared to U-937 cells (Figure 6: a, b, c). In ANOVA analysis of 3- separate experiments, M0 and M1 expressed significantly less CD14 by 2-fold decrease in M0, and 3-fold decrease was observed in M1 polarized cells (p 0.001, <0.001 respectively (Figure 6 d).

Phenotypic CD markers were determined on the U-937 and polarized macrophage

populations table1.

Table1.

Macrophage Differentiation CD Markers

MO-MQ membrane markers	Scavenger receptors	Membrane glycoprotein
1-CD14: LPS receptor mediates clearance of gram-negative pathogens [18]. 2- CD80 (B-7.1) and CD86 (B-7.2): Co receptor on APCs, transduce co-stimulatory signals to activate T cells [19, 20].	1- CD206: Macrophages mannose receptor (MMR). Expressed on macrophage but not monocytes identifies a variety of microbial carbohydrates [39,40]. . 2- CD163: hemoglobin-haptoglobin receptor. Expressed on monocytes and macrophage. CD 163 clear hemoglobin and regulate cytokine production by macrophages[23].	CD200R: expressed mainly by monocytes and neutrophils. The interaction between CD200R and CD200 limit and suppress macrophage induced inflammatory damage [37].

CD14 expression is enhanced in U-937 after chronic exposure to pyruvate containing medium.

After 20 passages in medium containing 2mg/ ml (11.1mM glucose) and 11mg/ ml pyruvate, the U-937 cells exhibited 5 –fold increase in the expression of CD14 when compared with passage one cells grown in medium containing 11.1 mM glucose without pyruvate (Figure 5). In one flow cytometry experiment Flow cytometry 83 % of passage 20 of U-937 cells grown in a medium containing 11mg/ml pyruvate expressed CD14 compared to 7% of passage one U-937 cells grown in a medium containing 11.1 glucose medium without pyruvate (Figure 7: a, b). Statistical significance was found by using ANOVA analysis (Sigma Plot) (p 0.001) (Figure 7 c). Chronic exposure to high glucose medium for two weeks or more augments CD14 expression in LPS- treated U-937 cells by 15- fold [24]. However, in the present study chronic exposure to pyruvate- containing medium for six weeks or longer enhanced CD14 expression by 5 fold in U-937 cells (Figure 7c).

M2 populations polarized with either IL-4 or IL-10 expressed lesser amounts of CD14 than did M2 cells induced by exposure IL-13.

M2 phenotype induced by IL-4 and M2 phenotype induced by IL-10 expressed significantly less CD14 compared to M2 cells induced by IL-13. In one flow cytometry experiment, 40 % of M2 cells induced with IL-4 and 35% of M2 (IL-10) expressed CD14 compared to 59% of M2 cells polarized with (IL-13). (Figure 8: a, b, and c). M2 cells induced by IL-13 exhibited a 2-fold elevation in the expression of CD14 compared to M2 (IL-4) and M2 (IL-4). One-way ANOVA (Sigma Plot) of three- separate experiments

demonstrated the statistical significance of this observation (p< 0.017, and p <0.02 respectively)(Figure 8d).

CD86 levels is upregulated upon differentiation and polarization treatments

CD86 expression could not be detected on U-937 cells by immunofluorescent staining. Appreciable immunofluorescent staining was observed in M0, M1, M2 induced by IL-4, M2 induced by IL-10, and M2 induced by IL-13 (Figure 9). In one representative experiment 36% of M0, 46% of M1, 76% of M2 (IL-4), 33% of M2 (IL-10), and 53% of M2 (IL-13) expressed CD86 compared to 11% of U-937 cells (Figure 10: a, b, c, d, e, and f). In an ANOVA analysis of 3- separate experiments, CD86 expression was increased by: 5-fold in M0 cells, 4 -fold in M1 cells, 7-fold increase in M2 cells polarized with (IL-4), 4-fold in M2 cells polarized with (IL-10), and 6-fold in M2 cells polarized with (IL-13) compared to U-937 cells. Sigma Plot (ANOVA analysis) showed this to be a significant increase (p <0.015, <0.004, 0.002, 0.028, and 0.010 respectively) (Figure 10 g).

Adding pyruvate to the medium did not affect CD86 expression in U-937 cells

Flow cytometry histograms showed comparable CD86 expression (11%) in passage one U-937 cells grown in pyruvate free medium and in passage 20 untreated U-937 grown in pyruvate- containing medium (Figure 11: a, b). No statistical significance in CD86 expression between these two passages was observed (p 0.665).

CD80 expression decreased in M0, M1, M2 (IL-10), and M2 (IL-13)

The results from one representative flow cytometry experiment showed a decrease in the

expression of CD80 to 43% by M0, 20% by M1, 39% by M2 induced by (IL-4), 25% by

M2 induced by (IL-10), and 35% by M2 induced by (IL-13) compared to 87% of U-937

cells (Figure 12: a, b, c, d, e, and f). ANOVA analysis of three-separate experiments

revealed that these differences were significant for all polarized cells (p <0.01) except M2

cells polarized with IL-4 (p 0.137) (Figure 12g).

M0 cells increased the expression of CD206 compared to control cells.

CD206 expression could not be detected on U-937 cells by immunofluorescent staining.

Appreciable immunofluorescent staining in M0, M1, and M2 (IL-4) was observed

(Figure 13), while M2 (IL-10) and M2 (IL-13) showed dim immunofluorescent

enhancement (data not shown). PMA treatment resulted in a significant increase of the

expression CD 206 in M0 when compared to U-937 cells. In one representative

experiment, flow analysis revealed CD206 expression was 9 % in U-937 cells while in

M0 cells were 73% (Figure 14: a, b). ANOVA analysis (Sigma Plot) of 3-separate

experiments showed this was statistically significant (P= 0.017) (Figure 14 g). The

expression of CD206 in M0 provides evidence of macrophage differentiation because

CD206 is present on macrophages but not monocytes [39][40].

**M2 induced by (IL-4) significantly expressed more CD206 than did M2 induced by
(IL-10) and U-937 cells.**

One representative experiment by flow cytometry showed an increase in the expression

of CD206 to 77% by M2 (IL-4), 17 % by M2 (IL-10) population, and 9% by U-937 cells

(Figure 14: a, d, and e). ANOVA statistics of 3-separate experiments showed 5 –fold

increase in CD206 expression in M2 polarized by IL-4 when compared U-937 cells,

while 3-fold increase when compared to M2 polarized by IL-10 (p 0.002, 0.016) (Figure 14 g).

Adding pyruvate to the medium did not affect CD 206 expression in U-937

Flow cytometry results showed comparable CD206 expression (9%) in both passage one U-937 grown in pyruvate free medium and passage 20 U-937 grown in pyruvate-containing medium (Figure 15: a and b). No statistical significant difference between the two group in CD206 expression was seen (p 0.282).

M1 phenotype exhibited noticeable less expression of CD163 and CD200R

U-937, M0, and M2 (IL-10) showed more expression of CD163 by immunohistochemistry than did M1 cells (Figure 12A). M2 (IL-4) and M2 (IL-13) showed dim fluorescence intensity (data not shown). In a representative flow cytometry experiment, 23 % of M1 population expressed CD163 compared to 70 % of U-937 cells (Figure 16: a, c). Following treatment with IFN γ/LPS treatment for 24 hours, M1 exhibited 3-fold decrease in CD163 when compared to U-937 cells (p = 0.011) (Figure 17 g). 20% M2 cells polarized with (IL-4), 40%M2 cells polarized with (IL-10), and 18% M2 cells polarized with (IL-13) expressed CD 163 (Figure 17: d, e, and f). In 3-separate experiments of flow cytometry, ANOVA showed no significance among the polarized cell populations (M1 or M2). The untreated unpolarized U-937 cells showed a significant increase in CD163 compared to M1 (p <0.01) (Figure 17 g).

Polarization into M1 phenotype was accompanied by a significant decrease in CD200R expression when compared to U-937 cells. In one representative flow cytometry

22

experiment, 11% of M1 population expressed CD200R when compared to 67% of U-937 cells (Figure18: c and a). In three- separate experiments ANOVA showed that M1 exhibited CD200R 3-fold less than U-937 cells (p = 0.049) (Figure 18 g). 27% of M0 cells, 31% of M2 polarized with IL-4, 37% of M2 polarized with IL-10, and 29% of M2 polarized with IL-13 expressed CD200R (Figure 18: b, d, e, and f). Again, no statistically significance differences were seen in CD 200r expression between the M1 or M2 groups.

After two weeks of growing U-937 cells in pyruvate free medium, CD 200R is down regulated in U-937 cells.

In one representative flow cytometry experiment 6% of U-937 expressed CD200R when they were grown in a pyruvate free medium for two weeks, compared to 87% of U-937 cells expressed CD200R when grown in a pyruvate- containing medium (Figure 19: a, b). ANOVA statistics of three-separate flow cytometry experiments showed that U-937 cells expressed 16-fold decrease in CD200R two weeks after eliminating the addition of pyruvate to the media (p = 0.011) (Figure 19 c). However, depriving U-937 cells of pyruvate for two weeks did not affect the CD80 expression (data not shown).

Discussion

In this study the U-937 human leukemic monocyte lymphoma cell line was differentiated into macrophages using phorbol 12- myristate (13) (PMA) and then polarized into (M1) and (M2) phenotypes according to specific microenvironments. The morphological characterizations between the two polarized phenotypes and unpolarized cells were distinguished as were the cell viability and cluster differentiation markers (CD) for each population.

U-937 monocytes are round, non-adherent, replicating cells, and after treating with PMA for 24 hours, 50% of the cells differentiated into macrophages (M0). As macrophages, they appeared as flat, large tightly adhering cells with a tendency to aggregate. IFN-γ and LPS were used to polarize the M0 cells to the M1 phenotype characterized by large irregularly shaped cells with pseudopodia and intracellular vacuoles; the M1 cells tightly adhere to the surface of the plastic dish. IL-4, IL-10, or IL-13 was applied separately to polarize M0 to the M2 phenotypes; M2 appeared round in shape and aggregated in large masses and also tightly adhered to the plastic surfaces of the culture dishes.

After differentiation of U-937 cells, the cell viability for each M0, M1, M2 (IL-4), M2 (IL-13), M2 (IL-10) decreased. PMA is known to augment cytotoxicity by disrupting the microtubule networks in Hela cells and HL-60 promyelocytic cell line [25] [26]. IFN-γ is also known to be toxic when combined with TNF [27]. TNF is one of the important secretions of M1 cells, and the combination of exogenous IFN-γ and secreted TNF alpha probably contributed to the decrease cell viability of the M1 cultures. Th2 cell-derived cytokines such as interleukin-4 (IL-4) and (IL-10) induce apoptosis in monocytes [28].

Obviously, these polarizing agents played a crucial role in decreasing the cell viability and induce apoptosis of the various polarized cell populations.

After treatment of U-937 cells with PMA and then polarization into the M1 phenotype by using LPS and IFN-γ, CD14 expression was significantly decreased. CD14 is part of the LPS receptor on monocyte/macrophage cells. LPS binds to CD14 and causes internalization of itself and CD14 [43]. M2 (IL-13) cells expressed CD14 more than M2 (IL-4) and M2 (IL-10) phenotypes. IL-13 enhances CD14+ cell monocyte to stimulate CD4+ T cells that eventually augmented its responses against chronic HIV-1 infection [32]. Our results suggest that IL-13 an important cytokine in enhancing the CD14 on M2 phenotype, which may reflect on the function of M2 macrophages to enhance and activate T cells against various pathogens.

U-937 is a monoblastic progenitor cell line that lacks CD14 expression; however, differentiating factors can induce its expression [28]. In the present study, CD14 expression of U-937 cells was increased by 5- fold after chronic exposure to medium containing pyruvate for six weeks or more (passage 20 or more). Nareika et al. (2008) found that glucose can enhance CD14 in LPS- stimulated U-937 cells through increasing nuclear factor KB and AP1 activities [24]. The medium that Nareika et al: (2008) used contained 25 mM glucose, while the medium used in the present study contained 11.1mM glucose and 11mg/ml pyruvate. Pyruvate is the end glycolytic product of glucose. Metabolically, Each glucose molecules yields two pyruvate molecules,. Eventually, pyruvate ends up as acetyl-co A or lactate, both of which are sources to generate ATP in the cell. Acetyl–co A enters the citric acid cycle, which is the crucial source of ATP (Figure 15(a)). Interestingly, pyruvate can also produce glucose through

gluconeogenesis. Gluconeogenesis is a reciprocal process of glycolysis and only occurs in the liver [29] (Figure 15b). However, it has been documented that 1,6-bisphosphatase gene is active in human alveolar macrophages. The product of this gene is the rate-limiting step of gluconeogenesis. Moreover, 25-dihydroxy vitamin D3, which is used for monocytic differentiation of cells from the human leukemia cell line such as HL-60 and U-937 elevates the expression of the 1,6-bisphosphatase gene [30]. Furthermore, it has been documented that imidazole pyruvate can rescue 1,6-bisphosphatase from inhibition in liver cells [31]. These observations suggest relationship between pyruvate, gluconeogenesis pathway affect CD14 expression inU-937 cells.

Addition of pyruvate enhanced expression of CD80 co-stimulatory molecule but not the CD86 co-stimulatory molecule. Differential expression of these molecules on macrophages has been described by others. For example, CD80 expression increased while CD86 was decreased in murine peritoneal macrophages [41, 42].

CD86 expression is up regulated in M0, M1, M2 (IL-4), M2 (IL-10) and M2 (IL-13). This up regulation is an evidence of differentiation into macrophages since it has been documented that U-937 cells express vey little CD86 [38]. U-937 cells in our research also expressed very low levels of CD86. In contrast to CD86, CD80 expression decreased after differentiation of U-937 cells to M0, M1, M2 (IL-10), and M2 (IL-13). These observations suggest that CD86 is a good marker for differentiation into macrophages.

PMA treatment caused a four- fold increase in CD 206 expression in U-937 cells indicating differentiation since CD206 is expressed in macrophages but not in monocytes

[39,40]. CD206 is important in innate and adaptive immune response. M2 cells polarized with IL-4 exhibited five-fold increase in CD206 expression when compared to U-937 cells. M2 polarized with IL-4 also exhibited three –fold increase in the expression of CD206 when compared to M2 cells polarized IL-10. These findings reveal the importance of the cytokine IL-4 to enhance CD206 in M2 macrophage phenotype.

U-937 cells expressed significantly higher level of CD163 when compared to the expression by M1 population. CD163, a hemoglobin haptoglobin scavenger receptor, is expressed on U-937 cells and also considered a specific marker of the M2 phenotype [33], [34], [35]. M1 polarized cells expressed small amount of CD163. It has been documented that IL-4, IL-13, TNF-α, and LPS/IFN-γ decrease the expression of CD163, and IL-10 increases its expression [33]. Our immunofluorescence findings are consistent with the previous study, which appreciable expression of CD163 on M2 cells polarized by IL-10, as well as by M0, and undifferentiated U-937 cells compared to M1. Immunofluorescence images of M2 (IL-4) and M2 (IL-13) showed low expression of CD163 (data not shown).

U-937 cells do not express CD200R but can be transfected to express it. In these transfection experiments, the greater expression of CD200R by U-937 cells correlates with decreases in expression of cytokines [36]. In the present study, undifferentiated U-937 cells grown in pyruvate-containing medium expressed appreciable level of CD200R. Ethyl pyruvate has been used as a potent anti-inflammatory drug in porcine toxic shock model to inhibit release of inflammatory cytokines [37]. The expression of CD200R by undifferentiated U-937 cells was appreciable but was decreased 16-fold when grown for two weeks in medium lacks pyruvate.

Table 3

Summary of CD Marker Expression on the Monocyte/Macrophage Populations*

Cells	Scavenger CD206	Scavenger CD163	Costimulator CD80	Costimulator CD86	LPS Receptor CD14	Inflammatory Inhibitor CD200R
U-937	12.7 ± 2.2	**50.9 ± 7.0**	76.2 ± 10.3	8.9 ±1.0	73.5 ± 6.9	48.7 ± 10.1
M0	**49.6 ± 11.9**	**24.6 ± 1.4**	**38.3 ± 4.6**	**45.7 ± 12.4**	**41.2 ± 5.5**	**26.3 ± 2.5**
M1	41.8 ± 7.3	16.3 ± 4.9	21.3 ± 3.3	39.1 ± 6.5	25.1 ± 4.2	18.2 ± 2.5
M2 (IL-4)	59.9 ±14.1	26.7 ± 12.1	47.7 ± 10.2	59.4 ± 6.7	32.5 ± 4.0	32.9 ± 9.6
M2 (IL-10)	24.3 ± 7.0	29.6 ± 4.6	35.1 ± 12.5	39.6 ±5.0	35.5 ± 3.0	26.1 ± 6.7
M2 (IL-13)	45.6 ± 2.1	23.6 ± 2.7	40.0 ± 3.1	57.5 ± 3.4	**58.9 ± 3.0**	30.1 ± 2.0

*Numbers represent means of 3 separate experiments ± s.e.m

Overall conclusions summarized in Table 3.

1. The undifferentiated U--937 cells expressed less CD206 and CD86 but greater amounts of CD163, CD80, and CD200R than did the differentiated U937 cells (M0 macrophages). These observations suggest that the differentiated M0 cells would be better at antigen presentation since they expressed a 6-fold increase the CD86 costimulatory molecule and half the amount of the CD80 costimulatory molecule as did undifferentiated U937 cells.

2. M1 polarized macrophages expressed lesser amounts of all CD markers than M0 cells which may reflect the production of toxic substances such as reactive oxygen molecules, nitric oxide and TNF-α. Both the differentiation and polarization processes caused decreases in cell viability no difference seen between M0 and M1 populations at the 24 hour observation time.

3. Marked differences in expression of these CD markers were obvious in the M2 subpopulations with the IL-4-polarized M2 cell showing marked elevations in expression of CD206 and CD86 and the IL-13-polarized M2 cells showing marked increase in expression of CD14.

28

These differences highlight the plasticity of the macrophage in different microenvironments and may be useful in interpreting experimental results in other systems. Reichard (2013) used murine macrophages (J774A) and polarized them into M1 by using IFN-γ and LPS, while IL-4 was used for polarization of the M2 phenotype. Similar to the observations of Reichard (2013), expression of CD86 (B7-2 co-stimulatory molecule and CD14 (LPS co-receptor) in the present study was not unique to the polarized populations. The lack of a unique CD specification between the M1 and M2 phenotypes in the current macrophage model could be related to the origin of the monocytes. The U-937 cell used in the present study were monocytic pleural effusion cells isolate from a 37 year-old male with histiocytic lymphoma (ATCCD CRL-2593.2) and may express CD markers different from those of peripheral blood monocytes. In contrast, Jaguin et al (2013) used human monocytes that purified from buffy coats of peripheral blood cells and differentiated them into macrophages human macrophage colony stimulating (M-CSF) [22]. IFN-γ and LPS were used to polarize the differentiated macrophages into M1 phenotype while IL-4 was used to polarize the macrophages into M2 phenotype [22]. Similar to the observation in the present study in which no difference in expression of CD206 was found between the polarized M1 and M2 phenotypes, Jaguin et al (2013) found the mannose receptor CD206 did not distinguish between M1 and M2 phenotypes of human macrophages. Jaguin and his colleagues also found CD80 is a specific marker for M1 phenotype and CD200R is a specific marker for M2 phenotype. Unlike Jaguin et al findings, unique expression of CD80 and CD200R molecules failed to discriminate M1 and M2 phenotypes in the present study.

In future studies it would be useful to determine whether the cytokines such as

29

IL-12 and IL-23 are produced by the M1 phenotype and the anti-inflammatory cytokine IL-10 is produced by the M2 phenotypes including the Il-10-polarized M2 cells. The microarray biochip analysis can provides cytokine profiles for each macrophage phenotype, this would allow a more discrete identification of the two phenotypes, and would provide definitive evidence of polarization. Small interfering RNAs (siRNAs) against CD14, CD200R, and CD80 could be used in undifferentiated U-937 cells to silence protein synthesis of these CD markers.
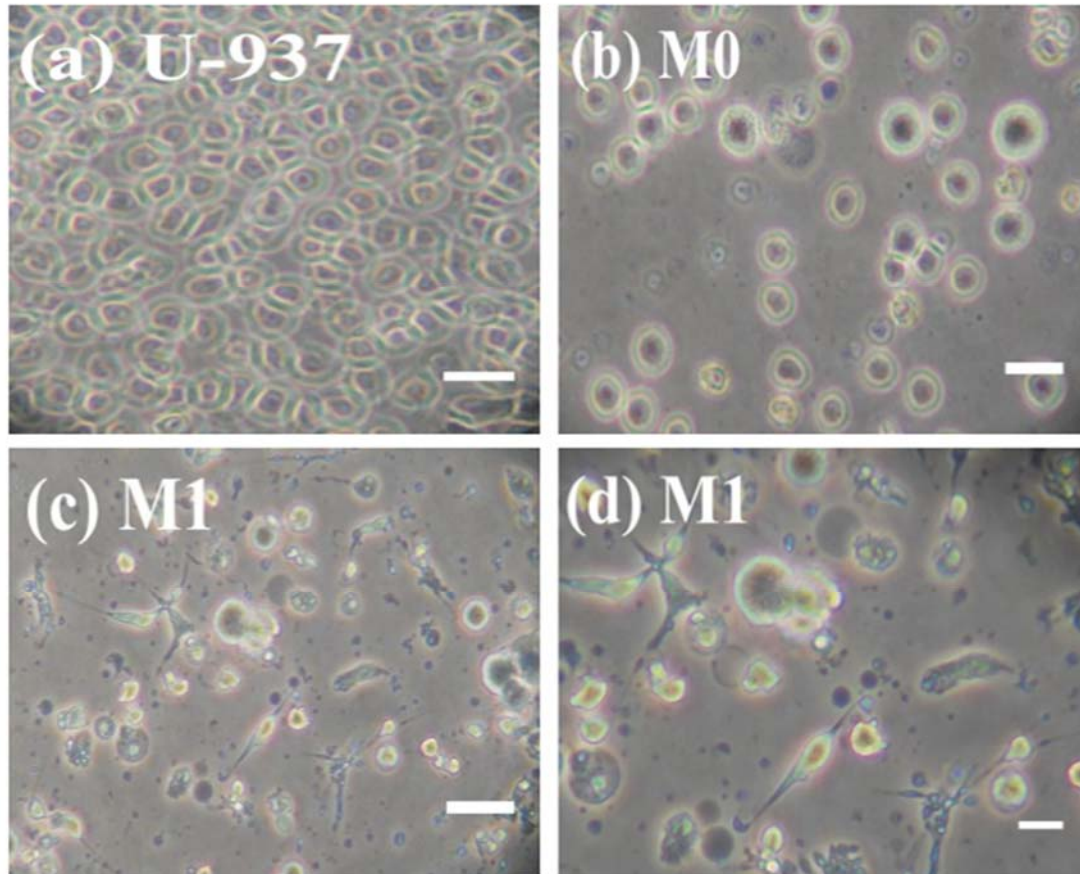
Figure 2: (a) U-937 cells monocytes are round non-adherent replicating cells with minimum changes in the morphology, (b) M0 (24 hour post PMA treatment) appeared flat, large, tightly adhering to culture plates. (c) and (d) M1 (24 hour after treatment with IFN-γ and LPS) exhibited pseudopodia and appeared large, and irregularly shaped with visible intracellular vacuoles. Cell confluency decreased upon differentiation to M0 cells and polarization to M1 cells. (Scale bar = 50μm).
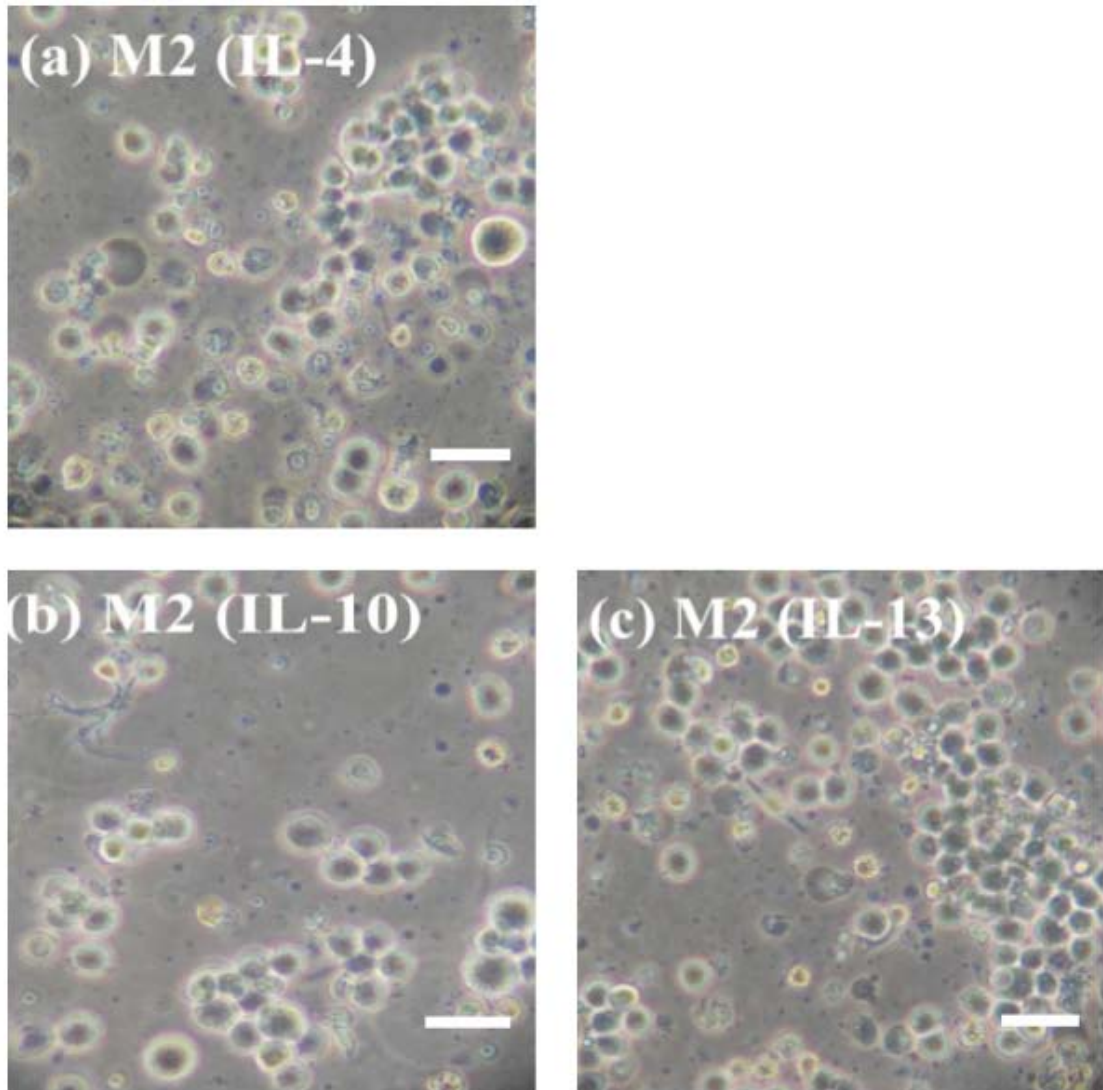
Figure 3: (a) M2 polarized by IL-4 for 24 hour, (b) M2 polarized by IL-110 for 24 hours, (c) M2 induced by IL13 for 24 hours. M2 macrophage populations appeared, large, rounded in shape, and tightly adhering to culture plates with few or no pseudopodia with tendency to aggregate in large masses. Cell confluency decreased upon polarization to M2 phenotypes. (Scale bar = 50μm).
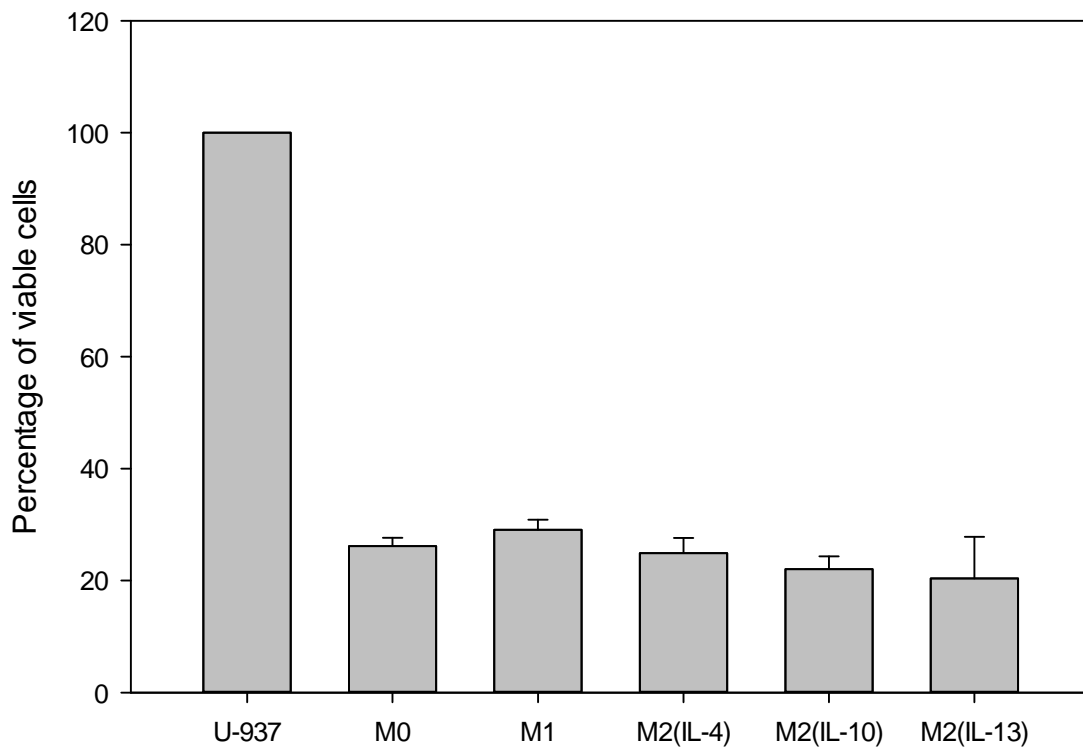
Figure 4: Cell viability in U-937 24 hours following differentiation and polarization treatment. There was a significant decrease in the cell viability after adding PMA and polarization agents. ANOVA analysis (Sigma Plot) showed the reduction is statistically significant (p <0.001).
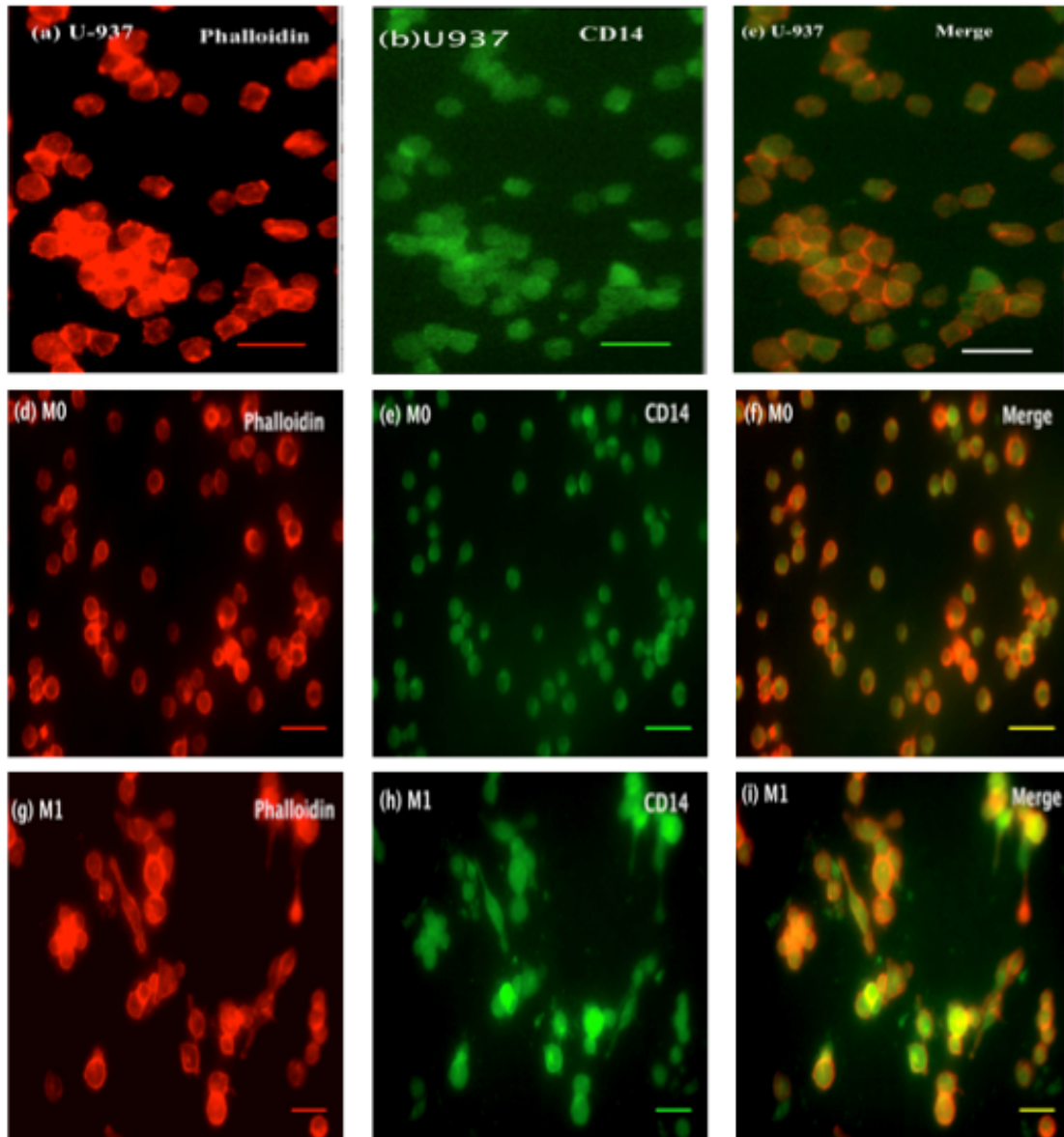
Figure 5: Immunofluorescent staining images of: (a) U-937 cells stained with Texas-Red Phalloidin X, (b) with anti-CD14 FITC-conjugated antibodies, (c) Phalloidin/ CD14 Merge. (d) M0 cells stained with Texas-Red Phalloidin X, (e) with anti-CD14 FITC-conjugated antibodies, (f) Phalloidin/ CD14 Merge. (g) M1 cells stained with Texas-Red Phalloidin X, (h) with anti-CD14 FITC-conjugated antibodies, (j) Phalloidin/ CD14 Merge. (Scale bar = 50μm).
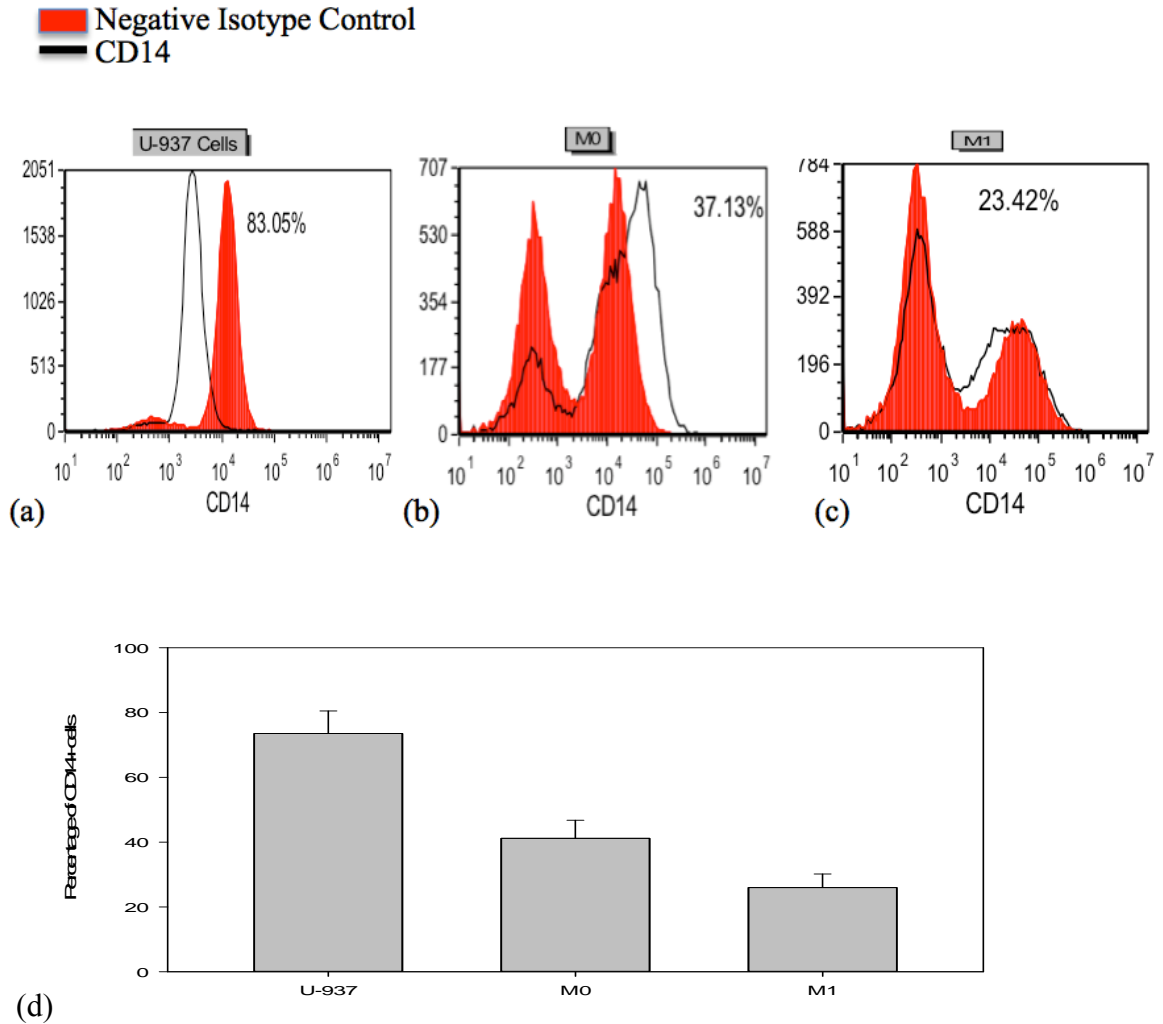
Figure 6: Flow cytometry histograms showing CD14 expression in: (a) U-937, (b) M0, (c) and M1. (d) Percentage averages of CD14$^+$ in U-937 cells, M0, and M1. A 2-fold decrease in CD14 expression in M0, and 3-fold decrease was observed in M1 polarized cells (p 0.001, <0.001 respectively). The experiments were done in triplicate.

(a) Passage one U-937

(b) Passage 20 U-937

(c)
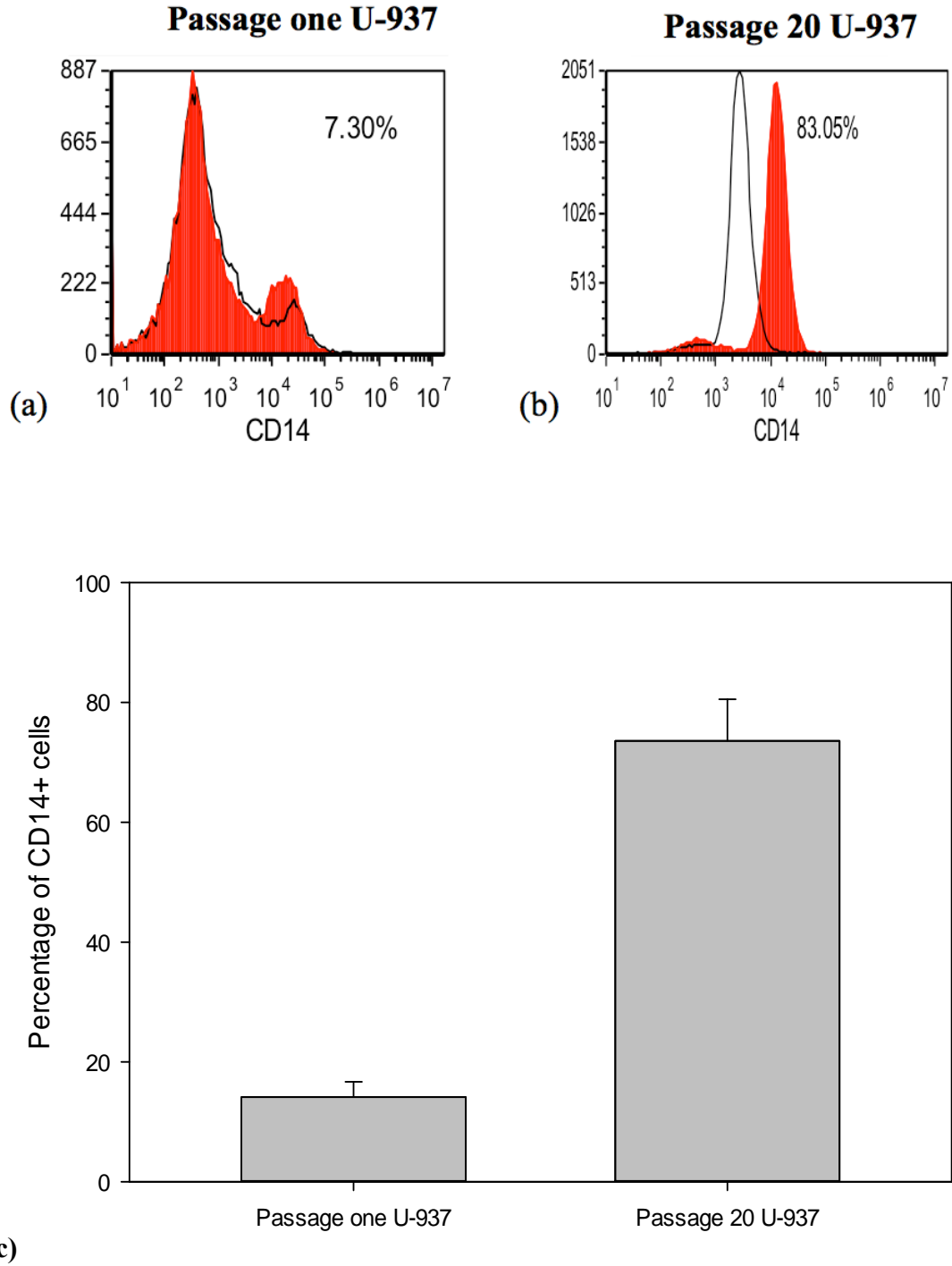
Figure 7: Flow cytometry analysis of U-937 cells grown in pyruvate- containing medium stained for six weeks or more (passage 20) (b) compared with passage one U-937 grown in pyruvate-free medium (b). Note that only 7.3% of passage1 stained positive for CD14 while 83.1 of passage 20 U-939 cells were positive for CD14. There was a 5-fold increase in CD14 expression in passage 20 of U-937 grown in pyruvate-containing medium (p<0.001). The experiments were done in triplicate.

36
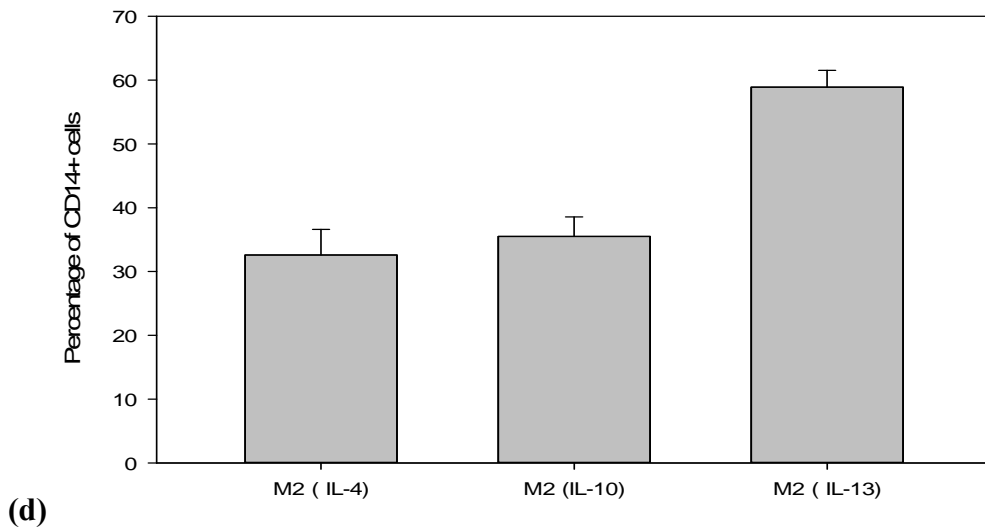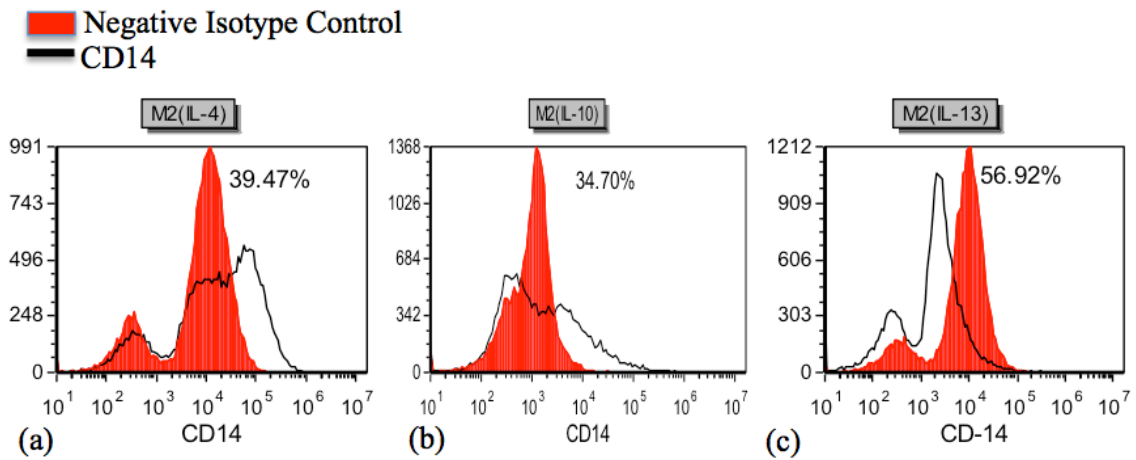
Figure 8: Flow cytometry histograms showing CD14 expression in: (a) M2 polarized with IL-4, M2 polarized with by IL-10, and M2 polarized with IL-13. (d) Percentage averages of CD14$^+$ in M2 (IL-4), M2 (IL-10), and M2 (IL-13). A 2-fold increase in CD14 expression in M2 (IL-13) when compared to M2 (IL-4) and M2 (IL-10) (p < 0.017, and p< 0.027 respectively). The experiments were done in triplicate.
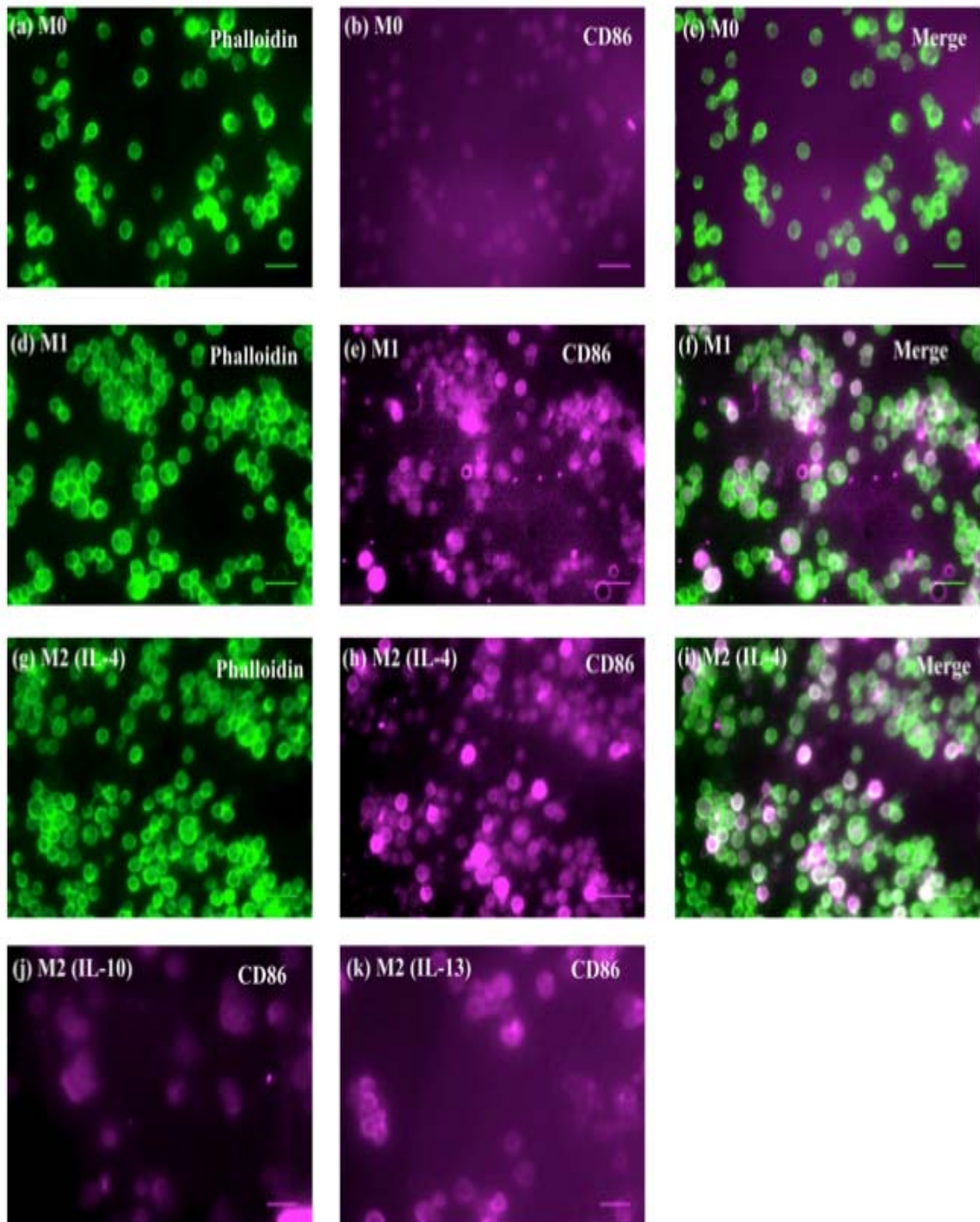
Figure 9: Immunofluorescent staining images of: (a) M0 cells stained with Texas-Red Phalloidin X, (b) with anti-CD86 Brilliant Violet 421-conjugated antibodies(c) Phalloidin/ CD86 Merge. (d) M1 cells stained with Texas-Red Phalloidin X, (e) with

anti-CD86 Brilliant Violet 421-conjugated antibodies, (f) Phalloidin/ CD86 Merge. (g) M2 cells polarized with IL-4 stained with Texas-Red Phalloidin X, (h) with anti-CD86 Brilliant Violet 421-conjugated antibodies, (i) Phalloidin/ CD86 Merge. (j) M2 cells polarized with IL-10 stained with anti-CD86 Brilliant Violet 421-conjugated antibodies, (k) M2 cells polarized with IL-13 stained with anti-CD86 Brilliant Violet 421-conjugated antibodies. (Scale bar = 50μm).
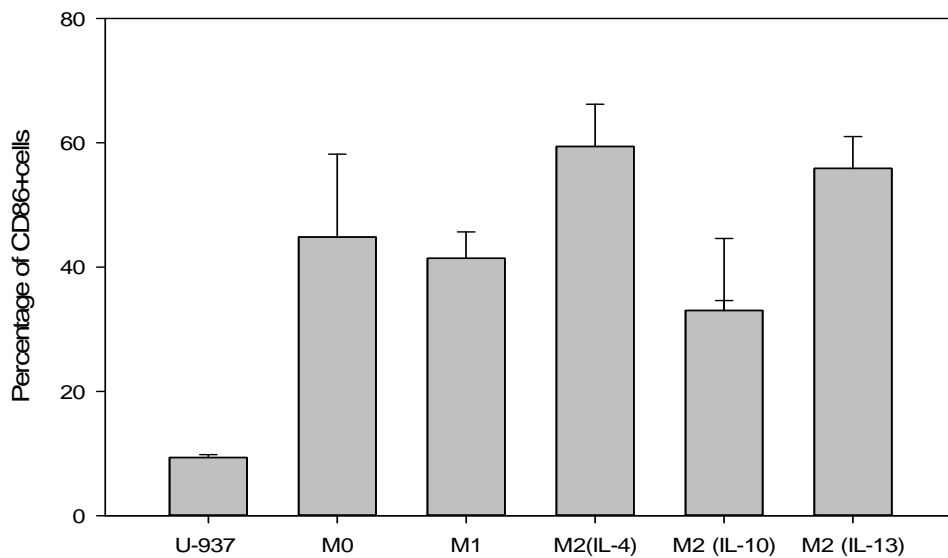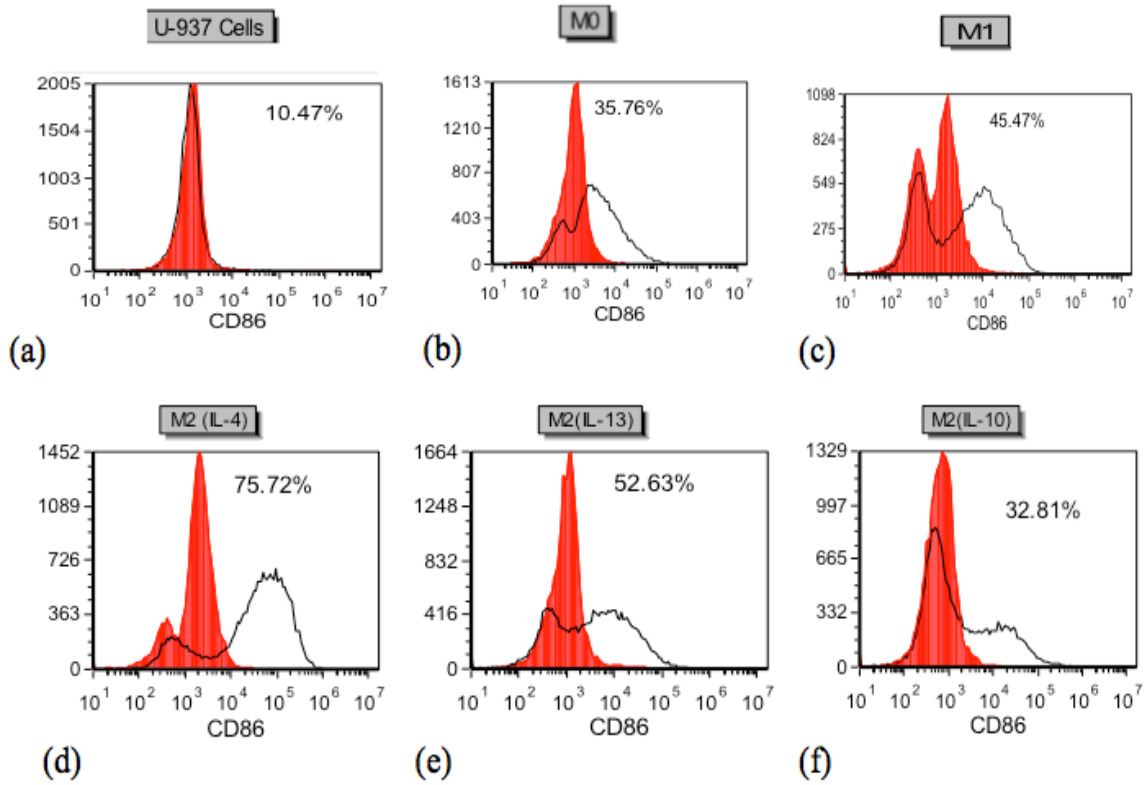
Figure 10: Flow cytometry histograms showing CD86 expression in: (a) U-937, (b) M0, (c) M1, (d) M2 polarized with IL-4, (e) M2 polarized with IL-10, and (f) M2 polarized with IL-13. (g): Percentage averages of CD86$^+$ in: U-937 cells, M0, M1, M2 polarized with IL-4, M2 polarized IL-10, M2 polarized with(IL-13. CD86

increased by: 5-fold in M0, 4-fold in M1, 7-fold increase in M2 (IL-4), 4-fold in M2 (IL-10), 6-fold in M2 (IL-13) when compared to U-937 cells. Sigma Plot (ANOVA analysis) proved the statistic significant of these observation (p 0.015, 0.004, 0.002, 0.028, 0.010 respectively). The experiments were done in triplicate.
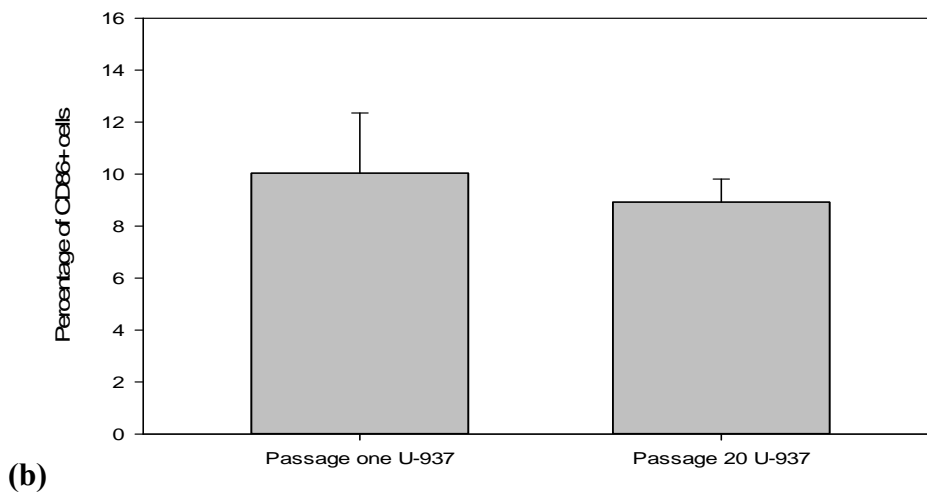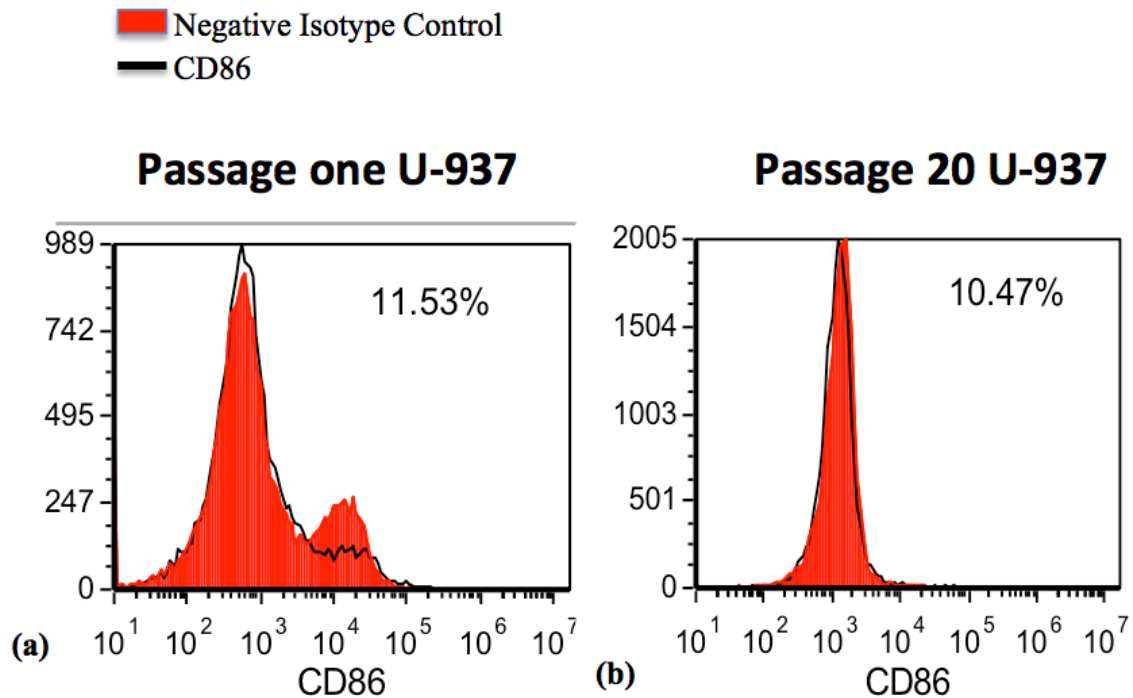
Figure 11: Flow cytometry histograms showing CD86 expression in: (a) passage one U-937 grown in pyruvate free medium, (b) passage 20 untreated U-937 grown in pyruvate- containing medium. (c) Percentage averages of CD86$^+$ in passage one grown in pyruvate -free medium and passage 20 untreated U-937 cells grown in pyruvate –containing medium N.S (p 0.665). The experiments were done in triplicate.
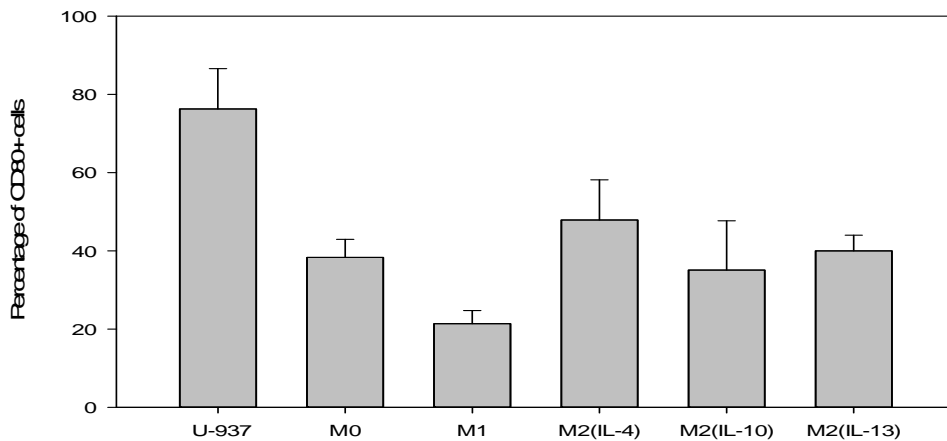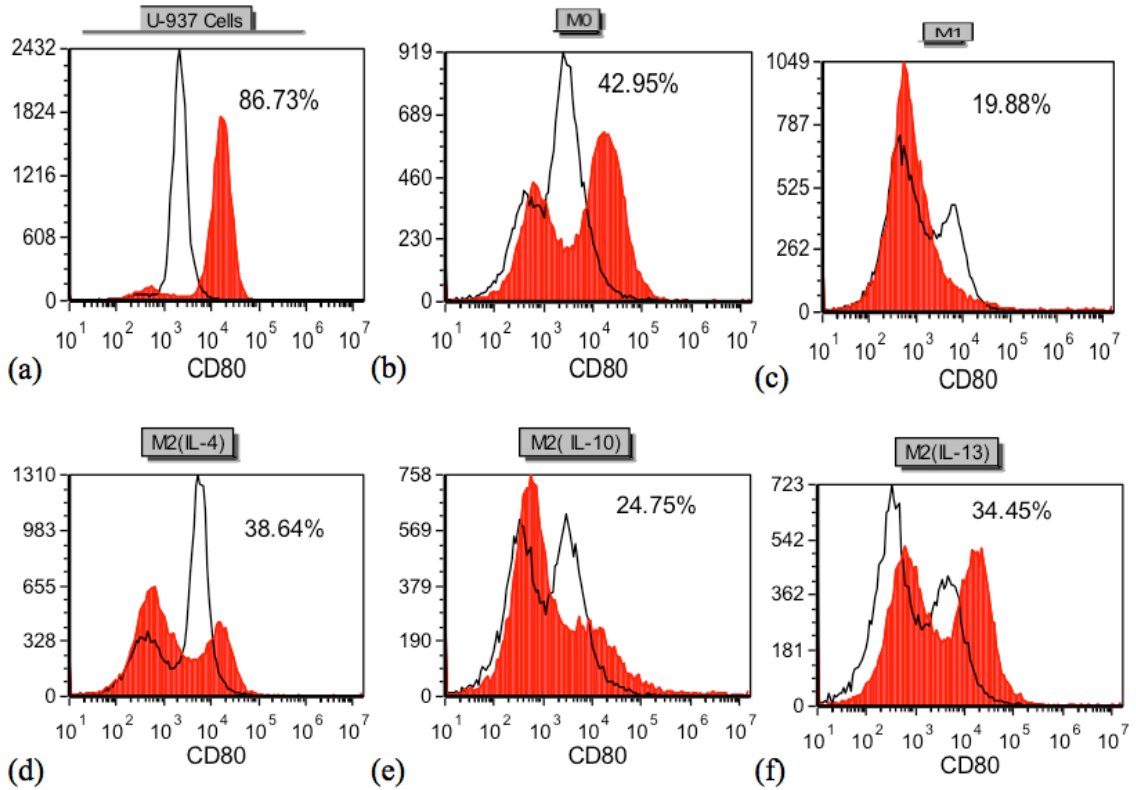
Figure 12: Flow cytometry histograms showing CD80 expression in: (a) U-937, (b) M0, (c) M1, (d) M2 polarized with IL-4, (e) M2 polarized with IL-10, and (f) M2 polarized with IL-13. (g): Percentage averages of CD80+ in: U-937 cells, M0, M1,
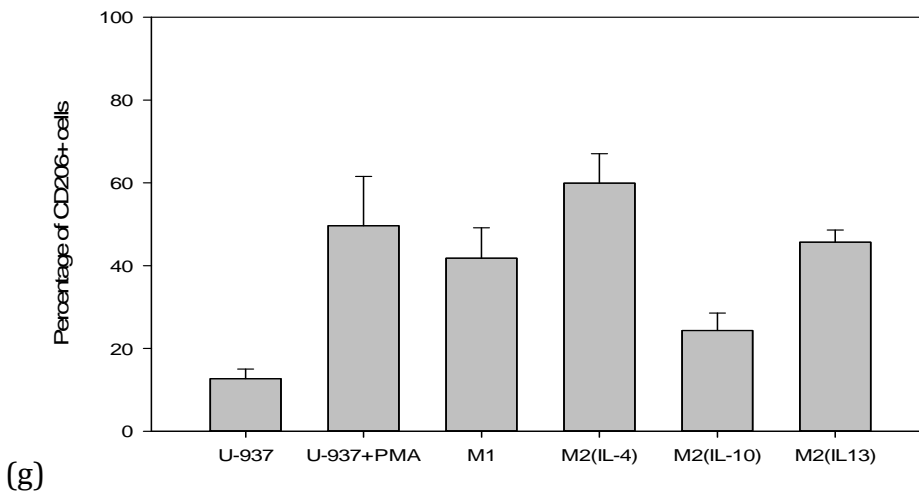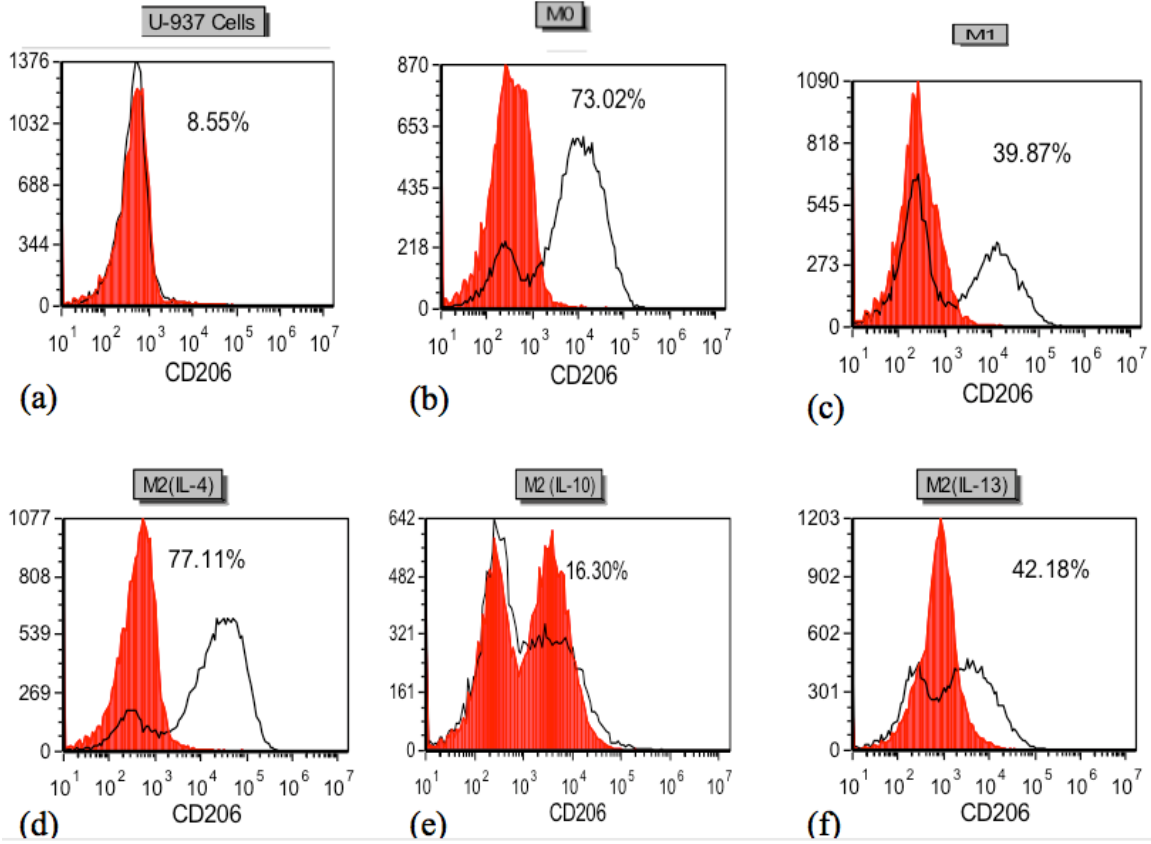
M2 polarized (IL-4), M2 polarized with IL-10, M2 polarized with (IL-13). (g) Percentage averages of CD80$^+$ following polarization 24 hours. ANOVA analysis of 3- separate experiments revealed that these differences were significant for all polarized cells (p <0.01) except M2 cells polarized withIL-4 (p 0.137). The experiments were done in triplicate.
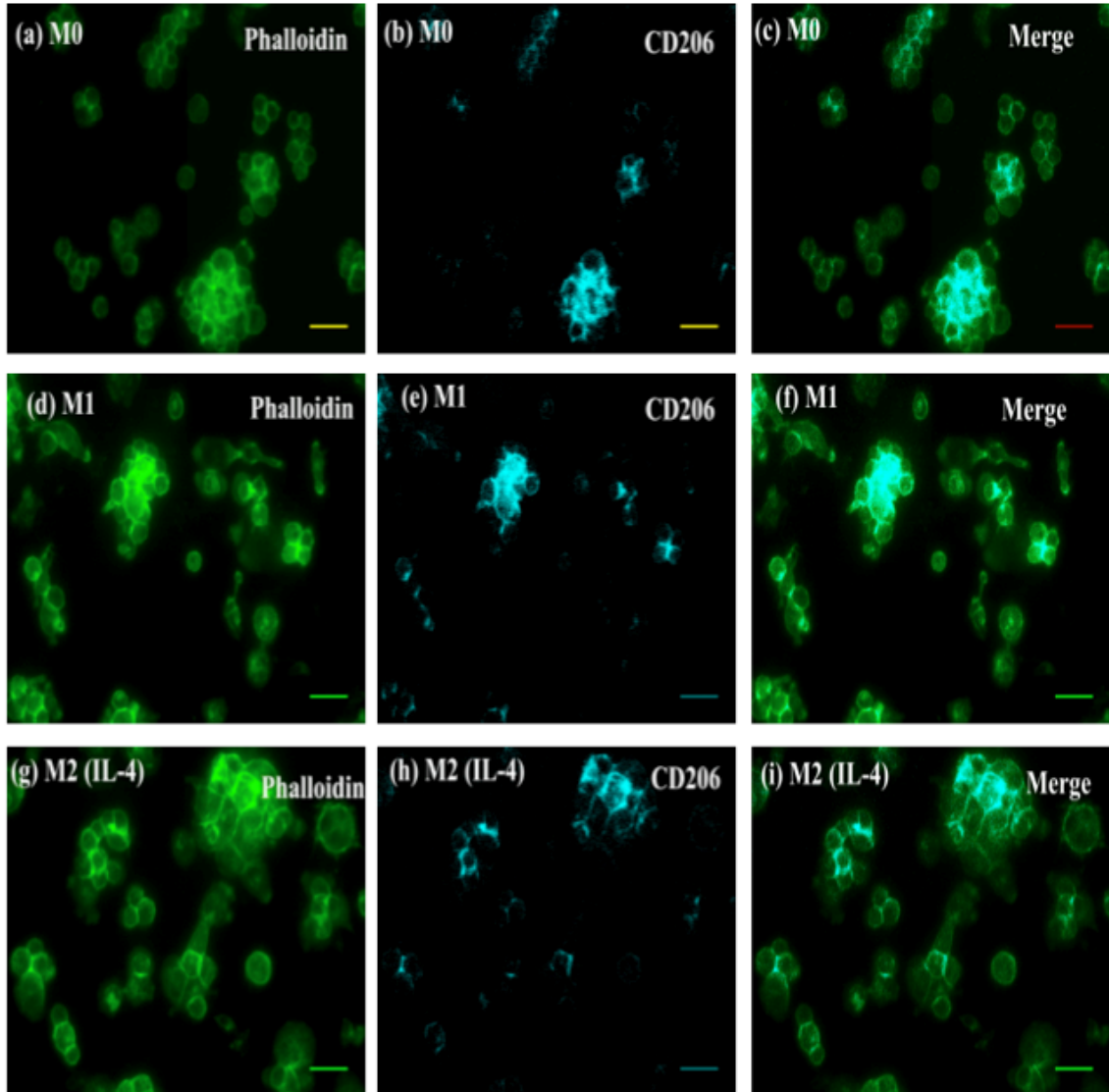
Figure 13: Immunofluorescent staining images of: (a) M0 cells stained with Texas-Red Phalloidin X, (b) with anti-CD206 APC-conjugated antibodies(c) Phalloidin/ CD206 Merge. (d) M1 cells stained with Texas-Red Phalloidin X, (e) with anti-CD 206 APC -conjugated antibodies, (f) M2 cells induced by IL-4 stained with Texas-Red Phalloidin X, (g) with anti-CD206 APC-conjugated antibodies, (h) Phalloidin/ CD206 Merge. (Scale bar = 50µm).
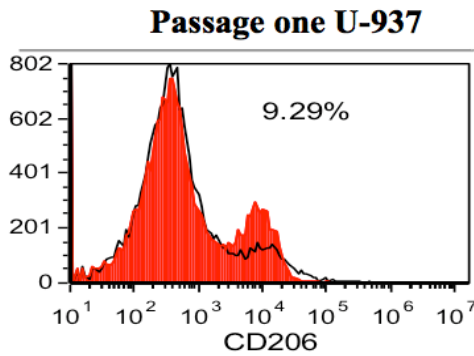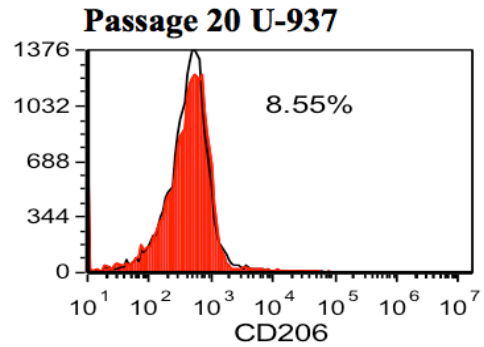
Figure 14: Flow cytometry histograms showing CD206 expression in: (a) U-937, (b) M0, (c) M1, (d) M2 induced by IL-4 , (e) M2 induced by IL-10, and (f) M2 induced by IL-13. (g): Percentage averages of CD206$^+$ in: U-937 cells, M0, M1, M2 polarized

with by (IL-4), M2 polarized withIL-10, M2 polarized with (IL-13. M0 cells exhibited 3- fold increase in the expression of CD206 when compared to U-937 cells (P 0.028). M2 cells induced by IL-4 exhibited 4-fold increase in CD206 expression when compared to U-937 cells (P-0.004). M2 induced by IL-4 also exhibited 3 –fold increase in the expression of CD206 when compared to M2 cells polarized with IL-10 (p 0.027). The experiments were done in triplicate.
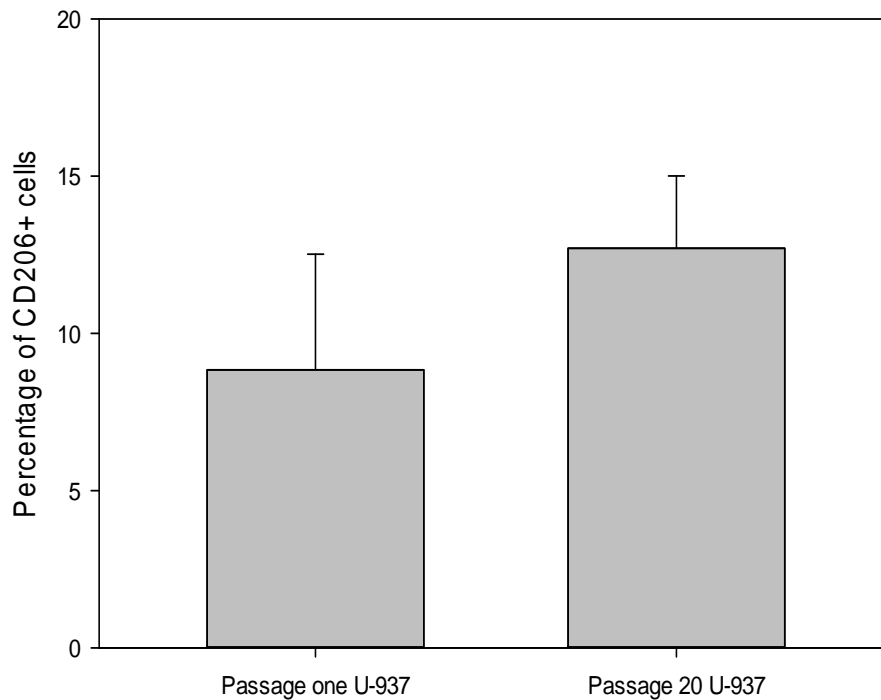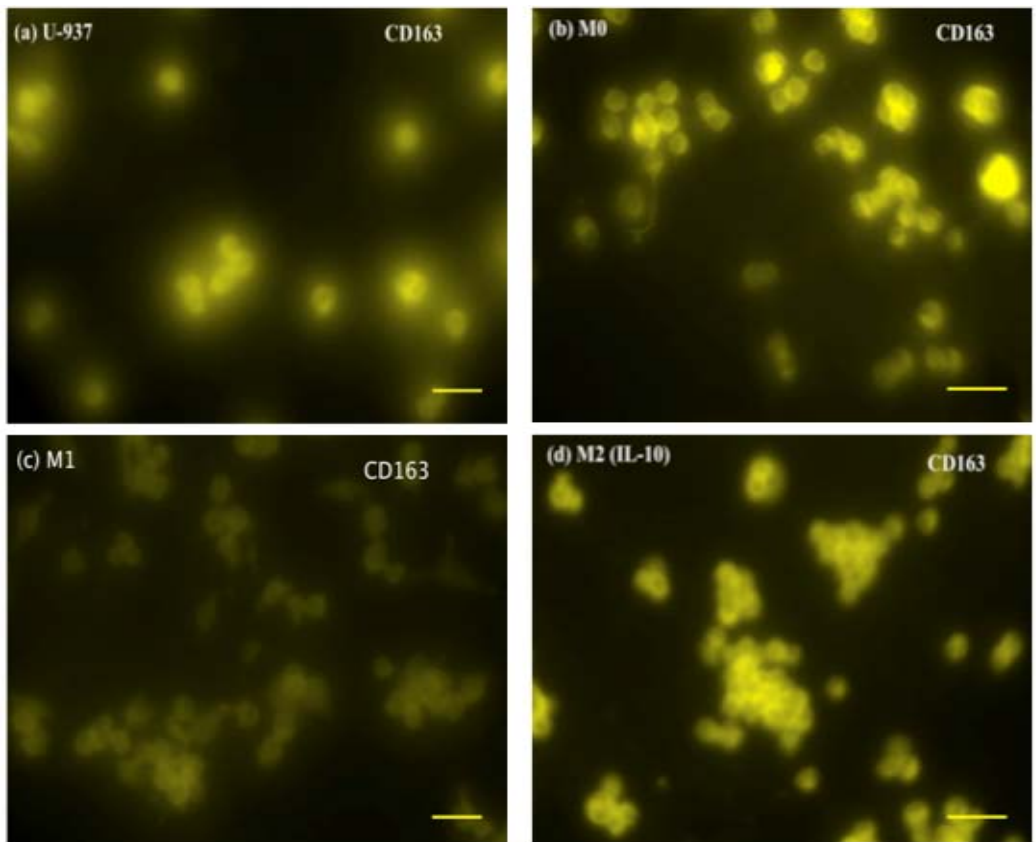
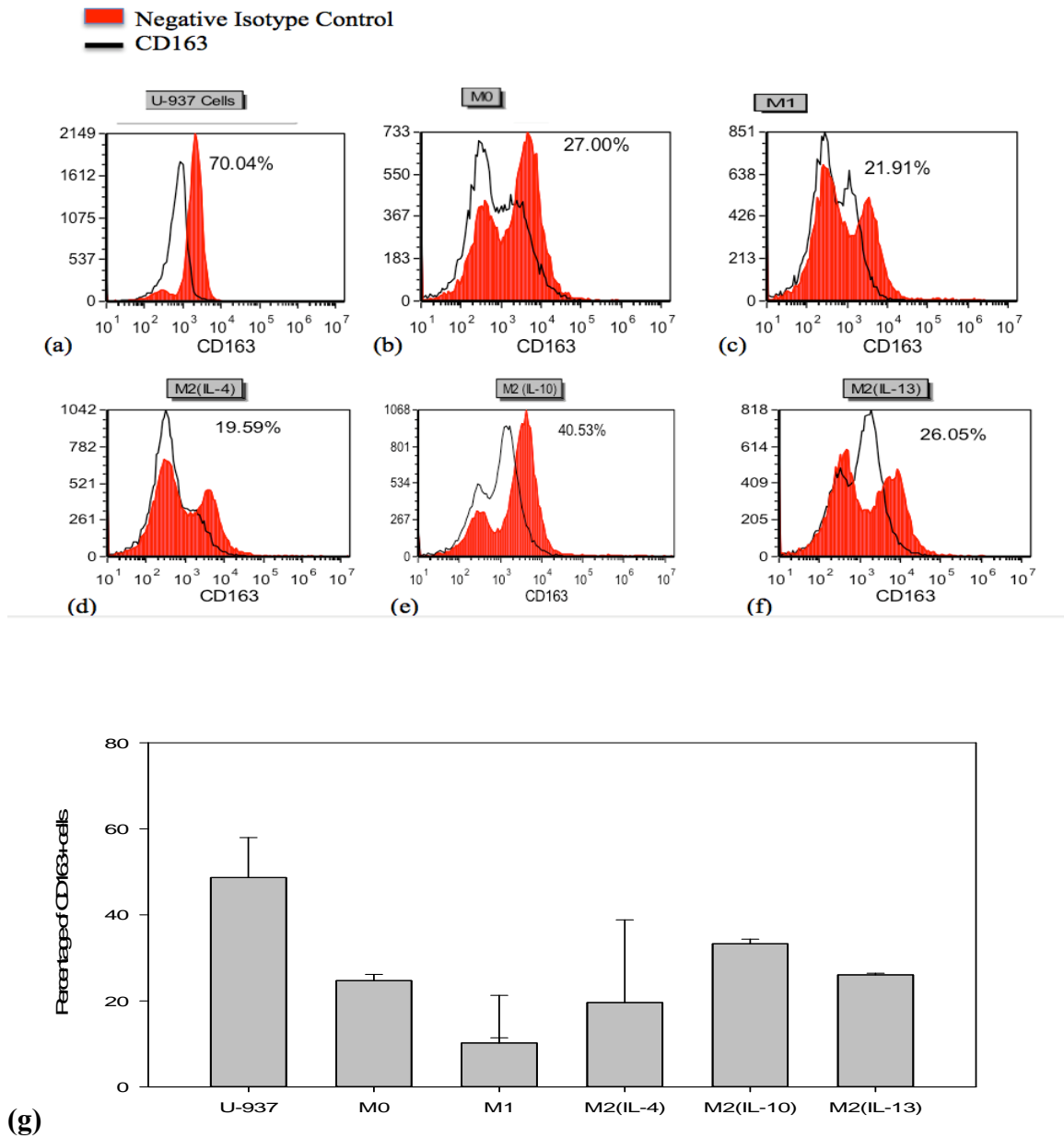Figure 15: Flow cytometry histograms showing CD206 expression in: (a) passage one untreated U-937 grown in pyruvate free medium, (b) passage 20 untreated U-937 grown in pyruvate- containing medium. (c) Percentage averages of CD206$^+$ in passage one grown in pyruvate -free medium and passage 20 untreated U-937 cells grown in pyruvate –containing medium N.S (p 0.665). The experiments were done in triplicate.

Figure 16: Immunofluorescent staining images of expression of CD163 in: (a) U-937 cells, (b) M0, (c) M1, (d) M2 (IL-10), d M2 (IL-4). (Scale bar = 50μm).

Figure 17: Flow cytometry histograms showing CD 163 expression in: (a) U-937, (b) M0, (c) M1, (d) M2 induced by IL-4 , (e) M2 induced by IL-10, and (f) M2 induced by IL-13. (g) : Percentage averages of CD163$^+$ in: U-937 cells, M0, M1, M2 induced by (IL-4), M2 induced by IL-10, M2 induced by (IL-13.)). In 3-separate experiments of flow cytometry, ANOVA showed no significance among the polarized cell populations (M1 or M2). The untreated un polarized U-937 cells showed a significant increase in CD163 compared to M1 (p <0.01).
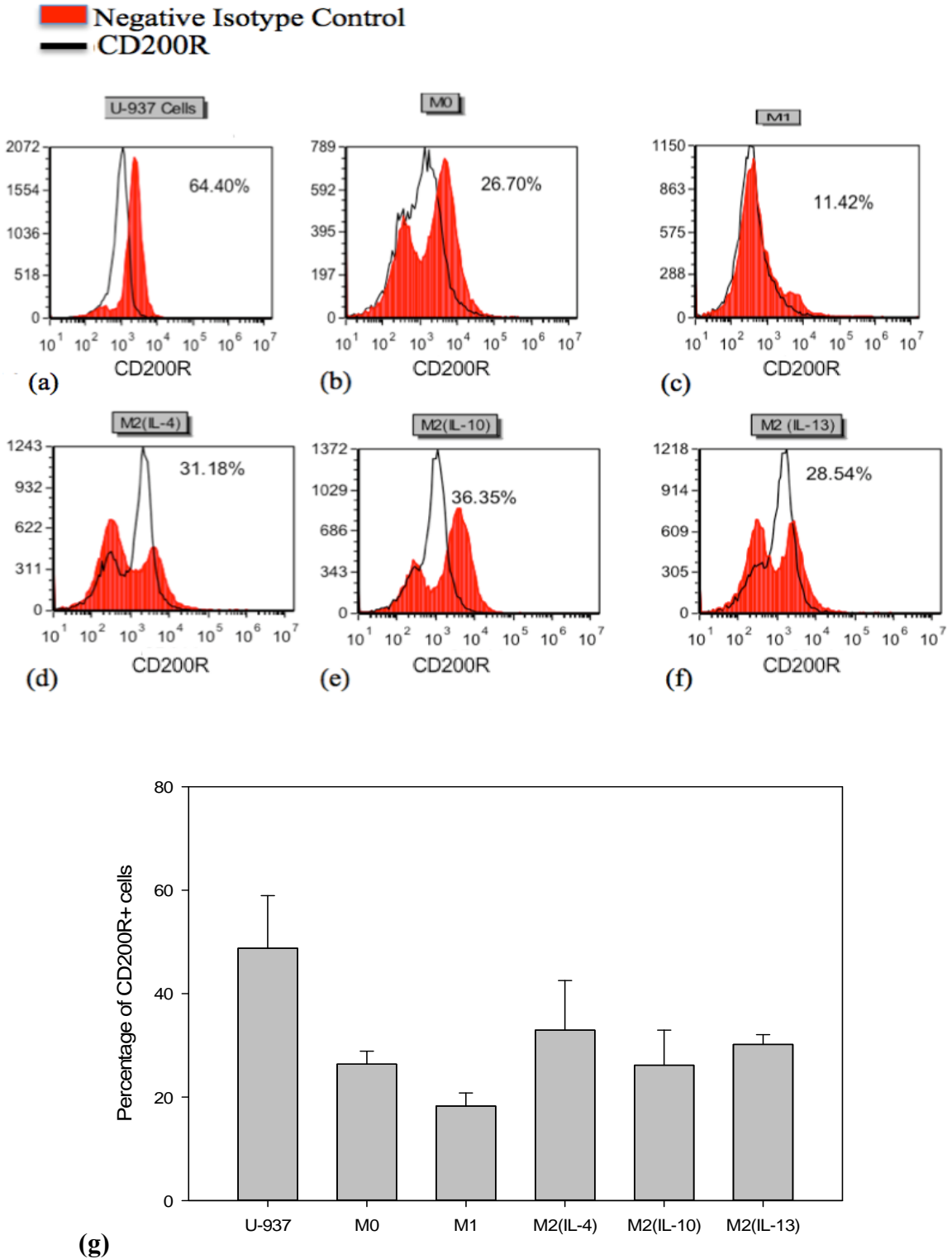
Figure 18: Flow cytometry histograms showing CD80 expression in: (a) U-937, (b) M0, (c) M1, (d) M2 polarized with IL-4, (e) M2 polarized with IL-10, and (f) M2 polarized IL-13. (g) : Percentage averages of CD200R$^+$ in: U-937 cells, M0, M1, M2 polarized with (IL-4), M2 polarized with IL-10, M2 polarized with (IL-13). In 3-

separate experiments ANOVA showed that M1 exhibited CD200R 3-fold less than U-937 cells (P 0.049). No statistically significance differences were seen in CD 200r expression in the M1 or M2 groups
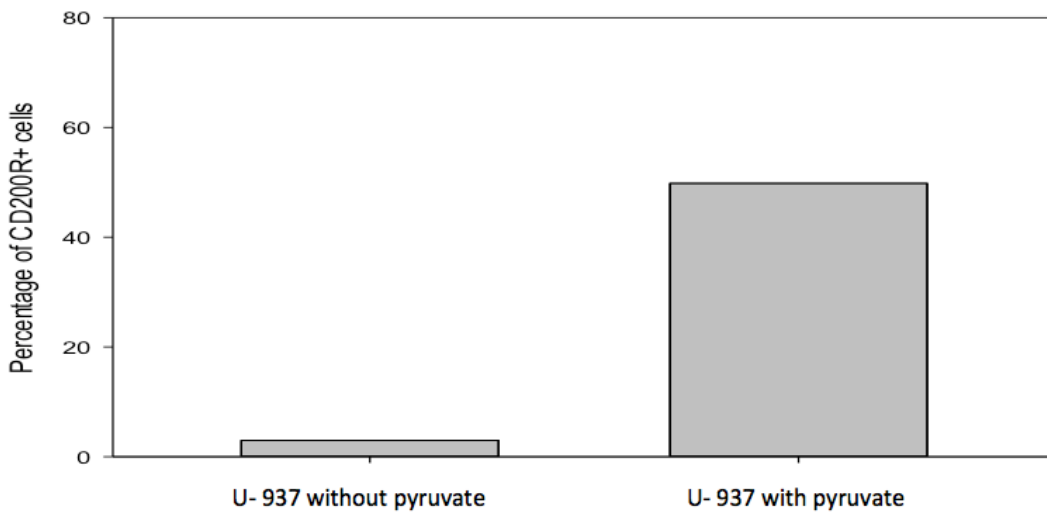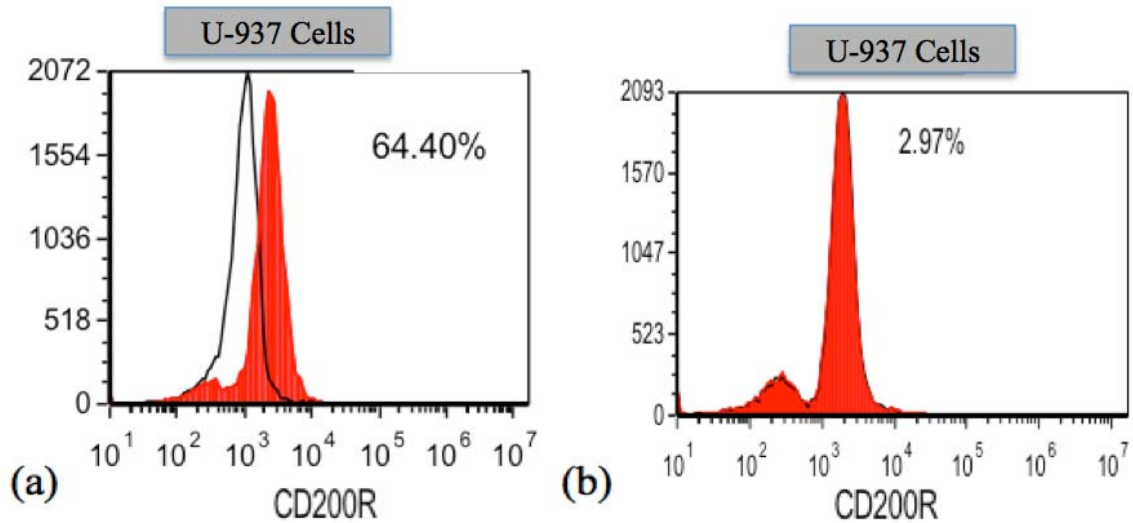
Figure 19: Flow cytometry histograms: CD200R expressed in: (a) U-937 cells grew in pyruvate- containing medium , (b) U-937 cells grew for two weeks after removing pyruvate from the medium, (c) Percentage averages of CD200R$^+$ in U-937 cells grew in pyruvate- containing medium and two weeks after removing pyruvate from the medium. ANOVA statistics of 3-separate flow cytometry experiments showed that U-937 cells expressed 16-fold decrease in CD200R after two weeks from stopping adding pyruvate to the media (p 0.011).
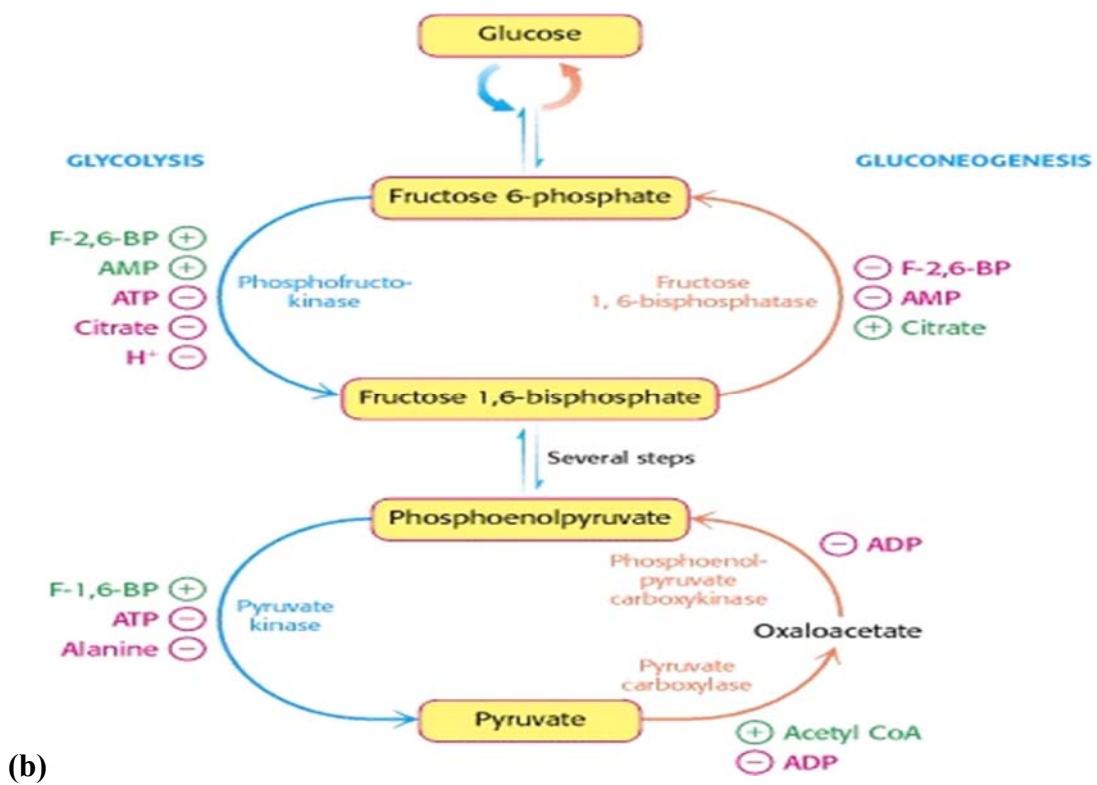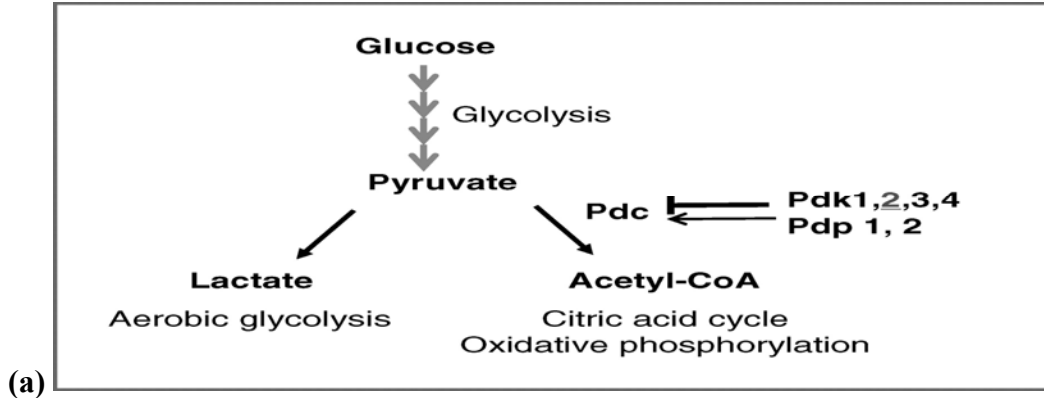
(a)

(b)

Figure 20: (a) Schematic diagram showing the source and the fate of pyruvate inside the cells adapted from (Contractor et al., 2012). (b) Schematic diagram showing pyrvate is part of both glycolysis and gluconeogensis adapted from (Berg et al., 2002) http://www.ncbi.nlm.nih.gov/books/NBK22423

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