Novel Mechanisms Underlying the Inflammatory Effects of Leptin and Low Dose Endotoxin

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ABSTRACT

Obesity over the last several has become a major health concern in our country as well as the world. Obesity is also one of the risk factors which lead to several inflammatory complications such as diabetes, artherosclerosis, etc. Two leading factors involved in the causes of inflammatory complications include leptin and low dose endotoxin lipopolysaccharide (LPS). However, the mechanism underlying the involvement of these two mediators is not clearly understood. The purpose of this study is to understand the mechanism underlying inflammatory complications caused by leptin and low dose endotoxin most recently coined metabolic endotoxemia. Interleukin-Receptor Associated Kinase 1 (IRAK-1) is an intracellular signaling component shown to activate NFκB which leads to the induction of proinflammatory mediators. Deletion of IRAK-1 in mice has beneficial effects in alleviating inflammatory complications and human variations in IRAK-1 gene are correlated with higher risks for inflammatory diseases. Therefore, we hypothesized that IRAK-1 is critically involved for the induction of proinflammatory mediators induced by leptin and low dose LPS. IL-6 mRNA levels were measured in THP-1 (human monocytic cells) and wild type and IRAK-deficient bone marrow derived macrophages (BMDM) challenged with different combinations of leptin and LPS. Data shows that leptin alone will not induce inflammatory mediators. However, increased induction of IL-6 was observed in a synergistic manner involving

both LPS and leptin in an IRAK-1 dependent manner causing a robust inflammatory response. With regard to the effect of low dose LPS, we observed that human monocytic cells treated with low concentrations of LPS showed a mild yet sustained induction of proinflammatory cytokines, which is contrast to the robust and transient induction of cytokines by a high dose LPS. To further determine the molecular mechanisms, we measured several key signaling molecules that include IRAK-1, IKKepsilon, and C/EBPdelta. Our study revealed a novel mechanism that appears to be distinct from the traditional NFκB pathway responsible for the effect of low dose LPS.

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All work in this dissertation was performed solely by Tamisha Y. Vaughan and shares contribution with my advisor Dr. Liwu Li.

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LIST OF ABBREVIATIONS

LPS	Lipopolysaccharide
IL-6	Interleukin-6
IRAK-1	Interleukin-1 Receptor Associated Kinase 1
TNF- α	Tumor Necrosis Factor-alpha
BMDM	Bone Marrow Derived Macrophage
IKK	I Kappa-B Kinase
NFκB	Nuclear Factor-KappaB
TLR	Toll-Like Receptor
PAMP	Pathogen Associated Molecular Patterns
IL-1	Interleukin-1 Receptor
TIR	Toll/Interleukin-1 Receptor
MyD88	Myeloid Differentiation Protein 88
TRAF	TNF Receptor Associated Factor
TRIF	TIR-domain-containing adapter-inducing interferon-β
JAK2	Janus Kinase 2
Ob-R	Full length Receptor
LBP	LPS Binding Protein
ELISA	Enzyme Linked Immunosorbent Assay
RT-PCR	Real-Time Polymerase Chain Reaction

Chapter 1: Introduction

The prevalence of obesity is increasing dramatically worldwide and has been linked to the pathogenesis of diabetes as well as several cardiovascular diseases [1, 2]. Recent estimates show that nearly two thirds of the U.S. adult population is now either overweight or obese [3]. Scientists have been studying the mechanism underlying human metabolism and insulin resistance with most attention on the biology of relationships between various human organs and cell systems. Gram-negative bacteria of the gut shed LPS once lysed which may ultimately lead to the above mentioned complications. This phenomenon has pioneered an increasing body of literature that directs the attention to the gut microbiota and its involvement in inflammatory complications starting at the innate immunity level. However, there are several missing links to better understand the mechanism involved. Therefore, this research was an attempt to bridge these scientific gaps.

The innate immune system is primarily responsible for the first line of defense protecting the body from foreign pathogens. It functions by recruiting a variety of cells like natural killer cells, dendritic cells and macrophages whose main responsibility is to engulf the foreign microbes. Macrophages have been identified to play a major role in the initiation and progression of diverse inflammatory diseases like obesity and diabetes [2, 4]. Recent studies have shown a close link between certain metabolic disorders and immune cell activity. Similar to macrophages, fat cells along with several others are capable of secreting diverse inflammatory mediators in response to their environment.

Many inflammatory mediators are elevated in obese individuals [2, 5]. Recent studies have demonstrated macrophage infiltration in white adipose tissue in obesity that contribute to the increased production of proinflammatory mediators such as Interleukin-6 (IL-6), Tumor Necrosis Factor (TNF-α).

LPS, a gram negative bacteria product, is known to be involved in several inflammatory complications by activating transcription factors such as nuclear factor kappa B (NFκB) through TLR4 which leads to the induction of proinflammatory cytokines [6]. TLR4, a key receptor of the innate immune system plays a central role in inflammatory immune responses. TLRs are key receptors in innate immunity and play vital roles in host protection against invading pathogens.

There has been evidence demonstrating that obesity is associated with a chronic low-grade inflammation characterized by abnormal cytokine production and activation of inflammatory signaling pathways in adipose tissues, which in turn increases the risk factor for insulin resistance [7-9]. These adipose tissues possess an endocrine function by synthesizing and secreting at least two main hormones, leptin and adiponectin. Excessive production of adipokines such as leptin in obese tissues may modulate inflammatory processes causing the above-mentioned complications such as diabetes and cardiovascular diseases. Macrophages are activated by a variety of stimuli in the course of an immune response and can exhibit unique and specific functional properties. One of the earliest activating signals comes from chemokines. However, phagocytosis itself is an important activating stimulus. They can be further activated by

cytokines secreted by T helper cells [IFN-gamma], mediators of the inflammatory response, and various microbial products (such as LPS) [10, 11].

TLR-4 once activated can trigger two diverse signaling pathways including MyD88 independent and MyD88 dependant [12, 13]. These two pathways involve different key players and are independently activated at the cellular membrane by LPS. Signaling through the MyD88 dependent pathway, involves important molecules IL-1 receptor associated kinases (IRAK-1) that regulates intracellular inflammatory signaling including macrophage functions in the innate immune response [14]. IRAK-1 has been linked to diseases such as atherosclerosis and other inflammatory infections [15, 16]. MyD88 dependent pathway currently serves as the traditional TLR4 signaling pathway. Not only is TLR4 MyD88 pathway able to recruit molecules which lead to proinflammatory response but it may interact with other parallel signaling pathways causing a more traumatic effect. However, the exact mechanism involving MyD88 independent pathways which is thought to be distinct to similar levels of LPS found in endotoxemia is not clearly understood.

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Chapter 2: Literature Review

Metabolic Endotoxemia and Signaling Crosstalks Involving Toll Like Receptor-4 (TLR4) Lead to Proinflammatory Complications

Abstract:

Toll-like receptors (TLR) play an active role in the recognition of foreign pathogens and function by its involvement in downstream signaling through adaptor molecules, transcription factors, cytokines and chemokines. Since its discovery, the TLR family has been shown distinct involvement in several pathogenic diseases. Metabolic endotoxemia has been most recently studied as an activator involved in obesity related inflammatory complications potentially through toll like receptors. This review focuses on the mechanisms in which toll-like receptors specifically TLR4 are involved in activating select pathways and ultimately inducing inflammatory responses that cause a cascade of complications to the host by altering innate immune responses.

2.1 INFLAMMATION BIOLOGY

2.1.1 Innate Immunity

The innate immune system is a primary defense mechanism against invading microorganisms. This mechanism serves as natural immunity meaning it is fully intact at birth. It takes place before the adaptive immune system has any knowledge of invasion. Cues sent by cells of the innate immune system activate the adaptive immune system to build up antibodies against future encounters. It is primarily important because of its ability to prevent, control and eliminate infection to the host. Studies have shown that inhibiting or eliminating any of several mechanisms of innate immunity increases the susceptibility to infections even when the adaptive immune system is intact [1, 2]. However, there have also been studies done which show inhibition of select proteins of the innate immune system and playing beneficial roles in reducing inflammatory responses [3-6]. Microbial substances that stimulate the innate immune system are pathogen-associated molecular patterns (PAMPs). Different classes such as viruses, gram-negative bacteria, gram positive bacteria etc. express different PAMP's. There are a variety of cell types expressing PAMP's including dendritic cells, macrophages, neutrophils, and endothelial cells [3, 7].

There are numerous receptors linked to the intracellular signal transduction pathways that activate various cellular responses including those that activate inflammatory responses. One major class of receptors that have been highly conserved throughout evolution is the toll-like receptors (TLR) [8-10]. These receptors have been known to interact with other receptors and cause downstream activation of a number of

inflammatory mediators which play a role in the pathogenesis of obesity, diabetes and inflammatory complications. However, the mechanism is not clearly understood and current knowledge is depicted in figure 2.1 and will be fully discussed in this review.

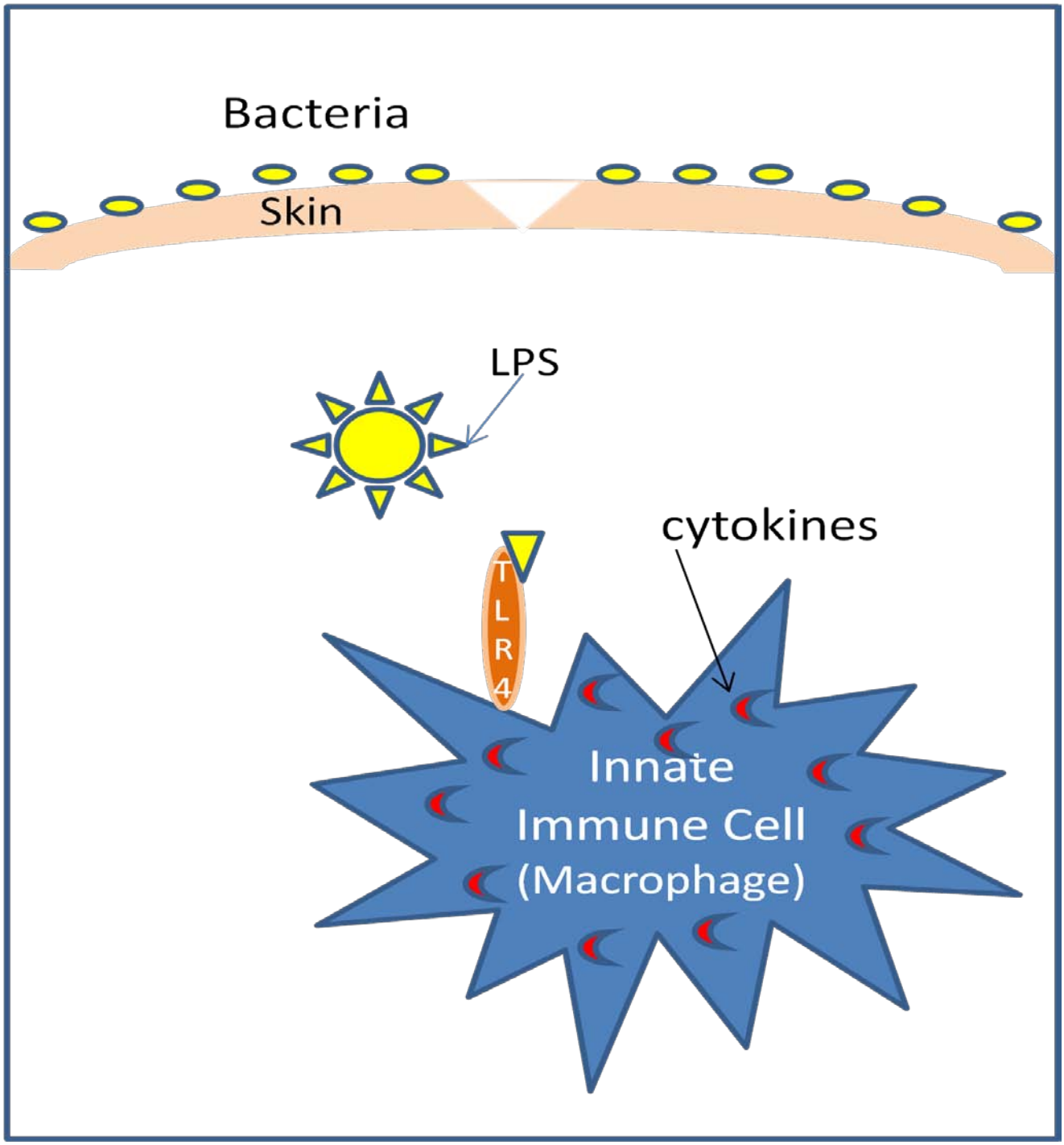

Figure. 2.1 Illustration of innate immune response

2.1.2 Obesity, Diabetes and Inflammatory Complications

Recent estimates show that nearly two thirds of the U.S. adult population is now either overweight or obese [11]. The metabolic effects of obesity have made this disease one of the most common risk factors for several inflammatory complications such as diabetes, hypertension, and artherosclerosis [12]. There has also been evidence demonstrating that obesity is associated with a chronic low-grade inflammation characterized by abnormal cytokine production and activation of inflammatory signaling pathways in adipose tissues [13]. However, there is currently no clear understanding of the mechanism. Studies suggest that the gut microbiota contributes towards the pathophysiological regulation of endotoxemia and sets the tone of inflammation for occurrence of diabetes and/or obesity [14].

Diabetes however, is a chronic metabolic disorder affecting more than 5% of the population [15]. The more prevalent form, type 2 diabetes, accounts for more than 90% of cases. The pathogenesis is very complex and involves progressive development of insulin resistance and a relative deficiency in insulin secretion, which may lead to overt hyperglycemia [15]. Based on these studies and several others, a later section will focus solely on endotoxemia and its contribution to inflammatory complications.

Inflammation plays a key role in several diseases from heart diseases, neurological diseases, respiratory diseases, arthritis as well as diabetes. The process is induced by microbial infection or tissue injury and its main function is to resolve the infection or repair the damage and return to a state of homeostasis [16]. Nevertheless, the term inflammation is often used to describe a series of signs and symptoms after injury which serves as a beneficial characteristic. These inflammatory processes can be initiated through a variety of mechanisms, which include the introduction of pathogens as well as challenges to the system through chemical, thermal, and mechanical stresses [17]. The identified changes are relayed to other cells by the production of secondary mediators such as cytokines, chemokines, complement proteins, and co-stimulatory factors. These signals are relayed, and then regulation of physiological responses occurs in the form of the four trademark inflammatory responses. The four hallmarks associated with microbial infection consist of redness, swelling, heating, and pain [17]. Importantly, inflammatory complications are the key to health disparities causing the nation a medical alarm.

2.1.3 Endotoxemia:

Type 2 diabetes and obesity have been closely associated to a low-grade inflammatory state when feeding a high-fat diet [18]. Very interesting studies have been done seeking a bacterially related factor that is able to trigger the development of high-fat diet-induced obesity, diabetes and inflammation [19, 20]. For the following reasons, LPS endotoxemia has served as the culprit for several health complications as previously mentioned. LPS is the most commonly used candidate as it is an

inflammatory compound of bacterial origin continuously produced within the gut and most likely involved in absorption during high-fat diet feeding. This endotoxin triggers the secretion of proinflammatory cytokines when it binds to the complex of CD14 and TLR4 at the surface of innate immune cells [21]. Increased circulating levels of inflammatory cytokines have also been associated with the pathogenesis of obesity and diabetes. In addition, LPS is continuously produced within the gut by the death of Gram negative bacteria and is physiologically carried into intestinal capillaries through a TLR4 dependent mechanism [22] and is transported from the intestine towards target tissues by a mechanism facilitated by lipoproteins [23-26]. The human gut microbiota has not been fully described, but it's clear that the human gut is home for a complex consortium of around 10^{13} to 10^{14} bacterial cells [27, 28]. An illustration of metabolic endotoxemia is depicted in figure 2.1.

Over the past five years, studies have highlighted some key aspects of the mammalian host-gut microbial relationship [14, 20, 29-34]. Ley, *et al.* demonstrated, in a rodent model, that obesity can be associated with an altered gut microbiota [35]. Interesting data suggest that high-fat feeding is associated with a higher endotoxemia in humans. So far, Backhed, *et al.* found that the mice raised in a germ free environment had about 40% less total body fat than mice with a normal gut microbiota [19]. Erridge, *et al.* has highlighted the putative role of a high-fat meal and development of metabolic endotoxemia. This study is the first to examine the kinetics of baseline endotoxemia concentrations in healthy human subjects [18]. Even in humans, plasma endotoxin levels are classically associated with sepsis and many studies have also reported that in

healthy subjects plasma endotoxin concentrations range from 1 to 200 pg/ml [20, 33, 36, 37]. It was found that a high-fat meal induces a metabolic endotoxemia which fluctuates rapidly in healthy subjects, from a very low concentration at baseline (between 1 to 9 pg/ml) to concentrations that may be sufficient to induce some degree of cellular activation in *in vitro* experiments [18]. Several cell types have been identified to be involved in the inflammatory processes. Among these, macrophage cells can be found in several locations of the body and as a result plays a significant role in the involvement of proinflammatory diseases. Therefore, biology of macrophages will be further discussed as most of our experiments have been done using these particular cells.

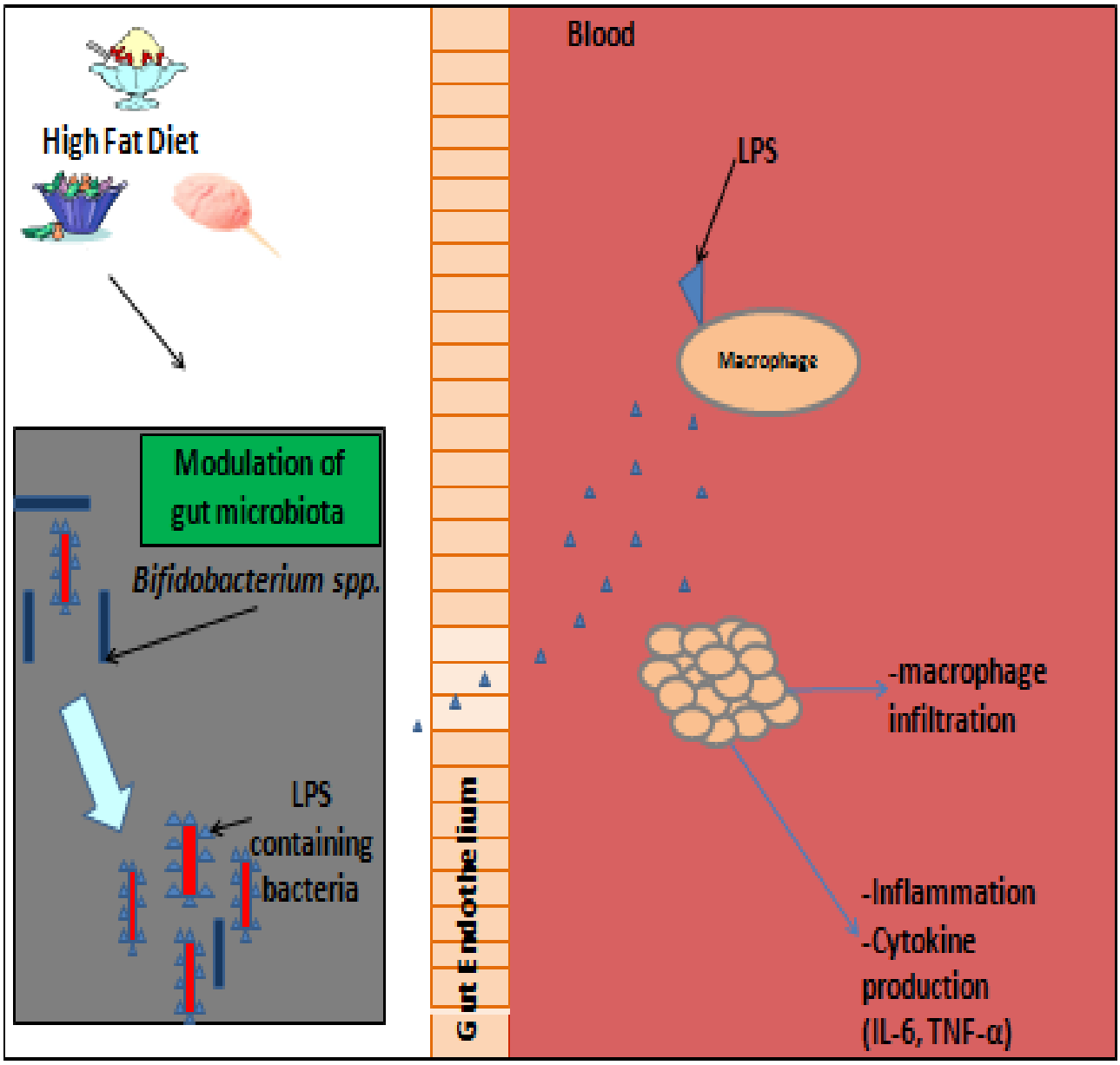

Figure 2.2 Illustration of metabolic endotoxemia

2.2 CELLULAR BASIS OF INFLAMMATION

2.2.1 Macrophage Biology:

Macrophages are key players in the immune response invaders such as infectious microorganisms. They have been defined as a type of white blood that ingests foreign material. The development of macrophages is in response to blood monocytes migrating into the tissues of the body and there they differentiate into several types of macrophages which each have special names. For example, blood monocytes which migrate to the liver are distinguished as Kupffer cells, neural tissue monocytes as microglia and to the bone as osteoclasts. These macrophages then help to destroy bacteria, protozoa, and tumor cells and also release substances that stimulate other cells of the immune cells. Being antigen presenting cells they carry the antigen on their surface and present it to a T cell. Since we are studying the effect of leptin in macrophage, it is important to mention in brief detail where they both have derived, adipose tissues.

The role of adipose tissue is energy storage, but there is increasing evidence that adipocytes and adipokines are involved in metabolic and inflammatory processes. White adipose tissue is now being recognized as an active participant in regulating physiologic and pathologic processes including immunity and inflammation. There has also been evidence demonstrating that obesity is associated with a chronic low-grade inflammation characterized by abnormal cytokine production and activation of inflammatory signaling pathways in adipose tissues [13]. Adipose tissue is a dynamic

endocrine organ that secretes several inflammatory and immune mediators known as adipokines.

2.2.2 Macrophage Cytokine Production

Macrophages are an important part of the secretory function of adipose tissue and the main source of inflammatory cytokines, such as TNF-α and IL-6. Cytokines are regulators of host responses to infection, immune responses, inflammation, and trauma. Some cytokines act to make disease worse characterizing them as proinflammatory, whereas others serve to reduce inflammation and promote healing making them anti-inflammatory. IL-6 and TNF are proinflammatory cytokines, and when administered to humans, produce fever, inflammation, tissue destruction, and, in some cases, shock and death. Attention also has focused on blocking cytokines, which are harmful to the host, particularly during overwhelming infection. The first indication for increased cytokine release in obesity was provided by the identification of increased expression of TNF-α, a proinflammatory cytokine, in the adipose tissue of obese mice in the early 1990s [38]. IL-6 has also been a very widely studied cytokine found to be highly elevated in humans with obesity and inflammatory complications. It plays important roles in host defense, acute phase reactions and immune responses. As both TNF-α and IL-6 are heavily studied cytokines in the innate immune response we will focus our attention on these studies as phenotypic readouts.

2.3 MOLECULAR SIGNALING PROCESSES REGULATING MACROPHAGE ACTIVATION

2.3.1 Toll-Like Receptors and Signaling

Toll-like receptors are type I transmembrane proteins of the Interleukin-1 receptor (IL-1R) family possessing an N-terminal leucine-rich repeat domain for ligand binding, a single transmembrane domain, and a C-terminal intracellular domain. Because of the high similarity of cytoplasmic portion of toll and IL-1 receptor, it was later named the Toll/IL-1 receptor (TIR) [9]. This family of receptors was originally identified as a *Drosophila* gene involved in the establishment of the dorsal-ventral axis during embryogenesis of the fly, it was later discovered that the Toll protein also mediated antimicrobial responses. They are expressed at the cell membrane and in subcellular compartments such as the endosome of different cell types such as *Caenorhabditis elegan*, *Drosopholia*, and mammals. A mammalian homologue of the Drosophila Toll receptor which was initially termed human Toll and is now known as Toll-like receptor 4 (TLR4), was identified. An active form of this receptor has since been known to activate transcription factor nuclear factor-kappa B (NF-κB), leading the expression of several proinflammatory genes. Since this discovery, a total of 13 mammalian TLR's, 10 in humans and 13 in mice have been identified. TLR's 1-9 have been conserved among humans and mice, while TLR10 is present only in humans and TLR11 is functional only in mice [9]. This family of transmembrane receptors gives rise to several inflammatory complications and diseases from heart disease, neurological disease, respiratory

disease, arthritis as well as diabetes. Interestingly, using gram negative bacteria and its endotoxin LPS have been the best studied model of innate immunity [39-41].

The activation of TLR signaling was originated from the cytoplasmic domain first revealed in the C3H/HeJ mouse strain possessing a point mutation which resulted in an amino acid change of the cytoplasmic proline residue to a histidine at the 712 position [42, 43]. Proline residue at position 712 is conserved among all TLRs with the exception of TLR3. However, this single amino acid change caused a dominant negative effect of TLR signaling [42]. The TLR pathways also have their own cascades for exhibiting their specific responses, which are characterized by many TIR domain-containing adaptors. For example, TLR2 specifically recognizes cell wall components such as gram-positive bacteria such as peptidoglycan, lipoteichoic acid and lipoprotein. In addition, TLR2 may also recognize LPS from *Leptospira interrogan and Porphyromonas gingivalis [41]*. However, other TLR family members are activated by a several different ligands including double-stranded RNA (TLR3), flagellan (TLR5), single-stranded RNA (TLR7&TLR8) as well as unmethylated CpG Oligodeoxynucleotide DNA by TLR9 [8, 9, 44]. Not only are these receptors ligand specific but they are cell specific and location specific by being expressed on the cell surface or intracellularly [45, 46]. Interestingly, though they are very distinct in recognizing ligands, some pathogens are known to trigger more than one TLR. In contrast, all TLRs utilize key adaptor proteins in its signaling transduction pathways. One molecule most of these receptors share in common is adaptor protein myeloid differentiation protein 88 (MyD88). In addition to the death domain located in its n-terminus, MyD88 possess a

TIR domain in its c-terminus which is able to associate with TLRs. To date TLR4 is the best characterized toll like receptor and is mainly activated by a component of gram-negative bacteria, LPS. TLR4, receptor responsible for the recognition of LPS, has been proposed to play integral roles in the relationship between obesity and inflammation.

2.3.2 Involvement of LPS in Inflammatory Complications

The activation of TLRs have been heavily involved in several inflammatory diseases such as chronic inflammatory bowel disease such as Crohn's disease as well as different types of cancers by activation of NFκB [47, 48]. All these complications can be regulated by alterations of TLR signaling [49-51]. In fact, disruptions to the TLR4 signaling pathway have been shown to decrease the susceptibility to infection. Understanding the involvement of TLR4 has become a hot topic as many think this may lead to several therapeutic interventions for so many medical conditions [51, 52]. As mentioned before, inflammation plays a key role in several diseases from heart diseases, neurological diseases, respiratory diseases, arthritis and diabetes which serve as a basis to better understand how modulation of LPS exposure may improve such complications.

2.4 CHRONIC INFLAMMATION ASSOCIATED WITH OBESITY

2.4.1 History of LPS

LPS has been closely associated with several inflammatory complications including endotoxemia, atherosclerosis, septic shock and Alzheimer's disease along with several other health complications [53-55]. The structure of LPS consists of polysaccharides, fatty acids and an O-antigen. However, the O-antigen may be smooth or rough which would determine the toxicity of LPS. Recognition of LPS by its binding protein (LBP) takes place at the cellular membrane with the help of CD14. CD14 then binds to MD-2 and forms a complex with the TLR4 receptor. Triggering of TLR4 results in the activation of two distinct intracellular pathways, one that relies on the common TLR adaptor MyD88 and one that is mediated by Toll/IL-1R domain-containing adaptor-inducing IFN-beta (TRIF) [56, 57]. TLR4 is the only toll like receptor family member other than TLR3 capable of signaling independent of MyD88. Studies have shown that the deletion of MyD88 does not completely abolish the response of TLR4 to LPS suggesting a signaling cascade independent of MyD88 [58]. Both pathways however distinctly activate NFκB which plays a major role in the activation and induction of inflammatory consequences and cytokines [59].

Traditional signaling through the MyD88 dependent pathway involves an important molecule called Interleukin-1 associated Kinase 1 (IRAK-1) that regulates intracellular inflammatory signaling including macrophage functions in the innate immune response [60]. IRAK-1 has been linked to diseases such as atherosclerosis and other inflammatory infections [6, 61-64]. The phosphorylation of IRAK-1 causes an interaction

with TNF receptor associated factor 6 (TRAF6) ultimately leading to the degradation of IκBα [65]. The potential function of IRAK-1 is not clearly understood. Studies in the past have linked IRAK-1 with activation of NFkB [66], STAT3 [60] and IRF7 [60]. It is therefore possible that IRAK-1 may associate with multiple signaling networks and perform multiple functions. Upon degradation of IκBα, two inflammatory transcription factors involved in inflammation NFκB and IRF-3, are then translocated into the nucleus [67]. The activation of this traditional pathway leads to the induction of proinflammatory cytokines such as IL-6 and TNF-α and also involves a negative feedback loop which causes IKBα, inhibitors such as suppressors of cytokines signaling (SOCS) as well as NFkB to reappear in the cytoplasm and interrupt the signaling pathway.

In contrast, the MyD88 independent pathway is initiated in a different manner. A very interesting study performed by Yamamoto showed that in TRAM-deficient macrophages LPS-induced IRAK-1 activation seemed to be similar to that of wild-type cells [68]. Their results show that TRAM is essential specifically for the TLR4-mediated MyD88-independent signaling pathway. TRAM interacts with TRIF to form a complex which leads to the recruitment of two kinases, IKKı also known as IKKε and TBK1. The activation of IKKε and TBK1 then are able to activate either IκBα leading to delayed phosphorylation of NFκB or IRF-3 which also serves a transcription factor leading to inflammatory complications. Still several questions arise regarding these two distinct pathways and the roles of these key molecules mentioned above. Nevertheless, it is thought that the MyD88 independent pathway is also capable of inducing inflammation

while the dependant pathway drives the inflammatory response to capacity as a result of NFκB activation [69].

2.4.2 Cross-Talk Between Lipopolysaccharide and Leptin

Adipose tissues possess an endocrine function by synthesizing and secreting at least two main hormones, leptin and adiponectin. Excessive production of adipokines such as leptin in obese tissues may modulate inflammatory processes causing complications such as diabetes [70]. Previous studies have revealed that leptin may activate macrophages, one of the key cell types of the innate immune system [71]. This may be responsible for propagating the inflammatory responses; however, the molecular mechanism remains unclear. Over the years studies have been done which lead many to believe leptin plays a dual role as an anti inflammatory as well as pro inflammatory mediator.

Leptin (product of the *ob* gene) is a 16 kDa adipocyte-derived protein, and mouse models that lack functional forms of leptin (e.g. *ob*/*ob* mice) are characterized by obesity due to hyperphagia [72]. Leptin has an important role in controlling appetite, but a great deal of interest has peaked in understanding the molecular basis of its actions. The cell-surface leptin receptor (ObR) shows a high degree of sequence similarity to members of the cytokine receptor superfamily and has signaling capabilities similar to IL-6 type cytokine receptors [73]. These receptors do not possess kinase activity themselves, but are commonly associated with other signaling molecules, such as the tyrosine kinase Janus kinase-2 (JAK-2), which are activated following binding of ligand

to the receptor. The ObR gene is widely expressed, and a number of splice variants exist. Most cell types express truncated forms that have cytoplasmic domains of only 30±40 amino acids, which are thought to be too short to mediate downstream signaling [74-76]. The full-length leptin receptor ObR has a 302-amino-acid intracellular domain which confers full signaling capabilities to the receptor. ObR was thought to be predominately expressed in the hypothalamus [75]; however, high levels of ObR have been detected in macrophages and others have demonstrated that Ob activates cognate signaling pathways in these cells [77]. This, combined with the fact that leptin has profound effects on macrophage phagocytic function, cytokine production [78, 79] and lipid metabolism [77], indicates that macrophages are a direct target for leptin. As cell lines from the hypothalamic ObR expressing neurons are not available, macrophage cell lines represent the best cell model for studying leptin receptor signaling in a natural cellular context.

Many ties have been made between leptin and inflammation solely based on increased circulating levels of leptin consistent with elevated levels of inflammatory cytokines such as IL-6 and TNF-α. However, no concrete evidence has shown that in fact the elevation in cytokine levels are correlated with circulating leptin. Understanding the intricate relationships between various molecules involved in TLR signaling and their positive or negative regulation has been a key focus for the development of effective therapeutics. Several studies have been done that suggest not only will TLR4 mediate signaling transduction but it will interact with other TLR family members and parallel receptors in inflammatory complications [80].

As mentioned previously, TLR4 is the only toll like receptor family member other than TLR3 capable of signaling independent of MyD88. Studies to understand the importance of MyD88 independent and dependent signaling pathways in the macrophages response to LPS will provide advanced knowledge of how TLRs interact to recognize microbial pathogens [81]. Ouyang et.al, found that TLR agonists can synergize in inducing inflammatory cytokines through both MyD88 and TRIF pathways [82]. Both TRIF and MyD88 are involved in downstream signaling of TLR4. Interestingly, our lab and others have shown that TLR4 may also interact with other receptors such as leptin receptor [83]. However, Leptin has been shown to induce high levels of IL-6 in combination with lipopolysaccharide.

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Specific Aims

Obesity and diabetes are two metabolic diseases characterized by insulin resistance and a low-grade inflammation. The metabolic effects of obesity have made this disease one of the most common risk factors for several diseases such as diabetes, hypertension, and artherosclerosis [1]. There has also been evidence demonstrating that obesity is associated with a chronic low-grade inflammation characterized by abnormal cytokine production and activation of inflammatory signaling pathways in adipose tissues [2]. These adipose tissues possess an endocrine function by synthesizing and secreting at least two main hormones, leptin and adiponectin. Recent evidence shows that leptin acts as a pro inflammatory cytokine. The understanding of leptin's traditionally identified biological function in regulating food uptake and energy expenditure is well known. However studies have shown that leptin's traditionally beneficial effect on macrophage cells has detrimental consequences in the presence of LPS. It has been shown that different inflammatory stimuli, including IL-1, IL-6 or LPS, regulate leptin mRNA expression as well as circulating leptin levels [3]. Recently, our lab found that leptin alone fails to induce proinflammatory cytokines. Furthermore, leptin is induced by inflammatory regulatory cells, suggesting that leptin expression could trigger or participate in the inflammatory process through direct paracrine and autocrine actions. We hypothesize that IRAK-1 may be involved in understanding leptins role in the inflammatory process.

IRAK-1 is a signaling molecule critically involved in regulating intracellular inflammatory signaling, including macrophage functions in the innate immune response. In a previous

study in our lab using IRAK-1 deficient mice, we demonstrated that IRAK-1 deficient mice were protected from high-fat diet induced insulin resistance and cardiovascular disease. Therefore, we hypothesized that IRAK-1 may be involved in modulating the differentiation of macrophages. In any case, the precise role of leptin in the development of inflammation remains incompletely understood. The objective of this study is to further understand the mechanism behind the leptin signaling pathway and IRAK-1 involvement.

In addition to the interaction of TLR4 and leptin in inducing inflammatory cytokines such as IL-6, it may also implement such complications by utilizing its unique function of activating both MyD88 dependent and independent signaling pathways [4, 5]. Both pathways are capable of activating key inflammatory transcription factor, NFkB. It is imperative to understand the activation of these two independent pathways as a way of LPS stimulation. We also hypothesized that IRAK-1 plays a role in downstream signaling involved in inflammatory complications independent of NFκB.

The specific aims of this project are:

SPECIFIC AIM 1: To define leptin's involvement in proinflammatory complications

Hypothesis: IRAK-1 is responsible for LPS and leptin to synergize in inducing IL-6.
Objective: Utilize both murine and human macrophage cells to determine the involvement of IRAK-1 on the induction of IL-6 and leptin's effect on the stability of IRAK-1.

SPECIFIC AIM 2: To examine the TLR-4 priming effect of LPS on inflammatory mediators.

Hypothesis: Low concentrations of LPS exacerbate inflammatory mediators through MyD88 independent TLR-4 pathway utilizing IKKε.

Objective: Human monocytic cells will be treated with varying concentrations of LPS to activate both independent and dependent pathways and measure protein expression of signaling molecule proteins and gene expression of proinflammatory cytokines.

Methods

Cell culture:

Human monocytic THP-1 are purchased from ATCC and grown in RPMI media 1640 (Invitrogen) containing 10% FBS, penicillin, and streptomycin (100 U/ml). Bone Marrow Derived Macrophages (BMDM) are prepared using wild type and IRAK-1 deficient C57BL6 chow fed mice. Briefly, mice were sacrificed and dissected. Using a 22 gauge needle suck up PBS into the syringe and pushed through the bone to remove the marrow. Using a sterile transfer pipette suck up the plugs of marrow and expel against the bottom of the petri-dish to break up clumps Transfer the media to a 50ml tube. Centrifuge at 12000 rpm for 8mins, 4°C, then remove the supernatant. Resuspend the pellet; add 5ml ACK buffer and let sit for 2mins and bring the volume up to 40ml with PBS. Filter the cell mixture (cell strainer 70um into a new 50ml tube) Centrifuge at 12,000 rpm for 8mins, 4°C, remove liquid. Resuspend cells in 12ml complete DMEM. Add 48 mL of the complete media (listed below) to each non-TC treated dish. Complete Media (filtered) consists of 180L-sup and 396DMEM. Add 2 mL of cells to each dish. Incubate. On the third day in culture add 25 mL complete media to each dish. On the 6th day in culture, there will be a monolayer, and macrophage can be harvested as follows: Aspirate medium and gently add back 25mls ice-cold PBS (Ca++/Mg++ free) by pipetting onto vertical of the dish. Refrigerate for ~ 15mins. Harvest cells by pipetting up and down (they come off relatively easily so harsh pipetting is unnecessary). Centrifuge at 12000 rpm for 8mins, 4°C, count cells and plate them. All experiments in this study are conducted after treatments using leptin, lps and a combination of leptin plus lps in

increasing concentrations ranging from 0.01-1mg/ml for the indicated time periods.

Real-time quantitative PCR

mRNA levels of inflammatory cytokines were measured in BMDM and THP-1 cells, $3x10^6$cells were cultured in the absence or presence of perspective treatments for documented incubation times. RNA was extracted from the cells using the Trizol reagent (Invitrogen, Carlsbad, CA, U.S.A) according to the manufacturer's protocol and cDNA was synthesized from 1.5 µg of RNA using the High Capacity cDNA reverse transcriptase kit protocol (Applied Biosystems). Real-time PCR was conducted using iQ SYBR Green Supermix (Bio-Rad). Amplification was performed on an iQ5 Multicolor Real-Time PCR detection system (Bio-Rad). mRNA levels are normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Sequences used are listed in Table 1.

Enzyme-Linked Immunosorbent Assay (ELISA)

Plasma IL-6 levels were detected using manufacturer protocol from Bio-Rad.

Protein Extraction and Western Blotting

Cells were treated with 100ng of the stimulants discussed for 0, 15, 30, and 60 minutes. Whole cell lysates were collected from both BMDM and THP-1 cells using 1X SDS lysis buffer. Samples are incubated on ice for 30 minutes then boiled at 100° for 10 minutes. Nuclear and cytoplasmic extracts were collected from THP-1 cells using 5 million cells per treatment according to lab protocol. Briefly, cells were harvested using 400µl Buffer A on ice and transferred to centrifuge tube. Samples were incubated on ice for 25 minutes followed by the addition of 16µl of 10% Triton X-100. They were then

vortexed and centrifuged at 4º C immediately for 10 minutes at 5,000 rpm. Supernatant (cytosol extract) was transferred to fresh centrifuge tube and stored at -80º C. The pellet was then resuspended in 50µl cold Buffer B and incubated on ice for 30 minutes with vortexing every 10 minutes. Centrifugation at 12,000 RPM for 20 minutes was completed before storing samples at -80ºC.

Western Blot

Protein samples are loaded into a protein minigel. The minigel is run at 100V until dye runs off in 1X SDS gel running buffer. The gels are then transferred to a membrane and run for 2 hours at 110V in transfer buffer with ice surrounding the apparatus. The membrane is then blocked for 1 hour at room temperature in 5% skim milk. After the blocking period, the membranes are exposed to the primary antibody diluted in 5% skim milk or BSA depending on antibody specifications overnight at 4°C on a rocker. The next day, the membranes are washed four times with TBST on a shaker at room temperature for 5 minutes each. Membranes are then exposed to specified secondary antibody for 1 hour at room temperature. After 1 hour, the membranes are then washed again (4X with TBST, 5 minutes per wash). The membrane can then be developed using the ECL kit from Amersham and chemiluminescence is detected.

5 X SDS sample buffer (20 ml) - store at -20°C:

-I M Tris-HCl, pH6.8	8 ml
-SDS	2 g
-glycerol	10 ml
-1% bromophenol blue	1.0 ml
-DTT	1.54 g

Protein minigel recipe:	**Separating gel (10%)**	**Stacking gel**
-30% acrylamide (29:1)	3.33 ml	650 µl
-10 % SDS	100 µl	50 µl
-1 M Tris-HCl	3.75 ml (pH 8.8)	630 µl (pH 8.8)
-H2O	2.92 ml	3.64 ml
-10% APS	50 µl	25 µl
-TEMED	10 µl	5 µl

10 X SDS gel running buffer (1 L):

-Tris base	30 g
-Glycine	144 g
-SDS	10 g
-18 MOhm H2O	1L

Western blot transfer buffer (1 L):

-Tris Base	2.9 g
-Glycine	14.5 g
-Methanol	200 ml
-18 MOhm H2O	800 ml

TBST:	**1X**	**10X**
-NaCl	150 mM	1.5 M
-Tris-HCl, pH8.0	10 mM	0.1 M
-Tween 20	0.05%	0.50%

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Chapter 3:

Mechanism Underlying the Inflammatory Effect of Leptin ǂ

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Running title: Mechanism Underlying the Inflammatory Effect of Leptin

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Abstract

Leptin, a key adipokine involved in regulating food intake and body weight, has been recently implicated in the exacerbation of inflammation. Elevated leptin levels in blood circulation are correlated with increased inflammation in obese individuals with cardiovascular complications. However, the underlying molecular mechanism is poorly understood. In this report, we demonstrated that leptin alone failed to induce the expression of inflammatory cytokines such as IL-6 in murine macrophages and human monocytic cells. Instead, leptin significantly augment the effect of LPS in inducing the expression of IL-6. The key inflammatory signaling molecule, IRAK-1, is partially involved in mediating the effects of both LPS and leptin. IRAK-1 deficient macrophages exhibit significantly lower expression of IL-6 following LPS or LPS plus leptin stimulation. Mechanistically, we observed that leptin increases the expression of IRAK-1 in both human monocytes and murine macrophages. Taken together, our data reveal that leptin primarily serves as a helper, instead of an initiator of inflammation during the pathogenesis of obesity related inflammation.

Introduction

Obesity, caused by high-fat diets and sedentary life styles, is taking an increasingly larger toll on current society [1-3]. However, it is not obesity per se, but rather the inflammatory complication accompanying obesity that contributes to significant morbidity and health concern. Severely obese individuals are prone to develop chronic inflammatory diseases including atherosclerosis, diabetes, and kidney dysfunction [4-6]. Furthermore, obese individuals are at a significantly higher risk of developing multi-organ failure and sepsis when facing systemic infection [2, 7, 8]. Consequently, intervention strategies targeting inflammation hold great potential in treating obesity-related pathological syndromes. However, the cause for obesity-associated inflammatory complication is not well understood.

Intriguingly, circulating levels of leptin increase dramatically in obese humans and mice, and have been closely associated with the risk of inflammatory complications including atherosclerosis [9, 10]. Functional leptin receptor is expressed in many cell types including monocytes and macrophages [11-14]. Limited studies regarding the effect of leptin on circulating leukocytes suggest that leptin might induce the expression of proinflammatorycytokines such as IL-6 and TNF-α [13, 15, 16]. However, the underlying molecular mechanism is not clearly understood.

To clarify whether and how leptin may either directly or indirectly contribute to the expression of proinflammatory mediators, we tested the expression of IL-6 in

macrophages challenged with different combinations of leptin and bacterial endotoxin LPS.

Materials and Methods

Cells and reagents

Human promonocytic THP-1 cells were purchased from American Type Cell Culture (ATCC), Manassas, VA and grown in RPMI media 1640 from Invitrogen, Carlsbad, CA, containing 10% FBS, penicillin, and streptomycin (100 U/ml). Bone Marrow Derived Macrophages (BMDM) were prepared using wild type and IRAK-1 deficient C57BL/6 chow fed mice. The isolation and culture of murine BMDM and maintenance of THP-1 cells were as previously described [17]. Macrophages were allowed to rest in 1% FBS DMEM for 4 hours before treatments.

Real-time quantitative PCR

To examine the effects of leptin on mRNA expression of inflammatory cytokines in BMDM cells, 3x10^6 cells were cultured in the absence or presence of increasing concentrations of leptin (0.01-1mg/ml) from Sigma-Aldrich and/or LPS, 100ng/ml from R&D systems for 4 hours, and cellular total RNA was prepared. RNA was extracted from the cells using the Trizol reagent from Invitrogen according to the manufacturer's protocol and cDNA was synthesized from 1.5 μg of RNA using the high capacity cDNA reverse transcriptase kit protocol (Applied Biosystems). Real-time PCR was conducted using iQ SYBR Green Supermix (Bio-Rad) and amplification was performed on an iQ5 Multicolor Real-Time PCR detection system (Bio-Rad). mRNA levels of TNF-α and IL-6 were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Table 1 contains the sequence of the primer sets used for each gene.

Protein extraction and western blotting

After treatment of leptin and/or LPS, cells were rinsed one time with ice-cold phosphate-buffered saline, solubilized in 1X SDS lysis buffer (1X SDS, cocktail 1,2,3), boiled at 100°C in dry bath for 10min, and centrifuged at 12,000 rpm for 2 min. The supernatant (cell extracts) was quantified and normalized to 60ug of total protein for each sample, and boiled for 10 min in SDS-PAGE sample buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 2% 2-mercaptoethanol, 10% glycerol, 0.005% bromphenol blue). The solubilized proteins were separated by 10 % SDS-PAGE, transferred to PVDF membrane (Bio-Rad), and detected by immunoblotting with the indicated antibody using ECL Plus western blotting detection reagents Kit (GE Amersham). Antibodies used in this study were purchased from Santa Cruz Biotechnology (IRAK-1 and GAPDH). They were both blocked in 5% milk in TBS-T. Protein expression for IRAK-1 and GAPDH was determined by overnight incubation at 4°C with a 1/1000 dilution of primary Abs (Upstate), followed by incubation for 1 hour at room temperature with a secondary anti-rabbit HRP Ab diluted at 1/10,000 (Jackson immunoresearch lab).

Enzyme-Linked Immunosorbent Assay

Plasma IL-6 levels from BMDM cells were measured using Bio-Rad manufacturer's protocol.

Results:

Leptin alone fails to induce proinflammatorygenes in humans and mice

To test whether leptin may directly induce the expression of proinflammatorymediators in monocytes or macrophages, we treated both BMDM and human THP-1 promonocytic cells with leptin ranging from 1 to 100 ng/ml. The levels of IL-6 were measured by real-time RT-PCR as described in the Materials and Methods. As shown in figure 1, there was no noticeable expression of IL-6 in murine BMDM or THP-1 cells treated with increasing concentrations of leptin (Fig. 3.1 A and B). In agreement with a previous study, we did detect the expression of leptin receptor within both BMDM and THP-1 cells (Fig. 3.1 C) [11-14]. As a positive control, LPS at a constant concentration of 100ng/ml shows a significant induction of IL-6 ($p<.0001$) (Fig.3.1 A and B). This indicates that leptin alone is not sufficient for robust induction of proinflammatory cytokines in both human THP-1 monocytic cells and murine macrophages.

Leptin primes macrophages to be more responsive to lipopolysaccharide

To further answer the question regarding how leptin may potentially contribute to excessive inflammation well-documented in obese individuals [9, 18-20], we examined whether leptin may enable the cells more responsive to LPS challenge. To test this, we treated the murine BMDM with either LPS alone, or LPS together with leptin. Intriguingly, we observed that there is a significantly elevated induction of IL-6 when cells were treated with leptin and LPS, as compared to the single LPS treatment (Fig. 3.2 A and Fig. 3.2 C). This phenomenon is similarly observed in human THP-1 cells (Fig. 3.2 B).

The priming effect of leptin is dependent upon IRAK-1

Since IRAK-1 is a well-characterized intracellular signaling component for LPS signaling, we tested whether IRAK-1 may be required for the priming effect of leptin. BMDM harvested from wild type (WT) and IRAK-1 mice were treated with LPS alone, or LPS plus leptin. The levels of IL-6 were measured by real-time RT-PCR analyses and ELISA. As shown in figure 3.3A and B, the levels of IL-6 induced by LPS or LPS plus leptin were significantly reduced in IRAK-1 deficient BMDM as compared to WT cells.

Leptin induces mRNA levels and elevated protein expression of IRAK-1

To further understand the mechanism underlying leptin-mediated augmentation of LPS response, we measured protein levels of IRAK-1 in macrophages treated with leptin in wild type BMDM cells and THP-1 cells. Intriguingly, we observed that leptin increased the message as well as protein levels of IRAK-1 in murine macrophages as well as human THP-1 cells (fig. 3.4).

Discussion:

In this current manuscript, we reported that application of leptin alone at physiological and pathological ranges (1-100ng/ml) does not induce the expression of proinflammatory mediators. Intriguingly, leptin primes macrophages to be more responsive to LPS in expressing IL-6. Leptin potentially exerts its role by inducing the expression of intracellular signaling kinase IRAK-1.

Our data help to clear the confusion regarding the role of leptin in causing inflammation. Although studies have indicated that elevated leptin levels in obesity are correlated with inflammation, whether leptin can directly induce the expression of proinflammatory mediators in macrophages and monocytes is not clear [13]. Zarkesh-Esfahani *et al*. previously reported that an extremely high concentration of leptin (over 250ng/ml) may induce the expression of TNF-α in human monocytes [21]. However, the authors acknowledged that humans rarely encounter leptin at such high concentration under normal conditions. In contrast, the circulating levels of leptin in healthy humans are well below 10ng/ml, and rises only to the range of 10-60 ng/ml in obese individuals [22]. Our data demonstrated that leptin within this concentration range will not induce the expression of TNF or IL-6 in either human monocytes or murine macrophages. Intriguingly, our result revealed that 10ng/ml leptin can significantly augment the effect of LPS in inducing the expression of IL-6. This is significant and helps to explain the phenomenon that obese individuals are at a higher risk of getting septic shock when encountering infection [18].

Our result also revealed that IRAK-1 is a key signaling component mediating the synergistic effect between leptin and LPS. Although IRAK-1 is a well-known molecule downstream of LPS-TLR4 pathway [23, 24], its regulation at the level of gene expression has not been well studied. Our data presented the first evidence that IRAK-1 expression can be induced by leptin. This suggests that obese individuals may have elevated levels of TLR4 signaling molecules due to leptin accumulation in circulation. As a consequence, this may explain the heightened inflammatory responses of obese individuals.

Questions remain regarding how IRAK-1 expression is modulated by leptin. A previous study indicates that IRAK-1 gene expression can be regulated by STAT3 [25]. Leptin is a known inducer of STAT3 activation, which may explain our observed phenomenon. Further molecular studies are warranted to examine the transcriptional regulation of IRAK-1 induced by leptin.

Our current report reveals an intriguing molecular mechanism underlying leptin-induced host inflammation, and suggests that strategies aimed at un-coupling the cross-talk of leptin and LPS signaling may hold promise in treating metabolic inflammation.

Acknowledgement
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Figure legend

Figure 3.1. *Leptin alone fails to induce IL-6 in human and murine macrophages.* Primary murine bone marrow derived macrophages (BMDM) from wild type mice (**A**), or human monocytic THP-1 cells (**B**) were treated with either leptin or LPS for 4 hours. The levels of IL-6 message were measured by real-time RT-PCR. The plot represents an average of four independent experiments. *** $p<0.0001$ is shown to be statistically significant by two paired student t-test. (**C**) RT-PCR analyses were performed to detect mRNA levels of leptin receptor (Ob-R) in BMDM from WT and IRAK-$1^{-/-}$ mice, as well as in THP-1 cells.

Figure 3.2. *Leptin primes macrophages and augments the effect of LPS in inducing the gene expression of IL-6.* WT murine BMDM (**A**) or human THP-1 cells (**B**) were treated with LPS plus leptin as indicated on the figure legend for 4 hrs. The levels of IL-6 message were measured by real-time RT-PCR. Enzyme Linked Immunosorbent Assay (ELISA) was performed using wild type BMDM cells (**C**). The plot represents an average of four independent experiments. ** $p<0.001$ is shown to be statistically significant by two paired student t-test.

Figure 3.3. *The priming effect of leptin depends upon IRAK-1.* WT or IRAK-$1^{-/-}$ BMDM were treated with LPS (100ng/ml), leptin, or leptin plus LPS as indicated on the figure legend. The levels of IL-6 message were measured by (**A**) real-time RT-PCR and (**B**) Enzyme-linked Immunosorbet Assay (ELISA). The plot for RT-PCR data represents an

average of four independent experiments. * $p<0.01$ is shown to be statistically significant by two paired student t-test.

Figure 3.4. *Leptin induces the expression of IRAK-1 in human and murine macrophages***.** (**A**) WT BMDM (above) or human THP-1 cells (below) were treated with leptin (100ng/ml) for 2 hrs. The levels of IRAK-1 protein were detected by Western blot. GAPDH served as a control. (**B**) THP-1 cells were treated with leptin (100ng/ml) for 2hrs. The levels of IRAK-1 message were measured by real time RT-PCR. *$p<0.05$ is shown to be statistically significant by two paired student t-test.

Figure 3.5. Proposed signaling illustration of leptin induced inflammation.

Gene	*Sequence*	*Species*
IL-6		
NM_000600	Forward: 5’ AAA TTC GGT ACA TCC TCG ACG GCA 3’	***Homo sapiens***
1201 bp	Reverse: 5’ AGT GCC TCT TTG CTG CTT TCA CAC 3’	
NM_031168	Forward: 5' ATC CAG TTG CCT TCT TGG GAC TGA 3'	***Mus musculus***
1087 bp	Reverse: 5' TAA GCC TCC GAC TTG TGA AGT GGT 3'	
TNF-α		
NM_000594	Forward: 5’ TCA ATC GGC CCG ACT ATC TC 3’	***Homo sapiens***
1669 bp	Reverse: 5’ CAG GGC AAT GAT CCC AAA GT 3’	
NM_013693	Forward: 5’CAC CGT CAG CCG ATT TGC 3'	***Mus musculus***
1619 bp	Reverse: 5’ TTG ACG GCA GAG AGG AGG TT 3'	
GAPDH		
NM_002046	Forward: 5’CAT GTT CGT CAT GGG TGT GAA CCA 3’	***Homo sapiens***
1310 bp	Reverse: 5’ AGT GAT GGC ATG GAC TGT GGT CAT 3’	
NM_008084	Forward: AAC TTT GGC ATT GTG GAA GGG CTC 3’	***Mus musculus***
1254 bp	Reverse: TGG AAG AGT GGG AGT TGC TGT TGA 3’	
Ob-Rb		
NM_001003680	Forward: 5' AGC CAA ACT CAA CGA CAC TCT CCT 3'	***Homo sapiens***
3096 bp	Reverse: 5' CAA TGG TGG GCT GGA CCA AGA AAT 3'	
NM_001122899	Forward: 5' GCA ACC CAC CAT GAT TTC ACC ACA 3'	***Mus musculus***
5571 bp	Reverse: 5' AGG ATT CCT GCC TCA CCA GTC AAA 3'	
IRAK-1		
NM_001569	Forward: 5' TGA GGA ACA CGG TGT ATG CTG TGA 3'	***Homo sapiens***
3589 bp	Reverse: 5' TCT GAG CAC AGT AGC CAG CAA AGT 3'	

Table 3.1

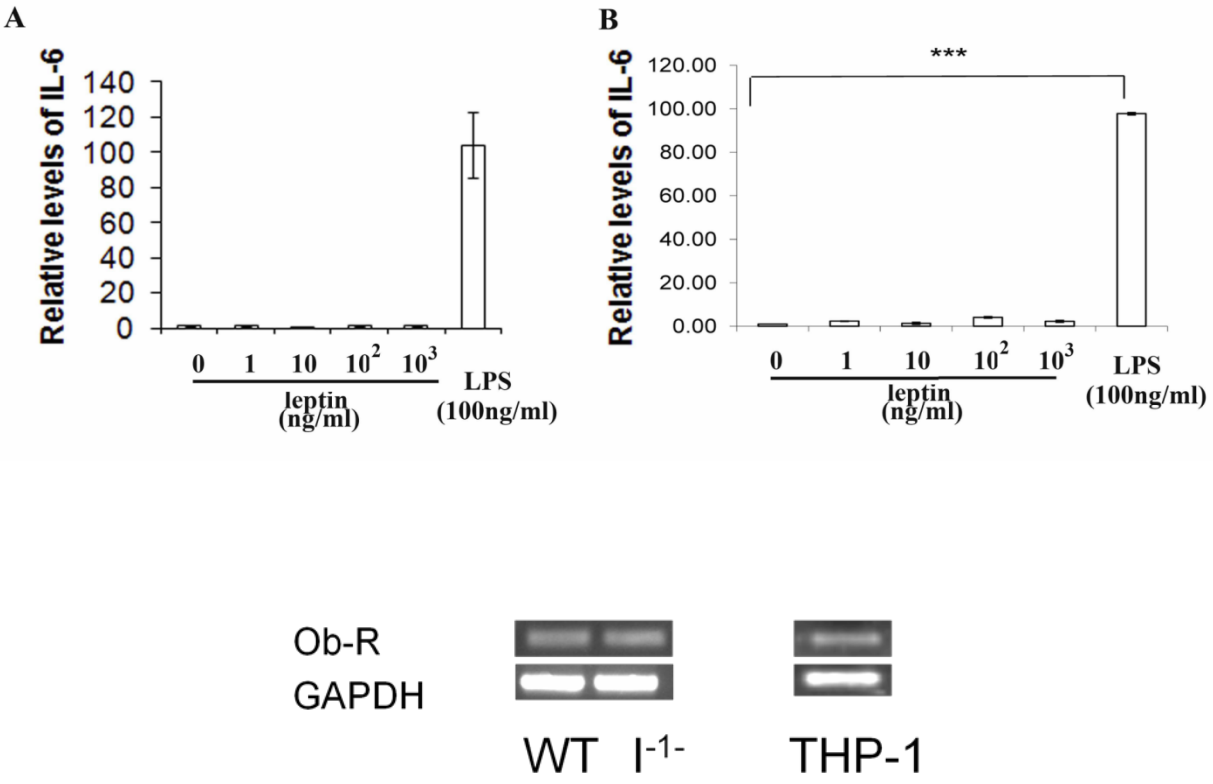

A
Relative levels of IL-6
140
120
100
80
60
40
20
0
0
1
10
10^2
10^3
leptin (ng/ml)
LPS (100ng/ml)
B
Relative levels of IL-6
***
120.00
100.00
80.00
60.00
40.00
20.00
0.00
0
1
10
10^2
10^3
leptin (ng/ml)
LPS (100ng/ml)
Ob-R
GAPDH
WT
I-1-
THP-1
C

Figure 3.1

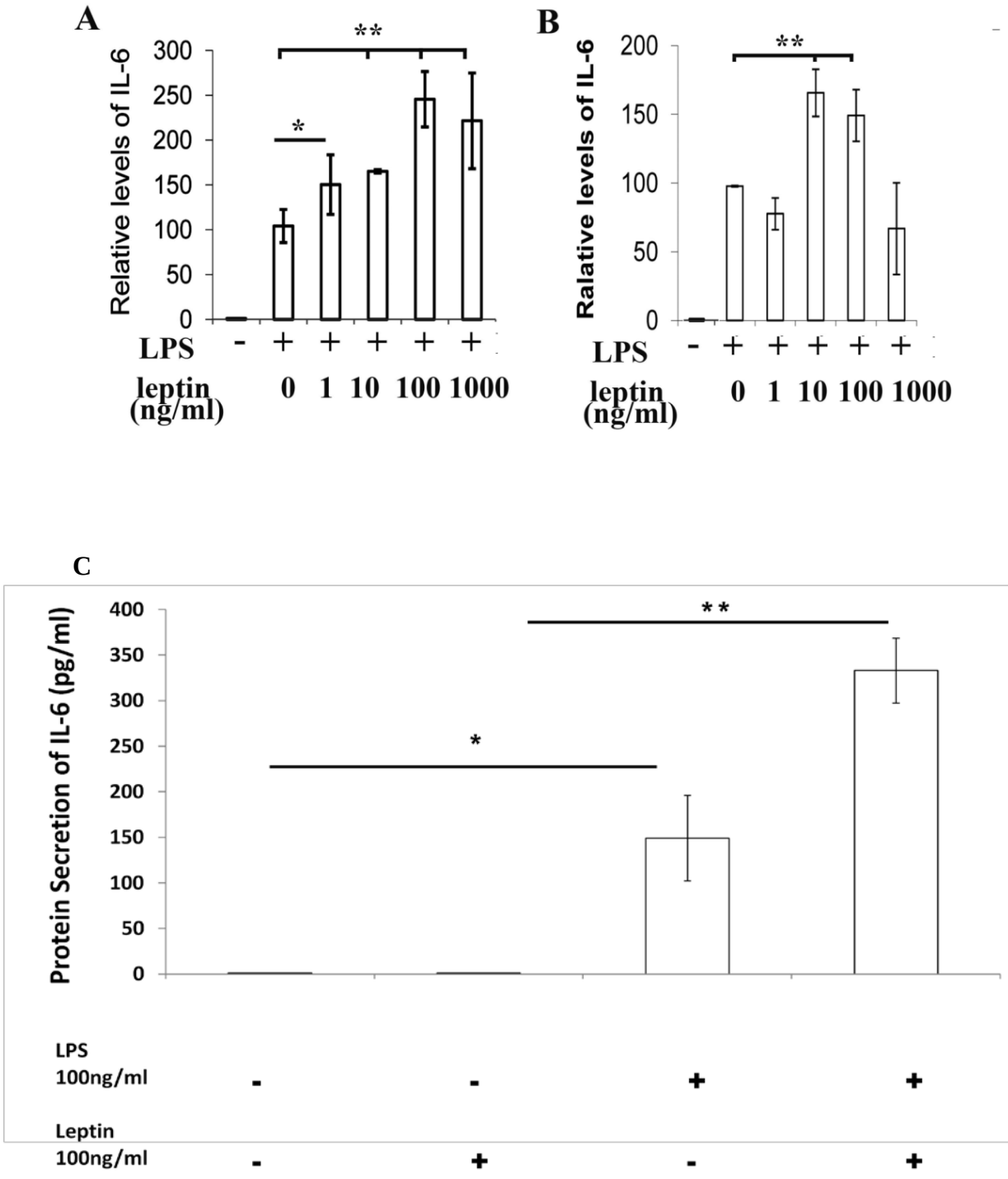
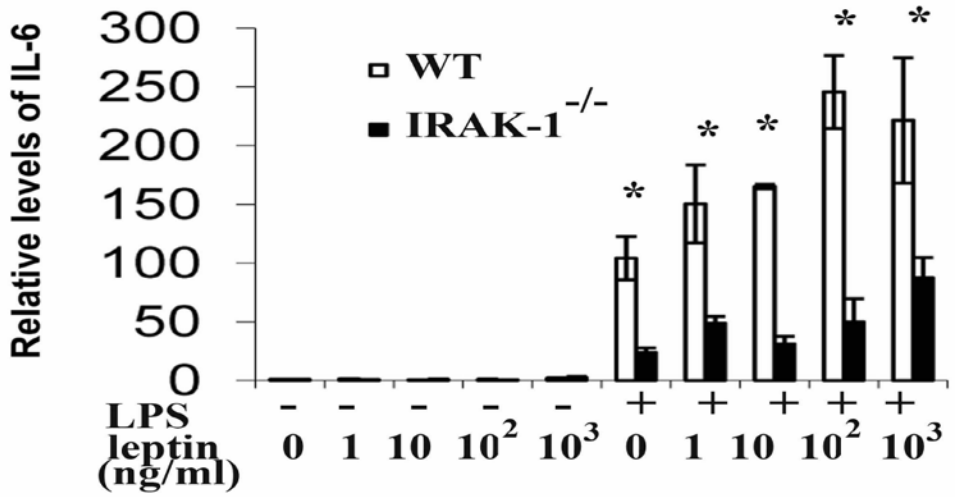
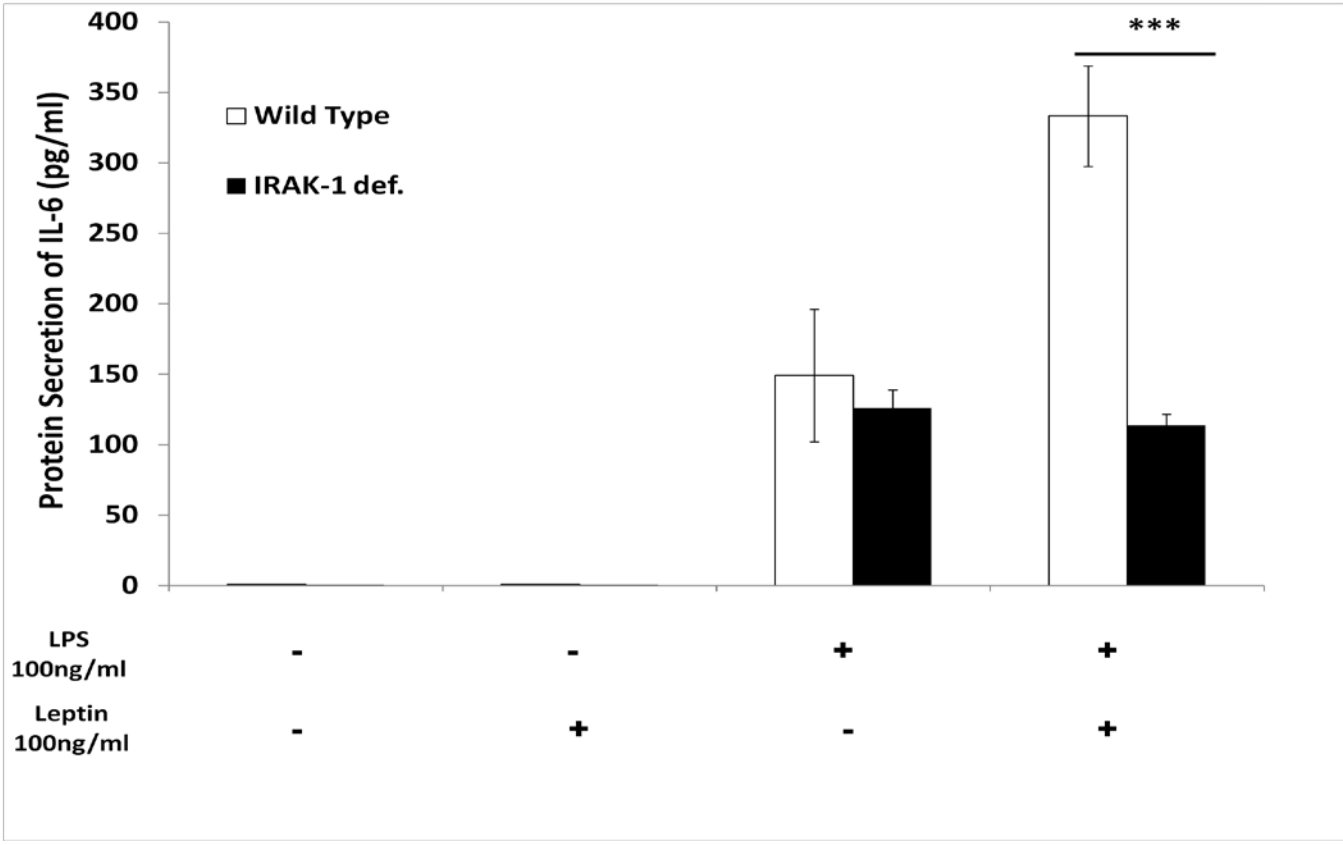

A
Relative levels of IL-6
300
250
200
150
100
50
0
**
*
LPS - + + + + +
leptin (ng/ml) 0 1 10 100 1000
B
Ralative levels of IL-6
200
150
100
50
0
**
LPS - + + + + +
leptin (ng/ml) 0 1 10 100 1000
C
Protein Secretion of IL-6 (pg/ml)
400
350
300
250
200
150
100
50
0
**
*
LPS 100ng/ml - - + +
Leptin 100ng/ml - + - +

Figure 3.2

A


Relative levels of IL-6
300
250
200
150
100
50
0
WT
IRAK-1 -/-
*
LPS
leptin (ng/ml)
- - - - - + + + + +
0 1 10 10^2 10^3 0 1 10 10^2 10^3


Protein Secretion of IL-6 (pg/ml)
400
350
300
250
200
150
100
50
0
Wild Type
IRAK-1 def.
***
LPS 100ng/ml
- - + +
Leptin 100ng/ml
- + - +

B

Figure 3. 3

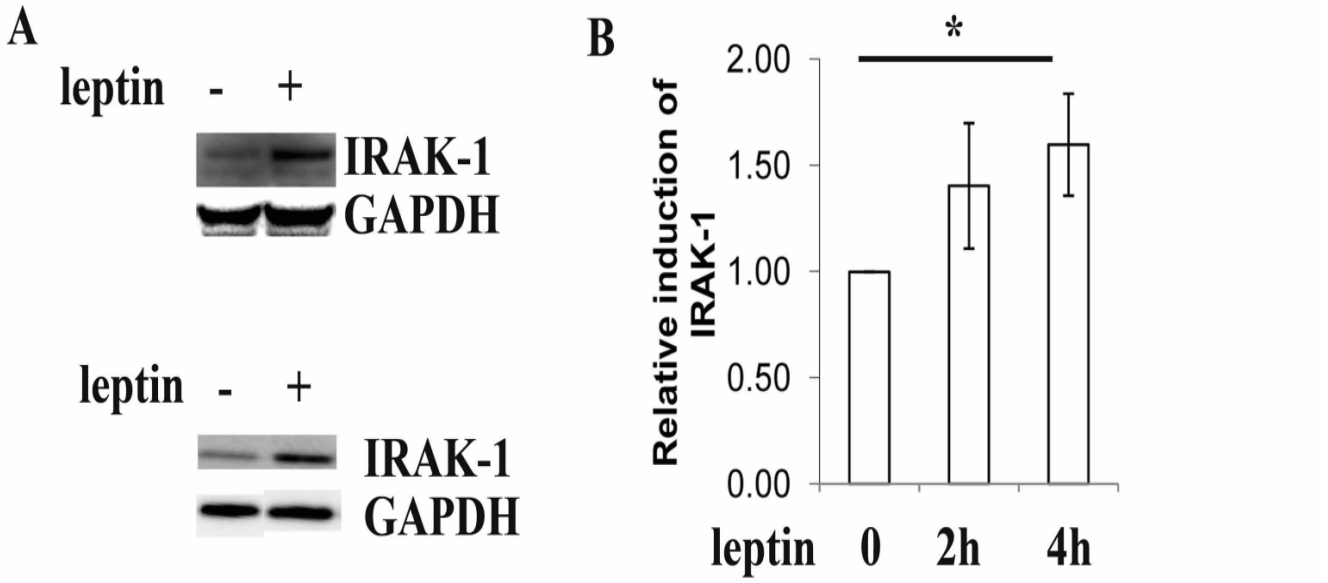

A
leptin - +
IRAK-1
GAPDH
leptin - +
IRAK-1
GAPDH
B
*
Relative induction of IRAK-1
2.00
1.50
1.00
0.50
0.00
leptin 0 2h 4h

Figure 3. 4

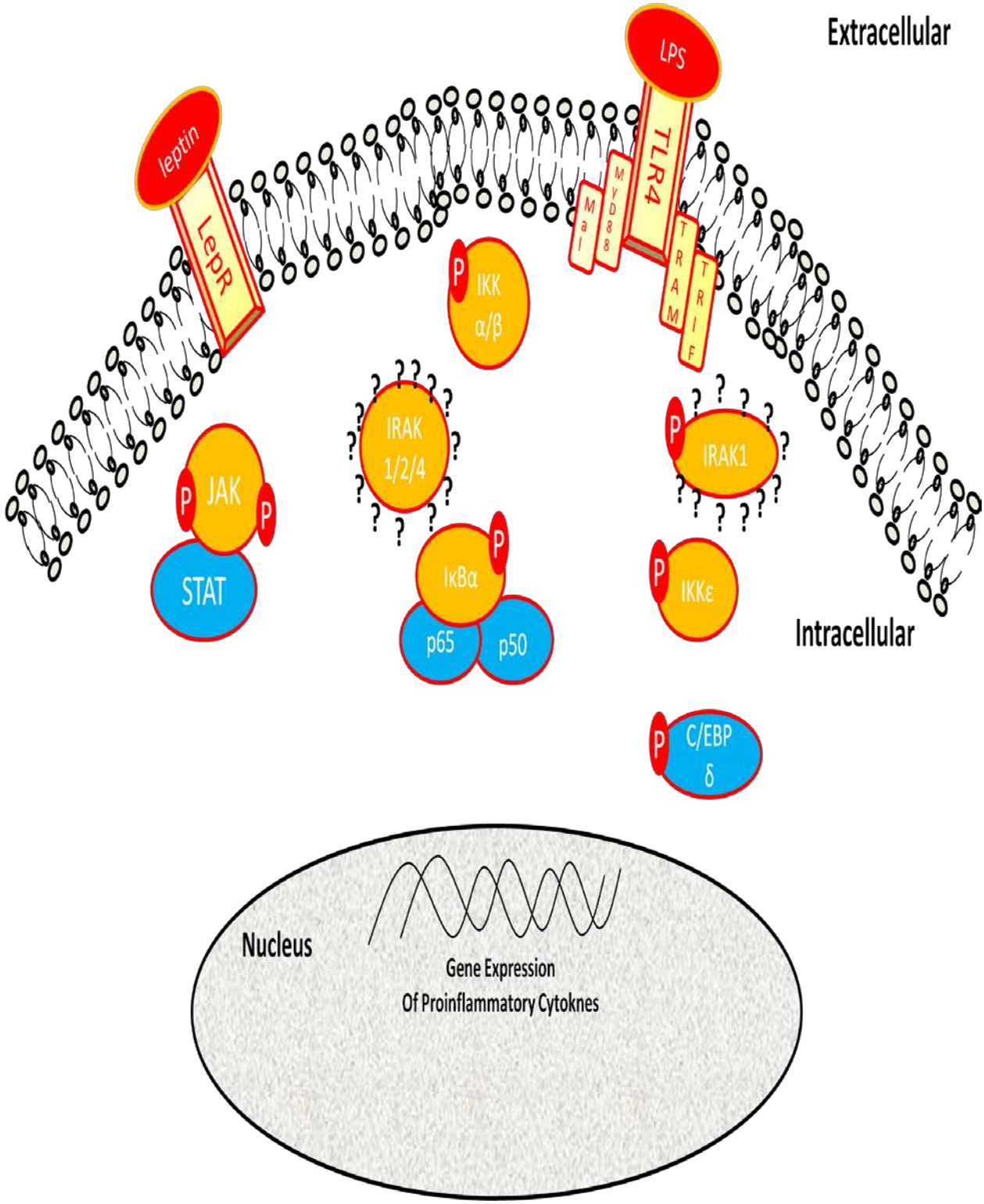

Extracellular
LPS
leptin
LepR
TLR4
Mal
MYD88
TRAM
TRIF
P
IKK
α/β
IRAK
1/2/4
P
IRAK1
P
JAK
P
STAT
P
IκBα
p65
p50
P
IKKε
Intracellular
P
C/EBP
δ
Nucleus
Gene Expression
Of Proinflammatory Cytoknes

Figure 3.5

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Chapter 4:

Novel Mechanism Underlying the Inflammatory Effect of Low Dose LPS ǂ

Running title: Novel Mechanism Underlying the Inflammatory Effect of Low Dose LPS

ǂ In preparation for journal submission

ABSTRACT

Metabolic endotoxemia, defined as circulating levels of endotoxin, has recently sparked interest for its involvement in diet-induced obesity. However, the mechanism involving circulating levels of LPS found the intestinal gut is not clearly understood. We hypothesized that LPS of the gut mediates obese phenotypes through a different mechanism studied in higher concentrations of LPS utilizing a pathway involving key adaptor molecule IKKε. Endotoxemia markers are molecules of the innate immune system which are produced by leukocytes including macrophages. To test this hypothesis, THP-1 cells (human monocytic leukemia cell line) were used to study downstream targets by western blot and proinflammatory cytokines were measured as a phenotypic readout using real-time PCR for both IL-6 and TNF-α. We conclude that metabolic endotoxemia signaling patterns is sufficient enough to induce and sustain inflammatory responses through TLR4 signaling pathway.

Introduction

As one of the most studied and potent immunostimulatory components of bacteria, lipopolysaccharide (LPS) has been a hot topic in several studies [1]. This particular endotoxin induces toxicity through increased signaling, triggering systemic inflammation as well as the release of several cytokines that are key inducers in metabolic disorders [2-4]. Its function has been under experimental research for several years due to its role in the activation of a number of transcription factors. Previous work has shown the involvement of LPS in chronic inflammatory responses such as obesity and diabetes. Studies show that injection of large doses of endotoxin in humans has been followed by severe hypertension and multiorgan dysfunction [5] . Interestingly, most reports in literature are centered on the involvement of LPS in sepsis and septic shock phenotypes. In contrast, administration of endotoxin in minute quantities may serve as a primer in triggering the release of proinflammatory cytokines.

Metabolic endotoxemia, which is defined by low levels of plasma endotoxin in the gut, has recently been coined over the last couple of years to be precursor for disease [6, 7]. This is a major concern as metabolic endotoxemia has been shown to produce metabolic disorders such as obesity and diabetes. An abundance of bacteria naturally exists in the intestinal gut. However, the microbiota of the gut is altered in response to diet choices and other addictions such as smoking and drinking. High fat diet changes the environment of the intestinal gut bacteria which feeds the LPS-containing bacteria and ultimately

dominates the surface. Previous studies have shown that in the absence of bacteria, high fat diet will not increase weight gain in comparison with non high fat diet [8, 9]. Recent publications by Cani *et.al* support the role of LPS/endotoxin in the pathogenesis of obesity and insulin resistance [6, 7, 10, 11]. The objective of this research was to identify key signaling molecules involved in the mechanism underlying inflammation due to metabolic endotoxemia.

Materials and Method

Cells and reagents

Human promonocytic THP-1 cells were purchased from ATCC, Manassas, VA, and grown in RPMI media 1640 from Invitrogen, Carlsbad, CA, containing 10% FBS, penicillin, and streptomycin (100 U/ml), maintenance of THP-1 cells were as previously described [12].

Real-time quantitative PCR

To examine the effects of both acute sepsis and chronic obesity LPS levels on mRNA expression of inflammatory cytokines in THP-1 cells, 3x10^6 cells were cultured in the presence of LPS (0.001-100ng/ml) from R&D systems and cellular total RNA was prepared. RNA was extracted from the cells using the Trizol reagent from Invitrogen, Carlsbad, CA, according to the manufacturer's protocol and cDNA was synthesized from 1.5 µg of RNA using the High Capacity cDNA reverse transcriptase kit protocol (Applied Biosystems). Real-time PCR was conducted using iQ SYBR Green Supermix (Bio-Rad) and amplification was performed on an iQ5 Multicolor Real-Time PCR detection system (Bio-Rad). mRNA levels of TNF-α, IL-6 and SOCS1 were normalized to GAPDH. Table 2 contains the sequence of the primer sets used for each gene.

Protein extraction and western blotting

After treatment of LPS (100pg/ml and 100ng/ml) and IKK inhibitors (200nM/ml)), cells were rinsed one time with ice-cold phosphate-buffered saline, solubilized in 1X SDS lysis buffer (1X SDS, cocktail 1,2,3), boiled at 100°C in dry bath for 10min, and centrifuged at 12,000 rpm for 2 min. The supernatant (cell extracts) was quantified and normalized to 60µg of total protein for each sample, and boiled for 10 min in SDS-PAGE sample buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 2% 2-mercaptoethanol, 10% glycerol, 0.005% bromphenol blue). Nuclear and cytoplasmic extracts were also collected from THP-1 cells using 8 million cells per treatment according to lab protocol. Briefly, cells were harvested using 400µl Buffer A on ice and transferred to centrifuge tube. Samples were incubated on ice for 25 minutes followed by the addition of 16µl of 10% Triton X-100. They were then vortexed and centrifuged at 4 degrees immediately for 10 minutes at 5,000 rpm. Supernatant (cytosol extract) was transferred to fresh centrifuge tube and stored at -80º C. The pellet was then resuspended in 50µl cold Buffer B and incubated on ice for 30 minutes with vortexing every 10 minutes. Centrifugation at 12,000 RPM for 20 minutes was then completed. The solubilized proteins were separated by 10 % SDS-PAGE and transferred to PVDF membrane (Bio-Rad). Membranes were blocked in 5% milk in TBS-T and incubated overnight at 4°C with a 1:1000 dilution of primary Abs, followed by incubation for 1 hour at room temperature with a secondary anti-rabbit HRP Ab diluted at 1/10,000 (Jackson immunoresearch lab). Protein expression was detected using ECL Plus western blotting detection reagents Kit (GE Amersham). Antibodies used in this study were purchased from Santa Cruz Biotechnology (IRAK-1, C/EBPδ and GAPDH) and Cell Signaling (IKBα and lKKε).

Results

Activation of acute sepsis and chronic obesity phenotype is LPS dose dependent

To confirm that metabolic endotoxemia is sufficient for the induction of chronic obesity but not to induce an acute response [6, 10, 13], mRNA levels of proinflammatory cytokines interleukin-6 (IL-6) and tumor necrosis factor (TNF-α) were measured in response to both high and low concentrations of LPS. Our data shows that after 100ng LPS treatments there is an abundant increase in cytokine production of 300-700 fold induction after only 2 hours; however prolonged time course reveals a reduction in cytokine expression (fig. 4.1. A and B). In contrast, treatment of low concentrations similar to the microbial gut flora, there appears to be a sustained amount of IL-6 and TNF-α over a period of time (fig. 4.1.C).

SOCS1 regulates cytokine production in response to LPS

While both high and low concentrations of LPS are able to induce cytokines involved in inflammation, the selective reduction of IL-6 and TNF-α only after high concentrations raises questions whether there are negative regulators involved in the signaling of this endotoxin. To better understand this theory, mRNA levels for suppressor of cytokines (SOCS1) were measured after treatment with both high and low LPS concentrations. Interestingly, we found that not only do low levels of LPS not alter the levels of SOCS1 in comparison with control but, 100ng LPS distinctly induces SOCS1 at the same time there is a reduction in the IL-6 and TNF-α production (fig. 4.2).

Selective activation of TLR4 classical pathway

The pathway activated by higher concentrations of LPS has been studied and are well understood. To better understand these pathways, it is necessary to measure expression of key signaling molecules involved in the classical TLR4 pathway. IKBα, a key molecule responsible for the activation and translocation of NFκB into the nucleus, plays a major role in this classical pathway. To test our hypothesis that low levels of LPS are signaling through an IKB independent pathway, IKBα protein levels were measured. Results confirmed that indeed there is a significant degradation and reappearance of IKBα only after 100ng LPS (fig. 4.3 A) and 100pg showed no significant change (fig. 4.3 B) in expression of this protein which confirms that only high concentrations will activate the IKB pathway.

IKBα independent pathway is activated in response to minute exposure to LPS

IRAK-1 has been shown by others to be degraded after high concentrations of LPS. Our data confirms this finding; in addition we also showed that low concentrations sustain IRAK-1 levels even at longer time courses. IRAK-1 degradation which is upstream of IKKε may cause altered signaling. To show IRAK-1 is necessary for IKKε expression, protein levels of IKKε were measured and showed no induced expression after high concentration treatments (data not shown). In fact, data shows that cells treated with low levels of LPS are sufficient to induce the expression of IKKε which confirms that this concentration of LPS signals through an IKB independent pathway. On the other hand, this finding doesn't give any insight to the involvement of IKKε on

gene expression. IKKε inhibitor shows a decrease in cytokine production after low dose treatments of LPS.

C/EBPδ plays a central role in IKKε signaling pathway

C/EBPδ, a transcription factor also involved in the induction of proinflammatory cytokines was also investigated. After low concentration LPS treatment for 8 hours there is elevated protein expression of C/EBPδ (fig.4.5 A). This confirms that low concentrations of LPS can induce expression of C/EBPδ but whether this activation is dependent on IKKε is not known. To test if IKKε is necessary for the activation of C/EBPδ, cells were pretreated with IKK inhibitors 30 minutes prior to 8 hour LPS treatments. Interestingly, no increase in C/EBPδ protein expression was detected after IKKε was inhibited.

Discussion

The molecular mechanism underlying the involvement of metabolic endotoxemia in the pathogenesis of inflammatory complications has been the focus of this manuscript. We report for the first time there are distinct signaling molecules involved in prolonged and sustained induction of proinflammatory cytokines using a low concentration of LPS similar to levels found in the intestinal gut. IRAK-1 is a well-known molecule downstream of LPS-TLR4 pathway [14, 15]. Intriguingly, 100pg/ml of LPS is sufficient to activate TLR4 and cause downstream activation of IRAK-1 in contrast to that of higher concentrations which has been shown by our group and others to cause degradation of this key protein. IRAK-1 may then phosphorylate IKKε which is only observed in the low concentrations of LPS. This is important because until now, the mechanism hasn't been understood.

Studies have indicated that elevated levels of LPS above 100ng/ml are correlated with sepsis and septic shock. Our data help answer the several questions of how LPS of the gut mediates an obesity phenotype However, with obesity serving as one of the leading causes of several complications such as diabetes and cardiovascular disease, it is significantly important to understanding the underlying problems that lie within the body. Backhead *et al*. previously reported that in germ-free mice fed with high-fat diet for six weeks show no increase in weight gain and therefore do not show an obese phenotype in comparison to their chow fed diet counterparts [8, 9]. These findings suggest that the gut microbiota is an important environmental factor that affects energy harvest from the diet and energy storage in the host. The molecular mechanism underlying the

involvement of the gut microbiota is not well understood. Our data demonstrated a distinct activation pathway independent of that which has been heavily associated with acute symptoms such as sepsis. Instead, we were able to show that 100pg/ml will not activate any key signaling molecules involved in the traditional TLR4 signaling pathway. Intriguingly, this amount of LPS subsequently leads to TLR4 activation and the expression of TNF-α and IL-6 in human monocytes. This is significant and helps to explain the phenomenon that addictions such as alcoholism, smoking, and high fat diet feeding leads to change in the gut microbiota and serves as precursor for long term inflammatory complications through LPS circulation.

Our results also reveal that NFκB; a key transcription factor involved in several inflammatory complications, is not only responsible for the induction of cytokines TNF-α and IL-6 but will also induce a negative feedback loop which consists of SOCS1 and IKBα. This data confirms previous data that SOCS1 can suppress TLR4 signaling and serves as the foundation to understand the different signaling pathways of LPS concentrations found endogenously in the gut of the body [16, 17]. Interestingly, this was only observed after treatments of 100ng/ml LPS which confirm an independent pathway using lesser LPS concentrations which explains the rapid induction and suppression of inflammation. However, we don't see this trend in low dose concentrations of LPS which suggest the potential involvement of another transcription factor. C/EBPδ, a transcription factor also involved in TLR signaling was then identified to be a key player in the endotoxemia effects. Nuclear extracts using only low dose LPS show translocation of C/EBPδ into the nucleus.

The mechanisms involving circulating levels of LPS signaling have not been extensively studied. Our current report adds to the body of knowledge to better understand molecular mechanism during metabolic endotoxemia. However, LPS may also be recognized by other TLR4 receptors located on the endosome in contrast to TLR4 receptors expressed on the cell surface. Therefore, further molecular studies are underway to focus on how low dose and high dose LPS induce different types of TLR4 configuration as well as defining novel transcription factors responsible for the induction of IL-6 under low dose LPS.

FIGURE LEGENDS

Figure 4.1 ***Proinflammatory cytokine production by LPS***

Human monocytic THP-1 cells were treated with 100ng/ml (fig. A and B) and 100pg/ml (fig. C) for 2, 4 or 8 hours and the levels of IL-6 and TNF-α were measured by real-time RT-PCR. The plot represents an average of four independent experiments. ** $p<0.01$, *$p<0.05$

Figure 4.2 ***SOCS1 regulates cytokine production***

Human monocytic THP-1 cells were treated with 100pg/ml and 100ng/ml for 8 hours and the levels of IL-6 and TNF-α were measured by real-time RT-PCR. The plot represents an average of four independent experiments. There was no statistical significance.

Figure 4.3 ***Downstream TLR4 molecules are dose dependently activated by LPS***

Protein levels of IKBα, IRAK-1 and IKKε protein were detected by western blot. Human THP-1 cells were treated with either 100ng/ml LPS (fig. A) or 100pg/ml LPS (fig. B). GAPDH served as a control.

Figure 4.4 ***Signaling effects of IKK inhibitors***

Cells were treated with 200nM IKK inhibitors and/or 100pg/ml LPS. IL-6 and TNF-α mRNA expression were measured in cells treated with LPS and/or IKK inhibitors.

Figure 4.5 ***LPS stimulation activates C/EBPδ***

Nuclear extracts and whole cell lysate from THP-1 cells treated with100pg/ml LPS (fig. A) and/or IKKε inhibitors (fig. B). Levels for C/EBPδ were detected using western blot. GAPDH served as the loading control.

Figure 4.6 ***Proposed illustration of signaling pathway involved in metabolic endotoxemia.***

LPS activation of both MyD88 dependent and independent pathways is concentration dependent. High concentrations activates a pathway involving a several readouts **1**)induction of proinflammatory cytokines in addition there is a negative feedback loop as a result of NFκB which involves, **2**) induction of suppressor of cytokines (SOCS1) and **3**) reoccurence of IκBα which will both translocate out of the nucleus back to cytoplasm and inhibit downstream signaling. Low concentrations of LPS cause activation of MyD88 independent pathway also responsible for induction of inflammatory cytokines utilizing another transcription factor, C/EBPδ.

Gene	*Sequence*
IL-6 NM_000600 1201 bp	
	Forward: 5’ AAA TTC GGT ACA TCC TCG ACG GCA 3’
	Reverse: 5’ AGT GCC TCT TTG CTG CTT TCA CAC 3’
TNF-α NM_000594 1669 bp	
	Forward: 5’ TCA ATC GGC CCG ACT ATC TC 3’
	Reverse: 5’ CAG GGC AAT GAT CCC AAA GT 3’
GAPDH NM_002046 1310 bp	
	Forward: 5’CAT GTT CGT CAT GGG TGT GAA CCA 3’
	Reverse: 5’ AGT GAT GGC ATG GAC TGT GGT CAT 3’
Ob-Rb NM_001003680 3096 bp	
	Forward: 5' AGC CAA ACT CAA CGA CAC TCT CCT 3'
	Reverse: 5' CAA TGG TGG GCT GGA CCA AGA AAT 3'
IRAK-1 NM_001569 3589 bp	
	Forward: 5' TGA GGA ACA CGG TGT ATG CTG TGA 3'
	Reverse: 5' TCT GAG CAC AGT AGC CAG CAA AGT 3'
SOCS1 N/A	
	Forward: 5' CAC GCA CTT CCG CAC ATT CC 3'
	Reverse: 5' TCC AGC AGC TCG AAG AGG CA 3'

Table 4.1

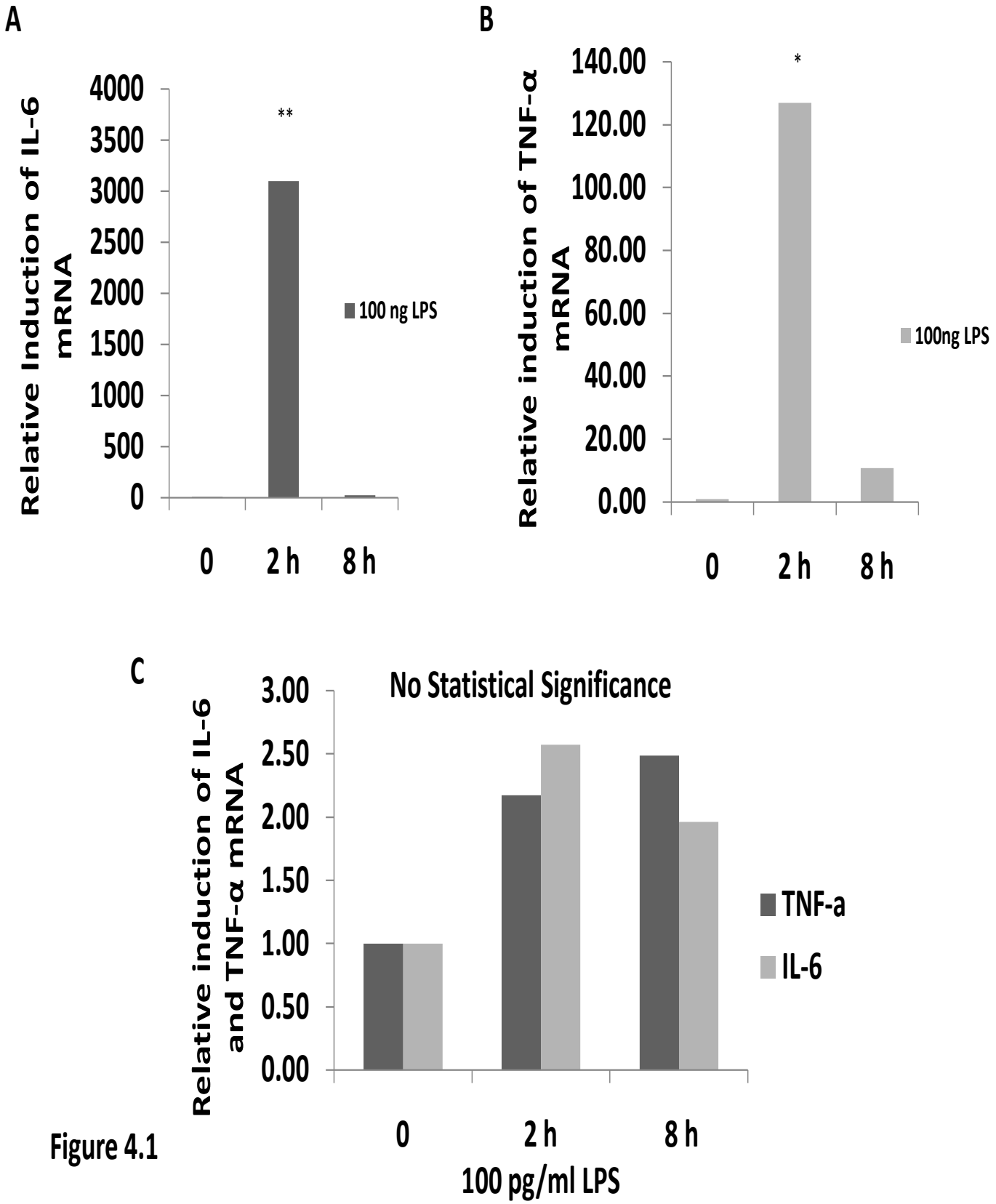
A
Relative Induction of IL-6 mRNA
4000
3500
3000
2500
2000
1500
1000
500
0
**
100 ng LPS
0
2 h
8 h
B
Relative induction of TNF-α mRNA
140.00
120.00
100.00
80.00
60.00
40.00
20.00
0.00
*
100ng LPS
0
2 h
8 h
C
No Statistical Significance
Relative induction of IL-6 and TNF-α mRNA
3.00
2.50
2.00
1.50
1.00
0.50
0.00
TNF-a
IL-6
0
2 h
8 h
100 pg/ml LPS

Figure 4.1

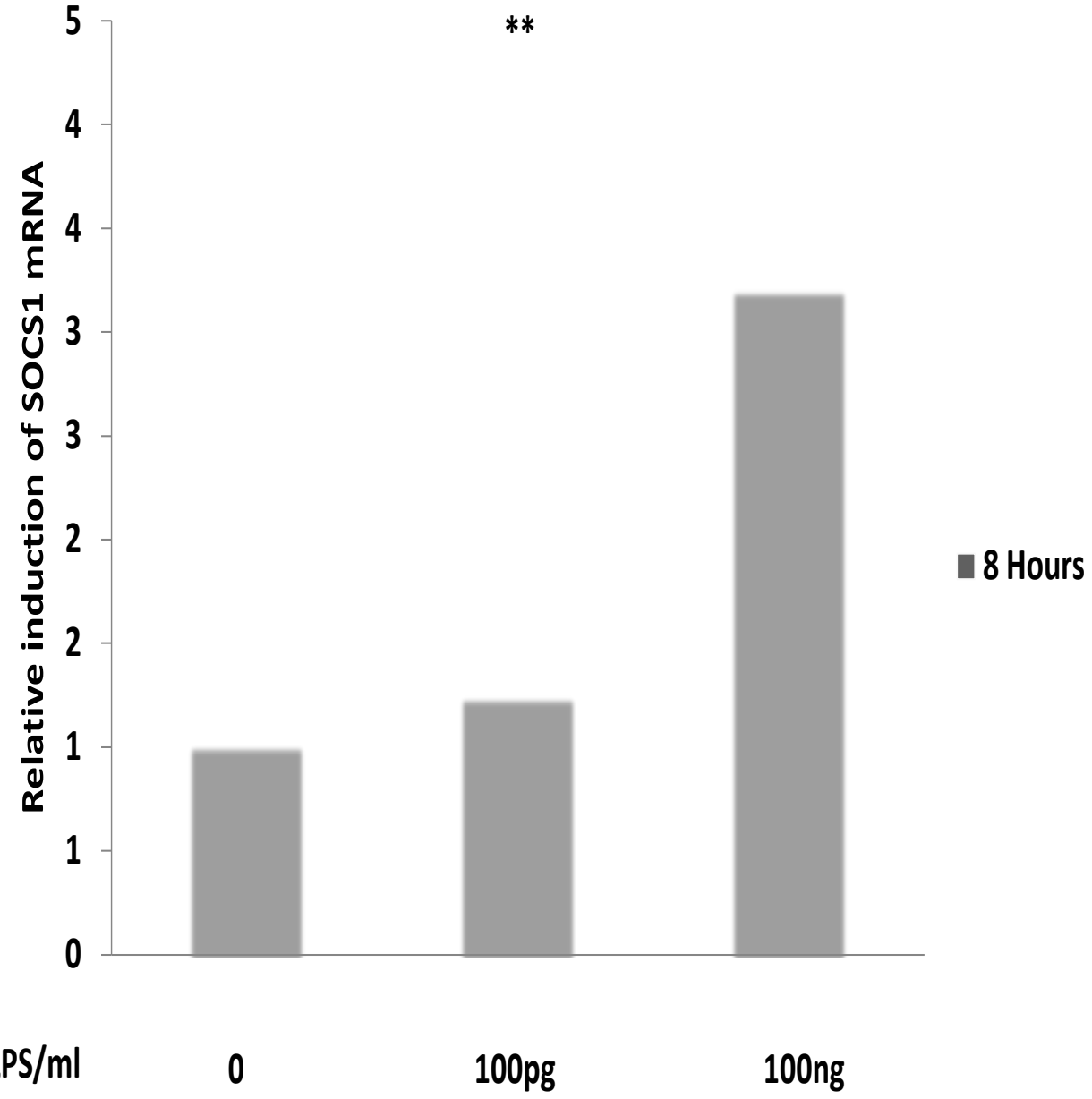
**
5
4
4
3
3
2
2
1
1
0
Relative induction of SOCS1 mRNA
8 Hours
LPS/ml
0
100pg
100ng

Figure 4.2

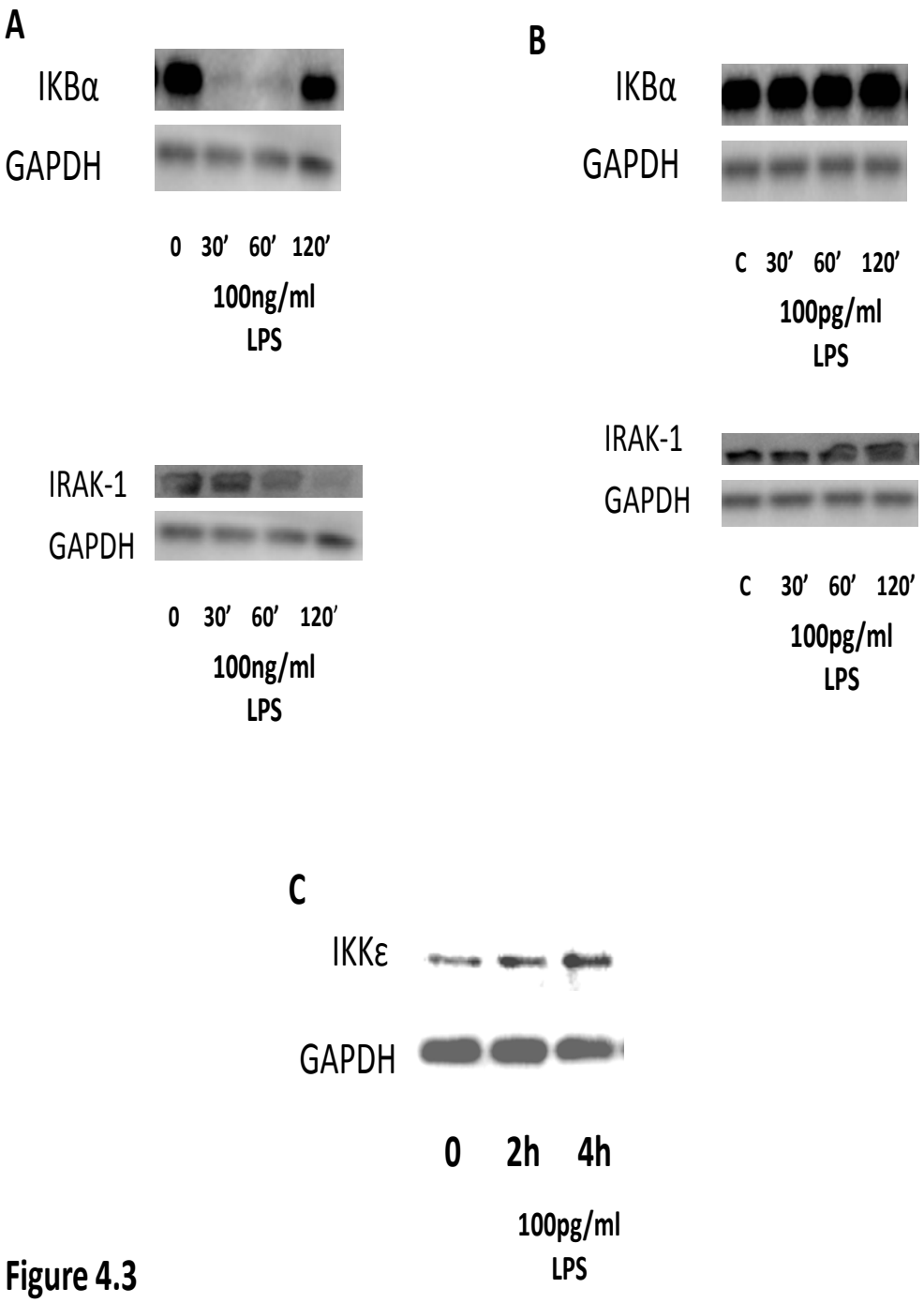
A
IKBα
GAPDH
0 30' 60' 120'
100ng/ml
LPS
IRAK-1
GAPDH
0 30' 60' 120'
100ng/ml
LPS
B
IKBα
GAPDH
C 30' 60' 120'
100pg/ml
LPS
IRAK-1
GAPDH
C 30' 60' 120'
100pg/ml
LPS
C
IKKε
GAPDH
0 2h 4h
100pg/ml
LPS

Figure 4.3

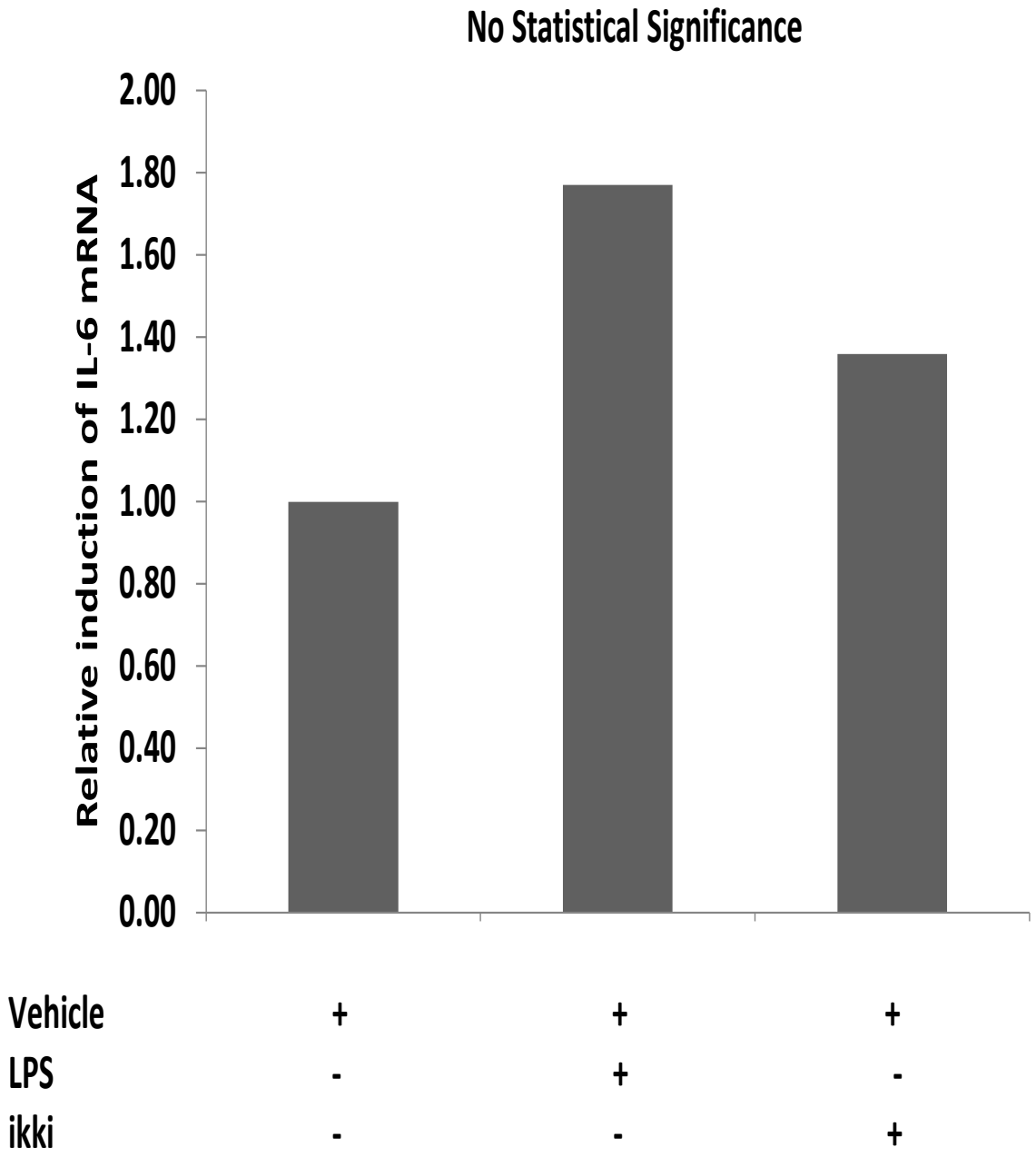
No Statistical Significance
Relative induction of IL-6 mRNA
2.00
1.80
1.60
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
Vehicle + + +
LPS - + -
ikki - - +

Figure 4.4

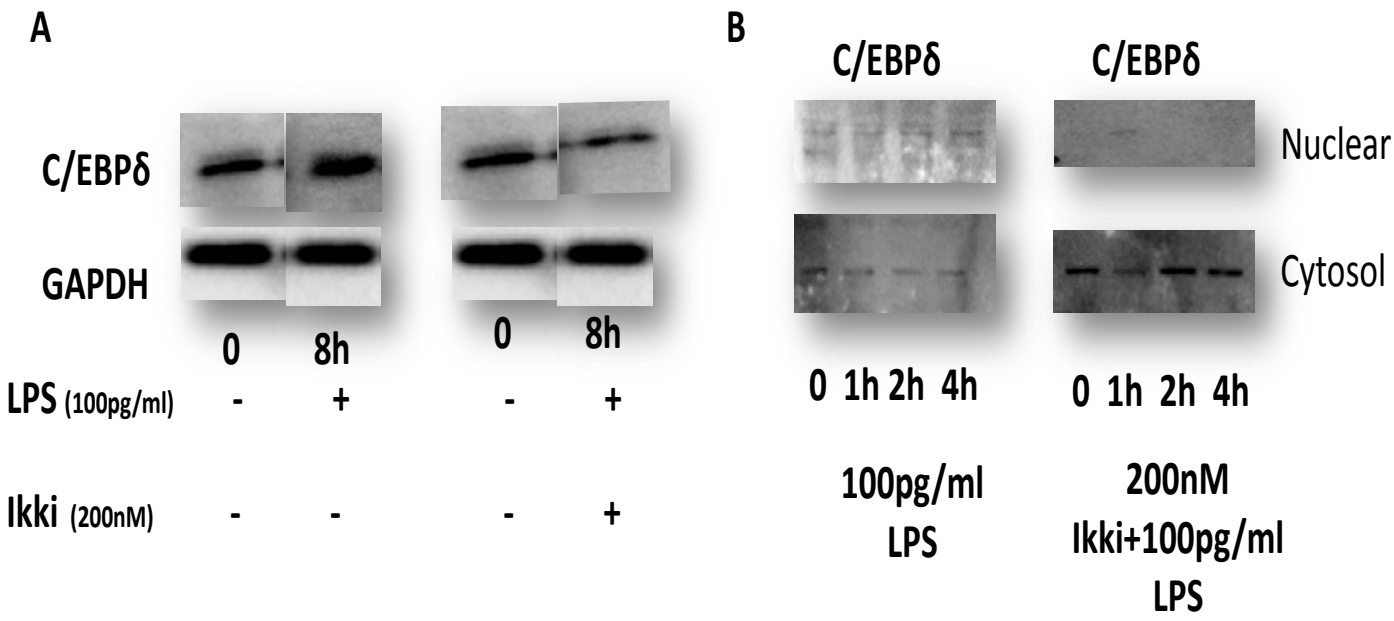
A
C/EBPδ
GAPDH
0
8h
0
8h
LPS (100pg/ml)
-
+
-
+
Ikki (200nM)
-
-
-
+
B
C/EBPδ
C/EBPδ
Nuclear
Cytosol
0 1h 2h 4h
0 1h 2h 4h
100pg/ml LPS
200nM Ikki+100pg/ml LPS

Figure 4.5

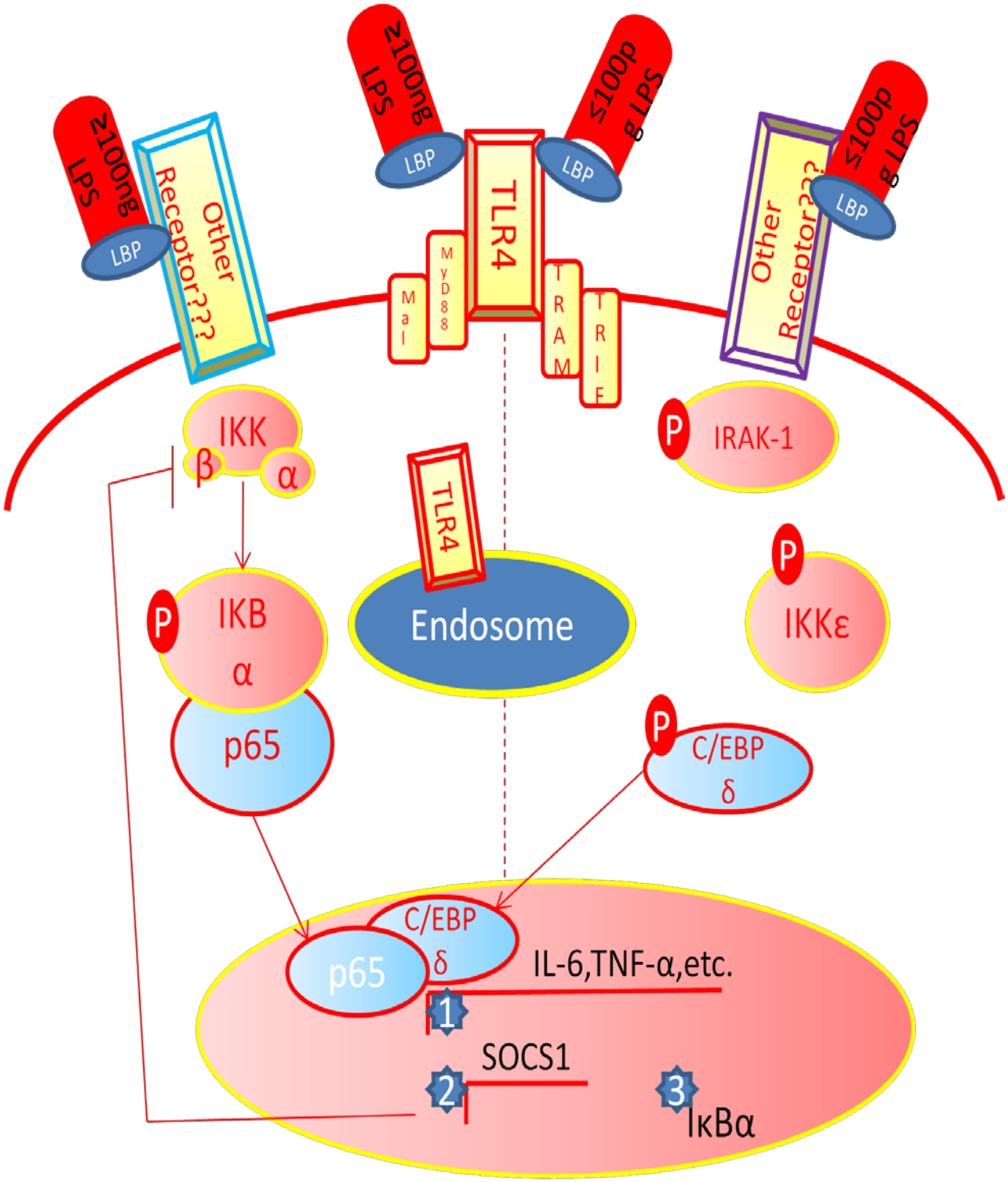
≥100ng LPS
LBP
Other Receptor???
≤100pg LPS
LBP
TLR4
Mal
MyD88
TRAM
TRIF
LBP
Other Receptor???
≤100pg LPS
LBP
IKK
β
α
P
IRAK-1
TLR4
P
IKB
α
p65
Endosome
P
IKKε
P
C/EBP
δ
C/EBP
δ
p65
IL-6,TNF-α,etc.
1
SOCS1
2
3
IκBα

Figure 4.6

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Chapter 6: Conclusions and Future Studies

The central objective of this project was to elucidate the involvement and interactions of toll-like receptor 4 in immune responses through LPS stimulation. Treatments in cell culture were done in both bone marrow derived macrophage as well as human macrophage cell lines to assess the several roles LPS plays during cell signaling processes involved in inflammatory responses.

Overall, with obesity issues being at the forefront of several health concerns, it is important to understand the underlying cellular and molecular mechanisms involved. The first set of conclusions contributes to the future of obesity related therapeutics. There have been many attempts to use leptin as a therapeutic intervention for weight loss with little knowledge of the alternative effects. However, we show that leptin will prime the cells in the body preferably in macrophage cells for inflammation via a mechanism that has not been studied. Our work suggest that LPS is in fact a part of this mechanism by synergizing with leptin to induce proinflammatory cytokines and ultimately leads to inflammatory complications such as obesity and diabetes. This information is novel as LPS is a very potent and endogenous endotoxin responsible for playing several roles in the innate immune system.

IRAK-1 which is a key adaptor protein involved in the innate immunity process plays a central role in the activation of several signaling processes. We show here for the first time leptins involvement in the innate immune system is by its interaction with IRAK-1 as well as its ability to initiate downstream signals by low dose LPS activation.

Not only is the priming effect of leptin and LPS dependent on IRAK-1 but importantly, leptin induces mRNA levels and increase protein expression of IRAK-1. The fact that there is an interaction between leptin and IRAK-1 gives insight to the question of how leptin is involved in the inflammatory process and the mechanism behind it. However, still several questions remain and need to be examined to understand the interaction between leptin and IRAK-1. Further molecular studies are warranted to examine the transcriptional regulation of IRAK-1 induced by leptin.

Intriguingly, IRAK-1 has not only played a significant role in unraveling the mystery of leptins involvement in inflammation, but an even important role in the TLR-4 MyD88 independent pathway. Limited studies have been done to examine the mechanisms involved in metabolic endotoxemia induced signaling. Our studies also report for the first time this pathway is activated by similar LPS levels found in the microbial intestinal gut and has been shown to be sufficient for the induction and sustainability of proinflammatory cytokines. Interestingly, IκBα independent pathway is activated in response to minute exposure to LPS.

Subsequently, our experimentation shows that minute levels of LPS are capable of priming the body for high-fat diet induced obesity complications, however several questions remain about how the gut microbiota contribute towards the pathophysiological regulation of endotoxemia and set the tone of inflammation for occurrence of diabetes and/or obesity. Scientists may take into consideration a key question of how we can manipulate the microbiotic environment to treat or prevent

obesity and type 2 diabetes. Thus, it would be useful to find specific strategies for modifying gut microbiota to impact on the occurrence of metabolic diseases while understanding the key protein-protein interactions involved in the pathway studied during this study.

Though the immunology field has come a long way over the years in its discovery of key receptors, agonists, transcription factors and interactions of pathways involved in the development of both stronger and weaker immune systems, still several new avenues have been opened. Our research findings play an intricate role in the enhancement of knowledge controlling our immune systems.