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Conversion of the U937 Monocyte into "Macrophage-Like" Populations Exhibiting M1 or M2 Characteristics

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CONVERSION OF THE U937 MONOCYTE INTO “MACROPHAGE-LIKE” POPULATIONS
EXHIBITING M1 OR M2 CHARACTERISTICS.

A thesis submitted in partial fulfillment
of the requirement for the degree of
Master of Science

By

BRADLEY M. SHARP
B.S., Ohio State University, 2009

2013
Wright State University

WRIGHT STATE UNIVERSITY

GRADUATE SCHOOL

May 6, 2013

I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION
BY Bradley M. Sharp ENTITLED Conversion of the U937 Monocyte into
"Macrophage-like" Populations Exhibiting M1 or M2 Characteristics BE ACCEPTED
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Master
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ABSTRACT

Sharp, Bradley M. M.S., Microbiology and Immunology Graduate Program, Wright State University, 2013. Conversion of the U937 Monocyte into “Macrophage-like” Populations Exhibiting M1 or M2 Characteristics.

The U937 cell line is an oncogenic human monocyte cell line. These monocytes have the potential of differentiating into either macrophages or dendritic cells (Lawrence et al., 2011). This differentiation pattern depends on the characteristics of the tissue microenvironment (Kigerl et al., 2009). PMA (Phorbol 12-Myristate 13-Acetate) is a phorbol ester capable of transforming monocytic cells toward the macrophage pathway. Upon treatment with PMA, U937 cells under-go a series of morphological and functional changes. Traditionally monocytic cell lines are used as a model of macrophage function, because current human macrophage cell lines require a T-cell conditioned growth medium and contact with irradiated peripheral blood leukocytes (PBLs) to propagate (Lee et al., 1997). The PMA-treated monocyte is referred to as “macrophage-like,” meaning that the properties of the transformed cell line are not yet fully understood (Dockrell et al., 2010). These macrophages are clinically significant for possible cancer immunotherapy experimentation. Recently, two divergent macrophage subsets have been identified: M1 (pro-inflammatory) and M2 (anti-inflammatory). These macrophages are differentiated based on the cytokines present in the extracellular matrix (Kigerl et al., 2009). In order for the U937 cell line to be represented as an effective macrophage model, the ability of the cell line to polarize must be analyzed. This study is a review of the features necessary to convert the human U937 cell line into M1 or M2 morphological and functional subsets.

TABLE OF CONTENTS

	Page
I. OBJECTIVE	1
II. BACKGROUND & INTRODUCTION.....	3
The Macrophage	3
The M1 Macrophage	5
The M2 Macrophage	6
Origin of the Monocyte	10
The Human Macrophage	12
III. CURRENT LITERATURE	18
Murine Macrophages	18
Cell Surface Markers.....	18
Cytokine Production.....	20
Transcription Factor.....	22
Iron Transport	25
Peripheral Blood Mononuclear Cells (PBMC's)	29
Cell Surface Markers	30
Cytokine Production	33
Transcription Factor	35
Iron Transport	36
U937 Cells	39
Cell Surface Markers	39
Cytokine Production	41
Transcription Factor	43
Iron Transport	46
IV. DISCUSSION	48
V. CONCLUSION	51
VI. FUTURE STUDIES	53
IV. REFERENCES	54

LIST OF FIGURES

Figure	Page
1. M1 and M2 Macrophage Polarization	7
2. Signaling Pathways of Macrophage Subsets	9
3. Lineage of the Monocyte	11
4. Representative Morphology of PMA treated U937 cells	13
5. CD11b Up-regulation with Transformation	15
6. Phagocytosis of PMA treated U937 cells	16
7. Cell Surface Markers of Polarized Murine Macrophages	20
8. Cytokine Profile Expression of Polarized Murine Macrophages	21
9. NF- κ B Activation Diagram	23
10. Electrophoretic Mobility Shift Assay (EMSA) of NF- κ B in RAW 264.7 Murine Macrophages After Stimulation with IL-4 and IL-10	24
11. SDS PAGE Analysis of Ferritin Levels in IFN- γ Stimulated Murine Macrophages	27
12. Effect of IL-4 and IL-13 on Murine Macrophage Ferritin mRNA Levels	29
13. Flow Cytometric Analysis of Human Peripheral Blood Mononuclear Cells (PBMCs) for CD206 and CD86 after Treatment with Anti- and Pro-Inflammatory Cytokines	32
14. Cytokine Profile Expression of Polarized Human PBMCs	33

LIST OF FIGURES
(Continued)

Figure	Page
15. EMSA of NF- κ B Activation in PBMCs treated with IL-10.....	35
16. Ferritin levels in PBMCs differentiated into macrophage subsets	36
17. Flow Cytometric Analysis of CD14 and CD86 in U-937 Cells Treated with PMA	39
18. Western Blot Analysis of CD206 Cell Surface Markers	40
Before and After PMA Stimulation	
19. IL-6 Expression in U-937 and THP-1 cells stimulated with	42
Lipopolysaccharide (LPS) or Anti-Inflammatory Cytokines	
20. EMSA of NF- κ B Expression in U-937 cells treated with IL-4	43
21. EMSA of NF- κ B Expression in U-937 cells treated with IL-13	45
22. Northern Blot Analysis of Ferritin levels in U-937 and HL-60	46
Cells Stimulated with PMA	
23. Western Blot Analysis of Ferritin levels in U-937 and THP-1	47
Cells Stimulated with PMA	

LIST OF TABLES

Table	Page
1. Heterogeneity of Macrophage Subsets	10
2. Ferritin content in polarized PBMC's	38
3. Summary Table	50

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I: OBJECTIVE

A self-replicating human macrophage cell line does not currently exist. Two human macrophage cell lines have been identified, 2MAC and K1m. These lines have been established spontaneously from the peripheral blood of disease-free individuals. However, growth requires culture with T-cell growth factor (TCGF)-conditioned media (Dialynas et al., 1997); (Lee et al., 1997). Cell propagation requires contact with a peripheral blood leukocyte irradiated feeder layer. Variations exist in the ability of the K1m cell line to propagate based on the source of the peripheral blood leukocytes used to prepare K1m media. Lee et al. were unable to explain these fluctuations (Lee et al., 1997). These cell lines are not frequently used in modern-day research. This lack of use is most likely due to the necessity for TCGF-conditioned media and irradiated PBL feeder layer to culture these cells. Investigators use the human U937 or THP-1 monocyte cell lines to study macrophage-like functions in cell culture (Dockrell et al., 2010). The objective of this study was to characterize the morphological and biochemical properties of the U937 monocyte after exposure to phorbol 12-myristate 13-acetate (PMA) and subsequent exposure to polarizing cytokines. Macrophages in solid tumors promoting angiogenesis and metastasis are of the M2 phenotype (Biswas et al., 2012). Understanding the factors for conversion of U937 cells to either M1 or M2 phenotypes will provide insight for potential therapeutic strategies. These strategies will be directed to converting the M2 anti-inflammatory macrophage into an aggressive cytotoxic M1

macrophage. The characteristics of M1 and M2 polarized subpopulations in the U937 cell line following PMA stimulation are currently being established (Abdulhadi, F and Bigley, N.J. Personal Communication. 2013). Thus comparisons will be made using characteristics of cytokine polarized murine macrophage cell lines and cytokine-treated peripheral blood monocytes from humans.

Bone marrow precursors give rise to myelomonocytic cells, essential components of innate immunity (Biswas et al., 2010). The monocyte-macrophage pathway of differentiation shows both plasticity and diversity (Biswas et al., 2010). This study focuses on information leading to the differentiation and polarization of cells belonging to this lineage and to characterize the potential polarization of the U937 cell line to either the M1 or M2 phenotype. It also focuses on literature describing M1/M2 polarizations of murine macrophage cell lines, other oncogenic monocytes (THP-1 and HL-60), and on cytokine-treated peripheral blood monocytes of humans.

II: BACKGROUND & INTRODUCTION

THE MACROPHAGE

Macrophages are immune cells which originate from blood-circulating monocytes. Monocytes roll along capillary walls until they arrive at infected tissue sites. Here they upregulate their cell adhesion molecules and squeeze through capillary walls as differentiated macrophage progeny (Parham, 2009). Macrophages are characterized by phagocytosis (the ability to engulf pathogenic organisms), antigen presentation (the ability to present antigenic components on the cell surface), cytokine release (pro-inflammatory/anti-inflammatory mediators), and chemotaxis (the ability to move in response to chemokines) (Sintiprungrat et al., 2010). These cells are also characterized by a large irregular shape and a vast cytoplasm filled with vacuoles. Also termed “scavenger cells,” these leukocytes envelop and digest other dead cells, debris, and pathogenic microorganisms. Macrophages are generally the first to arrive at infected tissue sites. Pathogens are engulfed and degraded intracellularly. Viral and bacterial proteins are internally processed and their peptides are presented extracellularly on MHC class I and II complexes, respectively, where they are exposed to T-cells in the lymph nodes (Parham, 2009). Along with neutrophils, macrophages are the first line in

immune defense; however excessive activity of the classical inflammatory macrophage can cause damage to the host through establishment of autoimmune and inflammatory diseases (Sintiprungrat et al., 2010).

Functional diversity and plasticity of macrophages have been implicated in various disease conditions (Biswas et al, 2012). Recently, two divergent macrophage subsets (M1 and M2) have been identified. These macrophages are differentiated by the cytokines present in the extracellular matrix. M1 (classical) and M2 (alternative) macrophages are functionally antagonistic to one another. During the initial stage of inflammation, M1 macrophages dominate the site of infection with anti-microbial activity. During resolution of inflammation, however M2 macrophages dominate the infection site. M2 macrophages release anti-inflammatory cytokines such as IL-10 which negatively regulate M1 activity and secrete products implicated in wound healing (Biswas et al., 2012). Uncontrolled expression of the M1 macrophage has damaging consequences. During sepsis, macrophages and monocytes are activated into hyper-inflammatory M1 phenotypes, releasing excessive levels of tissue damaging cytokines. Sepsis can lead to organ failure and hemorrhaging from blood vessel damage (Biswas et al., 2012). Over-expression of M2 macrophages has also been implicated in disease progression, specifically cancer. It is believed that macrophages switch from an M1 phenotype at the initiation of tumor growth to an M2 phenotype as the tumor becomes established (Biswas et al., 2012). The products secreted by the tumor itself polarize macrophages to an M2 form that supports angiogenesis. Thus M2 polarity contributes to tumor progression (Biswas et al., 2012). Polarity balance is essential to adequate

immune function. The ability of these cells to cross-polarize is inherently macrophage-like in nature. The properties surrounding macrophage polarization must be investigated in order to determine if macrophage cell lines such as U937 cells are accurate models of macrophage function. Biswas et al. and other researchers have outlined criteria to distinguish M1 macrophages from their M2 counterparts.

THE M1 MACROPHAGE

Upon interaction with antigen presenting macrophages, T-helper 1 cells release IFN- γ . In association with LPS, this cytokine is involved in a positive feedback loop which results in stimulation of an activated pro-inflammatory macrophage (Kigerl et al., 2009). These pro-inflammatory leukocytes or M1 macrophages produce high levels of IL-12, IL-23, TNF- α , IL-1, and IL-6 in addition to reactive nitrogen intermediates. These reactive intermediates are produced by catalyzing L-arginine with the inducible nitric oxide synthase (iNOS) enzyme. When released, nitric oxide is highly toxic to a variety of bacteria and viruses. Thus M1 macrophages are involved in the targeting of microbes and infected cells for destruction. M1 macrophages utilize the glycolytic pathway, down-regulate fatty acid oxidation, and up-regulate iron storage proteins, as assessed by high H-ferritin levels. With respect to gene up-regulation, NF κ B levels are increased in M1 macrophage colonies (Biswas et al., 2012).

THE M2 MACROPHAGE

Macrophage interaction with T-helper 2 cells results in a release of IL-4. IL-4 alone, or in association with IL-13, causes polarization of macrophages into an anti-inflammatory or M2 phenotype (Mantovani et al., 2006). M2 macrophages are characterized by a high production of IL-10 and upregulated scavenger, mannose, and galactose receptors. These M2 cells are involved in wound healing and repair (Mantovani et al., 2006). These macrophages utilize arginase-1 enzymes to catalyze L-arginine into ornithine and polyamines. M2 macrophages also utilize oxidative phosphorylation for metabolic purposes, up-regulate genes relating to fatty acid oxidation, and up-regulate iron export proteins, as assessed by high ferroportin levels. M2 macrophages up-regulate scavenging receptors; phagocytosis of apoptotic cells leads to polarization into an anti-inflammatory M2 phenotype. NF κ B inactivation has been shown to be a novel feature of M2 polarization. PPAR- α and PPAR- δ gene upregulation has been shown to be associated with M2 polarization (Biswas et al., 2012).

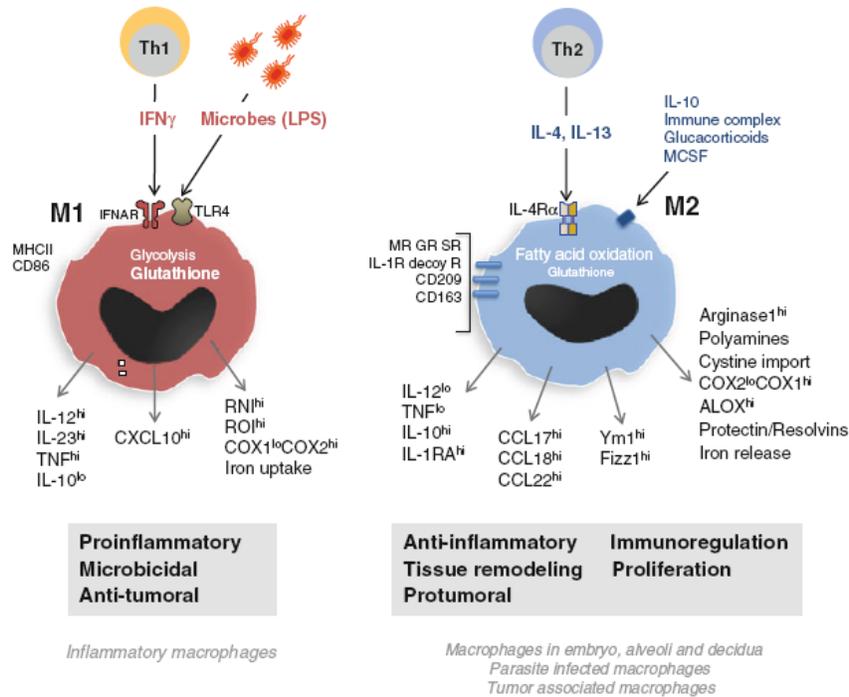


Figure 1: Polarization of M1 and M2 macrophages. M1 and M2 macrophages exhibit very different properties. Their polarization is driven by the surrounding microenvironment. M1 macrophage is rich in CD86, an MHC class II glycoprotein involved in antigen presentation. The M1 macrophage is also a high producer of pro-inflammatory cytokines such as IL-12 and TNF- α . M1 macrophages are characterized by high iron uptake, which is represented by up-regulation of iron storage molecules such as ferritin (ft). M2 macrophages are rich in mannose receptors (MR) such as CD206. They are characterized by production of anti-inflammatory cytokines, such as IL-10 and high iron release. Iron release is characterized through analysis of iron export proteins such as ferroportin (fpn). These are the primary characteristics that will be investigated for this study. Adapted from Biswas et al., 2012.

M1 and M2 macrophages also exhibit unique signaling pathways (Figure 2).

Lipopolysaccharide (LPS) stimulates Toll-like receptor-4 (TLR-4) to begin the transition into a pro-inflammatory M1 macrophage. LPS-stimulation results in downstream signaling to Toll/Interleukin-1-receptor-domain-containing adapter-inducing interferon- β (TRIF) and myeloid differentiation primary response gene-88 (MyD88) intermediate adapters. MyD88 activates intracellular kinases which lead to a signaling cascade

resulting in an activated nuclear factor-kappa-B (NF- κ B) transcription factor. NF- κ B controls the expression of pro-inflammatory cytokines tumor necrosis factor- α (TNF- α), interleukin-1- β (IL-1 β), interleukin-6 (IL-6), and interleukin-12 subunit p40 (IL-12p40). TRIF activates interferon-response factor 3 (IRF3). IRF3 activation results in expression of Interferon- α (IFN- α) and Interferon- β (IFN- β). Interferon- γ (IFN- γ) also encourages this M1 macrophage transition by activating signal transducers and activators of transcription 1 (STAT1). STAT1 up-regulates the expression of IFN-inducible chemokines which recruit other pro-inflammatory mediators to the site of interest (Biswas et al., 2012).

Interleukin-4 (IL-4) and Interleukin-13 (IL-13) stimulate the conversion to an M2 phenotype via the interleukin-4 receptor (IL-4R) and activate signal transducers and activators of transcription 6 (STAT6). STAT6 has been hypothesized to work in concert with other transcription factors such as peroxisome proliferator-activated receptor gamma (PPAR- γ) to produce an M2 macrophage (Biswas et al., 2012). These M2 signaling pathways are still vaguely understood. Macrophages with defective NF- κ B transcription factor molecules were shown to be immunosuppressive in nature. These macrophages were characterized by reduced inflammatory gene expression and endotoxin tolerance (Biswas et al., 2012). Variations in NF- κ B nuclear expression may shed a light on a transcriptional profile for M1 and M2 macrophages. The M1 macrophage would presumably be high in nuclear NF- κ B expression while the M2 macrophage would presumably be low in nuclear NF- κ B expression.

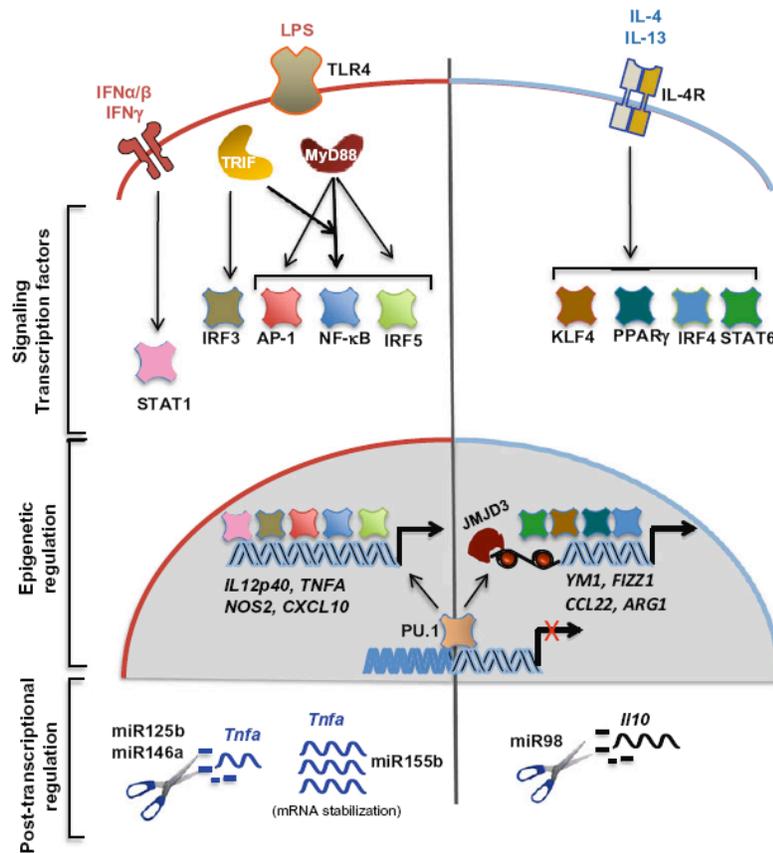


Figure 2: Signaling pathways of M1 and M2 macrophages. M1 and M2 macrophage activators follow very different intracellular signaling pathways. For M1 polarization, LPS utilizes the NF- κ B transcription factor. For M2 polarization, IL-4 utilizes the PPAR- γ transcription factor. This study will focus on NF- κ B activity and its ability to be suppressed by anti-inflammatory cytokines. Ideally, NF- κ B activity should be higher in macrophages stimulated with LPS rather than IL-4 or IL-13. Adapted from Biswas et al., 2012.

Table 1: Heterogeneity of macrophage subsets. The heterogeneity of phenotypes between M1 and M2 macrophages is summarized below. *Neisseria meningitidis* is an example of a response to a bacterial stimulus applied to the macrophage. Classically (M1) and alternatively activated (M2) macrophages exhibit different expression profiles. Adapted from (Taylor et al., 2005).

	Innate activation	Classical activation	Alternative activation
Stimuli	<i>Neisseria meningitidis</i> ^b	IFN- γ	IL-4/13
Effects on surface antigens	<ul style="list-style-type: none"> ↑ MARCO ↑ CD200 ↑ Co-stimulatory antigens (synergy with IFN-γ) 	<ul style="list-style-type: none"> ↑ MHC class II ↑ CD86 ↓ MR 	<ul style="list-style-type: none"> ↑ MR ↑ Dectin-1 ↑ CD23 ↓ CD14 CD163
Metabolic changes	<ul style="list-style-type: none"> ↑ iNOS Respiratory burst 	<ul style="list-style-type: none"> ↑ iNOS Respiratory burst 	<ul style="list-style-type: none"> ↑ Arginase
Secretory responses	<ul style="list-style-type: none"> Synergy with IFN-γ-induced responses 	<ul style="list-style-type: none"> Pro-inflammatory cytokine and chemokines (TNFα, IL-12, IL-1, IL-6, IP-10, MIP-1α, MCP-1) and low MW mediators 	<ul style="list-style-type: none"> IL-1RA IL-10 Chemokines (e.g., AMAC-1) Chitinase-like proteins (Ym1/2)
Functional effects of activation	<ul style="list-style-type: none"> Phagocytosis (by regulation of receptors) Pro-inflammatory signaling via TLR Enhanced costimulatory antigen expression 	<ul style="list-style-type: none"> ↑ Antigen presentation ↑ Killing of intracellular pathogens Pro-inflammatory 	<ul style="list-style-type: none"> ↑ Fibronectin and matrix associated proteins. Promotes cell growth, collagen formation, and tissue repair. ↑ Humoral/allergic immunity ↑ Parasite killing

ORIGIN OF THE MONOCYTE

An established human macrophage cell line is not currently available.

Researchers use oncogenic monocytes such as the U937 or THP-1 cell lines to mimic macrophage behavior. Monocytes are blood-circulating leukocytes. Monocytes express CD11b (an attachment integrin), and CD14 (an LPS receptor which associates with Toll-like Receptor 4 or 2 (TLR4/TLR2) and induces LPS stimulated signal transduction) (Taylor et al., 2005). They originate from haematopoietic stem cells from adult bone marrow.

When activated toward the monocyte lineage, these stem cells differentiate into Common Myeloid Progenitors (CMPs). These CMPs differentiate into Granulocyte and Macrophage Progenitors (GMPs). GMPs then follow a pathway into Macrophage and Dendritic Cell Progenitors (MDPs). Finally, the human monocyte develops from these MDPs (Figure 3). As shown in Figure 3, the haematopoietic stem cells must undergo a series of divisions before they are transformed into blood circulating monocytes (Chow et al., 2011). The monocyte has the potential to differentiate along a macrophage or dendritic cell pathway. Macrophage differentiation requires the activation of Runt-related transcription factor, or RUNX1, which encodes the ETS family transcription factor PU.1. PU.1 expression causes the monocyte to follow and maintain a macrophage differentiation pattern (Lawrence et al., 2011).

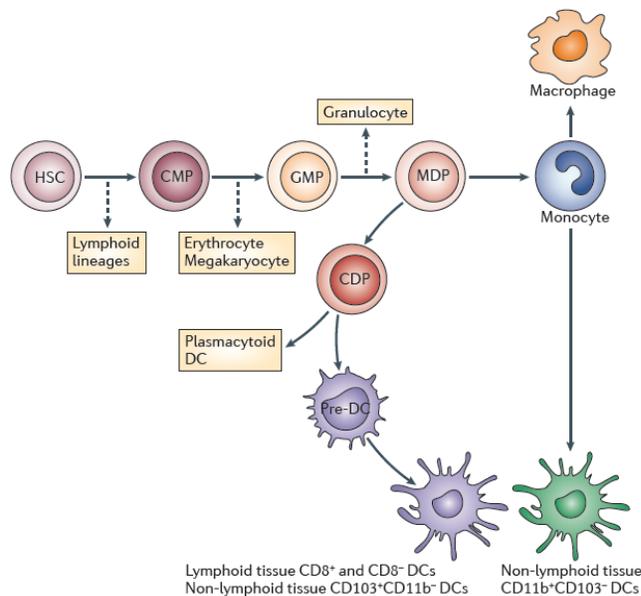


Figure 3: Maturation of a monocyte from a haematopoietic stem cell progenitor. Adapted from Chow et al., 2011.

THE HUMAN MACROPHAGE

Traditionally, U937 monocytes have been used as an in vitro model of macrophage function because primary human tissue macrophages cannot be replicated. When converted to a macrophage lineage, these cells exhibit characteristics which are similar to the human macrophage. The U937 cell line is a human hematopoietic cell line established from a 39 year old man with histiocytic lymphoma. These cells display properties of immature monocytes (Minafra et al., 2011). U937 cells are characterized by lysozyme production with no surface immunoglobulin (Ig) or immunoglobulin secretion. These cells are ovoid in shape with little to no variation in cell morphology. U937 nuclei are large and irregular in shape (Sundstrom et al., 1976). U937 cells have been widely used as an in vitro model through which to study cancerous cell behavior in haematopoietic patients (Minafra et al., 2011).

Phorbol 12-Myristate 13-Acetate (PMA), also known as TPA, is a phorbol ester used to convert monocytes into macrophages. With addition of the PMA reagent, the non-adherent U-937 cells up-regulate their cell adhesion molecules to attach to the flask surface and differentiate (Abbro et al., 2005). PMA-treated U937 cells express high levels of CD11b and protein kinase C (PKC) (Sintiprungrat et al., 2010). Upon PMA addition, beta-2 integrins (CD11a, CD11c, CD18 and CD11b) allow the monocytes to attach to the flask surface. PMA then activates the calcium and phospholipid-dependent isoforms of PKC and stimulates cyclic AMP metabolism, causing maturation into a macrophage-like morphology (Hass et al., 2011).

As shown below, U937 monocytic cells are transformed from a non-adherent, rounded, blood-circulating lymphocyte to an adherent flattened “macrophage-like” cell with PMA addition (Figure 4). Transformed cells develop elongated arms or pseudopods which increase surface area for antigen uptake. These physical characteristics can be used to differentiate microscopically between monocyte and macrophage morphologies; however, morphological differences alone are not sufficient for macrophage identification. Other morphologically similar subsets like dendritic cells may be misidentified based on morphology alone.

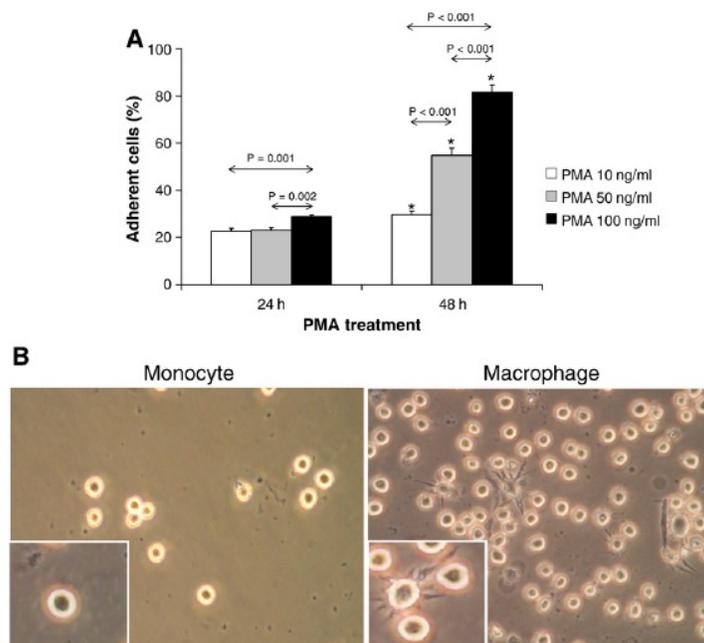


Figure 4: Change in morphology and adherence with increasing doses of PMA. (A) Sintigrungrat et al., exposed U-937 cells to varying concentrations of PMA (10ng/mL, 50ng/mL, and 100ng/mL) for different time points (24 hours and 48 hours). Adherent cells were counted for each test group. The 48-hour test groups were compared to their corresponding 24-hour test group for significant differences. Significance was identified by a p-value less than 0.05. (B) Microscopic pictures were taken of the untreated U937 cell (left) and the 48-hour 100ng/mL-treated macrophage-like cell (right). Adapted from Sintigrungrat et al., 2010.

Higher PMA concentration and longer culture time effectively increase cellular adherence of macrophage-like cells. A two-fold increase in adherence was observed in the 50ng/mL and 100ng/mL 48-hour test groups relative to the 24-hour test groups. Thus, a dosage of 100ng/mL and a 48-hour culture time results in the highest yield of adherent macrophage-like cells. Monocytes roll along capillary walls until they reach the site of infection. Monocytes up-regulate integrins for cellular attachment at infection sites. In the U937 cell line this dramatic increase in adherence most likely reflects an increase in CD11b expression. CD11b is an integrin associated with cellular attachment (Figure 5).

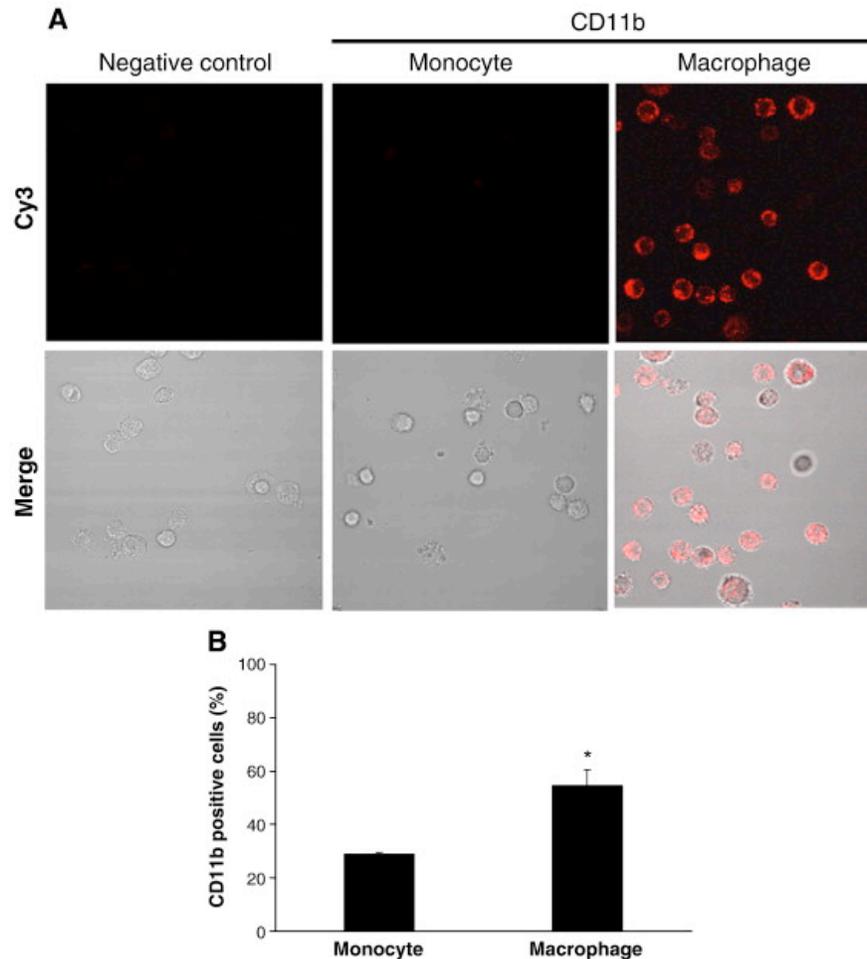


Figure 5: U937 cellular attachment may be associated with CD11b up-regulation. Sintiprungrat et al., exposed U937 monocytes to 100ng/mL PMA for 48 hours. Cells were incubated with monoclonal anti-CD11b antibody. Untreated (monocyte) and PMA-treated (macrophage) cells were then introduced to conjugated anti-Ig FITC antibody or conjugated anti-Ig Cy3 antibody. Images were taken of the PMA transformed and untreated cells with confocal fluorescence technology. Red fluorescence identifies CD11b positive cells. Flow cytometric analysis was performed on the untreated and treated subsets as well. Untreated cells were used as a control for the PMA-treated cells. Significance was identified by a p-value less than 0.05. Adapted from (Sintiprungrat et al., 2010).

PMA transformation was marked by increased cellular attachment and up-regulation of attachment integrin CD11b. A two-fold increase was observed of CD11b positive cells with addition of 100ng/mL PMA for 48 hours. Integrins allow the

monocyte to decrease its momentum along the capillary wall, transform into a macrophage, squeeze through endothelial cells and target pathogenic organisms. However, integrin attachment is not an exclusive feature of macrophages. Neutrophils and several other cells are responsible for inflammatory responses to pathogen intrusion. In order to understand if the PMA converted monocyte is able to functionally present itself as a macrophage, phagocytic ability must be analyzed.

One of the key components indicative of macrophage transformation is phagocytic ability. Macrophage cells are unique in their ability to envelop extracellular organisms and degrade them intracellularly. Reactive oxygen intermediates are stored in vesicles throughout the macrophage cytoplasm. These intermediates react internally with pathogens as they are engulfed through phagocytosis. Phagocytic ability can be analyzed by the ability of cells to internalize particles such as polyethylene. As shown in Figure 6, the converted U937 “macrophage-like,” cell is capable of enveloping polyethylene particles.

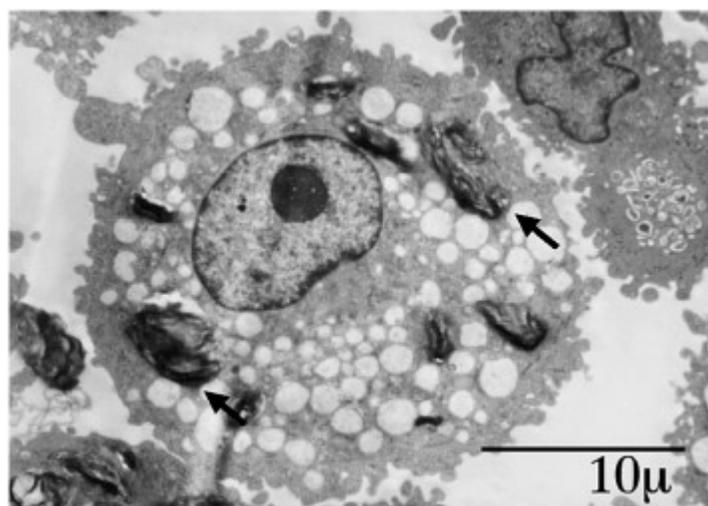


Figure 6: Phagocytosis of PE particles by the PMA converted U937 cell. Matsusaki et al., exposed U937 cells to 100nmol/L of PMA for 72 hours. Cells were then

introduced to 0.1 to 0.5mg/mL polyethylene particles and attached to a ventilated rotating device for 6 hours. After 6 hours, photos were taken of the transformed cells with an electron microscope. Adapted from (Matsusaki et al., 2007).

Macrophages are heterogeneous in nature and are known for their plasticity and diversity. In order to investigate if PMA conversion of U937 cells establishes an adequate in vitro model of macrophages, these cells must be analyzed with respect to polarization criteria. Current literature identifies two distinct macrophage subsets (M1 and M2). These subsets have been shown to have different physical and biochemical properties from one another. This study characterizes the U937 cell line with respect to properties previously investigated in murine cell lines and human peripheral blood mononuclear cells.

III: CURRENT LITERATURE

MURINE MACROPHAGES

Murine macrophage cell lines such as RAW 264.7 and J774.1 are well established in vitro models of tissue macrophages. These cell lines, derived primarily from BALB/c mice, have been successfully polarized using pro-inflammatory and anti-inflammatory cytokines (He et al., 2011) (Murphy et al., 2008) (Biswas et al., 2012). The characteristics of these polarized and un-polarized macrophages provide distinct properties which isolate subsets from one another. Characteristics have been outlined which indicate M1 versus M2 polarization in the murine model. These characteristics include cell surface markers, cytokine production, transcription factors, and iron transport (Biswas et al., 2012).

MURINE CELL SURFACE MARKERS

Cell surface markers are useful identification tags. These markers are often molecules that bridge the extracellular and intracellular environment. As signaling molecules, these cell surface proteins may serve to distinguish polarized subsets from one another. Two cell surface markers have been identified which isolate M1 macrophages from M2 macrophages. M1 macrophages have been shown to up-regulate CD86 protein levels (Kigerl et al., 2009) (Sun et al., 2012). CD86 is an MHC class II glycoprotein B7 co-stimulatory molecule involved with antigen presentation. M2

macrophages up-regulate the mannose receptor (MR) CD206 (Kigerl et al., 2009). Mannose receptors can recognize molecules such as sulfated sugars, collagen, and D-mannose terminal sugars. CD206 is mainly intracellular, with only 10-30% expressed on the cell surface of unstimulated macrophages. However, when naïve macrophages are exposed to IL-4, IL-13 and IL-10 they upregulate CD206. Conversely, CD206 is also down-regulated, but not abolished when macrophages are exposed to IFN- γ (Paveley et al., 2011). Thus CD206 up-regulation is indicative of M2 polarization, because IL-4 and IL-13 are M2 macrophage polarizing cytokines (Biswas et al., 2012).

In murine peritoneal macrophages, classically activated M1 macrophages (IFN- γ and LPS stimulated) were shown to express the greatest amounts of CD86 (Figure 7). There is a two-fold increase in CD86 expressed in the M1 macrophage population when compared to unstimulated macrophages. Alternatively activated M2 macrophages (IL-4 stimulated) were shown to express CD86 levels similar to that of unstimulated macrophages. Alternatively activated M2 macrophages were shown to express the greatest amounts of CD206, while classically activated M1 macrophages expressed CD206 levels similar to that of unstimulated macrophages (Figure 7). There is a five-fold increase in CD206 expressed in the M2 macrophage population when compared to unstimulated macrophages. In our lab at Wright State University, Adam Reichard was unable to find any distinctions between CD86 and CD206 on the surface of naïve and cytokine treated J774.1 macrophages (Reichard, A. and Bigley, N.J. Personal Communication. 2012). However, because CD86 and CD206 are prominent cell surface markers for polarization in current literature, they will be analyzed in this review.

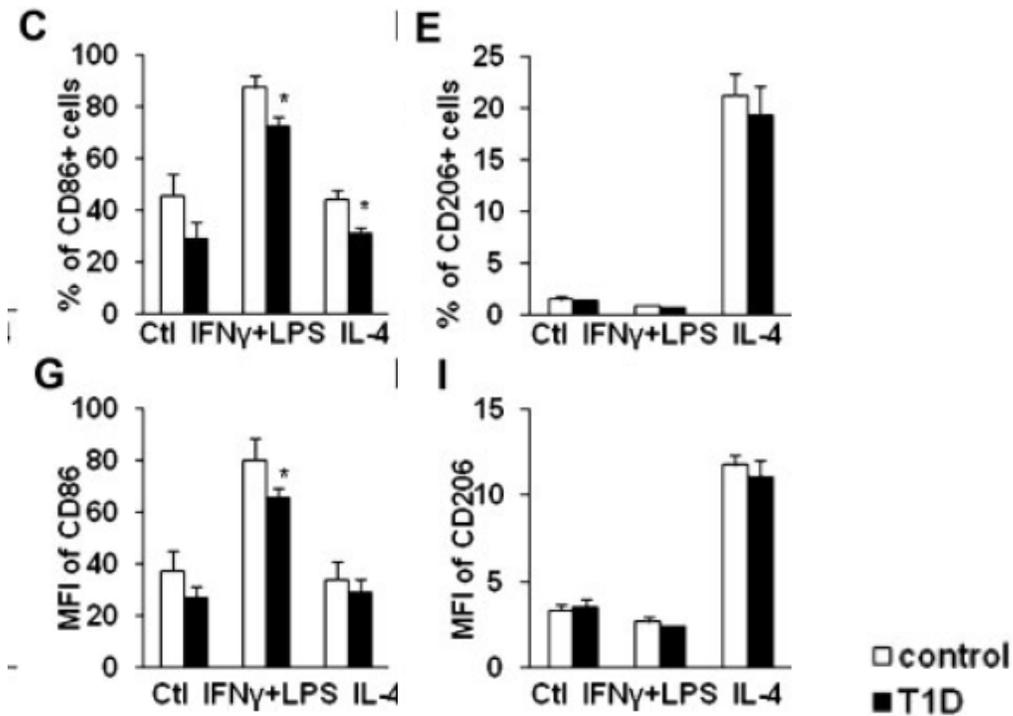


Figure 7: Flow cytometric analysis of macrophage subsets. Sun et al. collected mouse peritoneal exudate cells from 6 to 8 week old C57BL/6 mice. Cells were grown on 2% gelatin plates. Macrophages were isolated using flow cytometry for macrophage markers CD11b and F4/80. Control macrophages were compared to Type 1 Diabetic mouse subsets (T1D) to observe any changes in cell surface content with diabetic onset. Both subsets were subjected to treatment with IL-4 (1,000U/mL) or IFN- γ (50ng/mL) and LPS (1 μ g/mL) for 24 hours. Flow cytometric analysis was performed on macrophage subsets for cell surface markers CD86 and CD206. The mean fluorescence intensity and percent of CD86 and CD206 positive cells were analyzed. Adapted from Sun et al., 2012.

MURINE CYTOKINE PRODUCTION

M1 and M2 macrophages can be distinguished from one another based on the release of certain cytokines. Pro-inflammatory cytokines include IL-6, IL-12, and TNF- α . These cytokines are released in a greater amount from M1 polarized macrophages. The predominant anti-inflammatory cytokine is IL-10. This cytokine is released at greater levels from M2-polarized macrophages than M1-polarized macrophages (Biswas et al., 2012). Cytokines are environmental stimuli to cells of the

immune system. The signals triggered by these proteins enable immune cells to modify their behavior and composition based on their extracellular environment.

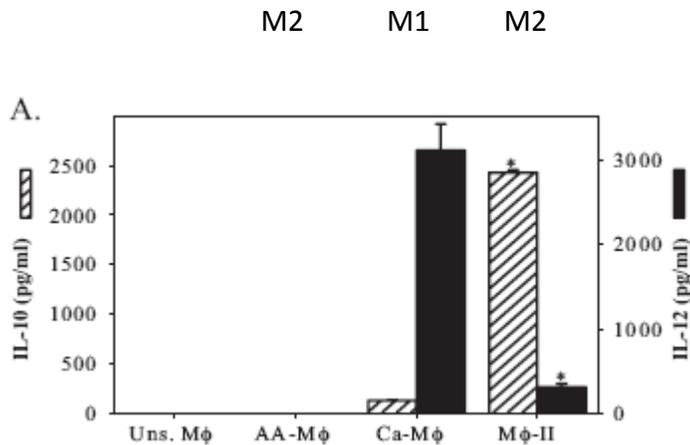


Figure 8: Cytokine profiles for polarized murine macrophage subsets. Edwards et al. extracted bone marrow derived macrophages from 6 to 8 week old BALB/c mice and subsequently polarized these cells using pro- and anti-inflammatory mediators. An M1 population was established by stimulating bone marrow derived cells with 100U/mL IFN- γ followed by 10ng/mL LPS (Ca-M ϕ). Two M2 populations were established by treatment of bone marrow derived macrophages with 10U/mL IL-4 (AA-M ϕ) or 10ng/mL LPS with immune complexes IgG and Ovalbumin (M ϕ -II). Sixteen hours after activation, macrophage supernatants were harvested and analyzed for IL-10 (anti-inflammatory) and IL-12 (pro-inflammatory) cytokines with ELISA. Adapted from Edwards et al., 2006.

M ϕ -II macrophages are currently categorized as M2 macrophages, because of their anti-inflammatory nature. This high IL-10 cytokine profile is indicative of M2 macrophage polarization. The alternatively activated macrophage population (AA-M ϕ) did not express IL-10 as expected. In fact, the classically activated macrophage population (Ca-M ϕ) expressed higher IL-10 levels than the alternatively activated M2 macrophage. This finding suggests that IL-10 release is dependent upon the polarizing stimulant. In this case, IgG and ovalbumin were necessary to produce an IL-10 secreting M2 macrophage. High IL-12 cytokine secretion is indicative of classically polarized M1

macrophages. Figure 8 illustrates the opposing nature of the production of these two cytokines in-vitro. The incidence of a pro-inflammatory cytokine release is accompanied with a decrease in anti-inflammatory cytokine release and vice versa.

MURINE TRANSCRIPTION FACTOR

NF- κ B is a transcription factor that has been implicated in macrophage polarization. NF- κ B consists of a hetero-dimeric molecule made up of p65 and p50 subunits. In the cytoplasm, NF- κ B exists in its inactive form as a multi-unit complex paired with the I κ -B α subunit. When activated by a pro-inflammatory cytokine such as TNF- α , the multi-unit molecule becomes phosphorylated, resulting in detachment of the p65 and p50 subunits and subsequent nuclear translocation. I κ -B α is ubiquitinated and targeted for proteasomal degradation. Nuclear NF- κ B binds to and transcribes gene sequences in the macrophage DNA. The transcription of these genes results in an inflammatory response (Ahn et al., 2005). The NF- κ B signaling cascade is shown in Figure 9. Pro-inflammatory behavior is typical of the M1 macrophage. Thus increased NF- κ B nuclear translocation is indicative of M1 macrophage polarization.

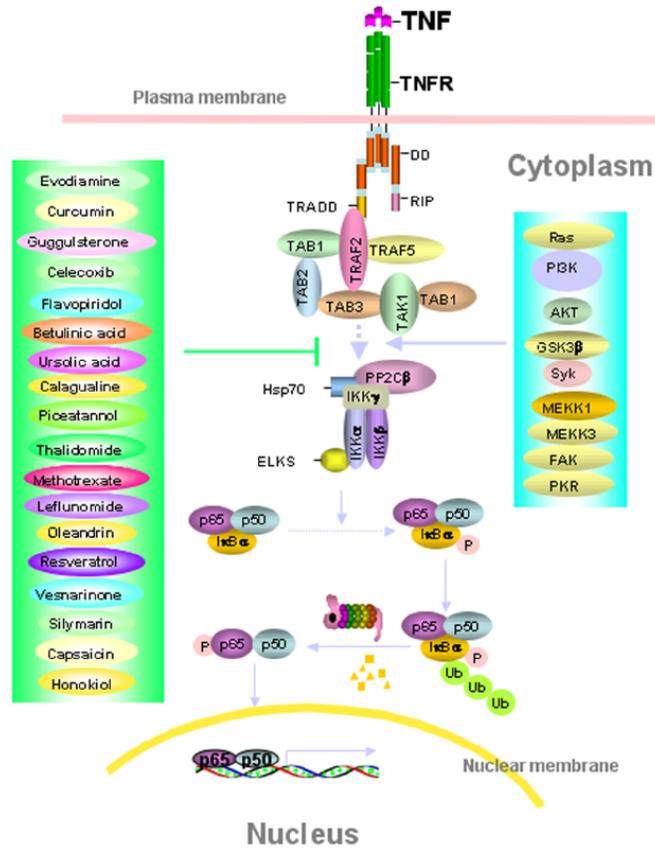


Figure 9: TNF- α mediated NF- κ B activation and nuclear translocation. Pro-inflammatory cytokine TNF- α interaction with the plasma membrane results in phosphorylation of the I κ B- α subunit of the NF- κ B heterodimer. This results in the proteosomal degradation of the I κ B subunit and subsequent translocation of the p65/p50 heterodimer into the nucleus. Therefore, free nuclear NF- κ B is an indication of NF- κ B activation. Adapted from Ahn et al., 2005.

The M1 macrophage is expected to be rich in free nuclear NF- κ B, because transcription of NF- κ B is necessary for the production of pro-inflammatory cytokines. The M2 macrophage is expected to have less nuclear NF- κ B, because these macrophages do not elicit pro-inflammatory responses. M1 and M2 macrophages use different activation pathways for transformation. The M1 activation pathway uses NF- κ B directly while the M2 activation pathway uses other transcription factors for

polarization. NF- κ B activation increased in RAW 264.7 macrophages stimulated with LPS (Figure 10). Nuclear NF- κ B levels were decreased with addition of anti-inflammatory cytokines IL-4 and IL-10. IL-4 was able to diminish NF- κ B concentration in the nucleus by 50% at a concentration of 100ng/mL (Figure 10). There appears to be a gel shift between where the NF- κ B arrow and the actual location of the band in the gel. This shift is not accounted for in the literature.

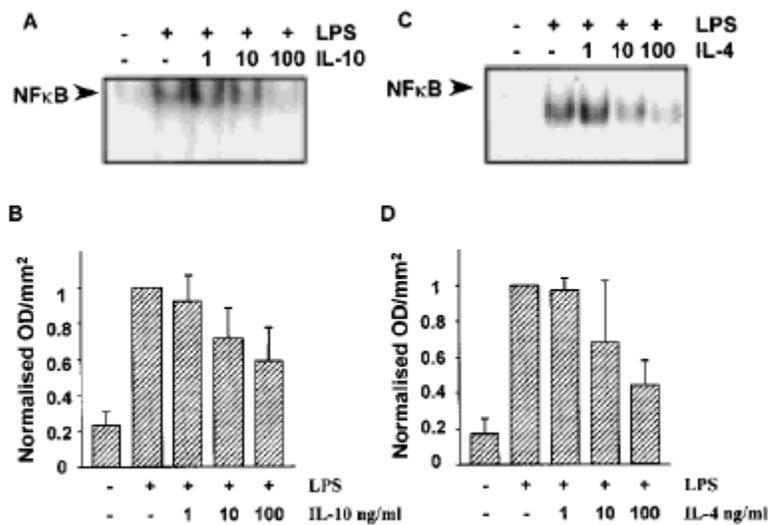


Figure 10: NF- κ B EMSA of nuclear extracts from RAW 264.7 murine macrophages treated with varying concentrations of IL-10 or IL-4. Clarke et al., stimulated RAW 264.7 macrophages with or without 10ng/mL LPS for 60 minutes. Macrophages were separated into testing groups based on the quantity of cytokine added after LPS stimulation. Macrophages were stimulated with or without 1, 10, or 100ng/mL of IL-10 (A) or IL-4 (C). Nuclear extracts were analyzed via an Electrophoretic Mobility Shift Assay. Each band represents positive binding of free nuclear NF- κ B. Densitometric quantification was performed on each test group and normalized to the LPS alone treatment group (=1) for (B) and (D). Adapted from Clarke et al., 1998.

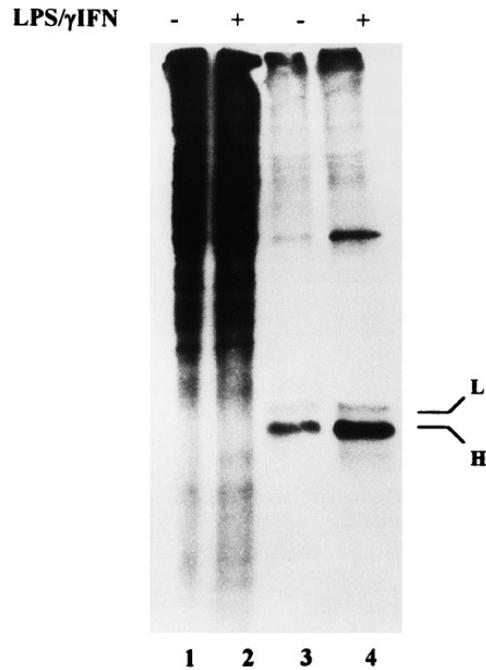
As shown in Figure 10A and C, NF- κ B activation can be modified by polarizing cytokines. Increased nuclear translocation of this transcription factor may be indicative

of an inflammatory macrophage response. This response can be decreased with addition of anti-inflammatory cytokines such as IL-4 and IL-10. Anti-inflammatory cytokines such as IL-4 use different signaling pathways than LPS or IFN- γ for activation (Figure 2). M2 polarization may inhibit NF- κ B activation through intra-cellular signaling. Addition of anti-inflammatory cytokines IL-4 and IL-10 may have initiated a phenotypic switch based on the change in nuclear NF- κ B composition.

MURINE IRON TRANSPORT

Iron metabolism is a fundamental process during innate immunity. Pathogenic organisms need Iron to reproduce and augment their virulence. Iron metabolism genes in macrophages can be regulated by cytokines. Thus cytokines can control the Iron concentration in a microenvironment by polarizing macrophages. Macrophages absorb iron from circulation to produce reactive oxygen intermediates. Reactive intermediates are responsible for the cytotoxic effects of the M1 macrophage. In addition, macrophage removal of iron from circulation prevents pathogenic utilization of the element. Both macrophages and bacteria use iron for energy production. Bacteria use their cell walls and macrophages use mitochondria. With mitochondria, mammalian cells can utilize oxidized iron for adenosine tri-phosphate (ATP) production via the electron transport chain. Macrophages possess the ability to store iron, which bacteria do not. These ferritin storage proteins enable the macrophage to have an advantage over bacteria in a microenvironment. M1 macrophages have been characterized as having high iron retention. M1 polarization resulted in an increase in cellular ferritin (Ft) protein levels (Recalcati et al., 2012)(Recalcati et al., 1998).

Ferritin is an iron storage protein that is found throughout the cell. Ferritin is found in the cytosol, nucleus, mitochondria, and lysosomes of the cell. A fully saturated ferritin molecule can hold up to 4,500 Iron atoms. Ferritin proteins are composed of an H and an L subunit. The H-subunit is held together primarily by hydrogen bonds while the L-subunit is held together primarily by salt bridges. H and L subunits come together to produce a 24-subunit ferritin molecule. The H-subunit contains a ferroxidase catalytic center which is absent in the the L-subunit. The ferroxidase center is where the process of iron storage begins. At this center, iron atoms are oxidized for processing. The L-subunit contains a nucleation site where oxidized iron atoms are arranged for iron storage. Recombinant H-subunit homopolymers were able to sequester and oxidize iron atoms seven times faster than recombinant L-subunit homopolymers. H-subunit rich ferritin proteins are found in lymphocytes such as macrophages and monocytes (Koorts et al., 2007). M1 macrophages need to oxidize iron to produce reactive oxygen intermediates. Ideally, M1 macrophages should favor an H-subunit rich ferritin profile for efficient processing of iron.



Subgroup	H-ferritin (ng/mg protein)	L-ferritin (ng/mg protein)
Control (no cytokines)	218 (+/-) 66	80 (+/-) 21
M1 (IFN- γ /LPS) 24 hrs	489 (+/-) 93*	102 (+/-) 47
* - significance (p-value less than 0.001 vs. control)		

Figure 11: SDS PAGE of H and L Ferritin levels after LPS/IFN- γ stimulation in J774.1 macrophages. J774.1 macrophages were polarized into an M1 phenotype with IFN- γ /LPS (lanes 2 and 4) or were left un-stimulated (lanes 1 and 3). Cells were also incubated with radioactive amino acids. In lanes 3 and 4, cellular extracts were introduced to recombinant antibodies against mouse ferritin H and L subunits. Equal amounts of radioactive proteins were loaded into the gel. Lanes 1 and 2 represent total protein aliquots in the supernatants of treated and untreated cells. Radioactivity was analyzed via fluorographic photography. H- and L- subunit quantities were analyzed via densitometric scanning techniques relative to the untreated macrophage population. The table represents an immunoassay of H- and L-ferritin subunits in this same experiment after cytokine stimulation. Cells were lysed and introduced to anti-H and anti-L polyclonal antibodies. These cells were not incubated with radioactive amino acids. Adapted from Recalcati et al., 1998.

Addition of IFN- γ and LPS (Lane 4) to J774.1 macrophages resulted in an increase in H-ferritin protein levels. A 2.5 fold increase was observed between untreated and pro-inflammatory treated in H-ferritin levels of J774.1 cells (Recalcati et al., 1998). Treatment with M1-stimulating cytokines resulted in increased H-ferritin production and priming for iron storage. Ferritin protein levels increased with transformation toward an M1 phenotype. The M1 macrophage presumably uses ferritin proteins rich in H subunits for efficient processing of iron and reactive intermediate production. The L-subunit is used more as a structural organizer for the iron atoms. Upregulation may not be essential for M1 polarization (Koorts et al., 2007).

Pro-inflammatory cytokines were shown to down-regulate ferroportin (Fpn) protein levels in macrophages. Ferroportin is a trans-membrane protein involved in Iron export. An increase in intracellular iron correlated with enhanced NF- κ B signaling and M2 macrophages were shown to have low iron levels (Recalcati et al., 2012). M2 macrophages are characterized by wound healing. Iron is an essential nutrient for tissue regeneration, specifically reconstruction of myofibers in skeletal muscle injuries. In periods of oxidative stress, the M2 macrophage phenotype is favored because these macrophages produce an iron rich extracellular environment (Corna et al., 2010). This iron rich environment is attributed to up-regulation of Fpn (Recalcati et al., 2012) and down-regulation of Ft levels (Weis et al., 1997). Thus M2 macrophages favor a lower intracellular iron concentration than their M1 counterparts.

To understand how inflammatory and anti-inflammatory cytokines regulate the nuclear environment of the macrophage, ferritin mRNA levels were analyzed. Cytokines

were shown to have little effect on H-ferritin mRNA levels (Figure 12). Anti-inflammatory cytokines were able to slightly decrease H-ferritin mRNA levels, suggesting that ferritin protein modification must take place after transcription. These data suggest that the macrophage naturally produces ferritin mRNA. The production of protein from this pool of mRNA is dependent upon the polarizing cytokines present around the cell.

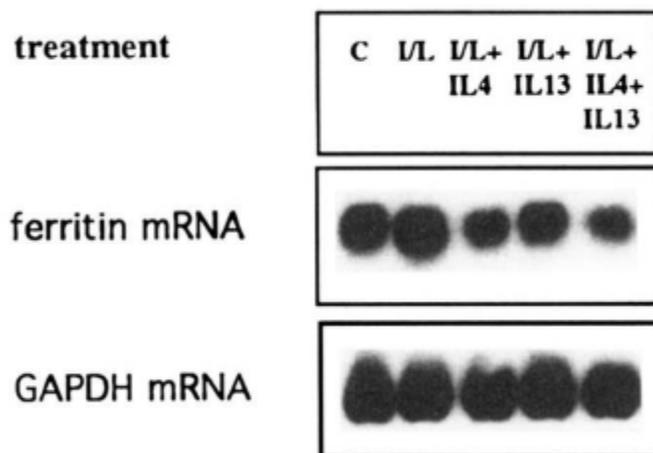


Figure 12: Ferritin mRNA levels in anti-inflammatory cytokine treated J774.1 macrophage cells. Weiss et al. stimulated J774.1 macrophages with LPS/IFN- γ alone in one treatment group (I/L). In three other treatment groups, macrophages were primed with IL-4, IL-13, or IL-4/IL-13 for 24 hours prior to LPS/IFN- γ treatment. The relative density of these bands was not determined. Adapted from Weiss et al., 1997.

HUMAN PERIPHERAL BLOOD MONONUCLEAR CELLS (PBMC's)

Peripheral Blood Mononuclear cells are taken from the venous blood of individuals and isolated by methods such as the Ficoll-Paque density gradient technique. These monocytes are naturally occurring lymphocytes transported through capillaries to sites of infection. Gender-specific variations between healthy male and female test groups were described. Lipopolysaccharide (LPS)-induced PBMCs produced significantly

more TNF- α and IL- β in cells from healthy male patients than in cells from healthy female patients similar in age (Imahara et al., 2005). Based on this cytokine release profile, PBMCs from males may have a stronger pro-inflammatory response than those from females. Differences were also observed in PBMCs based on age. LPS-induced neonatal monocytes released significantly less pro-inflammatory cytokines (TNF- α and IFN- γ) than healthy LPS-induced adult monocytes (Levy, 2005). Neonatal monocytes may be more susceptible to immune invasion than normal adult cells. It is important to recognize gender and age differences in order to exclude any faulty data.

PBMC CELL SURFACE MARKERS

A significant increase in expression of CD86 was seen in PBMCs treated with IFN- γ when compared to the cells only treated with medium alone (Ambarus et al., 2012)(Figure 13c). Unexpectedly, PBMCs treated with IL-4 markedly increased their CD86 content as well. The IL-4-treated group increased their CD86 levels with less significance than the IFN- γ -treated group. However IFN- γ and IL-4 subsets did not differ from each other as anticipated. PBMCs treated with IL-10, however, had significantly less CD86 than both the IFN- γ and IL-4 treatment groups. This suggests that IL-4 and IFN- γ polarize macrophage subsets which require CD86 for antigen presentation. There was a 10 fold increase of CD206 in PBMCs treated with IL-4 when compared to the cell only group (medium)(Figure 13f). IFN- γ treatment decreased CD206 content when compared to the cell only group. CD80 and CD64 were found to be better markers of M1 polarization, because they were only significantly up-regulated by IFN- γ treatment alone. CD80 was upregulated 8 fold greater than the control group. CD64 was

upregulated 45 fold greater than the control group. CD80 and CD86 are both B-7 co-stimulatory molecules which help to augment the T-cell activation signal. Both of these ligands produce an identical response in T-cells and bind with the same affinity to T-cell receptors (Lanier et al., 1995). CD80 seems to be unique to the IFN- γ -treated subset in humans. This finding suggests that human IFN- γ signaling may induce production of CD80 proteins. Murine and human macrophages may upregulate different cell surface proteins upon transformation into M1 and M2 subsets. CD86 is upregulated in the M1 and M2 polarized populations of human PBMCs. This cell surface receptor may be specific to the murine population. CD80 seems to be the M1 polarization specific cell surface marker in PBMCs. CD86 and CD80 are functionally similar, but they elicit different responses to IFN- γ . Unlike the PBMCs, CD200R levels were not affected by IL-4 in the mouse model (Koning et al., 2010). CD200R seems to be a specific M1 polarization marker for PBMCs. Although CD206 was significantly up-regulated with IL-4 treatment (10 fold), CD200R produced the strongest up-regulation with IL-4 treatment alone. A 30 fold increase in CD200R was observed over the other three treatment groups with IL-4 treatment (Figure 13d). CD200R up-regulation suggests that IL-4 polarization may slow intracellular signaling involving the mitogen-activated protein kinase (MAPK).

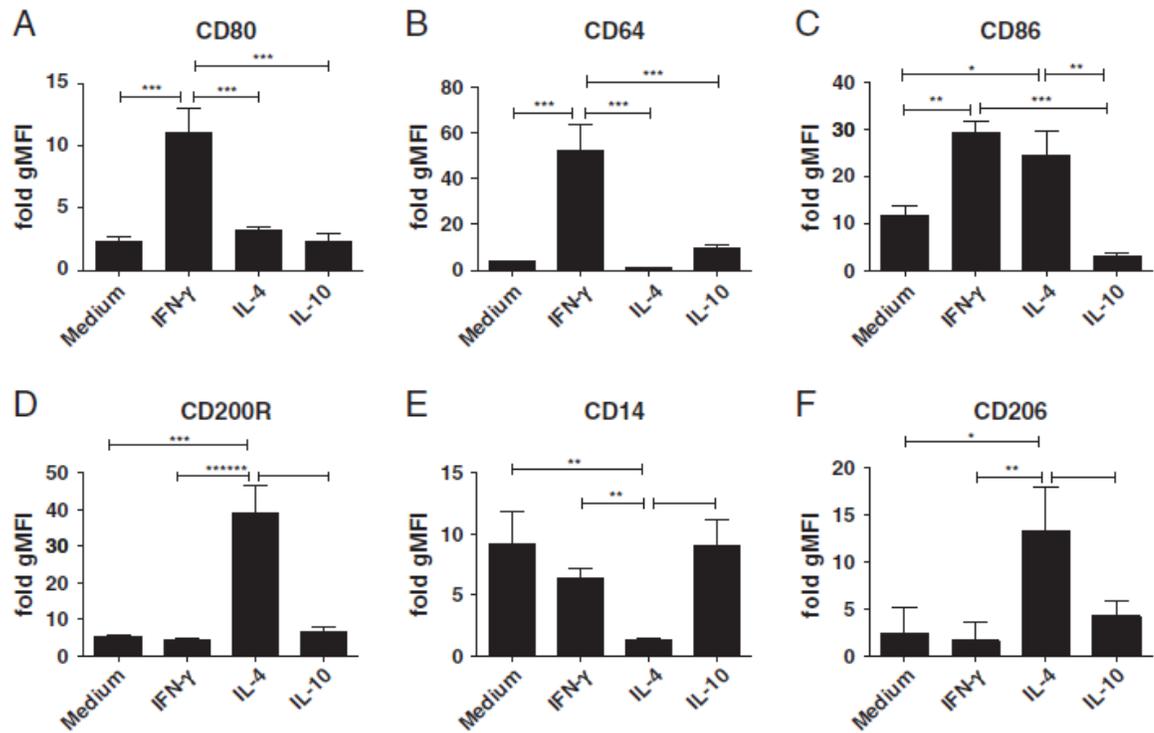


Figure 13: Flow cytometric analysis of CD206 and CD86 in PBMCs treated with anti-inflammatory (IL-4, IL-10) and pro-inflammatory cytokines (IFN- γ). Ambarus et al. extracted PBMCs from healthy donors. Age and gender values were not given. Data were expressed as a standard mean of values. A P-value was considered significant at a value less than 0.05. Monocytes were then isolated via Lymphoprep gradient centrifugation and subsequent Percoll gradient separation. Cells were polarized with IL-4, IL-10, or IFN- γ for four days and cell surface marker analysis was performed via flow cytometry techniques (Ambarus et al., 2012). Quantifications of CD206 and CD86 cell surface markers are shown in Figure 13c and f. In addition to CD206 and CD86, other cell surface markers were examined after polarization. These receptors include co-stimulatory molecules CD80 (a) and CD14 (e), high-affinity Fc γ receptor I CD64 (b), mitogen-activated protein kinase (MAPK) inhibitory receptor CD200R (d). Adapted from Ambarus et al., 2012.

PBMC CYTOKINE PRODUCTION

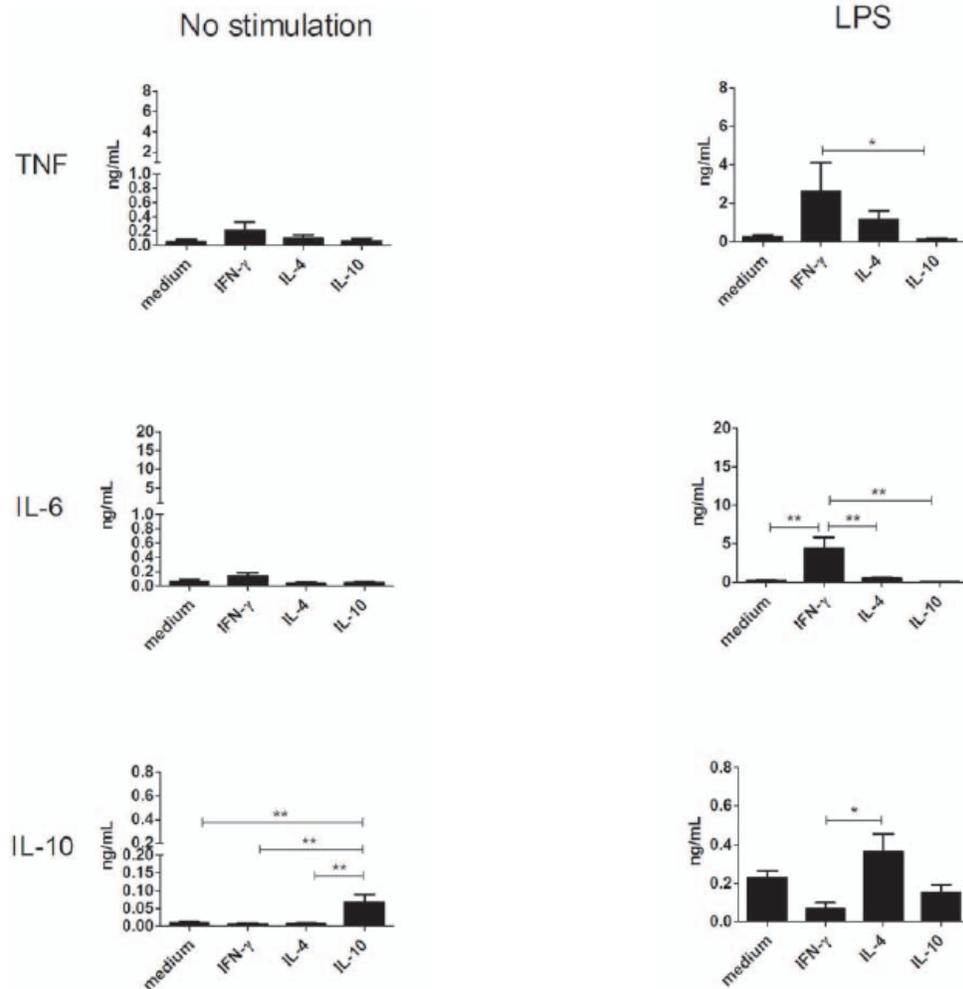


Figure 14: Cytokine profile expressions for PBMCs in various macrophage subset testing groups. Human peripheral blood monocytes were isolated from healthy volunteer blood samples using Lymphoprep and Percoll gradient separation techniques. Cells were cultured in Iscove's Modified Dulbecco's Medium (IMDM) and primed for 4 days with (50ng/mL) IFN- γ , (40ng/mL) IL-4, or (50ng/mL) IL-10. Cells were subsequently activated with (100ng/mL) LPS for 20 hours and cell supernatants were removed from each test group. Supernatants were finally analyzed with the Bio-Plex system for TNF- α , IL-10, and IL-6 cytokine levels. Bars represent the mean of six experiments. Significance was represented by a p-value less than 0.05. Adapted from (Santegoets et al, 2012).

PBMCs treated with IFN- γ expressed significantly higher levels of TNF- α and IL-6 than PBMCs treated with anti-inflammatory cytokines IL-10 or IL-4. A two-fold difference was observed in TNF- α levels between IFN- γ and IL-4 treatment groups. The IFN- γ treatment group produced five times the amount of IL-6 in all three treatment groups. Surprisingly, PBMCs treated with IL-4 expressed significantly higher levels of IL-10 than those treated with IFN- γ . The murine alternatively activated macrophage expressed very little IL-10 compared to the human peripheral blood mononuclear cell. In the murine model, LPS was not used for activation. LPS may be responsible for IL-10 production. Treatment of the monocyte with LPS alone resulted in upregulation of IL-10 (Santegoets et al., 2012). Stimulation by pro-inflammatory cytokines resulted in increased pro-inflammatory cytokine production (TNF- α and IL-6). Conversely, stimulation by anti-inflammatory cytokines decreased pro-inflammatory cytokine production and increased anti-inflammatory cytokine production in IL-4 treated cells.

PBMC TRANSCRIPTION FACTOR

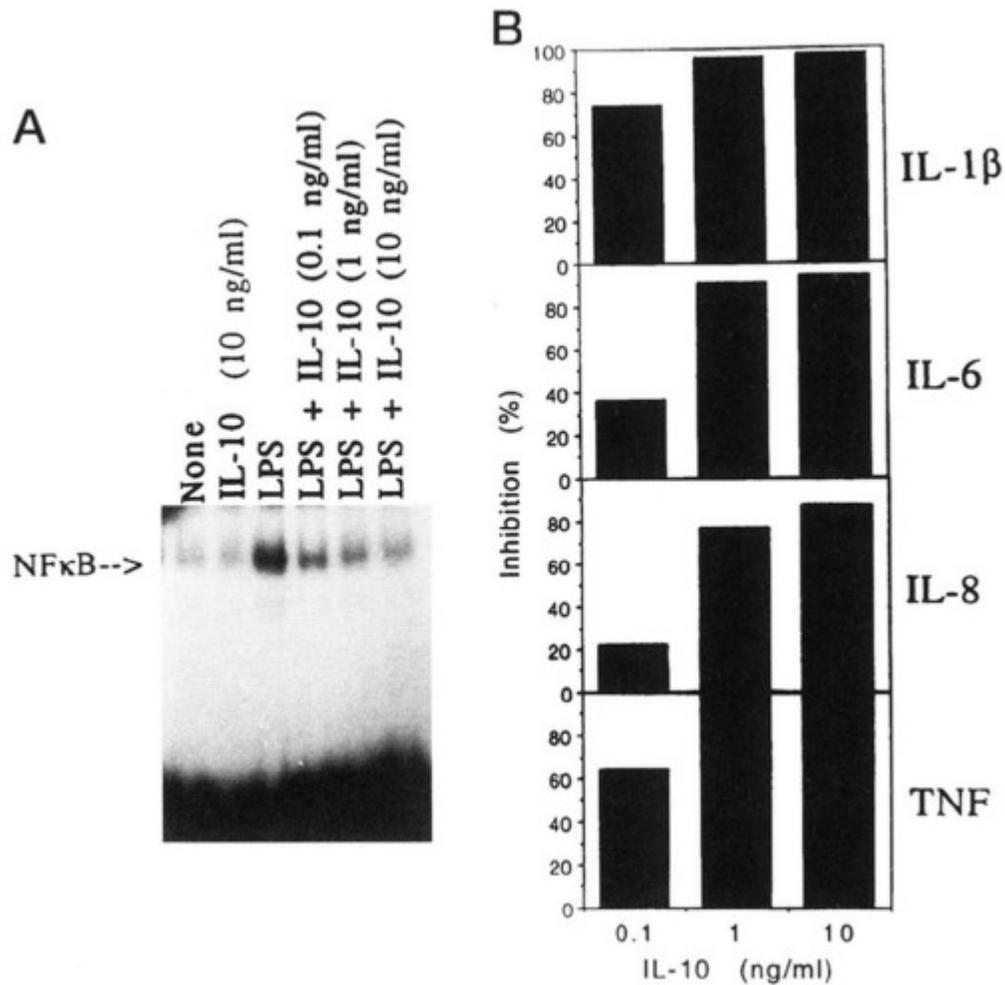


Figure 15: EMSA of NF-κB Activation in PBMCs treated with varying concentrations of IL-10. Wang et al. isolated human peripheral blood mononuclear cells from a healthy male donor using elutriation of peripheral blood. Monocytes were confirmed by immunofluorescence staining of the CD14 antigen. (A) Monocytes were incubated with 80ng/mL of LPS, 10ng/mL IL-10, or 80ng/mL LPS with 0.1ng/mL, 1ng/mL, and 10ng/mL IL-10 (5 minutes prior to LPS stimulation). Cells were incubated for 1.5 hours and nuclear extracts were removed. Nuclear extracts were loaded into an EMSA for the presence of NF-κB. NF-κB activation was measured by band thickness. (B) Aliquots of cell suspensions were obtained from each test group and incubated further for another 4 hours. Supernatants from cell cultures were analyzed for IL-1β, IL-6, IL-8, and TNF-α via an enzyme-linked immunosorbent assay (ELISA). Percent inhibition was calculated using LPS-treated cells as a control of 0% inhibition. Any decrease in cytokine levels was accompanied with an increase in inhibition up to a maximum 100%. Adapted from Wang et al., 1995.

As shown in Figure 15, increasing doses of IL-10 reduce NF- κ B expression substantially. This NF- κ B downregulation is accompanied by suppression of pro-inflammatory cytokine release. LPS stimulates all four cytokines (IL-1 β , IL-6, IL-8, and TNF- α). Addition of the anti-inflammatory cytokine IL-10 inhibits secretion of these cytokines (Ahn et al., 2005). NF- κ B translocation to the nucleus results in the production of these pro-inflammatory mediators. IL-10 must have the ability to regulate the activation of NF- κ B and cytokine levels. With addition of LPS, these cells could be converted into an M1 phenotype. This phenotypic switch may be accompanied by an M2 polarization with addition of IL-10. These cells become anti-inflammatory in nature based on their inhibition of pro-inflammatory cytokines and down-regulation of NF- κ B.

PBMC IRON TRANSPORT

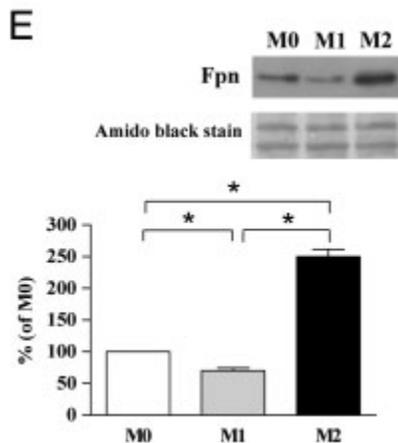


Figure 16: Ferritin immunoblot results of PBMCs isolated and converted into M0, M1, and M2 macrophages. Blood samples were isolated from healthy blood donors between the ages of 25 and 50. Participants were 59% male and had no previous iron deficiency disorders and normal serum iron levels. Monocytes were isolated by sequential centrifugation and cultured with 100ng/mL Macrophage Colony Stimulating Factor (M-CSF) for 6 days. Macrophages were then stimulated with either LPS plus IFN- γ (M1) or IL-4 (M2) for 72 hours. Immunoblot analysis was carried out on M0, M1 and

M2 subsets for membrane Ferritin (Fpn) using antibodies against the Fpn protein. Bars represent densitometric quantification of the blot. Significant values were contingent upon a p-value less than 0.001. To ensure equal protein loading, blots were stained with amido black. Adapted from Recalcati et al., 2010.

Immunoblot results indicate high membrane Ferritin levels in the IL-4 stimulated (M2) population. These results are significantly different than both the untreated (M0) and IFN- γ and LPS treated (M1) populations. Upon interaction with an acute phase protein hepcidin, ferritin blocks the release of iron from the macrophage. M2 macrophages favor an iron rich intracellular environment while M1 macrophages favor an iron-poor intracellular environment. By removing iron from the extracellular milieu, M1 macrophages are able to prevent pathogens from increasing their virulence. By increasing iron levels in the extracellular environment, M2 macrophages are able to help reconstruct damaged tissues. This is reflected in the high Ferritin protein levels expressed in the M2 subset. Cell lysates were examined using ELISA with antibodies against H- and L-ferritin subunits (Table 2).

Table 2: ELISA assay of H- and L-ferritin (Ft) subunits in cell lysates of polarized human macrophages. Blood samples were isolated from healthy blood donors between the ages of 25 and 50. Participants were 59% male and had no previous iron deficiency disorders and normal serum iron levels. Monocytes were isolated by sequential centrifugation and cultured with 100ng/mL Macrophage Colony Stimulating Factor (M-CSF) for 6 days. Macrophages were then stimulated with either LPS plus IFN- γ or IL-4 for 72 hours. Ferritin concentrations were determined by ELISA assays for the M0, M1, and M2 treatment groups with monoclonal antibodies against H and L-ferritin subunits. Table values represent mean quantities for each test group. Significant values were contingent upon a p-value less than 0.001 when compared to the M0 control. Adapted from (Recalcati et al., 2010).

	L-Ft (ng/mg)	H-Ft (ng/mg)
M0	1360 \pm 220	623 \pm 100
M1	1426 \pm 205	822 \pm 141*
M2	1345 \pm 211	548 \pm 99**

M1 macrophages have significantly higher levels of H-ferritin than M2 polarized macrophages. Ideally, M1 populations should be rich in ferritin and M2 populations should be rich in ferriportin. Human peripheral blood mononuclear cells (PBMCs) follow this same expression profile. M1 macrophages significantly increase their H-ferritin levels in response to pro-inflammatory cytokine treatment. M2 macrophages highly reduce their H-ferritin levels in response to anti-inflammatory cytokine treatment. The L-ferritin subunit is involved in the arrangement of iron atoms into a proper storage configuration. Macrophages generally should not need to upregulate L-ferritin levels because iron atoms are either exported or used for production of reactive intermediates. H-ferritin levels are increased or decreased based on the content of the

surrounding microenvironment. As Biswas et al. (2012) described, M1 macrophages favor iron acquisition while M2 macrophages favor iron release.

U937 MONOCYTES

U937 CELL SURFACE MARKERS

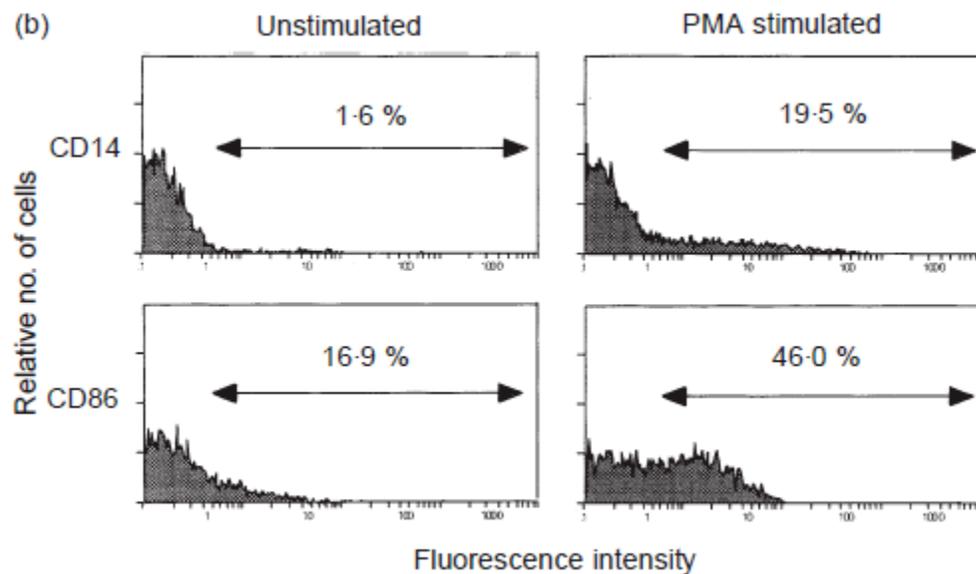


Figure 17: Flow cytometric analysis of CD14 and CD86 in U937 cells treated with PMA. Hida et al. exposed U937 monocytes to 10ng/mL PMA for 24 hours and flow cytometric analysis was performed for CD86 cell surface markers in untreated and PMA-stimulated subsets. Adapted from (Hida et al., 2000).

PMA treatment resulted in a 29.1% increase in CD86 MFI when compared to the untreated subset. These findings indicate that U937 monocytes express CD86 after PMA treatment (Hida et al., 2000). PMA treatment also resulted in a 17.9% increase in CD14 MFI expression when compared to the untreated subset. CD14 is an LPS receptor expressed mainly in monocytes and macrophages. CD14 associates with Toll-like Receptor 4 or 2 (TLR4/TLR2) and induces LPS stimulated signal transduction (Taylor et

al., 2005). CD14 expression is mildly increased with macrophage transformation. This up-regulation suggests that the converted U937 cell is more susceptible to LPS activation than the unstimulated U937 monocyte (Hida et al., 2000). Macrophages require a stimulus such as LPS to activate. The U937 cell is primed for M1 polarization according to the parameters examined by Biswas et al. (2012). PMA transforms the U937 monocyte into macrophage-like cells which are characterized by an increase in CD86 protein. This increase in co-stimulatory molecules ensures that the transformed cell is better prepared for antigen presentation. Biswas et al. classifies the M1 macrophage as rich in CD86 molecules (Biswas et al., 2012). An increase in these molecules is observed with transformation, but these transformed cells must be analyzed after treatment with polarizing cytokines to understand if they are upregulated in a pro-inflammatory environment.

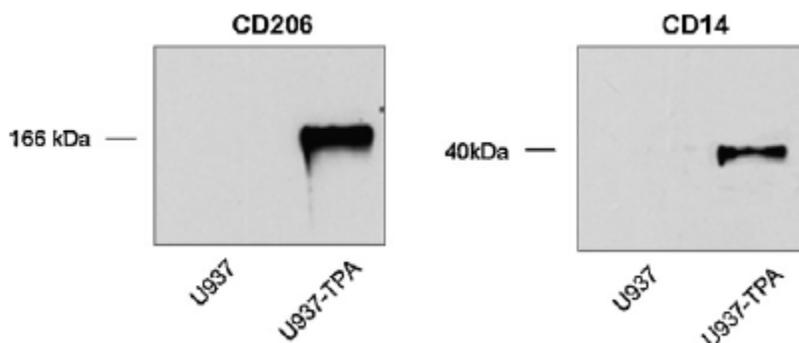


Figure 18: Western blot analysis of U937 cells before and after PMA (TPA) stimulation. U937 monocytes were exposed to 10ng/mL of PMA for 72 hours and cell lysates were analyzed for CD14 and CD206 protein expression with anti-CD206 and anti-CD14 monoclonal antibodies. Densitometric quantifications were not provided. Adapted from Minafra et al., 2011.

CD206 and CD14 protein expression is seen in PMA-treated cells, but not in untreated U937 cells (Figure 18). U937 monocytes are primed with CD14 and CD206 after PMA treatment. U937 monocytes were exposed to 10ng/mL of PMA for 72 hours and cell lysates were analyzed for CD14 and CD206 protein expression. PMA treatment stimulated an increase in CD206 and CD14 protein expression in these cells (Minafra et al., 2011). These converted macrophage-like cells are able to respond to opsonization of mannose-coated particles (M2-characteristic) and LPS-mediated activation (macrophage characteristics). These results need to be compared to cytokine stimulated treatment groups to fully understand if CD206 upregulation is observed in anti-inflammatory microenvironments.

U937 CYTOKINE PRODUCTION

Interleukin-6 (IL-6) is a potent pro-inflammatory cytokine. Several transcription factors regulate the mRNA production of this cytokine. One of these transcription factors is NF- κ B, which is linked to M1 polarization as previously described (Biswas et al., 2012). IL-6 secretion is increased in the M1 macrophage population. M2 polarizing cytokines inhibit the production of pro-inflammatory cytokines. In PBMCs, IL-10 stimulation resulted in downregulation of IL-6, and TNF- α (Ambarus et al., 2012); (Wang et al., 1995). The PMA-treated cell was able to modify its cytokine production based on the microenvironment (Takeshita et al., 1996) (Figure 19). IL-10 treatment resulted in a 40U/mL decrease in IL-6 content compared to the PMA and LPS induced subset in both U937 and THP-1 cells. IL-4 treatment resulted in a 35U/ml decrease in IL-6 in THP-1 cells and a 20U/mL decrease in IL-6 in U937 cells. These results suggest that the anti-

inflammatory cytokines IL-4 and IL-10 are able to shift the macrophage toward an M2 characteristic profile.

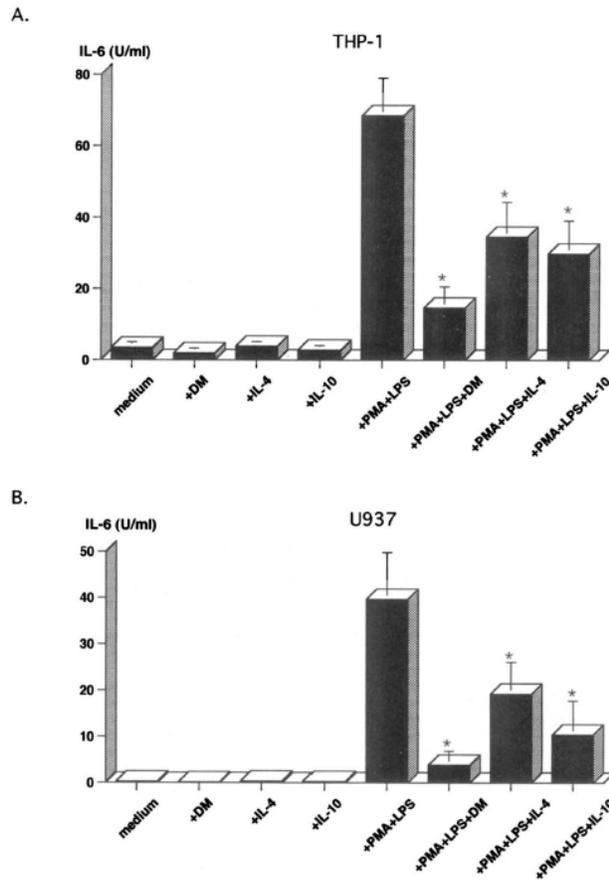


Figure 19: ELISA results of U-937 and THP-1 cell lines for IL-6 quantification following anti-inflammatory cytokine co-culture. Takeshita et al. used the U-937 and THP-1 cell lines. The monocytic cell lines were introduced to 1pg/mL PMA and 10µg/mL LPS for 48 hours with or without 10ng/ml IL-4, 10ng/mL IL-10, or a positive control (0.000001M) Dexamethasone (DM) (a known IL-6 inhibitor). Forty-eight hours later, cells were incubated with anti-IL-6 antibody. Supernatants were harvested and examined using ELISA for IL-6 quantification. Data are expressed as an average of samples. Significance was observed as values with a p-value less than 0.05. Adapted from Takeshita et al., 1996.

U-937 TRANSCRIPTION FACTORS

ELISA results demonstrated how U937 cells were able to reduce their pro-inflammatory cytokine release in response to anti-inflammatory cytokines IL-4 and IL-10 (Takeshita et al., 1996). NF- κ B is responsible for the activation of IL-6 transcription (Biswas et al., 2012). As expected, NF- κ B levels were able to be downregulated by anti-inflammatory polarizing cytokines IL-4 (Manna et al., 1998) (Figure 20) and IL-13 (Aggarwal et al., 1998) (Figure 21) in the U937 cell line. This evidence suggests that the U937 cell is able to respond to M2 polarizing cytokines. By modifying the nuclear NF- κ B content, U937 cells are able to downregulate their pro-inflammatory cytokine release and alter the state of their microenvironment. U937 cells are capable of the transition from a pro-inflammatory (IL-6 rich) to anti-inflammatory (IL-6 poor) environment.

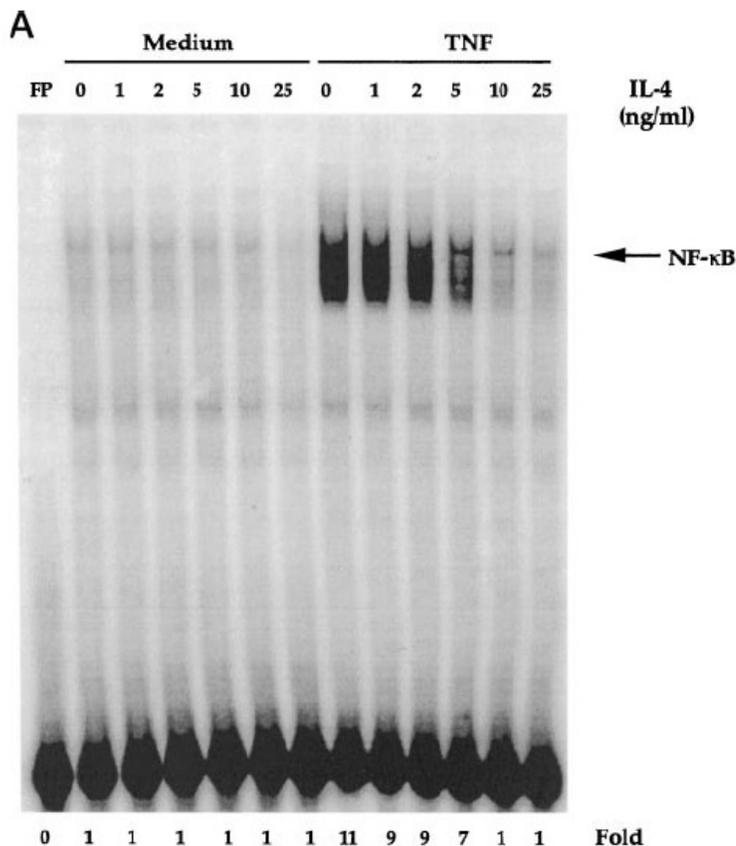


Figure 20: NF- κ B activation is inhibited by increasing amounts of IL-4. U937 cells were incubated with varying concentrations of IL-4 for 24 hours. This was followed by 100pM TNF- α treatment for 30 minutes. Cells were then examined for NF- κ B activation via an electrophoretic mobility shift assay (EMSA). This procedure was carried out by incubating 5 μ g of cellular nuclear extract with radioactive Phosphorus labeled NF- κ B oligonucleotides. Binding affinity was observed by band thickness. Adapted from Manna et al., 1998.

NF- κ B levels were reduced 10 fold when U937 cells were incubated with 10ng/mL IL-4. U937 cells were able to reverse the inflammatory (M1) polarizing effects of TNF- α by priming with IL-4. IL-4 is an M2 polarizing cytokine. These results demonstrate that U937 cells are able to shift their nuclear content in response to polarizing cytokine IL-4. This NF- κ B shift was also observed in IL-13-treated U937 cells (Aggarwal et al., 1998). Figure 21 shows the gradual decline of NF- κ B activation as IL-13 concentrations increase. There was a shift where the NF- κ B arrow and gel bands were located. This was not explained in the literature.

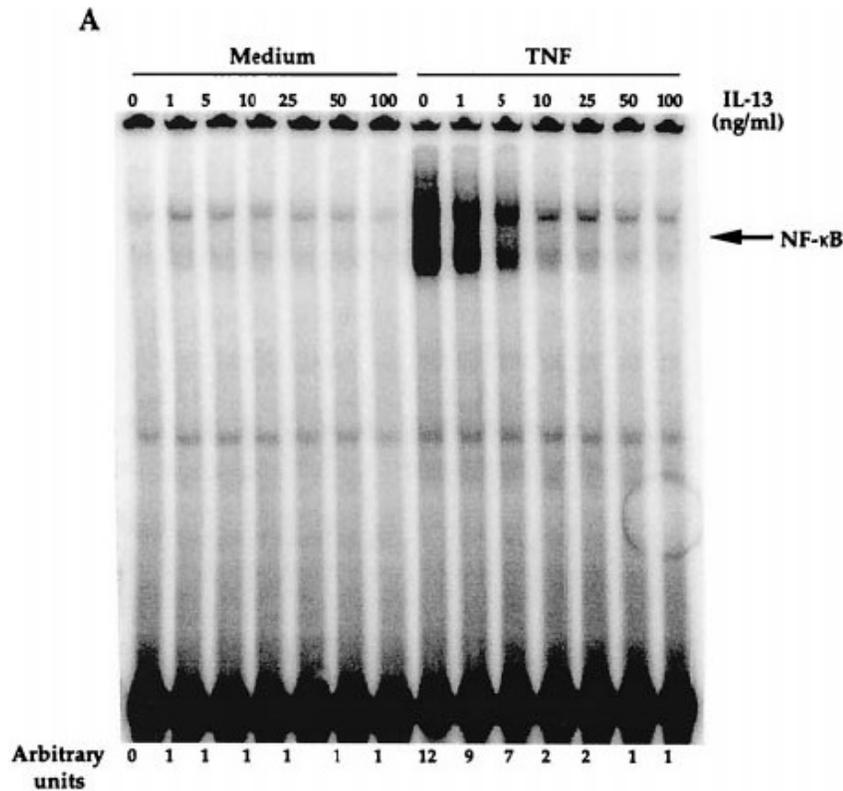


Figure 21: NF- κ B activation decreases with an increase in IL-13 concentration. Aggarwal et al. exposed U937 cells to varying concentrations of IL-13 for 24 hours. Treatment was followed by 30 minutes of incubation with TNF- α . Nuclear extracts were prepared and run through an electrophoretic mobility shift assay (EMSA). 4 μ g of nuclear lysates were used for this experiment. Adapted from Aggarwal et al., 1998.

Treatment of U937 cells with anti- and pro-inflammatory cytokines resulted in the response expected of polarizing macrophages. TNF- α treatment resulted in upregulation of NF- κ B activation (Manna et al., 1998); (Aggarwal et al., 1998). Manna et al. observed a 10 fold increase in NF- κ B activation. Aggarwal et al. observed an 11 fold increase in NF- κ B. Thus U937 cells are characterized by receptors for TNF- α to elicit a pro-inflammatory M1 response. Treatment with anti-inflammatory cytokines resulted in downregulation NF- κ B activation (Manna et al., 1998) (Aggarwal et al., 1998).

Aggarwal et al. observed an 11 fold decrease in NF- κ B activation when cells were primed with 50ng/mL IL-13 (Figure 21). U937 cells are characterized by receptors to IL-4 and IL-13 which are necessary for polarization into an M2 phenotype. Interaction of anti-inflammatory cytokines with the U937 cell results in decreased activation of NF- κ B and an essentially anti-inflammatory phenotype.

U-937 IRON TRANSPORT

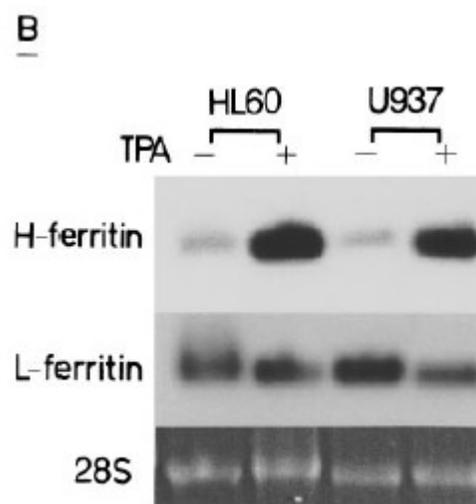


Figure 22: Northern Blot analysis of H- and L-Ferritin levels in HL-60 and U-937 cell treated with PMA. Pang et al. exposed U-937 and HL-60 cells to PMA (100ng/mL) for 24 hours. H- and L-Ferritin mRNA levels were analyzed via Northern Blot analysis. Ribosomal subunit 28S was used as a positive control. Quantitative results were not provided. Adapted from (Pang et al., 1996).

TPA/PMA conversion of the leukemic monocyte into a macrophage results in an increase in H-ferritin mRNA levels (Figure 22). Ferritin analysis demonstrates how the leukemic monocyte is able to prime itself for iron acquisition after TPA/PMA conversion with mRNA. Investigation of mRNA levels needs to be performed in order to examine if nuclear transcription is affected by these anti- and pro-inflammatory cytokines. Protein

levels cannot be determined by mRNA levels, as was demonstrated in the murine cell line. However, upregulation of H- and L-ferritin mRNA does make the U937 cell equipped to produce these proteins. Upon activation with a pro-inflammatory stimulant, U937 cells are able to upregulate H-ferritin protein based on the availability of H-ferritin mRNA. Ferroportin mRNA followed a different expression profile. PMA incubation only mildly increased ferroportin mRNA levels (Figure 23); (Knowles et al., 2006).

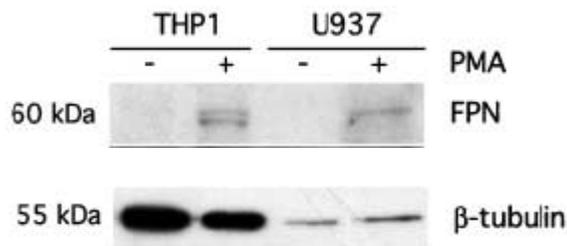


Figure 23: Western blot analysis of U937 and THP-1 ferroportin mRNA. Human THP-1 and U937 cells were cultured in growth medium with or without 20nmol/L PMA for 48 hours. Cells were assayed for ferroportin mRNA (FPN) via Western blot analysis. β -tubulin was used as a positive control. Quantitative data were not provided. Adapted from Knowles et al., 2006.

Fluctuations in mRNA levels do not necessarily reflect protein production; however, these fluctuations do provide insight into the ability of the U937 cell to prime itself for polarization. H-ferritin mRNA levels were increased with PMA treatment. Ferroportin levels mildly increased with PMA addition (Figure 23). These results suggest that the PMA-treated U937 cell is primed with both ferritin and ferroportin mRNA. It is essential to have data implicating protein quantities in these PMA-transformed cell lines to fully understand how the cell responds to the PMA stimulus.

IV. DISCUSSION

Incubation of the U-937 monocyte with PMA resulted in the production of cell with up-regulated macrophage components. Characterized by integrin-based cellular attachment and phagocytosis, this converted cell acts like the typical macrophage. Macrophages are also known for their plasticity and ability to switch phenotypes based on their surrounding micro-environment. Conversion was followed by up-regulation of both M1 and M2 macrophage factors. Cell surface markers CD86 and CD206 both increased with conversion to the macrophage-like morphology. These cell surface markers exhibited diverse expression profiles in polarized subsets of both murine and human peripheral blood mononuclear cells. M1 polarization produced a prominent CD86 or CD80 expression while M2 polarization produced a prominent CD206 expression. These results suggest that the converted U-937 monocyte is primed for either M1 or M2 polarization with respect to cell surface marker composition. PMA transformation primed U937 cells with H-ferritin and ferroportin mRNA. With respect to mRNA U937 cells are able to prime for protein production of both H-ferritin and ferroportin. Pro-inflammatory cytokine levels were able to be modified with polarizing cytokines in the U-937 cells like both murine and human PBMC subsets. PMA and LPS incubation resulted in high IL-6 production. IL-6 release was decreased by anti-inflammatory cytokines IL-4 and IL-10. Thus, the PMA-converted monocyte was able to mimic the ability of the macrophage to regulate cytokine production based on the

microenvironment. Finally, transcription factor activation of NF- κ B was able to be modified by cytokine incubation. Addition of TNF- α resulted in a prominent NF- κ B activation in murine and U937 models. Increasing doses of anti-inflammatory IL-10, IL-4 and IL-13 greatly reduced this activation, converting U937 cells to an M2-like phenotype. It would be interesting to investigate the PPAR- γ activation levels after IL-4 incubation in all models. Ideally, these two transcription factors (NF- κ B and PPAR- γ) should exhibit anti-parallel activation results when exposed to polarizing cytokines. These results indicate that a leukemic monocyte, such as U-937 is capable polarizing cells into different macrophage phenotypes.

Table 3: Summary Table of Murine Macrophage, Human PBMC, and U937 cells.

	M1-stimulated (IFN- γ , LPS, or TNF- α)	M2-stimulated (IL-4, IL-10, IL-13, or IgG/Ovalbumin)	PMA-stimulated
Murine Macrophage	<p>↑CD86 ↑IL-12</p> <p>↑NF-κB ↑H-ferritin</p>	<p>↑CD206 ↑IL-10</p> <p>↓NF-κB</p>	
Human PBMC	<p>↑CD80 ↑TNF-α</p> <p>↑CD86 ↑IL-6</p> <p>↑CD64 ↑NF-κB</p> <p>↓ferroportin</p> <p>↑ferritin</p>	<p>↑CD206 ↑CD200R</p> <p>↑CD86 ↓IL-1β</p> <p>↓IL-6 ↓IL-8</p> <p>↓NF-κB ↓TNF-α</p> <p>↑ferroportin</p> <p>↓ferritin</p>	
U937 cell	<p>↑IL-6 ↑NF-κB</p>	<p>↓IL-6 ↓NF-κB</p>	<p>↑Phagocytosis</p> <p>↑CD11b/Adherence</p> <p>↑CD14 ↑CD86</p> <p>↑CD206 ↑IL-6</p> <p>↑ferritin mRNA</p> <p>↑ferroportin mRNA</p>

V. CONCLUSION:

U937 cells mimic the functional plasticity to polarize into M1 and M2 phenotypes. These cells upregulate anti-inflammatory and pro-inflammatory cell surface markers to respond to various stimuli. PMA-transformed U937 cells can respond to mannose-coated particles with CD206 cell surface markers (M2 in nature). These cells also have the potential to present antigens on the cell surface based on the presence of CD86 cell surface markers (M1 in nature). LPS induces the release of pro-inflammatory cytokine IL-6 (M1 in nature). This cytokine release can be downregulated by anti-inflammatory cytokines IL-4 and IL-10 (M2 in nature). TNF- α induced activation of NF- κ B in U937 cells (M1 in nature). This activation was able to be reversed by IL-4 and IL-13 (M2 in nature). Finally, U937 cells were able to express mRNA of essential proteins (ferritin and ferroportin) for iron utilization. Bacteria and immune cells compete for intercellular iron. Serving both as an electron donor and acceptor, free iron utilization is essential to both bacterial and human cells. By binding free iron to transferrin the host cell successfully removes iron from infecting bacteria (Berlim, 2001). In this regard, transferrin is a host defense mechanism. These cells can modify their transcription factors, protein, and mRNA in response to different stimuli. Thus, U937 cells are able to be transformed into M1 and M2 macrophage subsets based on interaction with their microenvironment. There are advantages and disadvantages to using the U937 cell line. U937 cells reduce their proliferative capacity and c-myc

expressed after transformation with PMA. They are also restricted to monocyte to macrophage transformation (Minafra et al., 2011). Conversely, these cells produce identical copies with proliferation, unlike human PBMCs. PBMCs have variation from one cell to the next. U937 cells decrease variability based on their oncogenic nature. Overall, this study confirms that the U937 cell line can be manipulated by polarizing cytokines to study the beneficial and adverse effects of macrophages on tumor cells.

VI. FUTURE STUDIES

There are more research outlets to confirm the ability of the U937-transformed cell to polarize into M1 and M2 phenotypes. Researchers could analyze the ability of anti-inflammatory cytokine treated U937 cells to consume mannose coated particles against M1 and M1 populations. This experiment would show that the CD206 present on the surface of the U937 cell is able to function in a phagocytic manner. A phagocytosis assay would present data substantial to compare the polarized subsets. PMBCs expressed highest levels of CD80 in the M1 population and CD200R in the M2 population. Researchers should investigate the levels of these cell surface receptors in response to polarizing stimuli using Flow cytometry. It is also important to investigate ferritin and ferroportin protein levels in M1 and M2 polarized subsets of the U937 cell line. M1 macrophages are activated during the initial stage of infection. Ferritin up-regulation should be expected of these macrophages as an element of survival. Immunoblot analysis could be performed on these subsets with anti-H-ferritin and anti-L-ferritin antibodies to confirm the up-regulated expression of ferritin in the M1 macrophage. It would also be valuable to place these macrophages in varying iron concentrations in co-culture with bacteria cells to investigate which macrophage subset is best fit for survival in various conditions.

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