A Mechanism for the Metabolic and Inflammatory Alterations Associated with Low-dose Endotoxemia

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(Abstract)

Lipopolysaccharide (LPS), a Gram-negative endotoxin, has been well-established as the trigger for the effects of sepsis and septic shock through its binding with the innate immune receptor, Toll-like receptor 4 (TLR4). High doses of LPS signal through TLR4 to produce a massive release of pro-inflammatory cytokines including IL-6, TNFα, and other. Additionally, several recent publications have demonstrated severe metabolic alterations after LPS challenge, suppressing lipid oxidation and concurrently up-regulating glucose oxidation. Unfortunately, this switch in metabolism is inefficient for the great energy demands of the host during a systemic microbial infection which can result in vital organ failure.

Meanwhile, a novel concept in several chronic disease pathologies also implicates LPS, although at very low doses. The presence of subclinically elevated circulating endotoxin levels has been termed metabolic endotoxemia and is beginning to be investigated in disease pathologies including insulin resistance and type II diabetes, atherosclerosis, cancer metastasis and Parkinson's disease. These disease phenotypes all possess a component of chronic inflammation whose source has not largely been understood, but examining the effects of very low doses of LPS may provide vital information in understanding their etiologies.

However, most information on LPS signaling has been obtained using high doses of LPS (10-200ng/ml) while little to no studies have been published regarding the effects of very low doses of LPS (1pg-100pg/ml) on inflammatory and metabolic alterations. Thus, we use *in vivo* and *in vitro* models to determine that both IRAK1 and JNK are critical points of crosstalk downstream of TLR4 for the metabolic and inflammatory alterations associated with metabolic endotoxemia. Additionally, we observed significant down-regulation of nuclear receptors responsible for fatty acid metabolism, including PGC1α, PPARα, and PPARγ after very low dose LPS challenge. Further, we observe phenotypic changes in fatty acid oxidation and glucose oxidation, as well as subsequent changes in cytosolic acetyl-CoA levels and acetylation of pro-inflammatory transcription factor ATF2. Overall our studies point to several mechanisms of cross-talk between metabolism and inflammation and offer significant support to the concept of metabolic endotoxemia in the development of chronic disease.

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Dedication

I dedicate this work to my family and to the Lord. Thank you so much for all of your love and support, I am so blessed and so grateful for you all! Thank you to Jesus for leading me perfectly through all of the ups and downs and being with me always. To my husband Simon, who has been my source of unending support and encouragement through all the thick and thin. To my sister Sara, for being an ear to hear my heart, and to my mom for her countless unselfish sacrifices throughout my life. To my brother Seth for always making me smile and to Gary for your quiet but continual support. To uhmuhni, for your prayers and delicious food, and to my dad for all of the wonderful childhood memories. Also a huge thank you to all of my friends and family not mentioned here, I would truly not be who I am without you! A special thanks also to Cornerstone Christian Fellowship and Dwelling Place for being an amazing family away from home!

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Abbreviations

ABCA1	ATP-binding cassette transporter, subfamily A, member 1
ACoA	Acetyl Co-A
Apoe	Apolipoprotein E
ATF2	activating transcription factor 2
BMDM	bone-marrow-derived macrophage
CPT1α	carnitine palmitoyl transferase 1α
FAO	fatty acid oxidation
FFA	free fatty acid
GTT	glucose tolerance test
HDL	high density lipoprotein
HFD	high fat diet
iMM	immortalized murine macrophage
IR	insulin resistance
IRAK1	Interleukin-1 receptor associated kinase 1
JNK	c-Jun N-terminal kinase
LDL	low density lipoprotein
LPS	lipopolysaccharide
Mϕ	macrophage
MCAD	medium chain acyl dehydrogenase
MEF	murine embryonic fibroblast
ND	normal diet
NR	nuclear receptor

PBS	phosphate-buffered saline
PDK4	pyruvate dehydrogenase kinase 4
PGC1α	Peroxisome proliferator-activated receptor gamma coactivator 1α
PPARα/γ	Peroxisome proliferator-activated receptor α,γ
T2DM	type II diabetes mellitus
TG	triglyceride
THP1	human acute monocytic leukemia cell line
dTHP1	differentiated THP1 cell
TLR4	Toll-like receptor 4
VLDL	very low density lipoprotein

CHAPTER 1: A Review of Current Literature Regarding the Novel Concept of Metabolic Endotoxemia

1.1 Introduction

An emerging concept in the field of innate immunity is the implication of subclinical, persistent low-grade inflammation in the pathogenesis of several chronic diseases responsible for significant morbidity and mortality worldwide. In many of these models, the Gram negative bacterial endotoxin, lipopolysaccharide (LPS), has been targeted as a potential mediator of the underlying inflammatory conditions which give rise to disease. The phenomenon of subclinically elevated levels of endotoxin in the bloodstream has recently been termed “metabolic endotoxemia”. Many important disease pathologies are now being investigated under the lens of metabolic endotoxemia, including atherosclerosis, diabetes and insulin resistance, Parkinson’s disease, and cancer. Several hypotheses have been developed regarding the sources of this low-grade LPS exposure, including smoking, aging, chronic heavy alcohol consumption, high fat diet and periodontal disease. These sources of endotoxin will be discussed, as well as the consequences and implications of persistent, low-grade endotoxemia. Interestingly, most of the research regarding endotoxemia has been performed within the paradigm of sepsis and septic shock, therefore allowing a solid amount of information to be collected regarding the LPS signaling pathways and phenotypes of high-dose endotoxemia. Only recently has the concept of low-dose

endotoxemia begun to be accepted as a critical model for chronic disease etiology. Thus, the pathogenesis of low dose endotoxemia is just beginning to be explored. In this review we will examine the causes and consequences of low-dose endotoxemia, review the current understanding of LPS signaling, and address the studies necessary to further our understanding of this phenotype.

1.2 Sources of Low-Dose Endotoxemia

1.2.1 *Smoking*

It has widely been accepted that smoking increases the risks of chronic obstructive pulmonary disease and lung cancer (1). More recently, tobacco smoke has been implicated in several other respiratory illnesses and diseases, including asthma, influenza, and interstitial lung disease (1). Even so, there continues to be progress in the understanding of cigarette smoke composition and deleterious effects, though there is still much to learn. Interestingly, a handful of recent studies have indicated that the amount of bacterial endotoxins in cigarette smoke may be much greater than the small number of organisms isolated and cultured from cigarettes previously. For instance, a study in 2009 performed a microarray analysis on non-smoked cigarette samples and led to the identification of several Gram negative bacterial genuses, including *Clostridium*, *Klebsiella*, and *Pseudomonas* which were all identified in over 90% of the cigarette samples tested (1) These bacteria had not been previously isolated, but contribute to other current findings regarding cigarette smoke and endotoxin levels. For

instance, several studies have come forth showing a significant association between cigarette smoke and environmental LPS levels (3, 4). In terms of actual LPS exposure levels, Hasday's group was the first to demonstrate that bioactive bacterial endotoxin levels in both mainstream and second hand cigarette smoke ranged from 18± 1.5ng to 120± 64ng per cigarette (2). In this study, the differences in LPS plasma levels detected by LAL (limulus amebocyte lysate assay) were not different between smokers and non-smokers. However, the study demonstrated that the levels of LPS detected in the cigarette smoke were comparable to the levels required to cause chronic respiratory illness. Meanwhile, a separate study by Weidermann's group demonstrated an association between higher plasma endotoxin levels in ex-smokers and those with chronic infections than non-smokers and some current smokers, using the same LAL assays (5). Therefore, although a direct correlation between smoking and plasma LPS levels has not been robustly established, it is reasonably well-established that smoking increases environmental and respiratory endotoxin exposure and sets up a model of chronic inflammation coupled with decreased immune function. This may allow for increased incidence of chronic infection in chronic smokers, providing for prolonged or increased exposure of the patient to microbial antigens and endotoxin. This theory is in part supported by studies such as the studies by Doyle et al and Valenca et al, which found that chronic cigarette smoking alters the host defense system, allowing for increased incidence of respiratory infections (6, 7). Thus, smoking, either through increased LPS exposure or decreasing innate immunity functions, may allow for higher levels of LPS in the bloodstream, although at a subclinical level (5).

1.2.2 *Aging*

"Inflamm-aging" is a relatively new concept in the field of innate immunity, but together with several other studies, offers the idea that the process of aging is a source of progressive, low-grade inflammation. Data from several groups have demonstrated a consistent increase in the pro-inflammatory status of aged individuals (over 50 years old) compared with younger individuals (8, 9). "Inflamm-aging" has also been backed by other studies which demonstrate increased levels of pro-inflammatory cytokines in both aged mice and humans compared with their younger counterparts (10,11). Pro-inflammatory cytokines such as IL-6, as well as acute phase proteins are elevated in such individuals; these elevated levels have also been associated with increased levels of morbidity and mortality. Meanwhile, even in healthy centenarians, there is a steady rise in IL-6 levels (8). Thus, the inflamm-aging theory determines that ongoing environmental stresses, such as previous infection and other stressors cause the increase in these pro-inflammatory markers. Combined with an individual's genetic background, the compilation of environmental stressors over an individual's lifetime determine the degree of morbidity and time of mortality of that individual (8).

An emerging idea in regard to this phenomenon is the process of immune senescence that develops with age, largely due to the changes in neutrophil and macrophage functions such as phagocytosis and apoptosis (9). Although not inherently obvious, the gradual loss of innate immune function during the process of aging increases the risk of chronic infection and disease, therefore eliciting an increase in pro-inflammatory cytokines and mediators to persistent antigenic stressors. The partial inability of the

aged individual to completely rid themselves of such infections may then serve as a source of low-grade endotoxemia (9).

1.2.3 *Chronic Alcohol Consumption*

Chronic alcohol consumption and its correlation to alcoholic liver disease have been well-established. However, in examining the potential etiologies in the development of alcohol-induced hepatic disease, several studies have introduced the idea that chronic alcohol consumption causes changes in the bacterial flora of the gut and in the gut's mucosal integrity. These changes allow for an increase in endotoxin leaking from the gut and into circulation, setting up both a model of chronic inflammation and the background for the development of hepatic disease (12-17). In experiments with dogs and rodents, alcohol was administered orally at levels equivalent to those found in common alcoholic beverages. The mucosal integrity of the gut of these animals was significantly compromised upon histological evaluation and was most prominent in the duodenum (12, 13). These findings are in agreement with a second study which published correlations between alcohol consumption and duodenal bleeding (12). Meanwhile, the effects of chronic alcohol consumption on gut mucosal integrity have produced conflicting results, but several studies have demonstrated a significant increase in bacterial overgrowth and population differences after chronic alcohol consumption (12-14). It follows that loss of gut integrity also leads to increased gut permeability—which has been reported in several studies (12-17). Moreover, a study by Fukui in 1991 demonstrated transiently elevated levels of endotoxin in healthy and

alcoholic individuals after consuming an alcoholic drink (17). Another study by Parlesak in 1999 further demonstrated that the levels of endotoxin in the plasma of alcoholics were over five times greater than plasma endotoxin levels of healthy non-alcoholic controls. The group also determined that gut permeability in alcoholics was significantly increased compared to healthy individuals (16). Thus, alcoholism and chronic alcohol consumption can be considered a primary etiology for significant low-grade endotoxemia.

1.2.4 *High Fat Diet*

One of the prominent, emerging theories regarding the etiology of metabolic endotoxemia is the consumption of high fat diets. In this model, consumption of a high fat diet (HFD) is believed to facilitate gut absorption of endotoxin as well as cause gut flora changes that allow for endotoxin to enter systemic circulation. Several papers have been published examining the relationship between high fat diet and plasma endotoxin levels, as well as shifts in gut microbiota. A study by Cani et al in 2007 was the first of its kind to demonstrate that HFD fed to mice for 4 weeks caused the plasma LPS levels to rise 2-3 times higher in than plasma LPS levels in mice fed regular diet (18). To confirm these findings, the investigators went on to assess differences in absorption of LPS with a high fat content vehicle, oil, and a control vehicle, water. Mice administered LPS with oil orally demonstrated higher levels of LPS in the plasma compared to mice administered LPS with water. Thus, part of the detrimental effects of HFD appears to be the increased uptake of LPS into chylomicrons, which allow for its direct entry into

circulation (18). Interestingly, 76% of T2DM patients in one study demonstrated elevated levels of circulating LPS compared with non-diabetic matched controls. Thus, the presence and chronic induction of TNFα and other pro-inflammatory cytokines through TLR4 likely plays a major role in the development of T2DM.

Meanwhile, several other studies have examined the effects of HFD on gut microbiota, alluding to the hypothesis that changes in gut microbiota could allow for greater amounts of LPS to be released into circulation. Another study published by Cani et al demonstrated that HFD induced a significant decrease in both Gram negative and Gram positive cecal bacterial populations. Of particular note, cecal *Bifidobacteria* populations in mice fed HFD were significantly decreased compared to mice on a standard rodent diet. Mice administered *Bifidobacteria* prebiotics with the HFD did not develop endotoxemia, nor did they exhibit the levels of pro-inflammatory cytokines compared to mice fed HFD only (19). Further, a separate study by Manco et al was published a few years after the *Bifidobacteria* study, contributing to the theory that diet-induced changes in gut microbiota contribute to the development of metabolic endotoxemia and ultimately clinical signs of chronic disease. This newer study has focused on *Bacterioides* and *Firmicutes* populations, which were significantly different in lean versus obese individuals (20). Meanwhile, yet another study determined that high fat diet alone (regardless of whole body adiposity) can independently change the populations of these gut bacterial populations, as both wild type mice and mice with a gene deletion leaving them obesity-resistant demonstrated significant changes in gut bacterial populations, including the increase in *Firmicutes* and decrease of over 30 *Bacterioides* species (21). Moreover, when adult human fecal material is introduced into germ-free mice that are

subsequently placed on a high-fat, high-sugar diet a month after the colonization, both cecal and fecal bacterial contents are significantly different compared to mice fed a normal diet (22). One of the current theories regarding shifts in gut microbiota and the development of metabolic endotoxemia is that altered gut populations may affect the amount of lipid absorbed by the gut, therefore allowing greater amounts of LPS into circulation. Additionally, the differences in gut bacterial population dynamics and the general increase in bacterial load may also increase the risk of LPS "leaking" into the bloodstream.

1.2.5 *Periodontal Disease/Localized Chronic Infection*

Another increasingly recognized source of low-grade endotoxemia is that of periodontal disease. In Finland alone in a survey from the year 2000, 64% of adults had some form of periodontal disease and 21% had severe disease (32). Periodontal inflammation, or periodontitis, is caused by persistent bacterial infection in the tissues surrounding the teeth. The chronicity of the infection causes tissue damage and can ultimately result in tooth loss. Interestingly, the development of periodontitis is mainly associated with Gram negative bacteria (24). Importantly, a substantial number of studies have been published in the last ten years regarding the association of periodontal disease with other chronic systemic diseases, namely cardiovascular disease and Type II diabetes (25, 26). Studies previous to the turn of the millennium started documenting bacteremia associated with gingival inflammation, even dating back to the mid 1970's. These clinical correlations were observed under varying conditions, including dental

procedures, and toothbrushing in patients with both healthy gingiva and those with evidence of periodontal disease, although only transiently (27). Since then, several Gram negative bacterial genuses have been identified in these diseases, including *Porphyromonas* and *Actinobacillus* bacteria (28). Interestingly, as a side note, Porphyromonas species have been isolated from atherosclerotic lesions, although this will be discussed in a later section. However, it was not until 2002 that a study was published documenting for the first time a positive correlation between periodontal disease and low-grade levels of circulating endotoxin (29). In this study, plasma endotoxin levels pre-mastication were not statistically significant between patients with ranging degrees of periodontal disease (patients with concurrent diseases were excluded from the study). In patients with mild and moderate periodontal disease, levels of endotoxemia were slightly elevated after mastication. However, in patients with severe periodontal disease, the increase in plasma endotoxin levels was much greater and was statistically significant (29). A second study in 2004 confirmed the positive correlation between severity of periodontitis and circulating LPS levels, as well as its role in macrophage activation (30). A second study analyzing correlations between periodontitis and endotoxemia by Pussinen in 2007 demonstrated that patients with higher IgG levels against pathogenic periodontal bacteria also demonstrated higher levels of endotoxemia (31). As we will discuss later, elevated levels of both IgG and endotoxemia were positive and significant risk factors for cardiovascular disease.

1.3 Current Understanding in LPS Signaling

As mentioned previously, low-dose or metabolic endotoxemia is an emerging concept in the field of innate immunity and inflammation. Significant correlations have been established between the presence of elevated but subclinical levels of circulating endotoxin and chronic disease pathologies, including the obesity-insulin resistance-type II diabetes axis, atherosclerosis and cardiovascular disease (CVD), neurological degeneration diseases such as Parkinson's disease, and cancer metastasis and tumor growth. However, the majority of the literature has been focused on the paradigm of high dose endotoxemia which is associated with acute sepsis and septic shock. Thus, much of the LPS pathway detail and investigation has been examined under high dose LPS challenge. It follows that we will review what has been discovered in regards to LPS signaling through this high dose model, before addressing the potential differences in low-dose mechanisms in the next section of this review.

1.3.1 *TLR4 Structure*

It has been well-established that LPS signals through the innate immune receptor, TLR4, which is present at the surface of cells almost ubiquitously (36). The end effect is the activation of the transcription factors NFkB and AP-1, which lead to the increased expression of pro-inflammatory genes such as IL-1, IL-6, TNFα, and others. The Toll-like receptor itself is a mammalian homologue of the Drosophila Toll receptor. The mammalian Toll-like receptors (5 subtypes) all share basic structure homology. Mammalian TLRs all possess a similar structure containing an extracellular portion, a

trans-membrane domain, and an intracellular domain. The extracellular domain contains a leucine-rich repeat (LRR) which is accepted to be the method of pathogen detection. Meanwhile, the cytoplasmic portion of the mammalian TLRs share homology with the cytoplasmic portion of the IL-1 receptor, and this domain is termed the TIR domain, or Toll-IL-1 receptor domain. The TIR domain is responsible for the downstream activation of inflammatory pathways (35, 36).

1.3.2 *TLR4 Trafficking and LPS binding*

In circulation, the majority of LPS is bound to plasma lipoproteins. Healthy individuals tend to have LPS bound to a higher ratio of HDL, while LPS in sepsis patients exhibits increased binding to VLDL proteins (32). Interestingly, but for discussion later in this review, LPS bound to LDL and VLDL is more readily taken up by macrophages and causes their transformation into foam cells. Regardless, LPS binding protein (LBP), a lipid and phospholipid transfer protein, binds to this circulating LPS and transfers it to another accessory protein, CD14 (37). CD14 is a pattern recognition protein present in complex with TLR4-MD-2 as well as in circulation. CD14 is the accessory protein which recognizes antigenic patterns from both Gram positive and Gram negative bacteria (pathogen-associated molecular patterns, PAMPS), and is found in both soluble and membrane bound forms. Interestingly, both LBP and soluble CD14 (sCD14) are elevated in patients with chronic periodontitis (33).

Meanwhile, TLR4 is unique in its ability to signal from the cell surface as well as intracellularly (127). During the resting state, TLR4 traffics between the Golgi apparatus and the plasma membrane in a complex with accessory proteins MD-2 and CD14 (37).

MD-2 is a secretory accessory protein necessary for the TLR4 induced activation of NFkB (35) and is critical for the trafficking of TLR4 from the Golgi to the plasma membrane. MD-2 is also plays a role in determining the degree of cell responsiveness, as the number of TLR4 receptors present on the surface of the cell partially regulates the magnitude of the cell's response to LPS (37). Once the TLR4 complex reaches the surface and binds with circulating LPS, TLR4 undergoes tyrosine phosphorylation of its TIR domain at high doses of LPS. This phosphorylation is necessary for the signaling of TLR4 downstream to activate its target transcription factors NFkB, AP-1 and mitogen-activated protein kinases (MAPKs) (36), as it allows the TIR domain to interact with Mal and MyD88 proteins, activating the classical TLR4 pathway. Alternatively, TLR4 can be trafficked endosomally upon activation by LPS at the cell surface and lead to a different cellular response. These two pathways are discussed in more detail below.

It is also noteworthy to mention that at high to ultra-high concentrations of LPS (>1ug/ml), TLR4 signaling becomes CD14 independent (34). Further, several other molecules have been documented as associating with TLR4 during LPS challenge, including CD11b/CD18 (Mac-1), CD36 and Dectin-1 (36).

1.3.3 *The Classical vs. Alternative TLR4 activation pathways*

The classical activation pathway is dependent on the recruitment of the adaptor molecule MyD88 to the activated TLR4 complex (38). Through subsequent IRAK4/IRAK1 recruitment and phosphorylation, the activation of TRAF6 leads to the activation of NFkB. NFkB then promotes the transcription and expression of pro-inflammatory mediators such as IL-6 and TNFα (39-42). The classical activation

pathway is also responsible for the activation of MAPK pathways and the downstream activation of other pro-inflammatory transcription factors such as C/EBPδ (39). At high doses of LPS, IKK proteins catalyze the phosphorylation of the inhibitory subunits of NFkB (IkB), allowing for their disassociation and degradation. The p65 subunit of NFkB is then translocated into the nucleus to initiate the expression of pro-inflammatory mediators such as TNFα, IL-1β, IL-6, MCP-1 and others (40). Additionally, LPS can signal through the classical activation pathway, leading to the activation of c-jun N-terminal kinase (JNK), which is responsible for the activation of c-Jun, ATF2, and others (43).

Interestingly, TLR4 can also signal without the assistance of MyD88, through the recruitment of two other proteins, TRAM and TRIF. TRAM and TRIF appear to co-localize at endosomal membranes after interaction with activated TLR4 and it is now understood that the recruitment of TRAM and TRIF to TLR4 causes the activation of IRF3 and the production of interferon. These two cellular proteins associate with activated TLR4 after it has been internalized via endosome (37, 128, 129). This pathway also leads to the late activation of NFkB, as well as IFNβ (37, 44). This pathway has been termed the alternative pathway. Of note, the TRAM-TRIF endosomal pathway (also referred to as the MyD88-independent pathway) is believed to be responsible for MHC class II-like presentation, affording dendritic cell maturation and providing communication between innate and adaptive immune responses (37, 44).

Of note, it is also critical to note that TLR4 can also be activated by high levels of saturated fatty acids as well as LDL, although this will not be reviewed in detail (45-47).

1.4 Crosstalk between Metabolism and Inflammation

1.4.1 *Metabolic Regulators*

Nuclear receptors (NRs) comprise a large family of regulatory proteins which all share similar structural homology. There are three main classes of these receptors—the well-known glucocorticoid and sex hormone receptors, the orphan receptors to which regulatory ligands have not been identified, and the adopted orphan receptors, originally classified with the orphan receptors but to whom regulatory ligands have now been identified. The adopted orphan receptors briefly discussed below have been identified as being responsible for regulating inflammation that can be activated through TLR4, as well as regulating metabolism in terms of cholesterol regulation, lipid oxidation and glucose utilization (48. 49). Also to note, it is being increasingly recognized that NR functions can be very specific to the cell type, and that variations in cell type can elicit different behavior through TLR4 or NR activation (48).

1.4.2 *PPARs*

PPARs were only identified in the last 20 years and were first named for their activation by chemicals that induced peroxisomal proliferation in rodents (50). There are three main subtypes, α, β/δ and γ. All three subtypes are activated by fatty acids and their metabolites, including prostaglandins. However, while all three subtypes play roles in metabolism, inflammation, and differentiation, their tissue distributions are more specific and they each have a specific set of target genes. For instance, PPARγ is able to activate lipogenic genes and is essential for adipocyte differentiation as well as insulin

sensitivity (51). Meanwhile, the other subtypes promote lipid catabolism and are found in tissues with high β-oxidation activity such as heart, muscle, and liver (50, 52, 53). PPARs are also responsible for scavenging oxLDL and inhibiting inflammatory migration molecules (Hong). Additionally, PPARs have several accessory proteins that function as co-activators. These co-activator interactions are also dependent on PPAR subtype. For instance, PPARα and PPARγ both utilize PGC1α, an important co-activator for PPARα-regulated β-oxidation (50).

1.4.3 *PGC1α*

NR co-activators such as PGC1α play a critical role in the crosstalk between metabolism and inflammation. Not only does PGC1 serve as a co-activator to PPAR receptors, but it also directly regulates glucose oxidation through genes such as PDK4, the master switch between glucose and lipid oxidation. Of note, there have been a couple of studies demonstrating that both the p65 subunit of NFkB and PGC1α are able to mutually repress each other, and the activity is dependent on the degree of association between the two proteins. However this has yet to be further elucidated (54, 55).

1.4.4 *RARs/RXRs*

RARs and RXRs are another class of nuclear receptor whose ligand is retinoic acid (RA). RAR has three subtypes, α, β, and γ, while RXR subtypes include α, β, γ as well. RXRs will often heterodimerize with RARs to promote their binding to RA target genes. Deficiency in RAR/RXR leads to inhibited growth and developmental malformations.

Interestingly, since RXR can also heterodimerize with other nuclear receptors such as PPARs, the presence or absence of RA can influence its behavior (56). RXR itself plays a role in drug and fat metabolism, and its ability to heterodimerize with other nuclear receptors makes its function critical to a vast array of functions. It has also been documented that RARs can be involved in AP-1 and NFkB target gene inhibition at both the message and translational levels (56).

1.4.5 *LXRs*

LXRs are activated by cholesterol metabolites and functions to increase cholesterol efflux from cells through ABCA1 and ABCG1 genes (51). LXRs also heterodimerize with RXRs and are divided into α and β subtypes. LXRα is expressed in visceral organs and macrophages, while LXRβ is ubiquitously expressed (51).LXR activation results in increased HDL levels and reduced incidence of atherosclerosis. Additionally, LXR activation inhibits the expression of several pro-inflammatory mediators including iNOS and TLR4-induced inflammatory chemokines (51). In turn, LXR can be inhibited through LPS activation of IRF3 (57).

1.4.6 *Inflammatory Gene Trans-repression*

A novel, emerging concept related to crosstalk between inflammatory and metabolic pathways has recently begun to be identified. Relationships between specific nuclear receptors responsible for the regulation of metabolism and inflammatory transcription factors such as NFkB are beginning to be elucidated. It is now being accepted that many nuclear receptors including PPARγ and RAR serve as trans-repression

mechanisms to prevent inflammatory responses (48, 58, 59). The following nuclear receptors, their functions in metabolism, and their currently defined roles in transrepression are briefly discussed below. Interestingly, in the case of PPARs and LXRs, these NRs are able to heterodimerize with (retinoid x receptors) RXRs both in the presence or absence of ligand. In the absence of NR ligand, heterodimerization allows for the nuclear receptors to function as a trans-repression mechanism through their interactions with co-repressor complexes such as NCoR and SMRT. These nuclear receptors can also indirectly associate with genes downstream of NFkB and AP-1 pro-inflammatory transcription factors. Meanwhile, in the presence of NR ligand, heterodimerization also allows for the NR-induced recruitment of co-activators, allowing for the up-regulation of specific gene target expression (48).

In the case of pro-inflammatory genes downstream of TLR4, the sumoylation of several NRs including PPARγ and LXR have been demonstrated to suppress the inflammatory response through inhibition of NCoR ubiquitination. NCoR ubiquitination functions to allow the disassociation of the co-repressor from the inflammatory gene under repression. Thus, the binding of sumoylated NR to NCoR prevents NCoR ubiquitination, allowing for the repression of inflammatory genes to be maintained (60, 61). Functionally, the removal of NCoR from target pro-inflammatory genes downstream of activated TLR4 is dependent upon the p65 subunit of NFkB, which associates with IKKε to bind to NFkB target sites. Meanwhile, these sites are in close proximity to JUN on the AP-1 complex, allowing for JUN phosphorylation. This JUN phosphorylation then promotes the ubiquitination of NCoR (48).

The phenotype of NR trans-repression of pro-inflammatory genes is evident in the fact that PPARγ deficient animals exhibit a significant inflammatory status and predispose these animals to inflammatory disease (62).

1.4.7 *Nuclear Receptor Inhibition*

Reciprocally, NRs can be inhibited by activation of TLR4. Interestingly, in several clinical trials, PPARγ agonists were administered to patients with ulcerative colitis (UC) but showed little to no improvement in condition (63). In mouse studies as well, only prophylactic administration of PPARγ agonists was preventative of UC (62). Thus, there appears to be a balance between repression of pro-inflammatory genes and inhibition of nuclear receptor activity. In fact, TLR4 activation in one study has demonstrated an inhibition in PPAR mRNA synthesis through NFkB in murine macrophages. Additionally, the same group demonstrated that PPARγ deficiency in mice was alone enough to cause an increase in expression of pro-inflammatory mediators (62). Thus, the exact mechanisms of NR inhibition have yet to be deciphered, but it is clear that a strong inflammatory program is able to come back and inhibit NR expression and function. It is in this inhibition that the pathology of several chronic inflammatory diseases, including diabetes, insulin resistance, atherosclerosis, and others, may lie. Therefore, thorough future investigation into TLR4-induced NR inhibition is greatly warranted.

1.5 Low-dose Endotoxemia: A Novel Mechanism

Importantly, the inflammatory phenotype of metabolic endotoxemia is much different than that of high dose endotoxemia and septic shock. In high dose models, there is a large release of pro-inflammatory cytokines and chemokines, as well as adhesion molecules. However, the metabolic endotoxemia phenotype demonstrates “leaky”, low levels of pro-inflammatory mediators. Thus, the mechanism behind the two different phenotypes must be unique. As mentioned previously, high dose endotoxemia allows for the activation of a number of pro-inflammatory transcription factors including NFkB, AP-1, MAPKs, and C/EBPs. The high dose mechanism also possesses numerous negative feedback loops within the system in order to keep the inflammatory state in a relative check (64, 65). Interestingly, our group has just recently demonstrated that at very low concentrations of LPS (50-100pg/ml), the NFkB pathway is not activated, although there is still a significant yet smaller increase in pro-inflammatory mediators such as IL-6, MCP-1, and ET-1 (66). However, C/EBPδ is activated at this LPS dosage and is IRAK1-dependent. Interestingly, the activation of C/EBPδ was found to also be IKKε-dependent, as IKKε is downstream of IRAK1. Also of note, the expression of C/EBPδ is critical for the induction of IL-6 and TNFα, which are two cytokines whose chronic, low levels of expression are implicated in the development of insulin resistance, obesity and Type II diabetes.

Further, at this very low dose of LPS, our group has demonstrated the removal of nuclear receptors PPAR and RAR from the promoters of inflammatory genes in WT but not IRAK1$^{-/-}$ murine bone marrow derived macrophages (66). Of additional significance,

a separate paper by Wiesner's group has also demonstrated that levels of minimally oxidized LDL equivalent to that found in high fat diet feedings was also able to activate ERK1/2 and cause the activation of JNK. Importantly, Wiesner's group also determined that the combination of LDL plus an LPS component was able to synergize and cause a prompt and sustained activation of ERK phosphorylation and thus AP-1 gene transcription (67). Much more research must be performed to clarify the very-low dose LPS pathway and potential synergism with oxidized lipid, but these few published reports clearly demonstrate a difference in pathways between high dose and low dose LPS.

A handful of other studies have also contributed significant information in the understanding of the mechanism behind metabolic endotoxemia. For instance, Blomkalns et al demonstrated that at LPS concentrations down to 3pg/ml and consistently at concentrations down to 10pg/ml, there was a substantial increase in IL-8 expression and respiratory burst as low as 30pg/ml within human peripheral blood monocytes in culture (68). This group also demonstrated that these reactions were CD14 and TLR4 dependent.

Another important question lies in the fact that while high dose endotoxemia causes both pro- and anti- inflammatory mediator up-regulation, little is known about whether low-dose endotoxemia also up-regulates anti-inflammatory mediators as a balance for the low expression of pro-inflammatory signals. It appears from the phenotype that a constant exposure to very low doses of LPS allow for a small but significant increase in pro-inflammatory cytokines and chemokines which may or may not be at levels to elicit a negative feedback loop response. This also points to the chronicity of low-grade

inflammation observed in many of the disease pathologies that are beginning to be associated with very low dose LPS challenge. Thus, this model provides a possible explanation for the development of low-grade, chronic inflammation, at least in part due to the lack of an appropriate counter response through the inadequate expression of anti-inflammatory mediators and negative feedback loops. Much more research must be performed to determine the dynamics of very low-dose (metabolic) endotoxemia.

1.6 Metabolic Endotoxemia and Chronic Disease

1.6.1 *Diabetes/insulin resistance*

There have been many theorized etiologies behind the development of obesity and type II diabetes (T2DM). Recently, it is has been established that obesity and T2DM are often characterized by a generalized inflammatory state which induces insulin resistance before the onset of other clinical signs (126). Likely, a combination of genetics and environmental conditions (namely diet and exercise) are responsible for the onset of these diseases. High fat diet has been shown in several studies to increase circulating lipopolysaccharide (LPS) levels as well as cause the recruitment of pro-inflammatory macrophages and the formation of crown structures in adipose tissue (over 40% of adipose tissue cell content being macrophages) (69, 70). It is also now widely accepted that adipose tissue is not only a storage cell for lipid, but is a highly metabolic, highly secretory tissue capable of influencing the inflammatory state of the host. Elevated plasma endotoxin levels, as well as the secretion of pro-inflammatory

cytokines such as IL-1β, IL-6, and TNFα by both adipose tissue macrophages (ATMs) and adipocytes themselves are now being recognized as risk factors for T2DM (71, 130, 132).

1.6.2 *Inflammation and Obesity*

Although obesity is not synonymous with insulin resistance (IR), it is a recognized factor in the contribution to metabolic syndrome and other IR-related inflammatory diseases, including heart disease, hypertension, and T2DM, among others. Likely, a dual etiology between obesity contributions to systemic inflammation and systemic low-grade inflammation contributions to obesity exists. Recently, adipose tissue has begun to be recognized as not only a highly metabolic tissue, but also one that is highly capable of influencing an individual's inflammatory profile (69, 72, 73). Adipose tissue is composed of several fractions: the adipocytes themselves and the stromal vascular fraction (SVF) which contains red blood cells, endothelial cells, and macrophages (74). In the obese state, the increased amount of circulating FFAs in combination with the potential increase in circulating endotoxin, as well as the hypoxic state in adipocytes toward the center of the fat tissue is a substantial stimulus for inflammation. Crown-like structures develop in activated adipose tissue, composed of M1 macrophages (ATMs) expressing CD11c surface markers and surround necrotic adipocytes and residual extra-cellular fat droplets (71). A separate paper published by Suganami et al demonstrated significant paracrine effects between macrophages and adipocytes co-cultured together, where TNFα secreted by macrophages stimulated the secretion of pro-inflammatory chemokine MCP-1 in the adipocytes (75). In turn, this TNFα was demonstrated to be

derived from the pro-inflammatory adipocyte-induced release of FFAs, thus setting up a vicious cycle of inflammation within the two co-cultured cell types. Additionally, lipid accumulation in the liver of obese individuals stimulates the release of pro-inflammatory mediators IL-1β, IL-6 and TNFα from hepatocytes, which then systemically can provide an inflammatory environment contributing to and exacerbating the developing inflammation (76). Additionally, it appears that visceral fat is predisposed to exhibiting an inflammatory phenotype in response to lipid accumulation and adipocyte hypertrophy, although this has yet to be clarified (77).

As mentioned previously, the ingestion of a high fat diet (HFD) has been theorized to contribute to the movement of LPS across the gut mucosa and into circulation. Consumption of HFD also allows for the increase of circulating fatty acids (FAs), which together with LPS may synergize the activation of TLR4, which has been shown to be up-regulated in macrophages in conditions of obesity (67). The activation of the TLR4 pathway subsequently leads to the induction of pro-inflammatory cytokines (69). Of important note, several TLR4 pro-inflammatory pathways are MyD88-dependent. Deletion of MyD88 in the CNS limits diet-induced obesity and markers of insulin resistance (78). From another angle, the increase of FFAs in circulation also appears to activate adipocytes to secrete adipokines which stimulate macrophage migration to these tissues and influence polarization toward an M1 pro-inflammatory phenotype. These tissue macrophages subsequently become a potent source of pro-inflammatory cytokines, namely TNFα, IL-6 and IL-1β, ultimately resulting in the leakage of these cytokines into circulation. Furthermore, in conditions of obesity, it has been demonstrated that Th_1 cells residing in the stromal-vascular (SVC) portion of adipose

tissue are associated with macrophage accumulation and IR in adipose tissue, while T_{reg} cells which promote anti-inflammatory conditions are down-regulated in these stromal fractions (69).

1.6.3 *Inflammation and the Development of Insulin Resistance*

Normal insulin signaling consists of a diverse, complex array of interconnected metabolic pathways which are highly influenced by cross-talk with inflammatory pathways. Normally, insulin is released from the pancreatic β-cells and acts to decrease hepatic glycogenolysis and gluconeogenesis, while promoting glucose transport into glucose-dependent tissues such as skeletal muscle and adipose tissue through the GLUT4 transporter. Insulin also normally acts to decrease lipolysis in adipose tissue via de-phosphorylation and inactivation of hormone-sensitive lipase. In adipose, insulin promotes the breakdown of VLDL into FFAs, as well as inhibiting VLDL formation in the liver. As insulin is distributed throughout circulation, it binds to insulin receptors, causing their auto-phosphorylation and the activation of insulin receptor substrates (IRS). Downstream of these proteins are MAPK and PI3K, which are involved in growth effects and glucose metabolism, respectively. More specifically, PI3K leads to the downstream activation of Akt and phosphorylation of transcription factors responsible for regulating hepatic gluconeogenesis, as well as GLUT4 gene transcription through PPARγ. Thus, in the presence of low-grade chronic inflammation through LPS exposure and elevated FFAs, the activation of pathways downstream of TLR4 and the subsequent increase in pro-inflammatory cytokines interfere with insulin signaling through the inactivation of IRS (72, 76).

Interestingly, obesity can be uncoupled from IR through the inhibition of TLR4 or its downstream components, demonstrating the necessity for inflammation in the presence of obesity for the development of IR. Importantly, several reports have been published linking JNK activation to IR. JNK is a kinase downstream of TLR4 which leads to the activation of pro-inflammatory transcription factors such as ATF-2 and c-Jun (71, 79). Further, its deletion alleviates insulin resistance induced by HFD through the up-regulation of PDK4 and GS (glycogen synthase) activities (80). The inhibition of JNK allows for the switch from glucose oxidation to glucose storage through the up-regulation of PDK4, which acts as a master switch between glucose and lipid oxidation. PDK4 induction causes the inhibition of acetyl-CoA production (ACoA) in the mitochondria, thus decreasing cytosolic ACoA levels and causing the subsequent inhibition of ACoA-carboxylase (80). Additionally, JNK deficiency led to an increase in genes responsible for lipid oxidation including *Pgc1a*, which itself requires the co-activation of SIRT1 for the up-regulation of genes responsible for fatty acid β-oxidation (81). Besides JNK deficiency alleviating insulin resistance, several studies have also determined that deficiency in IKKβ is also significant in the prevention of IR (69, 76). IKKβ is a kinase upstream of NFkB whose activity results in the translocation of the p65 subunit of NFkB to the nucleus for pro-inflammatory gene transcription. However, the exact mechanisms behind JNK and IKKβ –induced insulin resistance has yet to be clearly understood.

To add to the complexity of the IR development, the interactions and balance between anti-inflammatory, catabolic programs with the pro-inflammatory programs of activated TLR4 in innate immune cells and adipocytes play a major role in determining the insulin

sensitivity of an individual. Co-repressor activity plays a major role in the relationship between TLR inflammatory transcription factor activation and nuclear receptor activity responsible for metabolic programs. In adipose tissue and macrophages, the expression of PPARs promotes an M2 polarization and is responsible for maintaining the basal anti-inflammatory tone of adipose tissue via promoting the co-repression of pro-inflammatory genes through co-repressors NCoR and SMRT. However, in our studies and in others, PPAR expression is inhibited after TLR4 stimulation by LPS, and complete knock out of this nuclear receptor promotes inflammation and IR (66). These interactions must be further studied to determine the threshold to developing insulin resistance through the activation of pro-inflammatory genes and down-regulation of these nuclear receptors.

Other factors also contribute to insulin resistance but will only be briefly mentioned in this review. For instance, the accumulation of high levels of glucose intracellularly, as well as the increased utilization of glycolytic pathways causing an increase in intracellular levels of hexosamines and AGEs may cause metabolic stress in the cells through the increase in ROS (reactive oxygen species) (76). Additionally, increase in intracellular lipids can cause direct lipotoxicity to cells not normally accustomed to high lipid accumulation and oxidative stress caused by increased flux of lipids going through β-oxidation can contribute to the activation of IR through mitochondrial and ER stress (76). Interestingly, both JNK and IKKβ activity is increased in situations of metabolic stress and thus may then induce an IR state through their phosphorylation of insulin receptor substrates (IRS) and their induction of pro-inflammatory pathways (79, 82).

1.6.4 *Atherosclerosis*

It has been well-established that endothelial dysfunction is the first step in the progression towards atherosclerosis. This dysfunction can be caused by a variety of conditions, including oxidized LDL (oxLDL), infection, free radical generation, hypertension and diabetes, among others. In a healthy individual, the endothelium plays several roles, including influencing vessel tone, coagulation status, and vessel permeability. When this is perturbed by the presence of chronic inflammation, significant changes in the endothelium result. Normally, nitric oxide (NO) is released by healthy endothelium, and possesses anti-thrombotic, anti-inflammatory properties. NO is normally also a major contributor to vascular tone. However, dysfunctional endothelium loses the ability to secrete NO, and the reduction in NO release from endothelial cells promotes the expression of endothelin-1 (Et-1), as well as encouraging leukocyte adhesion. Et-1 is a vasoconstrictor that is up-regulated in the presence of inflammation and has demonstrated increased expression in atherosclerotic plaques (83).

Sources of chronic inflammation that are attributed to endothelial dysfunction include infection, obesity, hypertension, and hyperglycemia. Further, several studies have demonstrated that chronic low-grade inflammation can be induced by a high fat diet (HFD) (67, 72, 84). Under HFD or other conditions of chronic low-grade inflammation, endothelial cells begin to express ICAM-1 and VCAM-1, which encourage the adhesion of monocytes and T cells to the endothelium. Once attached, these cells migrate to the intima of the blood vessel via the expression of MCP-1, a powerful chemoattractant for monocytes/macrophages (85). Here macrophages take on a pro-inflammatory phenotype and begin to up-regulate scavenger receptors for modified lipoproteins,

allowing for the uptake of these lipids and causing their subsequent transformation into foam cells (86). These foam cells demonstrate altered metabolism and efflux of lipid, thus resulting in gradual lipid accumulation. This accumulation then contributes to the dysfunction and eventual apoptosis of the cell and leads to the formation of the necrotic core characteristic of advanced atherosclerotic plaques. The monolayer of these foam cells and activated T cells that develops initially is termed the “fatty streak” (83, 86). Interestingly, several reports have demonstrated clinical associations between circulating low levels of endotoxin and risk of cardiovascular disease. The first of these studies was published only twelve years ago from an Italian cohort and found that subjects with circulating endotoxin levels at 50pg/ml or greater had a 3-fold greater risk of cardiovascular disease than those with circulating concentrations under 50pg/ml LPS (87). Further, a second major study demonstrated increased that circulating endotoxin levels among different ethnic groups correlated strongly with differences in risk factor for the development of cardiovascular disease amongst the different groups (88). Also of note, several studies have demonstrated that TLR4 up-regulation can be induced by mechanical stress, such as abnormal blood flow in endothelial cells which do not constitutively express TLR4. These endothelial cells capable of inducing TLR4 expression lie in the aortic trunk and arch, which is a common site for atherosclerotic plaque development (89, 90). Importantly, another study by Rice has demonstrated that even in healthy endothelium, LPS concentrations at 100pg/ml were enough to induce TLR4 expression in these cells, as well as increased expression of both MCP-1 and IL-8 (85).

The subsequent transformation of macrophages into foam cells is largely influenced by the cholesterol efflux transporter ABCA1. ABCA1 is part of the reverse cholesterol transport mechanism which is anti-atherogenic. Normally, accumulated cholesterol is transported out of macrophages to HDL or apoA-1 by several mechanisms, one of which is ABCA1-dependent. This is termed reverse cholesterol transport (RCT) (86, 91). The cholesterol is taken through the bloodstream by HDL to the liver where it is metabolized and excreted. However, in the context of inflammation, this process is severely inhibited. The exact mechanisms underlying the suppression of RCT are not completely understood, but it is well-established that ABCA1 is decreased in the presence of LPS (66, 86). ABCA1 is responsible for mediating the efflux of cholesterol and other lipids across cell membranes into HDL apolipoproteins. It is regulated upstream by nuclear receptors LXR and RXR. Additionally, ABCA1 expression is regulated by numerous cytokines. Inflammatory cytokines including IFNs, IL-1β, and platelet-derived growth factor are secreted by T cells present in the developing atherosclerotic lesion and have been shown to influence ABCA1 expression (91). Further, ROS generation as well as NFkB activation have also demonstrated ability to decrease ABCA1 promoter activity, thus revealing a link between increased inflammation and increased cholesterol/lipid build-up within cells (in particular, macrophages). On the contrary, studies examining anti-inflammatory cytokines such as IL-10 and TGFβ have been shown to increase ABCA1 expression levels (91). The exact mechanism behind ABCA1 suppression is not clearly understood but it has been noted that the nuclear receptors upstream of ABCA1 are down-regulated after LPS exposure (86). Additionally, our lab has demonstrated that ABCA1 down-regulation in the

presence of very low dose (50pg/ml) LPS is dependent upon IRAK1, an intracellular kinase downstream of TLR4 (66). Further studies must be performed to delineate the mechanisms behind ABCA1 and potentially LXR down-regulation. Meanwhile, ABCA1 is itself an anti-inflammatory mediator, through the prevention of inflammatory lipid exposure and direct activation of the STAT3 pathway (92). Thus, ABCA1 down-regulation by LPS not only contributes to foam cell formation, but also contributes to the decrease in anti-inflammatory mediator expression and the increase in inflammation through the inhibition of apoptosis in damaged cells.

The development of the fatty streak then matures into atherosclerotic plaque through the activity of damaged endothelial cells, foam cells, and T cells. T cells secrete TGFβ, as well as growth factors and fibrogenic mediators which cause smooth muscle cell migration and proliferation in the area of the fatty streak, as well as the development of thick extracellular matrix material. The cycle of monocyte and T cell recruitment continues as the smooth muscle cells continue to secrete chemoattractant molecules, which then stimulate further smooth muscle migration and proliferation (93). This cycle results in chronic inflammation and excess proliferation of fibrous tissue over the growing core of apoptotic macrophages and extracellular lipid. The pro-inflammatory macrophages at the core of the lesion continue to secrete inflammatory mediators, including matrix metalloproteinases (MMPs). These MMPs degrade the extracellular matrix of the plaque (termed the “cap”) and can lead to plaque rupture. Meanwhile, pro-inflammatory Th1 cells in the area also secrete IFNγ, which functions to decrease collagen formation and weakens the matrix which holds the plaque together. In fact, plaque rupture usually occurs in the area with the most active inflammation and

accumulation of macrophages, often resulting in death or severe morbidity due to the resultant myocardial infarction, coronary thrombosis and stroke (86, 93, 94).

1.6.5 *Parkinson's disease*

Parkinson's disease (PD) is a neurodegenerative disease characterized by loss of dopaminergic innervations from the substantia nigra to the striatum in the brain (95). Additionally, PD is characterized by deposition of amyloid and microglial aggregation and activation (96). As neuronal loss continues, patients clinically exhibit bradykinesia, tremors, and gait deficits. PD is typically observed in people over 60 years old and there are no curative treatments at this time (95). Recent studies have also begun to correlate the effects of LPS with the neuro-degeneration observed in Parkinson's diseases. However, the mechanisms behind the LPS-induced neuronal changes are still being deciphered. Thus far, several studies have been published demonstrating the ability of high doses of intra-cranially injected LPS to cause microglial activation and neuronal loss (95-98). However, these studies have not addressed the phenotype induced by very low systemic doses of LPS, although several have demonstrated the low affinity of LPS to cross the blood brain barrier (BBB). In a study by Qin et al, mice were injected intraperitoneally with a high dose of LPS (5mg/kg) (99). Plasma and tissue levels of TNFα rose quickly and subsided to similar levels as the control mice at 9 hours post injection. However, the protein levels of TNFα in the brain remained elevated up to 10 months post injection. Microglia also demonstrated a characteristic activation phenotype in the brain cortices, hippocampi, and substantia nigra (99). These findings support the findings of Pan et al in their discovery that TNFα crosses the blood-brain barrier and

TNFα receptors are necessary for an inflammatory response (100, 101). Qin et al also determined that TNFα challenge in the brain induced MCP-1, IL-1β, and p65 expression only in brains of mice with TNFα receptors (99).

Of note, the substantia nigra (SN) is an area of the brain dense with microglia, thus it has been hypothesized that the SN may be particularly vulnerable to inflammation and damage. Studies by both Gao and Qin have demonstrated an delayed onset of LPS-induced progressive loss of SN neurons after LPS injection (102, 103). In the context of metabolic endotoxemia, the chronic, low elevation in TNFα by LPS exposure may be a strong enough stimulus to cause chronic, low-grade activation of microglia, especially in regions of the brain containing a large proportion of these cells. This theory is strengthened by the fact that even a signal exposure to TNFα by microglia can cause a chronic, extended response in microglia (103). This low grade inflammation and chronic nature of TNFα expression in microglial cells likely contributes to neuronal dysfunction and cell death characteristic of the dopaminergic neurons in Parkinson's disease. However, more research must be performed to determine whether this is the case.

Other potential contributors to the exposure of microglia to TLR4 ligands may also lie in the ability of low-grade, chronic inflammation induced by very low doses of LPS (50pg/ml) to create inflammation and leakiness in the blood brain barrier (104). This could potentially also contribute to the activation of microglial cells and encourage chronic inflammation in the brain. Additionally, a recent publication has demonstrated that oxidized phospholipids which often circulate during conditions of chronic inflammation are also able to induce damage to neuronal cells (105). Thus, much has yet to be elucidated regarding the pathophysiology and etiology of PD, but the role of

low-grade LPS exposure remains a strong candidate for the initiation and progression of this disease.

1.6.6 *Cancer Metastasis*

A growing body of evidence suggests that chronic inflammation can play a major role in the promotion of several different types of cancer through low-grade, long term damage to DNA and normal cell processes. Many chronic bacterial infections causing cholecystitis, gastritis, and cystitis have been linked to the development of gall bladder cancer, gastric carcinoma, and bladder carcinoma (106). Thus, it appears that chronic, local, low-grade inflammation can promote cancer development in those areas. Meanwhile, ongoing research is being compiled regarding the relationship between the inflammatory statuses of cancer positive patients with rates of cancer metastasis. In this way, metabolic endotoxemia may play a role in the development of some cancers, although the role of chronic endotoxin exposure has not been investigated in the progression of cancer. Here we will discuss the potential contribution of metabolic endotoxemia, which creates a low-grade inflammatory state, in increasing metastatic risk.

Although a direct association between LPS and tumor growth and metastasis has not been clearly defined, several reports have published findings regarding the presence of TLR4 and the metastatic potential of the tumors involved. For one, a study by Huang et al demonstrated TLR4 activation by LPS in a number of different tumor tissue types (colon, breast, prostate, melanoma, and lung cancer cells) allowed for tumor progression and immune cell evasion, through the LPS-induced expression of

mediators (107, 125). To further demonstrate this principle, LPS-induced tumor supernatant inhibited the activation profiles of both dendritic and T cells (107). In vivo, TLR4 silencing caused a delay in tumor growth and an increase in survivability in mice (107).

An emerging theory in the field of cancer growth and metastasis also lies in the ability of the tumor to evade host immune response. One contributing factor to this theory lies in the discovery of single nucleotide polymorphisms (SNPs) among the TLR4 receptor. A review by El-Omar et al describes the SNPs in detail and will not be further described in this review. However, it is interesting to note that these TLR4 SNPs appear to be decrease LPS responsiveness (108), which may also contribute to a host's inability to attack tumor cells. To add, decreased TLR4 expression also decreases the ability of the host cells to present antigen and activate dendritic cells, thus allowing the tumor to remain un-harmed by the host immune system (109, 110). Another angle to the story lies in the ability of tumor cells to up-regulate IRAK-M activity and expression in human monocytes through their TLR4 and CD44 surface receptors. The ability of tumor cells to proliferate and metastasize partially lies in the tumor's ability to evade the host immune system. In this way, signaling through host TLR4 to selectively up-regulate IRAK-M allows for the monocytes to take an anti-inflammatory phenotype and results in their subsequent inability to detect and present antigen (111).

Meanwhile, LPS has been implicated as a ligand for TLR4 signaling in human colon cancer cells, as the gut represents a substantial source of LPS from the bacteria that reside there (112). Hsu's group also determined that TLR4 expression on colorectal cancer cells (CRC) was necessary to the metastatic potential of these tumor cells from

the gut to the liver in mice. TLR4-expressing CRC cells also exhibited greater affinity to adhere to several different cellular matrices, including endothelial cells and were more likely to be identified in hepatic sinusoids post LPS challenge. Further, LPS challenge in these cells stimulated β1-integrin function, which allows for the greater adhesiveness and metastatic potential of the CRC cells (112). Meanwhile, another study by Simiantonaki et al observed that both CD14 and constitutive TLR4 were non-determinant on CRC behavior. Rather, metastatic CRC tumors displayed a decrease in constitutive TLR4 expression (113). This may be due to the differences in tumor tissue type behavior and much more research is warranted, although it is clear that TLR4 plays a critical role in tumor metastasis.

A separate study by Sun et al demonstrated the beneficial effects of TLR4 pathway inhibition by rapamycin through the inhibition of IL-6 and PGE_2 production, as well as direct down-regulation of TLR4 and inhibition of NFkB (114). The inhibition of these factors likely contributes to the decrease in immune escape as well as metastatic potential in CRC cells. Additionally, TLR4 contributions to the proliferation and metastasis of several other types of cancers have also been documented. These include breast cancer, pancreatic adenocarcinomas, lung cancer, and melanoma. A breast cancer study by Gonzalez-Reyes et al observed an increase in protein and mRNA expression of TLR4 in ductal breast cancer cells among 74 women, although TLR4 expression was localized intracellularly and not on the cell surface. The group also observed a positive correlation between TLR4 expression and tumor size. Additionally, there was a significant positive correlation between TLR4 expression in host monocytes around the tumor and distant tumor metastasis (115). A specific

mechanism for glial tumor metastasis also requires the involvement of TLR4. In one study, glial tumor cells were inhibited from migration after blockade of TLR4 signaling. A reduction in HSP90-induced Ca^{2+} signaling was observed, thus pointing to a TLR4-dependent pathway in which binding of HSP90 to TLR4 allows for downstream increase of cytosol Ca2+ and release of ATP. These factors then promote cell migration and thus metastasis (116). Several other cancer types have also demonstrated TLR4 dependence, including pancreatic carcinomas, lung cancer, and melanoma (116-121). Therefore, it is apparent that TLR4 plays a critical role in the growth and metastasis of several types of cancers.

Of special note, a study in pancreatic adenocarcinoma elucidated a role for TLR2 in the growth and evasion of host immune responses (117). Interestingly, the activation of TLR2 allowed for the increase in ERK signaling without activation of NFkB. This specific pathway activation allowed for pro-tumorigenic mediators to be released, with little stimulus for host immune response. In correlation, as our lab has published previously, very low dose LPS through TLR4 also selectively activates C/EBPδ, resulting in low-grade chronic inflammation and activation of a select set of mediators without activating NFkB (66). Thus, potentially, very low doses of LPS may also elicit an NFkB-independent response in tumor cells, contributing to tumor growth and metastasis potential.

1.7 Missing Links

Since the concept of metabolic endotoxemia is a novel one, much information has yet to be obtained regarding the mechanisms and phenotypes involved. To add to the complexity of the phenotypes reviewed here, the behavior and wiring of different cell types likely also leads to a potentially wide variety of tissue-specific responses to very low dose LPS challenge. Additionally, the threshold doses of LPS necessary to induce pro-inflammatory mediators and inhibit nuclear receptors likely differ between cell types, while the doses which may allow for a solely pro-inflammatory program without the activation of anti-inflammatory mediators has yet to be determined. Additionally, the chronicity of the very low dose LPS responses must be distinguished, as well as the interactions between nuclear receptors and pro-inflammatory transcription factors. Moreover, detailed mechanistic work is necessary in order to further understand the dynamics of the TLR4 pathway in response to high dose versus low dose LPS. Greater understanding of these mechanisms and the phenotypes they elicit can provide critical information for future therapeutic targeting and intervention for several chronic diseases which contribute to significant morbidity and mortality worldwide.

1.8 Future Strategies/Conclusions

TNFα inhibitors have been tried in clinical trials and have met limited, if any success. This may be due to the differences in levels of TNF between plasma and tissue.

Whereas plasma levels may be low, TNF levels in the tissue compartments may not be affected by this TNF inhibitor and therefore, no beneficial effects are noted (122). Meanwhile, upregulating PPARs, such as PPARγ, has shown some success via TZD therapy, as PPARs promote an anti-inflammatory state, as well as increase the levels of the anti-inflammatory adipokine, adiponectin (48, 49, 123). Additionally, fatty acid metabolites such as prostaglandins have demonstrated an inhibition of NFkB and omega-3 FAs have also exerted anti-inflammatory effects in light of LPS stimulation (48, 49). Further, TLR4 inhibition is being examined as another potential therapy for many diseases, and may serve as a solid potential therapy in the future (64, 124, 131). In terms of specific therapeutic targets, since it is chronic low-grade inflammation in the presence of obesity that is essential for the development of IR and T2DM, it seems pertinent to address the inflammatory pathways. However, while directly inhibiting inflammatory pathways, it may also be effective to also examine the benefits of drugs that target the promotion of nuclear receptors downstream of TLR4, such as the PPARs and PGC1. If increased pharmacologically, nuclear receptor up-regulation could promote an anti-inflammatory state even in the face of TLR4 stimulation. Together in combination with drugs that target the inflammatory pathway, such as targeting IRAK1, then obesity, insulin resistance and T2DM may be successfully tackled. This may be possible through the reduction or inhibition of inflammation as well as the prevention of excess adiposity, since excess adiposity can lead to organ stress and inflammation. Of course, these treatments would be tailored to the patient and would need to be administered in combination with recommendations for exercise and dietary adjustments. Additionally, as studies increase on the factors necessary for IR

development, more specific targets may be identified and targeted, even at the gene level.

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CHAPTER 2: A Phenotypic Analysis of High Dose and Very Low Dose Endotoxemia in WT and IRAK1$^{-/-}$ mice.

2.1 Abstract

It has been well-established that lipopolysaccharide (LPS), a component of the Gram-negative bacteria, is a ligand for the innate immune receptor, Toll-like receptor 4 (TLR4) and that LPS is a critical trigger for the effects of sepsis and septic shock. Here, it has been determined that LPS not only elicits a cytokine storm of pro-inflammatory mediators, but it causes the suppression of lipid oxidation and the up-regulation of glucose utilization. This inefficient switch in metabolism often results in multiple organ failure and leads to significant mortality rates worldwide. Thus, therapies targeted solely at addressing inflammatory complications have met very limited success in the clinic. Meanwhile, a novel concept is now emerging that implicates LPS in not only significant disease pathology at high doses, but also at very low doses. The term metabolic endotoxemia has been coined recently and describes the subclinical elevation in circulating plasma endotoxin levels, which appears to have a distinct subset of effects that create a background of chronic inflammation and possibly metabolic changes as well. However, the cross-talk mechanisms between inflammation and metabolism downstream of TLR4 during both high dose and very low dose LPS challenge have yet to be understood. Particularly, the phenotype and mechanism between very low dose

LPS challenge have barely been examined. In this study, we determine the involvement of a key kinase, IRAK1, in the cross-talk between inflammation and metabolism in several *in vivo* models of high dose endotoxemia. Further, we examine *in vivo* the role of IRAK1 in the development of metabolic endotoxemia. Finally, we determine the *in vivo* phenotype of chronic low doses of LPS and high fat diet (HFD) on the development of insulin resistance.

2.2 Introduction

The role of lipopolysaccharide (LPS) in severe disease pathology has been clearly defined in the pathogenesis of endotoxemia and septic shock (7, 25). However, a novel role for very low doses of LPS is beginning to be implicated in the pathologies of several chronic inflammatory diseases, including diabetes and insulin resistance, atherosclerosis, and neurodegenerative diseases such as Parkinson's disease (18, 20, 23, 31). The correlation of very low doses of LPS and chronic disease is a novel concept and drastically challenges the previous understanding of TLR4 signaling. Thus far, the role of key mediators downstream of the innate LPS receptor, Toll-like receptor 4 (TLR4), have been examined under the conditions of high dose LPS challenge representative of severe endotoxemia and septic shock (6, 7, 11, 18). Much knowledge has been gained regarding the TLR4 pathway under these conditions, although the system of regulators downstream of TLR4 is complex at best and much has yet to be understood regarding the interactions of these proteins. For one, severe endotoxemia and septic shock continue to claim significant rates of morbidity and mortality worldwide. These syndromes are characterized by an acute cytokine storm of pro-inflammatory mediators, as well as severe metabolic alterations. In many cases, these severe alterations in inflammatory and metabolic parameters contribute to multiple organ failure (MOF) and boast mortality rates of up to 90% when four or more organs are severely impaired (6). Presently, target-directed therapies such as anti-TNFα therapies, have met

limited success and supportive treatment remains the mainstay of sepsis intervention (1, 2).

These treatments may be meeting limited success because of the current lack of understanding in the metabolic dysregulation involved in severe endotoxemia. For one, it has been documented that fatty acid oxidation (FAO) is severely impaired during high dose LPS challenge, while glucose oxidation up-regulation causes a quick depletion in energy stores, together contributing to MOF (6, 7, 9, 11, 15, 19, 25, 32, 33). These down-regulated metabolic regulators include nuclear receptors such as PPARs and PGC1, as their downstream targets are genes responsible for FAO regulation such as CPT1α and PDK4 (4, 5, 9, 10, 16, 24, 27, 30). Shut-down in FAO metabolism forces the host utilization of glucose, whose efficiency in ATP production is far less than that of lipid. Thus, the shift in metabolism encourages glucose depletion and a failure of vital organ tissues to gain the energy they need to function properly (6).

Meanwhile, one of the key regulatory kinases involved directly downstream of TLR4 is the interleukin-1 receptor associated kinase 1 (IRAK1) (8, 12, 21, 28), which contributes to the downstream activation of pro-inflammatory transcription factors NFkB (8, 28). The significance of IRAK1 in TLR4 signaling has been further supported by evidence that a variant haplotype of IRAK1 has demonstrated significant lethality after high dose LPS challenge (3). However, the involvement of IRAK1 in the metabolic alterations associated with severe endotoxemia has not yet been evaluated.

Additionally, as mentioned previously, LPS is also beginning to be implicated in several chronic inflammatory disease pathologies. The presence of subclinical levels of LPS have been demonstrated in the bloodstream of both people and animal models after high fat diet feeding, alcohol consumption, aging, and chronic infections such as periodontal disease. Importantly, the development of an inflammation-induced insulin-resistant state in the host has also been implicated in the development of these diseases. Further, slightly elevated levels of endotoxin have already been established as significant risk factors for the development of atherosclerosis (22, 31). However, the *in vivo* phenotype of chronic, very low dose LPS challenge has not been determined, nor the role of IRAK1 in these inflammatory and metabolic alterations. Therefore, this study aims to examine the role of IRAK1 as a point of crosstalk in the metabolic alterations associated with severe endotoxemia and then define a role for IRAK1 in very low dose LPS challenge. Lastly, we aim to determine the contribution of chronic, very low dose LPS challenge in the contribution to an insulin resistant state *in vivo*.

2.3 Materials and Methods

Reagents

LPS (E. Coli O111:B4) was obtained from Sigma. PPARα antibody was purchased from Santa Cruz Biotechnology. Primer sets were obtained from IDT.

Animal Studies

C57Bl/6 mice were purchased from Charles River laboratory. IRAK1$^{-/-}$ mice with C57Bl/6 background were kindly provided by Dr. James Thomas from the University of Texas Southwestern Medical School. All mice were bred and housed at Derring Hall Animal Facility, in accordance with approved Animal Care and Use Committee protocols at Virginia Polytechnic Institute and State University, with the exception of the WT mice for in the 2 month experiment, which were housed at the Life Sciences 1 vivarium at Virginia Tech, also in accordance with approved IACUC protocols.

High dose LPS challenge studies

WT and IRAK1$^{-/-}$ mice of matched age and gender were injected with PBS or 25mg/kg LPS intraperitoneally. Blood was drawn via cardiac puncture 16 hours post-injection and plasma was collected. Organ tissues were harvested and used for the described assays. The survival study was performed using WT and IRAK1$^{-/-}$ mice of matched age and gender (n=14 per genotype) injected with PBS or 25mg/kg LPS intraperitoneally. Once injected, mice were allowed free choice water and monitored at two hour intervals. Survival and mortality were observed and recorded over a period of 50 hours.

Apoe$^{-/-}$ in vivo studies

Apoe$^{-/-}$ mice were purchased from Jackson Laboratories and cross-bred with IRAK1$^{-/-}$ and IRAKM$^{-/-}$ mice kindly provided by Dr. James Thomas to produce Apoe$^{-/-}$/IRAK1$^{-/-}$, and Apoe$^{-/-}$/IRAKM$^{-/-}$ mice. All mice were housed and bred at Derring Animal Facility in accordance with approved institutional IACUC protocols at Virginia Tech. Mice of matched age and gender were placed on Western diet (HFD) for 2 months (Harlan

Teklad, 94059). PBS or LPS injections (1mg/kg) were performed intraperitoneally every seven days for the duration of the experiment. Plasma samples were harvested as mentioned previously.

Chronic very low dose LPS challenge in WT mice

C56Bl/6 mice of matched age and gender were placed on standard rodent chow (ND) or Western diet (HFD) for 2 months (Harlan Teklad, 94059) and injected with PBS or 5ug/kg LPS intraperitoneally every three days for 60 days. Blood was collected as described above and organs were harvested for the designed assays.

Histology

The kidneys and livers of each mouse were harvested and 3mm sections were placed in 10% neutral buffered formalin. The tissues were embedded in paraffin, sectioned, and then stained with Hematoxylin and eosin (H&E) stain for evaluation of tissue morphology and inflammatory cell infiltration (AML Labs, MD).

Free Fatty Acid (FFA) and Triglyceride (TG) Assays

Plasma free fatty acids were quantified using the Free Fatty Acid colorimetric assay from BioVision. 7ul of sample was measured against a standard of varying concentrations of Palmitic Acid (provided by the kit) and O.D. was measured at 570nm in a 96-well microplate reader. Plasma triglycerides were quantified in a similar manner using the Triglycerides colorimetric assay from BioVision, using 2ul sample per well and a triglyceride standard provided by the kit. Calculations for both free fatty acid and

triglyceride samples were performed using the slope of the standard curve and concentration was determined by dividing by the sample amount in ul to achieve nmol/ul.

Plasma triglycerides were quantified using a Triglyceride colorimetric assay kit from Wako. 2ul of sample was added to 300ul color reagent containing chromogen and measured against a standard of varying concentrations of Triglyceride Standard. The samples and standard placed in a 96-well plate were then incubated for 5 min at 37℃ per the kit instructions and measured on a microplate reader at 600nm absorbance. Calculations were made based off of the standard curve values and divided by the sample amount in ul to achieve mg/dl concentration values. The results were then multiplied by a factor of 0.011 to convert the concentration into nmol/ul.

Endotoxin Assay

A ToxinSensor™ Chromogenic LAL Endotoxin Kit was purchased from Genscript USA. Reagents and plasma samples were prepared in endotoxin-free pipet tips and vials. The assay was followed according to the manufacturer's protocol. Briefly, 100ul standard, sample and blank were dispensed into vials and added to 100ul LAL. Samples were vortexed and incubated at 37℃ for 45 minutes. Then, 100ul chromogenic substrate was added to each vial and mixed gently before additional incubation at 37℃ for 6 minutes. 500ul stop solution was then added and mixed gently before adding 500ul of a second color stabilizer. The vials were mixed again before adding 500ul of a third color stabilizer to the vials. Absorbance was read at 545nm, using distilled water as a blank.

Glucose Tolerance Test (GTT)

GTT was performed using a Kroger blood glucose reader and corresponding test strips. The reader was used as per the manufacturer's protocol. Mice were fasted overnight before being administered 10% D-glucose intraperitoneally (1U glucose/g body weight). Blood glucose was then measured at 0, 30', 60', and 120' in all mice.

Insulin ELISA

An ultra-sensitive mouse insulin ELISA kit from Crystal Chem, Inc was used to determine the fasting insulin levels of the mice. The wide-range protocol provided by the manufacturer was used. Briefly, the antibody-coated plate was incubated with 5ul sample or standard. The plate was then washed 5 times before adding anti-insulin enzyme conjugate. The plate was then incubated for 30 minutes before adding 100ul enzyme substrate to each well. The plate was protected from light and incubated for 40 minutes before the absorbances were read using a microplate reader at OD 450nm and 630nm within 30 minutes. OD 630nm readings were subtracted from OD 450nm readings per the manufacturer's instructions.

Real-time RT-PCR

Organs were homogenized in 1ml IsolRNA. Certified RNAse-free equipment and tubes were utilized. RNA purity was determined by 260/280nm absorbance readings of >1.6. Then, 1.5ug RNA from each sample was reverse transcribed using the High capacity cDNA Reverse transcription kit (Applied biosystems) in a mastercycler (Eppendorf). Subsequent real-time PCR was performed using SYBR green master mix (Bio-rad) in

an iQ5 thermocycler (Bio-rad). Each run was composed of 35-40 samples under the recommended real-time protocol and data was analyzed using the Δ-CT and ΔΔ-CT method after normalizing samples to GAPDH internal controls. Primer generation was from IDT primerquest and sequences were as follows:

Gene (*mus musculus*)	Primer Sequence (5'-3')
GAPDH fwd	TGT-GAT-GGG-TGT-GAA-CCA-CGA-GAA
GAPDH rev	GAG-CCC-TTC-CAC-AAT-GCC-AAA-GTT
CPT1-α fwd	CTC-AGT-GGG-AGC-GAC-TCT-TCA
CPT1-α rev	GGC-CTC-TGT-GGT-ACA-CGA-CAA
MCAD fwd	TCG-GTG-AAG-GAG-CAG-GTT-TCA-AGA
MCAD rev	AAA-CTC-CTT-GGT-GCT-CCA-CTA-GCA
PDK4 fwd	AGT-GAC-TCA-AAG-ACG-GGA-AAC-CCA
PDK4 rev	ACA-CAA-TGT-GGA-TTG-GTT-GGC-CTG
CEB/Pδ fwd	ACT-TCA-GCG-CCT-ACA-TTG-ACT-CCA
CEB/Pδ rev	TGT-TGA-AGA-GGT-CGG-CGA-AGA-GTT
IL-6 fwd	ATC-CAG-TTG-CCT-TCT-TGG-GAC-TGA
IL-6 rev	TAA-GCC-TCC-GAC-TTG-TGA-AGT-GGT
ABCA1 fwd	GGA-CAT-GCA-CAA-GGT-CCT-GA
ABCA1 rev	CAG-AAA-ATC-CTG-GAG-CTT-CAA-A

Protein isolation and Western Blot Analysis

Organ slices were homogenized in T-PER protein extraction reagent and protease inhibitor cocktail using a hand-held tissue homogenizer (Fisher) on ice before protein levels were measured by Bradford assay (Bio-rad). 1x SDS lysis buffer (80mM Tris-HCl (pH 6.8), 2% SDS, 50% glycerol) containing protease and phosphatase inhibitors (Sigma) was then added to 40ug protein of each sample before being run on an SDS-PAGE gel and western blots were performed as described previously. Western blot analysis of the protein samples was performed after running proteins on SDS-PAGE gels and transfer to PVDF membranes at 110V for 2 hours. Blots were developed using Amersham ECL Plus chemiluminescent detection system (GE Healthcare). The films were developed using FujiFilm Multi-gauge software and normalized against GAPDH levels.

Statistical Analyses

The results are expressed as means +/- standard deviations (SD). A log-rank test was used for evaluation of statistical significance for the mortality curve and one-way ANOVA was utilized for analysis of statistical significance in *in vivo* RT-PCR studies. P-values less than 0.05 were considered statistically significant.

2.4 Results

IRAK1 deletion is protective against mortality after a lethal dose of LPS.

To assess the overall functionality of IRAK1 deletion on survivability after inducing a severe endotoxemia, fourteen mice of WT and IRAK1$^{-/-}$ genotypes were injected intraperitoneally with PBS or 25mg/kg LPS. Mice were monitored every two hours for up to 50 hours post injection and their survival times were recorded. As demonstrated in Fig. 1, lethal LPS injection resulted in significant mortality in WT mice, with 90% succumbing to death over the 50 hour time period. In contrast, IRAK1$^{-/-}$ mice demonstrated a 50% survivability rate during the same observation period. The differences between the two genotypes were compared using the log-rank test, which showed a statistically significant difference ($p=0.018$) at the end of the 50 hour time period. These findings support the observations of another study which also demonstrated a higher survivability rate in IRAK1$^{-/-}$ mice compared to WT mice (Swantek, 2000).

IRAK1 deletion is protective against exhaustion of glucose after lethal LPS injection.

To further understand the protective effects of IRAK1$^{-/-}$ against endotoxemia-induced mortality, we examined the differences in glucose utilization between the two genotypes. Since a lethal dose of LPS is believed to cause a decrease in fatty acid oxidation (FAO) and an increase in glucose utilization, thereby exhausting glucose stores, we examined the blood glucose levels of WT and IRAK1$^{-/-}$ mice injected intraperitoneally with PBS or 25mg/kg LPS over a 6 hour time period to determine whether IRAK1 is involved in these metabolic alterations. As demonstrated in Fig. 2A, blood glucose levels became severely low in WT mice at 4 and 6 hours post injection,

and these levels were significantly lower than in IRAK1$^{-/-}$ mice at the same time points. Meanwhile, PBS control mice exhibited no significant differences in blood glucose levels among genotypes, and did not demonstrate the decrease in blood glucose levels observed in the LPS-treated mice (Fig. 2B).

Lethal injection of LPS results in significant elevation of plasma FFA and TG in WT but not IRAK1$^{-/-}$ mice.

Since IRAK1 deletion confers higher survivability and smaller changes in glucose utilization compared to WT mice, we further investigated whether IRAK1 deletion was also protective against changes in FAO. Elevations in plasma FFA and TG have been well-documented in endotoxemia and sepsis due to an increase in lipolysis and a decrease in FAO as well as FFA utilization in vital organs, contributing to energy depletion, morbidity, and ultimately, mortality in individuals with severe endotoxemia (15, 33, 34). As demonstrated in Fig. 3A, plasma FFA levels in WT mice were significantly increased after lethal LPS injection, whereas IRAK1$^{-/-}$ mice did not demonstrate a significant increase in plasma FFA after lethal LPS injection. Additionally, plasma TG levels were also significantly increased in WT mice after lethal LPS injection, while IRAK1$^{-/-}$ mice demonstrated a non-significant increase in plasma TG levels after lethal LPS injection (Fig. 3B).

Hepatic FAO is increased in IRAK1$^{-/-}$ mice after lethal LPS injection.

To confirm our findings regarding FAO differences in WT and IRAK1$^{-/-}$ mice, we performed a FAO assay in liver tissues collected 16 hours after intraperitoneal injection with PBS or 25mg/kg LPS. As demonstrated in Fig. 4, there was a non-significant trend for decrease in FAO in WT mice injected with LPS compared to PBS controls of the same genotype. Interestingly, IRAK1 deletion caused a significant increase in liver FAO in mice injected with LPS compared to PBS controls.

LPS treatment causes varying degrees of fulminant necrosis in the liver of WT and IRAK1$^{-/-}$ mice.

To determine whether IRAK1 deletion confers protection against inflammatory changes associated with high dose LPS challenge, WT and IRAK1$^{-/-}$ mice were injected with PBS or 25mg/kg LPS and liver tissues were harvested 16 hours post-injection. As demonstrated in Fig. 5A, WT livers injected with LPS exhibited severe neutrophilic infiltration and hepatocyte necrosis, as well as early thrombus formation in surrounding blood vessels. IRAK1$^{-/-}$ mice injected with LPS also demonstrated neutrophilic infiltration and hepatocyte necrosis, as well as early thrombus formation (Fig. 5B). However, there was a lesser degree of infiltration and necrosis in IRAK1$^{-/-}$ mice compared to WT mice. Meanwhile, PBS controls of both groups demonstrated normal liver morphology and blood vessel integrity (Fig 5C, D).

HFD plus very low dose LPS injection synergizes weight gain in Apoe$^{-/-}$ mice.

After examining the *in vivo* effects of IRAK1$^{-/-}$ deletion after lethal LPS challenge, we then decided to examine the effects of very low doses of LPS in a couple of *in vivo* models. Since there is a growing body of literature demonstrating the activation of TLR4 by HFD and very low dose endotoxemia as significant contributors to several chronic diseases including atherosclerosis, insulin resistance and obesity, we first examined the effects of HFD on body weight of Apoe$^{-/-}$ mice, as well as the combination of HFD and very low dose LPS. Apoe$^{-/-}$ mice were placed on ND or HFD for 2 months and injected with either PBS or 1mg/kg LPS every 7 days. As observed in Fig. 6, there is a trend, although not statistically significant, for increased body weight after HFD feeding in the Apoe$^{-/-}$ mice. Meanwhile, mice fed HFD and injected with low-dose LPS had significantly greater weight gain than mice on HFD alone and those mice on ND chow.

IRAK1 and IRAKM deletions are protective against glomerulosclerosis in Apoe$^{-/-}$ mice fed HFD.

To further study the effects of HFD on systemic function, we harvested kidney tissues from Apoe$^{-/-}$, Apoe$^{-/-}$/IRAK1$^{-/-}$, and Apoe$^{-/-}$/IRAKM$^{-/-}$ mice fed HFD for two months. Kidney tissues were harvested and processed with H&E stain to examine kidney morphology and integrity. Interestingly, HFD conferred a significant increase in percentages of abnormal glomeruli in all genotypes compared to ND control mice, although this increase in abnormal glomeruli was most pronounced in Apoe$^{-/-}$ mice (Fig. 7A). Additionally, Apoe$^{-/-}$ mice fed HFD demonstrated the most severe glomerular changes and the lowest percentage of normal glomeruli amongst the genotypes (Fig. 7B),

according to the grading scale (Fig. 7C). Samples of these sections are exhibited in Fig. 8, with Fig. 8A, B, and E revealing normal morphology and integrity from mice fed ND. Meanwhile, Fig. C demonstrates significant glomerulosclerosis indicative of insulin resistance in Apoe$^{-/-}$ mice, with a majority of the glomeruli showing varying degrees of lipid accumulation. Fig.D demonstrates a lesser degree of glomerular change in Apoe$^{-/-}$/IRAK1$^{-/-}$ kidney, similar to the glomerular changes observed in Fig. 8F from Apoe$^{-/-}$/IRAKM$^{-/-}$ kidney samples.

Plasma endotoxin levels are increased in Apoe$^{-/-}$/IRAK1$^{-/-}$ mice after HFD feeding.

One of the effects of HFD is believed to be the increase in circulating levels of subclinical endotoxin which then promotes low-grade chronic inflammation. Thus, we examined the levels of endotoxin in mice fed ND or HFD. In just 2 months of HFD feeding, we did not observe an increase in plasma endotoxin levels in Apoe$^{-/-}$ mice fed ND versus HFD. Intriguingly, however, we observed a significant increase in endotoxin levels in Apoe$^{-/-}$/IRAK1$^{-/-}$ mice fed HFD compared to ND controls (Fig. 9).

Very low dose LPS challenge causes an IRAK1 dependent increase in pro-inflammatory gene expression.

In order to control the exact amounts of endotoxin challenge *in vivo*, we moved from HFD feeding to a series of low-dose chronic injections of PBS or LPS (5ug/kg) every three days for 30 days in WT and IRAK1$^{-/-}$ mice. Here, very low dose chronic endotoxin

challenge alone significantly induced low levels of IL-6 expression, as well as pro-inflammatory transcription factor C/EBPδ in kidneys of WT mice. Interestingly, these increases were not observed in IRAK1$^{-/-}$ mice under the same challenge (Fig. 10A, B).

Very low dose LPS challenge causes an IRAK1 dependent decrease in genes involved in fat metabolism.

Since select pro-inflammatory gene expression was increased in WT mice but not IRAK1$^{-/-}$ mice injected with chronic very low doses of LPS, we decided to examine the effects of very low dose LPS on genes involved in fatty acid metabolism. We examined the expression of three genes, ABCA1, CPT1α, and MCAD in the WT and IRAK1$^{-/-}$ mice injected with 5ug/kg LPS every 3 days for 30 days. As observed in Fig. 11A, a decrease in ABCA1 expression was observed in WT mice after chronic LPS injection compared to PBS controls, although not statistically significant. Meanwhile, IRAK1 deletion conferred an increase in ABCA1 expression after chronic LPS injection compared to PBS controls. Similarly in Fig. 11B, CPT1α expression was significantly decreased in WT mice after chronic LPS injection compared to PBS controls, and IRAK1 deletion caused an increase in CPT1α expression in mice after chronic LPS injection compared to PBS controls. Finally, in Fig. 11C, MCAD expression exhibited similar trends to the other FA metabolic genes, but these changes were not statistically significant.

Signs of insulin resistance in WT mice fed HFD.

We then sought to examine the effects of HFD and chronic very low dose LPS in WT mice. Thus, WT mice were fed ND or HFD for two months, and were injected with either PBS or 5ug/kg LPS every three days for the duration of the diet feeding. As observed in Fig. 12A, a trend for increase in body weight was observed after HFD feeding and HFD plus LPS groups compared to ND controls, although these trends were not statistically significant. Meanwhile, in Fig. 12B, trends were observed for the beginning development of insulin resistance, as demonstrated from a glucose tolerance test. Here, the combination treatment of HFD plus very low dose LPS caused a delay in decreased blood glucose levels at 1 hour post glucose injection, while mice fed HFD alone exhibited the highest increase in blood glucose levels post glucose injection. Meanwhile, trends, although not statistically significant, were also observed in fasted insulin levels in the same group of mice (Fig. 12C). Fasting insulin levels were similar among mice fed ND and HFD; although there were increased levels of fasting insulin in the mice treated with the combination of HFD and very low dose LPS.

FAO gene expression is down-regulated after chronic very low dose LPS and HFD treatments.

Next, we sought to examine whether chronic HFD and chronic very low dose LPS would cause a decrease in FAO genes, since these changes contribute to altered glucose and

lipid metabolism. Again, mice were fed ND or HFD for two months and injected with either PBS or 5ug/kg LPS every three days for the duration of the feeding. As in Fig. 13A, CPT1α levels in the kidney were not affected by LPS treatment alone, but CPT1α levels were significantly decreased after HFD feeding and further by the combination of HFD plus LPS treatment. We also examined the expression of PDK4, which functions as a switch between glucose and lipid utilization. In accordance with other observations, LPS treatment alone, as well as HFD alone and HFD plus LPS treatment all caused a significant decrease in the expression of PDK4 (Fig. 13B) in WT mice. We also examined the expression of these genes in the brains these mice to get a picture of whole body metabolism. The expression of brain CPT1α and PDK4 were significantly decreased by LPS treatment alone, and exhibited similar decreases in expression after HFD treatment alone as well as the combination treatment of HFD plus LPS (Fig. 14A, B). Meanwhile, we also measured PDK4 expression levels in the liver of these mice to further understand the behavior of PDK4 in another vital organ. Here, PDK4 was not decreased after LPS treatment alone, but was significantly decreased after HFD treatment alone as well as the combination treatment of HFD plus LPS (Fig. 15).

TNFα expression is increased after HFD feeding in WT mice.

To get a picture of the inflammatory status of these mice, we also examined TNFα expression, since it is a known pro-inflammatory mediator present in insulin resistance. As shown in Fig. 14C, we found that kidney TNFα expression was significantly elevated after HFD feeding alone, but interestingly was not affected by LPS treatments.

PPARα protein levels are decreased after chronic very low dose LPS challenge as well as HFD feeding in WT mice.

Finally, we aimed to examine the protein levels of a nuclear receptor well-known to be involved in FAO regulation, PPARα. Thus, kidney protein lysates from mice fed ND or HFD for two months and simultaneously injected with 5ug/kg LPS every three days for 60 days were blotted for PPARα protein. As demonstrated in Fig. 16, HFD treatment alone was able to decrease PPARα expression, while the combination treatment of HFD plus LPS further decreased PPARα expression. Meanwhile, LPS treatment alone appeared to slightly decrease PPARα expression.

2.5 Discussion

We have demonstrated a significant protective effect of IRAK1 deletion against morbidity and mortality during severe endotoxemia, both in survivability and in vital metabolic organ function. IRAK1$^{-/-}$ mice exhibit improved survival rates compared to their WT counterparts and do not experience the severe depletion in blood glucose levels after a lethal LPS challenge. Additionally, IRAK1$^{-/-}$ mice do not demonstrate an increase in plasma FFA and TG levels post LPS challenge, pointing to a role for IRAK1 in metabolic functions downstream of TLR4. Furthermore, deletion of IRAK1 allows for an increase in FAO functions post LPS challenge. In addition to protection from

metabolic alterations, IRAK1 deletion also appears to be slightly protective against the degree of hepatic necrosis and neutrophilic inflammation present after lethal LPS challenge.

These findings regarding high dose endotoxemia confirm the results from a number of studies which have demonstrated an inhibitory effect of LPS on FAO (9, 15). Our findings also point to IRAK1 as a key mediator in these metabolic alterations. IRAK1 is a downstream kinase of LPS receptor TLR4, and now appears to have functionality in both inflammatory and metabolic arms after TLR4 stimulation. This study is the first to demonstrate that IRAK1 is an important downstream mediator involved in the suppression of FAO and the increase in glucose utilization after high dose LPS challenge. These alterations in metabolism likely play a critical role in the morbidity and mortality of sepsis patients, since a switch from FAO to glucose utilization provides a short burst of energy but ultimately results in the depletion of glucose stores and the subsequent inability of the body to provide adequate nutrition to its vital organs.

Meanwhile, the novel concept of metabolic endotoxemia has begun to implicate very low doses of LPS in the pathologies of several severe chronic diseases such as atherosclerosis and type II diabetes (13, 15, 20, 22). In a murine model of genetically altered lipid metabolism ($Apoe^{-/-}$), our data demonstrates a synergistic effect of HFD consumption with low dose LPS exposure (1mg/kg). Additionally, both IRAK1 and IRAKM deletions with an $Apoe^{-/-}$ background are significantly protective against the percentage of glomerular changes associated with insulin resistance as well as the

severity of these lesions. Further, it is interesting to note that the deletion of IRAK1 uncouples the ability of endotoxin to create an insulin resistant state. This is evidenced by the fact that Apoe$^{-/-}$/IRAK1$^{-/-}$ mice exhibited a significant increase in plasma endotoxin levels after 2 months of HFD feeding compared to Apoe$^{-/-}$ mice on the same diet, yet IRAK1 deletion was protective against glomerular lipid accumulation. These data point to IRAK1 as a key modulator of both inflammatory and metabolic alterations associated with low-dose endotoxemia, as glomerulosclerosis is an indicator of an insulin resistant state.

In another model of metabolic endotoxemia in mice of a C57Bl/6 background, we have further elucidated the role of IRAK1. In a controlled study where mice were exposed to exact amounts of LPS (5ug/kg) every three days for one month, we determined a significant role for IRAK1 in LPS-induced inflammation through the up-regulation of IL-6 and C/EBPδ expression in WT mice after chronic very low dose LPS injection, whereas IRAK1$^{-/-}$ mice under the same challenge did not demonstrate an increase in these gene expression patterns. Additionally, a role for IRAK1 in metabolic alterations was determined through the evaluation of gene expression for FAO genes. In kidney tissues of these mice challenged with very low dose LPS, the expression of several lipid metabolic genes including ABCA1 and CPT1α was inhibited in WT mice but not IRAK1$^{-/-}$ mice. Thus, our study is the first to determine that IRAK1 is a critical downstream kinase of TLR4 for both inflammatory and metabolic changes associated with very low, chronic challenge of LPS.

Finally, we sought to determine the role of metabolic endotoxemia itself on the development of chronic disease, specifically through the development of insulin resistance. Firstly, in C57Bl/6 mice fed HFD for 2 months in combination with PBS or 5ug/kg LPS injections, we determined that HFD and HFD in combination with very low dose LPS created a trend for increase in body weight, and that these two treatments also caused trends for insulin resistance through the increase and prolonging of blood glucose levels after glucose stimulation. Further, fasting insulin levels were increased in mice fed a HFD in combination with LPS challenge. We also observed a decrease in FAO gene expression of CPT1α and PDK4 from these mice in multiple vital organ tissues including kidney, brain and liver. This series of studies demonstrated the ability of chronic very low dose LPS alone to cause significant alterations in lipid metabolism. Additionally, we have demonstrated a role for PPARα *in vivo* in regulating lipid metabolism after chronic very low dose LPS challenge, in which HFD and HFD plus LPS cause a clear decrease in PPARα protein levels in the kidney. Furthermore, HFD in these mice was able to induce the expression of TNFα, which has been highly implicated in conditions such as insulin resistance and atherosclerosis.

Much more research must be performed to bring understanding to the mechanisms behind both high dose and very low dose LPS challenges. However, our study has proven a significant role for IRAK1 in both high dose and very low dose LPS challenges. We have demonstrated that IRAK1 represents a significant point of cross talk between inflammatory and metabolic pathways, as it plays significant roles in both scenarios, downstream of TLR4. Thus, our study provides an important potential future therapeutic

target for the development of successful sepsis treatments as well as the development of successful prevention and intervention strategies against the onset of chronic inflammation/metabolic alterations. Since past therapies which have been solely focused on targeting inflammatory cytokines and mediators have met very little if any success, treatments involving both interventions for inflammatory and metabolic branches of these diseases hold much promise (1, 2). As demonstrated in our study, IRAK1 serves as a critical point of cross-talk between these two pathways.

2.6 Figures and Legends

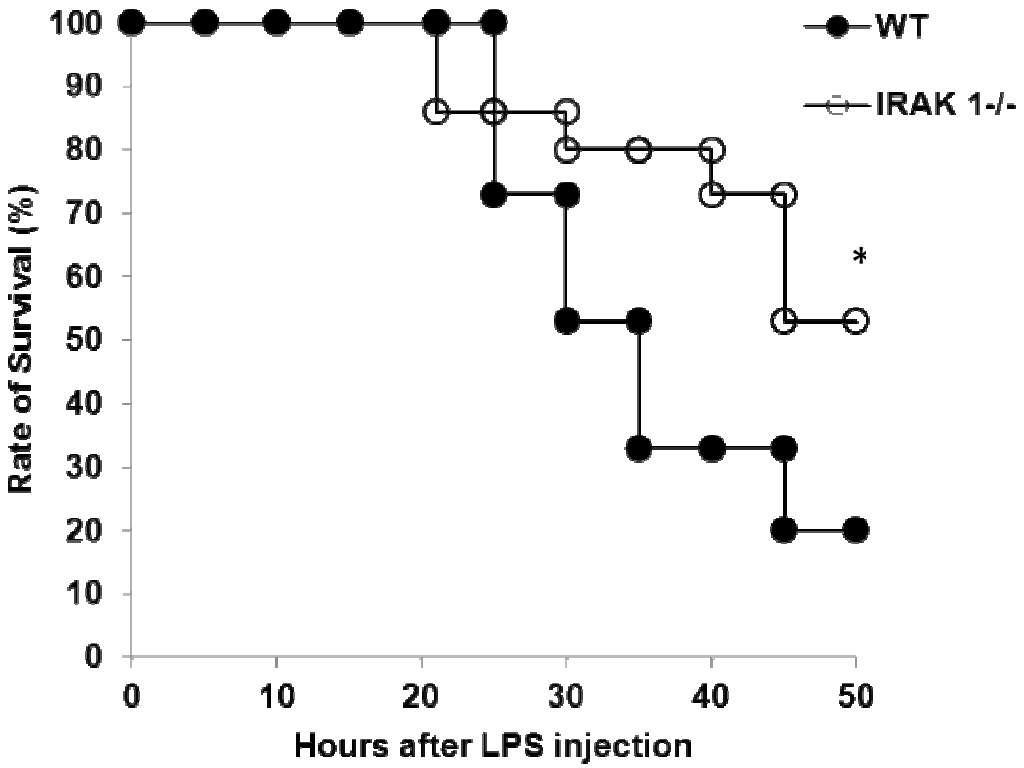

Figure 1: IRAK-1 deletion protects against LPS-induced mortality
The survival curve for WT and IRAK1$^{-/-}$ mice injected intraperitoneally with 25mg/kg LPS. Fourteen mice of each genotype were injected with either PBS or LPS. Survival was plotted against time (in hours) post-injection. Survival differences were analyzed using a log-rank test, *p=0.018.

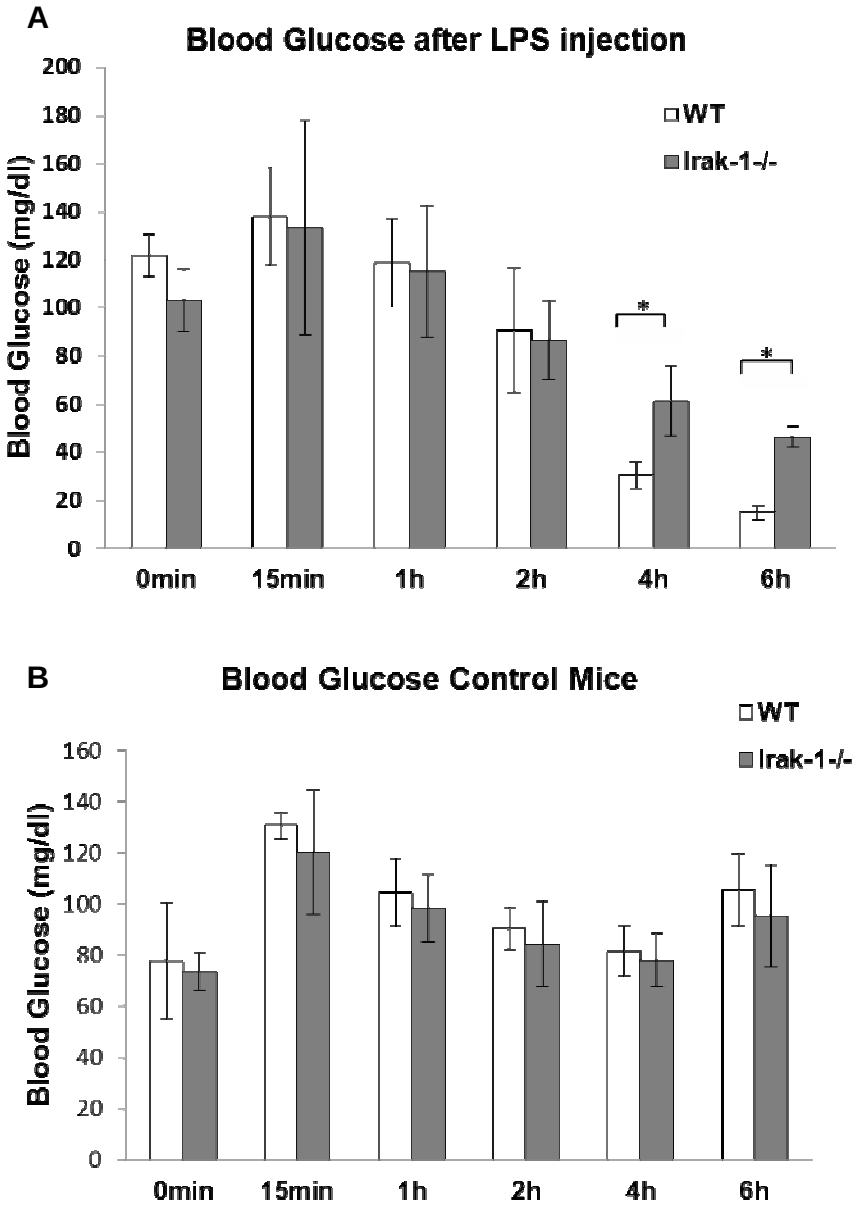

Figure 2: Blood glucose levels after lethal LPS injection or PBS control. WT and IRAK1$^{-/-}$ mice were injected intraperitoneally with either LPS (A) or PBS (B) as described in Figure 1(n=6 per group). After injection, blood glucose was monitored for a six hour time period. Student's t-tests were performed to determine statistical significance between genotypes, *p<.007.

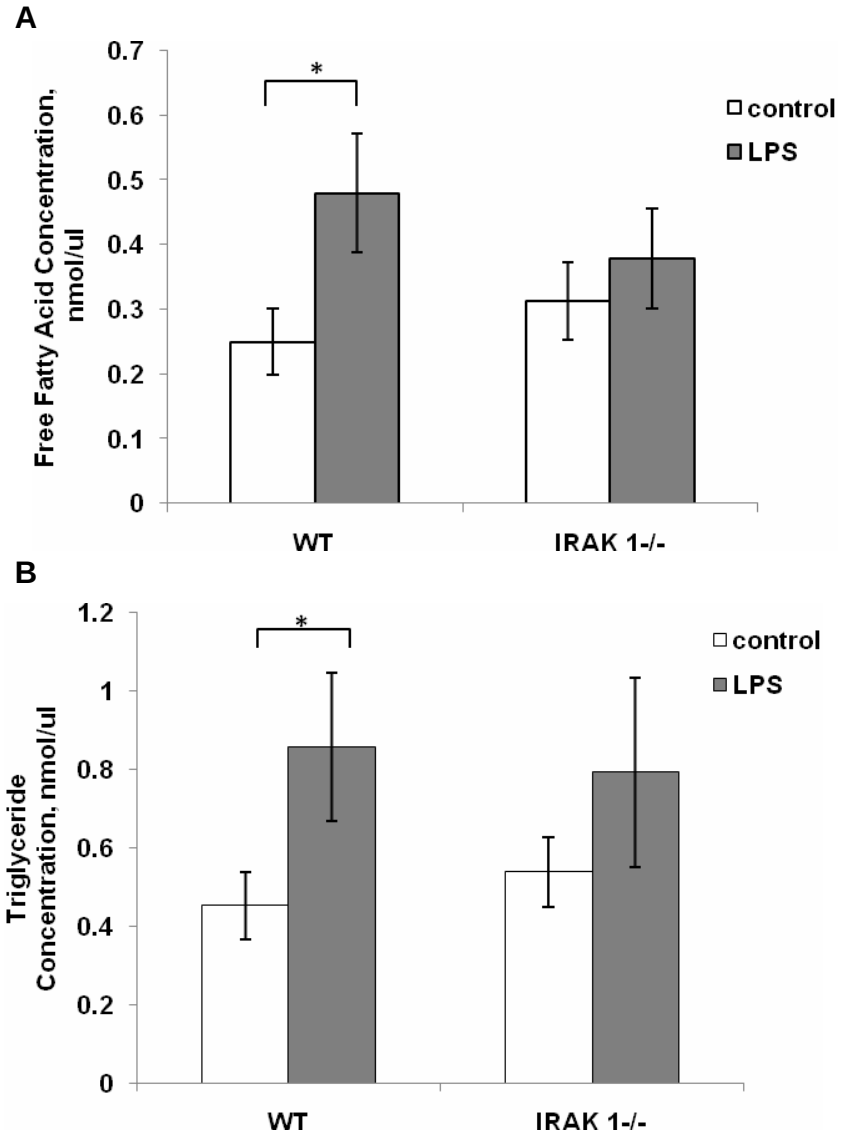

Figure 3: Increased plasma free fatty acid (FFA) and triglyceride (TG) levels in WT vs. IRAK1$^{-/-}$ mice after lethal LPS injection.

(A) Effect of IRAK1 deletion on plasma FFA levels in mice challenged with 25mg/kg LPS or PBS control. Plasma from mice (n=4 per group) was collected 16h post-injection. Results are representative of 3 independent experiments, *p<.005. (B) Effect of IRAK1 deletion on plasma TG levels after challenge with 25mg/kg LPS or PBS control. Plasma from mice was collected 16h post-injection and expressed as nmol/ul by multiplying mg/dl by a conversion factor of 0.011. Error bars are representative of standard deviation, *p<0.008.

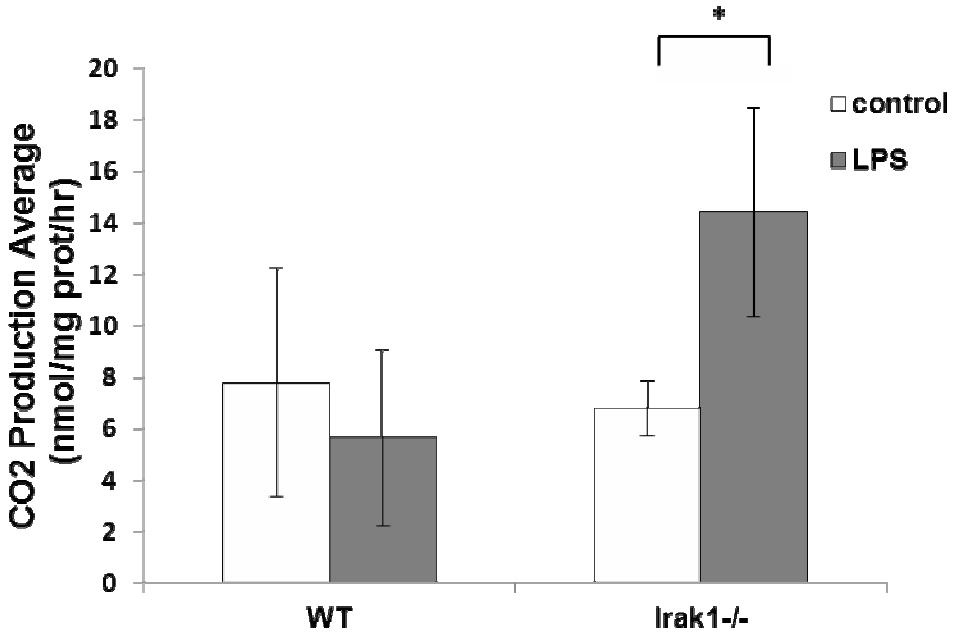

Figure 4: Effect of IRAK1 deletion on hepatic FA oxidation after lethal LPS injection.

Livers of WT and IRAK1$^{-/-}$ mice were immediately harvested 16 hours post-injection (n=5 per group) and processed with labeled CO_2 to quantify FAO activity (previously described in Materials and Methods section). Error bars are representative of standard deviation, *p<0.01.

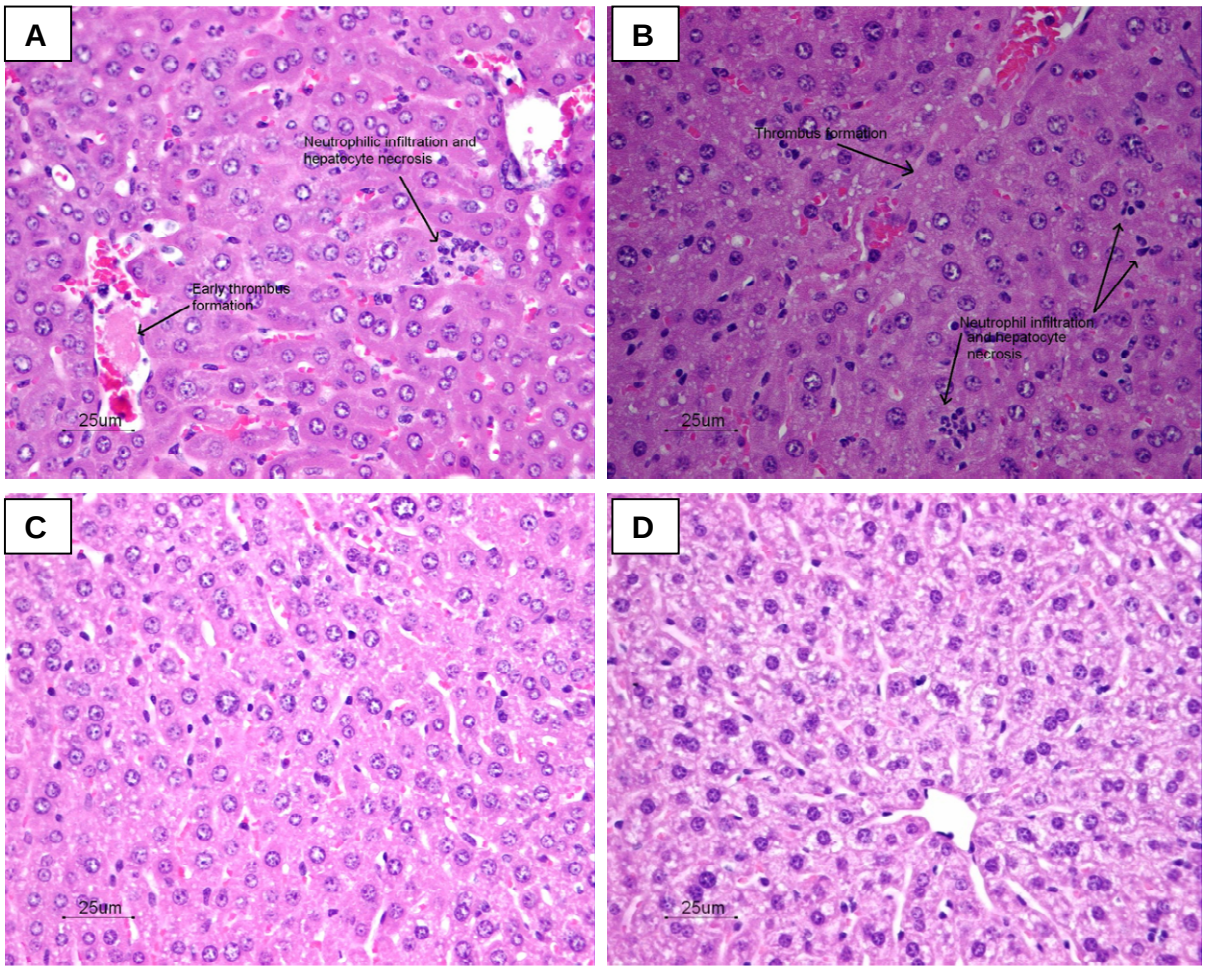

Figure 5: Effect of IRAK1 deletion on hepatic inflammatory changes, H&E liver sections.

Livers from WT and IRAK1$^{-/-}$ mice were harvested 16h post LPS injection (25mg/kg) or PBS (500ul), (n=5 per group). Sections were placed in 10% neutral buffered formalin and paraffin embedded before staining. (A) 400x Section from a WT liver sample demonstrating fulminant neutrophilic infiltration and hepatocyte necrosis (right arrow), as well as early thrombus formation (left arrow). (B) 400x Section from an IRAK1$^{-/-}$ liver sample also demonstrating thrombus formation and neutrophilic infiltration, as well as hepatocyte necrosis.(C) 400x Section from a WT control liver sample demonstrating normal hepatocyte morphology and no neutrophilic infiltration. (D) 400x Section from an IRAK1$^{-/-}$ control liver sample demonstrating healthy hepatocytes and absence of neutrophils.

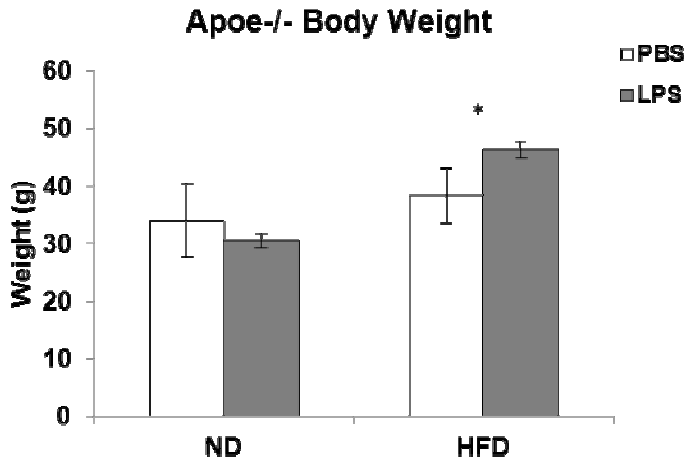

Figure 6: Apoe-/- body weight differences in ND and HFD mice.
Apoe$^{-/-}$ mice were placed on high fat diet (HFD) for 2 months and received weekly injection of 500ug/kg LPS or PBS control. Body weights for Apoe$^{-/-}$ mice were measured and compared among treatment groups. SD are represented by the error bars, and statistical significance determined at *$p<0.05$.

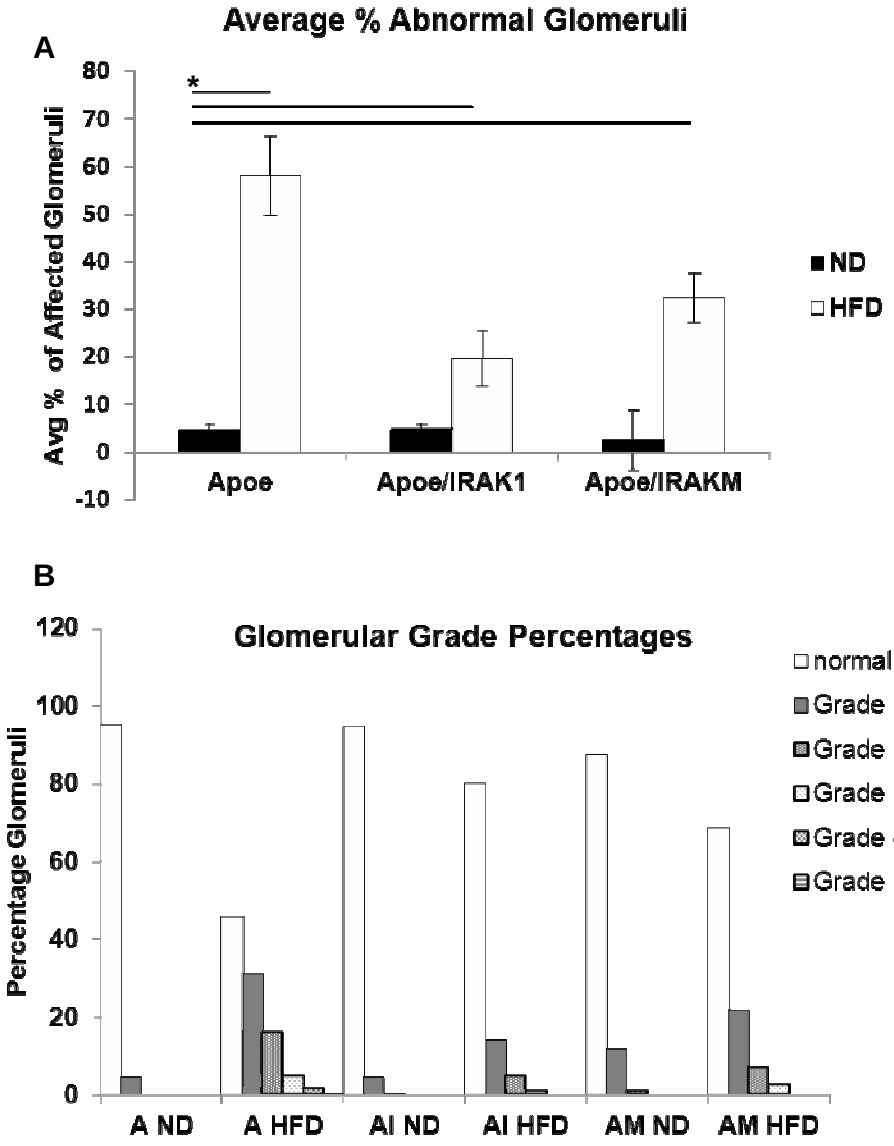

Figure 7: Differences in glomerular changes in Apoe$^{-/-}$, Apoe$^{-/-}$/IRAK1$^{-/-}$, and Apoe$^{-/-}$/IRAKM$^{-/-}$ mice.

Evidence of glomerular sclerosis was noted in H&E sections from the kidneys of Apoe$^{-/-}$, Apoe$^{-/-}$/IRAK1$^{-/-}$, and Apoe$^{-/-}$/IRAKM$^{-/-}$ mice placed on high fat diet for 2 months (n= 5 per group). (A) Percentages of total affected glomeruli were calculated using the total affected glomeruli divided by the total number of glomeruli per section (10 hpf). Statistical analysis was performed using one-way ANOVA, *p< 0.001. (B) Percentages of glomeruli affected according to the (C) grading scale for severity of glomerular damage.

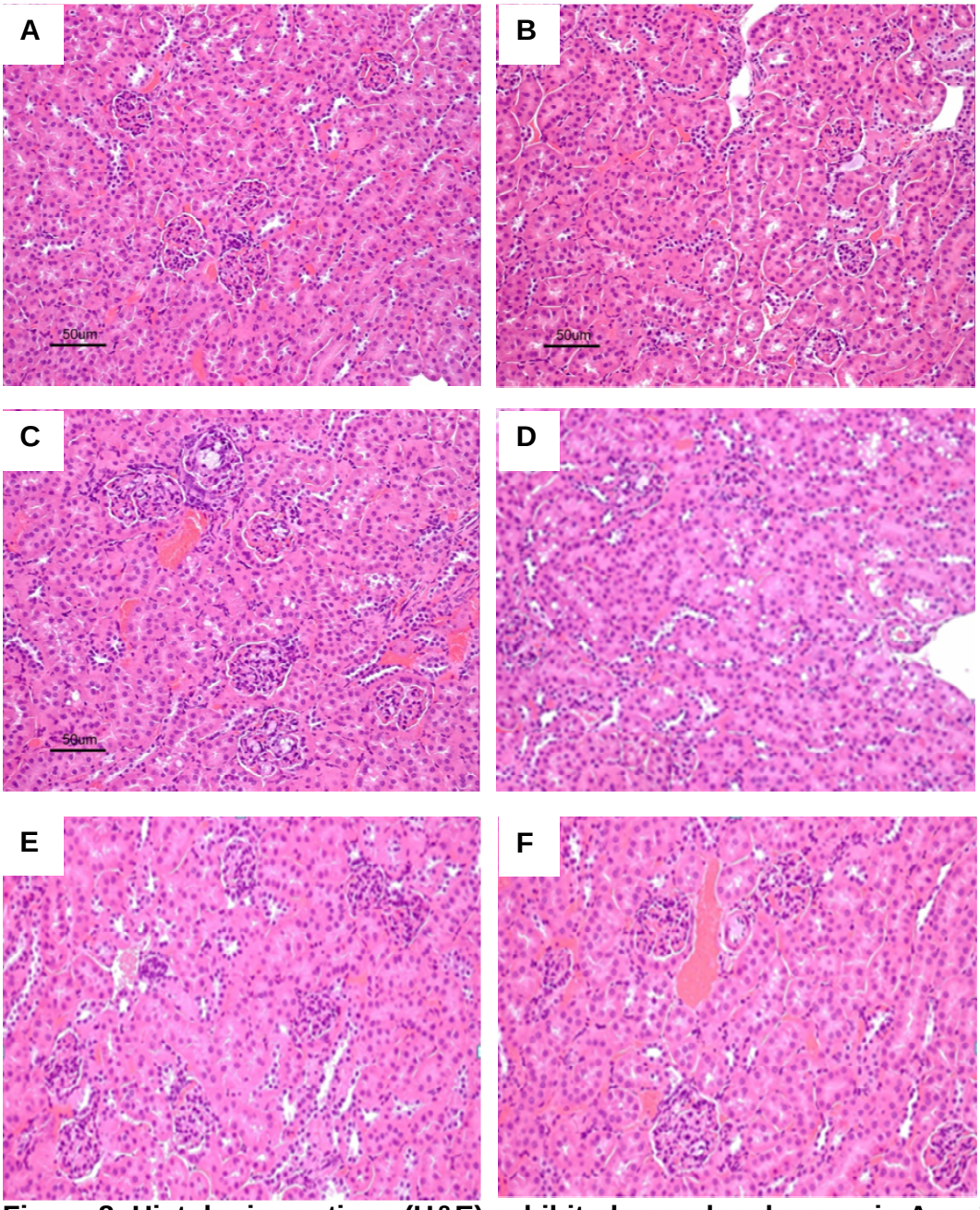

Figure 8: Histologic sections (H&E) exhibit glomerular changes in Apoe$^{-/-}$ vs. Apoe$^{-/-}$/IRAK1$^{-/-}$ mice.

Mice were fed normal diet (ND) or high fat diet (HFD) for 2 months before being sacrificed. Kidney sections were immediately placed in 10% neutral buffered formalin and paraffin embedded before sectioning and staining. Kidney sections from (A) Apoe$^{-/-}$ ND, (B) Apoe$^{-/-}$/IRAK1$^{-/-}$ ND, (C) Apoe$^{-/-}$ HFD, (D) Apoe$^{-/-}$/IRAK1$^{-/-}$ HFD, (E) Apoe$^{-/-}$ /IRAKM$^{-/-}$ ND and (F) Apoe$^{-/-}$/IRAKM$^{-/-}$ HFD mice are shown above.

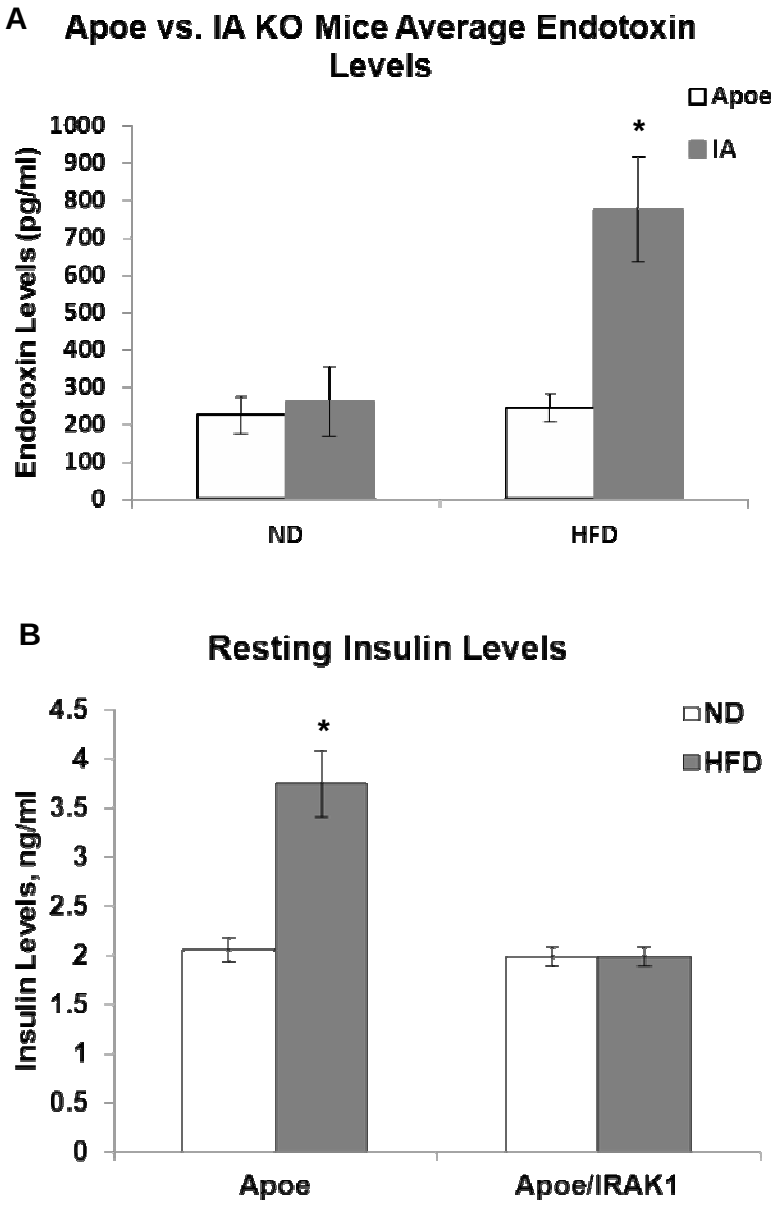

Figure 9: Endotoxin and resting insulin levels in Apoe-/- vs. Apoe-/-/IRAK1-/- mice (n=5 per group) after 2 months of ND or HFD.
Plasma from Apoe$^{-/-}$ and Apoe$^{-/-}$/IRAK1$^{-/-}$ mice was collected 2 months after being placed on normal diet (ND) or high fat diet (HFD) rodent chow (Harlan Teklad) (n=5 per group). Mice were anesthetized with isoflurane for blood collection via cardiac puncture. (A) LAL endotoxin assay (Genscript) was performed and quantified using a microplate reader. *P<0.0003. (B) Resting insulin levels in the same mice were measured using an insulin ELISA kit from CrystalChem, Inc. *P<0.005.

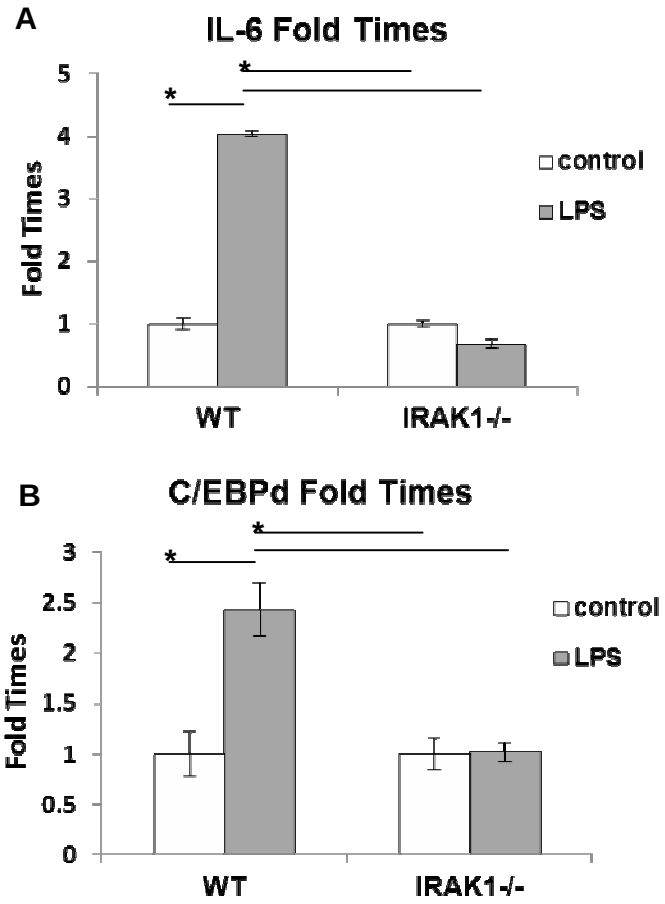

Figure 10: Effects of IRAK1 deletion on pro-inflammatory genes after chronic low-dose LPS challenge in mice.
WT and IRAK1$^{-/-}$ mice (n= 5 per group) were injected with 5ug/kg LPS every 3 days for 30 days. Kidney tissues were harvested immediately after sacrifice, placed in IsolRNA and homogenized, then stored at -80° C until RT-PCR analysis could be performed. Fold times were normalized to GAPDH message levels and (A) IL-6 and (B) C/EBPδ fold times were quantified. Statistical significance was calculated using one-way ANOVA, *p<0.001.

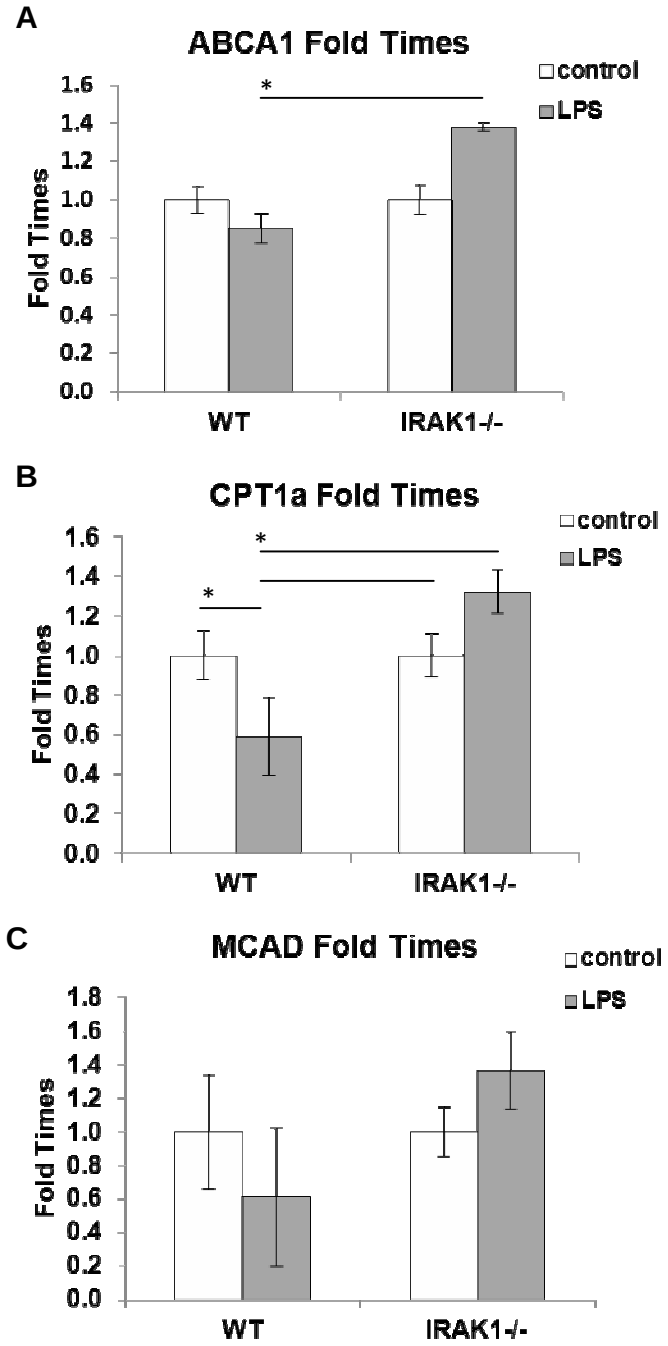

Figure 11: Effects of IRAK1 deletion on FAO genes after chronic low-dose LPS challenge in mice.

WT and IRAK1$^{-/-}$ mice (n=5 per group) were injected with 5ug/kg LPS every 3 days for 30 days. Kidney tissues were harvested immediately after sacrifice, placed in IsolRNA and homogenized, then stored at -80° C until RT-PCR analysis was performed. Fold times were normalized to GAPDH message levels and (A) ABCA1, (B) CPT1α, and (C) MCAD fold times were quantified. Statistical significance was calculated with one-way ANOVA, *p<0.05.

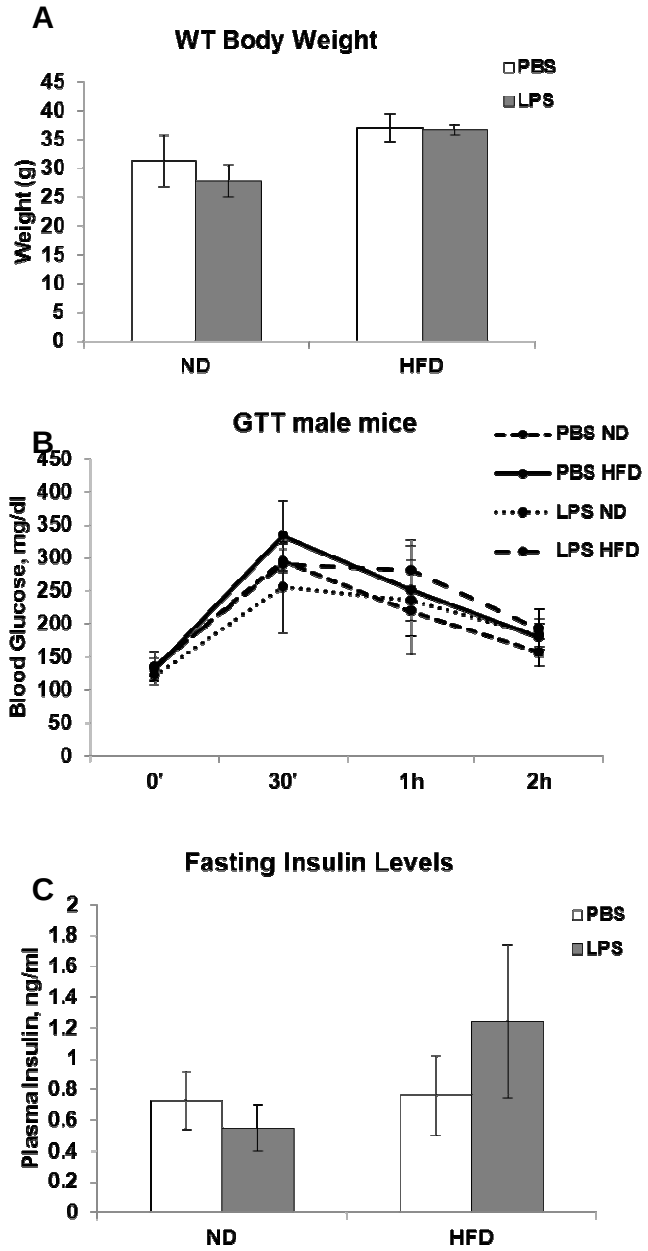

Figure 12: Signs of IR in glucose response and resting plasma insulin levels in WT mice.

WT mice were injected intraperitoneally with either 5ug/kg LPS or PBS every 3 days for 60 days. These mice (n=5-6 per group) were placed on either ND or HFD (Harlan Teklad) for the course of injections. (A) Body weights of mice in different treatment groups were measured and a (B) glucose tolerance test (GTT) was performed. (C) Plasma insulin levels were also measured by ELISA after overnight fast (n=5 per group). Error bars represent averages +/- SD.

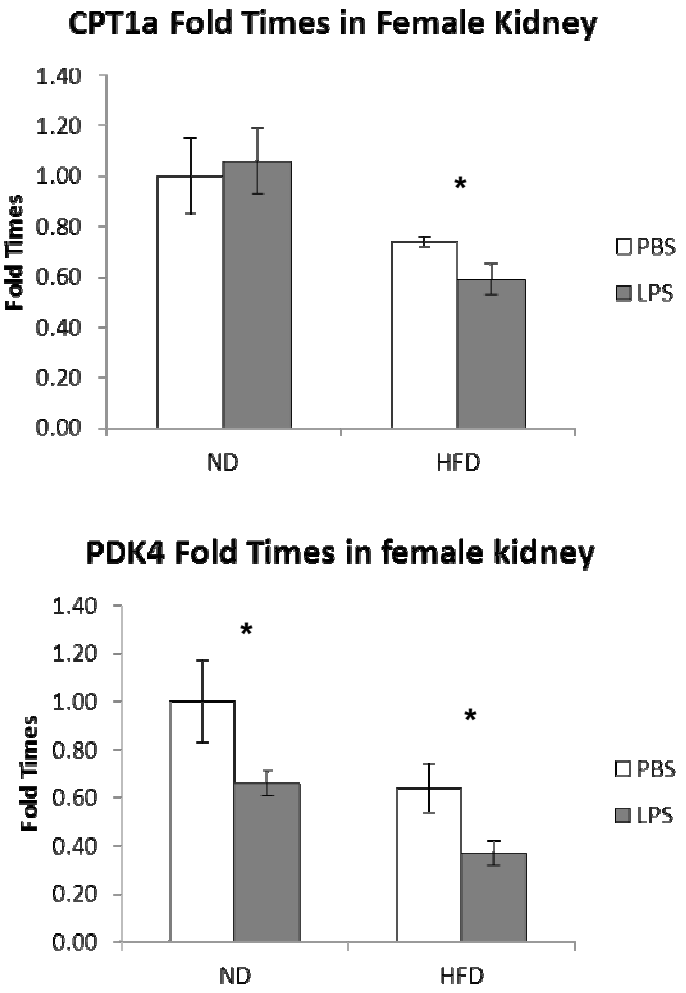

Figure 13: Gene expression levels of FAO genes in kidney tissues of WT mice. Kidney tissues from WT mice (n=6 per group) were harvested after 2 months of LPS or PBS injections and normal diet (ND) or high fat diet (HFD) treatments, as described previously. Tissues were homogenized in IsolRNA and stored at -80℃ until analysis could be performed. Gene expression was normalized to GAPDH fold times for both (A) CPT1α and (B) PDK4. Error bars represent averages +/- SD, *p<0.05.

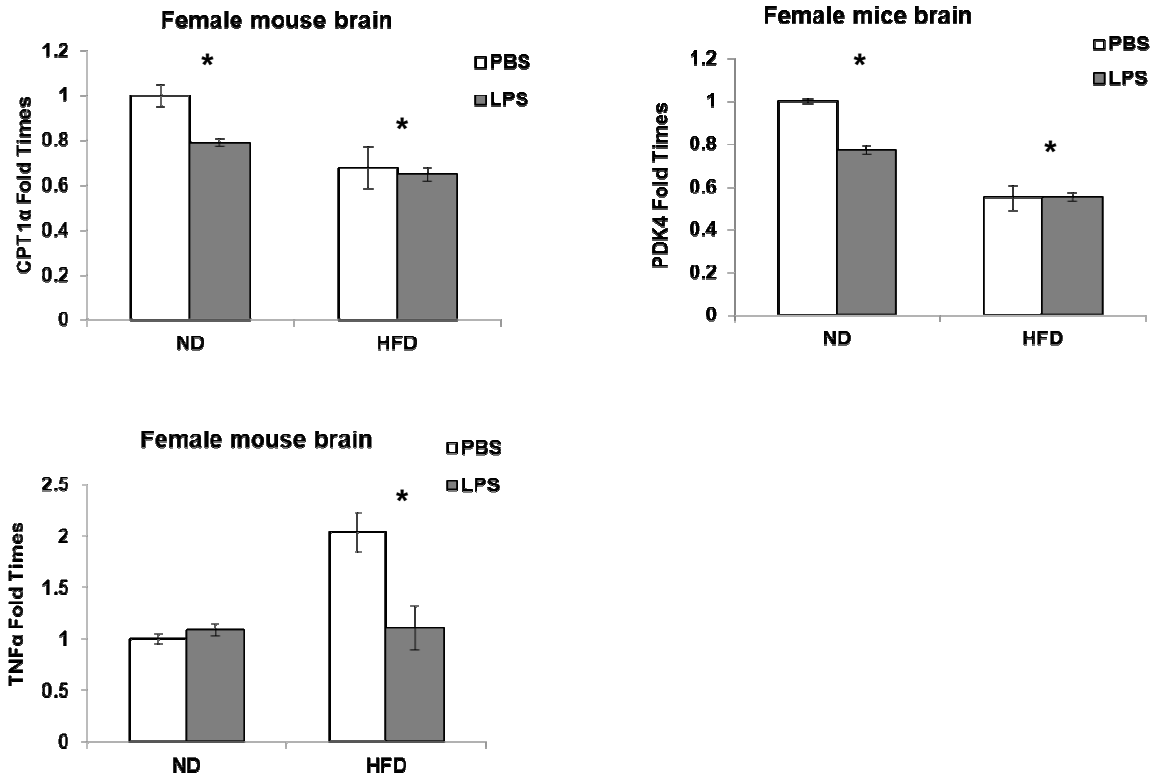

Figure 14: Gene expression levels of FAO genes in brain tissues of WT mice.
Brain tissues from WT mice (n=6 per group) were harvested after 2 months of LPS or PBS injections and ND or HFD treatments, as described previously. Tissues were homogenized in IsolRNA and stored at -80℃ until analysis could be performed. Gene expression was normalized to GAPDH fold times for both (A) CPT1α, (B) PDK4, and (C) TNFα. Error bars represent averages +/- SD, *p<0.05.

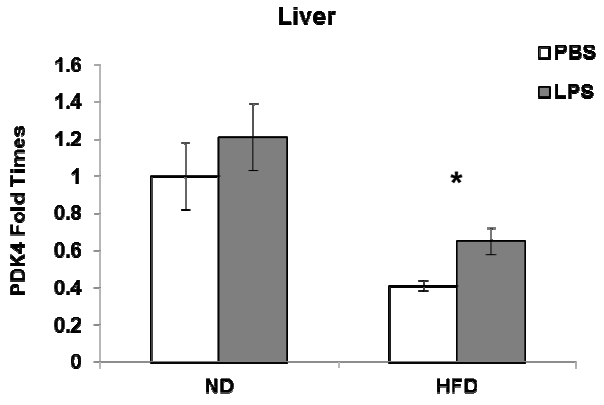

Figure 15: Gene expression levels of PDK4 in liver of WT mice.
Liver tissues of mice from the same 2 month experiment were harvested as previously described and stored at -80℃ until analysis. PDK4 levels were normalized to GAPDH expression as a control. Error bars represent average +/- SD, *p<0.05.

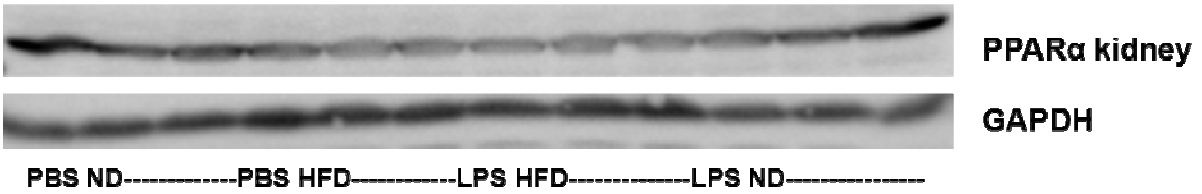

Figure 16: Protein levels of PPARα in WT mice on ND vs. HFD and PBS vs. LPS for 2 months.
Mice on ND or HFD were injected every three days with either PBS or 5ug/kg LPS for 60 days. The animals were sacrificed and protein from kidneys was harvested using T-Per extraction reagent and stored at -80℃ until further analysis. Protein levels of PPARα were analyzed between the different treatment groups via western blot.

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CHAPTER 3: A Mechanism for the Development of Metabolic and Inflammatory Alterations Associated with Metabolic Endotoxemia *in vitro*.

3.1 Abstract

Metabolic endotoxemia is a novel concept that is defined by subclinical elevations in circulating plasma endotoxin levels. It is beginning to be implicated as a potential etiology for chronic inflammatory disease development, including insulin resistance and type II diabetes, atherosclerosis, and Parkinson's disease. The low grade chronic inflammatory status established by very low doses of lipopolysaccharide (LPS) is produced through the Toll-like receptor 4 (TLR4), the innate immune receptor for LPS, just as in the case of sepsis and septic shock. However, in the case of metabolic endotoxemia, the phenotypes that result from TLR4 activation are drastically different. Instead of an acute burst of pro-inflammatory cytokines and chemokines, very low doses of LPS create a leaky, low grade expression of select pro-inflammatory transcription factors and cytokines. The mechanism behind this low grade endotoxemia has yet to be understood and further investigation is warranted. Meanwhile, it has also been established that high doses of LPS (10ng-300ng/ml) not only trigger significant inflammatory changes, but also cause metabolic alterations in the host. The switch from

lipid oxidation to glucose utilization can lead to glucose depletion and subsequent vital organ failure. However, whether the metabolic complications observed with high dose LPS challenge are also observed in very low dose LPS challenge has yet to be determined. Additionally, whether similar points of cross-talk exist between metabolism and inflammation downstream of TLR4 has also yet to be evaluated. Our study offers vital information in these regards. We determine that nuclear receptors and FAO genes are similarly suppressed at both high and very low doses of LPS, and that IRAK1 and JNK are key points of cross talk between metabolism and inflammation at very low doses of LPS. Further, our study demonstrates phenotypic changes associated with very low dose LPS challenge, including cytosolic acetyl CoA accumulation and changes in glucose oxidation after LPS challenge at 50pg/ml.

3.2 Introduction

The concept of metabolic endotoxemia is a novel one which has only recently begun to be implicated in severe disease pathologies including atherosclerosis, type II diabetes and insulin resistance, was well as cancer metastasis and Parkinson's disease (18, 28, 37, 39). Metabolic endotoxemia refers to subclinical, chronically elevated levels of endotoxin in circulation and its presence sets up a persistent, low-grade inflammatory state in the host. This low grade inflammation, along with other factors, can contribute to the development of insulin resistance and set the stage for the development of other chronic inflammatory diseases. Theorized sources of endotoxin include habits such as smoking, drinking, and consuming high fat diet (3, 4, 7). Additionally, increased but subclinical levels of plasma endotoxin have also been associated with aging and periodontal disease (26, 35).

Toll-like receptor 4 (TLR4) is the innate immune receptor for Gram negative bacterial endotoxin, namely, lipopolysaccharide (LPS), whose activation leads to the downstream activities of several pro-inflammatory transcription factors including NFkB, C/EBPδ, AP-1, and MAPKs at high doses of LPS (10-300ng/ml) (1, 2, 20). These transcription factors then up-regulate the expression of a variety of pro-inflammatory mediators including MCP-1, IL-6, TNFα, and endothelin-1 (ET-1), among others (1, 17, 19, 20). Normally, these pro-inflammatory mediators are suppressed at the gene level by a mechanism termed trans-repression. Here, nuclear receptors such as PPAR and PGC1

encourage the repression of pro-inflammatory genes by co-repressors such as NCoR (10, 15, 22, 29). However, TLR4 activation by high doses of LPS triggers the release of these nuclear receptors from gene promoters and promotes the activities of pro-inflammatory transcription factors. To add, these nuclear receptors which are also responsible for lipid and glucose metabolism are also inhibited at both the gene and protein levels (22, 24). Thus, at high doses of LPS, we and others have demonstrated a TLR4 dependent suppression in lipid oxidation and that this suppression is dependent on interleukin-1 receptor associated kinase-1 (IRAK1), a kinase downstream of the TLR4 receptor (22, 8).

Interestingly, the behavior of TLR4 signaling at very low doses of LPS (1-100pg/ml) appears to be quite distinct from high dose TLR4 activation, where instead of a burst of pro-inflammatory cytokines and subsequent resolution through the counter expression of anti-inflammatory mediators, very low doses of LPS cause a leaky, low grade release of pro-inflammatory mediators (22, 23). Additionally, differences in LPS concentration also appear to distinct downstream targets. In a recent published study by our lab, very low dose LPS selectively activated C/EBPδ, but not NFkB (23). It follows that the mechanism of signaling may be different in order to elicit these distinct concentration dependent phenotypes. However, these mechanisms have yet to be elucidated, and whether very low doses of LPS are able to create similar metabolic alterations to high doses of LPS has yet to be determined.

Therefore, in the current study, we examine the role of very low dose LPS on key fatty acid oxidation (FAO) genes including a gene regulating cholesterol efflux (ABCA1), a gene regulating mitochondrial β-oxidation (CPT1α), and a gene which acts as a switch between glucose and lipid oxidation (PDK4) in a variety of cell types (9, 12, 14, 21, 24, 30, 36). Additionally, since high doses of LPS cause metabolic alterations that are IRAK1 dependent, we aimed to examine the role of IRAK1 in metabolic alterations associated with very low dose LPS. Further, we aimed to elucidate the mechanism behind very low dose LPS effects on metabolism through examining the expression of nuclear receptors responsible for FAO gene expression (PPARα, PPARγ, PGC1α) as well as the activities of a pro-inflammatory cascade protein, JNK which has been implicated in the development of obesity and insulin resistance (13, 18, 21). Additionally, we aimed to examine phenotypic changes in metabolism utilizing a glucose oxidation assay in human differentiated THP-1 cells and demonstrate the pro-inflammatory effects of increased acetyl-CoA levels due to FAO inhibition.

3.3 Materials and Methods

Cell Culture

Murine embryonic fibroblasts and immortalized murine macrophages were maintained in DMEM (Invitrogen) with 10% FBS and 1% Penicillin-streptomycin (P/S). Cells were trypsinized once plates reached 80-90% confluence and reseeded into 6 well treated plates for RT-PCR experiments and 10cm treated plates for western blot experiments.

Cells were starved overnight in DMEM with 1% FBS and 1% P/S before the experiments were performed. THP-1 cells were maintained in RPMI supplemented with 10% FBS and 1% P/S and reseeded into untreated plates overnight in 1% FBS, 1% P/S before experiments were performed.

Briefly, bone-marrow derived macrophages (BMDM) were harvested from mice euthanized by cervical dislocation under isoflurane anesthesia. The femurs were collected aseptically and bone marrow was flushed out with PBS in a sterile environment. Cells were then placed in ACK lysis buffer and spun down to rid the samples of red blood cells. Cells were then placed in DMEM with 10% FBS and 1% P/S, supplemented with M-CSF filtered from cultured L929 cells. On day 3 of culture, cells were again supplemented with M-CSF and cultured until day 7. On day 7, BMDM were placed in 1% FBS, 1% P/S DMEM overnight before the experiment.

Real-time PCR

Cells were harvested in 1ml IsolRNA and isolated according to the manufacturer's protocol. Certified RNAse-free equipment and tubes were utilized. RNA purity was determined by 260/280nm absorbance readings of >1.6. Then, 1.5ug RNA from each sample was reverse transcribed using the High capacity cDNA Reverse transcription kit (Applied biosystems) in a mastercycler (Eppendorf). Subsequent real-time PCR was performed using SYBR green master mix (Bio-rad) in an iQ5 thermocycler (Bio-rad). Each run was composed of 35-40 samples under the recommended real-time protocol and data was analyzed using the Δ-CT and ΔΔ-CT method after normalizing samples to

GAPDH internal controls. Primer generation was from IDT primerquest and sequences were as follows:

Gene (*mus musculus*)	Primer Sequence (5'-3')
GAPDH fwd	TGT-GAT-GGG-TGT-GAA-CCA-CGA-GAA
GAPDH rev	GAG-CCC-TTC-CAC-AAT-GCC-AAA-GTT
CPT1-α fwd	CTC-AGT-GGG-AGC-GAC-TCT-TCA
CPT1-α rev	GGC-CTC-TGT-GGT-ACA-CGA-CAA
MCAD fwd	TCG-GTG-AAG-GAG-CAG-GTT-TCA-AGA
MCAD rev	AAA-CTC-CTT-GGT-GCT-CCA-CTA-GCA
PDK4 fwd	AGT-GAC-TCA-AAG-ACG-GGA-AAC-CCA
PDK4 rev	ACA-CAA-TGT-GGA-TTG-GTT-GGC-CTG
CEB/Pδ fwd	ACT-TCA-GCG-CCT-ACA-TTG-ACT-CCA
CEB/Pδ rev	TGT-TGA-AGA-GGT-CGG-CGA-AGA-GTT
IL-6 fwd	ATC-CAG-TTG-CCT-TCT-TGG-GAC-TGA
IL-6 rev	TAA-GCC-TCC-GAC-TTG-TGA-AGT-GGT
ABCA1 fwd	GGA-CAT-GCA-CAA-GGT-CCT-GA
ABCA1 rev	CAG-AAA-ATC-CTG-GAG-CTT-CAA-A

Human primers were utilized for THP-1 gene expression and primers were generated in the same manner as the murine primers, as follows:

Gene (*Homo sapiens*)	Primer Sequence (5'-3')
GAPDH fwd	CAT-GTT-CGT-CAT-GGG-TGT-GAA-CCA
GAPDH rev	AGT-GAT-GGC-ATG-GAC-TGT-GGT-CAT
CPT1-α fwd	ACA-GTC-GGT-GAG-GCC-TCT-TAT-GAA
CPT1-α rev	TCT-TGC-TGC-CTG-AAT-GTG-AGT-TGG
MCAD fwd	CTA-CCA-AGT-AGT-CCC-TGG-AAA-G
MCAD rev	TGT-GTT-CAC-GGG-CTA-CAA-TAA-G
PDK4 fwd	TTG-AGT-GTT-CAA-GGA-TGC-TCT-G
PDK4 rev	TGC-CCG-CAT-TGC-ATT-CTT-AAA-TA
ABCA1 fwd	GTC-CTC-TTT-CCC-GCA-TTA-TCT-GG
ABCA1 rev	AGT-TCC-TGG-AAG-GTC-TTG-TTC-AC

Western Blot

Isolation of whole cell lysate was performed by rinsing treated and control cells with ice cold PBS before lysing the cells with 1x SDS lysis buffer (80mM Tris-HCl (pH 6.8), 2% SDS, 50% glycerol) containing protease and phosphatase inhibitors (Sigma). Cytosolic and nuclear lysates were separated in other samples using Buffer A (10mM HEPES pH 7.9, 1.5mM $MgCl_2$, 10mM KCl, 0.2mM EDTA) to extract cytosolic lysate and Buffer B (20mM HEPES pH 7.9, 1.5mM $MgCl_2$, 0.4mM NaCl, 0.2mM EDTA, 25% glycerol). Western blot analysis of the protein samples was performed after running proteins on SDS-PAGE gels and transfer to PVDF membranes at 110V for 2 hours. Immunoblots were developed using Amersham ECL Plus chemiluminescent detection system (GE

Healthcare). The films were developed using FujiFilm Multi-gauge software and normalized against either B-actin or GAPDH levels. Antibodies were purchased from Santa Cruz Biotechnology, except for PPARγ and Phospho-PPARγ antibodies which were purchased from Millipore.

Immunoprecipitation Assay

Proteins were isolated using a standard protein lysate protocol (50mM HEPES pH 7.6, 150mM NaCl, 1mM EDTA, 0.5% NP-40) with protease and phosphatase inhibitors. Lysates were then incubated at 4℃ with primary antibody against ATF2 (rabbit monoclonal, Santa Cruz) under agitation for 2 hours. The lysates were then incubated with protein agarose beads (Santa Cruz) overnight at 4℃ on a rotator. The next morning, the beads were pelleted briefly and washed twice gently with cell lysis buffer. 2x SDS loading buffer was then added to each sample and boiled for 5 minutes. Samples were spun down and supernatants were then run on a 10% SDS-PAGE gel. After transfer to a PVDF membrane, samples were incubated with rabbit acetyl-lysine primary antibody and then anti-rabbit secondary antibody before being developed as mentioned in the Western blot protocol.

Glucose Oxidation Assay

THP-1 cells were seeded in 6-well plates and incubated with 100nM PMA for 24 hours to allow cells to differentiate and attach to the bottom of the wells before the assay. Cells were then placed in RPMI with 1% FBS, 1% P/S before the assay. […]. Glucose

oxidation was measured at 6 hours after treatment with LPS at 50pg/ml, LPS and DMSO vehicle, and LPS plus 50uM JNK II inhibitor (EMD chemicals).

Acetyl CoA Isolation

Cells were collected after treatment and washed with ice cold PBS before adding 1ml 1x cytosol extraction buffer (Mitochondria/Cytosol fractionation kit, Abcam). Cells were fractionated according to the manufacturer's protocol and cytosolic lysates were analyzed for acetyl CoA levels using the PicoProbe^{TM} Acetyl CoA Assay Kit, also from Abcam. Briefly, samples were then added to a 96-well plate (Costar) in duplicate. A CoA quencher was added for 5 minutes to the background samples before being incubated with a stop solution for an additional 5 minutes. The probe and associated enzyme mixes were added to each well and incubated at 37℃ before being read on a microplate reader at OD 535/589nm. Background readings were subtracted before analysis and results were extrapolated from a standard curve using the standard provided in the kit.

3.4 Results

Low and very low doses of LPS cause suppression of FAO genes in an IRAK1-dependent manner across several murine cell lines.

To assess the response of murine cells to low and very low dose challenges of LPS *in vitro*, we examined the gene expression levels of both CPT1α and PDK4, genes which

are both responsible for lipid metabolic activity in several different murine cell lines. Additionally, we aimed to determine whether these responses at low and very low doses of LPS were IRAK1 dependent. Thus, we utilized murine bone-marrow derived macrophages (BMDM), murine embryonic fibroblasts (MEF), and a murine immortalized macrophage (iMφ) cell lines to examine the effects of low and very low doses of LPS. As observed in Fig. 17A and B as well as Fig. 18 A and B, both CPT1α and PDK4 gene expression levels were significantly reduced after LPS stimulation with 1-10ng/ml LPS. Interestingly, IRAK1 deletion was protective against this reduction in gene expression for both CPT1α and PDK4. Thus, IRAK1 is a critical modulator downstream of TLR4 responsible for the down-regulation of both CPT1α and PDK4 at the gene level. Meanwhile, PDK4 levels were significantly reduced at very low doses of LPS (50pg/ml) in an IRAK1 dependent manner in BMDM, and both CPT1α and PDK4 levels at 50pg/ml LPS were significantly reduced in iMφ (Fig. 17C, 19A, 19C). Additionally, iMφ cells demonstrated a significant, dramatic decrease in the expression levels of ABCA1, a cholesterol efflux gene implicated in the pathogenesis of atherosclerosis (Fig. 19B).

Protein levels of nuclear receptors PPARα and PGC1α are reduced after very low and high doses of LPS.

Besides gene expression levels of FAO genes, we aimed to examine whether the protein levels of nuclear receptors upstream of these FAO genes were affected by very low and high doses of LPS. As observed in Fig. 20, a western blot of whole cell PPARα in MEF demonstrates a noticeable decrease in PPARα protein levels in WT cells at both

50pg/ml and 200ng/ml LPS. Additionally, IRAK1-/- cells appear to be protected from this decrease in PPARα protein levels at both 50pg/ml and 200ng/ml LPS.
Further, we examined protein levels of PGC1α, a co-activator for PPARα in nuclear lysate from iMφ cells. Similar to PPARα protein in MEF, iMφ protein levels of PGC1α were also decreased after both 50pg/ml LPS challenge and 200ng/ml LPS challenge.

Suppression of FAO gene expression is dependent on concentration in human THP-1 cells.

Next, we moved to a human cell line to determine whether the suppression observed in murine cell lines was similar to the behavior of a human monocytic cell line. Thus, we stimulated THP-1 cells with a range of concentrations of LPS ranging from 0.1pg/ml LPS to 100pg/ml LPS. Intriguingly, even minute concentrations of LPS down to 1pg/ml and 0.1pg/ml were able to suppress expression of CPT1α and PDK4, respectively (Fig. 22A, B).

Functional sensing of LPS on CPT1α and PDK4 expression levels in human THP-1 cells.

Further, we aimed to determine whether a lower concentration of LPS after initial challenge with 50pg/ml LPS would suffice to continue the suppression of CPT1α and PDK4 in THP-1 cells. Interestingly, the concentrations of LPS necessary to sustain suppression of CPT1α and PDK4 were 0.5pg/ml and 0.1pg/ml, respectively. Thus, THP-

1 cells were able to sustain suppression of these FAO genes at lower or equivalent concentrations of LPS after initial challenge with a very low dose LPS (50pg/ml) (Fig. 22C, D).

Chronicity of LPS response in THP-1 cells on FAO gene suppression.

We then moved to determine the duration of FAO gene suppression after very low dose LPS exposure. Out to 8 hours, 50pg/ml LPS was able to sustain significant suppression of both CPT1α and PDK4 in THP-1 cells. Additionally, we examined the effects of THP-1 cell differentiation on response to very low dose LP challenge, as well as low and high dose challenge. We observed the suppression of PDK4 at 100pg/ml LPS as well as 1ng/ml and 100ng/ml, demonstrating that behavior in differentiated THP-1 cells is similar to undifferentiated cells (Fig. 23 A, B).

Protein levels of nuclear receptors in response to very low dose LPS challenge in THP-1 cells.

We then aimed to examine the protein levels of both PGC1α, PPARγ and phosphor-PPARγ in THP-1 cells challenged with very low dose LPS, since these nuclear receptors are upstream of FAO genes. As demonstrated in Fig. 24A, even doses down to 50pg/ml LPS were able to suppress PGC1α protein levels. We also observed levels of PPARγ which appeared to be slightly decreased after 50pg/ml LPS challenge.

However, the phosphor-PPARγ levels appeared slightly increased after 50pg/ml challenge, offering the contribution of inhibition of protein function via phosphorylation.

After observing the suppressive effects of very low doses of LPS on FAO gene expression and nuclear receptor protein levels in THP-1 cells, we examined the role of cytosolic acetyl CoA levels and their potential contribution to transcription factor acetylation downstream of TLR4. In Fig. 25A, we determined that at very low dose LPS (50pg/ml), cytosolic levels of acetyl CoA were significantly increased at 6 hours post LPS challenge compared to control samples. Further, immunoprecipitation of ATF2 with an acetyl-lysine antibody demonstrates a slight increase in acetylation status of the pro-inflammatory transcription factor, thus elucidating downstream activity of TLR4 activation by very low dose LPS.

JNK phosphorylation contributes to TLR4 response to very low dose LPS.

To further elucidate the TLR4 pathway activated by very low dose LPS, we examined the levels of JNK phosphorylation after challenge of THP-1 cells with 50pg/ml LPS. We observed a marked increase in phosphor-JNK protein levels after very low dose LPS challenge, which was completely inhibited by the addition of a JNK inhibitor (Fig. 26A, B).

JNK is involved in the very low dose LPS induced up-regulation of glucose utilization in THP1 cells.

Finally, we aimed to examine the effect of very low dose LPS challenge on overall glucose oxidation levels in THP-1 cells and whether JNK was involved in the LPS-associated metabolic alterations. As demonstrated in Fig. 27, 50pg/ml LPS caused significant increase in glucose oxidation, while the addition of a JNK inhibitor caused a significant decrease in glucose oxidation.

3.5 Discussion

In a recent paper published by our lab, we demonstrated the ability of very low dose LPS to cause a significant increase in pro-inflammatory cytokine and chemokine gene expression levels; namely IL-6, MCP-1 (monocyte chemoattractant protein-1), and ET-1(endothelin-1) (22). Additionally, at high doses of LPS, we have observed a decrease in lipid oxidation and an increase in glucose utilization (22). However, the implication of very low dose LPS on metabolic alterations had not been determined. Thus, this study is the first to demonstrate the ability of very low doses of LPS to significantly suppress FAO gene expression as well as nuclear receptor protein levels in both murine and human cell lines. Gene expression levels of CPT1α, PDK4 and ABCA1 were all significantly suppressed after very low-dose LPS challenge in a variety of cell lines, including murine BMDM, MEF, iMφ, as well as both undifferentiated and differentiated

human THP-1 cells. In combination with our previously published low dose endotoxemia study, the findings in this study support the involvement of very low doses of endotoxin in chronic disease pathologies, including atherosclerosis and insulin resistance by causing alterations in both cellular inflammatory and metabolic statuses. Our western blot experiments also confirm our previous findings that very low doses of LPS are able to suppress protein levels of nuclear receptors responsible for FAO regulation, namely PPARα and PGC1α (23).

Further, we have determined some of the dynamics involved in the very low dose LPS-induced suppression of both CPT1α and PDK4 expression levels in human THP-1 cells as well as the effects of functional sensing of very low dose LPS on FAO gene expression. Since the exact doses of LPS necessary to elicit suppression of FAO genes had not yet been established, we performed a concentration curve with concentrations of LPS down to 0.1pg/ml. Surprisingly, extremely low doses of LPS were able to cause significant suppression in FAO gene levels, demonstrating a high degree of sensitivity of FAO genes to very low doses of LPS. This is a novel finding and warrants further examination *in vivo* to determine minimal concentrations needed in a host to elicit similar responses. A paper published by Shi et al has demonstrated the ability of very low levels of LPS in an *in vivo* mouse model to cause a solely pro-inflammatory program in circulating monocytes (32). However, more studies must be performed to confirm these findings. Another novel concept defined in this study is the effect of functional sensing of LPS on FAO gene expression. The theory of functional sensing determines that there are threshold concentrations that must be established before inflammatory or

metabolic alterations are observed. However, once that threshold concentration is reached, lower concentrations below that threshold are able to maintain the response. In our studies, we were able to demonstrate that the suppression of FAO genes upon initial exposure of THP-1 cells to 50pg/ml LPS was then sustained by lower concentrations of LPS for both CPT1α and PDK4. This finding has major implications for the prevention and intervention of chronic LPS-related diseases (as mentioned in Chapter 1), since FAO suppressive effects of LPS can be sustained at very low concentrations, even below the threshold concentration. In this way, functional sensing can contribute to the chronicity of disease.

Additionally, we have defined a critical role for IRAK1 and JNK in these metabolic alterations associated with very low dose LPS challenge. Previous studies published by our lab have demonstrated a critical role for IRAK1 in the selective activation of C/EBPδ after very low dose LPS challenge to activate a low grade chronic pro-inflammatory program. Of note, these studies also determined that very low doses of LPS do not induce the activation of NFkB or ERK pathways (23).This current study then contributes to the understanding of the critical role of IRAK1in very low dose LPS signaling, as we have observed significant protection from suppression of FAO genes and nuclear receptors in IRAK1$^{-/-}$ cells. These studies also serve to confirm the findings of our previously published observations that IRAK1 is necessary for the release of trans-repression mechanisms of nuclear receptors PPARα and RARα on the proximal promoters of pro-inflammatory genes (23). Further, we have determined that JNK is also activated after very low dose LPS challenge in human THP-1 cells and that

phosphorylation of JNK is critical in the regulation of cellular glucose oxidation. Thus, JNK serves as a point of cross-talk between inflammatory and metabolic pathways, since it has been well-established that JNK activation up-regulates expression of pro-inflammatory mediators.

To add, we have observed a novel pattern of increased cytosolic acetyl CoA levels and increased acetylation of the pro-inflammatory transcription factor, ATF2 after very low dose LPS challenge. Previous studies have demonstrated an increase in cytosolic acetyl CoA (ACoA) levels after inhibition of FAO due to the build-up of ACoA in mitochondria and subsequent delivery of this excess ACoA into the cell cytoplasm. We theorized that increased levels of cytoplasmic ACoA could provide a pool of ACoA for acetylation of genes, thus contributing to the regulation of gene expression. Our study is the first to demonstrate an increase in cytoplasmic ACoA levels after very low dose LPS challenge in THP-1 cells, as well as increased acetylation status of a pro-inflammatory transcription factor, ATF2 downstream of TLR4. Further studies are warranted in the examination of direct effects of cytoplasmic ACoA levels on transcription factor acetylation status.

Collectively, our study defines a unique phenotype and mechanism for the cellular response to very low doses of LPS. The paradigm that the LPS response is concentration-dependent is a novel one, and the findings in our study support the theory of a chronic alteration in inflammatory and metabolic host state in response to very low levels of endotoxin. Future studies must be performed to further decipher the

mechanism of this very low dose LPS pathway, but our study contributes significantly to the understanding of chronic low grade endotoxin induced alterations in inflammation and metabolism, and that these changes are IRAK1 dependent.

3.6 Figures and Legends

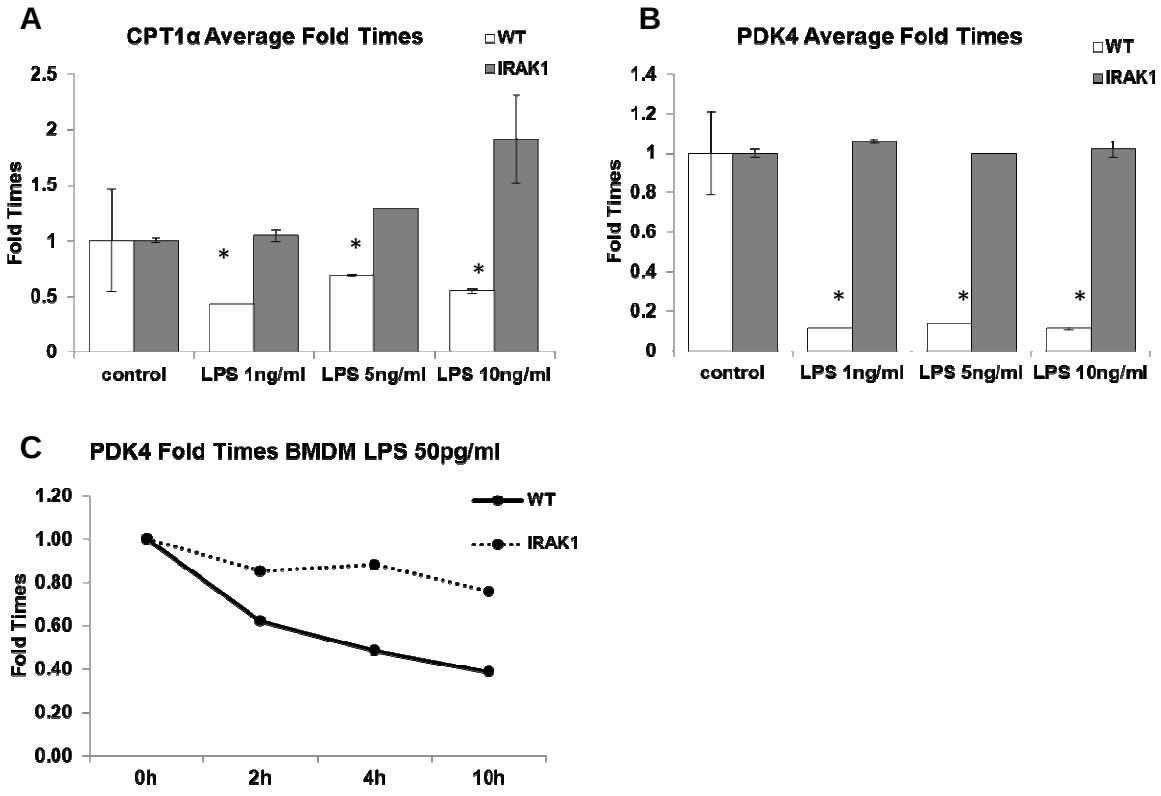

Figure 17: Expression levels of CPT1α and PDK4 in bone-marrow-derived macrophages (BMDM) harvested from WT and IRAK1$^{-/-}$ mice.
BMDM were harvested as described in the materials and methods section. Briefly, BMDM were starved overnight in DMEM (1% FBS) before being stimulated with varying concentrations of LPS. Gene expression was normalized to GAPDH fold times as an internal control. (A) CPT1α and (B) PDK4 expression was reduced in WT BMDM at concentrations as low as 1ng/ml, and this suppression continued at 50pg/ml LPS in (C) PDK4. Experiments were performed in triplicate. Error bars represent average fold times +/- SD. Results were considered statistically significant at *$p<0.05$ using Student's t-test.

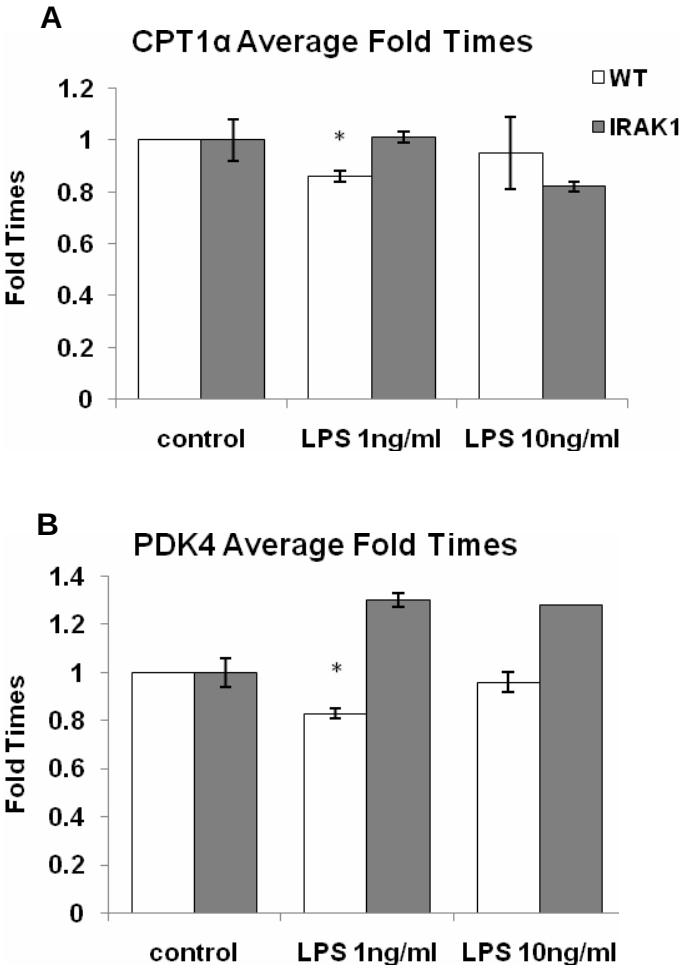

Figure 18: CPT1α and PDK4 gene expression in WT and IRAK1$^{-/-}$ murine embryonic fibroblasts (MEF).
WT and IRAK1$^{-/-}$ MEF were grown to 80% confluence and then placed in DMEM (1% FBS) overnight before being stimulated with varying concentrations of LPS. Fold times of (A) CPT1α and (B) PDK4 were examined at LPS concentrations down to 1ng/ml. Error bars represent average fold times +/- SD, results considered statistically significant at *$p<0.05$ using Student's t-test.

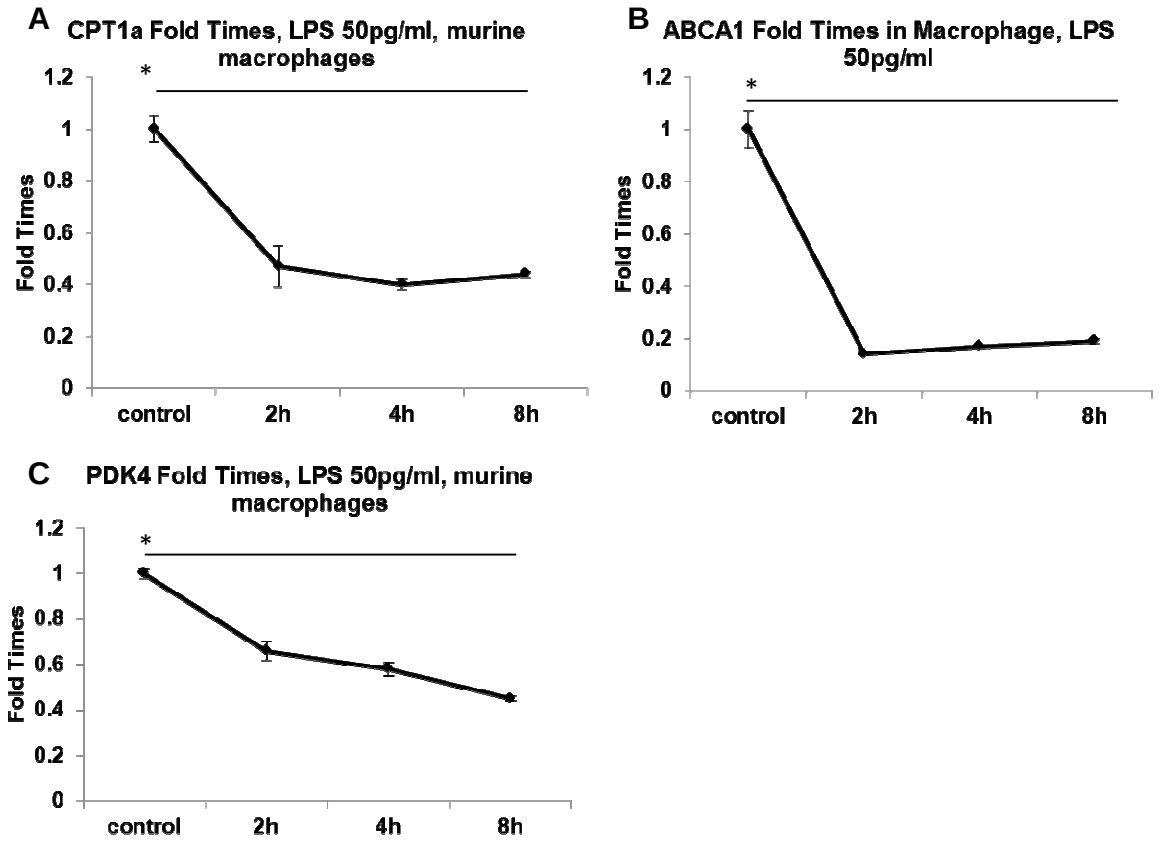

Figure 19: Expression of FAO genes vs. time after LPS challenge (50pg/ml) in murine immortalized macrophages.
WT and IRAK1$^{-/-}$ immortalized murine macrophages were grown to 80% confluence and then placed in DMEM (1% FBS) overnight before being stimulated with 50pg/ml LPS. Fold times of (A) CPT1α, (B) ABCA1, and (C) PDK4 were determined using GAPDH fold times as an internal control. Error bars represent average fold times +/- SD, results considered statistically significant at *$p<0.05$ using Student's t-test.

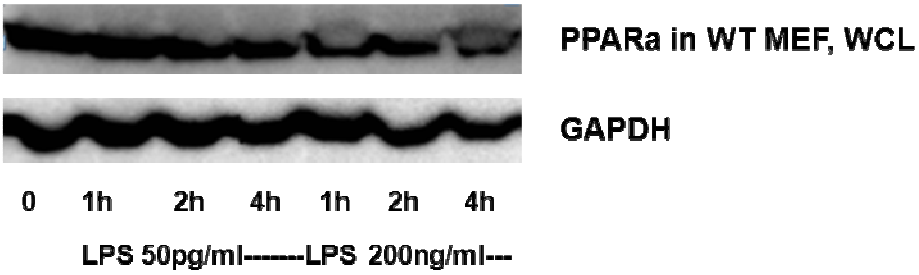

Figure 20: Protein levels of PPARα in MEF after challenge with 50pg/ml.
WT MEF were cultured and treated as previously described in the Materials and Methods section. Protein whole cell lysate was harvested using SDS lysis buffer after LPS challenge for 0', 1h, 2h, and 4h and then run on SDS-Page gel and blotted with an antibody to PPARα (rabbit).

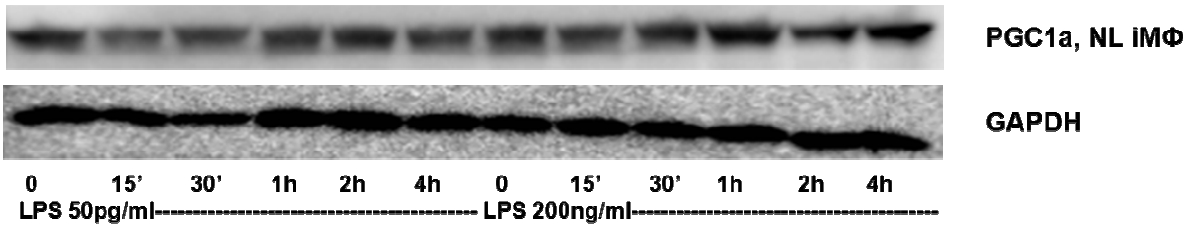

Figure 21: Protein levels of PGC1α in immortalized murine macrophages (iMM) after challenge with 50pg/ml or 200ng/ml LPS.
iMM were cultured and treated as previously described. Protein nuclear lysate was harvested with Buffer A and Buffer B before being quantified. Proteins were run on SDS-Page gel and blotted with an antibody to PGC1α at 0, 15', 30', 1h, 2h, and 4h time points.

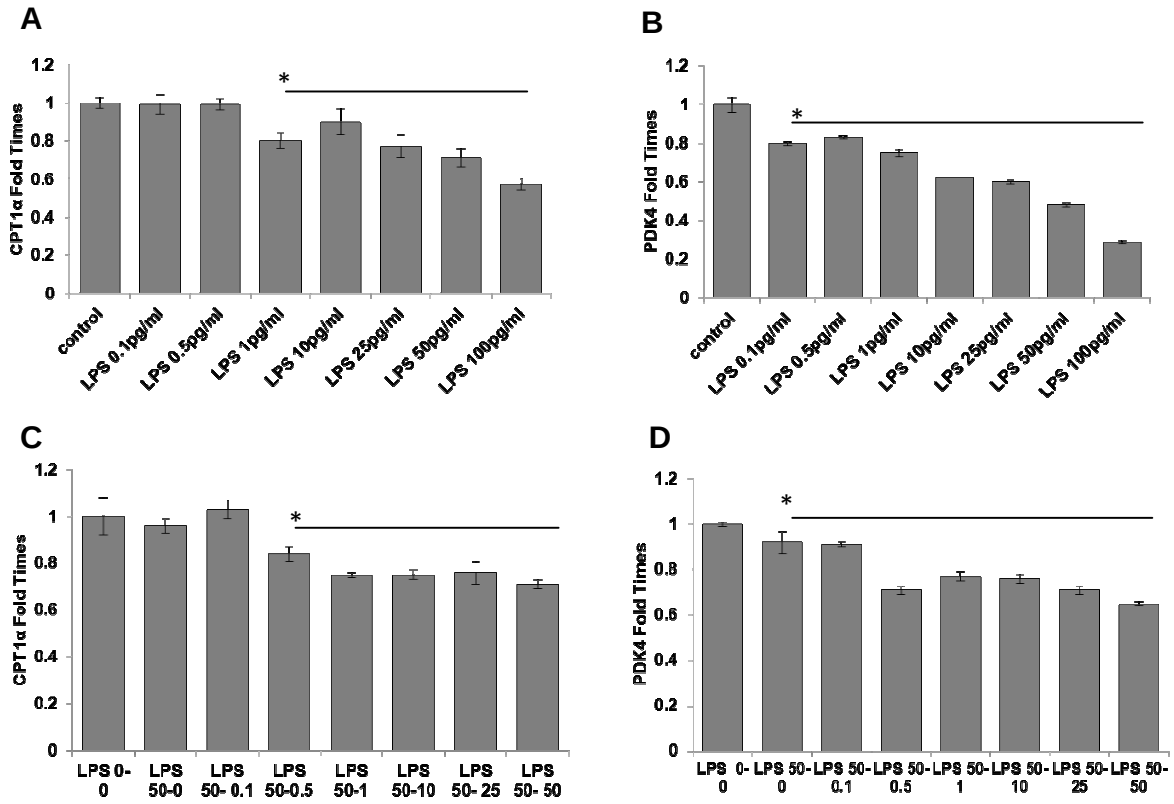

Figure 22: Concentration curve and functional sensing of LPS in THP1 cells. THP-1 cells were starved overnight in RPMI media (1% FBS) before being challenged with varying doses of LPS. Gene expression was normalized to GAPDH fold times for analysis. An LPS concentration curve was plotted for (A) CPT1α and (B) PDK4 expression. Additionally, a functional sensing analysis was performed on THP1 cells. Cells were stimulated with 50pg/ml LPS for 4 hours, washed twice with ice cold PBS, and then re-stimulated with varying concentrations of LPS. Fold times were analyzed as described above for (C) CPT1α and (D) PDK4. Error bars are representative of average fold times +/- SD and statistical significance was determined using Student's t-test at *p<0.05. Experiments were performed in triplicate.

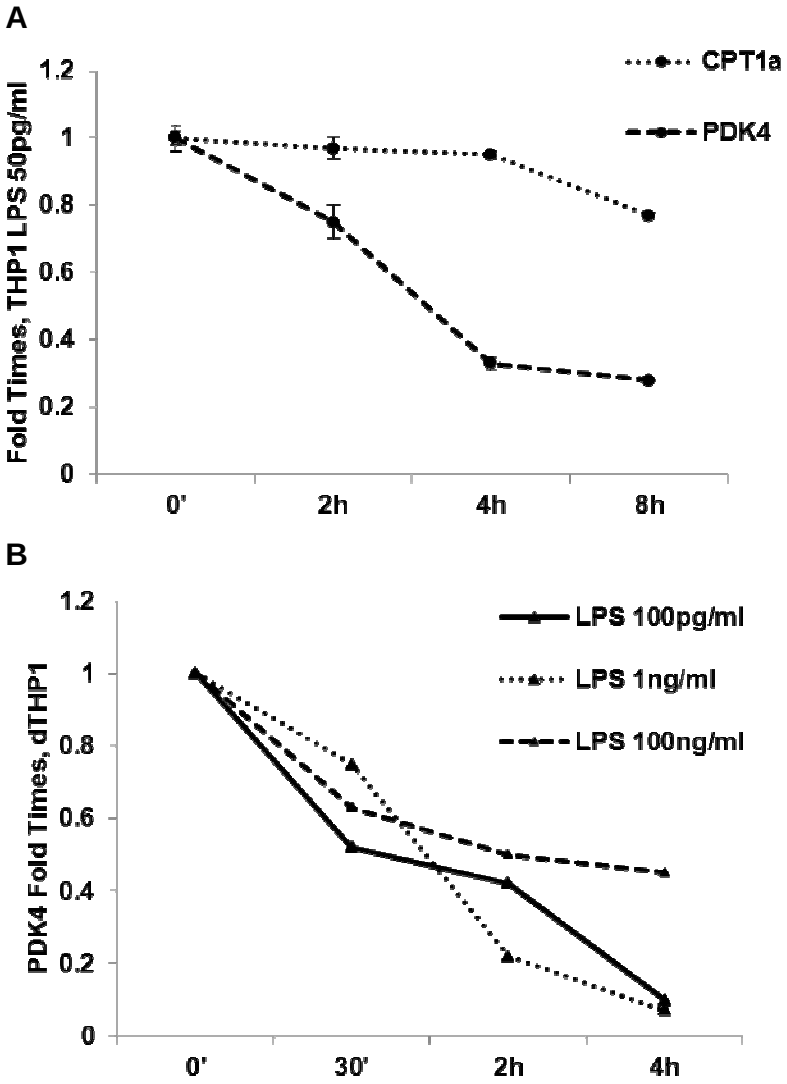

Figure 23: Time course for FAO gene expression after LPS stimulation in THP1 and dTHP1 cells.

(A) THP1 cells starved overnight in 1% FBS (RPMI) were challenged with 50pg/ml LPS for a time course, experiment repeated in triplicate. (B) THP1 cells were differentiated for 24h in 200nM PMA (dTHP1). Adhesion to the well was >80% before the experiment proceeded. dTHP1 cells were washed 3 times with ice cold PBS, then starved overnight in 1% FBS (RPMI) before being challenged with 50pg/ml-100ng/ml LPS.

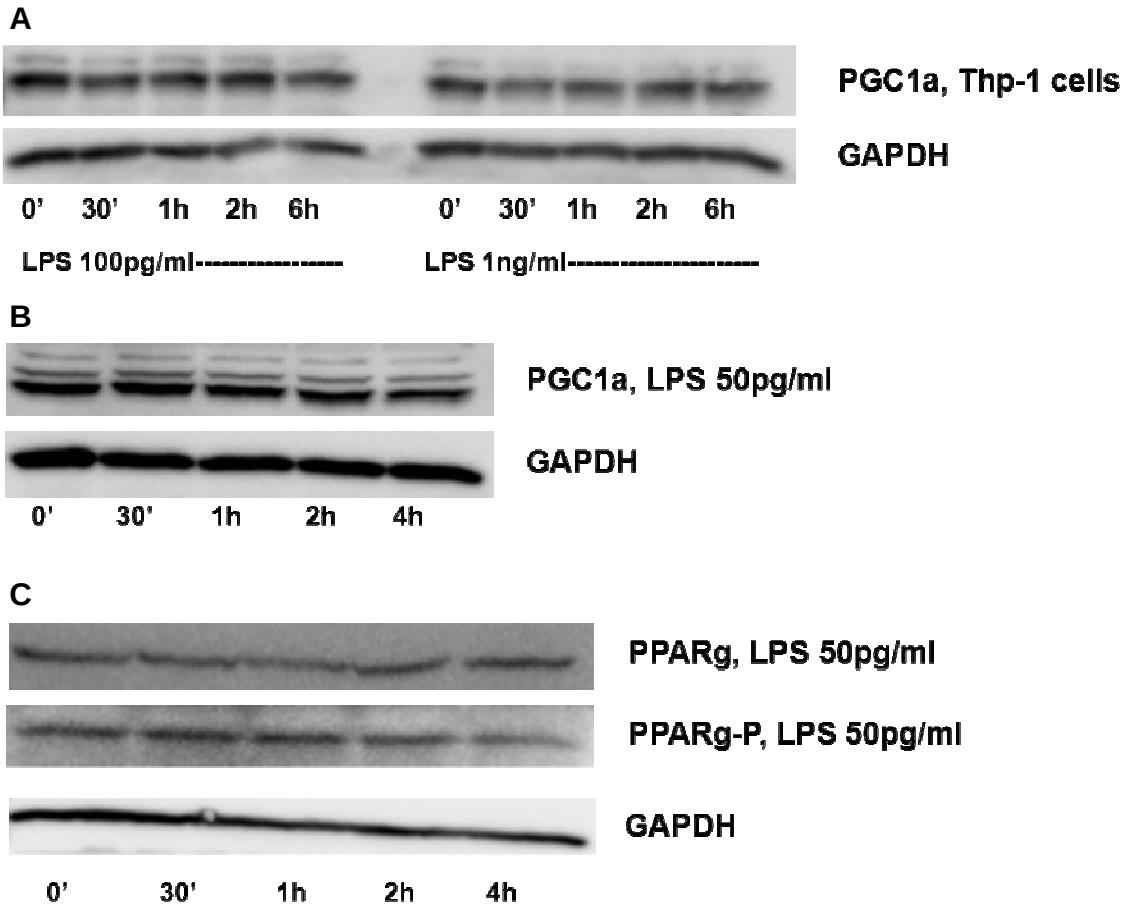

Figure 24: THP1 protein expression levels of nuclear receptors responsible for FAO metabolism regulation after challenge with low-dose LPS.
Whole cell lysate was harvested from THP1 cells challenged with 50pg/ml-1ng/ml LPS at 0', 30', 1h, 2h and 4h time points. PGC1α protein levels at LPS 100pg/ml (A) and LPS 1ng/ml (B) LPS 50pg/ml were determined, as well as PPARγ protein levels and phosphor-PPARγ protein levels at LPS 50pg/ml (C).

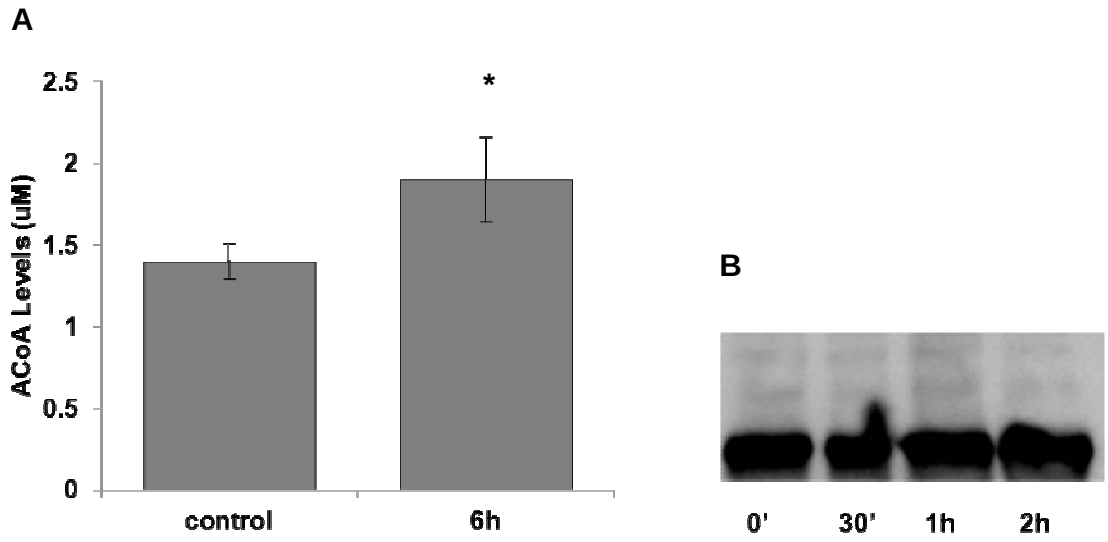

Figure 25: Acetyl-CoA levels and ATF2 acetylation in THP1 cells after stimulation with 50pg/ml LPS.
(A) Cytosolic lysate was extracted from nuclear and mitochondrial THP1 cell fractions using a fractionation kit from Abcam. Cytosolic Acetyl CoA levels were measured using the Acetyl CoA quantification kit from Abcam. Experiments were performed independently three times and error bars represent SD from the average. Statistical significance was performed using Student's t-test, *$p<0.037$. (B) ATF2 was co-immunoprecipitated with acetyl-lysine antibody in THP1 cells after stimulation with 50pg/ml LPS.

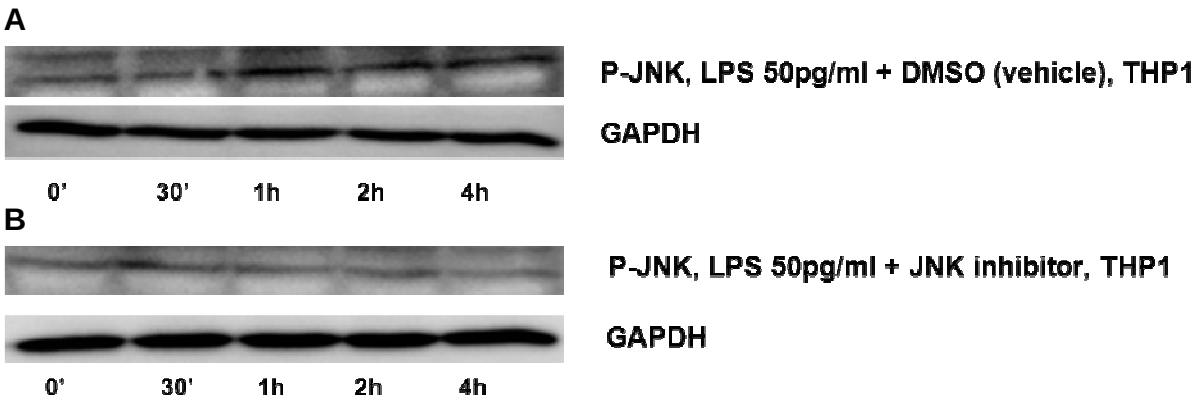

Figure 26: Phosphorylated JNK protein levels after LPS 50pg/ml challenge. THP1 cells were stimulated with (A) 50pg/ml LPS plus vehicle (DMSO) or (B) 50pg/ml LPS plus JNK inhibitor (Calbiochem) before whole cell lysate was harvested. Proteins were run on an SDS-page gel and western-blotted with antibodies to phospho-JNK.

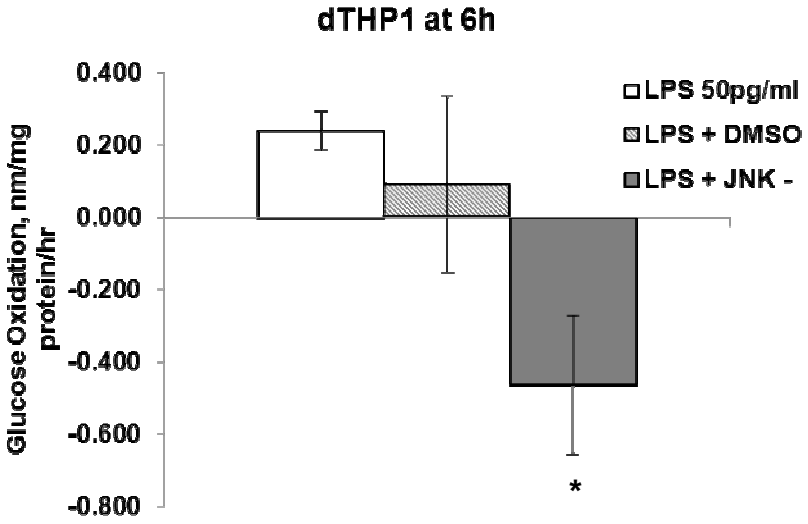

Figure 27: Inhibition of JNK on glucose oxidation after 50pg/ml LPS stimulation. THP1 cells were differentiated in 200nM PMA for 24 hours pre-assay to allow adhesion of the cells to the plate for the glucose oxidation assay. Glucose oxidation was generously performed by Ryan McMillan as previously described in the Materials and Methods section. Error bars are representative of SD from the average from three individual experiments. Statistical significance was assessed using one-way ANOVA, *p<0.007.

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CHAPTER 4: CONCLUSIONS

4.1 *Conclusions*

It has been well-established that high doses of circulating endotoxin in sepsis and septic shock contribute to extremely high morbidity and mortality rates worldwide. Originally the pathogenesis of the disease was thought to be due solely to the storm of pro-inflammatory mediators released through TLR4 activation by circulating LPS. However, more recently it has been established that LPS also triggers significant metabolic derangements at high doses. These metabolic derangements in combination with a strong inflammatory program contribute to MOF and high mortality. Thus, therapies targeted at intervention in pro-inflammatory changes such as anti-TNFα therapies have met limited success and treatment of sepsis and septic shock remain supportive at best. Thus, understanding the cross-talk between metabolism and inflammation downstream of TLR4 can provide identification of critical mediators that regulate both metabolic and inflammatory alterations.

In addition to high levels of circulating LPS, a novel concept has emerged pointing to the equally detrimental effects of very low levels of circulating LPS on inflammatory and metabolic host status. In the case of very low dose LPS, termed metabolic endotoxemia, the activation of TLR4 results in a leaky, low-grade inflammatory status in the host, as well as potential metabolic alterations. These changes are beginning to be challenged as likely etiologies for chronic inflammatory disease development, including insulin resistance and type II diabetes, atherosclerosis, cancer metastasis and even

Parkinson's disease. Thus, examining the phenotype and mechanism of very low dose LPS-TLR4 signaling is essential for future therapies targeted at prevention and intervention of these diseases. Therefore, the *in vivo* and *in vitro* work described in the previous chapters aims to identify a key point of cross-talk between metabolism and inflammation at high dose LPS, as well as define a phenotype for models of metabolic endotoxemia and identify whether similar points of cross-talk exist during very low dose LPS challenge.

4.2 *A Phenotypic Analysis*

Our results demonstrate a protective effect of IRAK1 deletion on mortality rates in mice with a C57Bl/6 background after lethal intraperitoneal injection of LPS (25mg/kg). Additionally, IRAK1 deletion was protective against the metabolic derangements associated with high dose endotoxemia, where IRAK1$^{-/-}$ mice did not demonstrate a depletion of blood glucose or elevation in FFA and TG after high dose LPS challenge. To further confirm these findings, FAO in the liver of IRAK1$^{-/-}$ mice was actually increased after LPS challenge. From an inflammatory standpoint, IRAK1 deletion also conferred a slight alleviation in hepatic necrosis and neutrophilic infiltration in livers of mice 16 hours after high dose LPS injection. Thus, it serves that IRAK1 is a critical point of cross-talk between metabolism and inflammation, and may be a valuable future therapeutic target in lowering mortality rates in sepsis patients.

Additionally, we found that HFD and very low doses of LPS synergize in the development of inflammatory and metabolic alterations in mice with an Apoe$^{-/-}$

background, and that again IRAK1 serves as a critical point of cross-talk between metabolism and inflammation, since IRAK1$^{-/-}$ mice were protected from severe glomerulosclerosis and insulin resistance despite demonstrating higher levels of plasma endotoxin after HFD feeding. To further understand the role of IRAK1 in inflammatory and metabolic alterations associated with very low dose LPS challenge, we examined the expression levels of pro-inflammatory transcription factor C/EBPδ and pro-inflammatory cytokine IL-6, which were significantly up-regulated after chronic injection with 5ug/kg LPS. Simultaneously, levels of several genes responsible for lipid metabolism including ABCA1, CPT1α, and MCAD were measured. IRAK1 deletion also conferred significant protection against LPS-induced suppression of these genes.

Further examining the phenotype of metabolic endotoxemia, we demonstrated trends for an increase in body weight and decreased glucose tolerance in WT mice chronically injected with very low dose LPS and/or fed HFD. Also, mice fed HFD alone or HFD in combination with very low dose LPS (5ug/kg) demonstrated significant suppression in CPT1α and PDK4 gene expression levels, while TNFα gene expression levels were increased after HFD feeding. Nuclear receptors responsible for metabolic regulation were also inhibited after HFD and HFD plus LPS treatments, thus demonstrating the significant alterations associated with both HFD feeding and very low dose LPS challenge.

The collection of this information demonstrates the wide range of LPS triggered effects that are highly dose dependent. However, in both high dose and very low dose LPS challenge, IRAK1 proves a significant point of crosstalk between metabolism and inflammation downstream of TLR4 and appears to significantly contribute to the

morbidity and mortality observed with a wide range of LPS concentrations (5ug/kg-25mg/kg). Importantly, HFD feeding alone and in combination with very low doses of LPS also appears to significantly alter the host metabolic and inflammatory status.

4.3 *A Mechanism for Metabolic Endotoxemia*

In order to examine the mechanisms behind metabolic and inflammatory alterations due to very low dose LPS challenge, we demonstrated significant suppression in FAO gene expression levels after challenge with a range of LPS concentrations (50pg/ml-10ng/ml). Additionally, protein levels of nuclear receptors which regulate these genes, namely PPARα and PGC1α, were decreased after very low dose LPS challenge. To investigate whether IRAK1 deletion conferred protection from these changes *in vitro*, IRAK1$^{-/-}$ cells were also treated in the same manner and demonstrated a protection against FAO gene suppression and decreases in nuclear receptor protein levels after very low dose LPS challenge.

Additionally, we sought to determine the minimum dose necessary to obtain FAO gene suppression in a human monocytic cell line (THP-1). Intriguingly, concentrations as low as 0.1pg/ml and 0.5pg/ml were able to suppress CPT1α and PDK4, respectively. This is a novel finding, as concentrations able to elicit FAO gene suppression had not yet been determined until now. Further, to understand the functional sensing of LPS on THP-1 cell FAO gene suppression at very low doses of LPS, we determined that these extremely low concentrations of LPS were able to sustain an initial suppression induced by 50pg/ml LPS. These findings are significant as they point to the ability of extremely

low doses of LPS (0.1-0.5pg/ml) to continue an original suppressive response in lipid metabolism elicited by very low dose LPS (50pg/ml).

We also examined whether as in the *in vivo* studies, nuclear receptors involved in metabolism regulation would also be affected *in vitro*. Indeed, PPARα, PPARγ, and PGC1α were all suppressed after challenge with 50pg/ml LPS in THP-1 cells and we demonstrated phosphorylation of PPARγ after the same challenge, thus suggesting a TLR4-induced phosphorylation of PPARγ to cause its inactivation. Additionally, protein levels of JNK (a pro-inflammatory kinase downstream of TLR4) were also phosphorylated after very low dose LPS, although this phosphorylation contributes to its activation. These findings demonstrate a post-transcriptional regulation of both pro-inflammatory and metabolic alterations after challenge with LPS as low as 50pg/ml. We then determined that JNK also plays a role in the cross-talk between metabolism and inflammation, as glucose oxidation is significantly increased in THP-1 cells after 50pg/ml LPS challenge, and that up-regulation is significantly suppressed after JNK chemical inhibition. Finally, we have demonstrated an increase in cytosolic acetyl-CoA levels post 50pg/ml LPS challenge in THP-1 cells. As demonstrated in our study, the excess of cytosolic acetyl-CoA induced by metabolic changes through very low dose LPS may contribute to acetylation of genes, including ATF2, whose acetylation has been demonstrated in our study after challenge with 50pg/ml LPS.

The information collected here is extremely valuable in understanding the mechanism of metabolic endotoxemia-induced alterations in metabolism and inflammation. Similarities appear to exist between high dose and very low dose LPS challenge, but there are several phenotypic differences which have yet to be fully understood. This study

contributes significantly, however, to the current understanding of very low dose LPS challenge and identifies two key contributors to the cross-talk between metabolism and inflammation downstream of TLR4—IRAK1 and JNK.

4.4 *Final Remarks*

Overall, these studies have demonstrated the ability of TLR4 to sense varying concentrations of LPS to elicit very different phenotypes. Additionally, we have identified IRAK1 as a critical point of cross-talk between metabolism and inflammation after LPS challenge at both high and very low doses of LPS, and that JNK appears to also play a significant role in this cross-talk at very low doses. We have identified the suppression of both nuclear receptors responsible for metabolism, as well as several genes responsible for lipid metabolism after both high and very low dose LPS challenge both *in vivo* and *in vitro*. Furthermore, we have demonstrated the ability of HFD feeding and very low dose LPS in the development towards an insulin-resistant state across several murine genotypes and significant very low dose LPS induced changes in both FAO and glucose oxidation. These studies demonstrate the ability of LPS to induce a state of metabolic endotoxemia characterized by significant alterations in both inflammatory and metabolic status. Importantly, these studies also point to IRAK1 as a future therapeutic target that may contribute to the alleviation and prevention of both severe acute illness as well as chronic inflammatory diseases, since it plays a role in both inflammatory and metabolic changes associated with the morbidity and mortality rates in these diseases.