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Pharmacologic Immunomodulation of Macrophage Activation by Caffeine

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A thesis submitted to the faculty of
Brigham Young University
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Master of Science

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ABSTRACT

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Caffeine is one of the most widely used neurostimulants in the world and there is considerable debate on its effect in immune cells. One of its main targets is proposed to be adenosine receptors which mediate an anti-inflammatory switch in activated immune cells while another target is phosphodiesterase where it acts as an inhibitor. In macrophages, caffeine has been shown to cause both pro-inflammatory (M1) and anti-inflammatory (M2) phenotypes. If the primary effect of caffeine on macrophages were to antagonize adenosine receptors we would expect cells exposed to caffeine to have a prolonged M1 response. However, we show that caffeine suppresses phagocytosis at physiological concentrations (an indicator of M2 phenotype). This suppression was reversed when macrophages were pretreated with protein kinase A inhibitor, suggesting that at physiological concentrations caffeine's phagocytic suppression may be due to its function as a phosphodiesterase inhibitor, pushing cells towards an M2 fate. However, mRNA expression profiling suggests that caffeine can modulate A_{2A} receptor expression and suppress MKP-1 expression, a hallmark of M1 macrophages.

Caffeine is, therefore an immunomodulator that can suppress or prolong inflammatory responses in macrophages, which may account for the abundance of contradicting evidence in the literature. Additionally, these effects are complicated by regular caffeine intake and fitness level, emphasizing that tolerance and immune robustness are important factors in macrophage activation.

Keywords: caffeine, macrophage, phagocytosis, adenosine receptors, inflammation

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INTRODUCTION

Macrophages

Macrophages are mononuclear immune cells derived from monocytes from the myeloid lineage which respond to stimuli and differentiate into immune effector cells. They perform a variety of functions ranging from the phagocytosis of debris and foreign particles to the repair and remodeling of damaged tissues.[11] Different subspecialties of macrophages exist in locations throughout the body: osteoclasts in the bone secrete massive amounts of acid and proteinases to degrade the matrix to release calcium ions when the blood calcium levels are low,[8] bone marrow-derived macrophages promote retention of hematopoietic stem cells and progenitor cells,[12] and lymph node subcapsular sinus macrophages neutralize invasion threats to the central nervous system (CNS) by neurotropic viruses.[31] Alveolar macrophages trap and eliminate debris in the lungs, and Kupffer cells in the liver help recycle red blood cells.[6] Microglial cells in the brain scavenge the CNS for infectious agents or damaged neurons to protect against disease. All of these subtypes are part of the mononuclear phagocyte system that helps maintain homeostasis in the body.

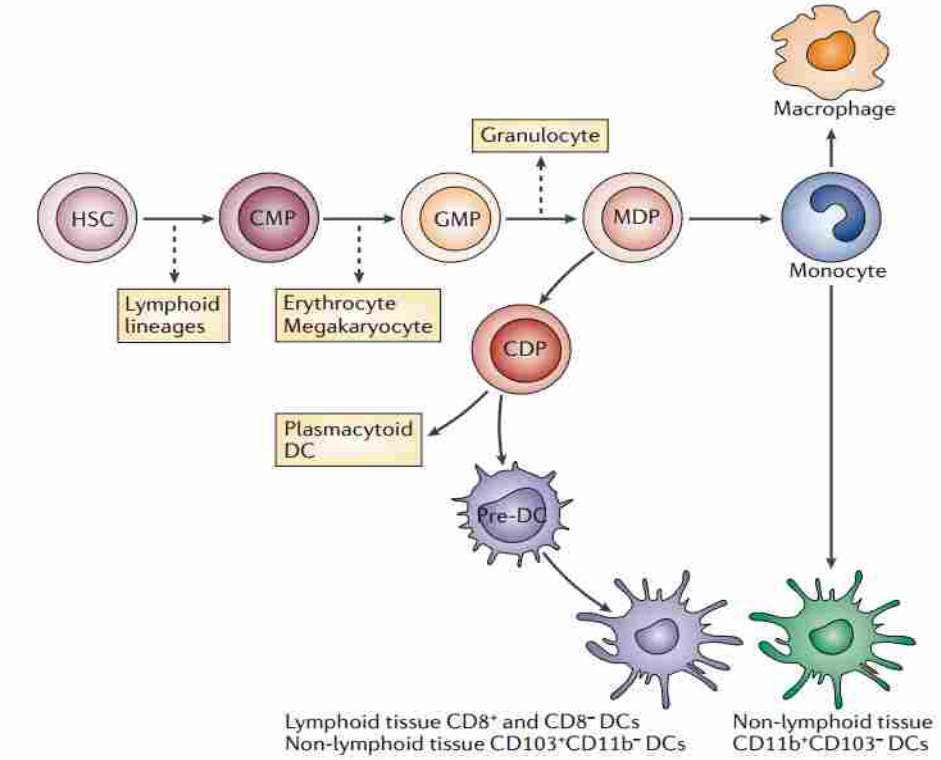


Figure 1 Hematopoietic lineage of monocytes, macrophages, and dendritic cells[12]

Macrophages express a variety of cellular markers including: CD14, CD40, CD64, F4/80 (mice)/EMR1 (human), lysozyme M, MAC-1/MAC-3, and CD68.[34] The most commonly used markers in biological assays are CD16 and CD14. CD16 is a low affinity Fc receptor that is expressed on natural killer cells, neutrophils, and monocytes. It binds the Fc portion of antibodies and activates antibody-dependent cell-mediated cytotoxicity.[33] CD14 is a co-receptor for toll-like receptor 4 (TLR-4) and binds lipopolysaccharide (LPS) with the help of LPS-binding protein (LBP). CD14 is expressed almost solely on macrophages, but is also found at a much lower concentration on neutrophils. The soluble form of the receptor is secreted in the liver and may confer LPS responsiveness to $CD14^-$ cells.[21] When LPS binds the CD14/TLR4 complex, MyD88 and TIRAP are recruited. This attracts IRAK1/4 and activates TRAF6, leading to the activation of IKK and MAPK. IKK then phosphorylates and degrades I-κB, leading to nuclear translocation of the transcription factor NF-κB. NF-κB activates pro-inflammatory pathways resulting in an pro-inflammatory response (Figure 2).[15]

Macrophage Functions

Traditionally, macrophages have been seen only as immune cells, and their homeostatic responsibilities have been overlooked. Mosser et al, proposed that macrophages exist in three main categories: host defense, wound healing, and immune regulation.[47] These categories can mix to create different subcategories of cells, which results in the variety of macrophage functions we see in vivo. The janitorial aspect of a macrophage's job may include clearing extracellular debris or recycling apoptotic cells. Often there is a danger signal in the debris that can activate macrophage's immune regulatory functions.

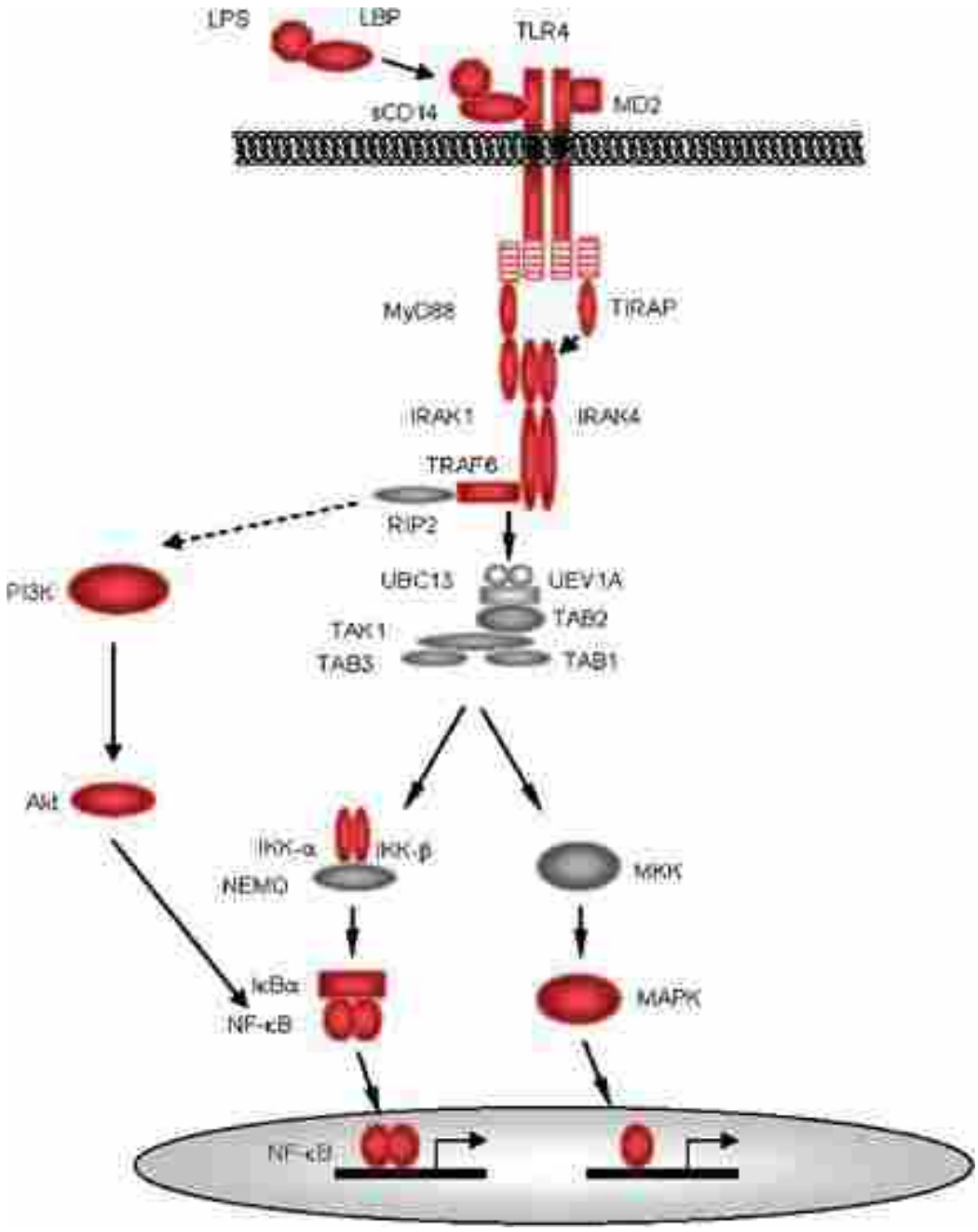


Figure 2 LPS stimulates MyD88 signaling[15]

When a danger signal is recognized, one of the first cellular responders are the tissue resident macrophages. Molecular signals from the pathogen called pathogen associated molecular patterns (PAMPs) are recognized by pattern recognition receptors (PRRs). One important class of PRRs is the toll-like receptor which, upon interaction with PAMPs, induces

NF-κB expression and MAPK pathway signaling. This signaling cascade stimulates the production and secretion of pro-inflammatory mediators and the recruitment and activation of other immune cells. Macrophages can also recognize opsonized pathogens and engulf and degrade them in intracellular compartments through a process called phagocytosis. Although phagocytosis has been observed since the groundbreaking studies by Metchnikoff in 1905,[18] there is still much unknown about the signaling pathways involved. When a pathogen becomes opsonized by antibody or complement proteins, macrophages can then recognize these pathogens via the complement receptor 3 (iC3b bound antigen) or Fc receptors (IgG bound antigen). There are three classes of Fc receptors: Fc-γ, Fc-α, and Fc-ε. The most important class for phagocytosis is Fc-γ which contains five subtypes: FcγRI (CD64), FcγRIIA (CD32a), FcγIIB (CD32b), FcγRIIIA (CD16a), and FcγRIIIB (CD16b), which all differ in affinity for IgG antibody due to structural variations. Upon binding, the receptors cluster to form lipid rafts with immunoreceptor tyrosine-based activating motif (ITAMs) that initiate an intracellular signaling cascade resulting in actin polymerization.[9]

The signaling cascade starts when (ITAM)-containing subunits are phosphorylated by members of the Src family. The phosphorylated receptors/ITAMs become docking sites for Syk, a tyrosine kinase. After docking, Syk activates downstream signals including phosphatidylinositol-3-kinase (PI3K), causing generation of 3'-phosphoinositides (PI3) at the phagosomal cup. 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.[40] Wiskott-Aldrich syndrome proteins (WASP's) act as molecular scaffolds by associating with PIP2 (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. [53] The Rho and Rac

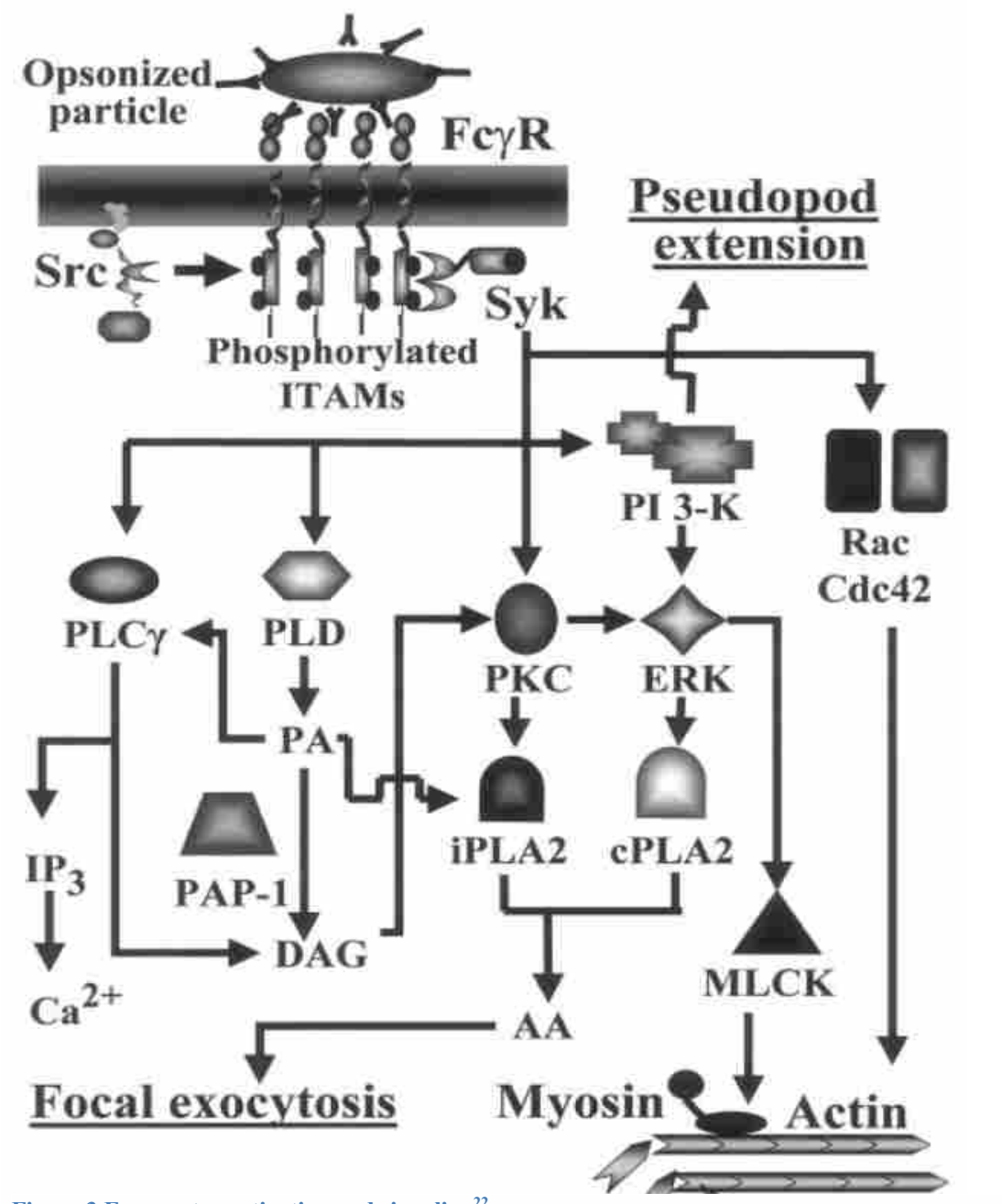


Figure 3 Fc receptor activation and signaling[22]

Fc receptor activation results in actin nucleation and polymerization. This is the basis of pseudopod formation which allows phagocytes to engulf extracellular pathogens for destruction in intracellular vesicles

GTPases are generally involved in all phagocytic activating pathways. Actin nucleation is the basis of forming pseudopod extensions (Figure 3).

Pseudopods form by actin microfilament polymerization and assembly. These "finger-like" projections extend, acting like probes by which they identify pathogens.[20] Once around a

pathogen, they fuse to form an endosomal compartment called a phagosome. If the pathogen is large, the endoplasmic reticulum can supply extra membrane to surround the object, possibly contributing to slower phagocytic rates (Figure 4).[1, 17] Beningo et al. recently revealed that mechanical properties of the target may also affect phagocytic rates, with macrophages favoring more rigid objects.[5] They also observed that engulfment of soft particles can be increased by activating Rac1and lysophophatidic acid, which suggests a Rac-1 dependent mechanosensory mechanism for phagocytosis. In our experiments we used latex microspheres engineered for low manufacturing diversity to eliminate bias due to mechanical properties.

After engulfment, the intracellular vesicle then fuses with a lysosome to form the phagolysosome which contains enzymes to lower the pH and degrade its contents. Among these enzymes is NADPH oxidase which generates reactive oxygen species that can be converted into oxidizing agents like hypochlorite that can damage pathogens. Degraded components of the vesicles are loaded onto major histocompatibility complexes and presented to T cells to activate the adaptive immune response. T cells in return secrete cytokines such as IFNγ to activate the macrophage. Macrophages can be further activated by their environment and by the nature of the pathogens they ingest. The extent of macrophage activation has recently been shown to play a major role in many diseases such as cancer, arthritis, sepsis, and autoimmune diseases.[2, 48] Extensive research on the signals involved in macrophage activation has revealed that at least two main subtypes of macrophages exist. Classically activated macrophages are stimulated by pro-inflammatory signals, while alternatively activated macrophages are usually stimulated by anti-inflammatory signals. These two phenotypes have also been termed M1 and M2, mirroring the T_H1 and T_H2 phenotypes of T cells.[45]

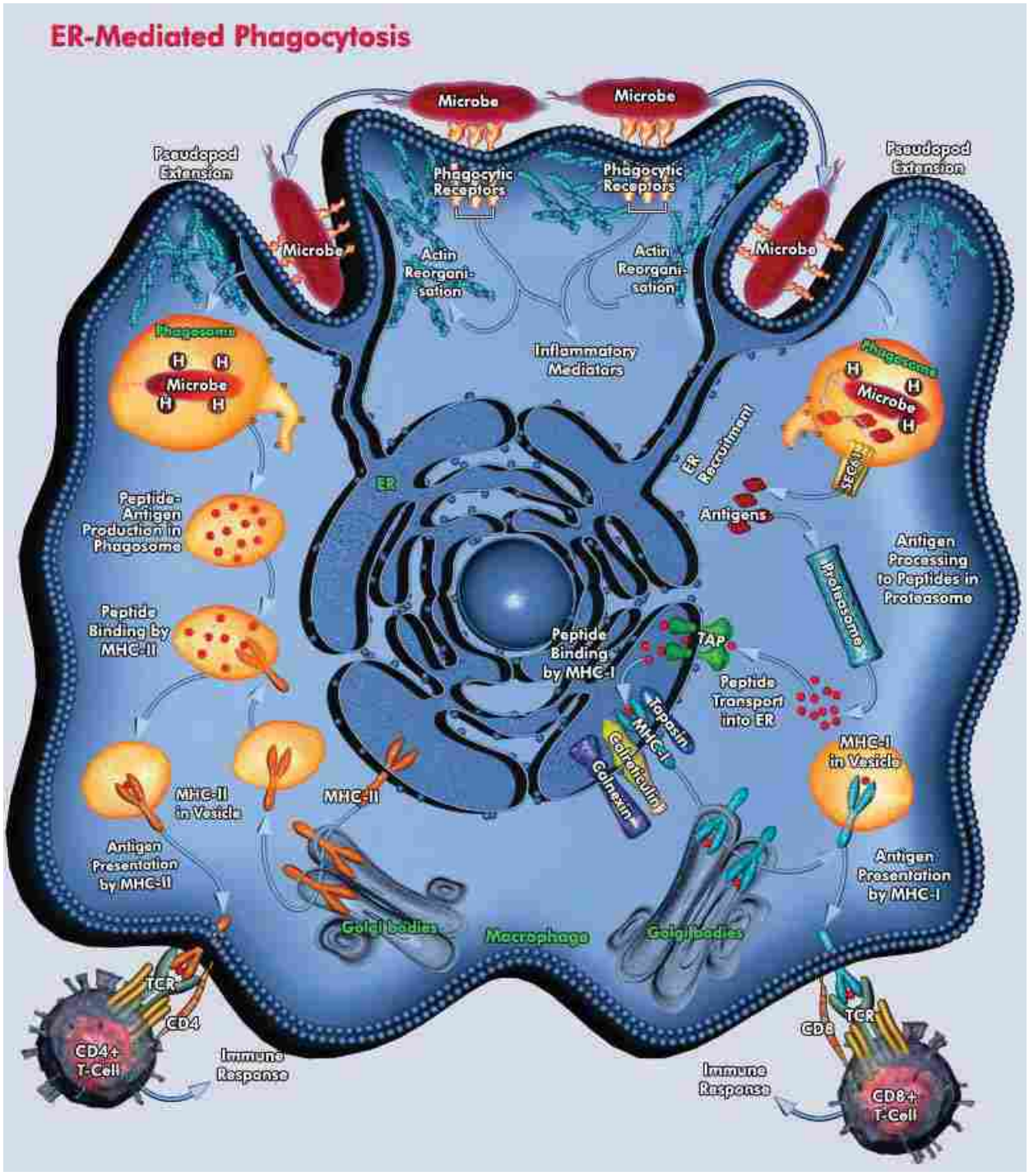


Figure 4 Endoplasmic reticulum supplies membrane for phagocytosis[54]

M1 and M2 Phenotypes

The M1 macrophage clears tissue of cellular debris and pathogens via aggressive phagocytosis and release of pro-inflammatory cytokines including: IL-1, IL-6, IL-12, IL-23, and TNF-α. They are also characterized by their ability to produce reactive oxygen and nitrogen intermediates (ROI, RNI), and an increased expression of MHC class II for more efficient antigen presentation (Figure 5).[44]

The M2 macrophage acts as a support cell by promoting the healing of damaged cells by angiogenesis and tissue remodeling via secretion of anti-inflammatory cytokines and by

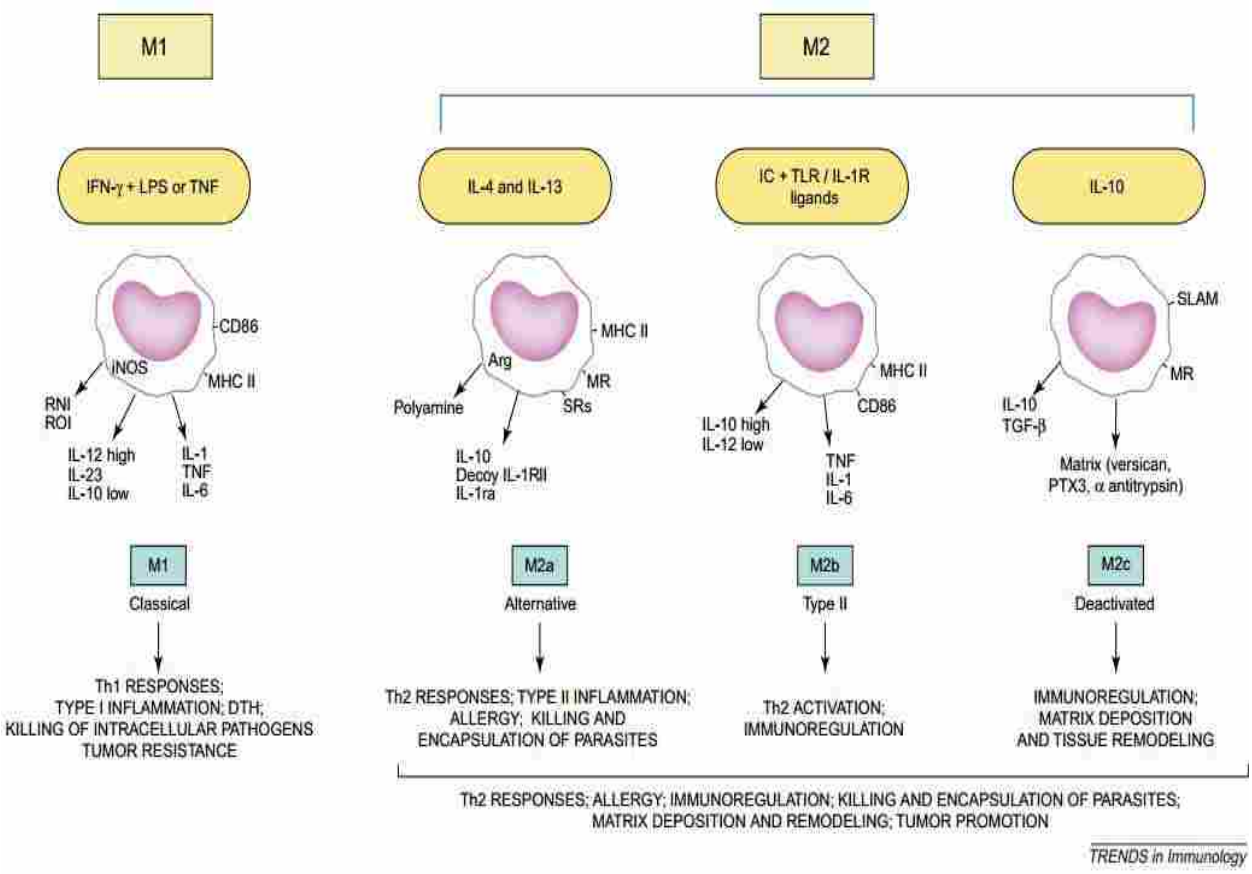


Figure 5 M1 and M2 stimulants and responses[44]

decreasing phagocytic activity.[44] The M2 group can be further subdivided by the mechanism of activation: M2a, M2b, and M2c. Il-4 and IL-13 drive M2a activation and type II immune responses, whereas TLR/IL-1R ligands activate the M2b suppression and immunoregulation. M2c macrophages are stimulated by IL-10, resulting in tissue remodeling and matrix deposition.[42]

While these two operationally useful polarities exist, it is widely believed that macrophages exist mainly in the continuum between these two phenotypes.[43] Regulation of this continuum involves complex processes that have been extensively studied, yet still remain relatively unclear. Recently it was shown that macrophages will respond to injury or infection by upregulating pro-inflammatory (M1) responses, but later switch to an anti-inflammatory (M2) response once the infection is under control.[14, 46] One proposed mechanism for this switch involves the activation of adenosine receptors in macrophages as they mature, to decrease the initial pro-inflammatory response and promote vascularization and wound repair.[14]

Adenosine Receptors

Adenosine receptors (ARs) are classed into four subtypes: A_1, A_{2A}, A_{2B}, and A_3 receptors. Each receptor type has a different affinity for adenosine and different signaling pathways, and not all cells express every subtype.[27] Activating the receptors leads to inhibition or excitation of adenylate cyclase (Figure 6-7) which can control the inflammatory response in a number of immune cells.[36] In macrophages, $A_{2A}R$ plays a critical role in regulating the angiogenic switch from M1 to M2 phenotypes. During an infection, over-activation of the inflammatory response would cause severe damage; therefore a negative regulatory system is needed to induce a switch from a pro-inflammatory response to an anti-inflammatory response.

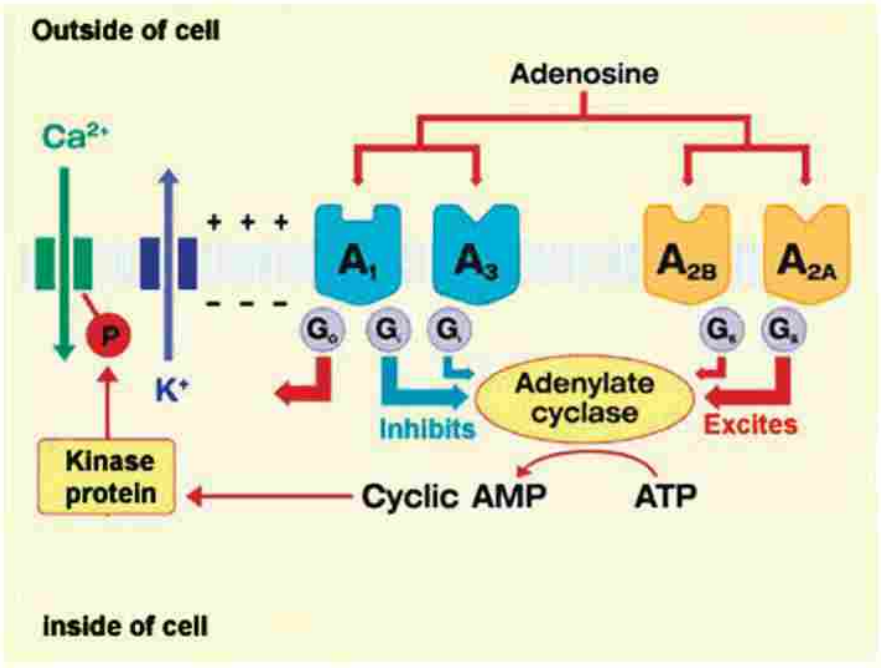


Figure 6 Action of adenosine in immune cells[19, 36]

(Top) Adenosine binds adenosine receptors which can excite or inhibit adenylate cyclase, thus increasing or decreasing concentrations of cAMP. cAMP levels can target kinase proteins like p38 MAPK (bottom) that can then release pro-inflammatory cytokines in macrophages.

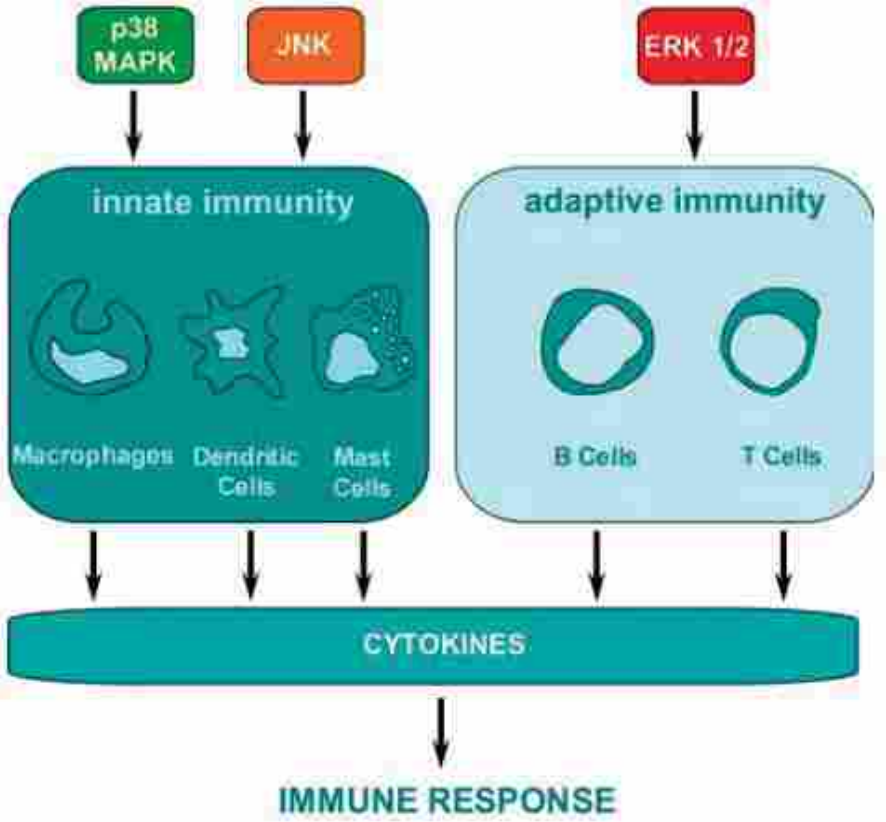


Figure 7 Action of MAPK in the immune response[25]

Adenosine release has been proposed as one of these negative feedback systems. Macrophages initiate inflammation through TLR-dependent activation to an M1 phenotype, but are then switched into an angiogenic phenotype by adenosine generated in response to hypoxia/ischemia within the wound area.[50]

This is accomplished when initial activation of macrophages by TLR agonists markedly induce expression of $A_{2A}R$ and $A_{2B}R$, essentially priming these macrophages to respond to increased local levels of extracellular adenosine and suppressing the pro-inflammatory response.[10] Activation of $A_{2A}R$ by adenosine inhibits phagocytosis and inflammatory cytokine production, and increases IL-10 and pro-angiogenic factor production (Table 1). Antagonists of A_1R and $A_{2A}R$ were recently discovered to have the ability to induce a pro-inflammatory or anti-inflammatory phenotype in microglial cells[26]. Additionally, it was shown that blockade of $A_{2B}R$ in mice enhances macrophage-mediated clearing of bacteria and increases TNF-α and IL-6 production.[4, 14] Analogs of adenosine and antagonists of its receptors are therefore of interest as therapeutics to regulate inflammation.

Table 1 Expression of adenosine receptors by subtype [25]

	A_1 receptor	A_{2A} receptor	A_{2B} receptor	A_3 receptor
Neutrophils		Diminished adhesion, activation		Stimulates chemotaxis
M1 macrophages		Inhibit phagocytosis, inflammatory cytokine production, increase IL-10	Stimulates IL-10	
M2 macrophages		Stimulates M2 macrophage differentiation Production of pro-angiogenic factors		

M1 and M2 macrophages both express A_{2A} receptors which have similar functions. In M1 macrophages A_{2A} receptor signaling inhibits phagocytosis and increases IL-10 production, whereas A_{2A} receptor signaling in M2 macrophages functions to perpetuate M2 activity.

Adenosine receptors and MAPK/MKP-1

Adenosine has also been shown to affect the mitogen-activated protein kinase (MAPK) pathway of inflammation. Knockout of $A_{2A}R$ results in prolonged mitogen-activated protein kinase (MAPK) phosphorylation, leading to increased activation of pro-inflammatory pathways.[61]

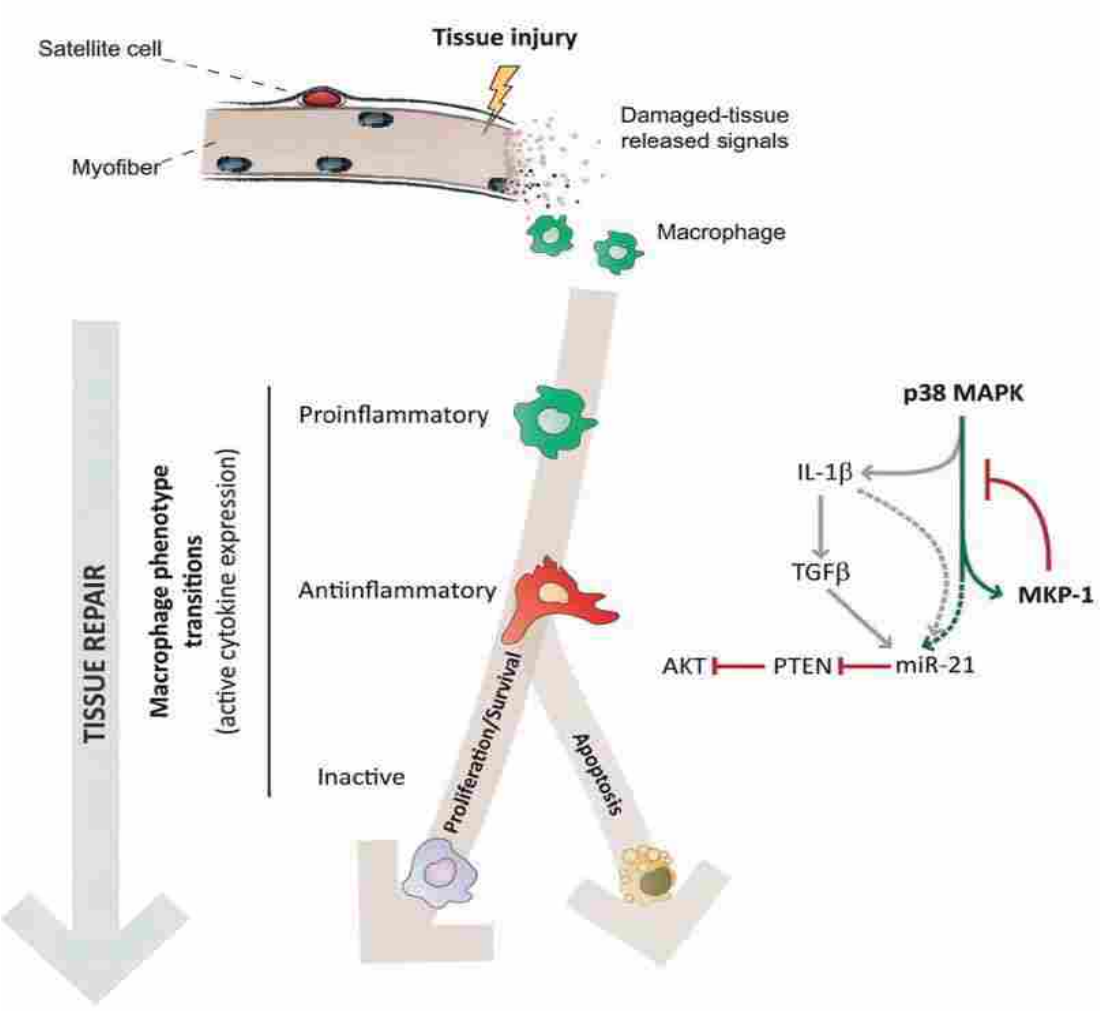


Figure 8 The transition from pro-inflammatory to anti-inflammatory state in tissue repair[52].
MAPK and MKP-1 regulate the angiogenic switch. Knockdown of A_2AR prolongs p38 MAPK phosphorylation, suggesting that adenosine signals through MPK-1 to activate an anti-inflammatory phenotype.

During wound repair in muscle cells, MAPK dephosphorylation by MAP kinase phosphatase-1 (MKP-1) induces an anti-inflammatory response (Figure 8). However, MKP-1 activation is delayed until approximately three days post infection.[52] It is possible that adenosine receptor

signaling activates MKP-1 activity, and that the prolonged MAPK phosphorylation in $A_{2A}R$ knockouts is due to lack of active MKP-1. We extended the observation window in our experiments to three days in order to study the effect of caffeine on MKP-1 activation.

Adenosine Receptors and Cancer

Extracellular adenosine accumulates in the tumor microenvironment due to hypoxia and inflammatory conditions. This accumulation may be inhibiting the cytotoxic capacity of immune cells,[38, 39, 49, 57] a phenomenon known as the Hellstrom paradox.[28] Hellstrom et al. noted that despite tumors being recognized by the immune system and generating anti-tumor T cells, the tumors still persist. Similar studies have shown that macrophages surround breast tumors, but are incapable of producing a cytotoxic response and instead promote angiogenesis and vascularization of tumor tissue.[7] Accumulation of adenosine in the tumor microenvironment may be contributing to this phenomenon. The source of excessive adenosine was, until recently, unknown. Wang et al. recently demonstrated that CD73, a hallmark of many cancers, acts at receptors $A_{2A}R$ and $A_{2B}R$, causing immunosuppression in the tumor microenvironment.[63] CD73, also known as ecto-5'-nucleotidase, is a membrane bound enzyme that catalyzes the conversion of purine mononucleotides to nucleosides, its main substrate being AMP. Adenosine generated by this enzyme acts by binding to adenosine receptors on surrounding cells resulting in tumor promotion.[62] Blockade of $A_{2A}R/A_{2B}R$ in mouse macrophages with $CD73^+$ tumors significantly decreased tumor growth and metastasis.[3] Knockdown of ARs in the tumor microenvironment decreases VEGF levels and angiogenesis in lung carcinoma, suggesting that adenosine signaling may be responsible for propagation of tumor growth factor signaling.[56] Therefore, antagonism of AR signaling by caffeine or its analogs may be of interest as a therapeutic in cancer.

Caffeine

Caffeine is the most widely used neuroactive compound worldwide.[24] It is an analog of adenine a member of the xanthine family of compounds. It is metabolized by cytochrome P450 oxidase into three metabolites also in the xanthine family: theophylline (4%), theobromine (12%), and paraxanthine (84%) (Figure 9). Theophylline and paraxanthine have neurotropic and

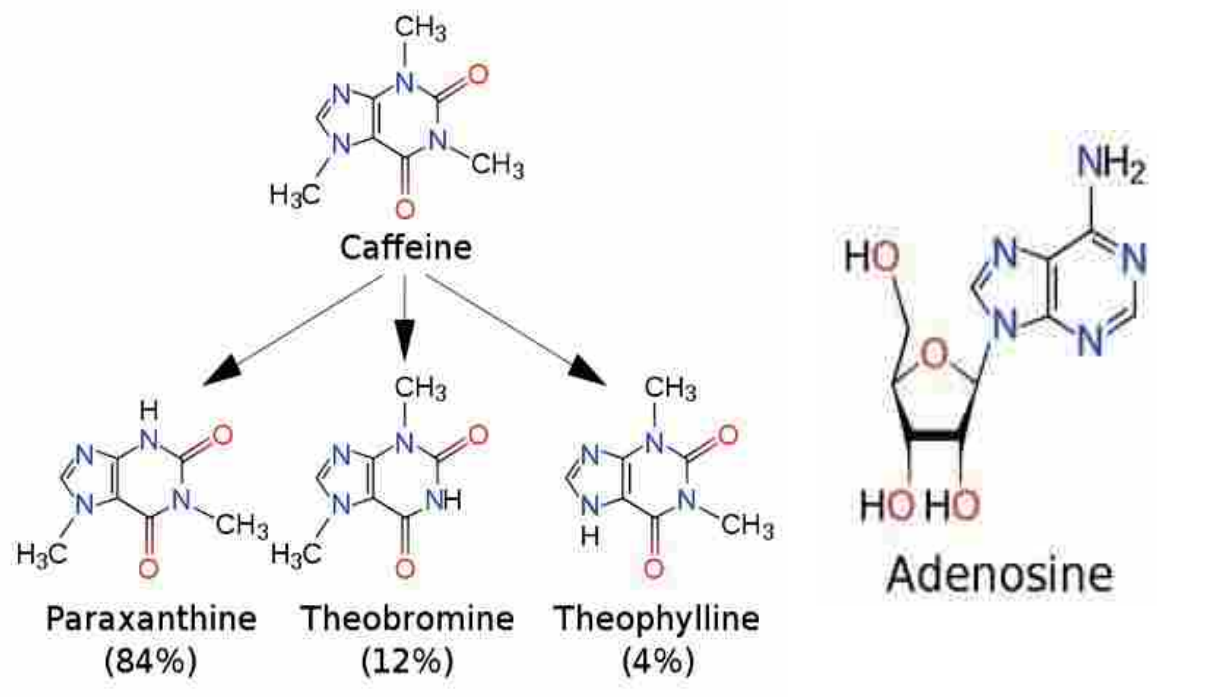


Figure 9 Caffeine and its metabolites.

Left, caffeine is metabolized into three metabolites by cytochrome P450 oxidase. Right, caffeine is an analog of adenine and may function as an antagonist at adenosine receptors. (Public Domain)

immunomodulatory activity similar to caffeine.[30, 58]

Caffeine's mechanism of action in immune cells has been an issue of debate for some time. Part of this debate is the result of a number of studies using supraphysiological concentrations, which are often rightfully disregarded. Some studies suggest that the upper limit

for caffeine concentration in serum is 75 μM.[16] Others suggest that this is an order of magnitude too low because of the low bioavailability of some forms of caffeine.[29, 55] Still, some researchers have argued that because its metabolites have identical bioactivity, the effective concentration of caffeine is much higher than both of these estimates.[32] For example, Van Furth et al. showed that theophylline, theobromine, paraxanthine and caffeine all have a suppressive effect on TNF-α production in human leukocytes.[60] In our experiments we used a range of concentrations in order to observe the immunological effect of caffeine at physiological as well as supraphysiological concentrations. The mechanism of action of these methylxanthines has been a subject of study for many years, yet still remains somewhat unclear.

There is a significant body of evidence to suggest that many of the physiological effects of dietary caffeine are mediated by antagonism of ARs.[41] Caffeine binds non-selectively to ARs with a K_i of 50-55 μM for A1 and A2 receptors. Binding of caffeine prevents the activation of ARs by adenosine. The activation of ARs on immune cells generally leads to the suppression of pro-inflammatory cytokines.[50] This suppression of cytokine production has been observed following stimulation with agonists of adenosine A_1, A_2, and A_3 receptors, as well as adenosine itself.[35, 37] Therefore if the primary effect of caffeine in macrophages was to antagonize ARs, we would expect caffeine to prolong pro-inflammatory responses. However, caffeine suppresses pro-inflammatory cytokine production in whole-blood macrophages.[30] Caffeine is also a phosphodiesterase inhibitor, increasing cAMP levels in cells and thus increasing activation of downstream targets like phosphokinase A (PKA). PKA belongs to a family of kinases that have two regulatory subunits controlled by cAMP binding (Figure 10). When cAMP levels are high, two cAMP molecules bind the regulatory subunits thus removing them from the active site allowing the catalytic subunits to interact with protein kinases to phosphorylate Ser or Thr

residues. The increased activation of PKA may be responsible for suppression of pro-inflammatory cytokine production in macrophages.[30]

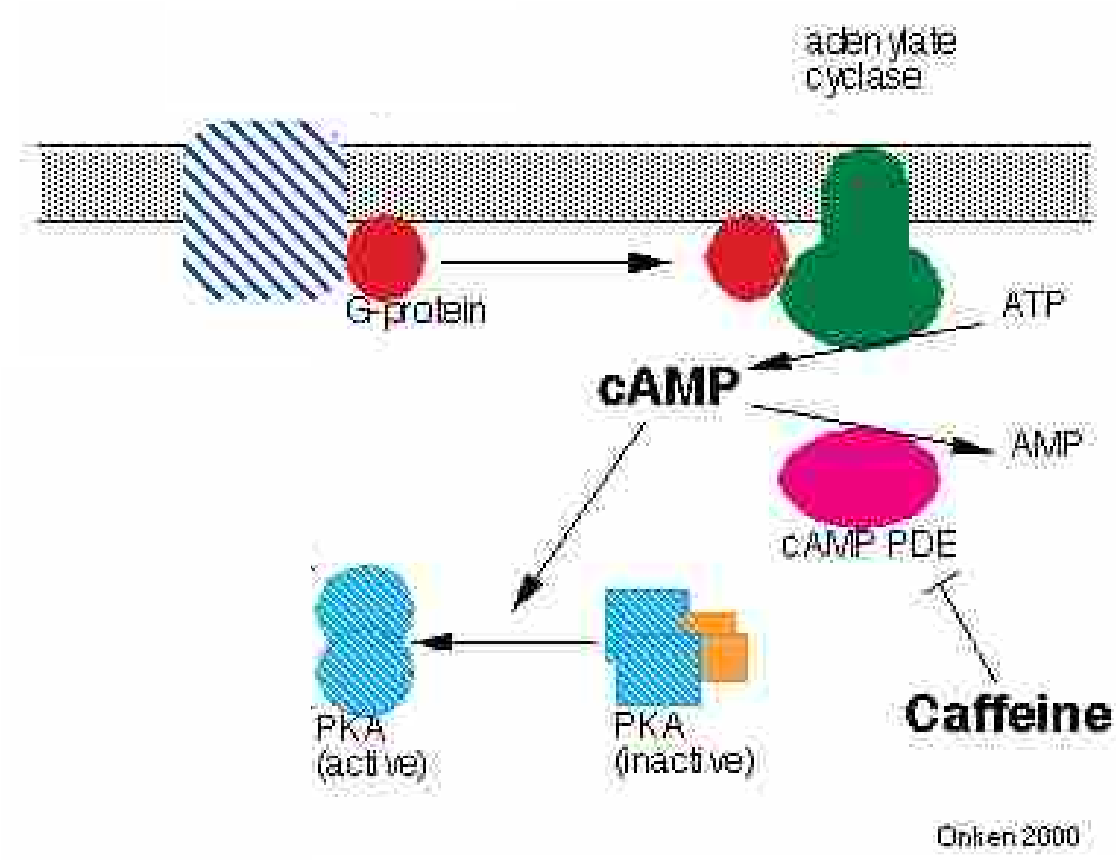


Figure 10 The phosphodiesterase inhibitor activity of caffeine[51]

G-proteins (adenosine receptors) activate adenylate cyclase, generating cAMP. Caffeine inhibits cAMP phoshodiesterase (cAMP PDE) leaving high levels of cAMP in the cell which is free to activate PKA phenotype.

Project Overview

In order to investigate this suppressive effect of caffeine, we studied the inflammatory profile of macrophages after exposure to caffeine and PKA inhibitor. Using a phagocytic assay and gene expression assays, we sought to elucidate caffeine's mechanism of action at a range of concentrations and time points. We hypothesized that caffeine would suppress phagocytosis at physiological concentrations and that this suppression is due to increased activation of PKA by accumulation of intracellular cAMP; thus the PKA inhibitor would negate the effects of caffeine.

We also hypothesized that at higher concentrations, caffeine will antagonize adenosine receptors and reverse phagocytic suppression due to PKA activation. We further hypothesized that adenosine receptor expression would increase with macrophage aging and that this increase in expression would contribute to the effects on phagocytosis.

MATERIALS AND METHODS

Tissue culture

Blood was drawn intravenously from 11 volunteers ages 20-30, and information was recorded on age, gender, caffeine intake, and exercise level (BYU IRB # X 14194). Patients who regularly consumed 400 mg/wk were considered regular caffeine users. Patients who exercised for 30 mins, more than twice a week, were considered regular exercisers.

Leukocytes were separated from whole blood using lymphocyte separation medium (STEMCELL Tech), then washed and resuspended in RPMI 1640 medium supplemented with 20% human serum from the blood donor. Cells were then seeded in 12-well plates at a concentration of 10^6 cells/mL and incubated (37°C, 5% CO_2) for 1h - 3 d. Adherent cells are widely accepted to be differentiated macrophages.[64] Cells collected after 1 h were termed "Day 0" and used as a baseline control for phagocytosis and adenosine receptor expression. Remaining cells were allowed to culture for 1 d or 3 d after isolation with or without PKA inhibitor (Rp-8-Br-cAMPS, Santa Cruz Biotech), final concentration 10^{-5}M for 0.5 h. Then caffeine-supplemented media (Sigma, CAS # C8960) was added, final concentration 35 μM-15 mM, for 2

h followed by LPS stimulation (1µg/mL) and further incubation (37°C, 5% CO_2) for 22 h. Cell viability was accounted for and no difference was seen between days.

Caffeine Concentration

Caffeine concentration was measured before and after cell culture to ensure uniform concentration across the 24 h incubation period. Caffeine's absorbance was read at 300 nm using a Synergy HT Multi-Mode Microplate reader (BioTek) at time zero, 2 h, and 24 h post incubation.

Phagocytosis Assay

After 24 h incubation with caffeine, 2 µm phycoerythrin (PE)-conjugated polychromatic red latex microspheres, or beads, (Polysciences, Inc.) were added to wells (~10^9 particles/mL) and allowed to incubate for 1 h. This concentration was chosen to ensure the beads were not a limiting factor in phagocytosis rates. The beads were suspended in fetal bovine serum (FBS) to allow proper phagocytosis by macrophages and to prevent beads from sticking to the cell membranes (Figure 11).

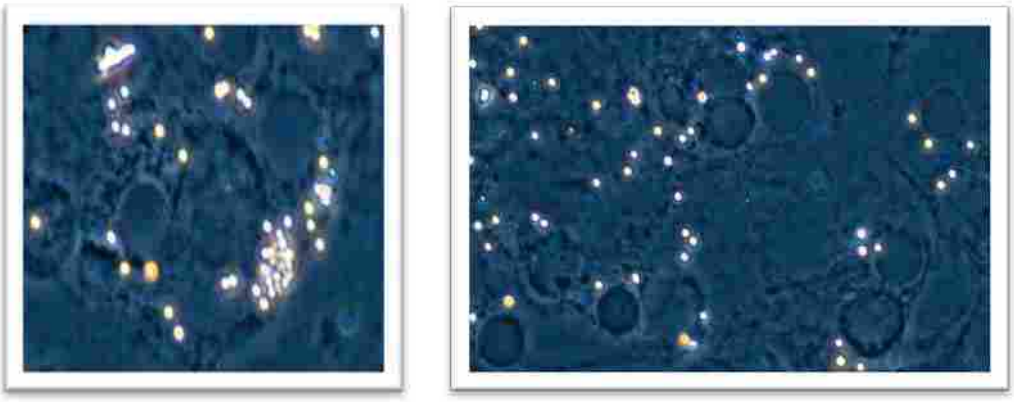


Figure 11 Fluorescent microscopy

(Left) beads without FBS; (Right) beads suspended in FBS, demonstrating how FBS prevents clumping and sticking of beads to cells.

Flow Cytometry Analysis

After 1 h, cells were placed on ice to stop phagocytosis and macrophages were isolated by their adherence to the plate. Macrophages were collected using a cell scraper, washed twice and stained with human CD14-APC/Cy7 antibody (BioLegend) for macrophage definition. After washing, samples were analyzed using a BD Accuri C6 flow cytometer and software. FL4-A represents $CD14^+$ events and FL2-A represents PE^+ events (Figure 12B,C). Macrophage phagocytosis was stratified by gating on PE^+ peaks representing 1, 2, and 3+ beads (Figure 12A). The "2 bead" and "3+ bead" populations are used in analysis of phagocytic aggressiveness and are used to calculate the percentage of cells that are "highly aggressive." This percentage was compared to all $CD14^+$ events to measure macrophage activation.

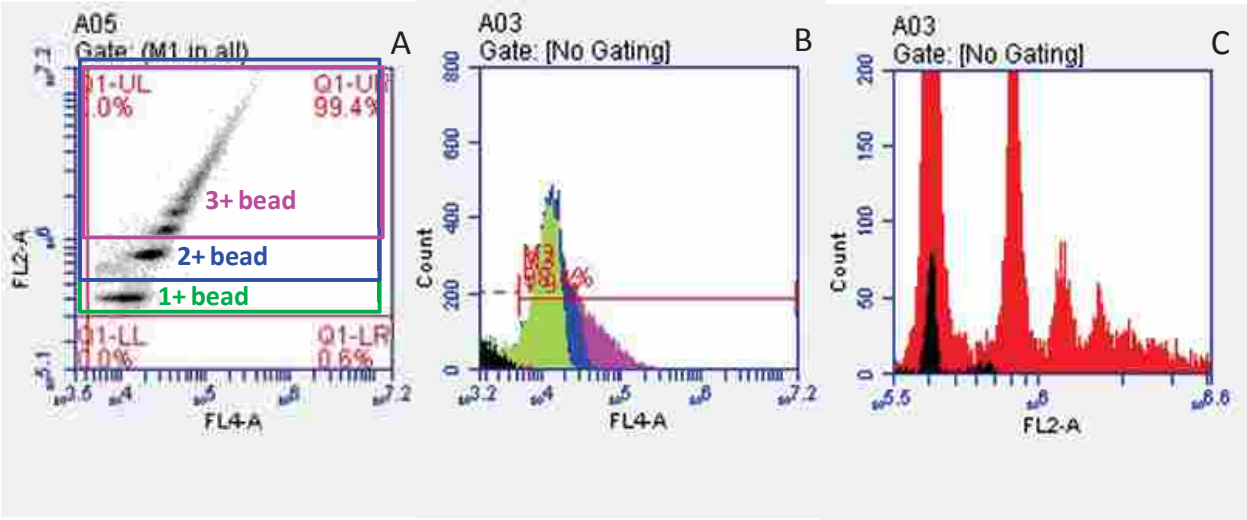


Figure 12 Flow cytometry analysis on BD Accuri software.

A) FL4-A represents CD14+ events, FL2-A represents PE^+ events. Distinct populations of cells engulfing 1, 2, and 3+ beads can be seen ascending in PEfluorescence. B) $CD14^+$ events are separated to show cells that are engulfing 1 bead (green), 2 beads (blue), and 3+ beads (pink). C) PE^+ events overlayed with $CD14^+$ in red. The 1 bead population shows slight contamination with non-$CD14^+$ events (black). This may be residual B cells which might have beads attached on the membrane. The 1 bead population was not used in analysis because of this contamination.

RT-qPCR

Macrophages were prepared in the same manner as for the phagocytosis assay. At the end of the 24 h incubation with caffeine, macrophages were isolated by their adherence and collected for lysis. RNA from lysates was isolated using RNAqueous kit (Ambion) and analyzed in our StepOne Real Time PCR System using qScript™ One-Step qRT-PCR Kit (Quanta Biosciences) and Taqman Gene Expression Assays (Life Technologies). ActinB was used as an endogenous control revealing the mRNA expression levels of adenosine receptor A_{2A}, and MAP kinase phosphatase (MKP-1).

Statistical Analysis

Results from gene expression analysis were analyzed using StepOne software, determining the fold change using the equation: fold change = $2^{-\Delta\Delta CT}$. Treatments for both gene expression assays and phagocytosis assays were analyzed and compared to controls using a beta regression controlling for factors of age, gender, caffeine intake, and exercise. Matched pair t-tests were used to further analyze the effect of each factor on macrophage aggressiveness.

RESULTS AND DISCUSSION

Caffeine Concentration

To ensure that caffeine concentrations remained uniform in culture throughout the experiment, caffeine's absorbance was read at 0 h, 2 h, and 24 h post-incubation. No significant difference was seen in caffeine concentration after incubation (Figure 13).

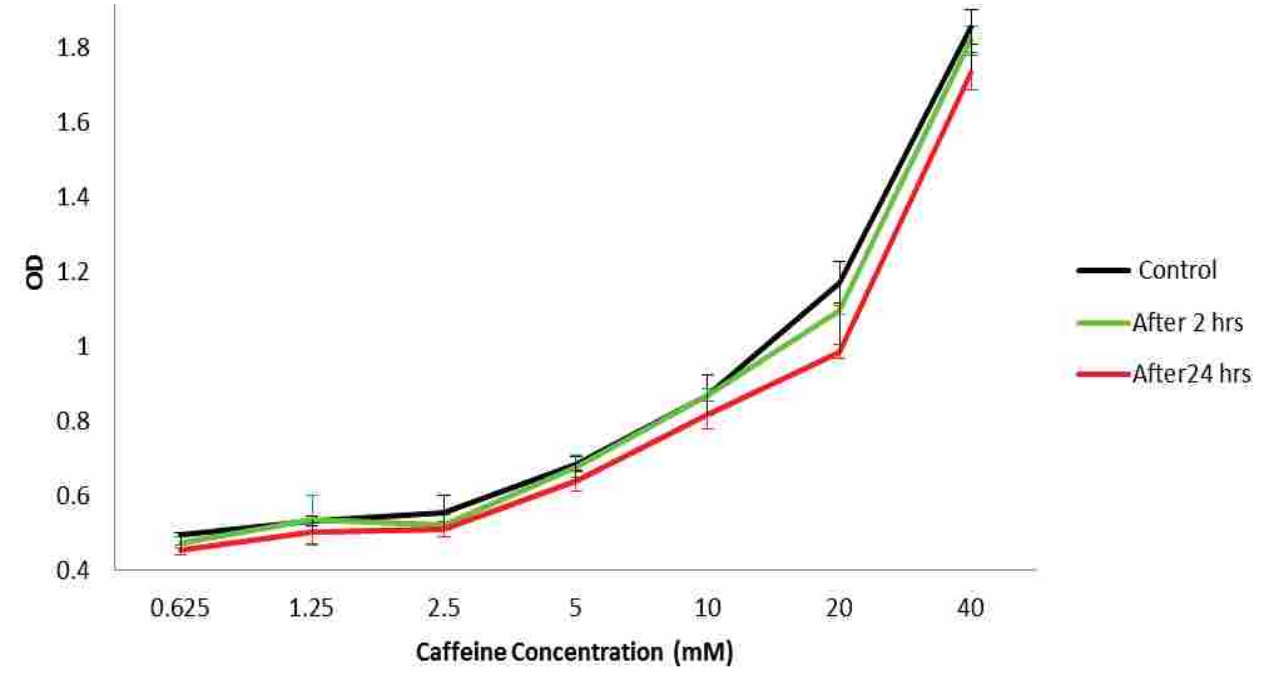


Figure 13 Caffeine concentration by absorbance.

Media from cell culture was sampled and measured using spectrophotometry to identify concentration of caffeine. Wavelength = 300 nm. All other variable besides caffeine concentration were kept constant. No significant differences were seen between time points, suggesting that caffeine concentrations remained relatively stable throughout the 24 h incubation.

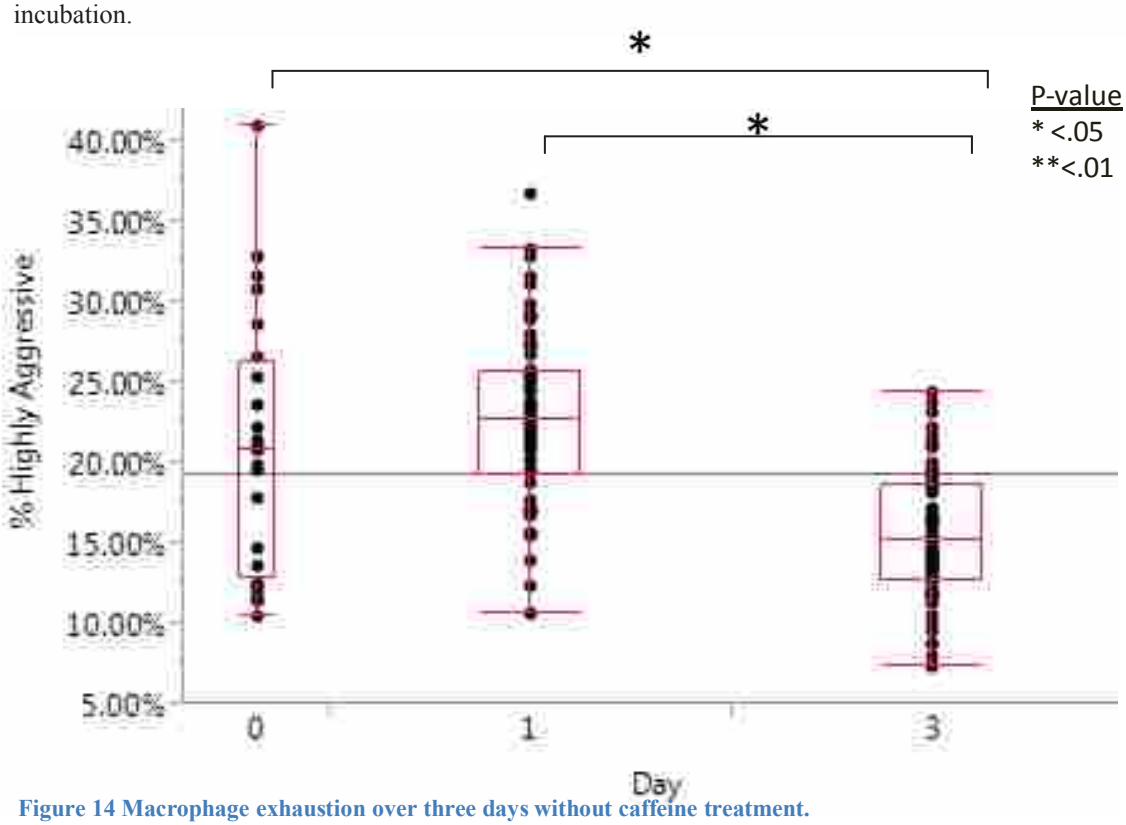


Figure 14 Macrophage exhaustion over three days without caffeine treatment.

Macrophage aggressiveness as measured by percent highly aggressively engulfing. Over three days, macrophage aggressiveness decreases significantly by 34.4% (p-value = 0.0003 , compared to control day 0. Data was generated using a paired t-test. n=11

Macrophage Exhaustion

In order to observe the phagocytic activity of macrophages as they mature, exhaustion was measured over three days using phagocytosis as an indicator of aggressiveness. Figure 14 shows that in non-caffeine-treated (control) macrophages, cell maturity reduced phagocytosis after three days. On day three total phagocytosis was reduced by 34.4% (p-value = 0.0003, 95% CI [13.8%, 37.6%]). This reduction confirms previous work showing that under homeostatic conditions macrophages shift to an M2 phenotype.[46]

Caffeine and Phagocytosis

Caffeine-treated macrophages were analyzed for aggressiveness based on the patient's caffeine intake, gender, age, and exercise level. PKA inhibitor was also added to determine caffeine's mechanism of action. A beta regression accounting for the overall effect of patient factors was performed (Figure 15). Caffeine's effect becomes more significant with cell maturity, with day three having the most profound effect. Pre-treatment with PKA inhibitor negates the effect of caffeine. This suggests that the effect on aggressiveness seen in caffeine-treated macrophages is due to the phosphodiesterase inhibitory activity of caffeine. Beta regression revealed that patient caffeine intake and regular exercise have a significant effect on macrophage response to caffeine treatment, while other factors had no significant effect or interaction.

Following the beta regression results, further analysis using a matched pair t-test was done to reveal the effect of patient caffeine intake and exercise for each day. Day 0 results showed no significant difference regardless of caffeine treatment, caffeine intake, or exercise level (data not shown).

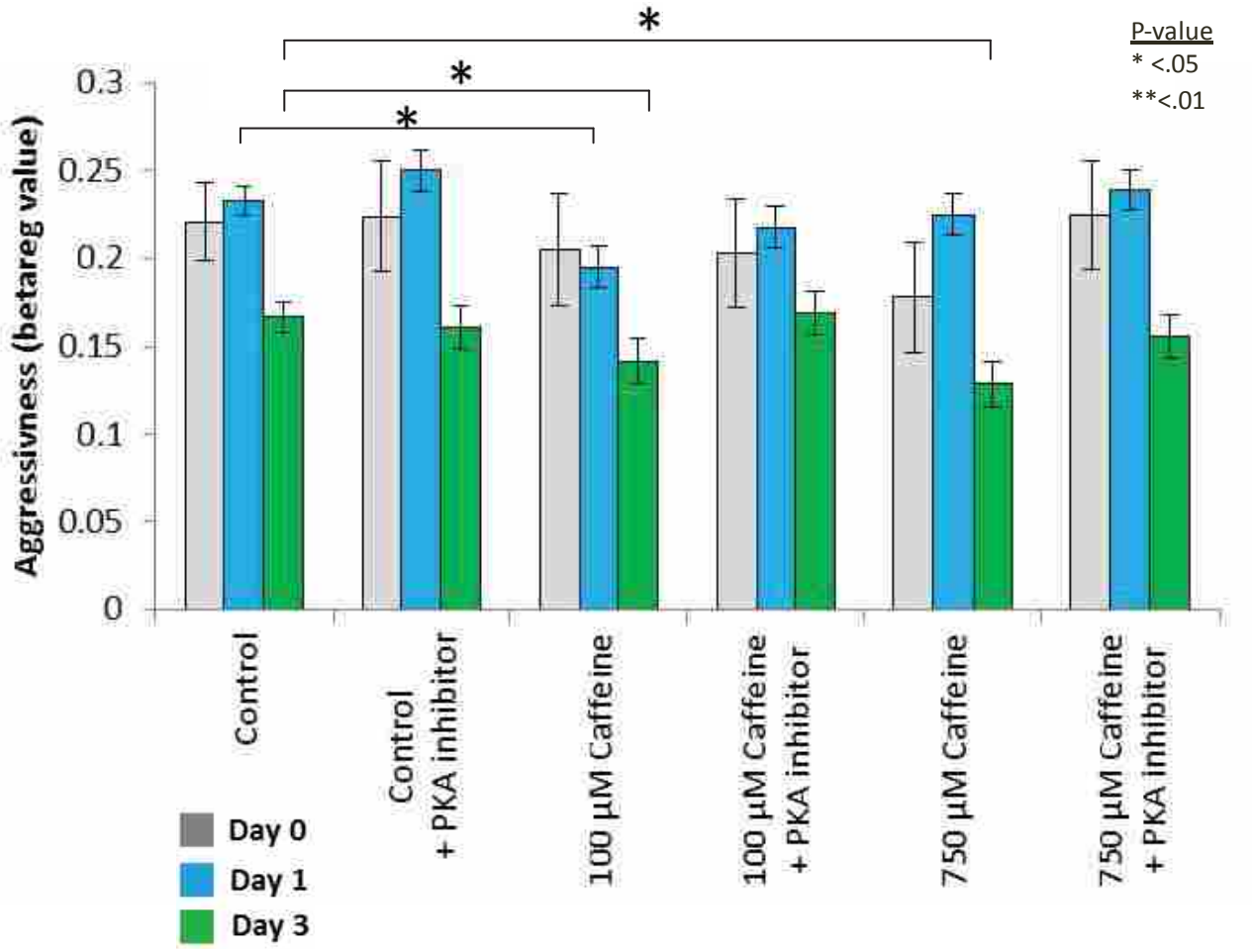


Figure 15 Beta regression on macrophage aggressiveness accounting for all patient variables

Beta regression accounting for gender, age, caffeine intake, and regular exercise was performed for each day of analysis. Bars represent the average beta regression value of aggressiveness. Day 0 (gray) showed no significant difference in any treatments with no factor having a significant effect. Day 1 (blue) showed a significant decrease in aggressiveness after 100 µM caffeine treatment (p-value = 0.0428), but addition of PKA inhibitor reversed this effect (p-value = 0.496). Day 3 (green) showed significant decreases in aggressiveness when treated with both 100 µM caffeine (p-value = 0.012) and 750 µM caffeine (p-value = 0.019). Addition of PKA inhibitor reversed this suppression in both concentrations (p-value = 0.92 and 0.509, respectively). n=11

Figure 16 shows the effect of caffeine treatment on non-caffeine drinkers' macrophages on day 1. Macrophages treated with 100 µM and 750 µM caffeine showed a 23.5% and 11.7% reduction in aggressiveness, respectively (p-value = 0.0480 and 0.0116, respectively). However, macrophages treated with 100 µM or 750 µM caffeine and PKA inhibitor (+pka_inh) showed no significant decrease in aggressiveness (p-value = 0.8773 and 0.8177, respectively).

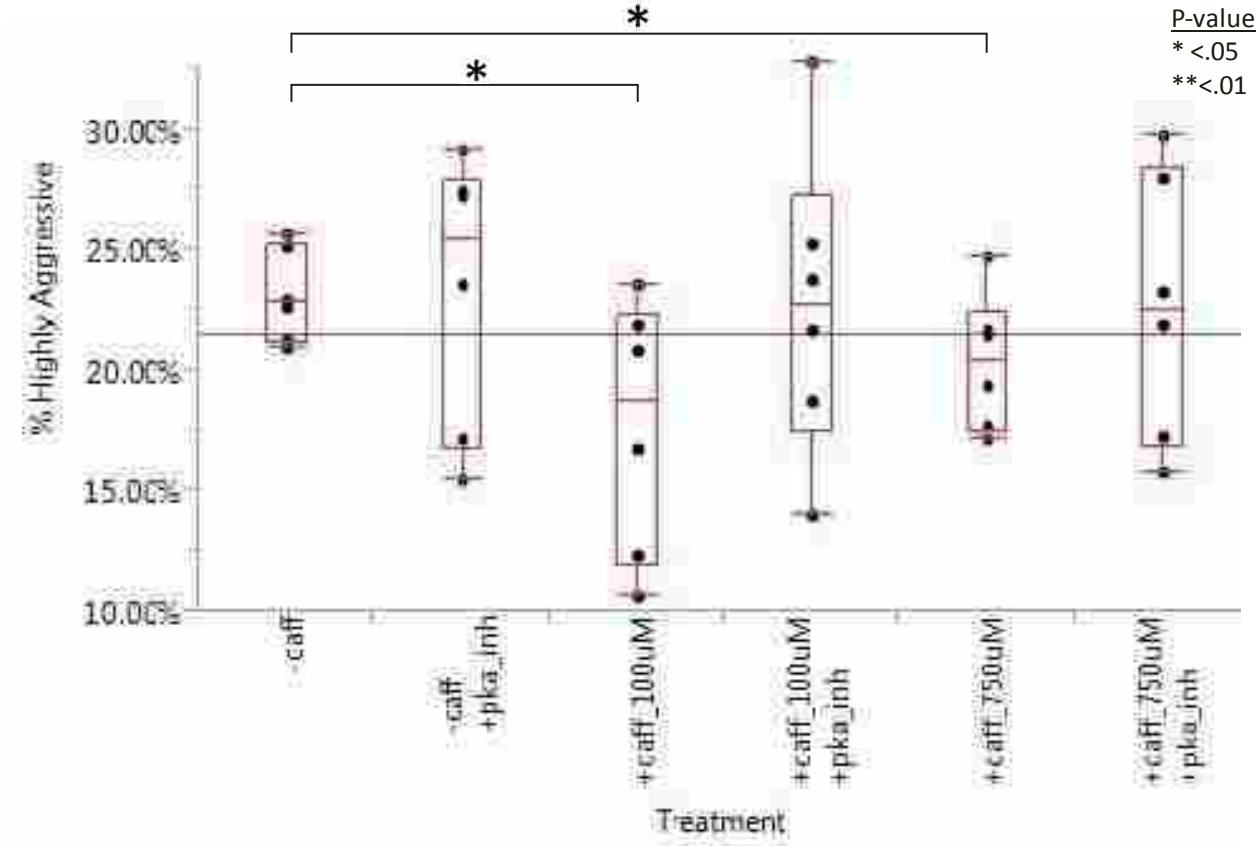


Figure 16 Caffeine's effect on day 1, non-caffeine drinkers.

Caffeine reduces macrophage aggressiveness in non-caffeine drinkers at 100 µM (p-value = 0.0480) and 750 µM (p-value = 0.0116). Differences report was generated using a matched pairs t-test. n=6

Figure 17 shows the effect of caffeine treatment on caffeine drinkers' macrophages on day 1. A significant difference in aggressiveness is seen in the non-caffeine-treated cells between the "-caff +pka_inh" and the "-caff" control. This may be due to residual caffeine in the serum of these patients.

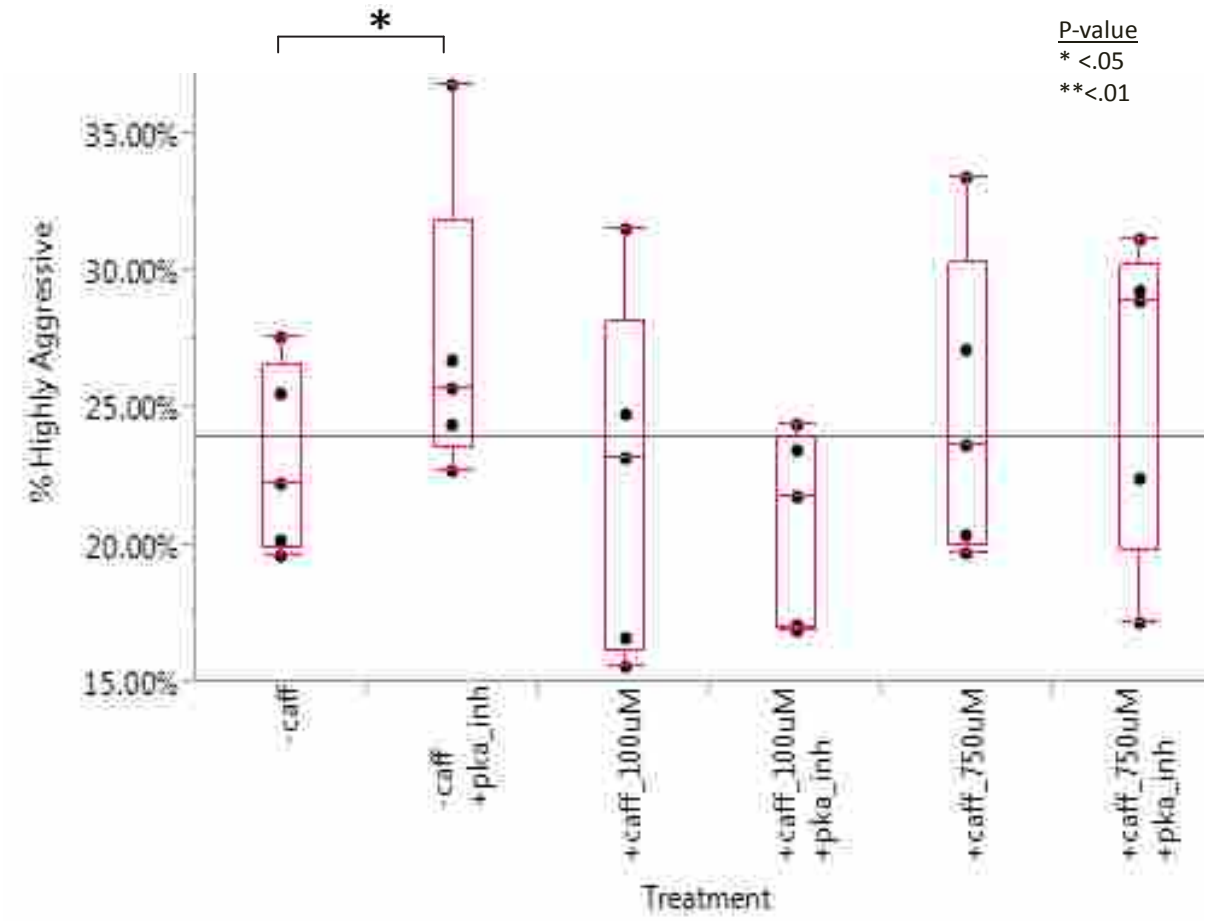


Figure 17 Caffeine's effect on day 1 in regular caffeine drinkers

With caffeine drinkers' macrophages, there was no significant difference in macrophage aggressiveness in any concentration or with PKA inhibitor except a small difference between the control and control + PKA inhibitor (p-value = 0.0439). This could be due to the inhibition of caffeine already in the serum of these patients. Data generated using a matched pair t-test. n=5

Figure 18 shows the effect of caffeine treatment on non-caffeine drinkers' macrophage aggressiveness after day 3 of incubation. Addition of 100 µM caffeine resulted in a 25.4% reduction in aggressiveness (p-value = 0.0314). Addition of 750 µM caffeine reduced aggressiveness by 36.9% (p-value = 0.0037). PKA inhibitor prevented the reduction caused by 100 µM caffeine (p-value = 0.2147), but was unable to completely reverse the effect of 750 µM caffeine, which still had a 21.9% decrease in aggressiveness compared to controls (p-value = 0.0117).

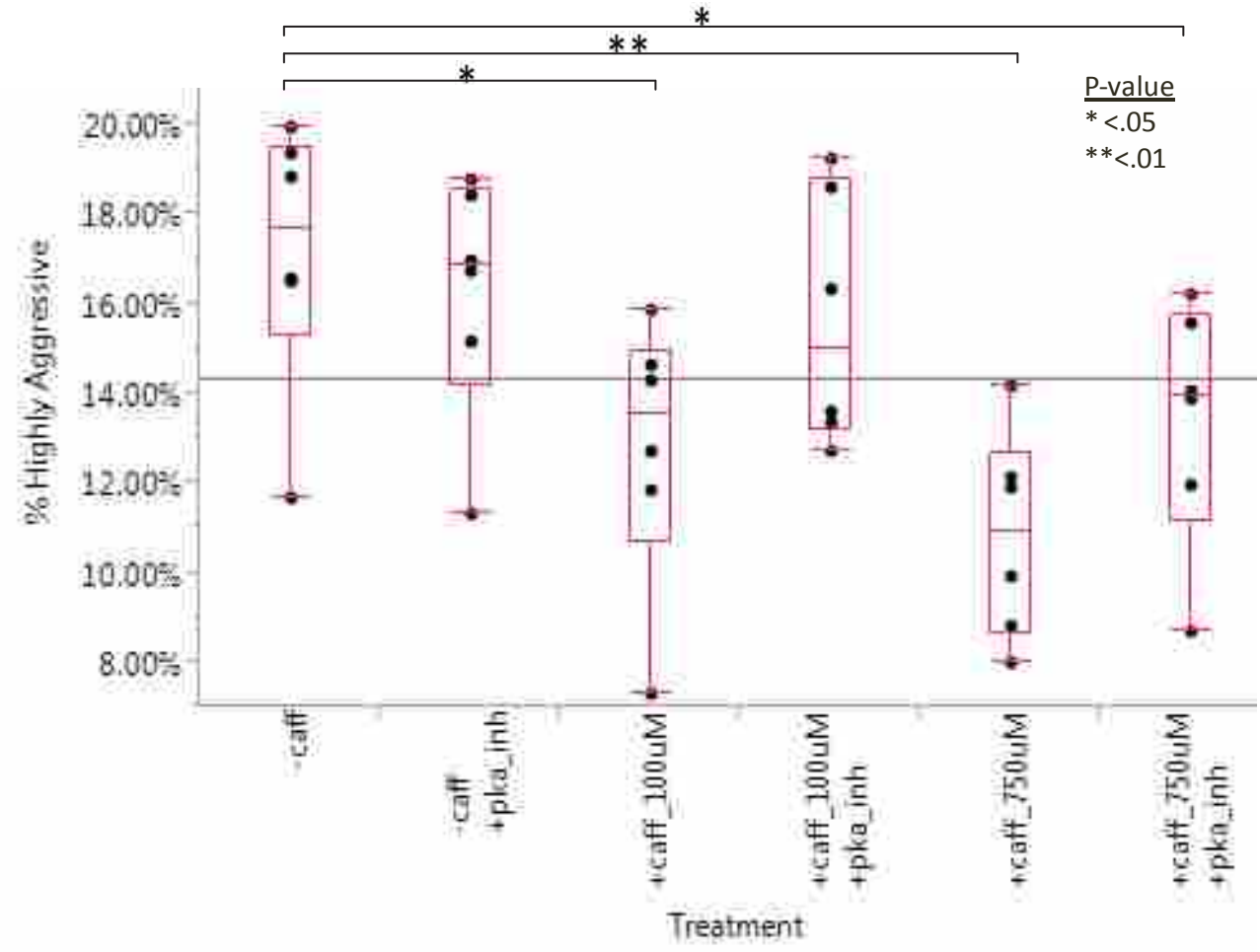


Figure 18 Caffeine's effect on day 3, non-caffeine drinkers

Non-caffeine drinkers' macrophages show significant reductions in aggressiveness when treated with either 100 µM (p-value = 0.0314) or 750 µM caffeine (p-value = 0.0037). Addition of PKA inhibitor reversed this reduction for 100 µM caffeine, but not totally for 750 µM caffeine (p-value = 0.0117). Data generated using a matched pairs t-test. n=6

Figure 19 shows caffeine drinkers' macrophage aggressiveness 3 d post stimulation and after caffeine treatment. No significant difference was seen in any treatment group. This confirms a form of caffeine tolerance that has been shown to exist in chronic caffeine drinkers.[13]

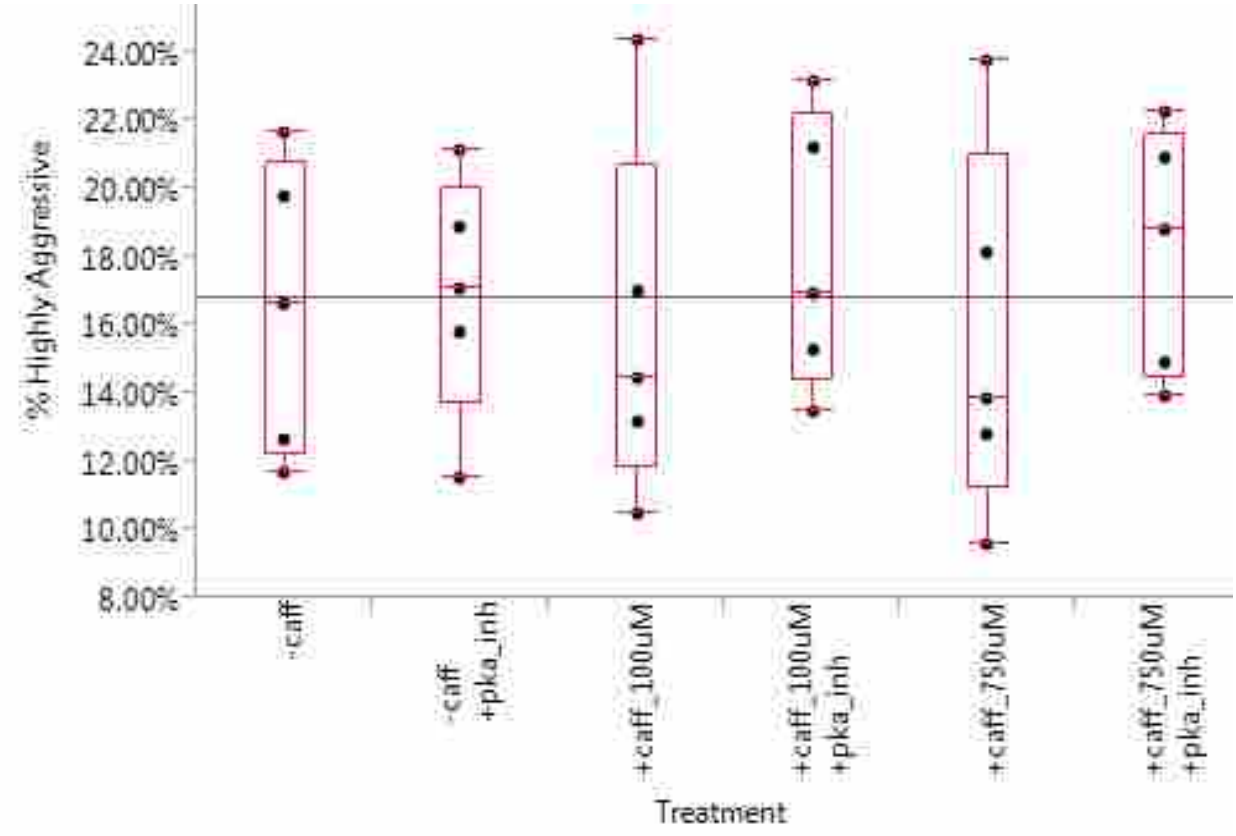


Figure 19 Caffeine's effect on day 3, caffeine drinkers

Caffeine drinkers' macrophages show no sensitivity to caffeine at 100 µM or 750 µM concentrations. PKA inhibitor also had no effect on these cells' aggressiveness. Data generated using a matched pair t-test. n=5

Exercise and Caffeine Suppression

Figure 20 and 21 show the effect of caffeine on macrophages in exercising versus non-exercising patients. There was no difference in macrophage aggressiveness between exercising and non-exercising patients. However, in non-exercising patients, macrophage aggressiveness significantly decreased after treatment with 100 µM and 750 µM caffeine by 23.8% (p-value = 0.0265) and 24.7% (p-value = 0.0215), respectively, and PKA inhibitor prevented this effect (p-value = 0.8687, and 0.2796, respectively). Caffeine intake was not suspected to be a confounding variable since both regular exercisers and non-exercisers both had an equal ratio of caffeine/non-caffeine drinkers and beta regression revealed no significant interaction. This finding adds confidence to recent suspicions that regular exercise may boost immune robustness and protect against immunosuppression by modulators such as caffeine.[59]

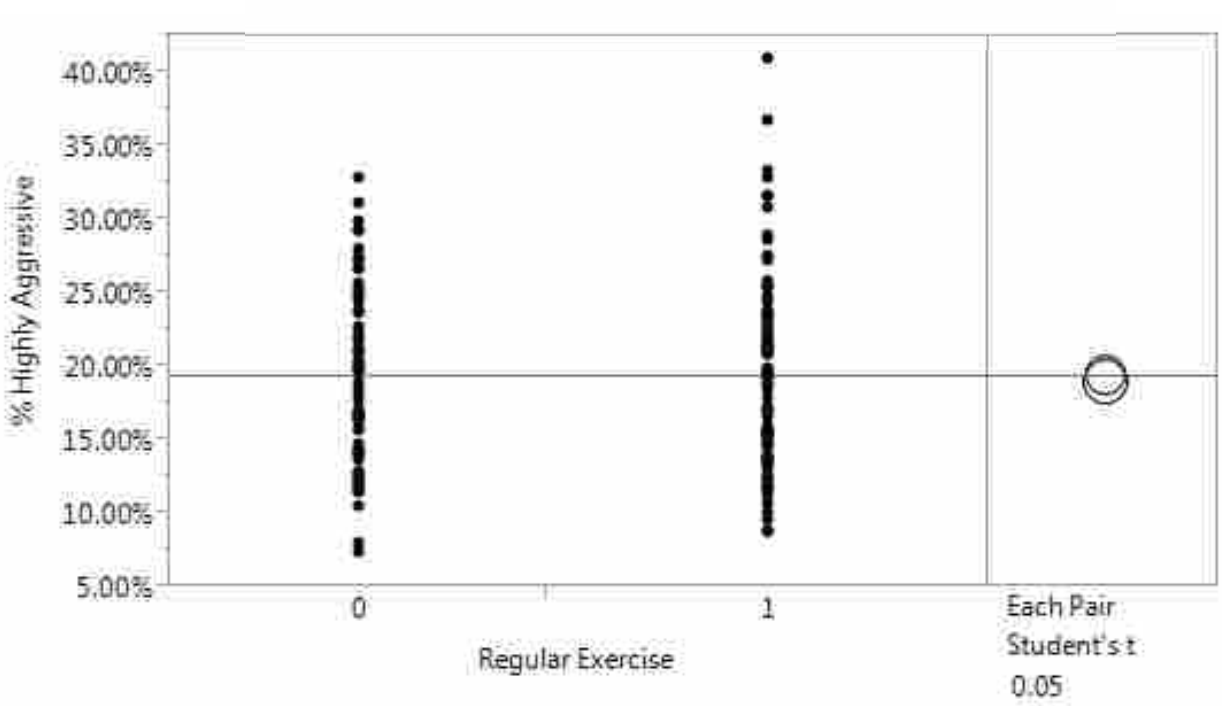
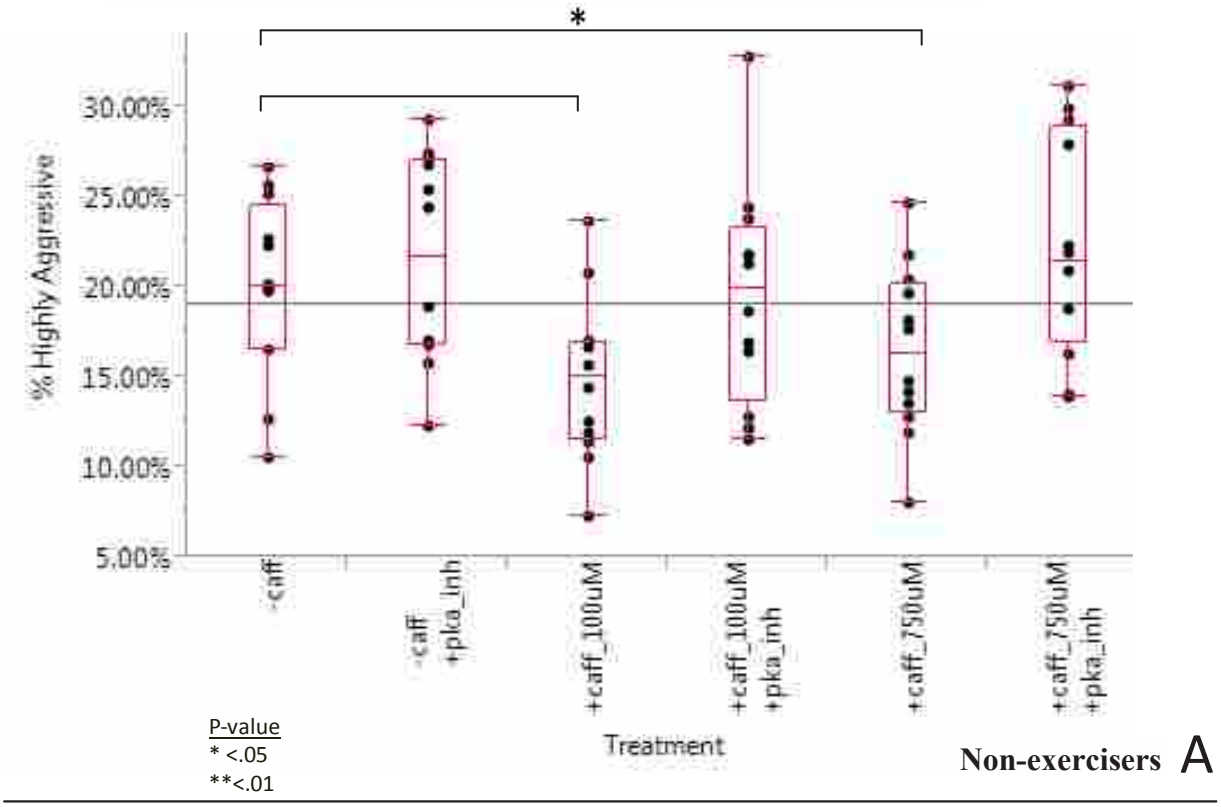


Figure 20 Effect of exercise on aggressiveness.

0= no regular exercise, 1= regular exercise. A studnent's t-test revealed no effect on overall aggressiveness was observed in exercising versus non-exercising patients (p-value = 0.5997). n=5(non -xerciser) and n=6(exerciser)



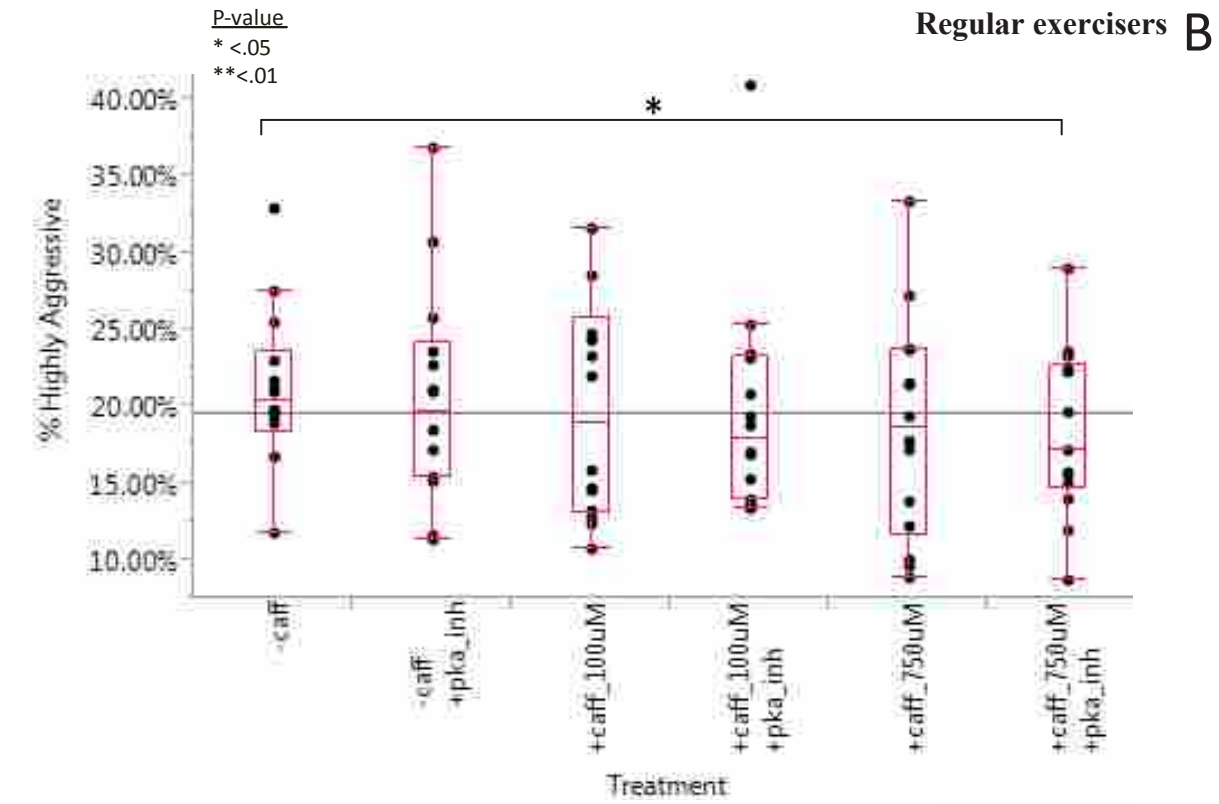


Figure 21 Effect of regular exercise on response to caffeine treatment

A) Caffeine has a significant effect on non-exercisers (p-value = 0.0207 at 100 µM, p-value = 0.0394 at 750 µM) and PKA inhibitor reversed the effect. However, in regular exercisers (B) caffeine had no significant effect. Data was combined across all days post stimulation to see an overall effect. Differences report generated using a matched pair t-test.

Adenosine Receptor Exhaustion

Adenosine receptor A_{2A} mRNA expression was measured in macrophages from three patients- one of which had recent caffeine intake- using an RT-qPCR gene expression assay. $A_{2A}R$ expression increased over three days by 2.29 fold/day (p-value <.0001) (Figure 22). The patient's caffeine intake had no effect. Addition of PKA inhibitor also had no effect on expression. Addition of PKA inhibitor was not further considered in analysis because of the lack of effect.

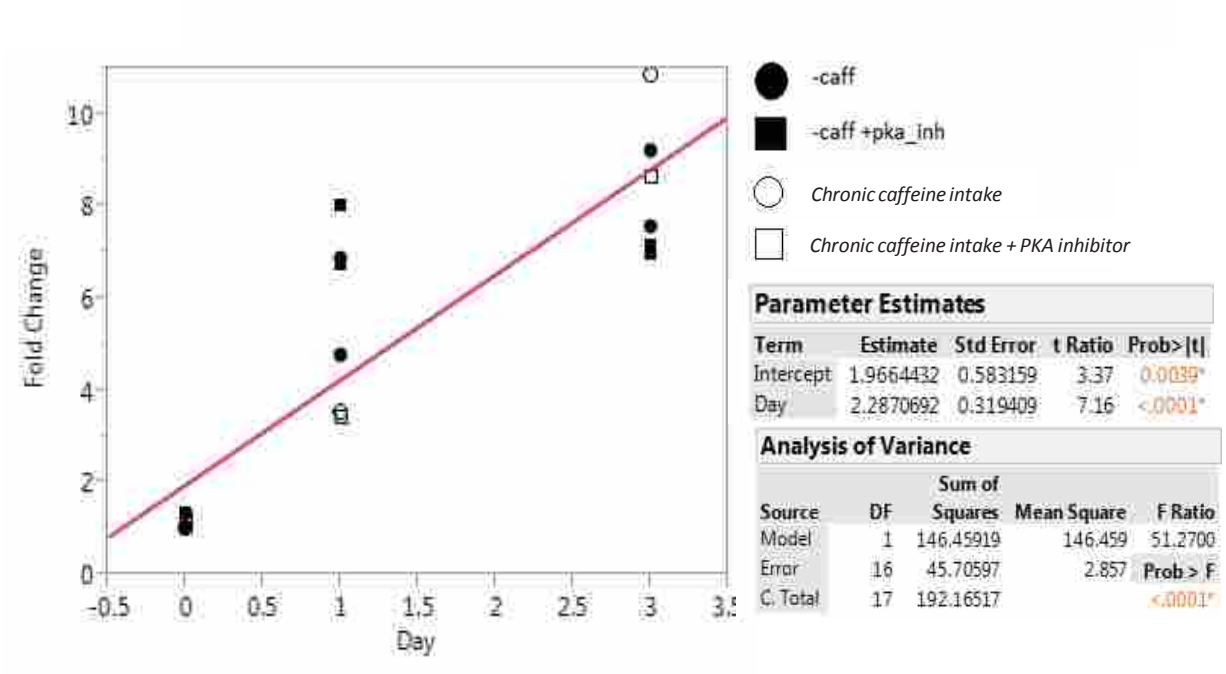


Figure 22 Adenosine receptor A_{2A} expression over three days without caffeine.
Adenosine receptor expression increases by 2.287 fold/day (p-value <.0001). Cells were not treated with caffeine, but were treated with PKA inhibitor. The inhibitor had no effect of adenosine receptor expression. Caffeine intake also had no significant effect on expression. n=3

Caffeine and Adenosine Receptor Expression

Caffeine treatment reduced $A_{2A}R$ expression on day three. 100 µM caffeine reduced $A_{2A}R$ expression by 7.6244 fold (p-value <.0001) and 750 µM caffeine reduced it by 8.1194 fold

(p-value <.0001). Addition of PKA inhibitor did not restore expression to normal levels (p-value = 0.0057 for 100 µM caffeine, p-value = 0.0327 for 750 µM caffeine).

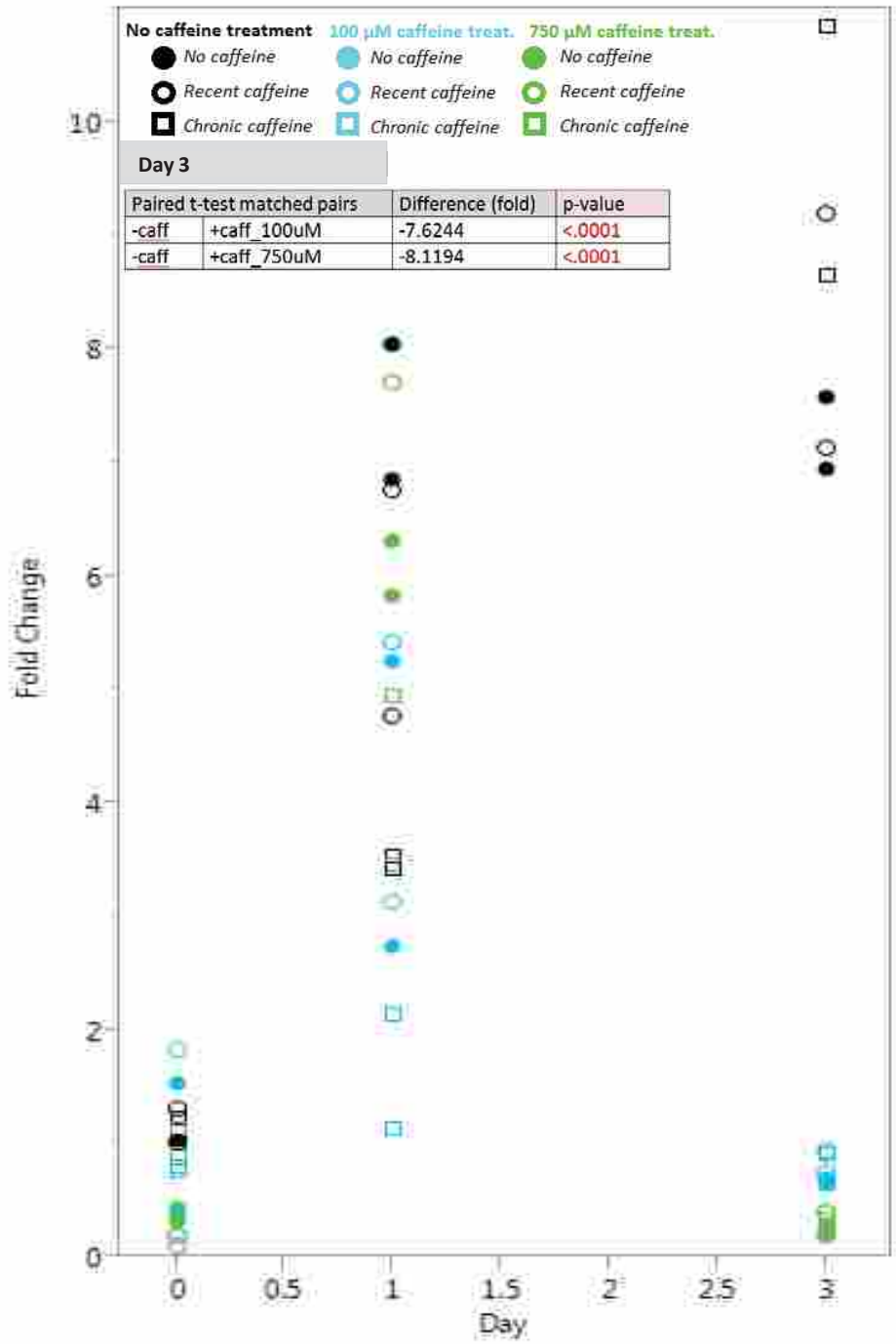


Figure 23 Adenosine A_{2A} receptor expression after caffeine treatment.

Adora2a expression in human macrophages over three days after 24 h treatment with 100µM caffeine (+caff_100uM), 750 µM caffeine (+caff_750uM). Caffeine control groups showed an increase in adenosine receptor expression whereas cells treated with 100 µM caffeine decreased on by 7.62 fold on day three. Cells treated with 750 µM caffeine decreased by 8.12 fold on day three. PKA inhibitor had no effect on expression. n=3

The reduction in expression in caffeine treated macrophages may be due to a negative feedback mechanism initiated by the blockade of $A_{2A}R$ signaling by caffeine. PKA inhibitor's failure to restore expression to normal levels suggests that decreased $A_{2A}R$ expression is not a downstream effect of phosphodiesterase inhibitor activity, but that caffeine is most likely binding antagonistically to adenosine receptors at physiological concentrations. The delay of this suppression until day three correlates with our work and work previously done showing that adenosine receptor expression increases with age.[23] Since signaling through adenosine receptors initiates an anti-inflammatory response, an increase in receptor expression would provide a default feedback mechanism to prevent tissue damage from prolonged exposure to pro-inflammatory attack.

MKP-1 Expression

MKP-1 mRNA expression was measured with the same protocol as $A_{2A}R$ mRNA expression as described above. PKA inhibitor had no effect on expression again and was excluded from further analysis for simplicity. Expression was significantly affected by chronic caffeine intake (p-value = 0.039). Chronic caffeine intake in one patient coupled with treatment of 750 μM caffeine resulted in up-regulation of MKP-1 on day three (Figure 24). This may be due to caffeine hyposensitivity in that patient, resulting in an increased stimulation of adenosine receptors and subsequently an increased activation of MKP-1. Excluding this, caffeine treatment reduced MKP-1 expression on days 1 and 3. This, along with a decreased $A_{2A}R$ expression profile, provides convincing evidence that caffeine is causing a prolonged inflammatory response. This would account for the prolonged MAPK phosphorylation seen by Wang et al.[61] However, this contradicts our phagocytosis data. We conclude that caffeine has both an anti-

inflammatory and a pro-inflammatory effect on immune cells. These effects may present as a phenotypically distinct macrophage exhibiting both pro- and anti-inflammatory characteristics. Such a phenomenon would fit the abundant, contradictory in-vivo and epidemiological evidence available on caffeine. Further study on the binding constants and IC_{50} value of caffeine in vivo would illuminate these complicated mechanisms and help us understand the overall profile of a

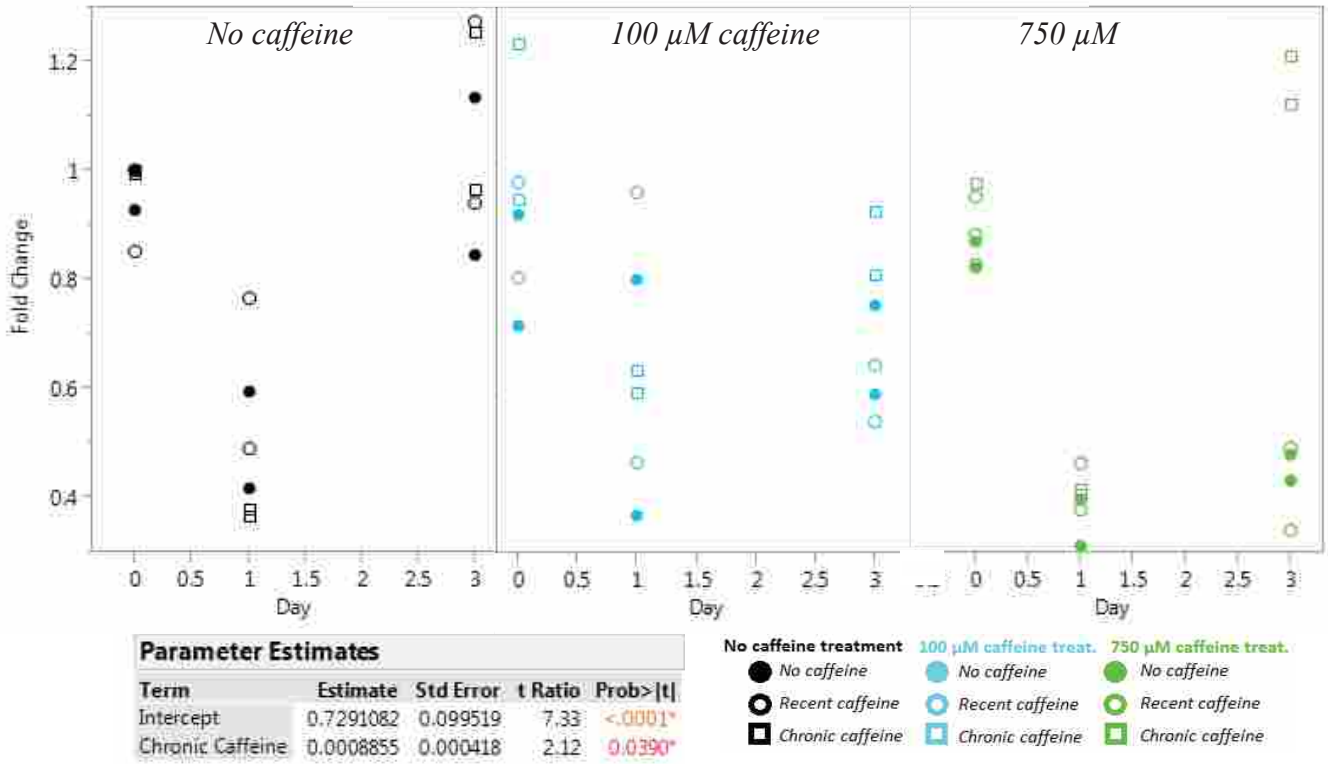


Figure 24 MKP-1 expression after caffeine treatment.

MKP-1 expression in human macrophages over three days after 24 hour treatment with no caffeine (black) 100µM caffeine (blue), or 750 µM caffeine (green). 24 h treatment with caffeine and PKA inhibitor began 24 h prior to the day of harvesting. Two outliers in the 750 µM group are from the chronic caffeine drinker that had had caffeine in the past 15 h. Addition of PKA had no effect on MKP-1 expression (data not shown). n=3

caffeine-treated immune system.

The regulation of macrophage activation is a complex process with many moving pieces that interact at varying time points and concentrations. Caffeine inhibits phosphodiesterase

causing suppressed phagocytosis, and antagonizes adenosine receptors causing pro-longed inflammatory responses (Figure 25). Both effects seem to be complicated by regular caffeine intake, while phagocytic suppression additionally depends on fitness level. Further study into caffeine as a modulator of the immune system will better inform the average consumer on the health risks and benefits of dietary consumption.

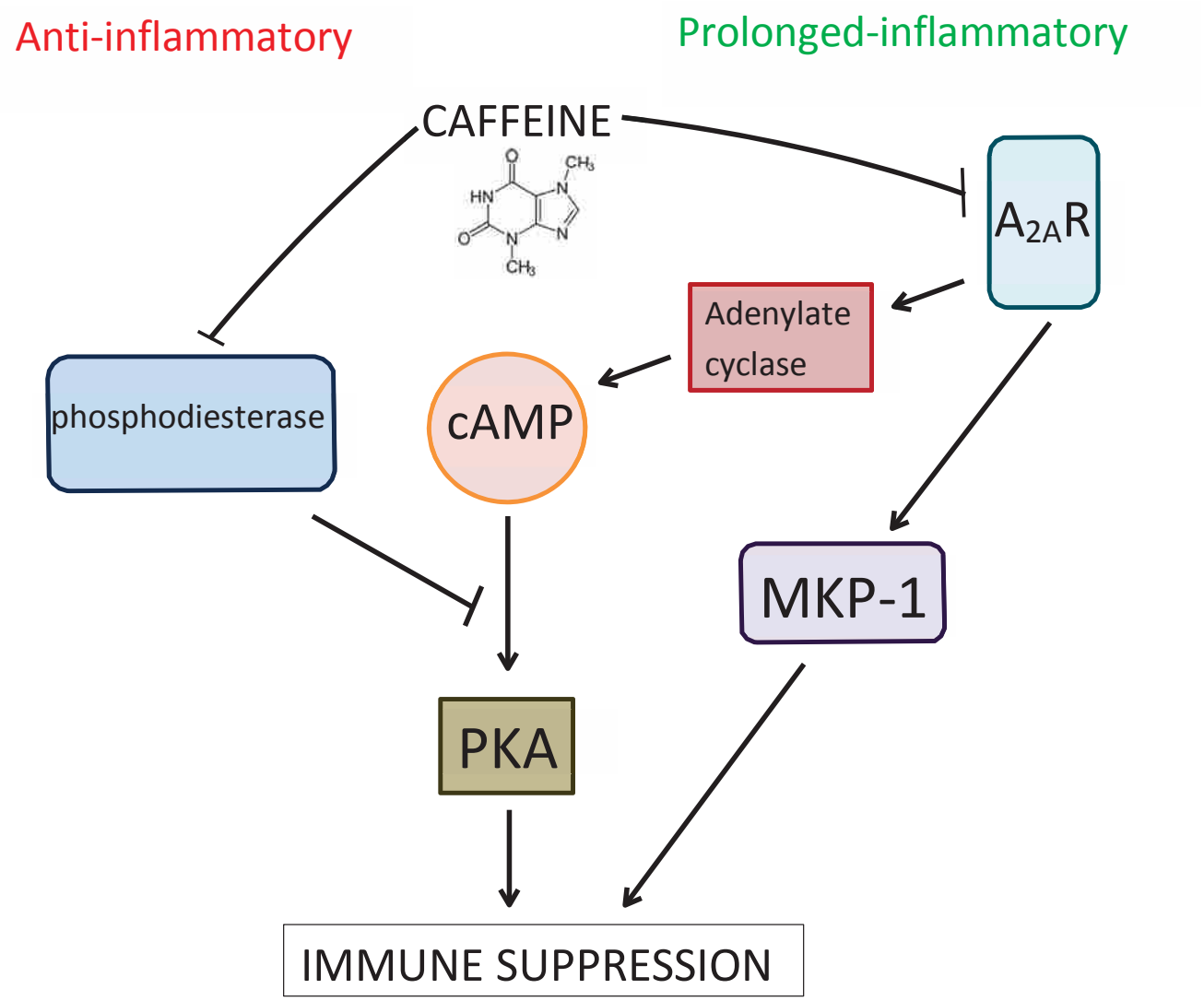


Figure 25 Caffeine's proposed anti-inflammatory and prolonged-inflammatory effects.

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