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Effects of Chemical Stimulation and Tumor Co-Incubation on Macrophage Activation
and Aggressiveness, Measured Through Phagocytosis and Respiratory Burst

Bo Marcus Gustafsson

A thesis submitted to the faculty of
Brigham Young University
in partial fulfillment of the requirements for the degree of
Master of Science

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ABSTRACT

Effects of Chemical Stimulation and Tumor Co-Incubation on Macrophage Activation and Aggressiveness, as Measured by Phagocytosis and Respiratory Burst

Bo Marcus Gustafsson

Department of Microbiology and Molecular Biology, BYU

Master of Science

Macrophages are a cornerstone in innate immunity, especially important in detecting and killing invading microorganisms. In tumor biology, the macrophages can contribute both to anti-tumor activity and tumor promotion depending on individual tumor microenvironment and therefore have a large impact on both tumor progression and prognosis.

Two of the most important functions of macrophages are the ability to phagocytose microorganisms and then kill them through the respiratory burst. Phagocytosis activates the respiratory burst, but the more subtle interactions between these processes are less known. Since phagocytosis and reactive oxygen species production are two attributes that change between the classically and alternatively activated macrophages we decided to compare these two functions in macrophages.

Activation of macrophages varies in terms of stimuli and effects. We specifically looked at macrophage activation by tumor cell lines and by chemical stimulation due to caffeine. We hypothesized that the level of oxidation would be directly linked to the level of phagocytosis. We assume that caffeine will increase activity in macrophages and that tumor cell co-incubation will decrease it.

We found that there is a high correlation between the level of engulfment and level of respiratory burst. Chemical stimulation with caffeine can lower aggressiveness of macrophages at lower concentration, raise it at higher concentrations and eventually become toxic to the cell. Co-incubation with leukemic cell lines, as well with necrotic cells, affected an increase in aggressiveness.

Keywords: macrophage, M1, M2, phagocytosis, respiratory burst, reactive oxygen species, caffeine, engulfment, rhodamine

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INTRODUCTION

Monocytes and macrophages

Monocytes are white blood cells that constitute up to 8% of the total leukocytes in the blood.

Monocytes are produced from monoblasts, hematopoietic stem cell precursors in the bone marrow, and are characterized by a large nucleus and cytoplasm as well as by many intracellular vesicles. After circulating in the blood for a few days, monocytes are stored in the spleen or migrate into the different tissues of the body and become dendritic cells or macrophages.

Monocytes are drawn to damaged tissue by chemotaxis in response to substances released by damaged cells, pathogens and other macrophages already at the site. Monocytes extravasate through the blood vessel wall to enter specific target tissues, where they then differentiate into macrophages and can survive for several months. Monocytes and macrophages are often referred to as mononuclear phagocytes. [1]

Macrophages are found in every tissue in the body and their main role is to phagocytose cellular debris, matured neutrophils and invading pathogens as well as stimulating other immune cells.

Depending on the tissue, macrophages have distinct specializations and are referred to as resident macrophages. In the brain, microglia act to clear debris and help repair sites of neurological damage. In the lung the main function of alveolar macrophages is to clear inhaled, non-infectious debris; this is under tight control to limit inflammatory responses to activate only in cases of infection so as not to compromise respiration. In the spleen and liver, sinusoidal lining cells and Kupfer cells act to clear dead and damaged erythrocytes and destroy microorganisms in the blood. In the bones, osteoclasts act to degrade bone during times of low plasma calcium concentrations. Macrophages provide a link between the innate immune system and the adaptive

immune system by presenting pathogen-derived antigens to T-cells and by releasing cytokines to help in their activation. [2]

As shown by their varied functions, diversity and plasticity are hallmarks of monocytes and macrophages. In response to different stimuli, macrophages undergo classical (M1) or alternative (M2) activation, with a varied number of phenotypes represented in between these two extremes. In vivo, different phenotypes co-exist in the same tissues, especially in tumor tissue. [3] Tumor-associated macrophages (TAM) exhibit a number of functions in tumors, such as promotion of tumor cell proliferation and angiogenesis, incessant matrix turnover and repression of adaptive immunity, which ultimately have an important impact on disease progression. [4] [Fig 1]

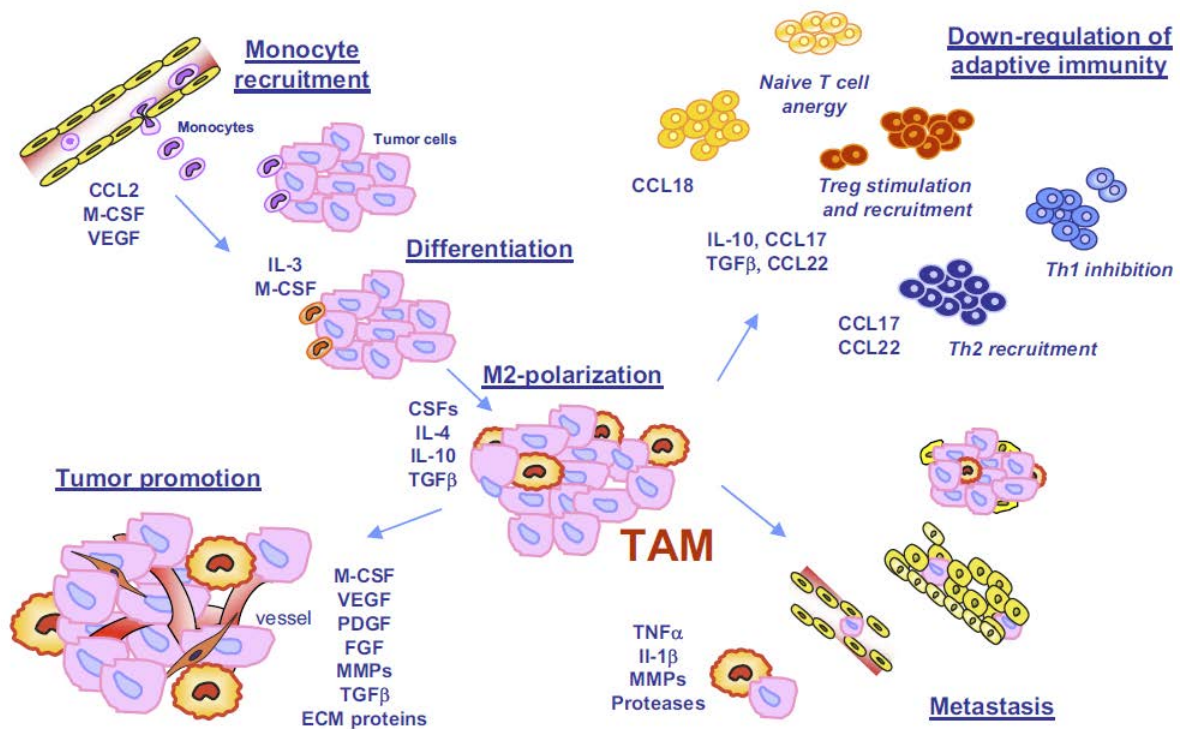


Figure 1 Different functions of tumor associated macrophages.[4]

Two functions of macrophages that are affected by phenotype changes are the levels of phagocytosis of microorganisms, tumor cells and cellular debris by those macrophages and their

production of reactive oxygen species (ROS) through the respiratory burst. These two processes are linked in that phagocytosis activates the respiratory burst, but depending on macrophage phenotype, phagocytosis can increase while ROS production decreases, or vice-versa. [5] In a classically activated (M1) macrophage, phagocytosis and ROS production is increased. The goal of these experiments was to examine the interplay between these two processes and see if the degree of activation changes with chemical and tumor cell stimulation, measured through fluorescent bead engulfment and rhodamine oxidation.

M1 and M2 phenotype

There are two distinct states of macrophage polarization: the classically activated macrophage phenotype and the alternatively activated macrophage phenotype, or M1 and M2, respectively. The idea that macrophages can be alternatively activated in addition to classical activation is relatively new. In 1992, Stein et. al. first proposed the concept of alternative activation through their work with interleukin 4 and mannose receptors. [6] In 1998, Sutterwala et. al. showed the role of Fc γ R signaling in inducing an M2-like polarization. [7] In 2002, Montavani et. al. proposed that M1 and M2 polarization mirrors that of T_H1 and T_H2 polarized cells. [8] In 2005, de Visser, et. al. provided evidence that B cells drive recruitment and protumoral polarization of myelomonocytic cells via the Fc γ R receptor pathway to drive inflammation and tumor progression in cancer. [9] Soon after that, the role of T_{reg} cells in macrophage polarization [10] and the role of NF- κ B in the tumor promotion and re-education of tumor-associated macrophages (TAMs)[11] expanded the understanding of macrophage phenotypes and their effects in cancer. Expanding on the concept of polarizing immune cells, more recent research has shown that neutrophils exhibit two distinct phenotypes (N1 and N2) in the context of tumor progression, mirroring macrophage polarization. [12]

M1 macrophages are activated by bacterial molecules such as LPS and interferon- γ (INF- γ), a T_H1 cytokine. M2 macrophages were originally discovered as a response to interleukin-2, a T_H2 cytokine, and later as a response to IL-13. [6]

M1 macrophages release higher amounts of proinflammatory cytokines like IL-12, IL-23 and TNF, and reactive nitrogen and oxygen intermediates. They have higher MHC II expression and co-stimulatory molecules, are more efficient antigen presenters, and exhibit higher lethal activity versus microbes and tumors. Characteristics of the M2 phenotype include higher radical scavenger expression, as well as mannose and galactose receptors and production of ornithine and polyamines through the arginase pathway. M2 macrophages also show a phenotype of low expression of IL-12 and high expression of IL-10. [13] They help regulate the immune system by promoting tissue remodeling and tumor progression, down regulating inflammation and aiding the T_H2 response. The chemokine profile of M1 includes CXCL9 and CXCL10, which attract T_H1 cells, while the M2 macrophages express the chemokines CCL17, CCL22 and CCL24. [14] M2 polarization can also be driven by CCL2 and CXCL4. [15] M1 is also linked to tumor destruction and clearance while M2 is linked to tumor progression. [5] In some tumors, a higher fraction of M1's is positively associated with longer survival. [16]

It is now recognized that there are many functional states of macrophages that fall in between the M1 and M2 phenotypes. Various other stimuli can create an 'M2-like' state where the macrophages share some but not all of the properties of IL-4 or IL-13 activated macrophages. [5] This is shown in Figure 2.

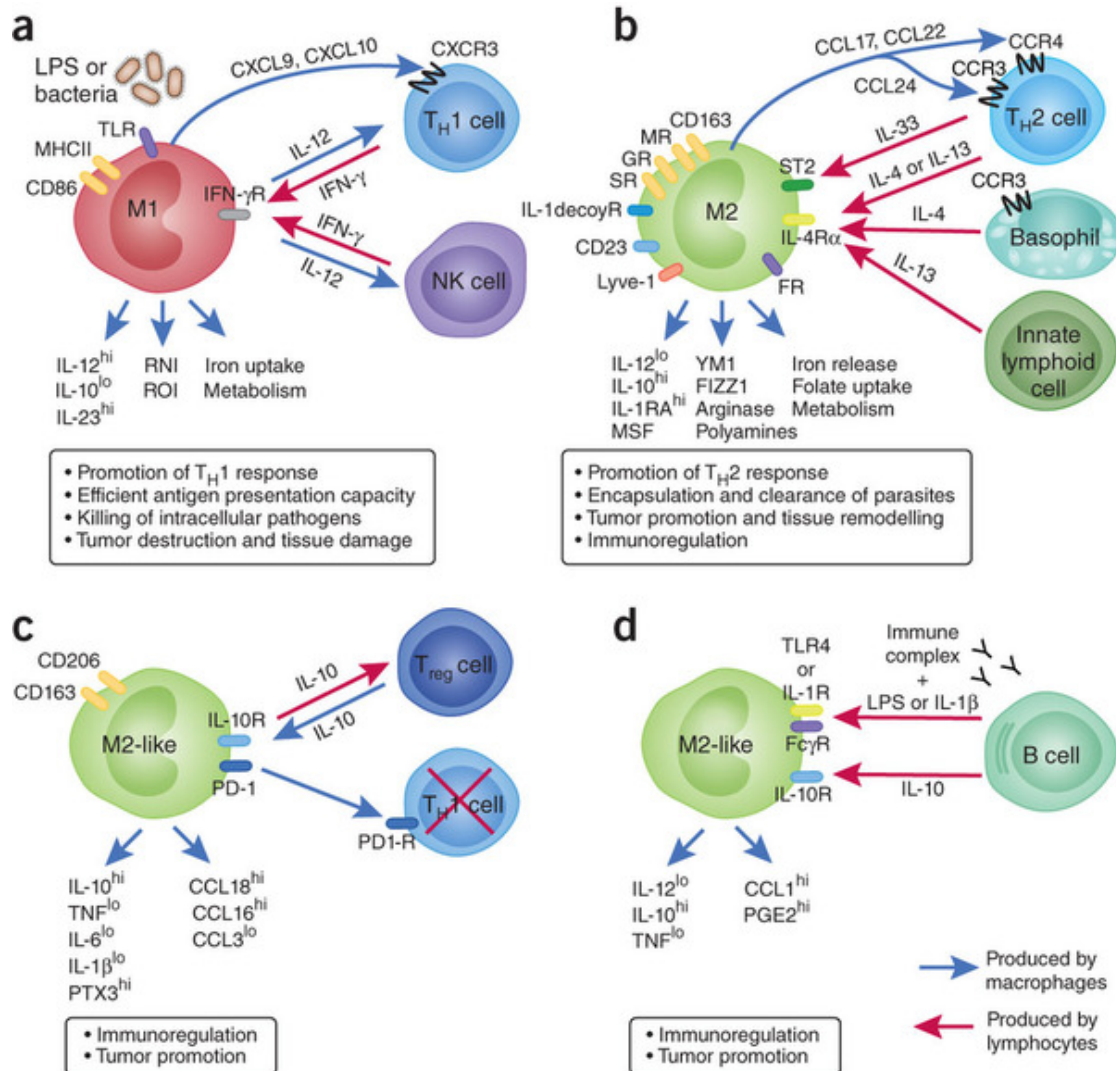


Figure 2 Macrophage phenotype signaling.

(a) M1-polarized macrophages and their crosstalk with TH1 and NK cells. (b) M2 polarization of macrophages driven by TH2 cells, basophils and innate lymphoid cells through their secretion of IL-4, IL-13 or IL-33. (c) M2-like macrophages polarized by interaction with Treg cells. (d) M2-like polarization of macrophages by interaction with B cells through antibody-mediated Fc γ R activation or cytokines. FR, folate receptor; GR, galactose receptor; IFN- γ R, IFN- γ receptor; IL-1decoyR, IL-1 decoy receptor; MHCII, major histocompatibility complex class II; MP, macrophage; MR, mannose receptor; SR, scavenging receptor; ST2, receptor; PGE2, prostaglandin E2; PTX3, pentraxin 3; RNI, reactive nitrogen intermediate; ROI, reactive oxygen intermediate.[5]

Certain diseases will give rise to overlapping phenotypes where both M1 and M2 genes are expressed [5], as well as macrophages that will start out as M1 and later shift to M2. [17]

Macrophages will interact with the different lymphoid subsets in both innate and adaptive immunity. M1 polarization is driven by and in turn amplifies the T_H1 response, aiding resistance

to intracellular pathogens and tumors. [18] The interaction between NK cells and macrophages become important in the shaping of immunosuppression in the placenta, as placental and embryonal macrophages have an M2-phenotype and their interaction with NK cells releases proangiogenic cytokines [19] and induces T_{reg} cells.[20] These interactions make sense because of the great need for establishing a blood supply in the placenta and to downregulate the immune response of the mother to the fetus. The chemokines released by M2 macrophages have corresponding receptors on T_{reg} cells, T_H2 cells, eosinophils and basophils and the recruitment of these cells amplify the T_H2 response. As well as recruiting T_{reg} cells, M2 macrophages drive differentiation of T_{reg} cells and T_{reg} cells in turn polarize macrophages into an M2 state. [10]

The molecular mechanisms of macrophage polarization are currently being researched.

Macrophages have both type I and type II IL-4 receptors that respond to both IL-4 and IL-13 (IL-13 response is only through type II receptors while type I receptors respond to both), activating Janus kinases and STAT6, a master regulator of M2 genes. [21] Chromatin remodeling also determines the direction macrophage activation will go. [22] Receptors, signals and transcription factors for the different macrophages phenotypes are shown in Figure 3.

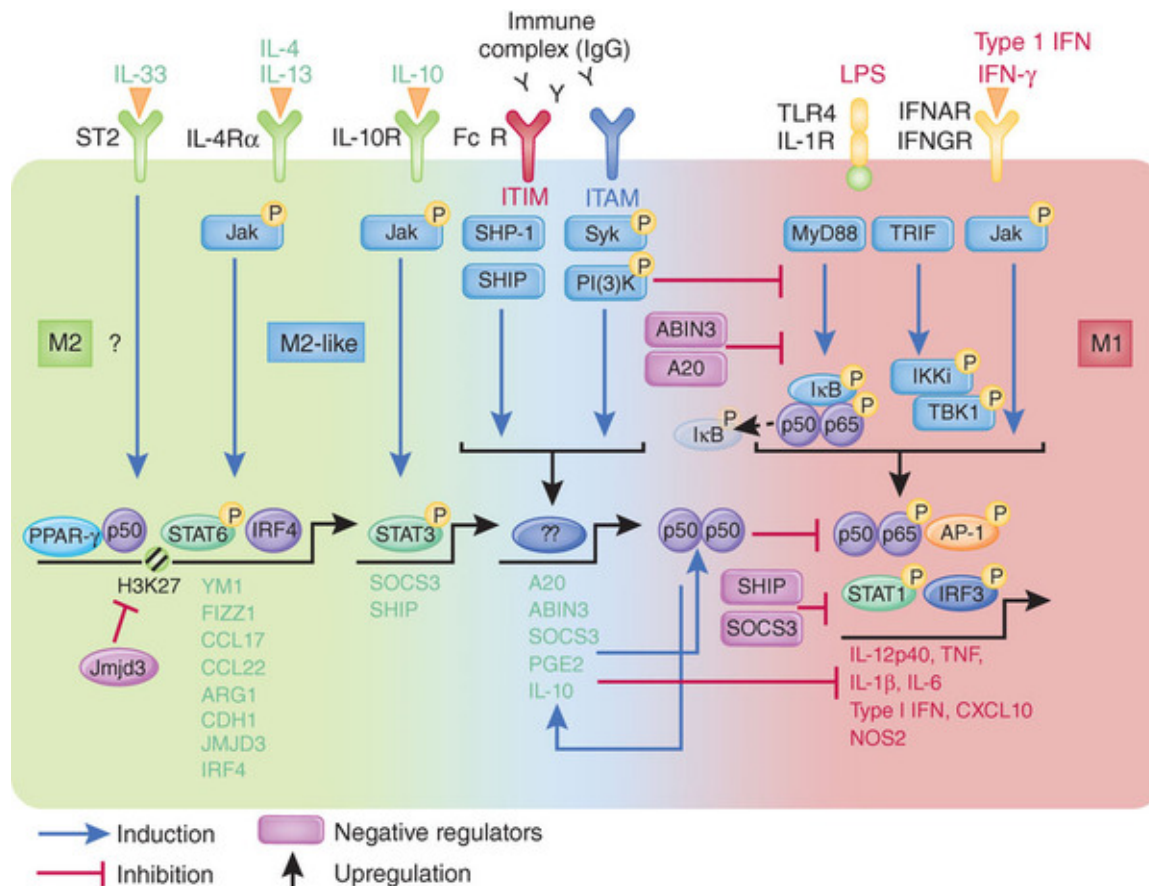


Figure 3 Macrophage phenotype intracellular signaling.

M1 stimuli such as LPS and IFN- γ signal through the TLR4, IFN- α , or IFN- β receptor (IFNAR) and IFN- γ receptor (IFNGR) pathways, inducing activation of the transcription factors NF- κ B (p65 and p50), AP-1, IRF3 and STAT1, which leads to the transcription of M1 genes (red lettering indicates molecules encoded). In contrast, M2 stimuli such as IL-4 and IL-13 signal through IL-4R α to activate STAT6, which regulates the expression of M2 genes (green lettering indicates molecules encoded). The regulation of these genes also involves JMJD3, IRF4, PPAR- γ and p50. IL-10 and immune complexes, plus LPS and IL-1, trigger M2-like macrophage polarization. IL-10 signals through its receptor (IL-10R), activating STAT3. Immune complexes trigger Fc γ R signaling, leading to the expression of molecules such as A20, ABIN3, SOCS3, prostaglandin E2 and IL-10, which negatively regulate the TLR4 and IL-1R and interferon-signaling pathway. Activatory and inhibitory Fc γ R signaling is initiated by activation of Syk-phosphatidylinositol-3-OH kinase (PI(3)K) and tyrosine phosphatase SHP-1-inositol phosphatase SHIP, respectively. Methylation of histone H3K27 is a post-translational modification linked to gene silencing. A20, deubiquitinating enzyme; ABIN3, A20-binding NF- κ B inhibitor; IgG, immunoglobulin G; I κ B, NF- κ B inhibitor; IKKi, inducible I κ B kinase; ITAM, intracellular tyrosine-based activatory motif; ITIM, intracellular tyrosine-based inhibitory motif; Jak, Janus kinase; TBK1, NF- κ B activator; TRIF, adaptor protein.[5]

More of the interaction between TAMs and tumor cells are being discovered. Macrophages isolated from mouse and human tumors generally have an M2-like phenotype. [23] The specific phenotype of the isolated macrophages is an IL-12^{lo}IL-10^{hi} phenotype. They have decreased reactive nitrogen species (RNS) expression, antigen presentation and tumoricidal activity, high

expression of angiogenic factors, metalloproteases, and cathepsins. [24-26] Some tumors, such as mammary tumors, contain macrophages where the phenotype is neither M1 nor M2. We should be careful to characterize whole tumor macrophage populations as it is also possible that the macrophage phenotype changes in different areas of the tumor. One study isolated high MHC class II macrophages with M1 markers that expressed antiangiogenic chemokines in normoxic (tissue with normal oxygen content) tumor tissue. This compared to macrophages isolated from hypoxic tumor tissue that showed low MHC class II expression, along with M2 markers, as well as exhibiting proangiogenic functions. [3] The tumor microenvironment contains a mixture of tumor cells and TAM, as well as different regions of normoxia and hypoxia, causing regions where necrotic and apoptotic tumor cells exist. The general response of macrophages to necrotic cells is higher activity, while lower activity is the response to apoptotic cells. [27] These findings were the reason we also wanted to look at the phagocytic and oxidative burst response of M1 macrophages to apoptotic and necrotic tumor cells in addition to co-incubation with normal tumor cells.

Phagocytosis

All cells can absorb molecules by the process of endocytosis in order to internalize substances that otherwise would not be able to cross the cell membrane due to size or polarity. Endocytosis is even used to recycle existing membrane proteins. Membrane proteins such as clathrin or caveolin are activated by receptors on the surface and cause invagination of the membrane which then pinches off to create an internal vesicle which enters the endocytic pathway by combining with structures called endosomes. Most vesicles from the cell membrane combine with early endosomes. A very mild acidity causes the ligands and receptors to separate and molecules can be shuffled off to other destinations or back to the membrane. Late endosomes are more acidic

(~pH. 5.5) and receive materials from different parts of the cell, including phagosomes, that will later be shuttled off to lysosomes. Lysosomes are acidic (~pH 4.8) and function to break down complex molecules into materials the cell can use through the action of hydrolytic enzymes. Phagocytosis is a complex process that allows for the engulfment of large particles ($\geq 0.5\mu\text{m}$) into vacuoles called phagosomes, sometimes larger than the phagocyte itself. Instead of membrane invagination, phagocytosis occurs by actin activation that results in pseudopod extension and particle engulfment. The phagosome then matures into phagolysosomes by fusing with early endosomes, late endosomes and lysosomes. Phagolysosomes are very acidic and packed with hydrolases that break down the engulfed particles.

Along with dendritic cells, and some B cells and epithelial cells, macrophages are classified as professional antigen-presenting cells (APC's.) APC's internalize antigens by phagocytosis or endocytosis, break them down into smaller pieces and then present those pieces of the antigen, bound to a class II MHC molecule, on their surface. The antigen-MHC II complex is recognized and bound by CD4 Helper T-cells and the T-cell is then activated by a co-stimulatory signal from the APC. Hydrolyzed peptides can also be transported to the cytoplasm by Sec 61 and eventually end up bound to class I MHC molecules and presented to CD8 cytotoxic T-cells. [28]

Neutrophils are sometimes included with macrophages and dendritic cells as professional phagocytes but not professional APC's, although they have been shown to help APC's with peptide presentation and T-cell activation. [29] Phagocytes also play a role in maintaining healthy tissues and remodeling by removing apoptotic cells and cellular debris. One good example of this is the removal of webbing between the fingers of a developing fetus.

Macrophages find their targets by extending actin-rich structures into their surroundings, actively probing the environment to more effectively find fast moving or poorly opsonized targets. Both

this probing and subsequent internalization happens through actin polymerization that is dependent on Rho-GTPases and phosphoinositides. [30] Receptors on these probes, or on the macrophage main body, recognize and bind to ligands on the target. Some of these receptors are pattern, or pathogen, recognition receptors (PRRs.) In insects and mammals, these are more specifically called Toll-like receptors (TLRs.) These bind conserved molecules on microbes called microbe-associated molecular patterns (MAMPs, classically called PAMPs but pathogen-associated has been changed in favor of microbe-associated, since microbes other than pathogens express these molecules.) Mannose is bound by mannose receptor, LPS by TLR 4, flagellin by TLR 5, viral double-stranded RNA by TLR 3, to mention a few. TLR binding and signal activation turn on the NF- κ B pathway. [31]

Targets bound by host serum factors, or opsonins, are said to be opsonized and are bound by opsonic receptors, typified by complement receptor 3 (binds iC3b) and Fc γ receptors (bind IgG.) The signals and events that lead to internalization are not the same for every receptor, but signaling will only start when multiple receptors converge, or cluster, in one region. It is not known how receptor clustering works, even though some have hypothesized that receptors and downstream signaling elements gather in lipid rafts. [32]

The Fc γ receptor, or FcR, pathway is one of the more studied signaling pathways associated with phagocytosis. Upon clustering of receptors, the receptors themselves, or associated immunoreceptor tyrosine-based activating motif (ITAM)-containing subunits, are phosphorylated by members of the Src family. The phosphorylated receptors/ITAMs become docking sites for Syk, a tyrosine kinase. Downstream of these events, phosphatidylinositol-3-kinase (PI3K) is activated, causing generation and accumulation of 3'-phosphoinositides (PI3) at the phagosomal cup, a crucial event necessary in phagocytosis. The phagosomal cup is a cup-

shaped structure, formed principally by invagination of the plasma membrane during the early stages of phagocytic uptake of particles by cells. Membrane that originates from other organelles may be added to it. [33] Wiskott-Aldrich syndrome proteins (WASP's) act as molecular scaffolds by associating with PIP₂'s (a phosphoinositide) on the membrane. When WASP binds both PIP₂ and the Rho GTPase Cdc 42, it activates the actin-nucleating function of Arp2/3. [34] The Rho and Rac GTPases are generally involved in all phagocytic activating pathways. Actin nucleation is the basis of forming pseudopod extensions.

It has often been assumed that the plasma membrane is the source for the membrane used in phagocytosis, but the plasma membrane does not account for the amount of membrane needed to enclose larger molecules. Cell function would also be greatly impaired if too much plasma membrane was disrupted for use in forming the phagosome. Current research shows that the ER-membrane contributes to the engulfment of large molecules, as well as some of the internal endosomes. [35] [Fig 4]

ER-Mediated Phagocytosis

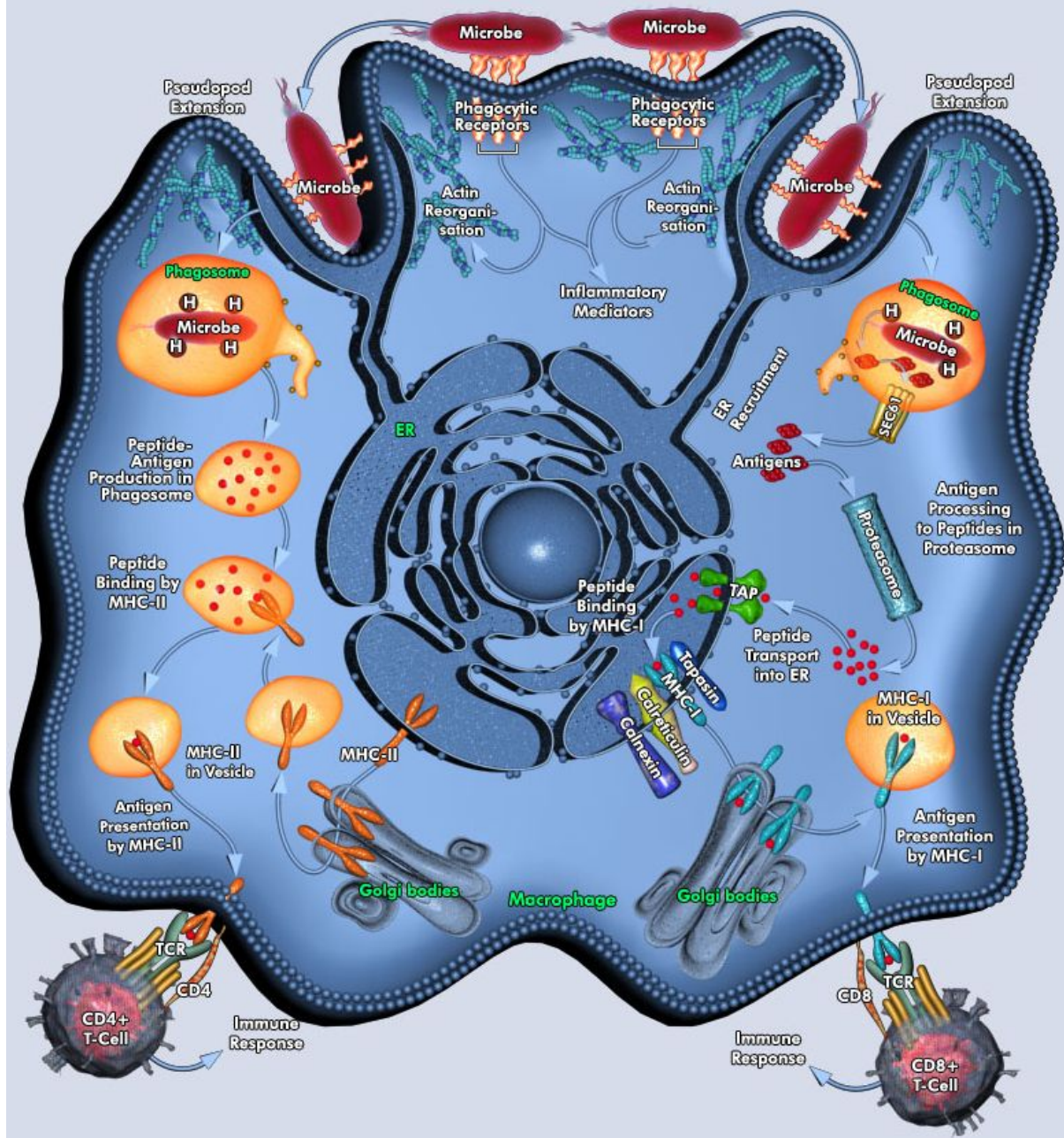


Figure 4. Phagocytosis and ER membrane.

Events in phagocytosis shown along with ER-membrane recruitment. Peptide processing in the antigen presentation pathways as well as interactions with T-cells shown. © QIAGEN 2003 - 2012. All rights reserved. [36]

Phagocytosis by professional phagocytes causes the activation of NADPH oxidase, causing the ‘respiratory burst’ and the production of reactive oxygen species. The respiratory burst is thought to be the main mechanism in killing phagocytosed microbes.

Reactive oxygen species and respiratory burst

Reactive oxygen species (ROS) include superoxide anion radical ($\bullet\text{O}_2^-$), hydroxyl radicals ($\bullet\text{OH}$) and hydrogen peroxide (H_2O_2). Since increased ROS production is a hallmark of the M1 macrophage phenotype, we decided to look more closely at its production in macrophages. H_2O_2 is a weak oxidizing agent and is less reactive than the free radicals, but has a relatively long half-life, good membrane permeability and higher intracellular concentrations which make it important as a potential second messenger. [37] Even though H_2O_2 by itself is not the most important reagent in the respiratory burst, the amount released is comparable to that of other ROS. It can be oxidized into the very reactive and toxic $\bullet\text{OH}$ in the presence of transition metals such as copper and iron through a reaction called the Fenton reaction. Cells contain natural antioxidant proteins that protect against the action of ROS, including superoxide dismutase ((SOD) converts $\bullet\text{O}_2^-$ into H_2O_2), catalase and glutathione peroxidase I (reduce H_2O_2), thioredoxin, and glutaredoxin. Thioredoxin and glutaredoxin are proteins that alleviate oxidative stress by reducing other proteins and are then in need of being reduced themselves. Glutathione (GSH) is oxidized into glutathione disulfide (GSSG), which is reduced back to GSH by GSH reductase, using NADPH as an electron donor. The ratio of GSSG/2GSH can serve as an indicator of the cellular redox state. [38]

Aerobic respiration and other cellular events produce ROS through normal cellular processes, but antioxidant proteins generally are enough to neutralize the ROS produced by these processes. [39] Excessive amounts of ROS can be produced by inflammatory processes, ionizing radiation and many chemotherapeutic drugs to the point where the ROS production exceeds the capacity of the antioxidant proteins. This causes oxidative stress, an imbalance between the oxidant

production and antioxidant capacity of the cell, which results in the cell no longer being able to prevent oxidative damage. [40]

ROS are produced by the professional phagocytes of the innate immune system, including neutrophils, eosinophils, monocytes and macrophages. The enzymatic complex that produces ROS in professional phagocytes is NADPH oxidase 2 (NOX2), part of a family of NADPH oxidases that consist of a transmembrane flavocytochrome that interacts with a variety of activating cytosolic proteins, including a small GTP-binding protein.[41] In NOX2, the flavocytochrome is gp91phox, the cytosolic proteins are p40phox, p47phox and p67phox and the GTP-binding protein is Rac. Some of the cellular components that participate in the various signals and events surrounding phagocytosis are also linked to NOX activation. Accumulation of phosphatidylinositols and phosphatidylinositol-3-kinase (PI3K), Rac and Rho proteins (which are also activated during phagocytosis) and their activity during NOX activation provides a link between the two mechanisms, helping the cells coordinate phagocytosis and microbial killing. This is shown in more detail in Figure 5.

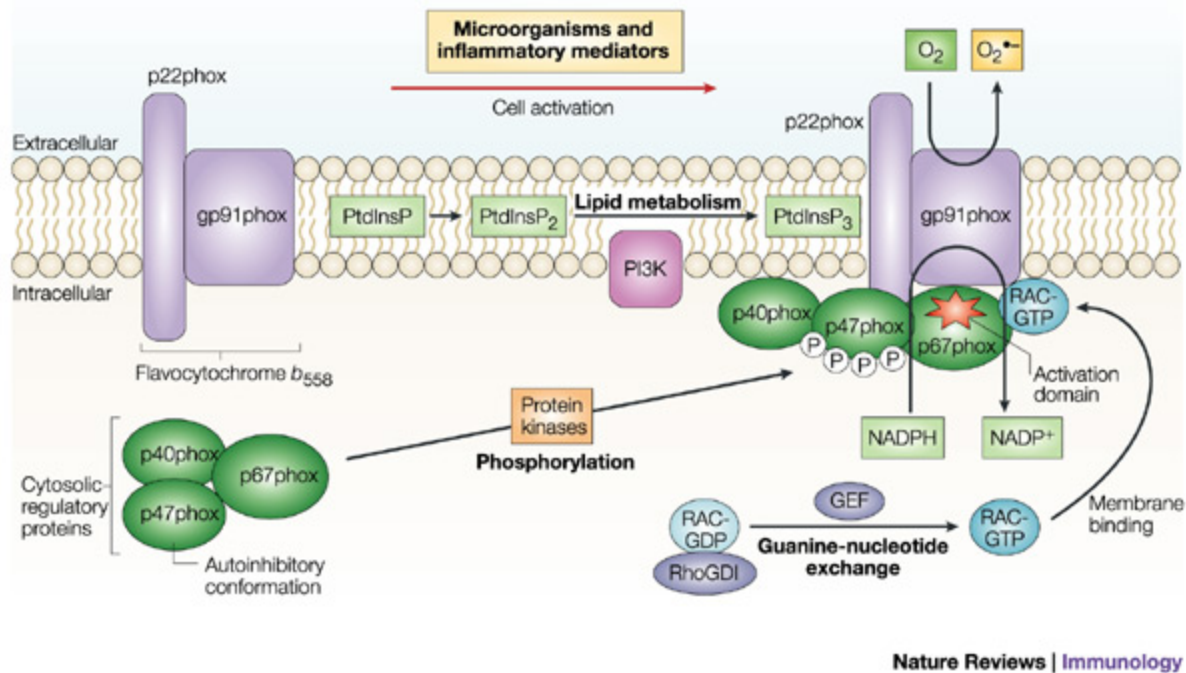


Figure 5 NADPH Oxidase complex in the phagosomal membrane.

Activation of the gp91phox system occurs by at least three signaling triggers that result in the assembly of cytosolic regulatory proteins (p40phox, p47phox and p67phox) with flavocytochrome b558 (comprised of the membrane-associated catalytic subunit gp91phox plus p22phox). These triggers involve protein kinases, lipid-metabolizing enzymes and nucleotide-exchange proteins that activate the GTPase Rac. The protein p47phox binds to p22phox and p67phox, as well as binding phosphatidylinositols together with p40phox in the membrane, and can be described as an organizer protein. [42]

NOX2 is assembled and activated on the membrane of the phagosome and generates superoxide inside the phagosome to help kill phagocytosed organisms and prevent damage to surrounding cells. [43] The receptors that are involved in respiratory burst, as well as phagocytosis, are the Fcγ receptor and the iC3b receptor. Some of the activating signals of superoxide formation, such as p38 MAPK and PI3K, also participate in phagocytosis signaling pathways, while PKC acts only on oxidase activation signaling. [44] Engagement of TLR can also influence the maturation of the phagosome through the MyD88 adaptor, a protein used by almost every TLR to activate the transcription factor NF-κB, and p38. [45] Thus phagocytosis and NOX activation shares activating receptors and activating signals as well as downstream components.

Mutations that result in a lack of NOX2 activity result in a disorder called chronic granulomatous disease (CGD). [46] The mutations that result in this disease are in the genes coding for component proteins of NOX and studies on patients with the disease helped in finding the identity and function of these proteins. The patients have a severely limited ability to kill pathogens and frequently contract severe, often fatal, infections. The clinical test for this disease involves the use of rhodamine, the same chemical used in our experiments to determine oxidative levels.

NOX2 is the main component responsible for the ‘respiratory burst.’ This is named after the magnified uptake of oxygen by neutrophils during phagocytosis. This ‘extra respiration’ was first noticed in 1932, but was first thought to provide additional energy for phagocytes. [47] Finding the protein complex responsible was complicated by how hard it was to isolate, how sensitive it was to salts and other molecules and not being able to function without a membrane. Eventually, a previously discovered b-type cytochrome was shown to be the mutated protein in many forms of GCD. [48] The cytochrome was called flavocytochrome b_{558} due to an absorbance maximum near 558nm. It’s a heterodimer, with the two subunits that co-purified with the heme group named gp91^{phox} and p22^{phox}, with two molecules of heme and one molecule of flavin adenine dinucleotide (FAD). [49] Both heme groups are located on p91^{phox}, which is heavily glycosylated, about 85-100kDa, N-linked glycoprotein with several transmembrane domains and a 65-kDa protein core. [50]

The respiratory burst is activated after a particle is phagocytosed and the vacuole has closed. [51] Several activating molecules cluster transiently at the phagosomal cup, such as different isoforms of PKC, diacylglycerol kinase (DGK) and p47^{phox}. [52] DGK produces phosphatidic acid, a signaling molecule and lipid intermediate, from diacylglycerol. Activation appears to require

phosphatidylinositol phosphates and there's a 20-30 second lag between activating stimulus and oxidase activity. [53] PI3K activity generates PIPs [54] that are bound by the phox homology (PX) domain on p40^{phox} and p47^{phox}, which helps organize the other proteins that do not contain a PX domain, as discussed earlier. Cytoskeleton rearrangement [55] and phosphorylation of the different NOX proteins[41] are also implicated in NOX activation.

Even with current research dedicated to the action of ROS and the respiratory burst, there is still not concrete evidence for how exactly microbes are killed by the respiratory burst. One problem being the difficulty in reproducing the staggering concentration of O₂⁻ produced in the phagocytic vacuole which is required for microcidal activity, in the region of 4 mol/L. [56] It has always been assumed that H₂O₂ is responsible for killing microbes, but studies show that *Salmonella* can easily neutralize the levels of H₂O₂ produced by neutrophils by the action of several scavenger proteins. [57] Because of the newer implications of ROS being critical for signaling and activation of other microbial factors, future research will probably be devoted in this direction. [58]

Another question about the respiratory burst that remains unanswered is how charge compensation is achieved in the vacuolar membrane. NOX transfers electrons unaccompanied by protons across the vacuolar membrane, creating a massive depolarization unless compensated. [59] The NOX complex itself is sensitive to depolarization, shutting down in case of too much charge buildup. [60] H⁺ and K⁺ are the only ions present at high enough concentrations for charge compensation and as such are likely candidates, but inhibiting the flow of either of these ions doesn't inhibit oxidase activity. [41] Current models make it difficult to answer these questions, but future research hopefully will.

High levels of ROS production have been shown to be damaging to the macrophage itself, inducing DNA fragmentation and apoptosis of the macrophage. [61] The presence of scavenger molecules inside and outside of the macrophages is likely a mechanism to maximize both ROS production and microbial destruction or tissue damage while minimizing harmful effects to the macrophage and healthy tissue.

Though ROS production is initialized by phagocytosis and the sharing of activating signals and downstream messengers provide some explanation of why the ROS production is linked to phagocytosis, other signals could explain why sometimes these processes are not always directly linked. To see if we could find ways to trigger different responses, we decided to find a chemical stimulus and compare that to the tumor cell stimulation of macrophages. For this, we chose caffeine.

Caffeine

Chemical stimulation, such as exposure to caffeine, can also affect ROS production and rates of apoptosis in macrophages in a dose dependent manner. [62] The main function of caffeine is as an inhibitor of the purine nucleotide adenosine and phosphodiesterase. Adenosine acts to suppress neurotransmitter release while phosphodiesterase breaks down cyclic AMP and their inhibition accounts for caffeine's role as a nervous system stimulant. Caffeine and its metabolite, theophylline, bind to the adenosine receptors as non-selective direct competitors to adenosine, binding the receptor without activating it. [63] The four different adenosine receptors all have caffeine as an antagonist. The receptors are A₁, A_{2A}, A_{2B} and A₃. The A₁ and A₃ receptors preferentially interact with G_s G-proteins and A₁ and A₃ with G_{i/o} G proteins. [64] The A_{2A} receptor is thought to have still undiscovered functions because of its ability to form heteromers

with other G protein receptors, such as dopamine and cannabinoid receptors. [65] One of the effects of A_{2A} has been shown to be attenuation of tissue-specific and systemic inflammatory response. [66] Binding to adenosine receptors explains some of the neurostimulatory effects, while other effects are possibly due to caffeine being a purine analog. Caffeine is broken down by hepatic P450 into paraxanthine, theobromine and theophylline. [67] Besides the main functions of caffeine, the secondary effects of caffeine are what made us choose it as a chemical stimulant for macrophages. It is a modulator of the innate immune systems and especially of macrophage subtypes such as microglia. [68] An increase in cAMP levels and prostaglandin production has also been shown in alveolar macrophages due to caffeine exposure. [69] These effects alone can have a significant impact on cellular activity. This is possibly because caffeine has been shown to inhibit the activity of PI3K. [70] PI3K activity can lead to delayed apoptosis and cell survival in tumors as well as other oncogenic effects. [71] This activity of PI3K is in addition to the role it plays in phagocytosis and respiratory burst activation, as previously discussed. Caffeine has many other effects on the cell that have been disputed because the concentrations used are sometimes much higher than possible under physiological conditions and different concentrations seem to have opposite effects. Depending on concentration and p53 expression, caffeine has been shown to induce, not induce or even protect against apoptosis. [72] Other studies show that at low concentrations (1-2 mM), caffeine can induce G1 arrest through p53 dependent action, [73] but at slightly higher concentrations (2-4 mM) it appears to block G1 arrest. [74] One major target of caffeine might be the DNA-damage sensing ATM/ATR protein kinases, even though this has been disputed as well. [75] Caffeine affects intracellular calcium release, which could be linked to immune function. [76] Increased calcium concentration, along with increased DAG, can act as activating signals for PKC.

Due to the link between macrophage phenotypes, engulfment and respiratory burst, our experiments will measure and compare these two cellular processes. Macrophages will be classically activated through LPS and exposed to a chemical stimulus (caffeine) as well as co-incubated with tumor cells to determine response.

Our experiments will focus on the effects on activated M1 macrophages caused by exposure to caffeine and tumor cell lines. We hypothesize that the level of phagocytosis and radical oxygen species production will be directly linked. We assume that activity will increase with exposure to caffeine and decrease with exposure to tumor cells, based on previous research or published material.

MATERIALS AND METHODS

Tissue culture: U937 monocyte and HL-60 promyelocytic cell lines were cultured in RPMI 1640 media supplemented with 10% bovine calf serum, 2 mM L-glutamine and 1.5g/L sodium bicarbonate. U-937 cells were resuspended in RPMI containing 200 nM phorbol 12-myristate 13-acetate (PMA) and cultured in cell culture flasks at 2×10^7 for 48 hours to stimulate macrophage differentiation. U937 cells differentiate into macrophages through exposure to PMA. Once differentiated, they show characteristics of whole blood macrophages (plating down in culture, pseudopodia, engulfment of foreign particles, irregular cell shape, etc.) MDA-MB-435 and MCF-7 breast cell lines were both maintained in DME high glucose media, while HT-29 and SW620 colon cell lines were maintained in McCoy media, L-15 Leibovitz media. All cells were incubated at 37° Celsius and 5% CO₂.

Macrophage separation and activation: Whole blood was drawn from volunteers under IRB approval number: [X 090128] and lymphocytes were separated with Lymphocyte Separation Medium (LMS, cellgro) according to the manufacture's protocol. LMS causes plasma, red blood cells and lymphocytes to separate based on density, leaving a middle layer of lymphocytes that can be easily removed (See Figure 6.)

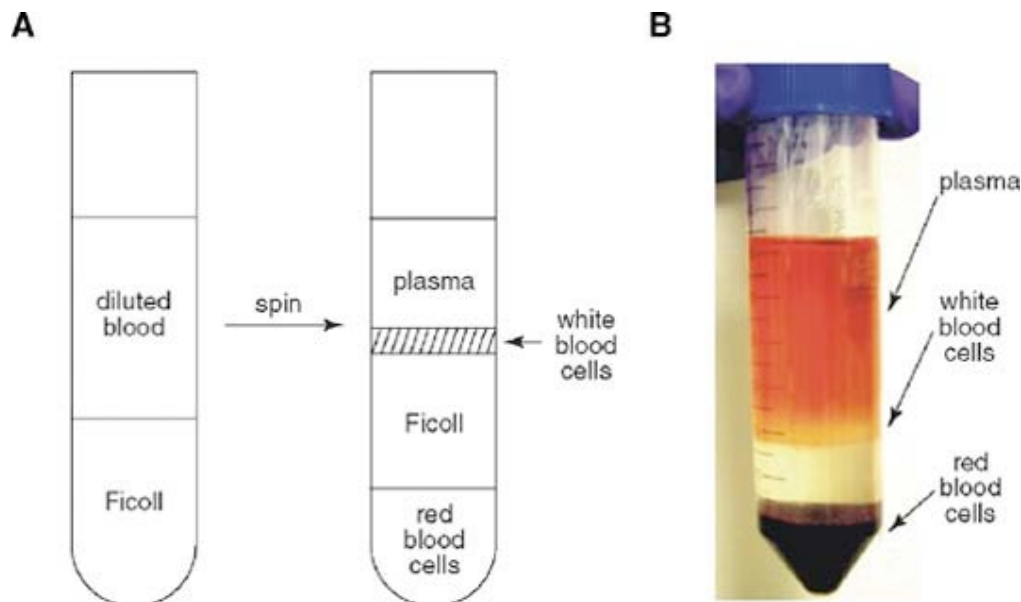


Figure 6 Separation of lymphocytes from whole blood. Whole blood after centrifugation in LMS. Shows density dependent separation of plasma, lymphocytes and red blood cells.

Lymphocyte layer is separated from mixture. The separated cells were then incubated in RPMI 1640 supplemented with 20% serum taken from the same volunteer. LPS was added at a concentration of $3\mu\text{g/mL}$ for 4 days to differentiate monocytes to macrophages. After 4 days, medium was discarded and 5 mL of PBS was added to the flask. Flask is then scraped with a cell scraper to remove adherent macrophages.

Culture and Co-culture: For spent media experiments, stained macrophages were allowed to incubate in spent media from each cancer cell line for 1, 12, and 24 hour time periods. For initial co-culture experiments with U937's, breast and colon cancer cells at a concentration of 1×10^6 cells/mL were seeded in 6 well plates and cultured for 48 hours. Following culture, stained macrophages were added to 6-well plates and allowed to incubate with cancer cells for 1, 12, and 24 hours. When using whole blood, activated whole blood macrophages were re-suspended in E-fluor 670 APC positive dye (eBioscience) and incubated for 20 min to allow staining unless no

co-culture was intended. Macrophages were then re-suspended in RPMI 1640 and plated in 12-well plates at a concentration of 0.5×10^6 cells/mL for 24 hours to allow adherence. For co-culture, unstained RAJI and HL-60 cells at a concentration of 0.5×10^6 cells/mL were added for 24 hours, so as not to exceed a total concentration of 1.0×10^6 cell/mL.

Caffeine: Caffeine solutions were prepared from caffeine powder (Sigma, CAS # C8960) dissolved in PBS and added to differentiated macrophages at the desired concentrations in medium for 24 hours.

Measuring oxidation levels: The chemical rhodamine (2-(6-Amino-3-imino-3H-xanthen-9-yl) benzoic acid methyl ester, Sigma CAT no. 62669-70-9) was used to measure the levels of respiratory burst in macrophages. Rhodamine enters the cell through the cell membrane and inside the cell is oxidized by H_2O_2 to create dihydrorhodamine (DHR), which fluoresces green at 560 nm. Once oxidized, DHR becomes positively charged and can no longer exit the cell. DHR was added to macrophages during bead incubation.

FACS analysis: Fluorescent readings were performed by a BD FACSCanto Flow Cytometer. For engulfment, 2.0 micrometer polychromatic red latex microspheres (or beads) and DHR were added for 1 hour to cells in a 12-well plate. Following engulfment, samples were washed three times in DPBS and resuspended in 300 μ l DPBS for flow cytometry analysis. Engulfment activity was measured by analyzing the total number of APC MØ's that engulfed PE-conjugated beads. Results were gated according to viable, APC-stained (if co-cultured) cells and divided by number of peaks corresponding to number of fluorescent beads.

RESULTS AND DISCUSSION

Initially, we wanted to use a plate reader to measure fluorescence due to DHR oxidation. We decided to abandon this method since DHR spontaneously oxidizes in solution. This problem is solved when reading fluorescence inside the cells individually by using a flow cytometer.

In our early experiments, there was a problem with non-specific bead binding to the outside of the cells. This was solved by incubating the beads in serum before addition to the 12-well plate.

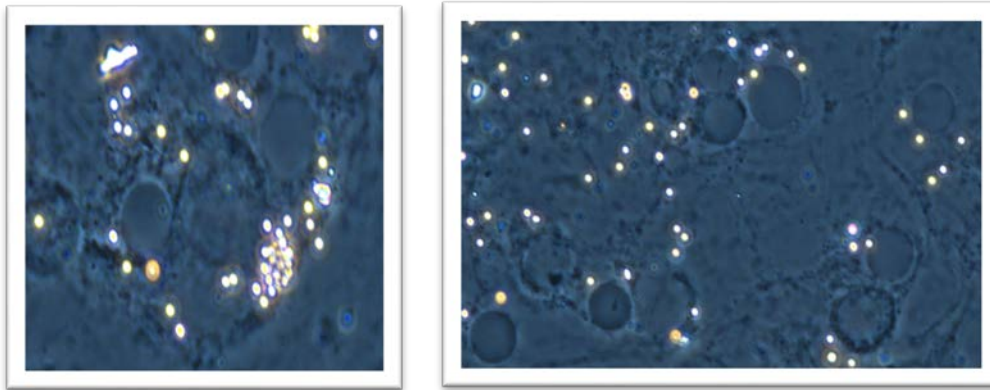


Figure 7a - Regular and serum coated beads with HT-29 cells.

Uncoated fluorescent beads on the left and serum coated beads on the right, incubated with HT-29 cells.

As seen in the fluorescent images in Figure 7a, non-specific surface binding of beads to cells was greatly reduced after incubating beads in serum. These results were also corroborated by using flow cytometry.

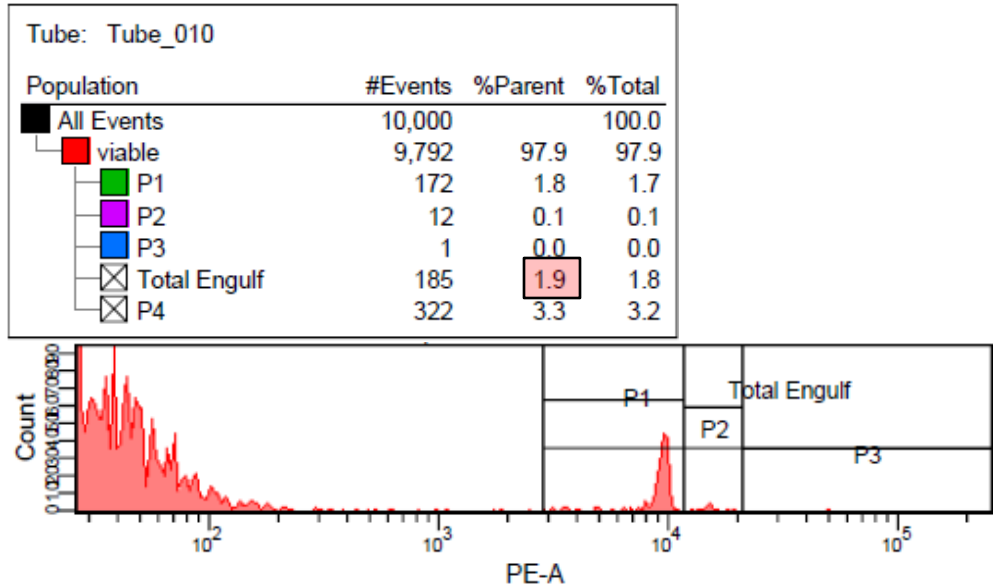


Figure 7b - HT 29 cell test sample with fluorescent beads.

Red box shows % of HT-29 cells that have fluorescent beads bound on the outside, indicating non-specific binding instead of engulfment. P1 and P2 show 1 and 2 beads binding, respectively.

The results in Figure 7b show that through incubating the fluorescent beads with fetal bovine serum, we can minimize non-specific binding to the outside of cells.

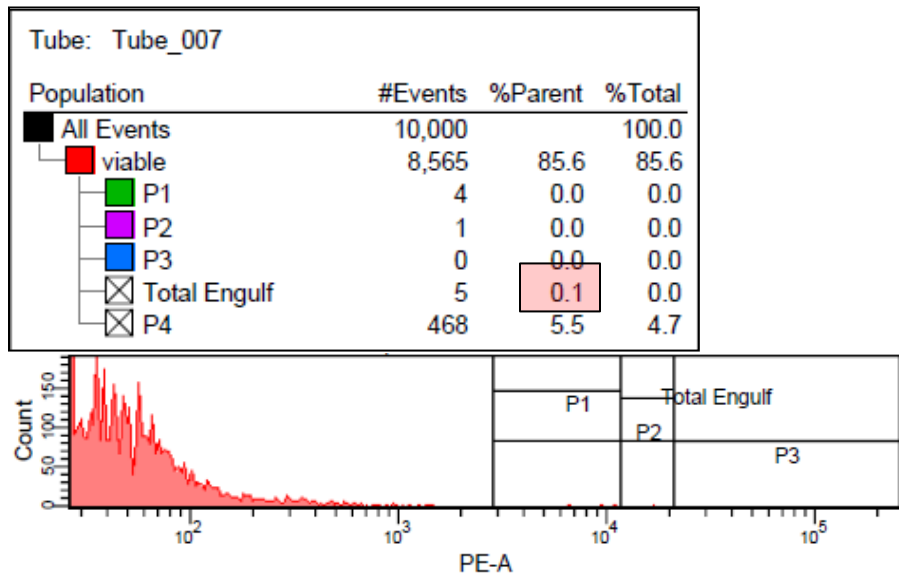


Figure 8 Macrophage (MO) negative control sample without beads.

Total engulfment shows the % of total macrophages (viable) that fall inside the gated area of the graph when no beads are present.

Figure 8, the negative control of macrophages incubated without any beads, renders an insignificant reading in the PE channel, the channel that reads bead fluorescence.

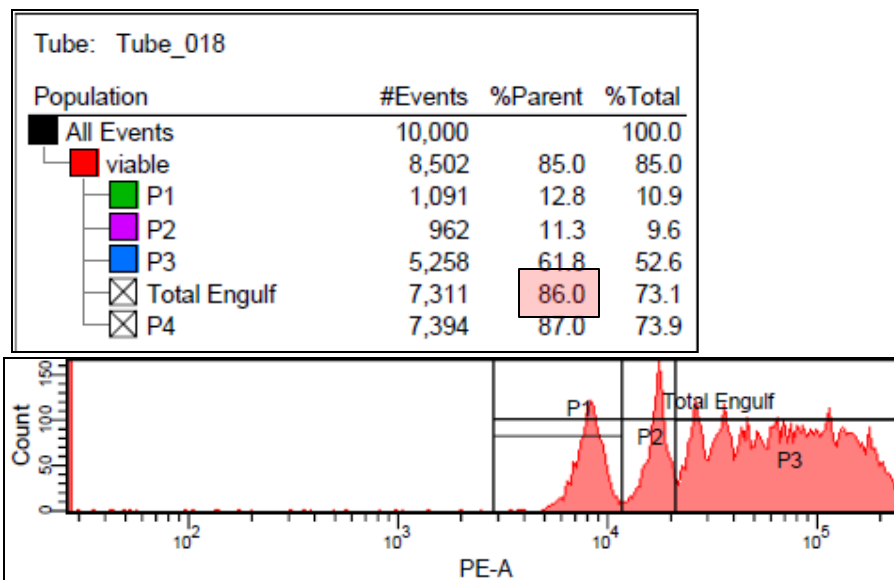


Figure 9 Positive control MO sample with beads.

Total engulfment shows the % of total macrophages (viable) that engulf 1 or more beads. P1, P2 and P3 show 1, 2 and 3+ beads being engulfed.

Results in Figures 8 and 9 indicate that the engulfment assay is working. Macrophages engulf beads while control cells do not. This can be seen from the insignificant binding of beads to non-macrophage cells compared to a high percentage of engulfed beads by macrophages. After solving the problem of non-specific binding, we moved on to finding a way to analyze the results and use them to measure the aggressiveness of macrophages. Readings from the flow cytometer showed separate peaks corresponding to the number of beads engulfed. These peaks were separated by gates and measured in each experiment.

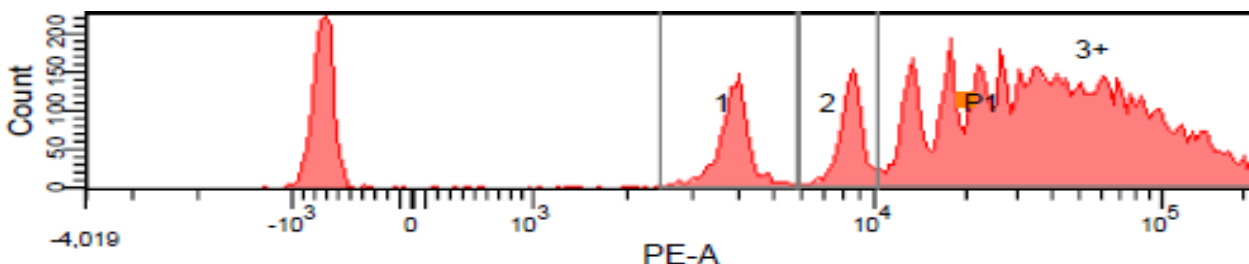


Figure 10 Fluorescent beads FACS reading and gating with 3 subpopulations.

Fluorescent beads show distinct peaks corresponding to 1, 2 and 3+ beads. Gray lines denote gates we used to separate the different populations of macrophages based on the number of beads engulfed.

Most experiments will use the method of dividing engulfing macrophages into three populations as shown in Figure 10.

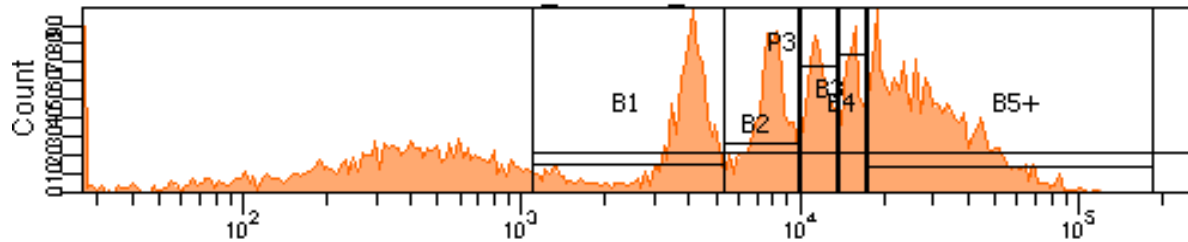


Figure 11. Fluorescent beads FACS reading and gating with 5 subpopulations.

Gated peaks correspond to 1, 2, 3, 4 and 5+ beads (B1, B2, B3, B4 and B5+, respectively.)

As can be seen in Figure 11, peaks can be resolved up to 5 beads before resolution becomes too small. Not all experimental runs will have such distinct peaks; therefore most experiments only measured 3 populations (1, 2 and 3+ beads,) as in Figure 10. Having developed a reliable engulfment assay, we had a decision to use either differentiated U937 cells or whole blood macrophages for our experiments. We performed an experiment that compared the activity of U937 derived macrophages to whole blood macrophages.

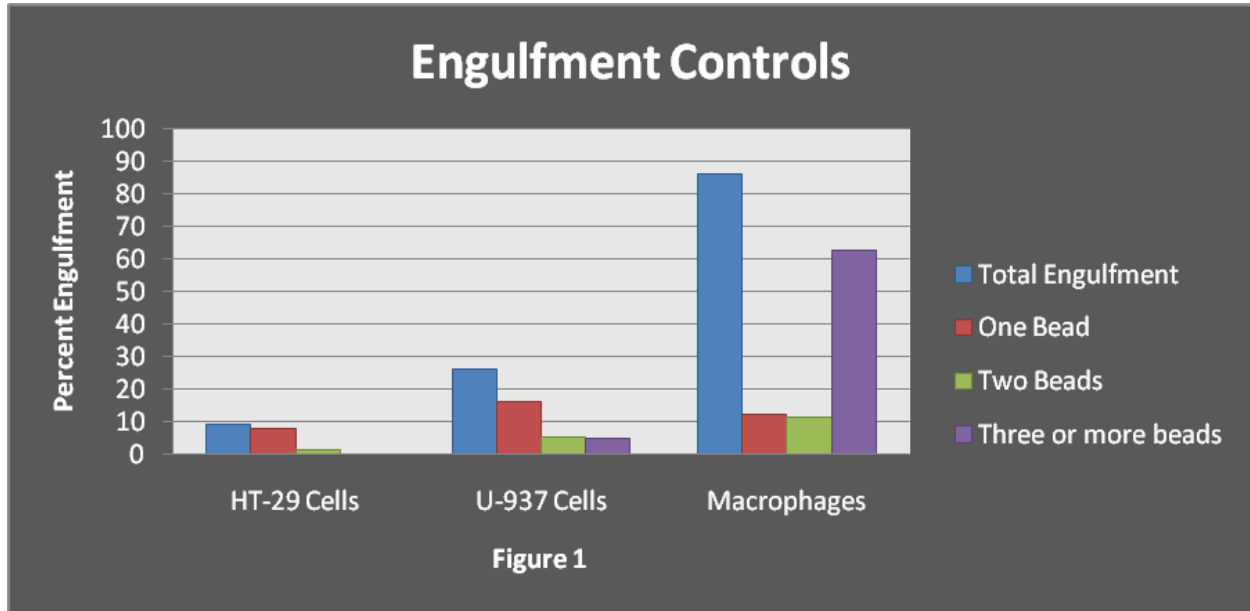


Figure 12. Comparison of whole blood and U937 derived macrophage engulfment.

Ability of U-937 stimulated macrophages (MØ's) to engulf latex beads was assessed. Beads were added to HT-29 cells, unstimulated U-937 cells, and MØ's. The total number of engulfing cells can be seen in orange. Of cells that engulfed, aggressiveness was measured as number of engulfing cells that engulfed 1, 2, or 3+ beads.

The experiment shown in Figure 12 indicates that at control levels, whole blood macrophages showed a larger total engulfment and a tendency to engulf more beads than U937's. In some experiments, we still used U937's for convenience, but later experiments used whole blood macrophages.

Our figures were gated to show the percentage of the population with increasing numbers of beads engulfed. Oxidation levels measured through DHR fluorescence for each peak were also graphed. Most experiments were only divided into 1, 2 and 3 or more bead populations and designated non-aggressive, aggressive and highly aggressive.

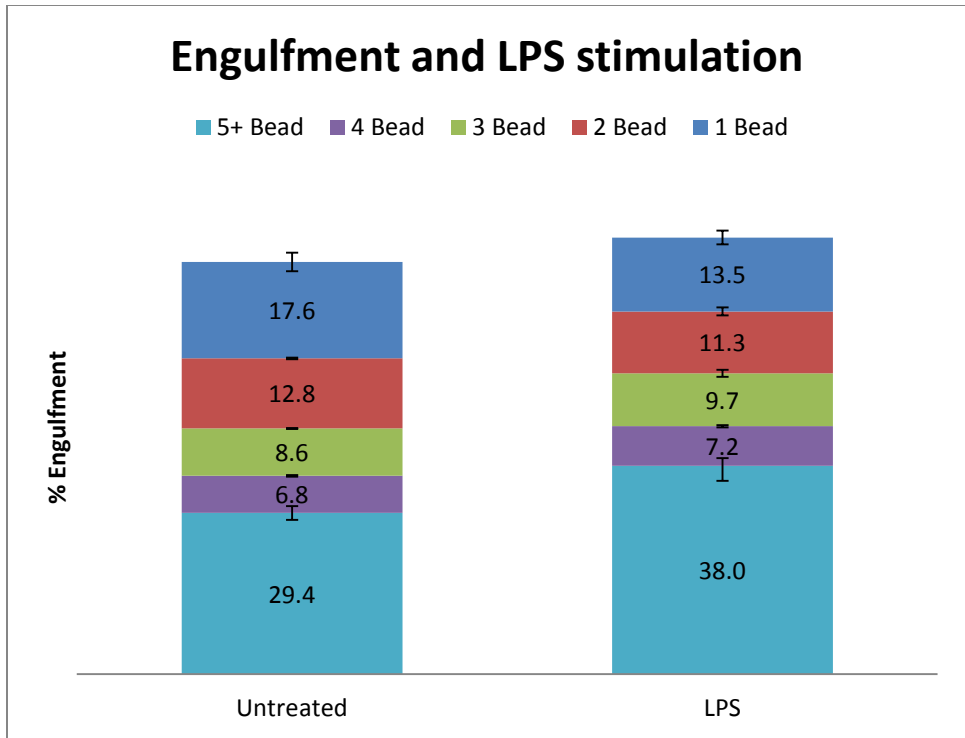


Figure 13 Normal and LPS treated macrophages and engulfment.

The percentages of macrophages with engulfed beads, separated by number of beads engulfed, in untreated state and LPS stimulated state. n=3. Error bars shown standard deviation.

The total height of the column in Figure 13 is due to total engulfment in the sample, while the individual stacks are from macrophage populations separated by number of beads engulfed.

These results reflect the mean of several experiments. The standard deviation for the most aggressive population (5+ beads engulfed) of the untreated cells is 1.27 and 2.76 for the most aggressive population of LPS treated cells. For untreated and LPS treated macrophages, total engulfment was 76.25 and 80.8 with a standard deviation of 1.34 and 1.41, respectively. This experiment shows a significant increase in the level of total engulfment as well as the percentage of aggressive macrophages with LPS stimulation. In a normal macrophage population, a larger portion of the population engages in one or two engulfment events, with each event after those involving decreasing portions of the population. Exposure to LPS will cause more macrophages to engulf 5+ beads.

Adding to our engulfment assay, we also wanted to measure dihydrorhodamine (DHR) fluorescence. DRH becomes fluorescent when rhodamine enters the cell and is oxidized by H_2O_2 , so DHR fluorescence becomes a suitable method to measure the amount of ROS release. We performed experiments where rhodamine dye was added to the engulfment assay.

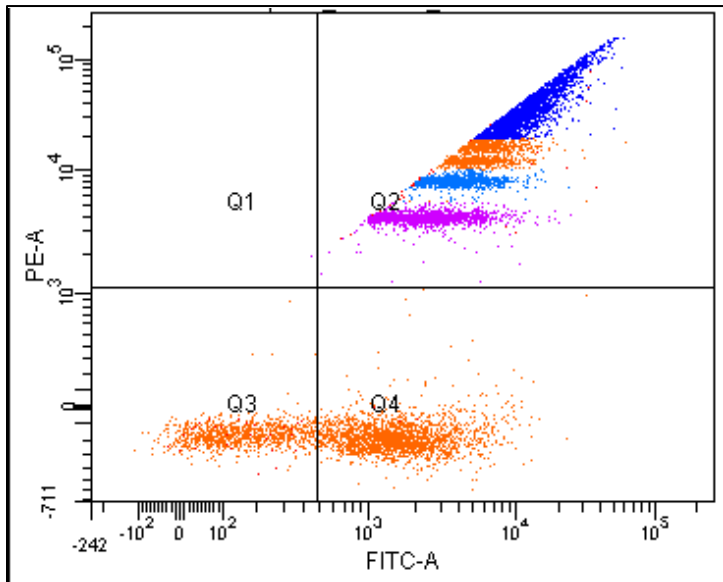


Figure 14. FACS reading of macrophages with fluorescent beads and DHR.

PE-A axis is the fluorescent reading for beads. The FITC-A axis reads the fluorescence for DHR.

The flow cytometry data in Figure 14 showed the correlation between bead engulfment and DHR oxidation. The Q2 quadrant shows macrophages that engulfed beads and also exhibited oxidized DHR. The Q4 quadrant in Figure 14 shows macrophages that have not engulfed any beads but still show DHR fluorescence. Reading in the Q4 quadrant but not in the Q1 quadrant shows that macrophages can produce ROS in response to LPS stimulation without any engulfment, but that engulfment does not happen without ROS production. This is according to expectations based on literature.

To find the correlation between DHR oxidation levels and the number of beads engulfed, DHR levels were read from different populations and averaged to find the general relationship of engulfment and oxidation.

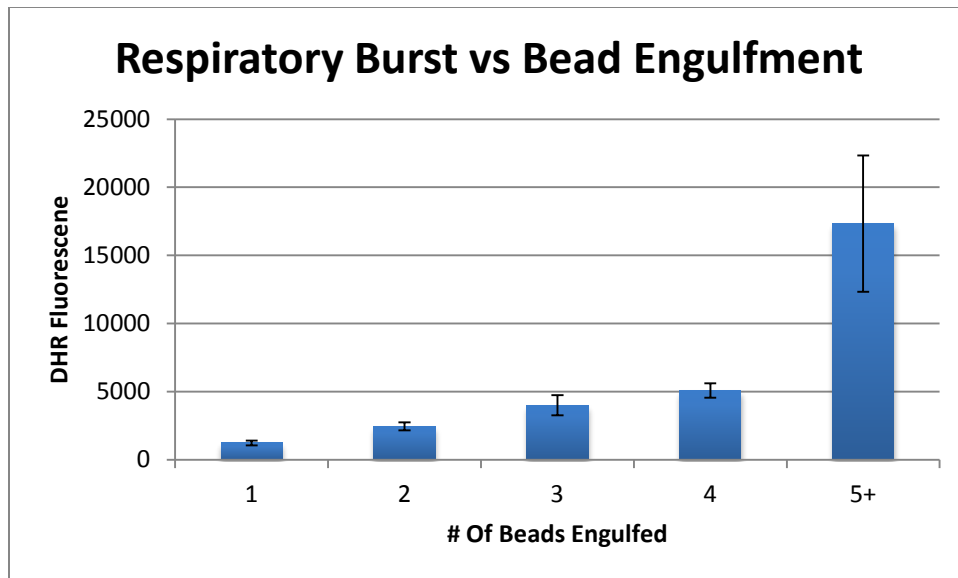


Figure 15. DHR fluorescence vs number of beads engulfed.

This graph shows the level of ROS, measured through DHR fluorescence for macrophages with different amounts of phagocytosed beads, as determined by DHR fluorescence, as well as the fluorescent average for all macrophages with engulfed beads. Error bars show standard deviation. n=19

Figure 15 shows that the first engulfment event causes an initial rise in oxidation levels, with each bead engulfed after that causing a significant but smaller rise in oxidation. The 5+ level of oxidation is much higher, but it is a compound of all macrophages with 5 or more beads engulfed, therefore we assume that a similar pattern of rising oxidation continues even with this population. Because of the variability of this population, the standard deviation is also much larger with larger sample size.

To find the best correlation between bead engulfment and respiratory burst, we plotted the 94 different points of both non aggressive, medium and aggressive populations of LPS stimulated macrophages against their level of oxidation.

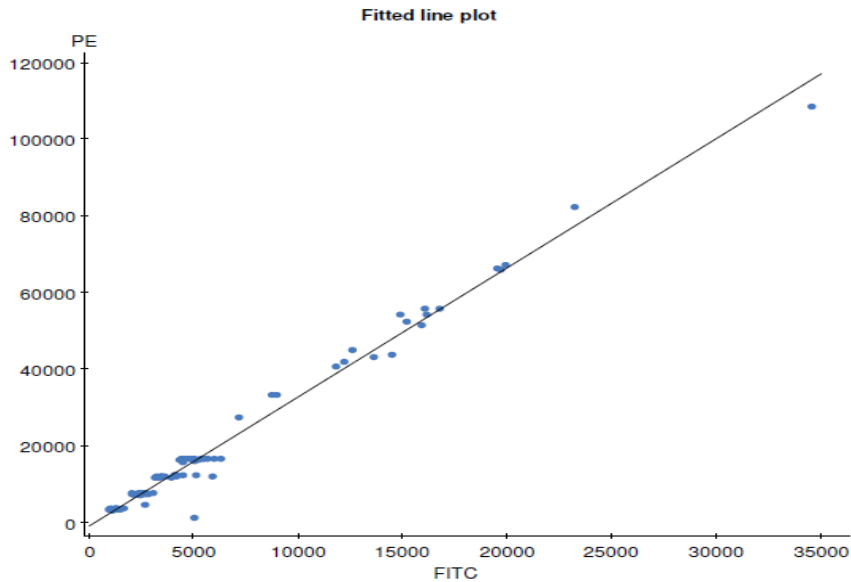


Figure 16. R-squared line for bead and DHR fluorescence.

Fitted line correlating the levels of PE fluorescence (beads) and FITC, the channel that DHR is read in. n=94.

In reading the PE channel, 1-2 beads usually falls below 10,000, 3-4 beads usually fall between 10,000 and 20,000 and 5+ beads usually falls around 40,000 units. In Figure 16, going up the line from the origin, you can see clusters of data points corresponding to 1 bead, 2 beads, 3 beads and 4 beads engulfed. The data points for 5+ beads engulfed are spread out along the line due to the variability in that group. The coefficient of determination, or R-squared value, for the fitted line is 0.983. Instead of using the number of beads to make the plot, we used the mean fluorescence of beads (read in the PE channel on the PE axis) and compared it to DHR fluorescence (read in the FITC channel on the FITC axis.) This removed the variability in the aggressive population and allowed us to find a better fitted line. We conclude that in LPS stimulated macrophages the level of oxidation is tightly linked to the level of engulfment.

Effects of caffeine on macrophages

We wanted to examine the effect of a chemical stimulus on engulfment and ROS production in macrophages. Experiments were performed with macrophages exposed to increasing levels of caffeine. Macrophages initially used were ones incubated after isolation for 72 hours, but later

experiments used macrophages incubated for 1, 24 or 72 hours. Bead engulfment by itself and bead engulfment with oxidation levels were measured.

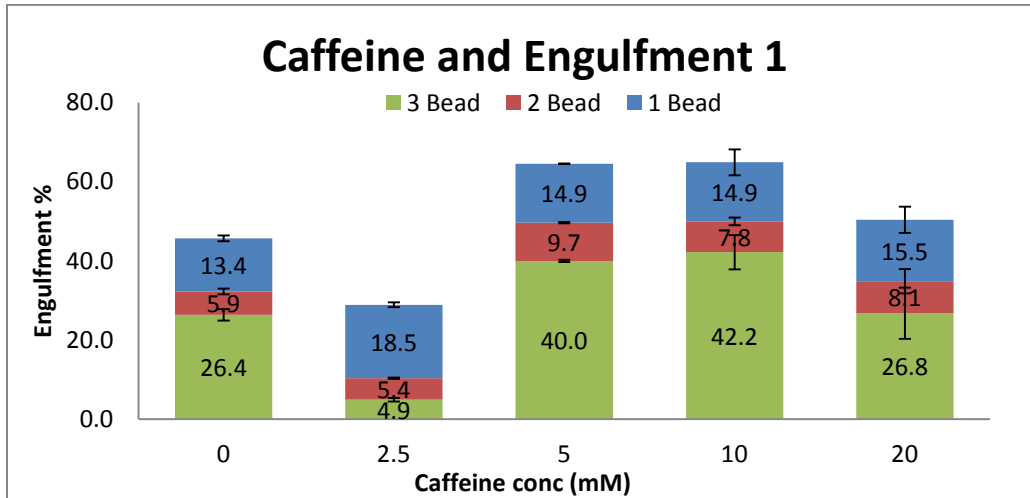


Figure 17. Macrophage Engulfment and Caffeine: Experiment 1.

This graph shows the effect on engulfment with increasing concentrations of caffeine. Error bars show standard deviation. n=2

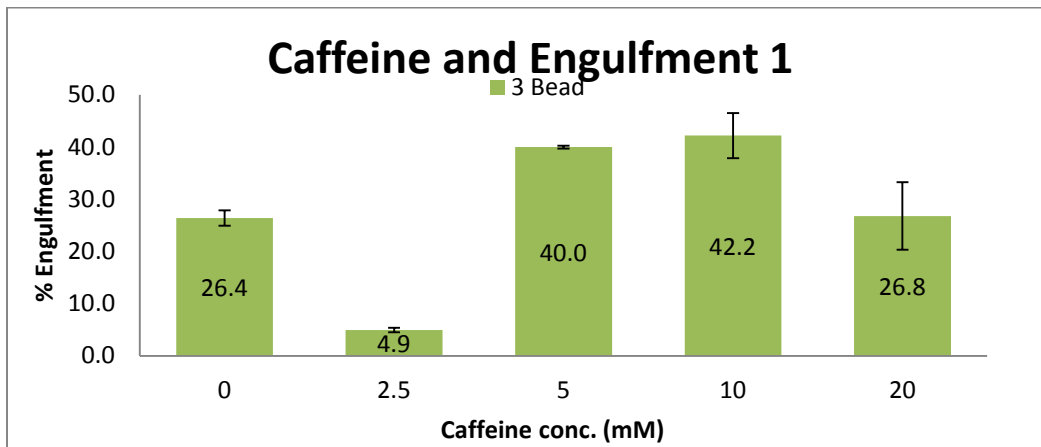


Figure 18. Engulfment and caffeine: Experiment 1 (3+ population only.)

Only the 3+ engulfing macrophages fraction is shown and all others removed. This was done to reduce clutter in the figure and only show the aggressive population with error bars. Error bars show standard deviation. n=2

The results in Figure 17 and 18 showed an initial drop in engulfment followed by a rise at higher concentrations. Between 5 mM and 10 mM of caffeine, there is no significant change. At 20 mM, engulfment again drops. DHR oxidation was also measured in this same experiment.

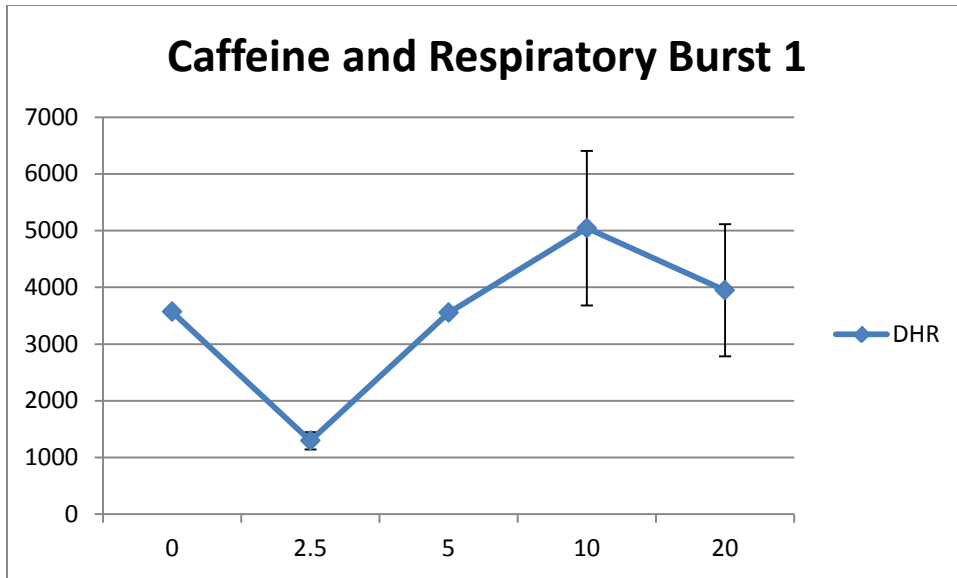


Figure 19. Caffeine and Engulfment: Experiment 1 – Respiratory Burst

This graph shows the levels of oxidation in whole blood macrophages read through DHR fluorescence with changing concentrations of caffeine. This data comes from the same experiment as Figure 17 and 18. Error bars show standard deviation of mean. n=2

Shown in Figure 19, DHR oxidation levels showed an initial drop with caffeine treatment and a later rise at higher concentrations. DHR fluorescence became more variable and had a larger standard deviation towards the higher concentrations. A non-significant drop in oxidation was noticed at 20 mM.

We repeated the same experiment using 6 different concentrations instead of 5. We increased the sample number between 0 and 2.5 mM and 10 and 20 mM concentrations.

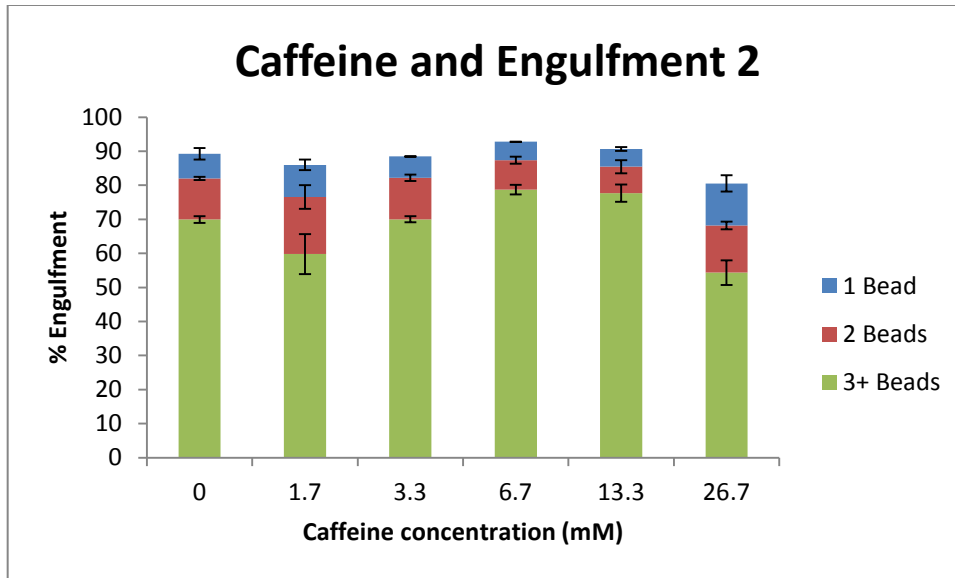


Figure 20 Caffeine and Engulfment: Experiment 2

Shows the effect on macrophage engulfment with increasing concentrations of caffeine. Error bars show standard deviation. n=4

The results in Figure 20 follow the same patterns as the ones in Fig 17 and 18. There is an initial drop in engulfment at low concentrations, a steady rise and then a large drop at 26.7 mM of caffeine.

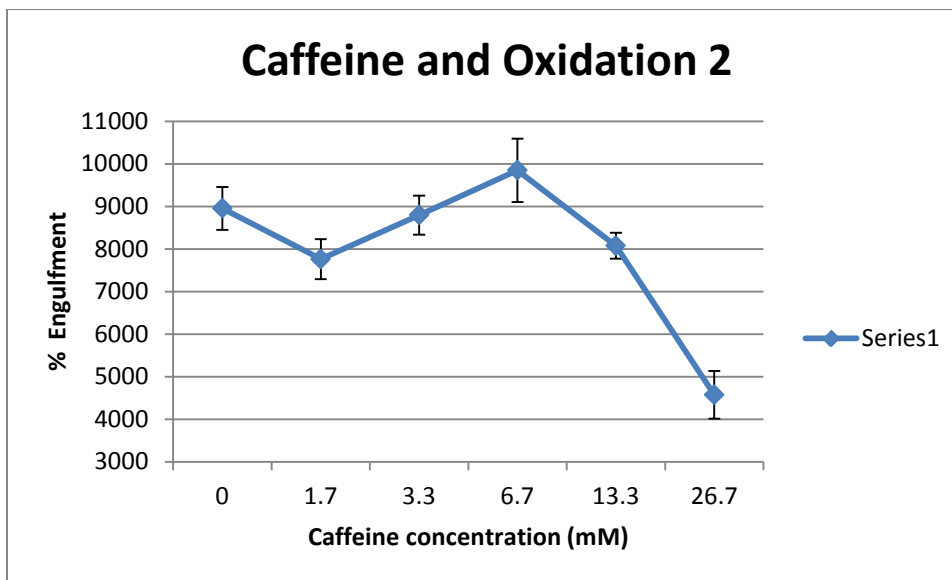


Figure 21. Caffeine and Engulfment: Experiment 2 – Respiratory Burst.

Shows the levels of oxidation in whole blood macrophages through DHR fluorescence in the same experiments as Fig 20 and 21. Total population mean oxidation was measured for each concentration. Error bars show standard deviation. n=4.

The respiratory burst patterns shown in Figure 21 followed a similar trend to engulfment. An early drop in engulfment was followed by a steady rise until a large drop at higher concentrations.

To further understand how caffeine affects engulfment and to see if results were immediate or delayed, we changed the amount of time macrophages from whole blood were incubated with LPS (allowed to mature) along with changing the concentrations of caffeine.

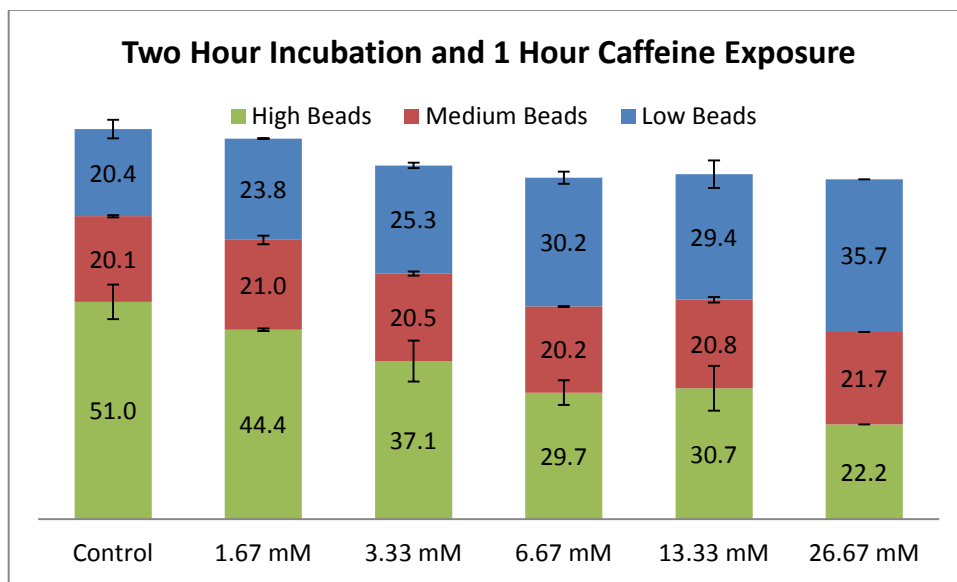


Figure 22. Caffeine and Engulfment: 1hr incubation and 1hr exposure. Macrophages incubated for 2hr and exposed to caffeine for 1hr. Error bars show standard deviation. n=2, except for 26.67mM where only one value was obtained.

The experiment in Figure 22 looked at effects of short exposure to caffeine in macrophages that had been stimulated for only a short time. Going from no caffeine to higher concentrations of caffeine, there is a steady decrease in both total engulfment and high engulfment in the exposed macrophages.

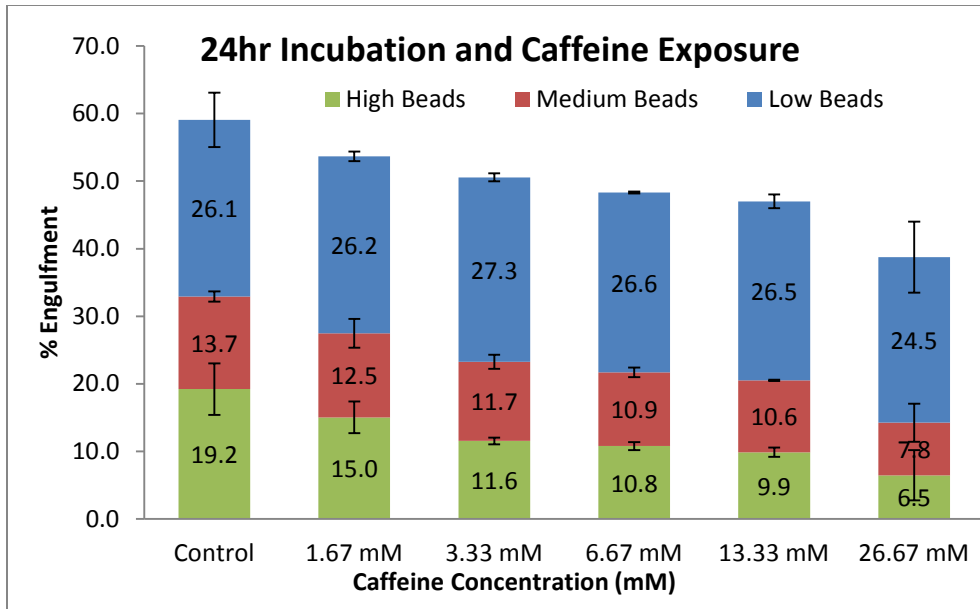


Figure 23. Caffeine and Engulfment: 24hr incubation and 24hr exposure.

Engulfment in macrophages incubated for 24hrs and exposed to caffeine for 24hrs. Error bars show standard deviation. n=2

Figure 23 shows the experiment testing macrophages that had been stimulated and exposed to caffeine for longer amounts of time. Again, a steady decrease in both total engulfment and high engulfment is seen. These experiments were compared to the standard testing procedure of incubating macrophages with LPS for 72 hours and exposing them to caffeine for 24 hours.

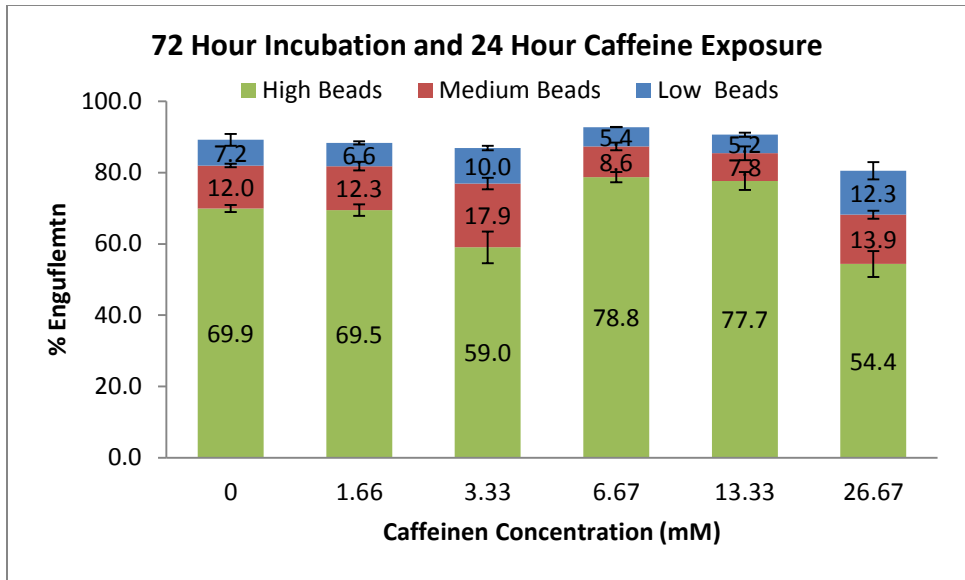


Figure 24 Caffeine and Engulfment: 72hr incubation and 24hr exposure.

Engulfment in Macrophages incubated for 72 hours and exposed to caffeine for 24 hours. Error bars show standard deviation. n=4

As seen in Figure 24, a greater effect was shown both when macrophages were incubated longer prior to caffeine exposure and with longer exposure to caffeine. Instead of seeing a steady decrease, we saw an initial decrease followed by a rise in engulfment, ending with a decrease again at the highest concentration. This indicates that maturation of macrophages is important in developing sensitivity to caffeine. The concentration of caffeine that had the largest effect was 6.67mM caffeine. From these results we decided to do an experiment that measured the effect of 5mM caffeine every three hours for 24 hours

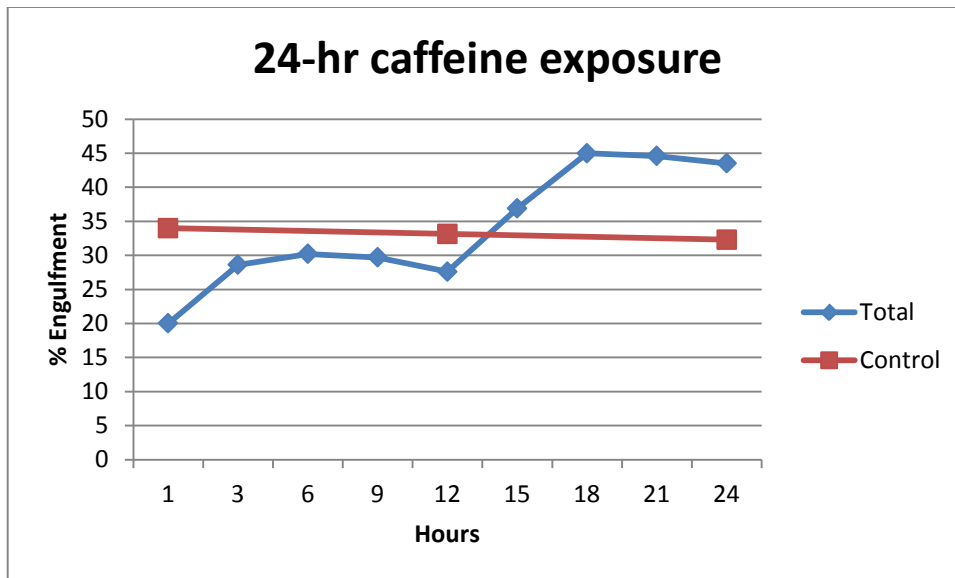


Figure 25. Caffeine and Engulfment: 24hr time lapse.
 Macrophages incubated for 72 hours and exposed to 5mM of caffeine for 1-24hrs.n=1

In the graph in Figure 25 we see an initial decrease in engulfment, followed by a significant increase between 12 and 18 hours. This initial experiment will need to be followed up by further experiments to validate the data.

The general trend for caffeine exposure will be a decrease in activity for both engulfment and oxidation between 0 and 5 mM concentrations of caffeine followed by an increase, sometimes above control levels, until 10 mM concentrations, after which activity drops. This corresponds to published research where low concentration (<5mM) lower ROS production, increased viability and decreased DNA fragmentation and apoptosis, whereas moderate concentrations (5-20mM) showed higher ROS activity and apoptotic rates, and high concentrations (>20mM) showed cytotoxic effects. [62] We assume that the drop in activity at high concentrations is due to the cytotoxic effects shown in literature. Engulfment and DHR oxidation levels followed the same pattern in all experiments and we conclude that these two cellular processes are directly linked.

Initial experiments in to find changes over time showed that there was an initial decreased response from macrophages to caffeine. Macrophage activity then increased over time, reaching a maximum at 18 hours and staying level until 24 hours. Caffeine’s effect on immune function might be more long term rather than immediate, suggesting immediate changes in second messengers but later changes in gene expression rather than cytosolic effects. This would have to be looked into further through genetic studies.

Tumor cell co-incubation

Having looked at engulfment and oxidation levels in macrophages, as well as the effects of chemical stimulus, we wanted to see the effect of co-culturing macrophages with cancer cell lines and we chose the cell lines RAJI and HL-60. RAJI is a lymphocyte-like hematopoietic cell line and HL-60 is human promyelocytic leukemia. Both cell lines grow in suspension.

Earlier experiments established that co-culture had a larger effect on macrophage engulfment than incubating with spent media from tumor cell lines.

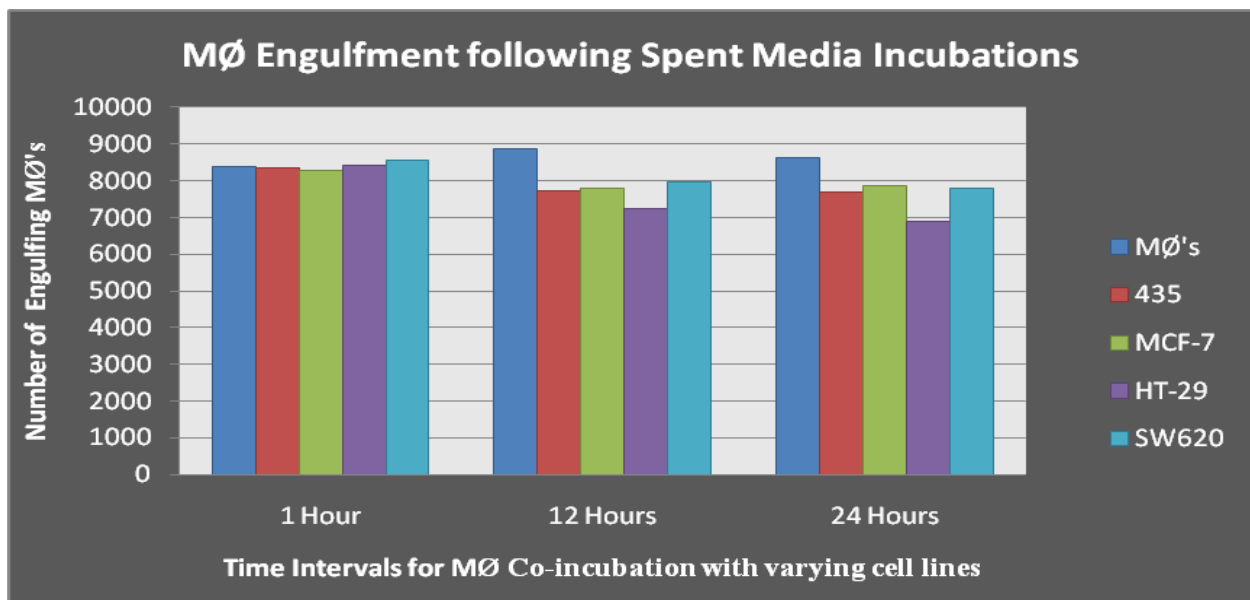


Figure 26. Macrophage engulfment following Spent Media Incubations.

Number of MØ's that engulfed beads after a co-incubation with spent media from the cell lines indicated in the legend for 1, 12, or 24 hours. MØ sample was the positive control and was not incubated with any other cell line spent media for the specified incubation time periods. A total of 10,000 MØ's were analyzed for each sample.

Incubating macrophages with spent media showed a small reduction in incubation, as shown in Figure 26. In the next experiment we co-incubated the cells with the macrophages instead of using the spent media.

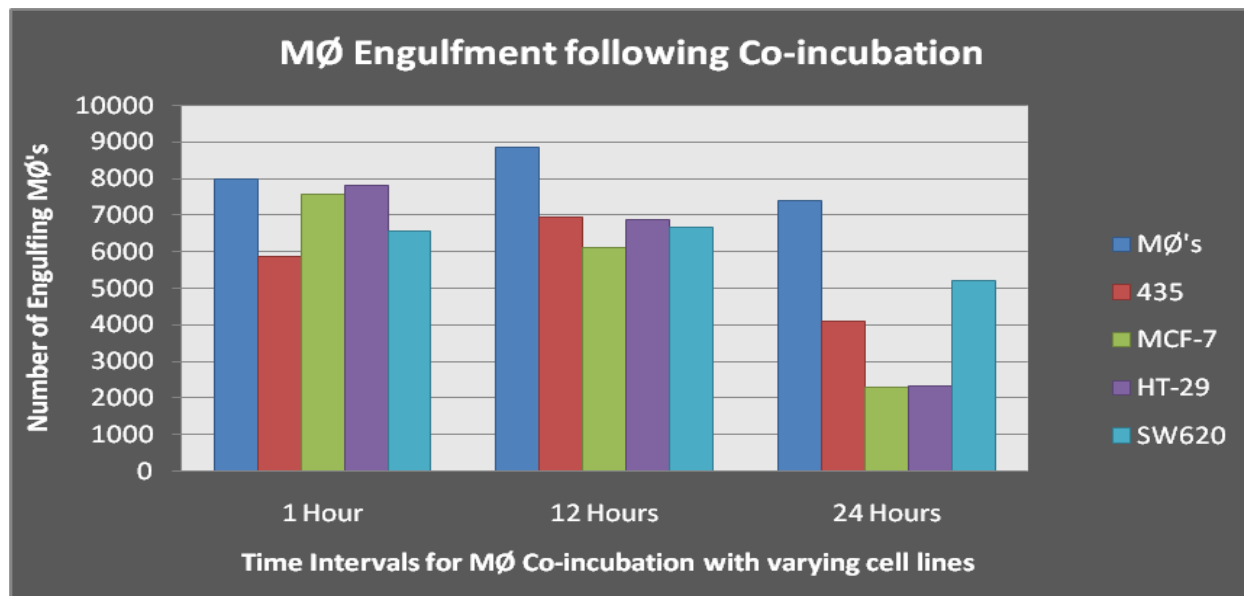


Figure 27. Macrophage Engulfment following Co-Incubation.

Number of MØ's that engulfed beads after a co-incubation with the cancer cell lines indicated in the legend for 1, 12, or 24 hours. MØ sample was the positive control and was not incubated with any other cell lines for the specified incubation time periods. A total of 10,000 macrophages were analyzed for each sample.

The results in Figure 27 show a small change in engulfment when incubating with spent media and a much larger change with co-incubation, especially after 24 hours. According to these results, we decided to use co-incubation for 24 hours in future experiments.

Effects of co-culture with RAJI cells

Macrophages were co-incubated with RAJI cells that had received (a) no treatment, (b) had been heat shocked or (c) freeze/thawed, producing apoptotic and necrotic cell fractions.

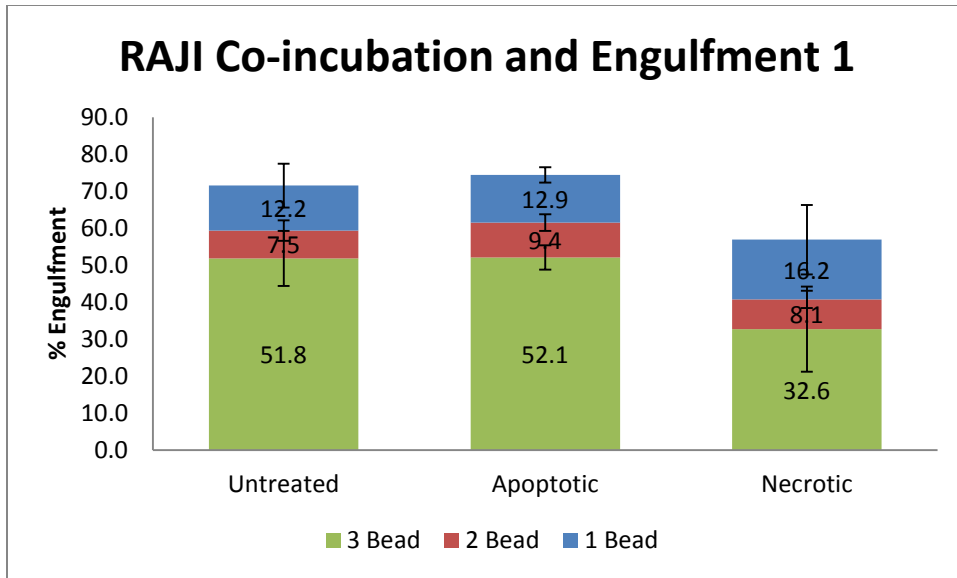


Figure 28. RAJI Co-Incubation and Engulfment: Experiment 1.

Engulfment by macrophages co-cultured with RAJI cells that are either untreated, were heat shocked for 1 hour (apoptotic) or killed through liquid nitrogen freeze/thaw (necrotic.) Error bars show standard deviation. n=2

From the results in Figure 28, we saw no change with apoptotic co-incubation and non-significant drop in the necrotic fraction. Due to the high initial values, we were concerned that it would be difficult to see an increase or a decrease of engulfment in our samples. We decided to reduce the time we incubated the macrophages from 2 hours to 1 hour, to start with lower initial values of engulfment. For comparison, we repeated the same experiment with 1 hour bead incubation, while also adding stimulation with LPS to induce an M1 response in the macrophages. In theory, this experiment would produce less variability when co-incubating with necrotic cells.

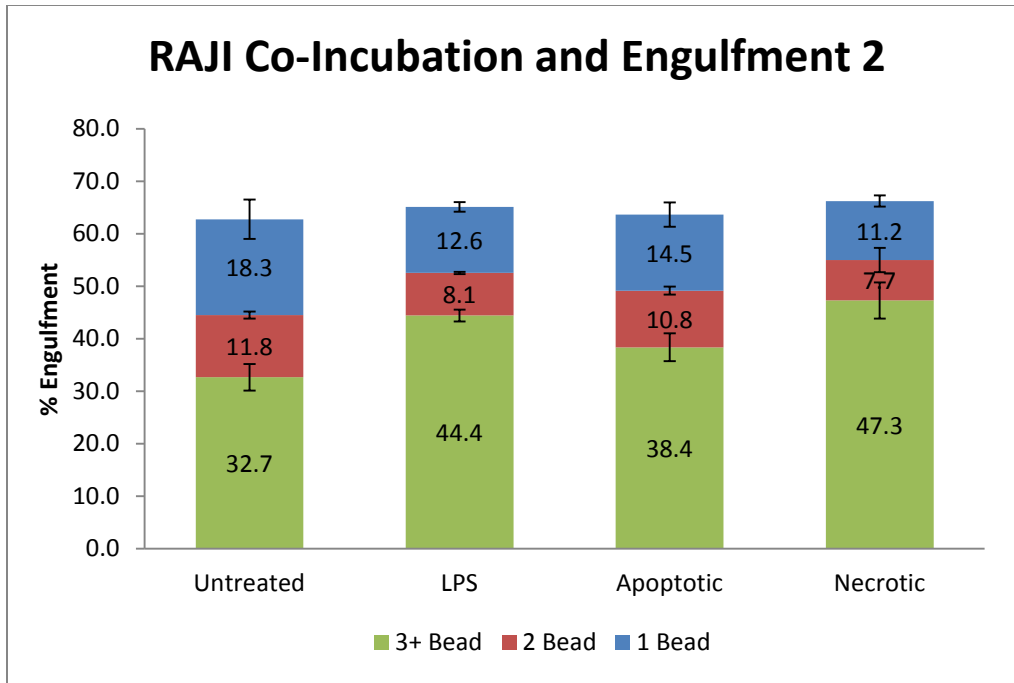


Figure 29. RAJI Co-Incubation and Engulfment: Experiment 2

Shows engulfment in macrophages co-cultured with RAJI cells that are either untreated, were heat shocked for 1 hour (apoptotic) or killed through liquid nitrogen freeze/thaw (necrotic,) similar to the experiment in Graph 15, while also adding LPS during untreated RAJI cell co-incubation. Error bars show standard deviation. n=3.

Figure 29 shows a significant increase in activity in the LPS treated and necrotic fraction.

Apoptotic co-incubation did not increase engulfment significantly. We conclude that necrotic cells have an activating effect similar to LPS on macrophages. Induction of apoptosis in RAJI cells proved difficult. We were never able to achieve more than 20-30% apoptosis in our heat shocked samples. Further work could investigate the HL-60 cell line as it has been reported to be an apoptotically sensitive cell line, exhibiting higher apoptotic induction. We performed experiments with the HL-60 cell line, but to date we have been unable to produce consistent values of apoptotic cell fractions to be used for experiments.

Effects of co-culture with HL60 cells

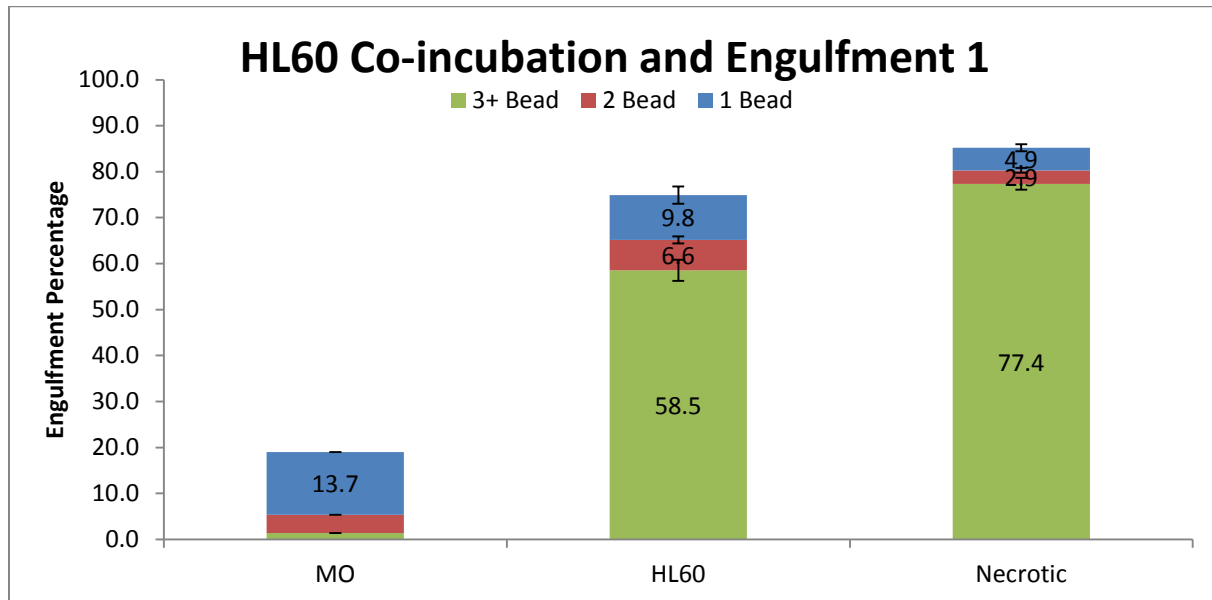


Figure 30. HL-60 Co-Incubation: Experiment 1.

Experiment was done to measure the effect of co-incubation with HL60 cell line. MO = macrophages, HL60 means co-incubation with HL60 cells. Necrotic means co-incubation with freeze-thawed HL60 cells. Significant increase in aggressiveness noted. A more detailed experiment was prepared to confirm this effect. Error bars show standard deviation. n=2

In our initial experiments, we first used a 40 min bead incubation time and the results in Figure 30 showed a significant increase in samples co-incubated with HL-60, especially in the necrotic fraction. It was concerning how low engulfment started out, so to get a better baseline, we decided to go back to 1 hour incubation along with adding LPS to our samples.

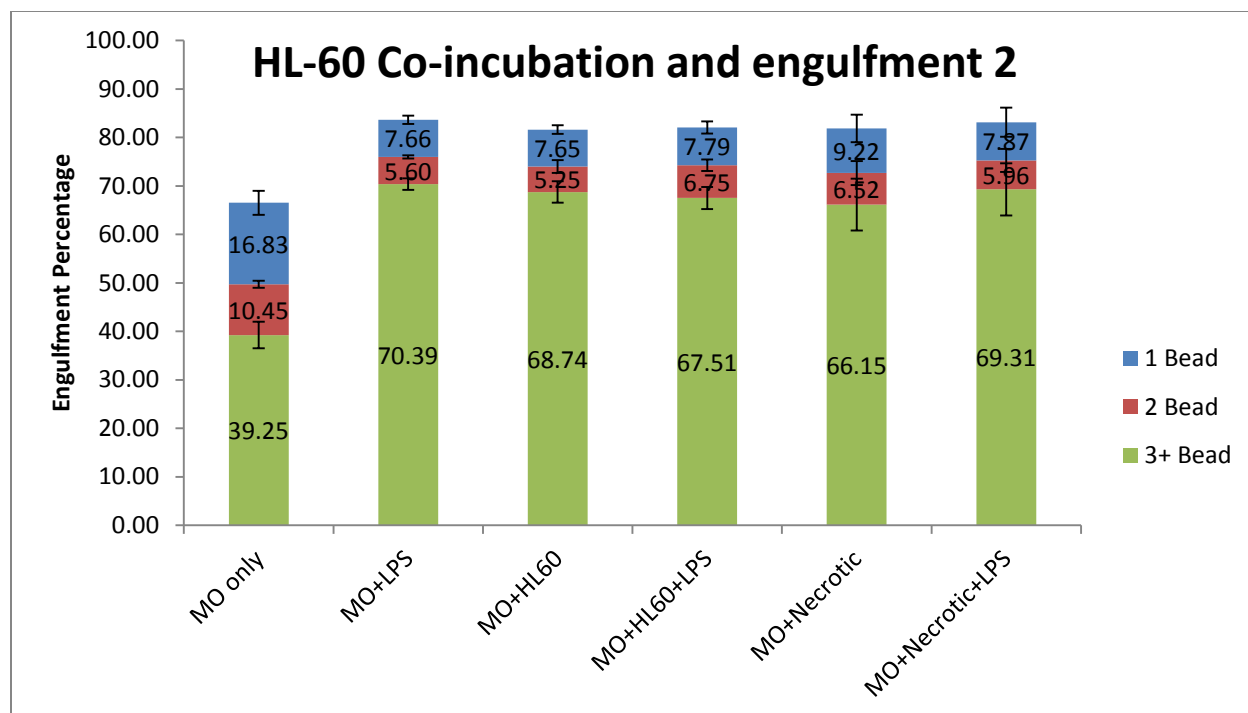


Figure 31 .HL-60 Co-Incubation and Engulfment: Experiment 2.

Macrophages only as the experimental controls, followed by macrophages stimulated with LPS. Co-cultures performed with normal and necrotic HL60 cells, with or without LPS. MO = macrophages, LPS = LPS added, HL60 = co-incubation with HL60 cells, Necrotic = Co-incubation with necrotic HL60 cells. Error bars show standard deviation. n=3

Co-culturing macrophages with the HL-60 cell line had similar effects on macrophage aggressiveness as exposure to LPS. Co-incubation with untreated HL60 cells, necrotic HL60 cells and addition of LPS to all samples showed a similar increase in engulfment. No significant difference was found in untreated HL-60 cell co-incubation and necrotic HL-60 cell co-incubation. This would indicate that both live HL-60 cells have an activating effect similar to that of necrotic cells on macrophage function. Ongoing research is looking further into the response to apoptotic and necrotic tumor cells as well as measuring oxidation levels.

Experiments showed a direct link between phagocytosis and respiratory burst levels in response to chemical stimulation with caffeine and tumor cell co-incubation. Possibly the linked response comes from the activating signals shared by phagocytosis and respiratory burst. Aggressiveness changed over time as macrophages were exposed to caffeine, possibly suggesting genetic

transcription changes in response, which merit further investigation. Future research into the specific cellular targets of caffeine, both those related and unrelated to adenosine receptors, might explain more clearly why caffeine has the effect it has. Transcriptional changes through genetic studies would be a good next step in our experiments. Further research would need to be performed to investigate the effects of caffeine on ROS production compared to engulfment over a 24 hour period.

Exposure to leukemic cell lines caused levels of aggressiveness similar to LPS activation, contrary to results gained with non-leukemic cell lines (unpublished data), showing a possible activating effect of leukemia on circulating macrophages, similar to activating effects between lymphocytes. It is also possible that the effect is due to the RAJI and HL-60 cell lines growing in suspension, whereas non-leukemic cell lines tested were monolayer cells. The activating effect of necrotic cells increased aggressiveness of macrophages, consistent with previous literature, [27] in a way similar to LPS stimulation. From our experiments with co-incubation of macrophages and tumor cell lines we conclude that even though macrophages are usually rendered less aggressive in the presence of cancer cells, certain types of cancer, specifically leukemic cancer cells, can have the opposite effect. Future experiments could look at cytokine profiles and transcriptional changes in both macrophages and tumor cell lines due to co-incubation. In addition to these experiments, reproducing the experiments to measure ROS production would also be necessary

Our hypothesis was that the level of oxidation would be directly linked to the level of phagocytosis. We assumed that caffeine would increase activity in macrophages and that tumor cell co-incubation would decrease it.

We found that there was a high correlation between the level of engulfment and level of respiratory burst. Chemical stimulation with caffeine lowered aggressiveness of macrophages at lower concentrations, raised it at higher concentrations and eventually became toxic to the cell. Co-incubation with leukemic cell lines, as well with necrotic cells, affected an increase in aggressiveness.

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APPENDIX

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Caffeine Affects Macrophage Engulfment

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ABSTRACT #171

Anti-inflammatory signals can modulate macrophage activation, reducing their phagocytic capabilities. Caffeine is a neurostimulant and has been suggested to be involved in modulation of the innate immune system. We used an engulfment assay to measure macrophage activity when exposed to caffeine at varying concentrations. Human leukocytes were isolated and incubated to allow for monocyte differentiation. Macrophages were collected and washed, then incubated with varying concentrations of caffeine (1-25 mM) for 24 hours. Cells were then treated with IPS, followed by incubation with fluorescent microspheres for two hours. Engulfment was measured using flow cytometry, indicating the phagocytosis of microspheres in integral ratios. It was observed that low and high concentrations of caffeine inhibited IPS-induced phagocytosis, however, at a concentration of 5-8 mM engulfment was significantly increased above the normal. These findings suggest that macrophage activation exhibits a threshold regulation.

INTRODUCTION

Macrophages are innate immune cells derived from the myeloid lineage. Macrophages perform a variety of functions, from phagocytosis to the repair and remodeling of damaged tissues. Two phenotypes have been proposed: M1 and M2. The M1 macrophage performs phagocytosis and releases pro-inflammatory cytokines. The M2 macrophage acts as a support cell by promoting the healing of damaged cells by angiogenesis and tissue remodeling.

Caffeine is a neurostimulant and has been suggested to be involved in modulation of the innate immune system. Previous research has demonstrated that caffeine has strong influences on cell-cycle checkpoints, has shown to increase cytotoxicity in combination with other DNA damaging agents and inhibiting DNA repair. Caffeine which is an analog of adenine has also been linked to tumor immunomodulation.

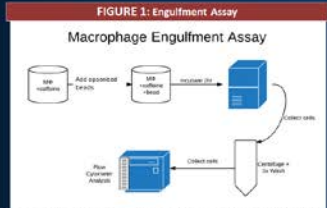


Figure 1: Engulfment Assay. Macrophages were incubated in the presence of IPS for 24 hours. Cells were exposed to fluorescent microspheres, 1 x 10⁶ cells/ml, 1000-2000 beads were suspended in each well and added to cells, and then filtered.

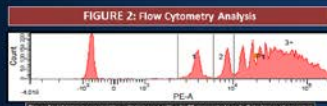


Figure 2: Flow cytometry analysis. Cells were exposed to IPS for 24 hours. Cells were then exposed to fluorescent microspheres, 1 x 10⁶ cells/ml, 1000-2000 beads were suspended in each well and added to cells, and then filtered.

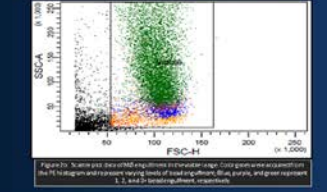


Figure 3: Scatter plot showing SSC-A vs FSC-H. The plot shows a dense population of cells in the upper right quadrant, with a smaller population in the lower left quadrant.

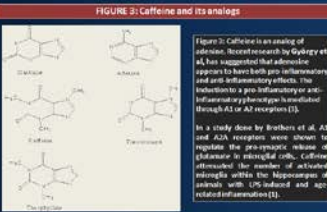


Figure 4: Caffeine is an analog of adenine. Recent research by Gylter et al. has suggested that adenine appears to have both pro-inflammatory and anti-inflammatory effects. This indicates to a pro-inflammatory or anti-inflammatory phenotype (modulated through A1 or A2 receptors [5]). In a study done by Brothers et al., A1 and A2A receptors were shown to regulate the pro-inflammatory release of cytokines in microglial cells. Caffeine attenuated the number of affected molecules within the hippocampus of animals with IPS-induced and age-related inflammation [6].

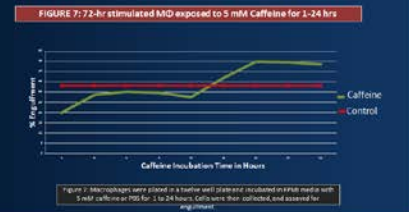
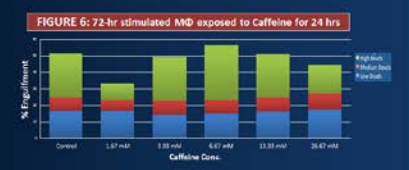
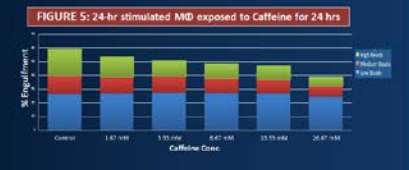
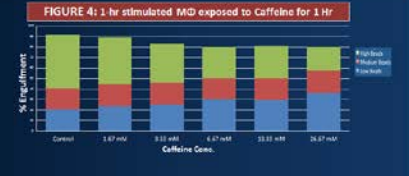


Figure 7: Macrophages were placed in a twelve well plate and incubated in RPMI media with 5 mM caffeine at 37°C for 1 to 24 hours. Cells were then collected, and assayed for engulfment.

MATERIALS AND METHODS

Preparation of Macrophages
 Leukocytes were separated from whole blood using lymphocyte separation medium, then washed and resuspended in RPMI 1640 medium which was supplemented with 20% human serum from the blood donor. Cells were incubated (37°C, 5% CO₂) for 1-72 hours. Differentiated monocytes were collected and seeded into 12-well plates with varying concentrations of caffeine-supplemented media.

Engulfment Assay
 After 1-24 hour incubation with caffeine, macrophages were stimulated with IPS and PE-conjugated microspheres were added and allowed to incubate for 2 hours.

Flow Cytometry Analysis
 Cells were collected, washed, and analyzed using BD FACSCanto flow cytometer. PE was gated to distinguish engulfment levels of macrophages.

CONCLUSION

Caffeine is the most widely used neurostimulant in the world, and it has been shown that 79% of the population in the United States drinks coffee at least occasionally. These results suggest that caffeine-induced regulation of macrophage engulfment is dependent on both macrophage differentiation and caffeine incubation time. Previous research has been done on caffeine suggesting it has an agonistic effect on the adenosine receptors, which is responsible for pro-inflammatory and anti-inflammatory modulation in microglial cells [1]. Other research suggests that at supraphysiological concentrations, caffeine stimulates the release of calcium [4], which may be responsible for increased activation in immune cells [2]. The time dependent effects of caffeine may also be due to the Ca²⁺ dependent activation periods in immune cells.

Engulfment has been shown to be related to activated phenotype in macrophages, these results indicate that caffeine may stimulate both pro-inflammatory and anti-inflammatory phenotypes. Macrophage phenotype has been implicated in a number of diseases, including cancer, and these results show potential for the therapeutic applications of caffeine and its analogs. Further research will be required to fully understand the regulatory mechanisms of caffeine on macrophages.

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