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EFFECT OF ADENOSINE AND CAFFEINE ON TOLL-LIKE RECEPTOR-4 (TLR-4)

by

SARA HISHAM MOODAD

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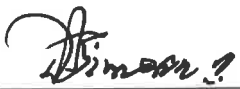
By
SARA HISHAM MOODAD

Approved by:

[Signature]

[Alexander Abdelnoor, PHD, Professor] Advisor
[Department of Experimental Pathology, Immunology and Microbiology]



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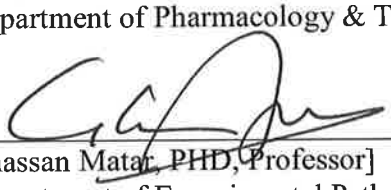

[Signature]

[Ghassan Matar, PHD, Professor] Member of Committee
[Department of Experimental Pathology, Immunology and Microbiology]

[Signature]

[Elias Rahal, PHD, Assistant professor] Member of Committee
[Department of Experimental Pathology, Immunology and Microbiology]

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AN ABSTRACT OF THE THESIS OF

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Background and aims: The role of Toll-Like Receptor-4 (TLR-4) in innate immunity and inflammation is well established. Lipopolysaccharide (LPS) a constituent of the cell wall of Gram negative bacteria is a ligand for TLR-4. Binding of LPS to TLR-4 activates both myeloid differentiation (MyD88) dependent and independent pathways leading to the production of excessive amount of pro-inflammatory cytokines, including Tumor Necrosis Factor-alpha (TNF-α) and Interleukin-12 (IL-12). The excess production of cytokines can lead to hypotension and shock, in some patients with Gram negative infections.

Previous studies have shown that endogenous adenosine, an anti-inflammatory agent, is released at sites of injury and inflammation thereby decreasing the excessive production of cytokines. On the other hand, caffeine, a non-specific adenosine blocker, has been reported in several studies to have opposing immune-modulatory effects.

In this study, the effects of caffeine and adenosine on TLR-4 in promoting or decreasing the production of TNF-α and IL-12 by LPS-stimulated monocytes was investigated.

Methods: Monocytes were isolated using Pluribead® kit from pooled blood obtained from ten volunteers. The monocytes were then incubated for 24 hours with LPS extracted from *Escherichia coli* (aTLR-4 ligand activator) , adenosine, caffeine and LPS extracted from *Rhodobacter sphaeroides* (LPS-RS, a TLR-4 ligand blocker), each alone or in different combinations. Later, the levels of pro-inflammatory cytokines TNFα and IL-12 were assessed using an Enzyme Linked ImmunoAssay (ELISA).

Results: Caffeine and adenosine significantly reduced the amount of TNFα and IL-12 produced by LPS-stimulated monocytes. Regarding non-stimulated and LPS-RS blocked monocytes, the presence of adenosine and caffeine significantly decreased TNFα levels produced by these cells but had little or non-significant effect on the levels of IL-12.

Conclusion: Both caffeine and adenosine block the production of the pro-inflammatory cytokines by LPS-stimulated monocytes. TLR-4, the LPS receptor, did not appear to be involved in the signaling pathway of caffeine and adenosine since blocking the TLR-4 receptor did not abolish the effects of adenosine and caffeine on cytokine production mainly TNFα.

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CHAPTER I

INTRODUCTION

Previous studies showed that endogenous adenosine, an anti-inflammatory agent, released by LPS-stimulated immune cells (monocytes, neutrophils, and dendritic cells) can bind to adenosine receptors present on these cells and decrease the amount of cytokines produced thereby decreasing the intensity of inflammation and tissue injury.

On the other hand, the role of caffeine, a non-specific adenosine blocker, in immunity and inflammation is still controversial. Some studies show that caffeine can decrease the production of cytokines, especially TNFα, IL12, and IL6 during sepsis and tissue injury while other studies suggest that it increases the intensity of inflammation.

The role of Toll-Like Receptor 4 (TLR-4) in innate immunity is well documented. During a Gram-negative bacterial infection, the LPS portion of the bacteria binds TLR-4 leading to the production of pro-inflammatory cytokines and the initiation of inflammatory response. Consequently, the role of TLR-4 in inflammation and its modulation has gained much attention lately.

In this study the effects of caffeine and adenosine on TLR-4 in promoting or decreasing the production of TNF-α and IL-12 by LPS-stimulated monocytes were investigated.

The significance of this study is that LPS-TLR-4 interaction is implicated in the etiologies of several diseases and syndromes including excessive inflammation and sepsis. Thus the results obtained on the effects of adenosine and caffeine on TLR-4 might have important implications on mechanisms involved in sepsis and tissue injury.

CHAPTER II
LITERATURE REVIEW

A. Adenosine:

The inflammatory response initiated by our body usually aims at elimination of foreign pathogens. However, as monocytes and polymorphonuclear cells fight pathogens, the overzealous release of cytokines, reactive oxygen species, and antibacterial granules to the extracellular milieu can result in tissue injury. This uncontrolled inflammation is implicated in the pathogenesis of several illnesses ranging from sepsis to chronic inflammatory conditions such as rheumatoid arthritis. To control inflammation, our body synthesizes anti-inflammatory agents that aim at decreasing the intensity of inflammation and the resulting tissue damage. One of these endogenous anti-inflammatory agents is adenosine. Adenosine is a purine nucleoside released at sites of injury during periods of stress and inflammation[1]. Referred to as "retaliatory metabolite", adenosine is produced by almost all the cells as a by-product of metabolism.

Normally, adenosine is present in the plasma in low concentrations of 600nM[1]. However, during systemic inflammation, hypoxia, or ischemia, adenosine concentration can increase dramatically[1]. A recent study showed that adenosine can increase up to 10 folds reaching a concentration of 4-10uM in septic patients. A higher concentration of 10-100uM was also reported in the synovial fluid of rheumatoid arthritis patients[2].

Adenosine is produced, intracellularly, by dephosphorylation of adenosine tri-phosphate (ATP) by an enzyme called 5'-nucleotidase (Figure 1). The release of adenosine extracellularly depends on the concentration gradient. When adenosine reaches a high concentration inside the cell, it is then transported to the extracellular matrix via specialized nucleoside transporters. Once released, adenosine can bind to its receptors present on different cell types such as monocytes, neutrophils, dendritic cells and lymphocytes[1].

The production of adenosine is limited via its catabolism by several enzymes such as the adenosine deaminase enzyme that deaminates adenosine into inosine which is further degraded to give uric acid. Another limiting enzyme is adenosine kinase which phosphorylates adenosine thereby producing ATP[1]. The presence of these two enzymes limits the plasma half-life of adenosine to 2 seconds. During metabolic stress and ischemia, the activation of 5'-nucleotidase enzyme is paralleled by suppression in the activity of adenosine kinase resulting in a higher concentration of adenosine intracellularly[1].

Another major pathway that increases the extracellular levels of adenosine during stress involves the release of the precursor adenine nucleotides (ATP, ADP, and AMP) from the cell followed by their degradation into adenosine through several ecto-nucleotidases. Ecto- nuceotidases include Nucleoside Triposphate Dephosphorylase (NTPD ase) enzyme (CD39), which dephosphorylates ATP and ADP into AMP and 5'-ectonucleotidase (CD73) which degrades AMP into adenosine[1].(Figure 1).

Adenosine is produced by most cells; however, neutrophils, endothelial cells, and nerve terminals have been reported as major sources of extracellular adenosine[3].

Platelets producing ADP at sites of injury can also contribute to adenosine production by the dephosphorylation of the produced ADP[3].

The physiological effects of adenosine result from binding to extracellular adenosine receptors and activating intracellular signaling pathways. The ability of adenosine to function as an extracellular signaling molecule was first described by Drury and Szent-Gyorgyi who showed that adenosine, present in ischemic heart muscle, acts as a vasodilator and exhibits a negative inotropic effect, decreasing myocardial contractility [1,3]. Later, various studies demonstrated the presence of adenosine in ischemic tissue including infarcted heart leading to a hypothesis that adenosine plays a protective role during ischemia by decreasing the heart pumping and increasing the coronary blood flow. After collecting evidence that adenosine plays a similar protective role in other ischemic tissues including the brain, kidney, and skeletal muscle, the term "retaliatory mediator" was adopted to describe adenosine [3,4].

Adenosine plays a protective role by binding to specific extracellular receptors. So far, four types of adenosine receptors have been identified, these are; A_1, A_{2A}, A_{2B}, and A_3[5] (Table 1). All adenosine receptors belong to the G-protein coupled receptor (GPCR) family. However, each receptor associates with a different G-protein and therefore initiates a different signaling cascade[5].

Adenosine A_1 receptor is coupled to Gi and Go proteins. Signaling via A_1 receptor results in adenylyl cyclase (AC) inhibition, Ca^{2+} channels inactivation, K^+ channels activation, as well as dose-dependent activation of mitogen-activated protein kinases (MAPK). A_1 receptors, expressed by most cell types, exhibit high expression in the tissues of the brain, spinal cord, adrenal gland, and eye [5,6].

A_{2A} receptors are coupled to Gs stimulatory proteins. Binding of adenosine to A_{2A} receptor results in opposite effects to that seen in A_1 stimulation. Subsequently, A_{2A} stimulation results in activation of adenylyl cyclase, leading to the release of the second messenger cAMP. Moreover, an increase in the level of inositol triphosphate has been reported after A_{2A} stimulation suggesting that this receptor affects phospholipase activity. Despite signaling via a different pathway from A_1, A_{2A} also stimulates MAPK. This stimulation, however, is not dose dependent. Rather, it is cell-type dependent. This has been shown during the study of A_{2A} signaling in different cell types. For instance, when HEK293 cells are transfected with A_{2A}, the activation of MAPK was due to activation of phospholipase C and ras protein but not due to Gs. On the other hand, when CHO cells were transfected with A_{2A}, MAPK activation was due to a signaling pathway initiated by Gs . Therefore, binding of adenosine to A_{2A} receptors can initiate different signaling pathways depending on the cell type. A_{2A} receptors are mainly expressed on the surface of leukocytes, brain, heart, blood vessels, and platelets [4,5,6].

Like A_{2A} receptors, A_{2B} receptors are also coupled to Gs proteins. Thus, signaling via these receptors results in an increase in adenylyl cyclase and cAMP production. A_{2B} is mainly expressed on the surface of cells of the colon, cecum, ovary, lungs and urinary bladder. Unlike A_1 and A_{2A} which stimulate the ERK pathway of MAPK, A_{2B} can stimulate the three different pathways of MAPK; ERK, P38 and JNK. This activation was shown by the study of A_{2B} receptors on the platelet surface where it resulted in a dose dependent activation of all MAPK pathways resulting in mast cell activation and IL-8 release. Recent studies suggest that A_{2B} might also signal via pathways other than Gs as blocking the Gs pathway did not block MAPK activation by A_{2B}[5,6].

The last adenosine receptor is A_3. A_3 receptor couples with both the Gi protein, resulting in AC inhibition and decrease in cAMP production, and the Gq protein causing an increase in inositol triphosphate, DAG, and intracellular Ca^{2+} levels. Like A_1, and A_{2A} receptors, A_3 receptor activates only the ERK pathway of MAPK. A_3 receptor is expressed by several cell types mainly mast cells, thyroid glands, and testes[6].

1. The role of adenosine in immunity:

The protective role of adenosine is mediated via three mechanisms. First adenosine inhibits the parenchymal cell functions thus decreasing the energy demand of the hypoxic tissue. A clear example is the negative inotropic effect of adenosine on ischemic heart. Second, adenosine causes vasodilation resulting in increased blood and nutrient flow to the ischemic tissue thereby creating a favorable environment for hypoxic cells. Third, adenosine plays an immune-modulatory role by decreasing inflammation[1].

The role of adenosine as an anti-inflammatory agent has gained much interest lately .Early observations demonstrated that activated neutrophils and endothelial cells release high amounts of adenosine *in vitro*[1]. Studies exploring the role of adenosine on the immune system were performed in 1983 where Cronstein et al. showed that adenosine prevents the production of superoxide anions (O2-) by neutrophils stimulated with different chemo-attractants[7]. This study was later confirmed by several laboratories where the addition of adenosine deaminase, an enzyme that degrades adenosine, to stimulated neutrophils increased the release of oxygen species. The effect of adenosine on degranulation of neutrophils, however, remains controversial. Several laboratories

reported that adenosine blocks the degranulation of neutrophils while other studies showed that adenosine produces no such effect[1,3].

The effect of adenosine on adhesion and phagocytosis was also assessed. Adenosine, when present at low concentration, activates A_1 receptors and promotes both phagocytosis and the adhesion of stimulated neutrophils to cultured endothelial cells. However, when present in higher concentrations, adenosine has an opposite effect; it binds adenosine A_2 receptors and blocks both phagocytosis and adhesion of neutrophils to the endothelium[1,3]. Studies involving the use of adenosine A_1 and A_2 receptor agonists confirmed the above results.

Further studies demonstrated that adenosine inhibits neutrophil's secretion of cytokines and adhesion molecules by binding to its A_{2A} receptor. These studies were confirmed using A_{2A} agonists such as CGS-2168038where the addition of these agonists blocked the adhesion of neutrophils *in vitro*. This anti-inflammatory role of adenosine was also established in monocytes, macrophages, Mono Mac 6 cell lines and other cells[8]. A study by Hamano et al. on LPS- stimulated PBMC showed that binding of adenosine to its A_{2A} receptors inhibited the expression of intercellular adhesion molecule 1 (ICAM-1) on PBMC and decreased TNFα production by these cells as well[4]. Another study by McColl et al.in 2006 reported that modulation of A_{2A} receptor increases pro-inflammatory cytokines and chemokine expression in LPS-stimulated mononuclear cells and neutrophils[9].

Activation of A_{2A} receptors in LPS- stimulated dendritic cells resulted in decreased production of IL-12. Being a major cytokine in innate immunity, decreasing IL-12 provides evidence in support of the anti- inflammatory role of adenosine[10]. A

study by Hasko et al. assessed the effect of adenosine A_{2A} agonist on RAW 264.7macrophages and endotoxemic mice. This study showed that while adenosine decreased the production of TNF-α and nitric oxide in both LPS- primed macrophages and LPS-injected mice, it increased the levels of IL-10 [11]. The elevation of IL-10 , an immune-suppressive cytokine, further supports the function of adenosine as an anti-inflammatory molecule. Combined together, these studies show that adenosine acts as anti-inflammatory agent by decreasing phagocytosis, adhesion, and the production of inflammatory cytokines and reactive oxygen species by stimulated cells.

Besides binding to the different innate immune cells described above, adenosine can also bind to its A_{2A} receptors on endothelial cells thereby decreasing the expression of adhesion molecules such as vascular cell adhesion molecule-1 (VCAM-1). Moreover, adenosine blocks the secretion of IL-8, an important chemoattractant for neutrophils, by endothelial cells thus decreases the recruitment of neutrophils to the site of inflammation [6,8].

To identify the effects of the other adenosine receptors (A_1, A_{2B}, and A_3) on innate immune cells (monocytes, macrophages and neutrophils) function and on inflammation, agonists and antagonists of these receptors were used. For instance, the presence of A_1 agonists demonstrated an increase in chemotaxis and phagocytosis by stimulated neutrophils. In contrast, A_{2B} receptor activation decreased the expression of MHCII molecules in macrophages and inhibited the production of inducible nitric oxide synthase (iNOS)[10]. Binding of adenosine to A_3 receptors also results in decreased production of TNFα and IL-12 by LPS-stimulated monocytes[10]. On the other hand, various studies reported that A_{2B} and A_3 receptors can cause degranulation of mast cells and thus can have a pro-inflammatory role[5]. Binding of adenosine to these two receptors

on the surface of mast cells results in release of histamine, and chemokines. Moreover, stimulation of A_{2B} receptors increases the release of IL-8 a chemokine that attracts neutrophils to site of injury[4,5]. Therefore, while activating A_{2A} receptors results in an anti-inflammatory effect, binding of adenosine to other receptors either promotes or decreases inflammation.

Being immune-modulatory, adenosine A_{2A} receptors are perfect targets in therapeutic treatment of inflammatory conditions including sepsis.

B. Caffeine:

Caffeine is the most popular CNS stimulant and the number one consumed psychoactive drug. It belongs to the methyl-xanthine family of drugs and was first described in 1821 by French chemist Robiquet[12]. Being present in coffee, tea, as well as medicinal products, consumption of caffeine is common worldwide. Despite being a psychoactive drug, caffeine is considered to be generally safe by the FDA; where the toxic doses of caffeine are almost 20 folds higher than the regular daily consumption which is below 500mg. Caffeine can be potentially fatal at doses higher than 10g/ day[13].

In addition to its role as a stimulant, caffeine affects the endocrine, respiratory, gastrointestinal, and cardiovascular system[12]. Recently, caffeine and its metabolites have gained much interest regarding their effect on the immune system. Accumulating evidence shows that caffeine and other methyl-xanthines can regulate both the innate and adaptive immune response[12].

Caffeine is a non-selective adenosine blocker. It antagonizes all four adenosine receptors. It is believed that at normal concentrations the major effects of caffeine are

exerted via adenosine receptors inhibition[12]. In addition to blocking adenosine, caffeine is a weak phosphodiesterase (PDE) inhibitor.

Regarding the effect of caffeine on the immune system, some studies demonstrated that caffeine and its metabolite para-xanthine inhibit the chemotaxis of neutrophils and monocytes. Other studies reported that the release of inflammatory cytokines: TNFα, IL-12, and IL-6 by monocytes, decreases in the presence of caffeine[12]. The mechanism by which caffeine may have decreased cytokine production is unclear. Some studies suggested that it might be due to inhibition of phosphodiesterase (PDE) enzyme[13,14]. This was based on studies demonstrating a decrease in the synthesis of TNFα and IL-12 in addition to suppression of chemotaxis by stimulated neutrophils after exposure to phosphodiesterase inhibitors such as rolipram[14]. PDE inhibitors, in general, prevent the degradation of cAMP and result in activation of PKA which in turn phosphorylates cAMP-response element binding protein (CREB)[13]. The later then translocates to the nucleus and modulates the transcription of many genes including that of pro-inflammatory cytokines [13,14]. The above studies thus suggested that the inhibition of PDE, although weak, by caffeine might result in an inhibitory effect on the monocytes and neutrophils.

In contrast to the above, it was previously established that most, if not all, of the effect exerted by caffeine is due to adenosine receptor blockage[12]. Adenosine, as discussed earlier, exerts mainly an anti-inflammatory effect when binding to its receptors. Based on that, some studies stated that the presumed PDE-effect of caffeine on immunity is probably due to the use of caffeine at a concentration higher than the normally consumed one[12]. This claim was supported by studies indicating that caffeine concentrations close to the normally consumed ones do not decrease the cytokine

production by monocytes and natural killer (NK) cells. A study done by Meiners et al, in 2004, also showed that caffeine used in a concentration relevant to normal consumption had no effect on the production of TNFα and IL-12 by stimulated monocytes[15]. An *in-vivo* randomized study in 2011 involved inducing experimental human endotoxemia in 43 volunteers with or without administration of caffeine. Afterwards, the levels of endogenous adenosine, TNFα, and IL-6 produced were assessed. This study reported that during experimental endotoxemia, an increase in adenosine, cytokines, renal and endothelial injury occurs and that the co-administration of caffeine does not affect the level of injury or the level of cytokines *in vivo*[16]. A study by Ohta et al. also demonstrated that caffeine can worsen acute liver injury, via attenuating the physiological immunosuppressive mechanisms[17].

Building on the above data, it can be concluded that the effect of caffeine on the immune system is still debatable. While most studies indicate a possible immune-modulatory role of caffeine, other studies show an implication of caffeine in increasing the intensity of inflammation.

C. Lipopolysaccharide (LPS)

LPS is a constituent of the outer cell wall of Gram negative bacteria and is considered as the major virulence factor in these bacteria[18]. It is referred to as an endotoxin since it is released into the circulation only upon lysis or death of the Gram negative bacteria[19,20]. As the name implies LPS consists of a polysaccharide portion and a lipid portion[19]. The polysaccharide part is made up of an inner core, outer core, and O-antigen while the lipid portion is called lipid A[19,21].

Unlike the O-antigen which is highly variable and antigenic, Lipid A constitutes the conserved pattern of LPS that is recognized by Pattern Recognition Receptors (PRR) such as Toll-Like receptor-4 (TLR-4)[19,20]. Lipid A is the toxic part of LPS and accounts for most of the inflammatory response directed against LPS [18]. This was demonstrated by studies showing that isolated lipid A can produce the same inflammatory response as LPS[22]. Lipid A consists of fatty acid residues connected to a backbone of two phosphorylated glucosamine sugars[20,21]. The number of acyl groups present depends on the bacterial species from which Lipid A was extracted and dictates whether LPS will function as TLR-4 agonist or antagonist[23,24]. Variation in the degree of Lipid A acylation affects the binding of LPS to TLR-4[23]. In general, Lipid A moieties having six or more acyl groups result in an optimum inflammatory response[23,25]. In contrast, hypo-acylated LPS having penta-acylated and tetra-acylated Lipid A blocks the normally initiated inflammatory response[25]. For instance, LPS extracted from *E. coli* consists of hexa-acylated Lipid A and is responsible for the broad spectrum of biological activity of LPS[21,25]. Its biological activity includes activation of the complement system and coagulation system, and initiating signaling via the TLR-4 all leading to products involved in inflammation[8,26,27]. On the other hand, LPS extracted from *Rhodobacter sphaeroides* (LPS-RS) consists of penta-acylated lipid A[28,29]. LPS-RS binds to TLR-4, does not initiate a signaling pathway and prevents hexa-acylated LPS from initiating a signaling pathway [28,29]. Moreover, some Gram negative bacteria such as Shigella, normally possessing hexa-acylated LPS, can decrease the number of acyl chains in their Lipid A portion as an attempt to evade the immune system[23].

D. Toll-Like Receptors:

The initial response to Gram negative bacteria starts by the recognition of conserved motifs called Pathogen Associated Molecular Patterns (PAMPS) present in the bacteria by innate immune system receptors referred to as Pattern Recognition Receptors (PRR). These receptors can recognize different bacterial molecules such as LPS, flagellin, and lipoteichoic acid [8,25] . One family of PRR that gained much interest in the past few years is the Toll-Like Receptor (TLR) family.

1. *Toll Receptors (TR)*

Toll receptors were identified in the fruit fly *Drosophila melanogaster*[30]. The Toll protein was found to be crucial for the dorsal-ventral patterning during the embryonic development of the fruit fly[30,31]. Toll receptor is type I integral membrane receptor characterized by having three domains; an extracellular domain with leucine-rich repeats (LRR), a single transmembrane helix domain, and an intracellular domain with a cysteine-rich carboxyl terminus. The intracellular domain, homologous with the mammalian interleukin 1 receptor, is referred to as intracellular Toll/interleukin-1 receptor- like (TIR) domain[8, 30,32,33]. In 1996, Lemaitre et al. showed that mutations in the Toll receptors make the fly vulnerable to fungal infections by suppressing the production of antifungal peptide drosomycin thus the importance of Toll proteins in the fly innate immunity was established[34]. So far, ten *Drosophila* Toll receptors (dToll) have been identified. Only two, the Toll protein and its relative called the 18 wheeler, play a role in the immunity of the fly against pathogens. The rest of the proteins are probably implicated in the development of the *Drosophila*[33].

2. *Toll-Like Receptors (TLR)*

In 1996, the first human homolog of the *Drosophila* toll proteins referred to as Toll Like Receptor-1 (TLR1) was cloned[33]. In the next year, Medzhitov et al. discovered the second human toll like receptor, now designated as Toll Like Receptor-4 (TLR-4), and showed that the constitutive expression of TLR-4 activates the Nuclear-Factor kappa-B (NFkB) and results in an increase in inflammatory cytokines. Medzhitov's work was the first to suggest a possible role of TLR in the innate immune response[33].

Using genome sequencing 13 TLRs have been identified so far in the mammalian genomes; 10 were sequenced in humans and 13 in mice. The structure of human TLR resembles that of dToll in having a LRR extracellular domain, a helical transmembrane domain, and an intracellular cysteine rich domain called TIR with the ability to activate NFkB [8,33]. The extracellular domains of TLR have a solenoid structure resembling a horseshoe. These receptors can recognize different conserved patterns present in variable pathogens[30] (Table 2) .Binding of TLR to their ligands activates different pathways resulting in release of cytokines involved in the generation of the inflammatory response and the adaptive immune response.

Based on Northern blot and messenger RNA (mRNA) expression analysis, TLR were found to be expressed in a number of cell types including Antigen Presenting Cells and lymphocytes. TLR3, TLR7, TLR8, TLR9, and TLR13 are present within the endosome/lysosome compartment in the cytoplasm, whereas the other TLRs are expressed on the cell surface[35,36].

3. *Toll like receptor-4 (TLR-4):*

TLR-4 receptors are mainly expressed on the surface of macrophages and dendritic cells. Non immune cells such as epithelial, endothelial and smooth muscle cells can also express TLR-4[35]. Besides recognizing LPS from Gram negative bacteria, TLR-4 can bind other ligands including the fusion protein present in the respiratory syncytial virus (RSV), heat shock proteins, nickel, as well as molecules from *M. tuberculosis*[25].

In addition to its role in the innate immune response, TLR-4 is implicated in the pathogenesis of several autoimmune diseases and chronic inflammatory conditions such as contact dermatitis, rheumatoid arthritis, colon cancer, and liver cirrhosis [37,38,39].

TLR-4 is under tight endogenous regulation at different levels starting from its biosynthesis, vesicular trafficking, and signaling up to its degradation in the lysosome[35,37]. Glycosylation and proper folding of TLR-4 are crucial for its cell surface expression and require a set of adaptors and accessory proteins. It was noted that the absence of any adaptor protein results in decreased cell surface expression of TLR-4 and thus lower response to LPS[40]. A failure in TLR-4 regulation occurs in endotoxin shock leading to systemic inflammation or even death[37].

TLR-4 are usually expressed on the cell surface; however, upon synthesis, a small part of these receptors can go to the endosome compartment in the cytoplasm forming an intracellular pool of receptors. These receptors can recognize Gram negative bacteria, such as *E.coli*, that are phagocytized into the phagocyte's cytoplasm[37].

Activation of TLR-4 by LPS is controlled by internalization of the whole receptor complex thus terminating the signal[37].

E. LPS- TLR4 interaction:

Being amphipathic in nature, LPS molecules tend to aggregate in aqueous environments when present in a concentration above their critical micellar concentration[33]. LPS aggregates first bind to lipopolysaccharide binding protein (LBP) present in the serum. LBP breaks down LPS aggregates into monomers and delivers it to both the membrane and soluble CD14 molecules[33].

After degrading the LPS aggregates into monomers, LBP transfers the monomeric molecules to the CD14 receptor. CD14 exists in two forms; serum sCD14 and membrane mCD14[25,41]. sCD14, present in serum, confers LPS-responsiveness to mCD14-negative cells. For instance, endothelial and some epithelial cells, CD14-negative, can respond to LPS due to the presence of sCD14 that substitutes the mCD14. sCD14 binds LPS delivered by LBP, then the sCD14/LPS complex attaches to the surface of CD-14 negative cells [25,41]. On the other hand, mCD14 is present on cell surfaces mainly myelo- monocytic cells, B cells, microglial cells and other cells [8,30].

mCD14 was first thought to be the major LPS receptor; however, lacking trans-cellular and intracellular domains precluded the idea of CD14 transmitting signal inside the cells and producing response alone. After the discovery of the defected TLR-4 gene in CH3/Hej mice and its correlation with the LPS-resistance exhibited by these mice, scientists identified TLR-4 as the LPS receptor and presumed that CD14 binds LPS monomers then forms a complex with TLR-4 receptor thereby transmitting the signal[32,42].

TLR-4 is thus crucial for LPS signaling. Any mutation in lps gene in TLR-4 results in loss of LPS response. However, experiments showed that the overexpression

of TLR-4 and CD14 in HEK 293T cells was not sufficient to induce a response when challenged with LPS[25,43]. Thus, the presence of an additional crucial molecule for signaling was proposed. This molecule is called Myeloid Differentiation protein (MD-2)[43].

MD-2 is a small protein lacking a transmembrane domain. It binds TLR-4 on the cell surface forming MD-2/TLR-4 complex. MD-2 is a β-cup folded molecule made up of two anti-parallel β-sheets folded in a way forming a hydrophobic pocket to which LPS can bind[30]. Binding of LPS induces dimerization of two MD-2/TLR-4 complexes forming an M-shaped hetero-tetramer. The dimerization of TIR intracellular domains of both TLR-4 allows the recruitment of downstream adaptor molecules and thus initiates signaling transduction[30,37].

F. TLR-4 signaling:

Since the intracellular domain of TLR-4, also called TIR domain, shares a great degree of homology with that of IL-1 receptor (IL-1R), it is believed that TLR-4 shares the same signaling pathway as IL-1 receptor. This pathway is referred to as myeloid differentiation 88 (MyD88) dependent[25,44].(Figure 2)

Following the binding of LPS to MD-2/TLR-4 complex and the homo-dimerization of TLR-4, intracellular signaling is initiated. (Refer to Figure 4). A cytoplasmic adaptor protein, MyD88, is recruited. Binding of MyD88 to TLR-4 recruits kinases called IL-1 receptor-associated kinase 1 and 4 (IRAK1, IRAK4). These kinases bind to MyD88, undergo phosphorylation, and then dissociate from the receptor

complex. Once free, IRAK units interact with another adaptor protein called TNF-receptor-activated factor 6 (TRAF6)[32,35,37]

The IRAK-1/IRAK-4/TRAF-6 complex then associates with a membrane protein called TAK-1 and TAK binding proteins TAB1 and TAB2.The formed complex leads to the activation of nuclear factor kappa-B (NFkB) [8,30,35] . NFkB induces the transcription of several genes encoding for pro-inflammatory cytokines such as TNFα, IL1β, IL6, IL12 and other cytokines. On the other hand, TRAF6 can also bind and activate mitogen-activated protein kinases (MAPK) pathways[8,25].

On the other hand, a study made by Kawai et al. showed that knocking out MyD88 in mice did not block the nuclear translocation of NFkb where a delayed activation of NFkB and MAPK was obseved[45]. Thus Kawai suggested that an alternative MyD88 independent pathway exists.

Regarding the MYD88 independent pathway,(Figure 2) binding of LPS to the TLR-4 receptor complex causes the recruitment of TIR domain-containing adaptor inducing IFN-β (TRIF). TRIF binds to TLR4 and activates interferon regulatory factor 3 (IRF3) which translocates to the nucleus and up-regulates several genes including IFNα and IFNβ genes. Once produced, IFNαand β, type one interferons, bind to their interferon α/β receptors and cause the transcription of interferon inducible genes. In addition to producing type one interferons, MyD88 independent pathway also results in late phase activation of NFkB and production of inflammatory cytokines[32,44].

Thus by activating both the MyD88 independent and dependent pathways, LPS leads to an inflammatory response that, if not controlled, can lead to systemic inflammation and potentially shock.

G. Important cytokines released upon TLR-4 activation:

1- Tumor necrosis factor (TNF-α):

Tumor necrosis factor (TNFα) is a pro-inflammatory cytokine involved in systemic inflammation and produced by several cell types such as macrophages, and neutrophils. Previous studies have demonstrated that TNFα produced by mononuclear cells plays an important role during LPS induced-sepsis. During sepsis, a marked increase in TNFα occurs which contributes to its pathogenesis. Immunization against TNFα protected LPS-injected mice from endotoxin (LPS) shock[40]. Thus TNFα has been used in several *in vitro* and *in vivo* studies as a marker to assess the severity of sepsis and other inflammatory conditions.

2- Interleukin 12 (IL-12):

IL12 is an early pro-inflammatory cytokine produced mainly by macrophages/monocytes in response to infection. IL12 is a paramount cytokine that links the innate and the adaptive immune response. During sepsis, activated macrophages release IL12 to stimulate an adaptive response by lymphocytes[46].

H. Sepsis and Septic shock:

The normal function of the immune response is to protect the host via eradication of pathogens. However, the improper regulation of the immune system can result in an uncontrolled inflammatory response to pathogens leading of sepsis. Sepsis is

defined as a systemic inflammation accompanied by a presumed or known site of infection [47].

Sepsis may be induced by different pathogens; viruses, fungi, and bacteria[48]. However, it is mainly caused by Gram negative bacteria. In case of sepsis, binding of LPS, from Gram negative bacteria, to TLR4 results in an exaggerated inflammatory response leading to excessive production of pro- inflammatory cytokines [2,25]. Excess cytokines result in vasodilation, hypotension, in addition to tissue and multi-organ damage[2].

The incidence of sepsis increased over the last few decades where it is currently considered as a major cause of death in the intensive care units in the United States [49] . Recent epidemiological studies showed that severe sepsis occurs in 300/100,000 patients annually with 25% mortality rate[47]. The treatment of sepsis is quite expensive and costs around $14 billion in United States each year [47,50] , thus sepsis is considered as an important medical and public health problem.

Due to the implication of LPS-TLR-4 in the pathogenesis of several inflammatory conditions, including sepsis, the significance of this study is that the results can be later translated to such inflammatory conditions. Identifying whether caffeine can decrease or increase inflammation and whether TLR-4 is implicated in caffeine and adenosine signaling may help in developing new therapeutic approaches targeting sepsis.

CHAPTER III
MATERIALS AND METHODS:

A. Sample collection:

Ten volunteers older than 18 years of age were recruited for the purpose of the study after obtaining the Institutional Review Board (IRB) approval. All 10 volunteers were asked to refrain from caffeine consumption for at least 48 hours before blood withdrawal to insure that previous high plasma caffeine levels could not interfere with the purpose of the study. After reviewing and signing the consent forms, 3 to 4 ml of blood collected from each volunteer were pooled. Monocytes were separated from the pooled blood by a procedure described below.

B. Separation of Monocytes

Human monocytes were separated directly from the pooled blood using the PluriBeads® M- kit (PluriSelect Life Science, Leipzig, Germany). This kit follows the principal of non-magnetic separation of monocytes from blood, PBMC, or tissue. It relies on using non- magnetic monodispersed micro-particles (beads) coated with CD14 monoclonal antibodies. These antibodies are specific for CD14 markers present on the surface of monocytes. PluriBeads® are designed to be larger than blood cells, thus preventing their phagocytosis by the Macrophages. During incubation of the PluriBeads® with blood, the anti CD14 antibodies conjugated to the pluribeads bind to the CD14 markers on monocytes; increasing their size and thus inhibiting their passage

through the cell strainer while allowing the rest of the cellular components to go through.

1. ***Reagents and kit contents:***

- M-Pluribeads® suspension containing pluriBeads with anti-CD14 antibodies
- Wash buffer
- Incubation buffer
- Stabilization buffer
- Detachment buffer
- M-Pluri Strainer
- M-pluri Connectors with luer-lock

2. ***Protocol of separation:***

a. <u>Preparation of sample material</u>

- Blood from 10 volunteers was pooled in 50ml sterile conical tube
- 50ul Stabilization buffer was added for each 1 ml blood
- Stabilized blood was diluted with wash buffer (2ml wash buffer for each 1 ml blood)
- The diluted blood was centrifuged at 300g for 10min with no brake
- Plasma and supernatant were detached to 1 cm above the blood cells
- The procedure of dilution and centrifugation was repeated again
- The concentrated cells obtained were used to separate the monocytes

b. Binding of target cells:

- M-pluriBeads suspension was added to the concentrated cells in 50 ml centrifuge tube. (50ul of pluriBeads were added for each 1 ml blood -considering the original volume of blood used)
- The cells were incubated up to 30 minutes with continuous mixing.
- Pluri strainer filter provided by the kit was attached to new 50ml centrifuge tube
- The Pluri strainer was first washed with 1ml wash buffer
- After washing, the sample was poured directly onto the strainer. The beads with the target cells were retained on the strainer while the other blood components passed through.

c. Detachment of target cells:

- A new 50 ml centrifuge tube was used, to which a Leur lock supplied by the kit is connected. Leur lock is a lock which when closed prevents the flow of fluid to the underneath tube.
- The Pluri strainer with the target cells was placed over the 50 ml tube with the Leur lock.
- 1ml wash buffer was added to the walls of the strainer.
- 1ml detachment buffer was added
- Then, the strainer was incubated for 10 min with gentle swirling every 2 minutes.
- After incubation, 1ml wash buffer was added again.

- Cells were separated from the beads by pipetting the sample up and down 10 times using a 1 ml pipette
- The leur –lock was opened allowing the detached cells to pass through into the centrifuge tube.
- The strainer was washed 10 times with 1ml wash buffer to insure total collection of detached monocytes
- The suspension was poured into a 15ml tube and centrifuged at 300g for 10 minutes with no brake to separate the monocytes from the detachment buffer and wash media
- The supernatant was discarded and the monocyte rich pellet was then reconstituted in the previously prepared culture media.

C. Preparation of reagents:

1. E. coli LPS :

Ultrapure lipopolysaccharide from E.coli 111:B4 strain was purchased from invivogen ((3950 sorrento Valley Blvd. suite 100, San diego CA-92121-USA). LPS suspension was prepared by reconstituting the LPS powder in 1ml of endotoxin-free water provided by the supplier resulting in an LPS concentration of 5mg/ml. The LPS suspension was then diluted and a concentration of 0.1ug/ml was used.

2. Rhodobacter sphaeroides LPS:

Ultrapure LPS from *Rhodobacter sphaeroides* (LPS-RS) is a TLR-4 antagonist. LPS-RS was purchased from Invivogen (3950 sorrento Valley Blvd. suite 100, San diego CA-92121-USA). LPS-RS suspension was prepared by reconstituting 1 ml of the

lyophilized powder in 1.5ml endotoxin- free water. The concentration of LPS-RS suspension obtained was1mg/ml. The reconstituted suspension was later diluted where a concentration of 1ug/ml was used.

3. Adenosine:

Adenosine 04036 was purchased from sigma (3050 Spruce Street, Saint Louis, MO 63103, USA) in the form of a white powder. Based on manufacturer's directives, 7g of adenosine were dissolved in 100ml sterile water yielding an adenosine solution of 0.07mg/ml. The concentration of adenosine used was adjusted to 100M.

4. Caffeine:

Caffeine was purchased from sigma (3050 Spruce Street, Saint Louis, MO 63103, USA) in the form of a white powder. Eighty mg of caffeine was constituted in 5ml sterile water yielding a caffeine solution of 16mg/ml concentration. The caffeine concentration used was 100M.

5. Culture medium:

RPMI 1640 medium containing 1% pen-strep, 1% L-Glutamine, and 10% heat-inactivated FBS was used.

D. Monocyte culture:

- The Separated monocytes were transferred to the prepared culture media; and were counted using a Neubauer chamber.

- The cells were then cultured using a 48 well plate; where 0.5 ml of culture media containing 2x 10^5cells/ ml were added to each well
- LPS, LPS-RS, Caffeine, and Adenosine were added to the wells according the pattern represented in Table 3. Each sample was run in duplicate.
- Wells were incubated for 24hrs with corresponding stimulators or inhibitors.
- After incubation, the content of the each well was collected, centrifuged at 3oog for 10 min and supernatant was obtained.

E. Determination of Tumor Necrosis Factor alpha (TNF*α*) levels in cell-culture supernatant:

Abcam TNF alpha Human ELISA kit (Ab46087) (abcam company) was used. Standards, samples, control and blank were all run in duplicates. The kit was used according to the manufacturer's instructions that are briefly described below

1. Reagents:

- TNF alpha standard (800pg/ml)
- Biotinylated anti-TNF alpha antibody
- Biotinylated antibody diluent
- Streptavidin-HRP
- HRP diluent
- Chromogen TMB substrate solution
- Stop reagent
- 10x standard diluent buffer
- 200x Wash buffer

- Control vial
- TNF alpha microplate (12 x 8 well strips)

2. Protocol:

- Wash buffer and standard diluent buffer were diluted using distilled water.
- Control solution was prepared by reconstituting control vial in standard diluent buffer
- Serial dilutions of standard were prepared (standards 1-6) starting with a 1:2 dilution using standard diluent buffer,
- Blank was considered standard zero (standard diluent buffer alone)
- 100 ul of standards, samples, control, and blank were transferred into each well of the ELISA microplate accordingly.
- 50ul of 1x Biotinylated anti-TNF alpha was added to all wells.
- The ELISA microplate was covered and incubated for 3 hours at room temperature.
- After incubation, the microplate was washed three times using wash buffer.
- 100ul of streptavidin- HRP solution was added to each well.
- The ELISA microplate was covered and incubated for 30 min at room temperature.
- The wells were again washed with buffer to remove excess unbound material.

- 100ul of chromogen TMB substrate solution was added to all wells while avoiding direct exposure to light.
- The plate was incubated in the dark for 12-15 minutes at room temperature.
- Color development was blocked by adding a stop solution
- Measurement of the absorbance was performed at a wavelength of 450nm using the BIO-Tek/ELx800 micro-plate reader.

F. Determination of the Interleukin 12 (IL-12) levels in cell culture supernatant:

Abcam IL-12 human ELISA kit (purchased from abcam) was used. All standards, sample, control, and blanks were assayed as duplicates. The protocol used in the ELISA technique for quantification of the IL12 cytokine levels in cell-culture supernatant was similar to that used to quantify TNF alpha levels but with reagents specific for IL12 provided by the kit.

G. Statistical analysis:

The unpaired student T-test was implemented to assess the sample variations using the Graphpad online software. Groups were compared to the LPS-stimulated monocytes and control unstimulated monocytes. Results were considered to be statically significant when P value was ≤ 0.05.

CHAPTER IV
RESULTS

A. Tumor Necrosis Factor (TNF-α) :

The results obtained are presented in Figure 3.

In control non-stimulated monocytes, the TNFα levels was 129.34pg/ml. The addition of adenosine or caffeine resulted in a significant decrease in TNFα levels reaching 34 pg/ml and 4.34pg/ml respectively as compared to non-stimulated monocytes alone(P values= 0.0021, and 0.0018 respectively). The incubation of adenosine and caffeine together with non-stimulated monocytes resulted in a pronounced significant decrease in TNFα to 2.15pg/ml as compared to non-stimulated monocytes alone.(P value= 0.0017)

LPS stimulated monocytes caused a significant increase in TNFα production (391.84pg/ml) compared to the control non-stimulated monocytes (129.34pg/ml).

The addition of adenosine to the LPS-stimulated monocytes significantly decreased TNFα production by more than six folds as compared to the LPS stimulated monocytes alone; it decreased from 391pg/ml to 59.9pg/ml (P=0.0024). Additionally, When LPS stimulated monocytes were incubated with caffeine, TNFα production was significantly reduced to 41.21pg/ml. (P=0.0022)

When caffeine and adenosine were both added to the LPS- stimulated monocytes, TNFα production was barely detectable; levels were 1.21pg/ml as compared to 391.84pg/ml in LPS- stimulated monocytes alone. (P=0.0017).

When TLR-4 receptors expressed by monocytes were blocked with LPS-RS, TNFα production was significantly decreased from 129.34pg/ml in non-stimulated cells to 53.71pg/ml in LPS-RS blocked monocytes. The addition of adenosine to LPS-RS blocked monocytes caused a further significant decrease in TNFα levels from 53.71pg/ml to 20.28pg/ml.(P= 0.0019). Caffeine added to LPS-RS blocked monocytes resulted in a significant reduction in TNFα levels from 53.71pg/ml in LPS-RS blocked monocytes to 0.075pg/ml.(P=0.0017). The incubation of both caffeine and adenosine with LPS-RS blocked monocytes also decreased TNFα levels significantly by more than ten folds (4.34pg/ml) compared to LPS-RS blocked monocytes (P=0.0018).

It is worth noting that, in all of the above scenarios, caffeine was decreasing TNF-α levels more than adenosine.

B. Interleukin 12 (IL-12) :

IL-12 levels obtained are shown in Figure 4.

Non-stimulated monocytes produced 3.01pg/ml of IL-12. The addition of adenosine, caffeine, or both molecules to non-stimulated monocytes did not result in significant changes in the levels of IL-12 produced; the levels produced were 3.7pg/ml, 2.7pg/ml, and 2.87pg/ml respectively.(P>0.05).

Stimulation of monocytes by LPS significantly increased the levels of IL-12 produced by almost 2 folds from 3.01pg/ml in control non-stimulated monocytes to 5.19pg/ml in LPS-stimulated monocytes. The addition of adenosine or caffeine to the LPS-stimulated monocytes significantly decreased IL-12 levels from 5.19pg/ml to 3.82pg/ml and 3.7pg/ml respectively (P=0.0225, and 0.0373). When both adenosine

and caffeine were added to the LPS stimulated monocyte culture, IL-12 levels were further significantly decreased to 3pg/ml. (P=0.0097)

The blockage of TLR-4 with LPS-RS decreased the levels of IL12 produced from 3.01pg/ml in non-stimulated monocytes to 2.5pg/ml in LPS-RS blocked monocytes. However, the addition of adenosine, caffeine, or both reagents to LPS-RS blocked monocytes did not result in changes in the IL-12 levels as compared to the pre-produced level of IL-12 produced by LPS-RS blocked monocytes alone. IL-12 levels were 3.12pg/ml, 3.127pg/ml, and 3.03pg/ml respectively.(P>0.05).

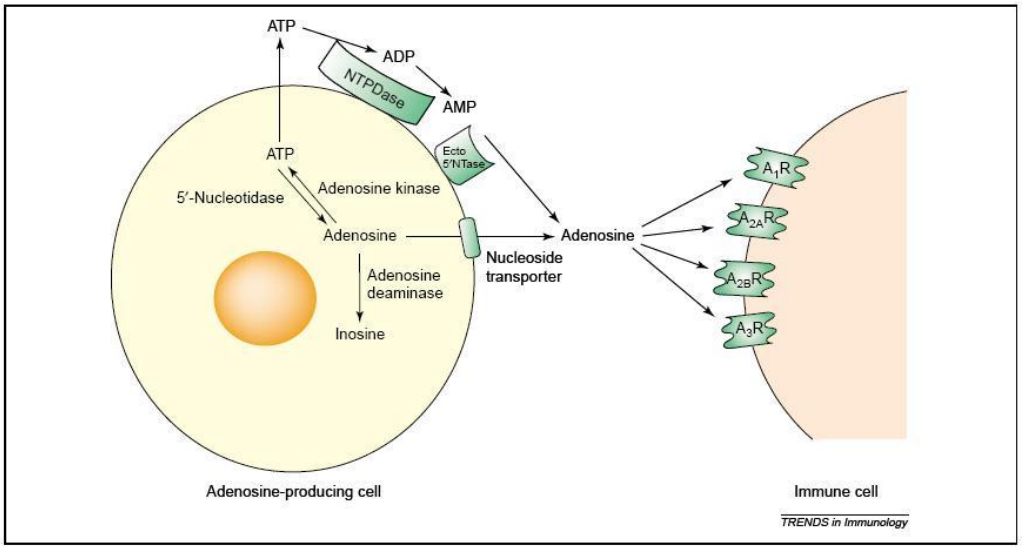

Figure 1. The synthesis and metabolism of adenosine[1]. Adenosine is produced intracellularly from ATP by 5'Nucleotidase enzyme and released via nucleoside transporter. Adenosine synthesis is limited by 2 enzymes; Adenosine deaminase which degrades adenosine into inosine and adenosine kinase which converts it into ATP. Extracellularly adenosine is produced from ATP via NTPDase and Ecto5'NTase enzymes. Adenosine produced then bind to its receptors (A_1 , A_{2A}, A_{2B}, and A_3) on surface of immune cell such as monocytes.

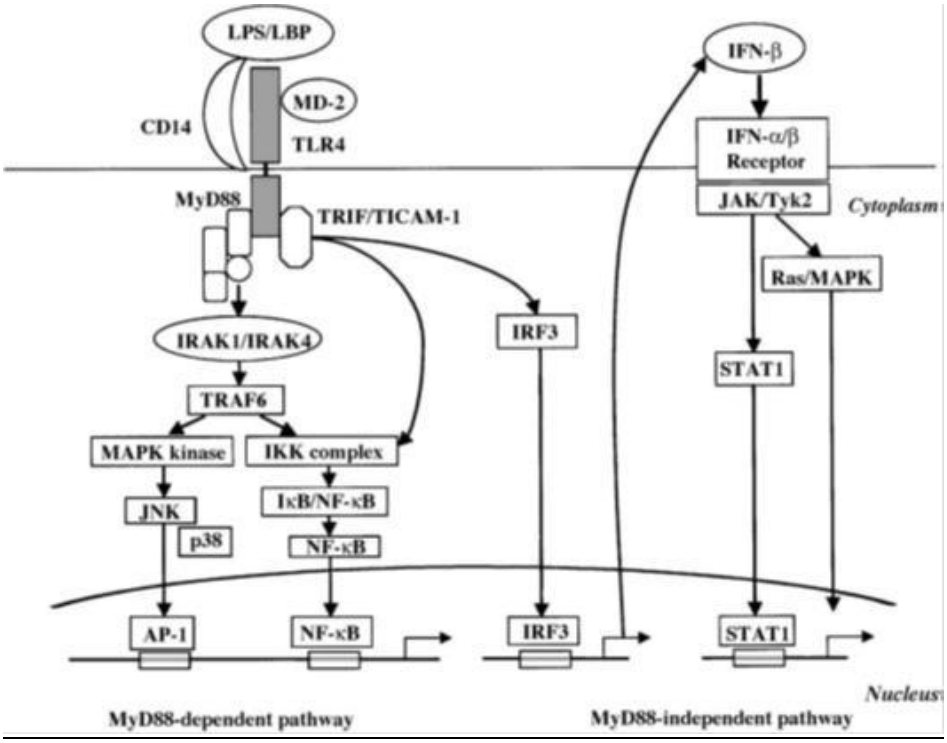

Figure 2. The downstream signaling pathways initiated by the activated TLR-4[24]. Binding of LPS to TLR-4 initiates MyD88 dependent pathway which involves the recruitment of IRAK1/IRAK4 kinases and TRAF6 leading to the activation of MAPK and IKK complex. Activated MAPK results in activation of transcription factor AP-1 while activated IKK leads to NF-kB activation. NF-kB transcribes genes responsible for pro-I nflammatory cytokine production. MyD88 independent pathway is also activated. It involves early activation of IRF3 leading to production of IFNα and IFNβ and Late phase activation of NF-kB leading to cytokine production. The released IFNα/β bind to their receptors and initiate Jak-STAT signaling pathway. (LBP: LPS Binding Protein. IRAK: IL-1 Receptor-Associated Kinase.TRAF6: TNF-Receptor-Activated Factor 6. MAPK: Mitogen Activated Protein Kinase. IKK: Inhibitor of kappa-B (IkB) Kinase. NF-kB: Nuclear Factor kappa B)

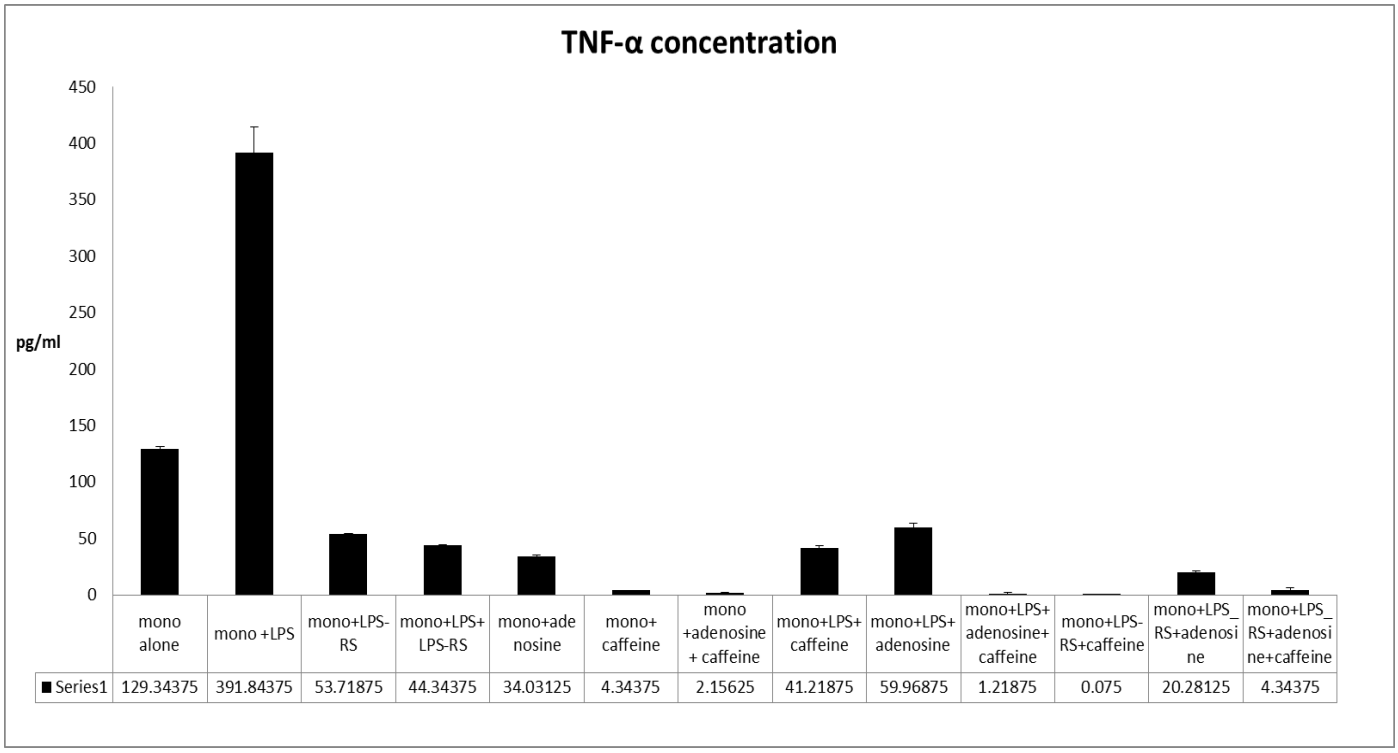

Figure 3. Supernatant TNF-α levels determined by ELISA. Monocytes were incubated for 24 hours alone, with the presence of LPS, LPS-RS, caffeine, and/or adenosine. After incubation, supernatant was used to assess the TNF-α level in each well.

Legend to **Figure 3**

Mono= Monocytes

LPS-RS= TLR-4 antagonist

LPS= TLR-4 agonist

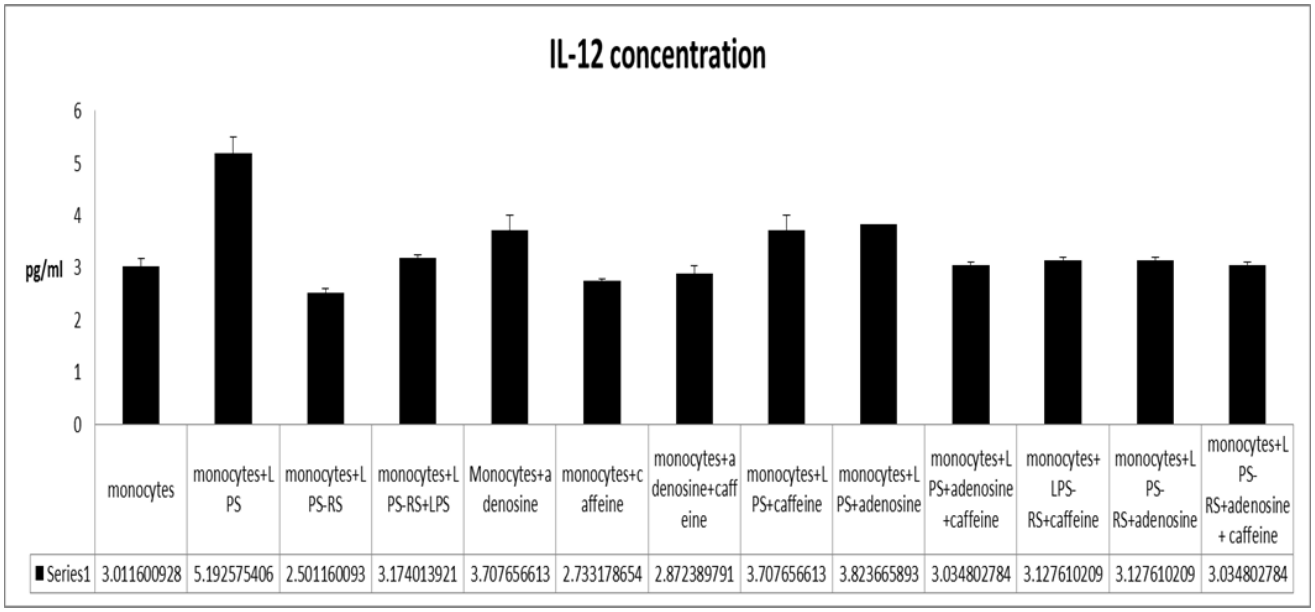

Figure 4. Supernatant IL-12 levels determined by ELISA. Monocytes were incubated for 24 hours alone, with the presence of LPS, LPS-RS, caffeine, and/or adenosine. After incubation, supernatant was used to assess the IL12 levels in each well.

Legend of **Figure 4**

Mono= Monocytes

LPS-RS= TLR-4 antagonist

LPS= TLR-4 agonist

Receptor	A_1	A_{2A}	A_{2B}	A_3
G-protein coupling	G_i and G_o	G_s	G_s and G_q	G_i and G_q
Main Effect on immune cells*	Promotes adhesion, phagocytosis, and chemotaxis	Decrease adhesion phagocytosis, and Cytokine release**	Decrease MHCII. Increase IL-8. Mast cell degranulation	Decrease IL-12 and TNFα levels. Mast cell degranulation
Intracellular effects upon receptor activation	↓cAMP,↓Ca^{2+} ↑ K^+,↑ MAPK	↑ cAMP, ↑ MAPK	↑cAMP, ↑MAPK	↓cAMP ↑ Ca^{2+},↑ MAPK
Tissues with high receptor expression	Brain, eye, spinal cord, adrenal gland	Brain, heart, blood vessels platelets	Colon, lungs Cecum, ovary	Mast cells, thyroid gland, Testes

Table 1. Adenosine receptors and their characteristics. *Immune cells: Innate immune cells (monocytes, macrophages, neutrophils, and dendritic cells) and lymphocytes. All Adenosine receptors are expressed on these immune cells. **Cytokine: decrease pro-inflammatory cytokines such as TNFα, IL-12, IL-6.

Abbreviations: cAMP: cyclic Adenosine Mono Phosphate, MAPK: Mitogen-Activated Protein Kinase.

Toll Like Receptor (TLR)	Ligand
TLR-1	Lipo-peptides and similar lipid molecules
TLR-2	Lipo-peptides and similar lipid molecules
TLR-3	DsRNA
TLR-4	LPS
TLR-5	Flagellin
TLR-6	Lipo-peptides and similar lipid molecules
TLR-7	ssRNA
TLR-8	ssRNA
TLR-9	Non-methylated DNA (CpG motif)
TLR-10	No ligand identified
TLR-11	Profillin
TLR-12	Profillin
TLR-13	23S rRNA

Table 2. The mammalian TLR and their ligands.

Abbreviations: DsRNA: double stranded RNA present in some viruses. LPS: Lipopolysaccharide. ssRNA: single-stranded RNA.

well 1: Monocytes alone	**well 2:** Monocytes + LPS (TLR-4 agonist)
Well 3: Monocytes + LPS-RS (TLR4 blocker)	**Well 4:** Monocytes +LPS +LPS-RS
Well 5: Monocytes + adenosine	**Group 6:** Monocytes + caffeine
Well 7: Monocytes + adenosine + caffeine	**Well 8:** Monocytes + LPS + caffeine
Well 9: Monocytes + LPS + adenosine	**Well 10:** Monocytes + LPS + adenosine + caffeine
Well 11: Monocytes + LPS-RS + caffeine	**Well 12:** Monocytes + LPS-RS + adenosine
Well 13: Monocytes +LPS-RS + adenosine +caffeine	

Table 3. The constituents of the different wells in the 48-well plates. Monocytes were incubated at a concentration of 2x 10^5 cells/ml. All wells were incubated as duplicates.

CHAPTER V
DISCUSSION

In the presence of Gram negative bacteria, TLR-4 recognizes LPS and initiates an inflammatory response in an attempt to eliminate these bacteria. While inflammation is usually beneficial, excess inflammation can result in tissue injury, hypoxia, or sepsis. To control the inflammatory response, adenosine is released at sites of tissue injury and inflammation where it acts as an endogenous anti-inflammatory agent. It has been reported that adenosine decreases TNF-α and IL-12 production in LPS-stimulated monocytes and thus suppresses inflammation[4,8,9,11].

Adenosine exerts its anti-inflammatory role by binding to receptors present on monocytes[5]. Four adenosine receptors have been discovered so far. These are the A_1, A_{2A}, A_{2B}, and A_3 receptors[5]. In this study we wanted to assess whether TLR-4, a receptor for the inflammatory agent LPS, would be affected by adenosine signaling or if signaling of adenosine is only via adenosine receptors. Our results showed that the addition of adenosine to LPS-stimulated cells resulted in a significant decrease in the production of both IL-12 and TNFα as compared to LPS-stimulated monocytes alone. Such results are consistent with previous studies indicating that adenosine is an anti-inflammatory agent that decreases the production of pro-inflammatory cytokines by LPS-stimulated monocytes[4,8,9]. On the other hand, the addition of adenosine to LPS-RS blocked monocytes as well as to non-stimulated monocytes significantly decreased the levels of TNFα but showed no significant effect on the levels of IL-12 released by these cells. Such results suggest that TLR-4 is probably not implicated in the signaling of

adenosine since the blockage of TLR-4 by LPS-RS or the lack of its stimulation in non-stimulated monocytes did not prevent adenosine from exerting its anti-inflammatory role and decreasing TNFα levels.

Caffeine, the popular psychostimulant, is a non-specific adenosine blocker that antagonizes all adenosine receptors. Previous studies investigating the role of caffeine in immunity were controversial; while several studies agreed that caffeine decreases inflammation[12,13], other studies presented evidence indicating that caffeine increases tissue injury, inflammation, or even plays no significant role in immunity at all[15,16,17]. Therefore, we first aimed to assess the exact role of caffeine in promoting or decreasing inflammation. Moreover, knowing that caffeine has no identified receptors yet, we investigated whether TLR-4 plays any role in caffeine signaling. Our results showed that when caffeine was added to LPS stimulated monocytes, it appeared to possess an anti-inflammatory role by significantly decreasing the levels of both TNFα and IL-12 produced by these cells. These findings are in agreement with previous studies suggesting that caffeine decreases cytokines production in LPS-stimulated monocytes and might play an anti-inflammatory role[13,14] . On the other hand, the addition of caffeine to LPS-RS blocked monocytes and to non-stimulated monocytes resulted in a significant decrease in TNFα levels but had little effect on the levels of IL-12 released by these cells. In addition, when comparing the effect of caffeine to that of adenosine in both LPS-stimulated monocytes and LPS-RS blocked monocytes it was noted that caffeine decreased TNFα levels much more than adenosine. These results suggest that caffeine plays an immuno-modulatory role via decreasing the production of cytokines by LPS-stimulated monocytes. Moreover, TLR-4 is not involved in caffeine signaling

since the addition of caffeine to non-stimulated monocytes and LPS-RS blocked monocytes still resulted in the decrease in cytokine production, mainly TNFα.

Additionally, knowing that Adenosine is an anti-inflammatory molecule and that the major effect of caffeine is exerted by blocking adenosine receptors[12], we thought of trying a combination of adenosine and caffeine. We expected that, when incubated together, caffeine would antagonize the effects of adenosine and result in an increase in the levels of IL-12 and TNFα produced by LPS-stimulated monocytes. However, our results showed that upon incubation of both reagents, caffeine did not inhibit the anti-inflammatory role of adenosine. On the contrary, the addition of both adenosine and caffeine to LPS-stimulated monocytes resulted in a further reduction in levels of TNFα and IL-12 as compared to the effect of either reagent alone. Regarding LPS-RS and non-stimulated monocytes, the addition of both adenosine and caffeine resulted in a marked decrease in TNFα levels but did not affect the levels of IL-12.

It had been noted that in LPS-RS blocked monocytes and non-stimulated monocytes, the addition of adenosine, caffeine, or both reagents decreased TNFα levels significantly but did not result in significant changes in IL-12 levels. This can be due to two reasons: first the levels of TNFα produced by monocytes are usually several folds higher than the levels of IL-12 released by these cells. Therefore, upon blockage of TLR-4 a decrease in TNFα level is expected to be more pronounced than a decrease in IL-12 levels. The second may be that the production of IL-12 by monocytes is regulated by different mechanisms than TNFα. This was suggested by Aste-Amezaga et al. who demonstrated that the expression of IL-12 genes, at the transcription levels, is controlled by mechanisms different than those regulating TNFα production[52].

Our results suggest that caffeine plays an anti-inflammatory role by decreasing the levels of cytokine production in stimulated monocytes. However, this anti-inflammatory role does not appear to involve the blockage of anti-inflammatory adenosine receptors. It probably involves a pathway not related to adenosine or maybe complementing that of adenosine. This requires further research to determine the exact mechanism of cytokine reduction caused by caffeine.

One of the previous studies suggested that the reduction of cytokines by caffeine can be due to increase in cAMP levels and activation of PKA [13]. However, other studies indicated that the elevation in cAMP is due to PDE inhibition which is unlikely to occur at normal physiological concentrations of caffeine[12]. Another study by Verani et al. involved administration of caffeine at a dose of 400–600 mg/day, equivalent to almost 10-11 cups of coffee daily, for 1–2 weeks. This study reported that caffeine resulted in up-regulation of A_{2A} receptors present on neutrophils[51]. It had been previously shown that the anti-inflammatory role of adenosine is mainly exerted through binding to A_{2A} receptors[9]. Thus, up-regulation of A_{2A} receptors by caffeine can possibly explain the decrease in cytokine production observed in the presence of caffeine. However, despite being anti-inflammatory, adenosine can sometimes promote inflammation by binding to its other receptors; A_1, and A_{2B} receptors[5]. Additionally, activation of A_3 receptors was also linked to an increase in inflammation by causing mast cell degranulation in asthmatics[5]. Being a non-selective adenosine blocker, another possible mechanism by which caffeine modulates the inflammation can be via blocking A_1, A_{2B}, or A_3 adenosine receptors.

Based on the above, further studies are needed to confirm which mechanism of action is the one responsible for the observed immune-modulatory role of caffeine.

Future studies can involve the investigation of cAMP/PKA pathway using caffeine concentrations relevant to normal consumption as well as testing the role of caffeine on other adenosine receptors such as A_1, A_{2B}, and A_3.

In conclusion, caffeine, the most popular psychostimulant and non-specific adenosine blocker, caused a decrease in cytokine production in LPS-stimulated monocytes. This identified role of caffeine can be translated to cases of severe inflammation and sepsis where administration of coffee and other caffeine-rich products can be beneficial. The exact mechanism by which caffeine decreases cytokines production is still unclear. TLR-4, the LPS receptor, did not appear to be involved in the signaling pathway of caffeine and adenosine since blocking the TLR-4 receptor did not abolish the effects of adenosine and caffeine on cytokine production mainly TNFα. Future studies should focus on investigating the possible mechanisms by which caffeine immune-modulates the inflammatory response.

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