Molecular Mechanisms Governing Persistent Induction of Pro-Inflammatory Genes by Lipopolysaccharide

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ABSTRACT

Low dose endotoxemia is caused by several health conditions including smoking, alcohol abuse, high fat diets, and aging. Several studies have correlated low dose endotoxemia with increased risks of atherosclerosis, diabetes, and Parkinson's disease. Unlike high doses of endotoxin which induce a strong but transient induction of pro-inflammatory mediators, low doses of endotoxin result in a mild but chronic induction of pro-inflammatory genes. The central hypothesis of our study was that if low doses of endotoxin are capable of inducing mild prolonged inflammation, then a unique signaling circuit must be utilized.

In the first study, the molecular mechanisms for the persistent induction of lipocalin 2 (LCN2) in response to 100 ng/mL of lipopolysaccharide (LPS) in kidney fibroblasts was examined. It appears that the intracellular signaling network responsible for the persistent induction of LCN2 requires both activator protein-1 (AP-1) and CCAAT/enhancer binding protein delta (C/ebpδ). Interleukin-1 receptor-associated kinase 1 (IRAK-1) is critical for LCN2 expression.

In the second study, the molecular mechanisms governing the persistent induction of interleukin 6 (IL-6) upon a 50 pg/mL challenge of LPS in macrophages was examined. At this dose, only the persistent activation of cJun N-terminal kinase (JNK) and C/ebpδ was observed. IL-6 transcription requires the transient recruitment of activating transcription factor 2 (ATF2) and the persistent recruitment of C/ebpδ to the *IL-6* promoter.

In the third study, the molecular mechanisms that mediate LPS-induced priming was examined. The results demonstrate that macrophages are able to sense their prior history of exposure to LPS that result in either a priming or tolerance phenotype upon a secondary challenge of LPS. Results suggest that this sensing mechanism involves cross-talk between IRAK-1 and phosphoinositide-3-kinase (PI3K).

Collectively, these studies indicate that JNK and C/ebpδ are the primary players responsible for the persistent expression of pro-inflammatory genes during low dose endotoxemia. IRAK-1 is a key intracellular signaling kinase that mediates signaling at low doses of LPS. IRAK-1 is not only critical for low dose induced expression, but also for LPS-induced priming. This research has revealed a novel signaling pathway that could provide new molecular targets for drug development against chronic inflammatory diseases.

DEDICATION

I would like to dedicate this work to my wife and son. Jessica, thank you so much for supporting me throughout my graduate studies. Your sacrifices did not go unnoticed! You have been an amazing wife and mother to our son. Zachary, thank you so much for flipping our world upside down. Just when I thought we had everything figured out, God blessed us with you. Being your father has been an experience I will always cherish.

Finally I would like to thank God for designing such an amazing world filled full of things to learn and discover. I feel blessed to be gifted with the task of solving the mysteries of your creations. The more I learn and discover, the more humble I become.

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INTRODUCTION

1.1 Background

Low dose endotoxin in human blood arises due to adverse health conditions including chronic smoking, drinking, high fat dietary habits, and aging. Compelling clinical studies performed in humans and experimental animals indicate low-dose endotoxemia is closely correlated with elevated risks of inflammatory diseases including atherosclerosis, diabetes, and Parkinson's disease. In contrast to high dose endotoxin (bacterial lipopolysaccharide-LPS) which can induce a robust and transient expression of pro-inflammatory mediators, low dose endotoxin causes mild yet prolonged induction of pro-inflammatory mediators. The underlying molecular mechanism governing low dose endotoxemia is poorly understood and even less studied. This review will provide a critical analysis regarding our current understanding of low dose endotoxemia and its pathological consequences.

1.2 Causes of Low Dose Endotoxemia

Endotoxin or LPS is the biologically active component responsible for the induction of a mild but chronic inflammatory state seen in low dose endotoxemia. It is clear that a multitude of poor lifestyle choices all contribute to the progression of this disease. These habits include but are not limited to the heavy consumption of alcohol, smoking, high fat diets, and periodontal disease. Regardless of choice, we will all be subject to a low grade inflammatory state from tissue deterioration due to aging. From here on when low dose endotoxemia or subclinical doses are mention, we are referring to concentrations much less than 100 pg/mL, unless otherwise noted.

1.2.1 Alcohol

The consumption of alcohol in humans is associated with the onset of many human diseases including alcoholic liver disease, cardiomyopathy, and brain injury. The root cause to these diseases seems to be due to the increased levels of circulating endotoxin seen in individuals who abuse alcohol. There is very little convincing evidence linking alcohol to promoting or suppressing bacterial growth within the gut. However, some unusual growth patterns do immerge. Increased permeability of large molecular weight molecules through the epithelial barrier due to chronic alcohol feeding was observed by several groups in the 1970s. Within the last ten years, investigators been able to show that alcohol feeding also increase the permeability of endotoxin through the intestinal barrier [1-4]. Not surprisingly, several labs have also been able to show that the degree of alcohol consumption is directly correlated to the amount of circulating endotoxin regardless of any disease manifestation [5-7]. Further supporting the involvement in alcohol induced leakage of endotoxin into circulation in humans is a finding by Fukui et. al. showing a decrease in plasma endotoxin levels when alcohol was no longer consumed[5]. Once endotoxin is leaked out into circulation it is capable of causing wide spread low grade inflammation. Alcohol induced inflammation is strong linked to metabolic endotoxemia and liver disease.

1.2.2 Smoking

Smoking as long been a risk factor associated with many serious human diseases. Not until recently has it become apparent that a wide range of bacteria are found in cigarettes. Using 16S rRNA based cloning techniques researchers were able to detect the presence of a wide array of bacterial species, include many known human pathogens. Many of these organisms are known

to cause diseases like pneumonia and foodborne illness [8]. Not only is endotoxin detected in extremely high levels (6-9 ug of LPS per 1 gram of tobacco) in un-burnt tobacco, but it is also an active component in smoke. A single smoked cigarette contains between 75-120 ng of biologically active LPS [9]. Cigarette smoke indoors has also been shown to increase the concentrations of airborne endotoxin nearly 120 times [10, 11]. Obviously the presence of LPS in the lung tissue itself is not sufficient to drive signaling processes that could result in inflammation. However, several researchers have shown that smoking also compromises the integrity of the endothelial barrier within the lung. A study done by Peerschke et. al. convincingly demonstrated that the smoke along with sheer stress within in the lung leads to an increase in deposition of complement on the surface of human endothelial cells [12]. In addition the nearly 3000 other chemicals and heavy metals within smoke particulates are also capable of causing significant lung tissue damage. Another study also shows that cigarette smoking can cause the activation of a nuclear factor kappa B (NFkB), a well-known LPS-inducible transcription factor [13]. With all the evidence considered, it is clear that smoking not only introduces endotoxin into lung tissue, but it is also capable of activating pro-inflammatory signaling pathways.

1.2.3 Obesity and High Fat Diet

Like smoking, consumption of diets high in fat as also been linked to many human diseases. With obesity becoming a world-wide epidemic, its contribution to human disease cannot be overlooked. Obesity causes major changes in the microbial population within an individual's gut, it seems to be a major contributor to metabolic endotoxemia, and is strongly linked to a chronic low-grade inflammatory state. A diet rich in fats not only causes elevated levels of circulating fatty acids, but it also shift the bacterial population within the gut to a more Gram

negative state [14]. In addition, research has also shown that high fat diets cause a 2-3 fold increase in circulating LPS [14]. The mechanism for the leakage is currently under investigation, but it is thought that leakage occurs via weakened tight junctions between endothelial cells or by chylomicron-facilitated transport [15-17]. Indeed, there is data showing that high fat feeding in mice causes a decrease in the expression of proteins associated with the formation of the tight junction [18, 19]. High fat diets have also been shown to cause an increase in glucose tolerance, increased levels of macrophage infiltration in adipose tissue and markedly higher levels of pro-inflammatory markers all of which was lost when cluster of differentiation 14 (CD14), a key LPS signaling molecule, was knocked out [18]. The gut microbiota also plays a major role in contributing to disease. Mice breed in a sterile facility also fed with a high fat diet did not exhibit any of the symptoms of disease, but upon the introduction of bacteria from other mice characteristic metabolic endotoxemia ensued [20]. The message is clear, the gut microbiota and high fat feeding cause a significant increase in circulating levels of endotoxin and collectively contribute to low-grade inflammation.

1.2.4 Periodontal Disease

Periodontitis or inflammation around the teeth is strongly associated with a risk of developing more serious life threatening diseases like atherosclerosis, diabetes, arthritis, or more acutely; preterm labor [21-28]. Periodontal disease is characterized by the colonization of bacteria in pockets of the gums or teeth that eventually lead to bone loss and potential loss of teeth. In the mouth, bacteria are capable of forming thick biofilms that are very resistant to antiseptics, antibiotics, and mechanical removal. Several of the bacteria associated with this disease are Gram negative in nature, including the *Porphyromonas* species. These bacteria have routine and easy access to the circulatory system during mechanisms that can cause lesions like the chewing

of food or during teeth cleaning. The exacted mode of entry into circulation has not been well defined, but studies have shown that individuals with severe periodontitis have elevated serum levels of LPS [29, 30]. The persistent nature of periodontal disease and bacterial colonization make for conditions that would allow for the shedding of bacterial into circulation to occur consistently for a long period of time.

1.2.5 Aging

Aging is accompanied by an increase in the likelihood to develop many inflammatory-linked diseases such as cardiovascular disease, neurological disorders, and increased susceptibility to infection and sepsis. It is widely accepted that these diseases are more likely to develop because of the immune-compromised state of the elderly population. Immunosenescence is a term that refers to this form of immune dysregulation. Aging is also associated with 2-4 fold increases in circulating inflammatory mediators such as IL-6, tumor necrosis factor alpha (TNF α), and C-reactive protein (CRP) [31-33]. Although there is an overall immune dysfunction, it has been shown that neutrophils and macrophages functions are specifically reduced in the elderly. Both macrophages and neutrophils exhibit compromised ability to migrate to chemotactic signals, have decreased expression of toll-like receptors and consequently have milder levels of signal transduction activation[34-36]. Several studies were able to show that elderly individuals have significantly elevated levels of circulating endotoxin (7+/-4 pg/mL)[37-39]. Taken together, it is apparent that aging results in immune dysregulation that contributes to the progression of many human diseases.

1.3 Consequences of Low Grade Inflammation

Multiple risk factors or life style choices that are linked to chronic low grade inflammation have been summarized above. Collectively this inflammation has been linked and can lead to the development of more serious diseases like atherosclerosis, diabetes, and Parkinson's disease. The following is a summary of these diseases and their correlation with LPS-induced low grade endotoxemia.

1.3.1 Atherosclerosis

The disease atherosclerosis is initiated by the dysfunction of the endothelial layer lining the inner arterial wall. Damage that occurs to this layer can be caused by a wide array of stimuli such as oxidized low density lipoprotein due to high fat diets, free radicals due to smoking, high blood pressure, and the presence of microbes. Normally the endothelium does not bind circulating white blood cells, but upon its activation, endothelial cells express receptors like intercellular adhesion molecules (ICAMs) and vascular adhesion molecules (VCAMs), that allow for extravasation to occur. Upon their attachment several chemoattractant proteins recruit the migration of monocytes to the site of damage. Once monocytes are at the site of damage, they mature into macrophages. These macrophages then begin to ingest and store lipids causing them to turn into macrophage foams cells. This forms the first lesion on the arterial wall. The lesion eventually becomes a plaque as a result of a vicious cycle of inflammation, fibrosis, and lipid deposition. Interestingly, the first evidence that subclinical endotoxin (LPS <50pg/mL) plays a role in the progression of atherosclerosis was shown by Willeit et. al. in 1999 [40]. Another study found that circulating LPS concentrations ranged between 6 and 209 pg/mL and that individuals with concentrations greater than 50 pg/mL were at much higher risk of developing

atherosclerosis[41, 42]. In addition, several labs independently documented that toll-like receptor 2 and toll-like receptor 4 have been shown to advance the progression of this disease [43-46]. Although subclinical doses of endotoxin are not the sole contributor to atherosclerosis progression, it is clearly linked to the progression of the disease.

1.3.2 Diabetes

Currently there are two recognized forms of diabetes, type I and type II. Both forms of diabetes are a result of immune dysfunction. However, type II diabetes is the predominate form accounting for nearly 90% of all cases. Type II diabetes is also reversible with diet and exercise. An obesity induced inflammatory state seems to be the primary cause of insulin resistance, hence type II diabetes. As previously reported, high fat diets cause an increase in adipose tissue. Adipose tissue in obese individuals is heavy infiltrated with activated macrophages that are major sources of pro-inflammatory cytokines like TNFα and IL-6. These adipose tissue macrophages (ATMs) actively recruit additional macrophages to the adipose tissue contributing a chronic inflammatory state [47]. Inflammation that proceeds collectively activates JNK, AP-1, and NFkB which are capable of phosphorylating insulin receptor substrate proteins (IRS), insulin receptors which create a state of overall insulin resistance [48-50]. Similar to atherosclerosis toll-like receptor 2 (TLR2) and toll-like receptor 4 (TLR4) have been linked to the progression of diet induced obesity and insulin resistance using a TLR4 knock out mouse model. Investigators have shown that high levels of saturated fatty acids seen in adipose tissue along with LPS are capable of initiating inflammation via TLR4 signaling pathways [49, 51]. Further supporting the contribution of LPS to the progression of diabetes and the insulin resistant state is the fact that LPS itself is capable of inducing a reversible insulin-resistant state [52]. Just like observations made in obesity, individuals with type II diabetes all showed significantly higher levels of

circulating LPS [53]. Although the exact contribution of LPS to the insulin-resistant state seen in diabetes is not clear, the correlation of elevated levels of circulating LPS as well as the potential of LPS to directly cause insulin resistance via TLR4 makes for a strong case that low dose endotoxemia may be the underlying player.

1.3.3 Parkinson's Disease

Parkinson's disease is a disease of the nervous system that is typically seen in individuals over the age of 60 [54]. The disease is characterized by the loss of motor function leading to tremors, rigidity, and defective gait. Parkinson's disease is progressive in nature with no known therapies. Although the exact mechanisms that cause Parkinson disease is unclear, many studies have shown that LPS-induced inflammation in neuron neighboring microglial cells is capable of causing neural degeneration. A new cell culture based system was able to demonstrate that LPS is capable of inducing neurodegeneration 10 days post stimulation [55]. Similar studies were performed *in vivo* also showing that LPS administered directly in the brain caused significant irreversible loss of neurons 21 days post injection [56]. In addition, injections given to mice intraperitoneally also induced a significant loss of neurons 7-10 months post injection [57]. Pro-inflammatory mediators such as TNF α and interleukin 1 beta (IL-1β) not only pass through the blood/brain barrier with high efficiency but also remained elevated only in the brain for duration of the entire study [58]. Interestingly, another group was able to show that TNFα neutralizing antibodies were able to reduce neuron loss [58]. LPS has been shown to be a poor penetrator of the blood/brain barrier [59]. So in the context of low grade endotoxemia, it seems that the effects of chronic inflammation and Parkinson's disease is more than likely mediated directly by ability of pro-inflammatory cytokines such as TNFα to efficiently pass through the blood brain barrier to act on cells neighboring neurons [60].

1.4 Current Understanding of LPS/TLR4 Signaling

It is clear that LPS/TLR4 signaling is involved in the initiation and progression of many diseases. As previously mentioned, these diseases seem to be due to chronic low grade inflammation in response to extremely low levels of endotoxin. Very little is known about signaling mechanisms that occur in response to low doses of endotoxin. Signaling mechanisms downstream of high doses of LPS have been very thoroughly investigated. The following is a review of our current understanding of LPS/TLR4 signaling that occurs at high doses >1ng/mL of LPS.

1.4.1 Lipopolysaccharide

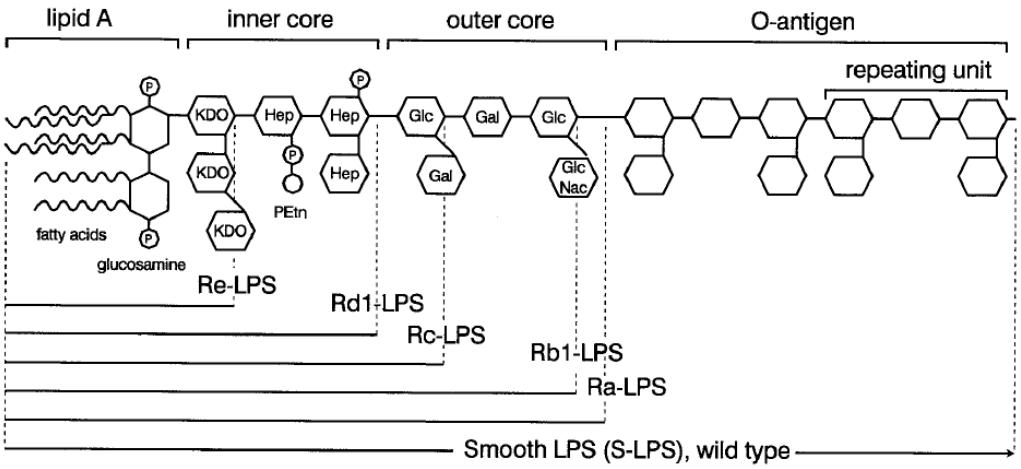

Figure 1.1: Structure of LPS [64]

Lipopolysaccharide (LPS) is a major component of cell walls in Gram negative bacterium [61]. When LPS is incorporated into the bacterial cell wall it is not immunogenic, but when LPS is released from the bacterium upon bacterial death it is capable of initiating inflammation [62, 63]. LPS consists of 4 primary parts; lipid A, the inner core, the outer core, and the O-antigen [64]. The inner core and the outer cores are composed of several different sugars only found in

bacteria. Lipid A is the toxic moiety of LPS [65]. There is a large variation in the acylation pattern, length of fatty acid residues, and the number of fatty acids depending on the source of the lipopolysaccharide. The O-antigen is attached to the terminal sugar of the outer core, extending from the bacterial surface. Like lipid A, there is a high degree of variability in the composition and length of the sugar units. It is the o-antigen that gives bacteria their smooth appears on an agar plate [64]. Of the four components of LPS, lipid A is the component required to initiate signaling by its receptors: TLR4, cluster of differentiation 11 b (CD11b/MAC-1), and scavenger receptors [66-68].

1.4.2 Toll-like receptor 4

TLR4 is part of a large family of receptors that collectively recognize conserved components of various foreign invaders like fungi, bacteria, and viruses [69]. TLR4 is composed of an extracellular domain with leucine rich-repeats (LRR) that is responsible for LPS binding [70]. LPS binding with its receptor requires LPS to interact with LPS binding protein (LBP), CD14, and myeloid differentiation factor-2 (MD-2). LBP is an acute phase protein that directly binds the lipid A region of LPS [71]. Once this complex is formed it easily associates to CD14; either the membrane bound form mCD14 or the soluble form sCD14. CD14 then facilitates the transfer of LPS/LBP to the TLR4/MD2 complex [72]. Upon formation of this complex, TLR4 forms a homodimer and a signal is propagated to its intracellular domain known as the Toll/Il-1 receptor domain (TIR). The TIR domain has three critical regions required for the recruitment of five known adaptor proteins: myeloid differentiation primary response gene 88 (MyD88), TIR domain-containing adaptor protein (TIRAP or Mal), TIR domain-containing adaptor inducing IFN-β (TRIF), TRIF-related adaptor molecule (TRAM), and sterile α and HEAT-Armadillo motifs-containing protein (SARM) [69].

1.4.3 TLR4 Intracellular Adaptor Proteins

The combination of adaptor proteins recruited to the TIR domain of TLR4 determines the signaling pathways downstream that are utilized. These adaptor proteins are also recruited to the TIR domain of other TLRs, not just TLR4. Interestingly, TLR4 is the only TLR to use all five adaptor proteins [69]. MyD88 has been shown to be required for the induction of pro-inflammatory cytokines in response to LPS stimulation in macrophages; however, the expression of Type I interferons was not affected [73, 74]. Mal was first identified using computer software that was scanning for proteins that contained a TIR domain [75]. Mal is known to facilitate the association of MyD88 to the TIR domain of TLR4 [76]. TRAM was also discovered using computer scanning technology [77]. Like Mal, it is also required for MyD88 dependent signaling [78]. SARM is a newly discovered protein that seems to be involved in inhibiting TRIF-mediated signaling, but its exact function remains unclear [79].

1.4.4 MyD88 dependent and independent TLR4 signaling

The MyD88 dependent signaling pathway is also known as the 'classical TLR4 pathway'. MyD88 has a TIR domain that allows it to associate with the intracellular domain of TLR4, but it also has a death domain (DD) that allows it to associate with other proteins which also contain a DD. The best recognized DD-containing protein that has been shown to be necessary for signaling to proceed immediately downstream of MyD88 is IL-1 receptor-associated kinase-4 (IRAK-4). Loss of IRAK-4, like the loss of MyD88, causes a severe loss of pro-inflammatory cytokine production [80]. Several other IRAK molecules have been shown to associate with IRAK-4. This includes IL-1 receptor-associated kinase-2 (IRAK-2) and IRAK-1 [81, 82]. Unlike IRAK-4 deficient macrophages, macrophages lacking IRAK-1 only show a partial defect

in pro-inflammatory cytokine production [83]. TNF receptor-associated factor 6 (TRAF6) is also required downstream of IRAK-4 for the expression of pro-inflammatory mediators. TRAF6 then activates transforming growth factor-β-activate kinase 1 (TAK1), which then in turn activates I-kappa-B kinase (IKK) and mitogen activated protein kinase (MAPK) [84, 85]. IKK can then phosphorylate I-kappa-B (IkB) the inhibitor of NFkB, thereby activating NFkB. Collectively, these signaling events leads to the rapid induction of many transcription factors, NFkB, C/ebps, and AP-1, which control the expression of thousands of pro-inflammatory genes including TNFα, IL-1, IL-8, IL-6, lipocalin 2 (LCN2), and monocyte chemoattractant protein-1 (MCP-1).

Unlike the classical TLR4 pathway, the MyD88 independent signaling pathway or the 'alternative TLR4 pathway' is mediated by the interaction of TRIF to the TIR domain of TLR4. TRIF is then able to recruit TRAF3 [86]. TRAF3 can then associate with TRAF family-member associated NFkB activator (TANK), TANK binding kinase 1 (TBK-1), and I-kappa-B kinase i (IKKi) [87]. Together, TANK, TBK-1, and IKKi modulate the activation of IRF3 and late-phase activation of NFkB and MAPK/AP-1 primarily mediating the expression of type I interferons and interferon-inducible genes needed for host defense against viral and bacterial challenges [88, 89].

1.4.5 Negative Regulation of TLR4 signaling

In order for the inflammatory process to cease, LPS/TLR4 signaling also induces the expression of many negative regulators. Suppression of signaling occurs at many levels. Notably, the binding of LPS with TLR4 can be suppressed by complexes that act as homologs of TLR4 such as radioprotective 105 (RP105), ST2L, and SIGIRR. RP105 can form a complex with MD-1, a

homolog of MD-2, and directly interact with TLR4 to prevent LPS binding [90]. ST2L and SIGIRR are transmembrane homologs of IL-1 receptor. They can compete with and sequester many of the adaptor proteins required for signaling including MyD88 and Mal [91, 92]. Several LPS-inducible molecules can suppress signaling. These include suppressor of cytokine signaling 1 (SOCS1), MAPK phosphatase 1 (MKP-1), IkB, PI3K and activating transcription factor 3 (ATF3). SOCS1 is capable of suppressing LPS signaling by targeting IRAK-1 [93]. The exact mechanism in which SOCS1 suppresses cytokine signaling remains unclear, but it is clear that without SOCS1 enhanced phosphorylation of several pro-inflammatory kinases (P38, JNK, IkBα) is observed [94, 95]. MKP-1 has been shown to dephosphorylate both JNK and P38 causing a reduction in pro-inflammatory gene expression [96]. IkB is a suppressor of NFkB signaling. Normally, LPS induces the phosphorylation of IkB which leads to its degradation. This causes the release of NFkB, allowing it to translocate to the nucleus to initiate gene transcription. NFkB regulates its own deactivation by inducing the transcription of its own negative regulator IkB. ATF3 negatively regulates several pro-inflammatory genes, most notably IL-6. It functions by binding to the promoter region of the gene and restricting access for other transcription factors that normally activate transcription thereby suppressing its induction [97]. PI3K are activated in response to LPS and have been shown to suppress the induction of pro-inflammatory cytokines by suppressing IRAK-1, P38, JNK and ERK [98]. Clearly the loss of PI3K causes enhances TLR signaling and expression of pro-inflammatory mediators [99]. Many more mechanisms for suppression of LPS/TLR4 signaling suppression exist, but for the purposes of this review they will not be discussed in detail, but readers are referred to an excellent review [100].

1.4.6 IRAK-1

Due to the involvement of IRAK-1 in several of the signaling pathways described here in, its involvement in TLR4 signaling will be reviewed briefly. IRAK-1 was first identified by Cao et. al. [101]. IRAK-1 is directly regulated by phosphorylation, ubiquitination, and summoylation. IRAK-1 has been shown to be phosphorylated by IRAK-4 [102]. Following this phosphorylation event, IRAK-1 is then capable of self-phosphorylation. This self-phosphorylation can then lead to subsequent ubiquitination and degradation. This may serve as a negative feedback mechanism to prevent excessive inflammation [103]. IRAK-1 can also be summoylated which leads to its nuclear localization where it contributes to the activation of signal transducer and activator of transcription 3 (STAT3) [104]. To this regard, IRAK-1 has been shown to be involved in the regulation of several transcription factors, including NFkB, NFAT, STAT3, and interferon regulatory factor 3/7 (IRF3/7). It is important to note that IRAK-1 has not been implicated in the classical activation of NFkB which involves the phosphorylation and degradation of its inhibitor IkB, but rather by directly phosphorylating NFkB [105, 106]. IRAK-1 has also been shown by immunoprecipitation to directly bind IKKε contributing to its subsequent activation [107]. Another group convincingly demonstrated that IKKε directly activated C/ebpδ by phosphorylation [108]. Taken together, there seems to be strong evidence linking IRAK-1 to the activation of C/ebpδ. In addition, mutual inhibition of IRAK-1 and PI3K has been shown to exist [109, 110]. This inhibition suggests that IRAK-1 may be essential for TLR4 dependent cross-talk that allows for cells to mount very specific and fine-tuned response to an LPS challenge.

1.5 Factors Influencing LPS sensitivity

Sensitivity to lipopolysaccharide is highly context dependent. A wide range of factors can influence sensitivity to LPS including age, gender, tissue type, and species. Several studies have been done to access the contribution of age on the immune response when challenged with endotoxin. A study done in 1996 found that old mice were ten times more sensitive to endotoxin. Upon LPS challenge older mice exhibited an increase in TNFα, nitric oxide production, and mortality [111]. On the other hand, a study done by Pedersen's group convincingly demonstrated that in humans elderly patients have a decrease in cytokine production, particularly TNFα and Il-1β, when whole cell blood extracts are challenged with a septic dose of LPS [112]. Together this data suggests that their might also be differences in LPS sensitivity from species to species. To this regard, several papers published by Urbaschek in the 1970's and 1980's clearly demonstrated a range in LPS sensitivity in the same cells of different species [113-115]. Perhaps the most convincing of which is a study in which they tested the LPS sensitivity in Kupffer cells of a guinea pig, hamster, mouse, and rat. In this study Urbaschek et. al. concluded by a mortality study that guinea pigs were the least sensitive to LPS and rats were the most sensitive [116]. The gender of the individual challenged with LPS also affects one's sensitivity to endotoxin [117]. A study done on human volunteers was able to show that whole blood samples taken from males produced higher levels of pro-inflammatory cytokines like TNFα, IL-1β, IL-6, and IL-8 than their female counterparts [118]. This data suggests that male linked hormones such as testosterone might synergize with TLR signaling.

Although there is no definitive study investigating the sensitivity of endotoxin in various tissues throughout the body, the literature suggests that such a phenomena exists. Clearly differences in immune cell infiltration, cellular tissue composition, as well as cellular expression of endotoxin

receptors would all contribute to differences in sensitivity. In the case of sepsis, endotoxin becomes disseminated throughout the entire body. This circulating LPS is able to come into contact with many more immune cells that are rich in receptors for LPS and very effective producers of pro-inflammatory mediators; thus resulting in a powerful transient cytokine storm. The same situation would occur during low dose endotoxemia, but inflammation would be very subtle and chronic in nature. On the other hand, during localized infection, such as a kidney infection, there are much higher concentrations of endotoxin present due to the heavy load of infectious bacteria. Even though the levels of endotoxin are extremely high, the tissues seem to respond to the challenge much like what is observed during low dose endotoxemia. The inflammation is typically mild compared to sepsis and can become chronic if the bacterium is able to colonize the host. All in all, it is clear that many factors influence sensitivity to LPS. All of these factors must be considered when assessing LPS-induced inflammation.

1.6 Known molecular mechanisms of low dose endotoxemia

Although the concept of low dose endotoxemia has been around for a long time, very little is known about the molecular mechanisms that result in a pro-inflammatory response that is mild but chronic in nature. As discussed previously, many factors can cause the leakage of endotoxin into circulation like obesity, high fat diet, smoking, excessive alcohol consumption, and aging. At high doses, LPS is capable of causing a robust immune response that involved NFkB, C/ebps, AP-1, and MAPKs. Together these players induce the expression of thousands of pro-inflammatory genes. Compensatory mechanisms are also induced that keep inflammation controlled. Recent evidence published from our lab has shown that low doses of endotoxin (100 pg/mL) fail to activate NFkB, but instead selectively removes nuclear suppressors (peroxisome proliferator activated receptor/PPARα and/or retinoic acid receptor/RARα) from the promoters

of IL-6, Endothelin-1, and monocyte chemotactic protein 1 (MCP-1) in murine bone-marrow derived macrophages (BMDMs). In addition, low doses of endotoxin selectively activate C/ebpδ in an IRAK-1/IKKε signaling cascade [107]. Another group showed that LPS concentrations as low as 3 pg/mL were capable of inducing measurable levels of Il-8, with statistical significance at 100 pg/mL in peripheral blood mononuclear cells (PBMCs). Using blocking antibodies they were able to link the induction of Il-8 at low doses of LPS to specifically to TLR4. Concentrations as low as 10 pg/mL were capable of inducing a superoxide respiratory burst [119]. Outside of these two publications little else is known about the molecular mechanisms underlying subclinical/low dose endotoxemia.

1.7 Missing Links

Since the concept of low dose endotoxemia is so cutting edge, many questions need to be addressed. Clearly this disease involves many different tissues and cell types throughout the body. Understanding exactly how each tissue type responds to low doses of LPS will be important to understanding the progression of this disease. More work needs to be done to better understand how LPS leakage occurs. Is there something that we can do to prevent leakage from occurring? Furthermore, the true sensitivity of each cell type needs to be addressed. What is the lowest dose that can induce the chronic expression of pro-inflammatory cytokines and does this dose vary depending on the cell/tissue type? Determining the exact mechanisms that lead to a mild and chronic induction of pro-inflammatory genes is also very important. Are these programs the same in a mouse and a human? Mechanistic discoveries could allow for the development of drugs that could slow the progression or development of low dose endotoxemia linked diseases.

Chapter 2:

MOLECULAR MECHANISM UNDERLYING PERSISTENT INDUCTION OF LCN2 BY LIPOPOLYSACCHARIDE IN KIDNEY FIBROBLASTS

MOLECULAR MECHANISM UNDERLYING PERSISTENT INDUCTION OF LCN2 BY LIPOPOLYSACCHARIDE IN KIDNEY FIBROBLASTS

2.1 Abstract

The neutrophil gelatinase-associated lipocalin 2 (LCN2) is a critical inflammatory mediator persistently induced during endotoxemia, contributing to tubular damage and kidney failure. The intracellular process responsible for persistent induction of LCN2 by LPS is not well understood. Using primary kidney fibroblasts, we observed that LPS-induced LCN2 expression requires a coupled circuit involving an early transient phase of AP-1/cJUN pathway and a late persistent phase of C/ebpδ pathway, both of which are dependent upon the IRAK-1. Using Chromatin immunoprecipitation analysis (ChIP) we observed transient binding of AP-1/cJUN to the promoters of both *TNFα* and *C/ebpδ*. On the other hand, we only observed persistent binding of C/ebpδ to its own promoter and not on *TNFα*. Blockage of new protein synthesis using cyclohexamide significantly reduced the expression of *C/ebpδ* as well as *LCN2*. Also by ChIP analyses, we demonstrated that LPS recruited C/ebpδ to the *Lcn2* promoter in WT, but not IRAK-1 deficient fibroblasts. *In vivo*, we observed elevated levels of LCN2 in kidneys harvested from LPS-injected WT mice as compared to IRAK-1 deficient mice. Taken together, this study has identified an integrated intracellular network involved in the persistent induction of LCN2 by LPS.

2.2 Introduction

Initially identified in neutrophils as a gelatinase-associated small protein [120], LCN2 was recognized as an innate defense molecule by sequestering and depleting iron-containing siderophores and curbing bacterial growth [121]. Recent studies reveal that LCN2 can be potently induced by inflammatory stimulants and widely expressed in vital organs and tissues such as kidney, heart, and brain [122-125]. Elevated level of LCN2 in kidney is a well-recognized marker for both chronic kidney diseases, reflecting the extent of kidney damage [126, 127]. LCN2 is not only a marker, but also a key contributor of kidney disease, as reflected by the alleviation of chronic kidney damage in mice with *Lcn2* gene deletion [125].

Bacterial endotoxin (lipopolysaccharide-LPS) is a potent inducer of LCN2 [128-130]. Elevated levels of endotoxin are seen in both acute and chronic conditions. Acute endotoxemia leads to septic shock and multi-organ damages including acute kidney failure [131]. In contrast, chronic endotoxemia associated with obesity, aging, and other adverse health conditions can lead to persistent inflammatory complications and chronic diseases [18, 130, 132]. LCN2 levels persist during both acute and chronic phases of kidney damage, and may serve as a key propagating factor for chronic kidney disease [125]. However, the molecular mechanism underlying the persistent expression of LCN2 is not well understood.

Studies using other inflammatory stimulants such as IL-17 and IL-1β indicate that the induction of LCN2 requires multiple transcription factors including AP-1 and C/ebpδ [128, 133]. AP-1 activation by LPS is transient and responsible for the transient induction of pro-inflammatory mediators [134]. On a separate report, C/ebpδ was implicated in propagating the persistent activation of TLR4 pathway [135].

Based on these observations, we examined the molecular circuit underlying the persistent induction of LCN2 by LPS in kidney fibroblasts. We identified that IRAK-1, a key TLR4 intracellular signaling component, is involved in coordinating both the transient activation of AP-1, as well as the persistent activation of C/ebpδ, which are collectively responsible for the sustained expression of LCN2.

2.3 Materials and Methods

2.3.1 Reagents

LPS (*Escherichia coli* 0111:B4) and cycloheximide (CHX) were obtained from Sigma Aldrich. Anti-LCN2 antibody was purchased from R&D Systems. Anti- IkBα antibody was purchased from Cell Signaling. Anti-Lamin B (ab-16048) was purchased from Abcam. Anti-C/ebp δ (M-17), anti-cJun (H-79), anti-p65 (F-6), anti-GAPDH (FL-335), anti-IRAK-1 (F-4), and anti-β-actin (C-4) antibodies were from Santa Cruz Biotechnology. All primers were ordered from Integrated DNA Technologies™.

2.3.2 Kidney Fibroblast cultures

Both WT and IRAK-1 $^{-/-}$ C57/BL/6 mice were housed and bred in the Derring Hall Animal Facility in compliance with approved Animal Care and Use Committee protocols at Virginia Polytechnic Institute and State University. Murine kidney fibroblasts were isolated as previously described [136]. Fibroblast cells were grown in 50:50 DMEM/F12 #10-092-CV (Mediatech™, Inc., VA) supplemented with 10% FBS #SH30071.03 (Hyclone™) and 1% Penicillin/Streptomycin at 37°C with 5% CO_2.

2.3.3 Real time RT-PCR (qRT-PCR)

Total RNAs were harvested from murine kidney fibroblasts treated with or without LPS using Trizol® Reagent (Invitrogen) according to the manufacturer's protocol. cDNAs were generated using the High Capacity cDNA Reverse Transcription Kit (Applied BioSystems™, Foster, CA). Real-time PCR analyses were performed using the iQ Sybr® Green Supermix (BioRad™ Laboratories, Hercules, CA) on an IQ5 thermocycler (BioRad). The relative levels of transcripts were calculated using the ΔΔCt method using *GAPDH* as the internal control. The relative levels of mRNA from the untreated samples were adjusted to 1 and served as the basal control value. The following primers were used perform qRT-PCR: mouse *LCN2* forward: 5'- TTT CAC CCG CTT TGC CAA GT-3', reverse: 5'-GTC TCT GCG CAT CCC AGT CA-3'; mouse *C/ebpδ* forward: 5'-ACT TCA GCG CCT ACA TTG ACT CCA-3', reverse: 5'-TGT TGA AGA GGT CGG CGA AGA GTT-3'; mouse *GAPDH* forward: 5'-AAC TTT GGC ATT GTG GAA GGG CTC-3', reverse: 5'- GGA AGA GTG GGA GTT GCT GTT GA-3'; mouse *TNFα* forward: 5'-AGC CGA TGG GTT GTA CCT TGT CTA-3', reverse: 5'-TGA GAT AGC AAA TCG GCT GAC GGT-3'; mouse *IL-6* forward: 5'-ATC CAG TTG CCT TCT TGG GAC TGA-3', reverse: 5'- TAA GCC TCC GAC TTG TGA AGT GGT-3'; mouse *SOCS1* forward: 5'-AGT CGC CAA CGG AAC TGC TTC TT-3', reverse: 5'-ACG TAG TGC TCC AGC AGC TCG AAA-3'; and mouse *ATF3* forward: 5'-TCA AGG AAG AGC TGA GAT TCG CCA-3', reverse: 5'-GTT TCG ACA CTT GGC AGC AGC AAT-3'.

2.3.4 Western Blot Analysis

Isolation of whole cell, cytoplasmic, and nuclear lysates were performed as described previously [137]. Fibroblasts were lysed on ice using lysis buffer (10mM HEPES, pH 7.9, 1.5 mM MgCl2, 10% Triton X-100, 10 mM KCl, 0.5 mM EDTA, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 1ug/mL leupeptin, 1ug/mL pepstatin) for 30 minutes. Samples were centrifuged at 5000 rpm for 10 minutes at 4^0C. The supernatants were saved as the cytoplasmic extract and the pelleted nuclei were re-suspend and lysed in a high salt buffer (20mM HEPES, pH 7.9, 1.5 mM MgCl2, 0.4 M NaCl, 0.2 mM EDTA, 0.5 mM dithiothreitol, 1mM phenylmethylsulfonyl fluoride) on ice for 30 minutes. The samples were then centrifuged at 12,000 RPM for 20 minutes at 4^0C and supernatant kept as the nuclear extract. Protein samples were analyzed by Western Blot as described before. Images were quantified with Fujifilm Multi Gauge software normalized against β actin, GAPDH, or Lamin B.

2.3.5 ChIP assays

Protein and chromatin from treated cells were cross-linked using 1% formaldehyde solution in complete media for 15 minutes with gentle rocking every 3 minutes at room temperature. Cells were washed twice with cold PBS and treated with a glycine solution for 5 minutes to stop the cross-linking reaction. The cells were then lysed in an ice cold buffer containing SDS and protease inhibitor cocktail. The samples were then subject to sonication in an ice water bath to shear the chromatin for 6 minutes (30 seconds on and 30 seconds off). The sheared chromatin was processed using Chip-IT Express Kit #53008 from Active Motif™. The immune-precipitated chromatin was analyzed by PCR using primer pairs that span the promoter regions of either *Lcn2, C/ebpδ, and TNFα*. The primer sequences are as follows: mouse p*LCN2*

forward: 5'-TGA CCC ACA AGC AGT GCC CTG T-3', reverse: 5'-ACT TGG CAA GAT TTC TGT CCC-3'; mouse p*C/ebpδ* forward: 5'-ACA AAC AGG AAG GAG GGA AG-3', reverse: 5'-TCC AAG TTG GGC TGT CA-3'; and mouse p*TNFα* forward: 5'-AGG AGA TTC CTT GAT GCC TGG GT-3', reverse: 5'-TTT CTG TTC TCC CTC CTG GCT AGT-3'.

2.3.6 In vivo LPS injection

Female IRAK-1 $^{-/-}$ and WT C57/BL/6 mice of 12 weeks old received intraperitoneal injections with 25 mg/kg of LPS. Six hours after the injection, mice were sacrificed and the tissues harvested for subsequent analyses.

2.3.7 Statistical Analysis

Data significance was determined using the Student's t-test. P-values less than 0.05 were considered statistically significant as indicated by an asterisk (*).

2.4 Results

2.4.1 LPS induces persistent expression of LCN2 in kidney fibroblasts in an IRAK-1 dependent fashion.

We first compared the expression profiles of selected inflammatory genes in fibroblasts stimulated with LPS. As shown in **Figure 2.1A**, the induction of *Tnfα* message by LPS was transient, peaked at the 2 hour time point post challenge, and returned to basal values at the 16 hour time point. In contrast, the expression of *Lcn2* mRNA and protein persisted throughout the course of LPS treatment (**Figure 2.1B/C**). Because IRAK-1 is one of the key downstream components of TLR4 pathway, we then evaluated whether IRAK-1 is involved in both the

transient induction of *Tnfα* and persistent induction of *Lcn2* message. As shown in **Figure 2.1**, IRAK-1 deficient kidney fibroblasts have significantly less induction of both *Tnfα* and *Lcn2*.

2.4.2 LPS induces transient activation of AP-1 and persistent induction of C/ebpδ in kidney fibroblasts.

To determine the potential mechanism of LCN2 persistent induction in kidney fibroblasts by LPS, we examined the activation status of relevant transcription factors. Thus, we examined the status of NFκB, AP-1 and C/ebpδ in WT and IRAK-1 deficient kidney fibroblasts. As shown in **Figure 2.2A**, LPS treatment caused no apparent decrease in the levels of Iκbα in WT fibroblasts, indicating a lack of robust NFκB activation by LPS in kidney fibroblasts. Further supporting this claim, we observed no apparent induction of nuclear p65/RelA protein levels by LPS (**Figure 2.2B**). In contrast, we observed a rapid yet transient induction of AP-1/c-Jun (**Figure 2.2C**). C/ebpδ, on the other hand, exhibited a steady and persistent induction induced by LPS (**Figure 2.3**). The induction of c-Jun and C/ebpδ are significantly reduced in IRAK-1 deficient cells, indicating the IRAK-1 is required for this process (**Figure 2.3**).

Based on above observation, we hypothesize a coupled step-wise process leading to the persistent activation and induction of C/ebpδ. LPS may first induce an initial C/ebpδ expression primarily through a transient activation of AP-1 and residual NFκB in the nucleus. Subsequently, newly synthesized C/ebpδ can auto-induce its own expression and contribute to a steady induction of C/ebpδ and its downstream target gene LCN2. To test this hypothesis, we performed ChIP analysis to determine the recruitment of c-Jun and C/ebpδ to the promoter of *C/ebpδ*. As shown in **Figure 2.4**, LPS transiently increased the recruitment of c-Jun to the promoter of *C/ebpδ* (**Figure 2.4A**) and the promoter of *Tnfα* (**Figure 2.4B**), peaking at 4 hr for

C/ebpδ and 1 hr for *Tnfα* promoter, and dropping off at the 8 hr time point. In contrast, increased recruitment of C/ebpδ at the 8 hr time point was evident on the promoter of *C/ebpδ*, but not *Tnfα*.

2.4.3 Both De novo synthesis and subsequent activation of C/ebpδ is required for the expression of LCN2

To further confirm that *de novo* synthesis of C/ebpδ is involved in LPS-induced LCN2 expression, we applied CHX, a known protein synthesis inhibitor, to the culture medium. As shown in **Figure 2.5A**, CHX significantly reduced LPS-mediated induction of *Lcn2* message, suggesting that new protein synthesis is required for the induction of *Lcn2* gene expression. In contrast, CHX has no inhibitory effect on LPS-mediated expression of *C/ebp*δ message (**Figure 2.5B**). This is in agreement with a previous finding that the expression of C/ebpδ and its subsequent transcriptional activation are two separate events controlled by two distinct upstream processes [108]. To further confirm the role of C/ebpδ in LPS induced expression of *LCN2*, we examined the recruitment of C/ebpδ to the promoter of *Lcn2* following LPS challenge. As shown in **Figure 2.5C**, LPS dramatically elevated the recruitment of C/ebpδ to the *Lcn2* promoter in WT cells.

2.4.4 The lack of inducible negative regulators contributes to prolonged expression of LCN2 in kidney fibroblasts upon LPS stimulation

Through the classical NFkB pathway, LPS is known to induce negative regulators that subsequently turn off the expression of pro-inflammatory genes. Among the negative regulators, SOCS1 suppresses the upstream LPS signaling pathway by inhibiting IRAK-1 [93]. On the other hand, ATF3 is shown to compete and suppress the activity of C/ebpδ [97, 135]. Given the fact that we did not observe noticeable activation of the NFkB pathway, we hypothesize that LPS

may not be able to induce related negative regulators in kidney fibroblasts. Indeed, we demonstrated that LPS failed to induce the expression of *Socs 1* and *Atf 3* (**Figure 2.6A**). As a consequence, we observed that there was no apparent degradation of IRAK-1 protein in kidney fibroblasts following a 4 hr LPS treatment (**Figure 2.6B**). Taken together, our data indicate that LPS treatment in kidney fibroblasts failed to induce negative regulators that modulate the persistent activation of C/ebpδ, leading to a sustained induction of its downstream gene *Lcn2*.

2.4.5 <u>In vivo</u> induction of LCN2 expression in an IRAK-1 dependent fashion

To test the *in vivo* relevance of our finding, we examined the expression of LCN2 in kidney tissues from WT and IRAK-1 deficient mice following LPS challenge. As shown in **Figure 2.7**, LPS injection induced significant induction of *Lcn2* messages (**Figure 2.7A**) as well as LCN2 protein (**Figure 2.7B**) in kidney tissues harvested from LPS-injected WT mice. In contrast, the levels of *Lcn2* messages and LCN2 protein were significantly lower (~90% less in messages, ~30% less in proteins) in kidneys harvested from IRAK-1 deficient mice injected with LPS.

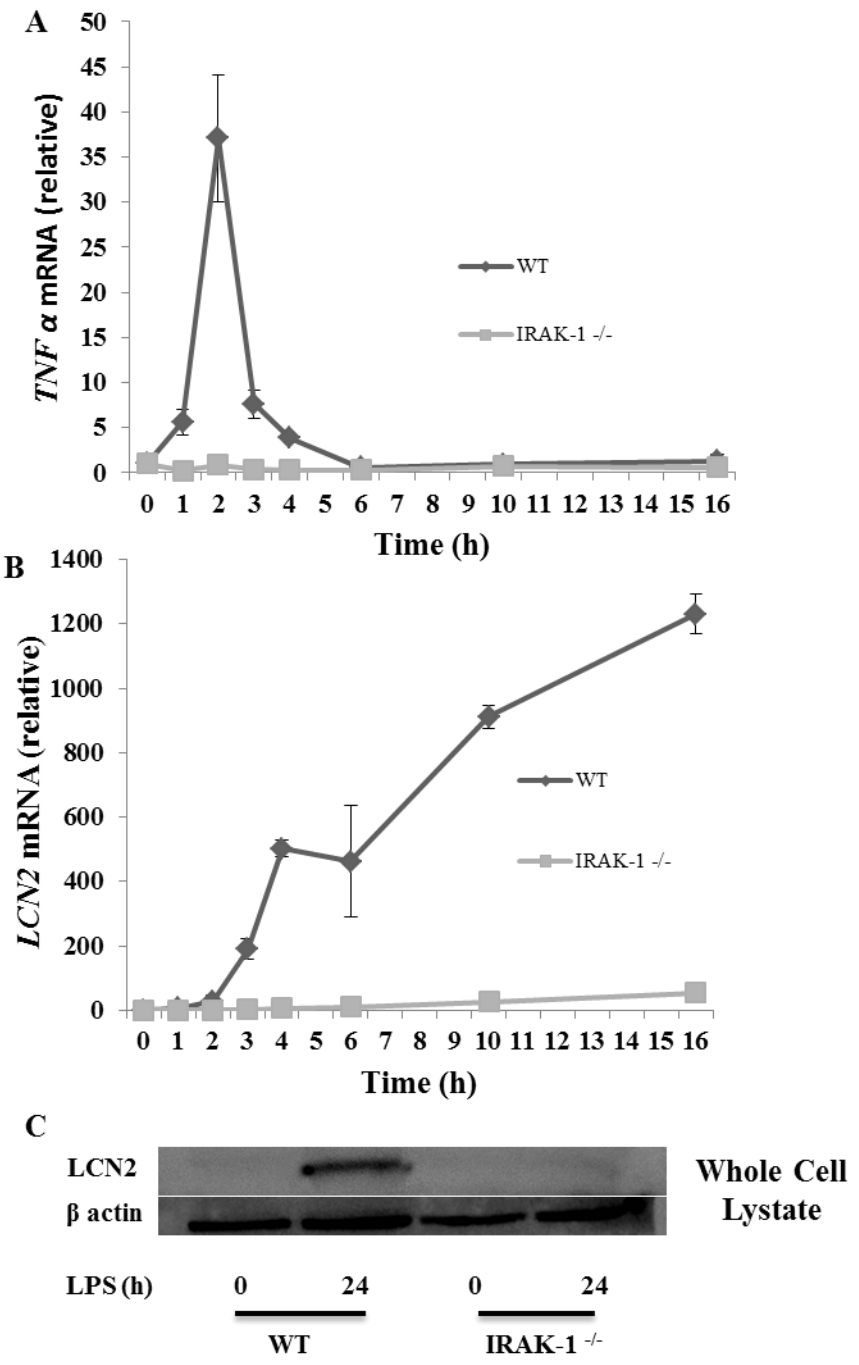

Figure 2.1: LPS stimulation induces a persistent induction of LCN2 in kidney fibroblasts.

(A) LPS (100ng/mL) induces a transient induction of *Tnfα* mRNA. (B) LPS (100ng/mL) induces a persistent induction of *Lcn2* mRNA Transcript levels were measured by qRT-PCR as described above. The results are expressed as means +/- standard deviation performed in triplicate. (C) LCN2 protein levels also persist after 24 hours of LPS stimulation. Whole cell protein levels were measured by western blot as described above.

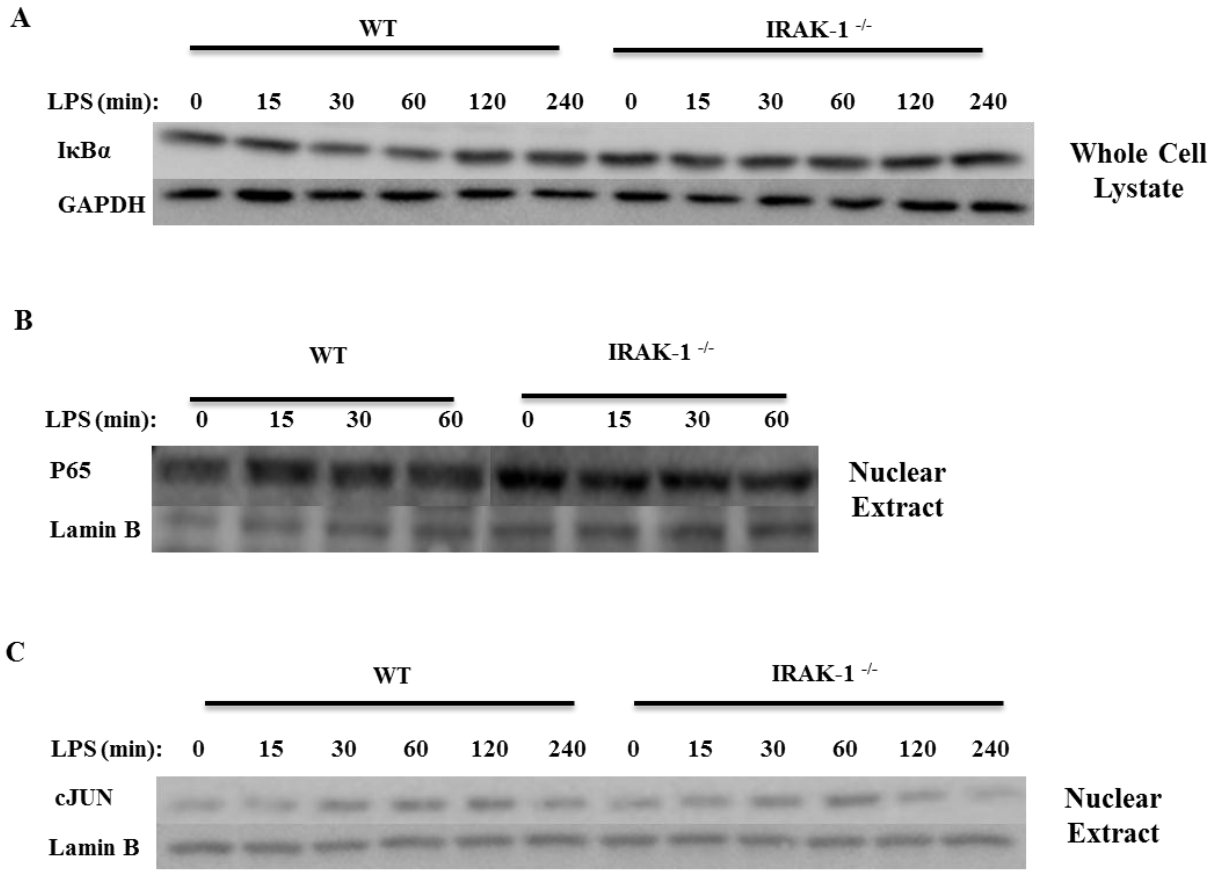

Figure 2.2: LPS induces transient activation of AP-1 in kidney fibroblasts

WT and IRAK-1$^{-/-}$ fibroblasts were treated with LPS (100ng/ml) for the indicated time periods. (A) LPS fails to induce IkBα degradation with or without IRAK-1. Whole cell lysates were harvested and analyzed by Western blot using an IκBα specific antibody. (B) LPS fails to induce p65 nuclear translocation in WT and IRAK-1 deficient kidney fibroblasts. Nuclear cell lysates were harvested and analyzed by Western blot using an anti-p65 antibody. (C) LPS induces the expression of AP-1/c-Jun. Whole cell lysates were harvested and analyzed by Western blot.

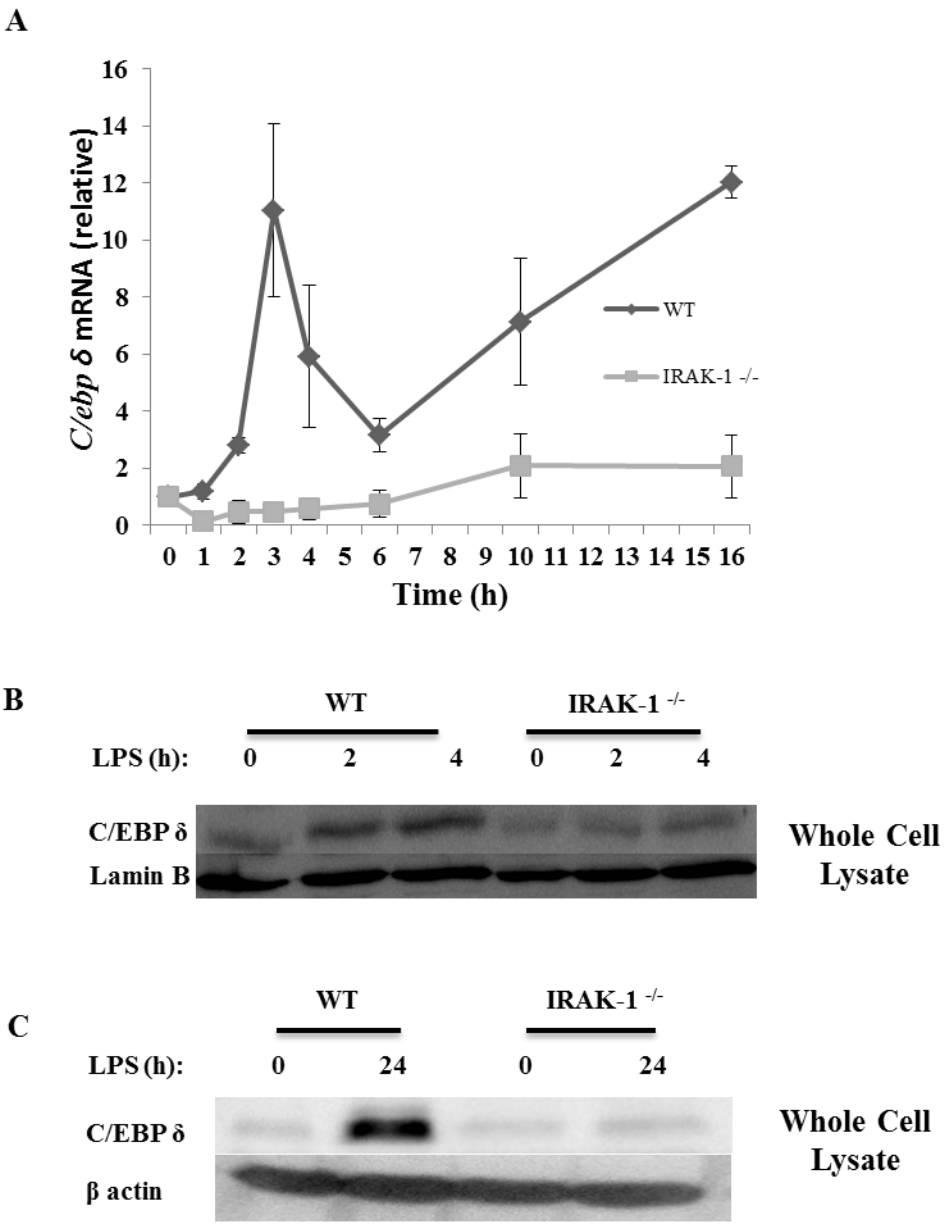

Figure 2.3: LPS induces a persistent activation of C/ebpδ in kidney fibroblasts.

(A) LPS induces a persistent increase of *C/ebpδ* message dependent upon IRAK-1. (B-C) LPS induces a persistent increase in C/ebpδ protein dependent upon IRAK-1. Total RNAs were harvested. Real-time quantitative RT-PCR analysis was performed to determine the levels of *C/ebpδ* message. Whole cell lysates were harvested and analyzed by Western blot. Data shown were the representation of three independent experiments.

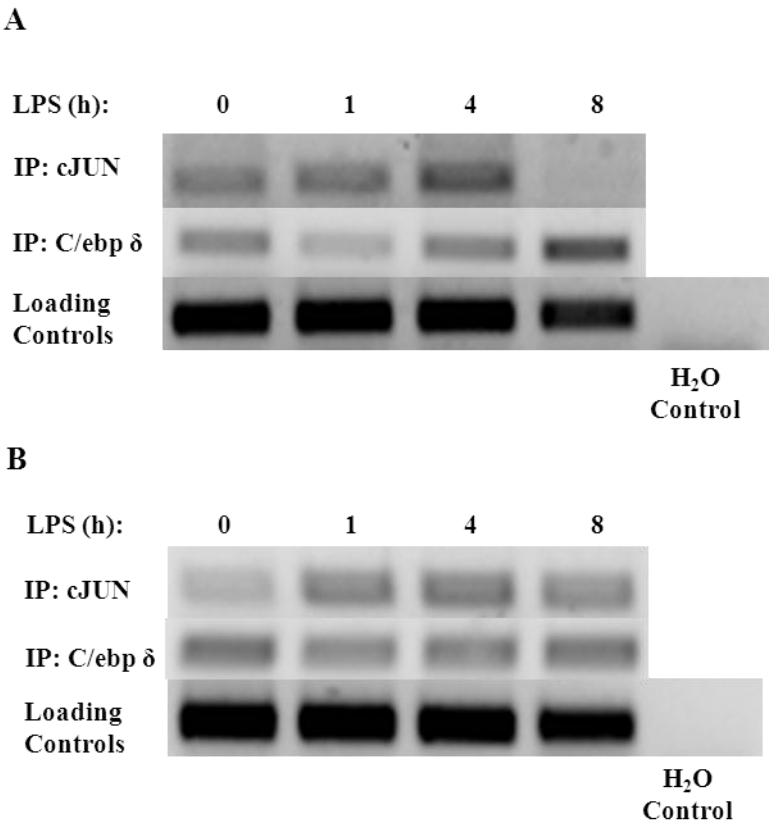

Figure 2.4: LPS induces transient recruitment of c-Jun to the promoters of *Tnfα* and *C/ebpδ*, and induces persistent recruitment of C/ebpδ to the promoter of *C/ebpδ*.

WT kidney fibroblasts were treated with or without LPS for a time course. Nuclear lysates were then subjected to ChIP analysis to examine relative binding to the promoter of *C/ebpδ* (A) or *TNFα* (B). Data shown were the representation of three independent experiments.

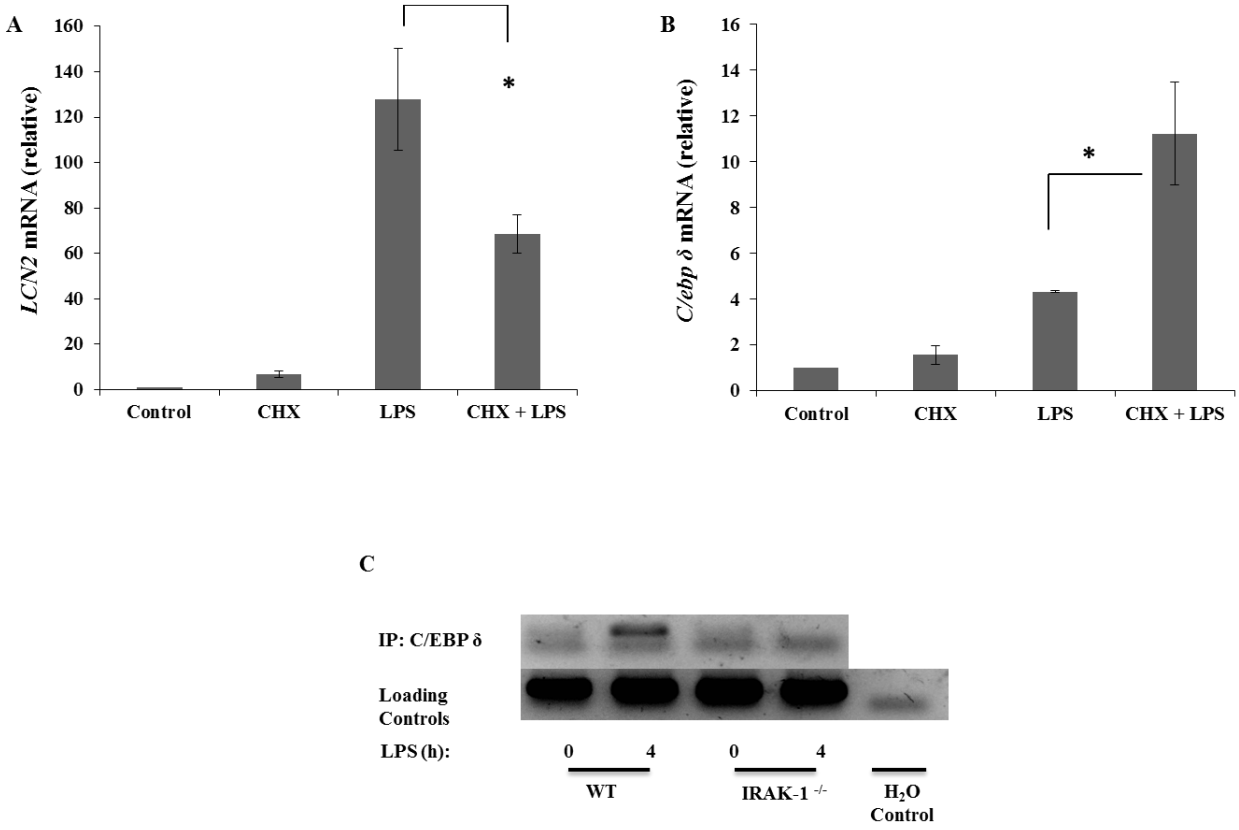

Figure 2.5: ***Lcn2*** **transcription requires new protein synthesis, but** ***C/ebpδ*** **does not. C/ebpδ is recruited to the promoter of** ***LCN2*** **upon LPS stimulation in an IRAK-1 dependent manner.**

(A CHX, a protein synthesis inhibitor, blocks *Lcn2* transcription upon LPS stimulation. (B) The induction of *C/ebpδ* does not require new protein synthesis. (B) Wild-type kidney fibroblasts were untreated (DMSO) or pretreated with CHX 5ug/mL for 1 hour. Immediately following pretreatment, the cells were stimulated with or without LPS for 4 hours. Transcripts were quantitated using qRT-PCR and standardized using *Gapdh* as the internal loading control. Each experiment was performed in triplicate. *P< 0.05 (C) The recruitment of C/ebpδ to the promoter of LCN2 upon LPS stimulation depends upon IRAK-1. Nuclear lysates were subject to ChIP analysis using a C/ebpδ specific antibody to determine relative binding.

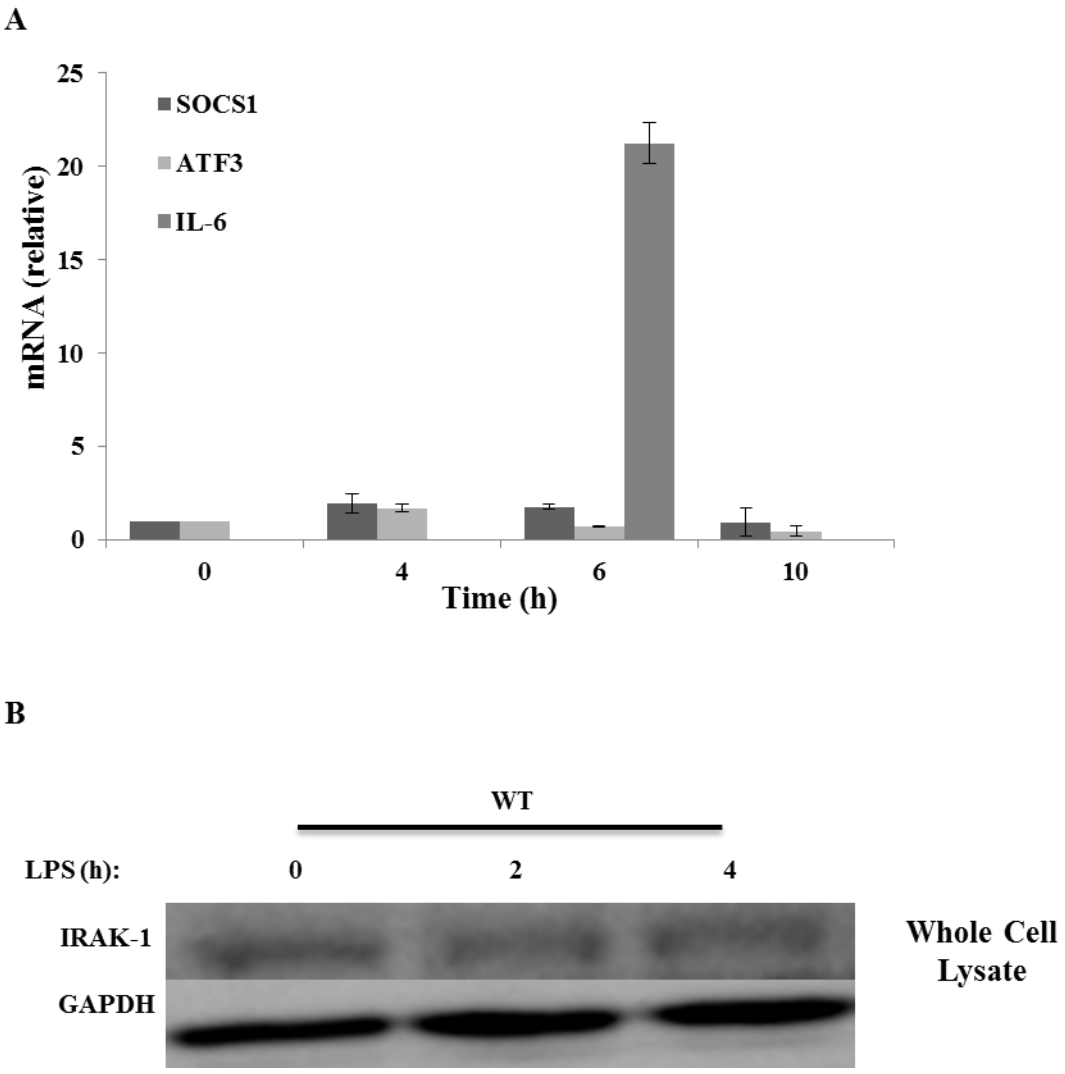

Figure 2.6: *Socs1* and *Atf3*, negative regulators of TLR4 signaling, are not induced and IRAK-1 remains intact upon LPS stimulation in kidney fibroblasts.

(A) The expressions of *Socs1* and *Atf3* were not induced after stimulation with LPS. Wild-type kidney fibroblasts were either untreated or treated with 100 ng/mL LPS for 4, 6, or 10 hours. *Socs1*, *Atf3*, and *IL-6* transcripts were measured by real time RT-PCR assays and standardized against *Gapdh* levels. Each experiment was performed in triplicate. Data is depicted as means +/- standard deviation. (B) LPS does not cause the degradation of IRAK-1. Wild-type kidney fibroblasts were either untreated or treated with 100ng/mL LPS for either 2 or 4 hours. Whole cell lysates were harvested and analyzed by Western blot with IRAK-1 specific antibodies.

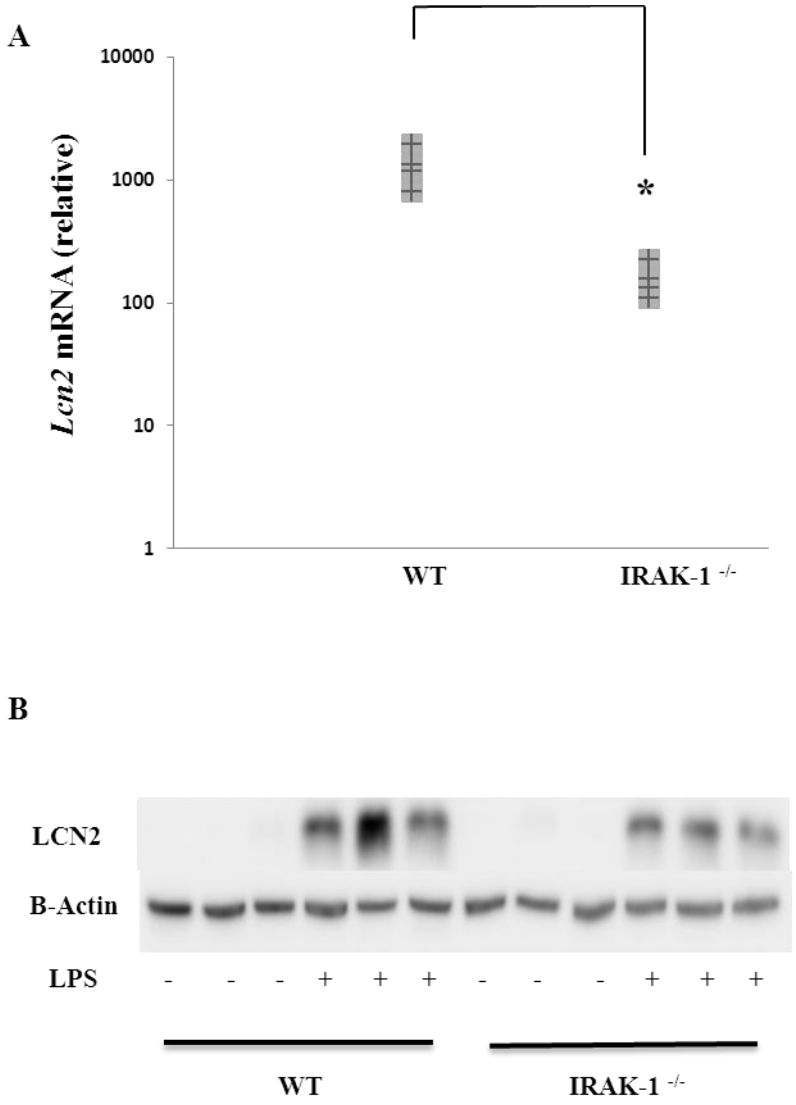

Figure 2.7: Loss of IRAK-1 causes decreased expression of *Lcn2* mRNA and protein *in vivo*.

WT and IRAK-1$^{-/-}$ C57/BL/6 female mice of 12 weeks old (6 each) were intraperitoneally injected with either 30 mg/kg of LPS or PBS for 6 hours. (A) Kidney tissues were extracted and subject to RNA extraction. *Lcn2* transcripts were measured by qRT-PCR assays and standardized against their respective controls (mice receiving PBS injections). Data is depicted as three separate mice (both WT and IRAK-1$^{-/-}$). *$P<0.05$ (B) Protein lysates were extracted from the kidney tissues and subjected to Western blot. Blots were analyzed using LCN2 specific antibodies. The same blot was probed with β-actin as a loading control.

2.5 Discussion

We have identified that LPS can induce a persistent expression of LCN2 in kidney fibroblasts. The intracellular signaling circuit responsible for the persistent induction of LCN2 involves dynamic interaction among AP-1 and C/ebpδ. An early phase of a transient AP-1 activation helps to usher in a late phase of C/ebpδ activation. IRAK-1 is responsible for the propagation of both phases necessary for the persistent induction of LCN2.

Our study sheds light on the dynamic and functional circuitry within cells when challenged with bacterial endotoxin. Although empirical knowledge reckons that integrated intracellular circuits instead of linear cascades are more capable of interpreting and responding properly to environmental cues including LPS, almost all existing studies have adopted conventional approaches in defining the signaling cascades downstream of LPS and TLR4 receptors [138]. To this regard, a large number of signaling molecules and pathways have been discovered that can be activated by LPS [139]. However, how these signals are properly integrated and give rise to distinct cellular responses is not clear. This work builds upon conventional information uncovered in the area of LPS signaling within host cells, and reveals an integrated circuit that couples a transient activation of AP-1 with a sustained activation of C/ebpδ.

Our effort is made possible by the integrated approach combining experimental tests and computational analyses. Taken into consideration of the experimental data, we have employed a differential equation-based approach to analyze time-dependent activation of AP-1 and C/ebpδ, as well as expression of *Tnfα* and *Lcn2* (data not shown). As a consequence, genes primarily under the control of AP-1 such as *Tnfα* experience a transient pattern of expression. The expression of *C/ebp*δ, on the other hand, is initially activated by AP-1, and subsequently

sustained by its own auto-activation [108, 135]. Due to the transient nature of AP-1 activation, we observed a transient dip in the expression of *C/ebpδ* (around the 4 hr time point). Subsequent self-activation of C/ebpδ sustains the continued expression of *C/ebpδ* and its downstream gene *Lcn2*.

It is likely that there will be significant perturbations as well as additional connections surrounding this circuit in different cell types. Consequently, this may allow distinct outcomes in different cell types in response to bacterial endotoxin. For example, the expression of C/ebpδ in macrophages induced by LPS eventually subsides, due to a novel negative feedback initiated by ATF3 [135]. In contrast, we observed that LPS failed to induce *Atf3* in kidney fibroblasts, which may explain the sustained expression and activation of C/ebpδ. Furthermore, a high dose LPS (100ng/ml) causes IRAK-1 degradation in macrophages, which can further dampen the signals leading to C/ebpδ activation [140]. Instead, we observed that IRAK-1 is relatively stable in kidney fibroblasts treated with 100ng/ml LPS. Our recent study indicates that a much lower LPS dose (~100pg/ml) induces a persistent activation of C/ebpδ in macrophages, similar to the effect seen in this study using fibroblasts with a high dose LPS. Further studies employing systems biology approach are clearly warranted to clarify the differential sensing mechanisms of LPS signal strength in different cell types. On a separate note, the integrated systems approach will likely explain the complex phenotypes such as LPS priming and tolerance exhibited in cells treated with either a low or a high dose LPS.

Our findings bear significant clinical implication during the pathogenesis of chronic and persistent inflammatory diseases. Local tissues and fibroblasts encountering bacterial endotoxin may exhibit persistent activation of the C/ebpδ circuit without a dampening mechanism. As a consequence, inflammatory mediators such as LCN2 may accumulate and lead to chronic tissue

damages including kidney failure. Strategies targeting at this functional circuit may hold promise in designing therapeutic strategies to combat chronic inflammatory diseases.

Chapter 3:

MECHANISM FOR PERSISTENT EXPRESSION OF IL-6 IN MACROPHAGES

MECHANISM FOR PERSISTENT EXPRESSION OF IL-6 IN MACROPHAGES

3.1 Abstract

Subclinical doses of endotoxin are strongly correlated with many human diseases. The chronic expression of pro-inflammatory mediators seems to be the underlying cause of disease. In this study, we examined the molecular mechanisms that result in the induction of *IL-6* and *C/ebpδ* in bone-marrow derived macrophages (BMDMs) after treatment with low dosages of LPS. Unlike a transient expression of pro-inflammatory cytokines seen upon high doses of LPS, low doses of LPS cause a mild but persistent expression of *IL-6* and *C/ebpδ*. Low dosages of LPS fail to induce the classical NFkB signaling pathway, but rather selectively activate JNK and C/ebpδ. Using ChIP, we were able to show that C/ebpδ and ATF2 are recruited to the promoters of *IL-6* and *C/ebpδ*. In addition, the binding of C/ebpδ to the promoters appears to be persistent in nature. Also by ChIP analysis, the binding of ATF2 at low dosages of LPS does not require JNK phosphorylation. The recruitment of C/ebpδ and ATF2 happens in a MyD88 dependent fashion. Further supporting the persistent phenotype, subclinical doses of LPS fails to induce MKP-1, SOCS1, and ATF3, classical negative regulators of classical TLR4 signaling. The sensitivity of murine macrophages to a single challenge of LPS seems to be around 25 pg/mL *in vitro*; however, once the signaling machinery is primed by this initial dose for 4 hours, a significantly lower dosage is required to sustain the persistent induction of *IL-6*. Taken together, this data has revealed a narrow signaling pathway utilized by subclinical dosages of LPS that may prove essential in the development of drugs to combat chronic inflammatory human diseases.

3.2 Introduction

A large body of evidence suggests that low circulating levels of endotoxin may be the underlying cause of many chronic human diseases. Research suggests that unhealthy lifestyle habits such as smoking and a high fat diet contribute to slightly elevated levels of circulating endotoxin [8-10, 14, 15, 18]. In addition, aging and obesity have been linked to leakage of endotoxin through the intestinal barrier and into circulation [37, 38]. Indeed, low grade endotoxemia (<1 ng/mL) may be the root cause for diseases such as diabetes, atherosclerosis, and Parkinson's disease [40, 41, 49, 51, 55, 58]. High concentrations of circulating endotoxin are observed in severe cases of sepsis and infection. At these concentrations (>1 ng/mL), endotoxin is a potent inducer that results in a powerful cytokine storm that ultimately leads to widespread host tissue damage and in severe cases death. On the other hand, low grade endotoxemia causes a very mild induction of inflammation that appears to be chronic in nature. Over time these persistent low levels of endotoxin are capable of disrupting normal cellular function. Even though low grade endotoxemia has been linked to so many human diseases, the mechanisms that contribute to the phenotype are poorly understood.

A lot of effort has been put into understanding how high dosages of endotoxin activate cellular signaling machinery to initiate inflammation. Endotoxin or lipopolysaccharide (LPS) is capable of triggering inflammation using a wide array of signaling pathways. LPS first binds to its receptors TLR4, MAC-1, and/or CD11 with a complex that includes several adaptor molecules such as MD2, LBP, and CD14 [69]. Together these elements activate an intracellular signaling cascade that ultimately leads to the activation of several transcription factors that regulate the expression of nearly 3,000 genes. Transcription factors from the C/ebp, NFkB, and AP-1 families are among the most well-known inducers of inflammation [64, 141]. Collectively

these players, at high doses of LPS, are capable of initiating inflammation and also clearing inflammation.

IL-6 is a high inducible pro-inflammatory cytokine that is capable of directly causing fever and has been shown to be elevated in most inflammatory linked diseases. IL-6 transcription has been shown to be regulated by a variety of transcription factors including C/ebpδ, AP-1, and NFkB [123, 142]. A majority of published studies have focused on doses of endotoxin typically exceeding 10 ng/mL. However, a recent study has indicated that low doses of endotoxin are sufficient to cause the removal of PPARα, a nuclear receptor, from the promoter of IL-6 [107]. Normally, nuclear receptors suppress or prevent the transcription of pro-inflammatory genes in the absence of any infection. Taken together, this data suggests that a low dose of endotoxin is sufficient to remove the suppressive mechanism and allow gene transcription to occur.

Despite our understanding of signaling mechanisms downstream of LPS, very little is known about how sub-clinical doses initiate inflammation. It is clear however, that low circulating levels of endotoxin are strongly correlated to many human diseases and are capable of initiating a mild inflammatory response. In this study, we examined the molecular mechanisms that regulate low dose mediated expression of *IL-6* and *C/ebpδ* in murine bone-marrow derived macrophages. Intriguingly, we observed that sub-clinical doses of endotoxin cause the persistent induction of *IL-6* and *C/ebpδ*. Our research suggests that JNK, ATF2, and C/ebpδ are key players involved in the persistent and chronic inflammation seen during low dose endotoxemia.

3.3 Materials and Methods

3.3.1 *Reagents*

LPS (*Escherichia coli* 0111:B4) and DMSO were obtained from Sigma Aldrich. JNK II (SP600125) inhibitor was purchased from Calbiochem. Anti-IkBα (#9242), pJNK (#9251), pERK (#4370), pP38 (#9211), pATF2 (#9225) antibodies were purchased from Cell Signaling Technology. Anti-Lamin B (ab-16048) was purchased from Abcam. Anti-C/ebpδ (M-17), anti-MKP-1 (M-18), anti-IRAK-1 (F-4), anti-p65 (F-6), anti-GAPDH (FL-335), and anti-ATF2 (C-19) antibodies were from Santa Cruz Biotechnology.

3.3.2 *Isolation of Bone-Marrow Derived Macrophages (BMDMs) and Cell Culture*

Wild-type C57BL/6 mice were purchased from Charles River Laboratory. IRAK-1 $^{-/-}$ C57/BL/6 mice were provided by Dr. James Thomas from the University of Texas Southwest Medical School. WT and IRAK-1 $^{-/-}$ mice were housed and bred in the Derring Hall Animal Facility in compliance with approved Animal Care and Use Committee protocols at Virginia Polytechnic Institute and State University. MyD88$^{-/-}$ bones were kindly provided by Dr. Michael Fessler's group at the National Institute of Health. Bone-marrow derived macrophages (BMDMs) were harvested as previously described [107]. Macrophage cells were grown in DMEM (Invitrogen^{TM}, Inc., CA) supplemented with 10% FBS #SH30071.03 (Hyclone^{TM}), 1% Penicillin/Streptomycin (Invitrogen^{TM}, Inc., CA), 1% L-Glutamine (Invitrogen^{TM}, Inc., CA), and 30% L-929 conditioned medium as a source of m-CSF at 37°C with 5% CO_2. On the third day of culture, cells were fed 25mL of fresh medium. On the seventh day, the cells were harvested for experimentation. All cells were starved overnight with DMEM containing 1% FBS before treatment.

3.3.3 *Real time RT-PCR (qRT-PCR)*

Total RNAs were harvested from BMDMs treated with or without LPS using Trizol® Reagent (Invitrogen) according to the manufacturer's protocol. cDNAs were generated using the High Capacity cDNA Reverse Transcription Kit (Applied BioSystems™, Foster, CA). Real-time PCR analyses were performed using the iQ Sybr® Green Supermix (BioRad™ Laboratories, Hercules, CA) on an IQ5 thermocycler (BioRad). The relative levels of transcripts were calculated using the ΔΔCt method using *GAPDH* as the internal control. The relative levels of mRNA from the untreated samples were adjusted to 1 and served as the basal control value. The following primers were used perform qRT-PCR: mouse *Gapdh* forward: 5'- AAC TTT GGC ATT GTG GAA GGG CTC-3', reverse: 5'- TGG AAG AGT GGG AGT TGC TGT TGA-3'; mouse *IL-6* forward: 5'-ATC CAG TTG CCT TCT TGG GAC TGA-3', reverse: 5'- TAA GCC TCC GAC TTG TGA AGT GGT-3'; mouse *C/ebpδ* forward: 5'- ACT TCA GCG CCT ACA TTG ACT CCA-3', reverse: 5'- TGT TGA AGA GGT CGG CGA AGA GTT-3'; mouse *MKP-1* forward: 5'-TGC TGG AGG GAG AGT GTT TGT TCA-3', reverse: 5'-ACT CAA AGG CCT CGT CCA GCT TTA-3'; mouse *SOCS1* forward: 5'-AGT CGC CAA CGG AAC TGC TTC TT-3', reverse: 5'-ACG TAG TGC TCC AGC AGC TCG AAA-3'; mouse *ATF3* forward: 5'-TCA AGG AAG AGC TGA GAT TCG CCA-3', reverse: 5'-GTT TCG ACA CTT GGC AGC AGC AAT-3'.

3.3.4 *Western Blot Analysis*

Isolation of whole cell, cytoplasmic, and nuclear lysates were performed as described previously [143]. Briefly, macrophages were lysed on ice using lysis buffer (10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10% Triton X-100, 10 mM KCl, 0.5 mM EDTA, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 1ug/mL leupeptin, 1ug/mL pepstatin) for 30 minutes. Samples

were centrifuged at 5000 rpm for 10 minutes at 4^0C. The supernatants were saved as the cytoplasmic extract and the pelleted nuclei were re-suspend and lysed in a high salt buffer (20 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 0.4 M NaCl, 0.2 mM EDTA, 0.5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride) on ice for 30 minutes. The samples were then centrifuged at 12,000 RPM for 20 minutes at 4^0C and supernatant kept as the nuclear extract. Whole cell lysates were obtained using RIPA buffer. Protein samples were analyzed by SDS-PAGE and Western Blot. Images were quantified with Fujifilm Multi Gauge software normalized against β actin, GAPDH, or Lamin B.

3.3.5 *ChIP assays*

Protein and chromatin from treated cells was cross-linked using 1% formaldehyde solution in complete media for 15 minutes with gentle rocking every 3 minutes at room temperature. Cells were washed twice with cold PBS and treated with a glycine solution for 5 minutes to stop the cross-linking reaction. The cells were then lysed in an ice cold buffer containing SDS and protease inhibitor cocktail. The samples were then subject to sonication in an ice water bath to shear the chromatin for 10 minutes (30 seconds on and 30 seconds off). The sheared chromatin was processed using Chip-IT Express Kit #53008 from Active Motif™. The immune-precipitated chromatin was analyzed by PCR using primer pairs that span the promoter regions of either *IL-6* or *C/ebpδ*. The following are the primer sequences used to amplified the enriched chromatin samples: promoter of mouse *C/ebpδ* forward: 5'-ACG GTT CAC TAG TTC TGG TCT CG-3', reverse: 5'-TTT TCT AGC CCC AGC TGA CGC-3'; promoter of mouse *IL-6* forward: 5'-TCC CAT CAA GAC ATG CTC AAG TGC-3', reverse: 5'-AGC AGA ATG AGC TAC AGA CAT CCC-3'.

3.3.6 *Statistical Analysis*

Data significance was determined using the Student's t-test. P-values less than 0.05 were considered statistically significant as indicated by an asterisk (*).

3.4 Results

3.4.1 Low and high dosages of LPS induces two very different inflammatory responses in bone-marrow derived macrophages (BMDMs).

We first compared how the dosage of LPS effects the expression of *IL-6* and *C/ebpδ*, well-known inducers of inflammation. As shown in **Figure 3.1A**, low dosages (50 pg/mL) of LPS causes a persistent induction of *IL-6* and *C/ebpδ* message. Message levels remained significantly higher than the basal levels after 8 hours of LPS stimulation. In contrast, high dosages (100 ng/mL) of LPS induced a transient expression of both *IL-6* and *C/ebpδ* (**Figure 3.1B**). Message levels peaked around 4 hours for both genes and then returned near basal levels after 8 hours of stimulation. Because NFkB is one of the key transcription factors associated with the induction of pro-inflammatory genes in response to LPS, we analyzed the stability of its inhibitor IkBα. As shown in **Figure 3.2**, 50 pg/mL of LPS is not sufficient to induce the degradation of IkBα. However, 100 ng/mL of LPS is capable of causing the rapid degradation of IkBα after 30 minutes of stimulation.

3.4.2 Low dosages of LPS cause the persistent activation/phosphorylation of JNK, ATF2, and C/ebpδ.

To determine the potential mechanism that causes the persistent induction of *IL-6* and *C/ebpδ* in BMDMs, we examined the activation status of mitogen-activated protein kinases (MAPK) at low

and high doses of LPS. As shown by others, high dosages of LPS are capable of inducing a transient phosphorylation of JNK, ERK, and P38 (**Figure 3.3A**). On the other hand, low dosages are only capable of causing the activation/phosphorylation of JNK, not ERK. (**Figure 3.3A**). Furthermore, this phosphorylation event seemed to persist longer, than the phosphorylation that occurred in response to the high dose. Even after 1 hour, JNK phosphorylation levels are significantly higher than resting levels. To determine the transcription factor downstream that JNK may be responsible for the persistent induction of *IL-6* and *C/ebpδ*, we focused on ATF2. As shown in **Figure 3.3B**, ATF2 was also persistently phosphorylated in response to low dosages of LPS. Further supporting our previous finding, nuclear levels of C/ebpδ also persisted in response to low dosages of LPS and became transient upon stimulation of high dosages of LPS (**Figure 3.3C**).

3.4.3 Sustained JNK activation contributes to the expression of IL-6 upon low dosage LPS stimulation.

To confirm that JNK plays a critical role in the expression of IL-6 in response to low doses of LPS, we applied a phospho-JNK specific inhibitor to the culture medium. As shown in **Figure 3.4A**, the JNK inhibitor (SP600125) was capable of blocking the phosphorylation of JNK upon the stimulation of low dosages of LPS at a concentration of 10 uM. Further suggesting the importance of JNK in the expression of *IL-6* at low dosages, we observed a significant reduction of *IL-6* expression when the BMDMs were pretreated with SP600125 for 1 hour following a 6 hour low dose stimulation (**Figure 3.4B**).

3.4.4 The absence of inducible negative regulators contributes to the sustained and persistent induction of IL-6 following treatments with low dosages of LPS.

Through the classical NFkB pathway, LPS is known to induce negative regulators that are capable of shutting down the expression of pro-inflammatory genes. Of the many negative regulators we examined the expression of MKP-1, SOCS1, and ATF3. MKP-1 has been shown to suppress ERK and JNK activation. SOCS1 has been shown to suppress the LPS signaling pathway by inhibiting IRAK-1. ATF3 competes with C/ebpδ to suppress its activity. Since we did not observe the activation of NFkB at low dosages of LPS, we hypothesized that LPS may not be capable of inducing these regulators that collective shut down signaling. This would explain why the induction of pro-inflammatory genes is able to persist. As shown in **Figure 3.5A**, *MKP-1* message is not induced above basal levels after 4 hours of stimulation with 50 pg/mL of LPS. Further supporting this, by Western blot, MKP-1 protein levels are also not induced by low dosages of LPS. In addition, we did not observe an induction of *SOCS1* (**Figure 3.6A**) or *ATF3* (**Figure 3.6B**) message upon stimulation with 50 pg/mL of LPS. As a consequence, we did not see any degradation of IRAK-1 after low dose LPS treatment (**Figure 3.7**). Together, our data indicates that 50 pg/mL LPS treatment fails to induce negative regulators that normally modulate the induction of *IL-6* and *C/ebpδ* causing their expression to persist.

3.4.5 ATF2 is involved in the initial induction of both C/ebp δ and IL-6, but C/ebp δ is the key player that is involved in the sustained induction upon a low dose treatment of LPS.

Many transcription factors are known to regulate *C/ebp δ* and *IL-6* message. Among these are C/ebp, AP-1, and NFkB. To test the involvement of these transcription factors in the expression

of *IL-6* and *C/ebpδ* ChIP analysis was performed. As shown in **Figure 3.8**, low dosages of LPS cause the persistent recruitment of C/ebpδ to its own promoter; however, following treatment with high dose C/ebpδ binding is transient. The binding of ATF2 is transient following LPS treatment with low or high dosages. In agreement with our previous findings, binding of p65 was only detected after stimulation with 100 ng/mL of LPS. Similarly, as shown in **Figure 3.9**, C/ebpδ was also recruited in a persistent fashion to the promoter of *IL-6*. No binding of NFkB/p65 was detected upon treatment with low dosages of LPS and ATF2 was also recruited in a transient fashion regardless of the dose of LPS. Taken together, our data indicates that both ATF2 and C/ebpδ seem to be involved in the induction of *C/ebpδ* and *IL-6* in response to low dose LPS treatment.

3.4.6 JNK does not appear to be responsible for the activation of ATF2 upon the stimulation of BMDMs with low dosages of LPS.

Previous studies have indicated that JNK is upstream and capable of phosphorylating ATF2 upon LPS stimulation. To confirm this signaling pathway at low dosages of LPS, we applied a phospho-JNK inhibitor to the culture medium and then examined the recruitment of ATF2 to the promoter of both *C/ebpδ* and *IL-6* by ChIP analysis. As shown in **Figure 3.10**, the binding of ATF2 still occurs to the promoter of *C/ebpδ* (**Figure 3.10B**) and *IL-6* (**Figure 3.10A**) when the phosphorylation of JNK in inhibited. This data suggests that something other than JNK must be responsible for the activation of ATF2 at low dosages.

3.4.7 MyD88 is a critical adaptor molecule needed for the recruitment of C/ebpδ, ATF2, p65 to both C/ebpδ and IL-6.

Others have shown that MyD88 is a key adaptor molecule downstream of LPS/TLR4 that is required for the expression of *C/ebpδ*. To test whether this adaptor molecule is involved in signaling processes downstream of low dosages of LPS, we used MyD88 deficient BMDMs and performed ChIP analysis to examine the recruitment of C/ebpδ, ATF2, and p65 to the promoters of *C/ebpδ* and *Il-6*. When MyD88 is lost, no binding of C/ebpδ, p65 or ATF2 is detected on the promoter of *C/ebpδ* (**Figure 3.12**) or IL-6 (**Figure 3.11**).

3.4.8 Sensitivity to LPS

To determine the lowest dose of LPS that is needed to induce a detectable induction of *IL-6* a dose analysis by qRT-PCR was performed. As shown in **Figure 3.13**, IL-6 message levels were significantly induced above the basal level at a dose between 10-25 pg/mL after 6 hours of LPS treatment. On the other hand, when the cells have been pretreated with a low dose of LPS and then exposed to a secondary dose of LPS for 6 more hours, a much lower dose of LPS was needed to sustain the induction of *IL-6* (**Figure 3.14**). A secondary dose as low as 5 pg/mL was sufficient to drive the induction of *IL-6* above cells that only received the initial pretreatment dose.

3.4.9 IRAK-1 is required for the sustained phosphorylation of JNK.

We hypothesized that IRAK-1 might be a critical node downstream of LPS/TLR4 that is required for low dose signaling events to allow for the persistent expression of pro-inflammatory mediators. To examine whether IRAK-1 is a critical upstream kinase in this signaling cascade, IRAK-1 deficient BMDMs were used to determine whether or not JNK phosphorylation requires

IRAK-1. As shown in **Figure 3.15**, loss of IRAK-1 leads to a very short and transient phosphorylation of JNK. JNK phosphorylation peaks at 15 minutes upon low dose treatment and then returns to resting levels after 30 minutes. JNK phosphorylation is significantly ablated and not capable of being sustained when IRAK-1 is loss compared to wild-type BMDMs.

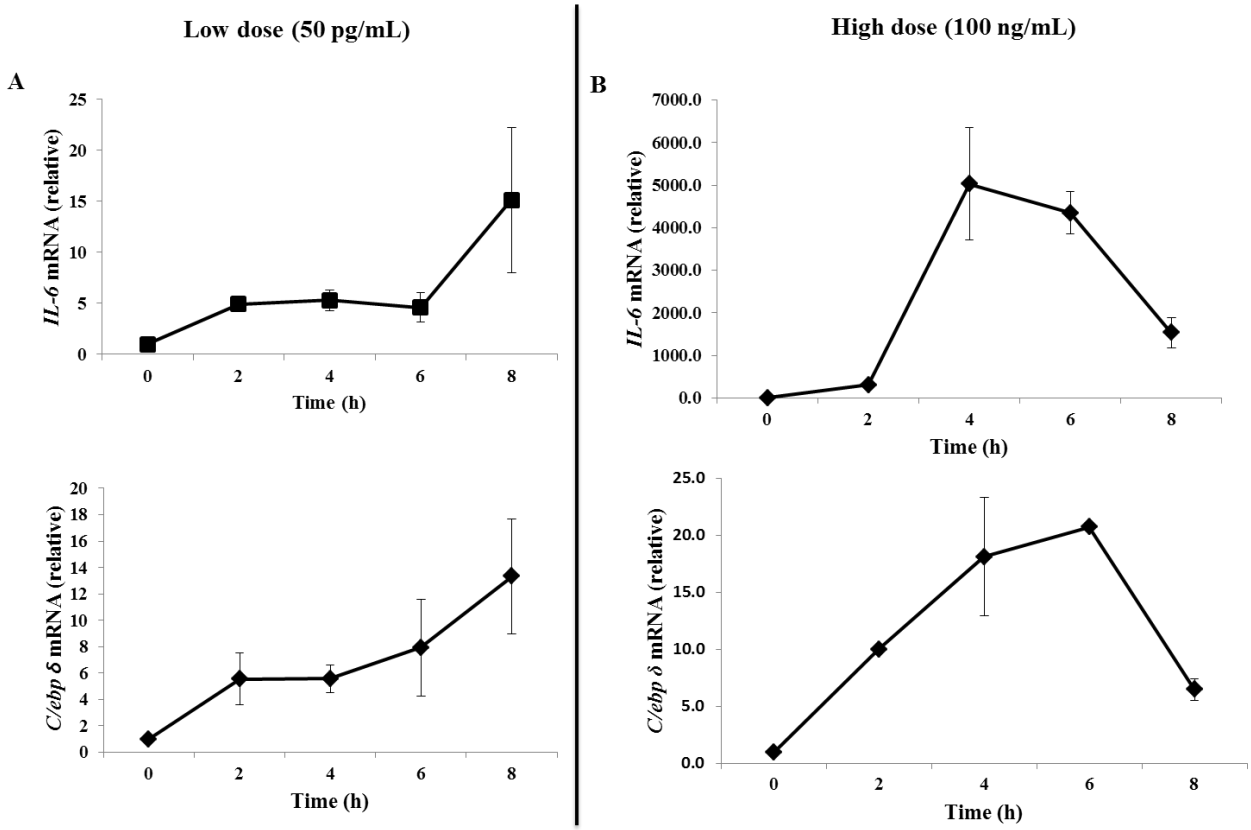

Figure 3.1: Low doses of LPS cause a persistent induction of *IL-6* and *C/ebpδ*.

WT BMDMs were treated with or without a low dose (50 pg/mL) of LPS (A) or a high dose (100 ng/mL) of LPS (B) for 2, 4, 6, 8 hours. Total RNA was isolated and transcript levels were measured by qRT-PCR as described previously. The results are expressed as means +/- standard deviation performed in triplicate.

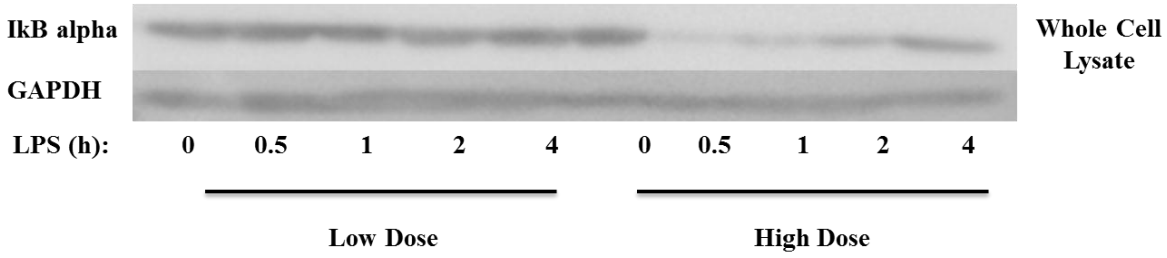

Figure 3.2: Only high doses of LPS cause the degradation of IkB α.

(A) LPS is only capable of causing the degradation of IkB α at high dosages (100 ng/mL), not at low dosages (50 pg/mL). Whole cell protein levels were measured by Western blot.

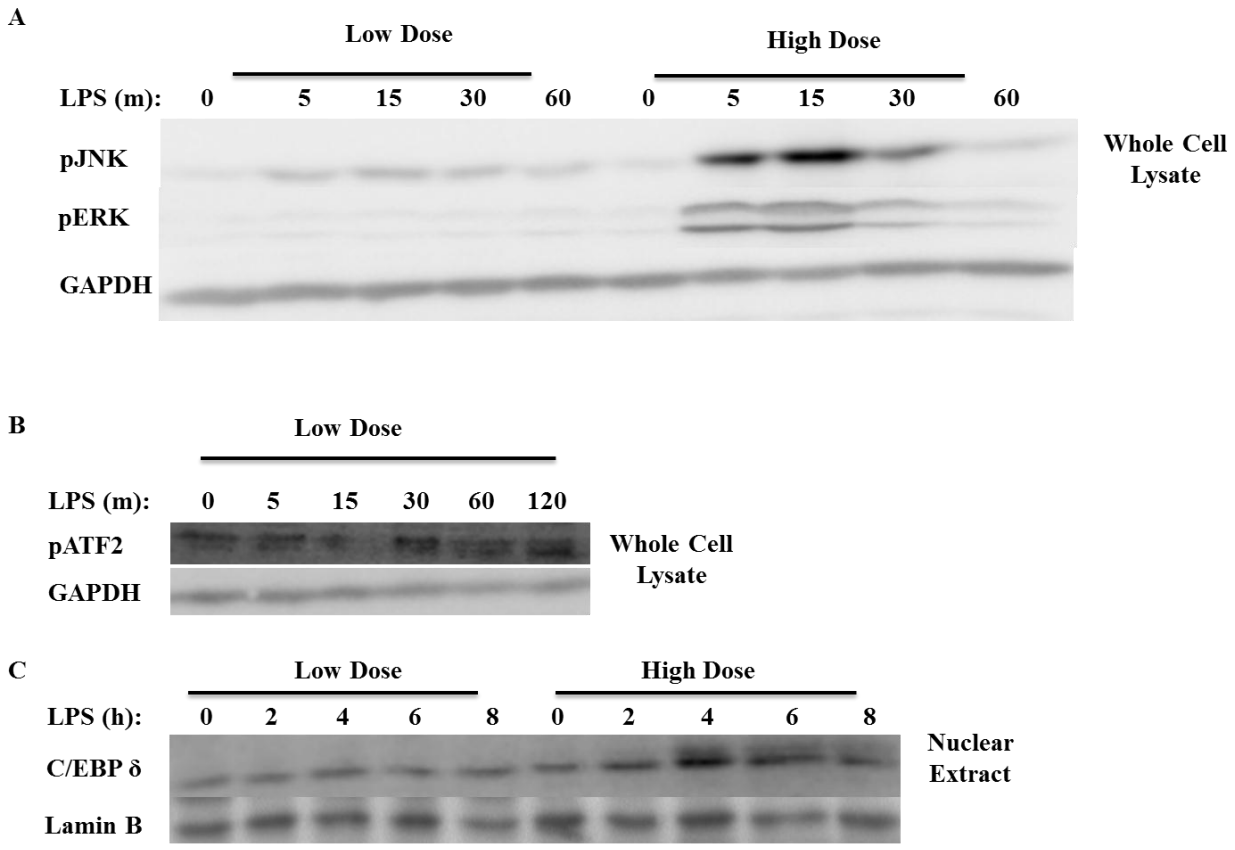

Figure 3.3: Low dosages selectively cause the persistent expression of pJNK, pATF2, and C/ebpδ.

(A) Low doses of LPS (50pg/mL) only result in the persistent phosphorylation of the MAPK JNK. At high doses (100ng/mL), LPS is capable of transiently phosphorylating JNK and ERK. (B) Low dosages of LPS cause the persistent phosphorylation of ATF2 (C) Both low and high doses of LPS are capable of causing the nuclear translocation of C/ebpδ. The nuclear levels of C/ebpδ persist at low dosages and are transient at high doses. Whole cell and nuclear protein levels were analyzed by Western blot. These are representative blots from three independent experiments.

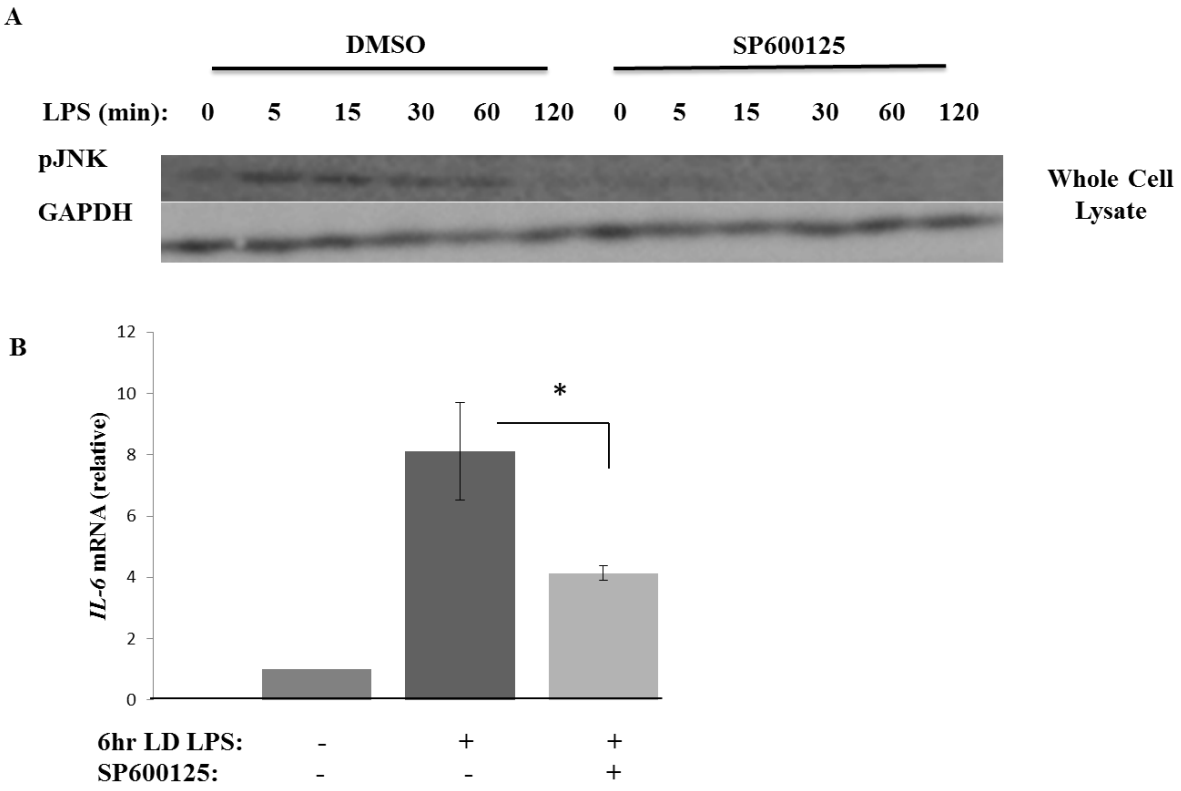

Figure 3.4: pJNK is required for the induction of *IL-6* at low doses of LPS.

(A) SP600125 (10 uM) is capable of blocking the phosphorylation of JNK. Macrophages were pretreated for 1 hour with SP600125 or DMSO before LPS stimulation. Whole cell protein lysate was analyzed by Western blot using an anti-pJNK antibody. (B) pJNK is required for the transcription of IL-6 at low dosages (50 pg/mL) of LPS. Real-time quantitative RT-PCR analysis was performed to determine the relative levels of *IL-6* message. The results are expressed as means +/- standard deviation performed in triplicate. *Statistically significant difference determined by Student's t-test ($P<.05$)

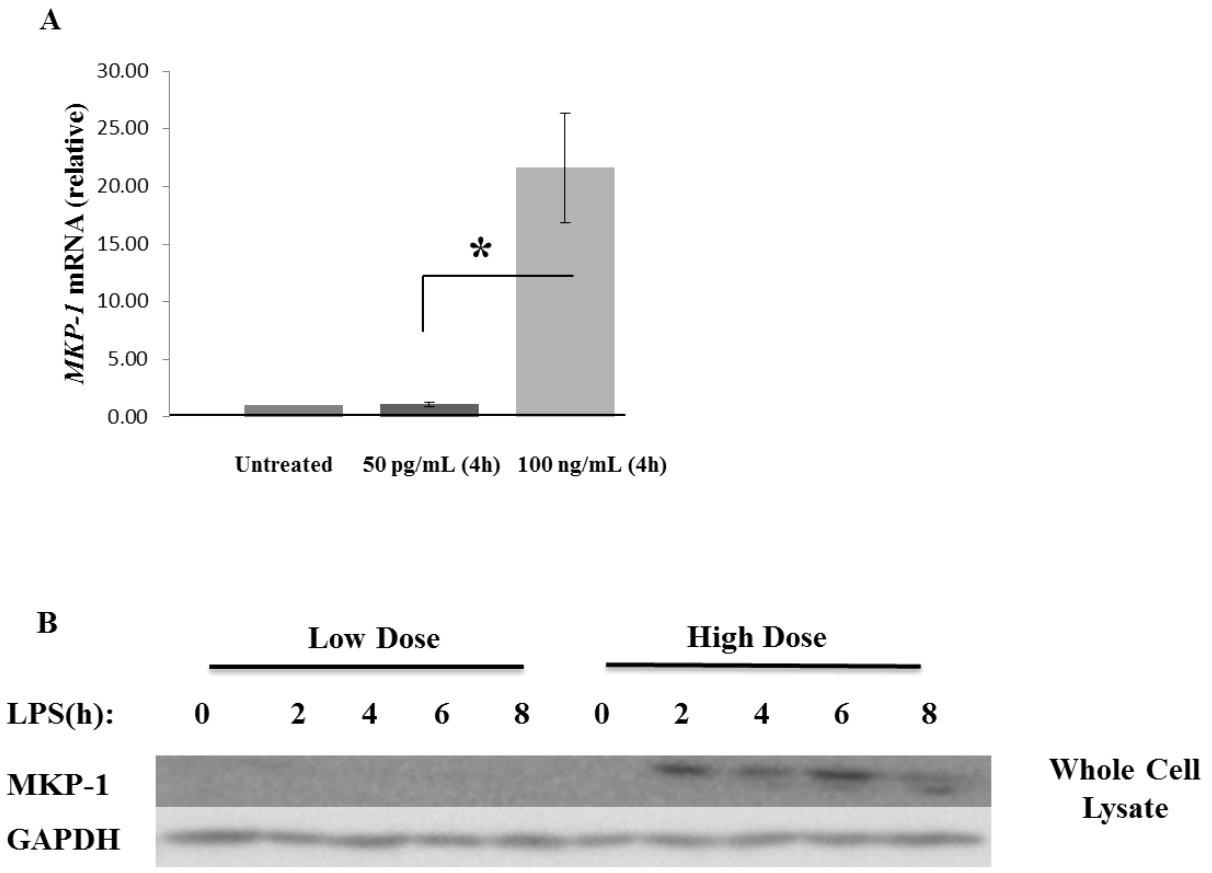

Figure 3.5: MKP-1 is not expressed after low doses of LPS, only after high doses.

(A) A low dosage of LPS fails to induce the transcription of MKP-1. Real-time quantitative RT-PCR analysis was performed to determine relative levels of *MKP-1* message. The results are expressed as means +/- standard deviation performed in triplicate. (B) Low dosages also fail to cause the expression of MKP-1 protein levels. Whole cell protein was analyzed by Western blot using an anti-MKP-1 antibody. *Statistically significant difference determined by Student's t-test ($P<.05$)

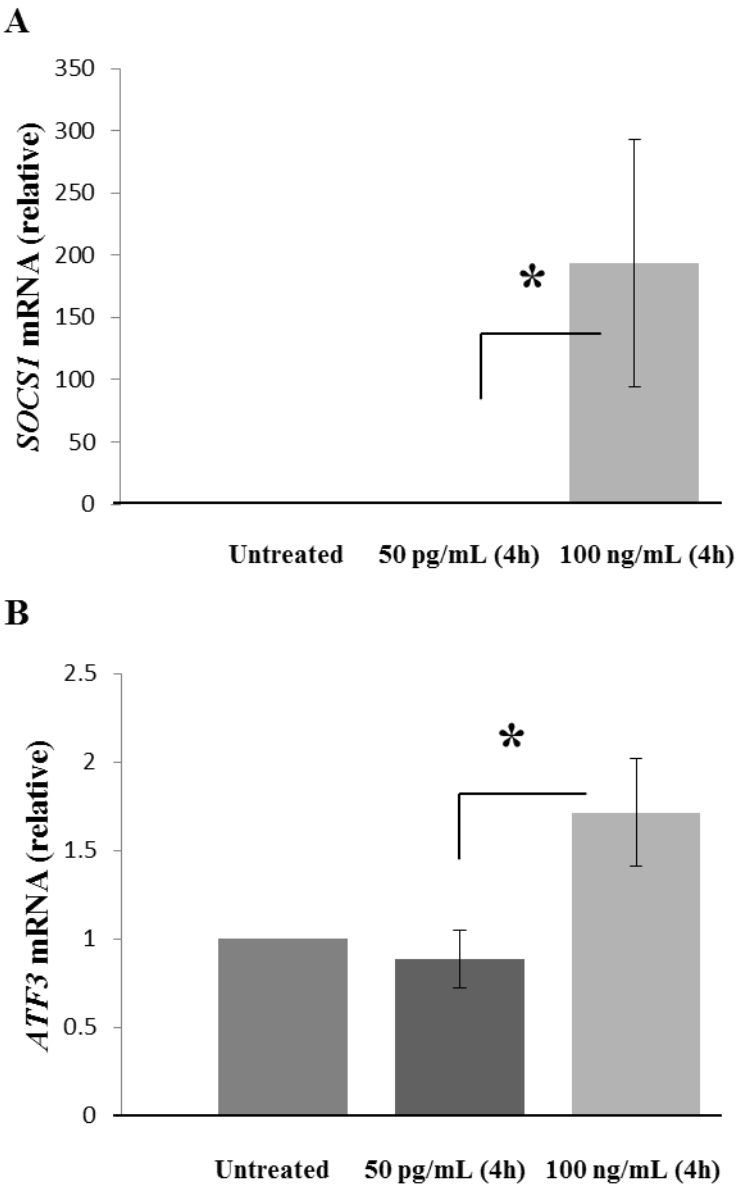

Figure 3.6: Low dosages of LPS will not induce *SOCS1* or *ATF3* message levels.

(A) The transcription of *SOCS1* requires high dosages of LPS (100 ng/mL). (B) The transcription of ATF3 also requires high dosages of LPS. Real-time quantitative RT-PCR analysis was performed to determine relative levels of *SOCS1* and *ATF3* message. The results are expressed as means +/- standard deviation performed in triplicate. *Statistically significant difference determined by Student's t-test ($P<.05$)

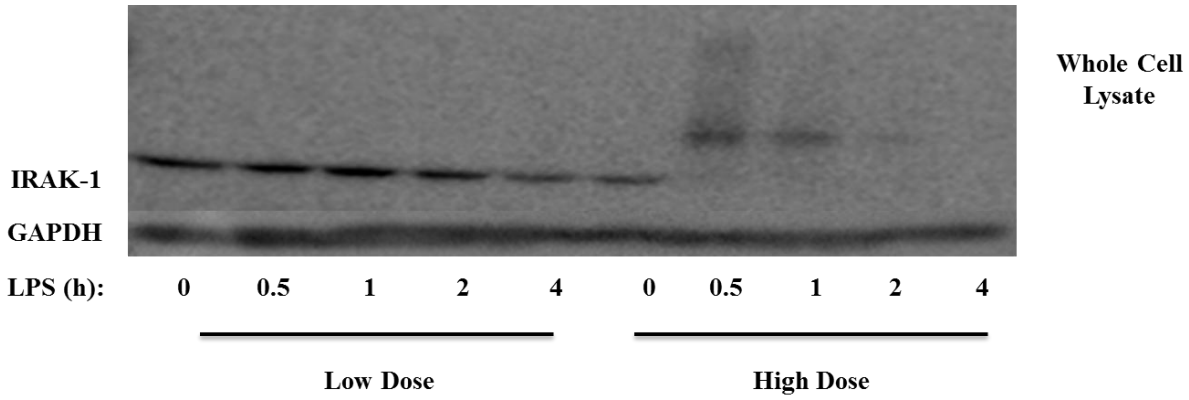

Figure 3.7: IRAK-1 is not degraded at low dosages of LPS.

IRAK-1 is targeted for degradation upon stimulation with high doses of LPS (100 ng/mL). IRAK-1 protein levels are capable of persisting upon treatment with low levels of LPS (50 pg/mL). WT BMDMs were treated with or without LPS for 0.5, 1, 2, and 4 hours. Whole cell lysate was analyzed by Western blot using an anti-IRAK-1 antibody. GAPDH serves as a loading control. The Western blot is a representative gel from three independent experiments.

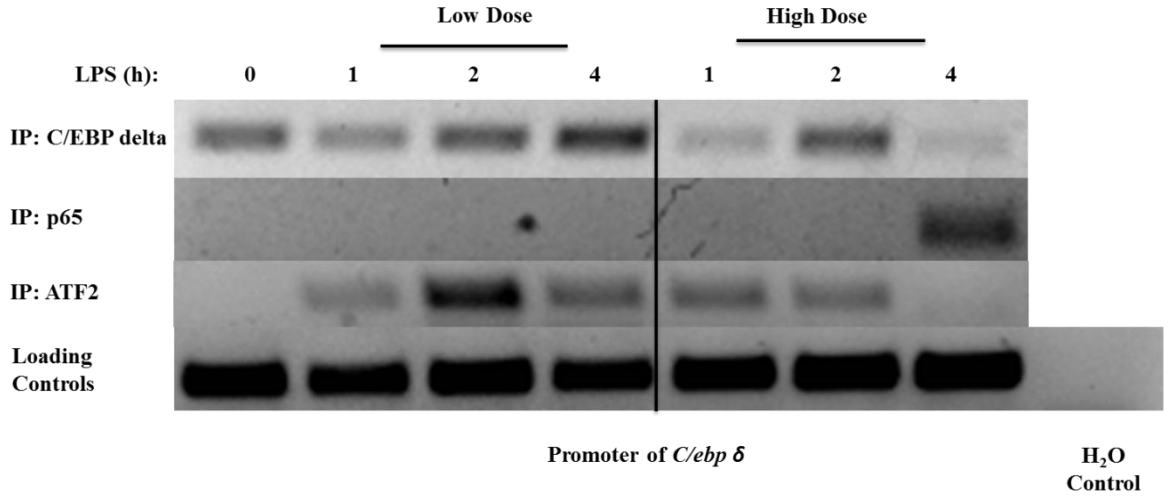

Figure 3.8: A low dose of LPS induces the persistent recruitment of C/ebpδ and ATF2 to the promoter of *C/ebpδ*.

Low doses cause the persistent binding of C/ebpδ and ATF2 to the promoter of C/ebpδ. These two transcription factors also bind upon stimulation with high doses of LPS, but in a transient manner. No binding of p65 was detected upon stimulation with low dosages of LPS. Wild-type BMDMs were treated with either low dosages (50 pg/mL) or high dosages (100 ng/mL) of LPS for a time course. Nuclear lysates were then subjected to ChIP analysis.

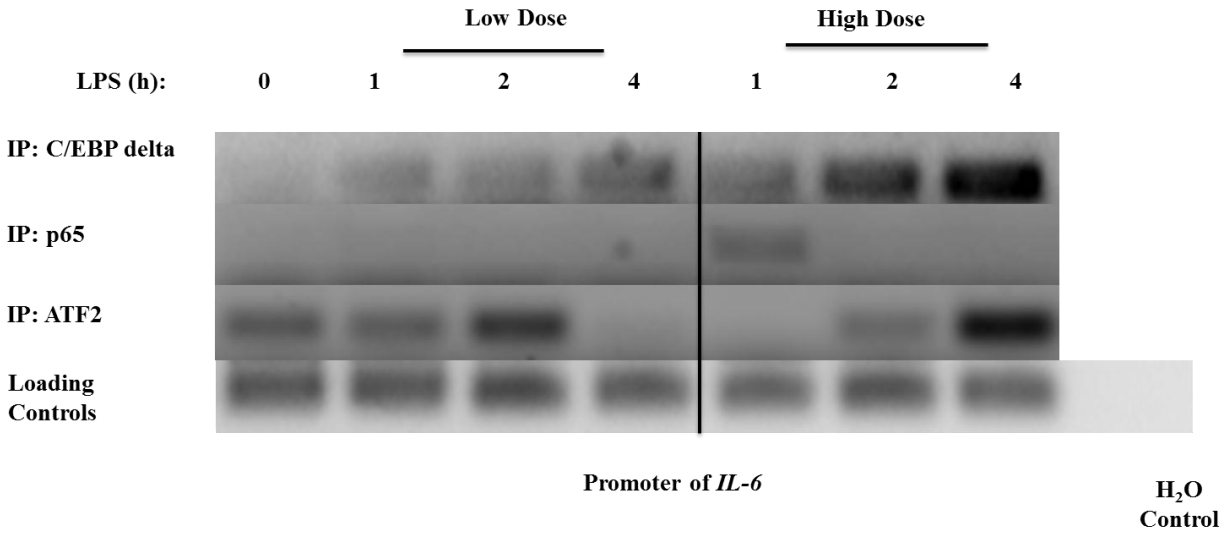

Figure 3.9: Low doses of LPS induce the persistent recruitment of C/ebpδ and transient recruitment of ATF2 to the promoter of *IL-6*.

Low doses cause the persistent binding of C/ebpδ and to the promoter of *IL-6*. ATF2 also binds upon low dose stimulation of LPS, but in a transient manner. No binding of p65 was detected upon stimulation with low dosages of LPS, only at high dosages. Wild-type bone marrow derived macrophages were treated with either low dosages (50 pg/mL) or high dosages (100 ng/mL) of LPS for a time course. Nuclear lysates were then subjected to ChIP analysis.

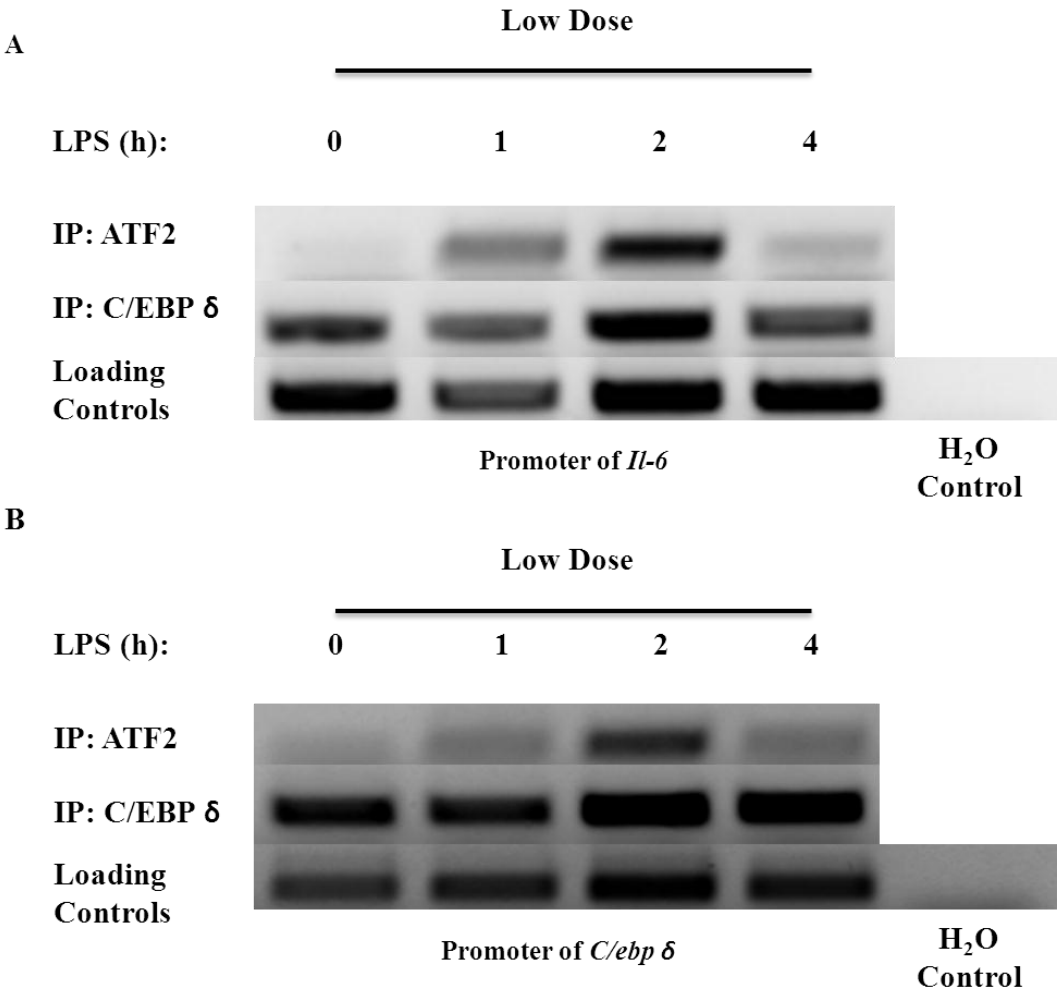

Figure 3.10: JNK is not required for ATF2 recruitment to the promoter of *IL-6* or *C/ebpδ*.

The inhibition of JNK phosphorylation does not prevent the transient binding of ATF2 to the promoters of *IL-6* (A) or *C/ebpδ* (B). Wild-type BMDMs were pretreated with a 10 uM solution of SP600125 for 1 hour. Following pretreatment the cells were treated with or without 50 pg/mL LPS for a time course. Nuclear lysates were harvested and analyzed by chromatin immune-precipitation.

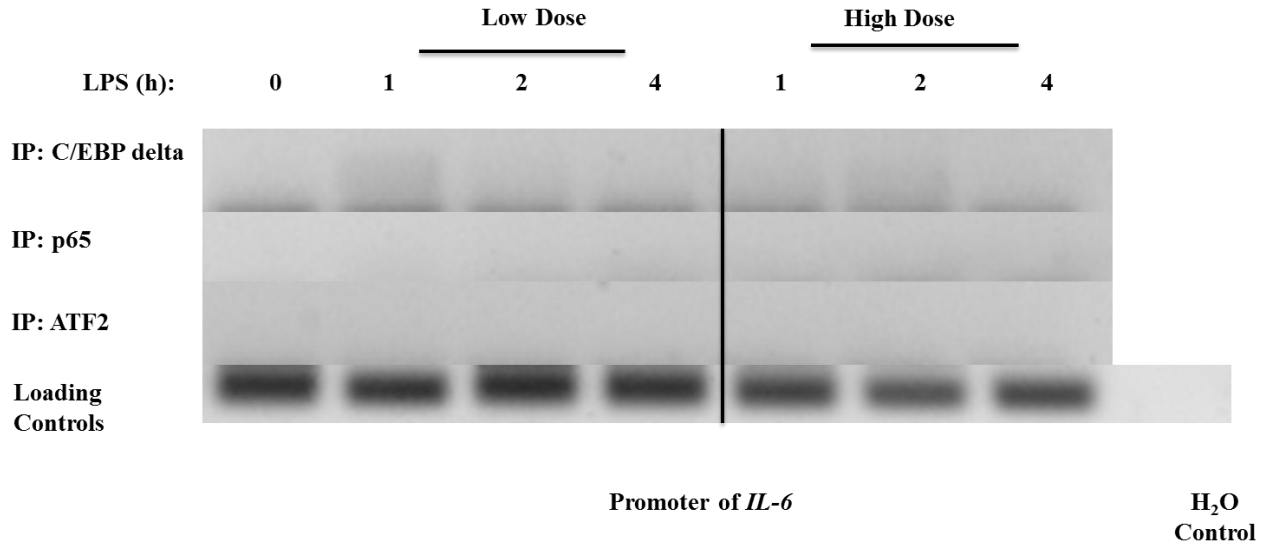

Figure 3.11: MyD88 is required for the binding of C/ebpδ, ATF2, and p65 to the promoter for *IL-6*.

The binding of C/ebpδ, ATF2, and p65 to the promoter of *IL-6* requires MyD88. MyD88 deficient BMDMs were treated with either low dosages (50 pg/mL) or high dosages (100 ng/mL) of LPS for a time course. Nuclear lysates were then subjected to ChIP analysis.

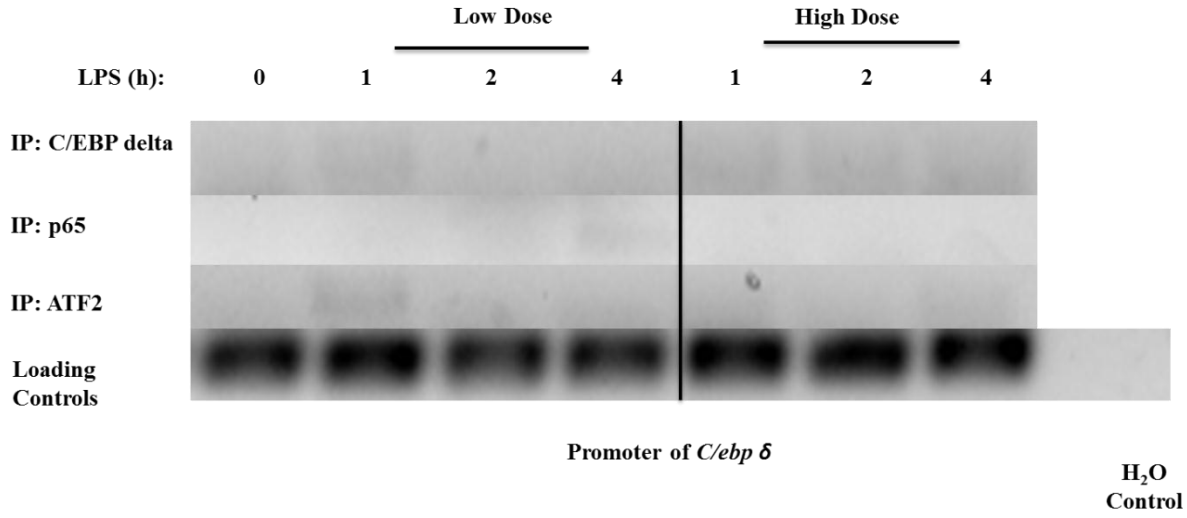

Figure 3.12: MyD88 is required for the binding of C/ebpδ, ATF2, and p65 to the promoter for *C/ebpδ*.

The binding of C/ebpδ, ATF2, and p65 to the promoter of *C/ebpδ* requires MyD88. MyD88 deficient BMDMs were treated with either low dosages (50 pg/mL) or high dosages (100 ng/mL) of LPS for a time course. Nuclear lysates were then subjected to ChIP analysis.

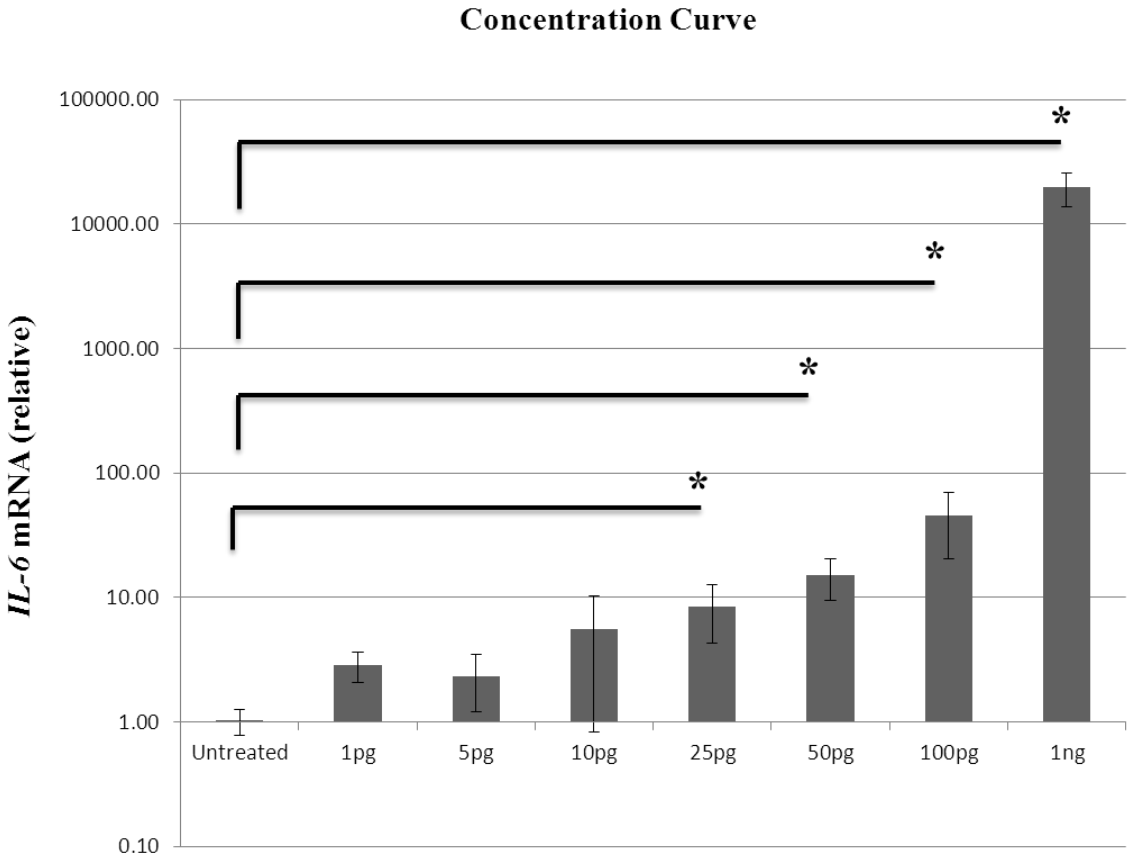

Figure 3.13: The expression of IL-6 requires a dosage of LPS between 10-25 pg/mL.

The minimal dose needed to induce the transcription of IL-6 is between 10-25 pg/mL. Doses lower than 10 pg/mL fail to induce the transcription of IL-6 beyond normal resting levels. WT BMDMs were treated with varying doses of LPS for 6hours. Total RNA was isolated and subjected to real-time quantitative RT-PCR analysis to determine the relative levels of *IL-6* message. The results are expressed as means +/- standard deviation performed in triplicate. *Statistically significant difference determined by Student's t-test ($P<.05$)

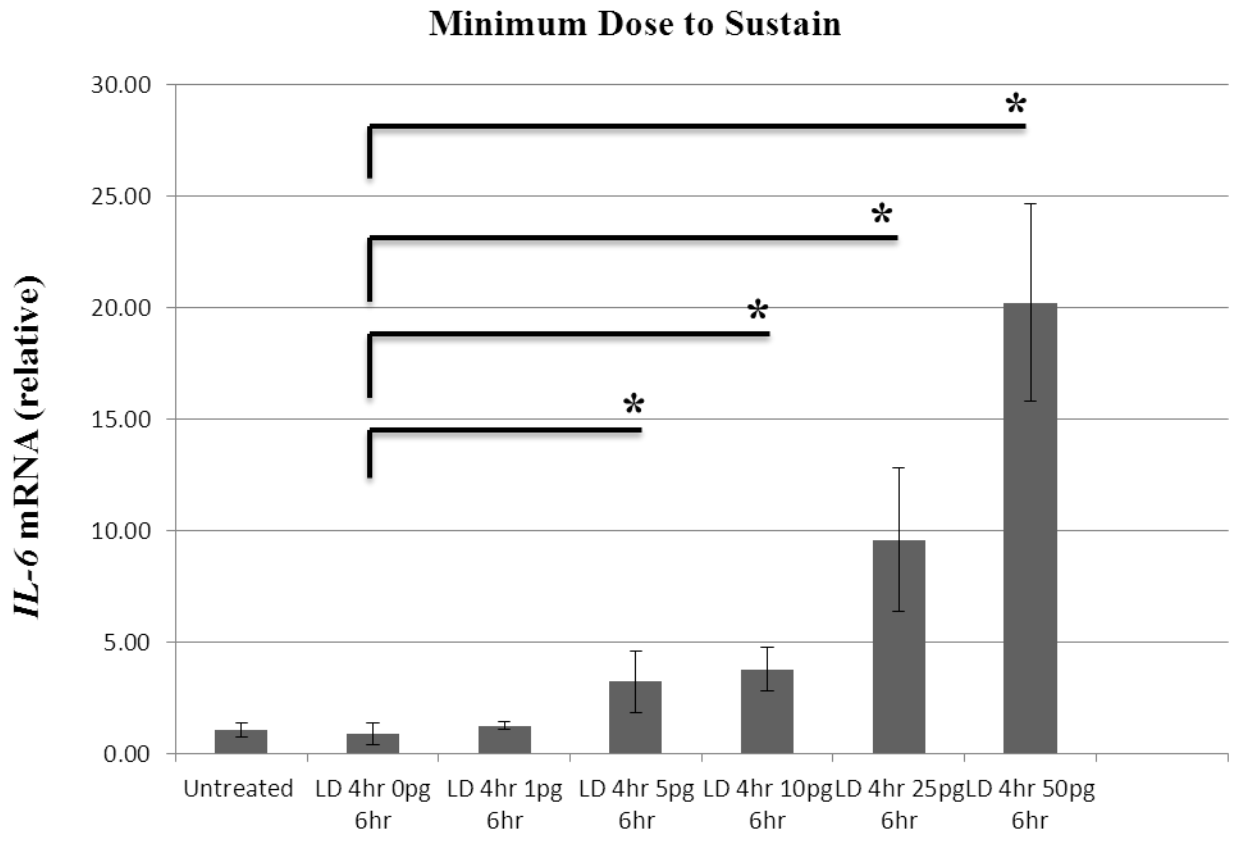

Figure 3.14: Following an initial priming dose of LPS, the minimum dose to maintain the persistent expression of IL-6 is significantly lower.

All doses exceeding 5 pg/mL were capable of sustaining a persistent expression of *IL-6* message. WT BMDMs were primed with a low dose (50 pg/mL) of LPS for 4 hours, then washed two times with PBS, and finally treated with a second varying dose of LPS for an additional 6 hours. Total RNA was isolated and subjected to real-time quantitative RT-PCR analysis to determine relative levels of *IL-6* message. The results are expressed as means +/- standard deviation performed in triplicate. *Statistically significant difference determined by Student's t-test ($P<.05$)

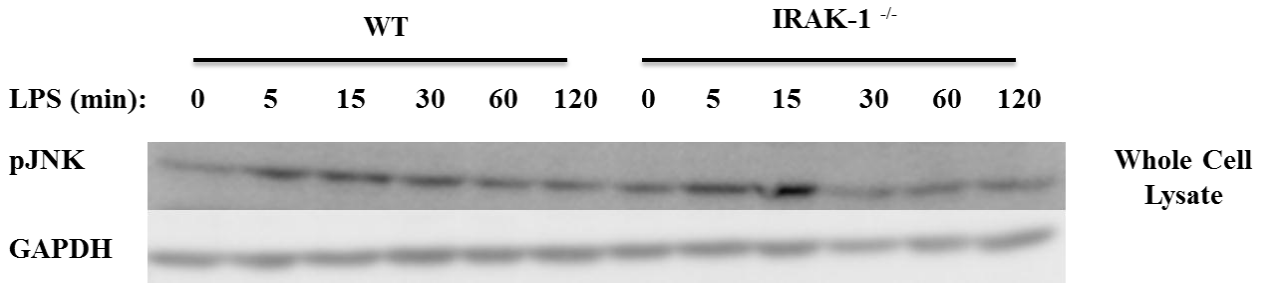

Figure 3.15: Persistence of pJNK by low dosages of LPS requires IRAK-1.

When IRAK-1 is lost, JNK becomes phosphorylated in response to low doses of LPS in a transient manner. WT and IRAK-1$^{-/-}$ BMDMs were treated with or without a low dose (50 pg/mL) of LPS for a time course. Whole cell lysates were analyzed by Western blot using an anti-pJNK antibody. The Western blot is a representative blot from three independent experiments.

3.5 Discussion

In this study, we identified novel signaling pathways in response to low dose endotoxin in murine BMDMs. Previous studies have shown that high doses of LPS cause a strong but transient induction of pro-inflammatory genes such as IL-6, TNFα, IL-1, and MCP-1 [144]. This transient activation is due to several signaling pathways, but primarily the MAPK pathway and the NFkB pathway. The induction of these genes is quickly down regulated by numerous LPS-inducible negative regulators including SOCS1, ATF3, MKP-1, and IkBα [100]. On the other hand, low doses of endotoxin cause a very mild induction of inflammatory genes [14, 107]. These low levels of endotoxin and inflammation have been observed in many human diseases including diabetes, atherosclerosis, and cancer [40, 49, 55, 145].

Previous studies in our lab, have documented that subclinical doses (<1 ng/mL) of LPS fail to activate the NFkB signaling pathway and ERK. In addition, they were able to show that subclinical doses are sufficient to causing the removal of suppressive molecules like nuclear receptors and cause the activation of C/ebpδ [107]. In this study, we did identify JNK, a key component of the MAPK signaling pathway, as a critical component required for the low dose induction of IL-6. Aderem et al. also convincingly showed that C/ebpδ, upon its activation, is capable of triggering its own expression forming a positive feedback loop [141]. To the best of our knowledge, this is the first documented instance *in vitro* showing that subclinical doses of LPS cause a persistent induction of both IL-6 and C/ebpδ. Taken together, this data suggests that subclinical doses of endotoxin activate a very narrow signaling pathway that requires JNK and C/ebpδ.

Further supporting the persistent phenotype observed during low dose endotoxemia, we were able to show that SOCS1, MKP-1, and ATF3, down regulators of inflammation, are not expressed. Using chromatin immunoprecipitation we observed that C/ebpδ and ATF2 are key transcription factors that are recruited to the promoters of both *IL-6* and *C/ebpδ*. Due to the transient binding nature of ATF2 and the persistent binding nature of C/ebpδ, we hypothesize that C/ebpδ that is primarily responsible for the persistent expression. Unlike previous findings, we observed an apparent disconnect between JNK and ATF2. When JNK phosphorylation was block we still observed the binding of ATF2 to the promoters of both IL-6 and C/ebpδ. Perhaps another member of the AP-1 family of transcription factor may be the target of JNK; like JunB, cJUN, or cFos. Another explanation is that another MAPK is activating ATF2 in conjunction with JNK. Similar to signaling that occurs at high doses of endotoxin, we did observe that the signaling mechanisms downstream of low dose endotoxin require the upstream adaptor molecular MyD88. We were able to detect a significant expression of IL-6 in response to concentrations of endotoxin as low as 25 pg/mL in murine BMDMs. Intriguingly, we did notice that following an initial dose of 25 pg/mL of LPS for 4 hours, a dose as low as 5 pg/mL of LPS was sufficient to sustain the persistent induction of IL-6. Therefore, mechanisms must exist that allow for macrophages to 'remember' their prior history of LPS exposure. The duration of this memory and key players still needs to be addressed.

Our study also sheds light on a potential unique role for IRAK-1 in macrophages challenged with low levels of LPS. Normally upon a high dose of LPS, IRAK-1 is quickly poly-ubiqinated and shuttled to the proteasome for degradation, however during a challenge with low levels of LPS IRAK-1 this is not seen [103]. IRAK-1 is a key signaling kinase downstream of many toll-like receptors. Its role has been quite controversial, but it clearly plays a significant

role in inflammation. When IRAK-1 is lost, in general, inflammation is significantly reduced and less inflammatory-linked diseases are observed. So in a system where low levels of LPS are present, IRAK-1 remains intact allowing for it to continue initiating inflammation. This might be one of the key molecules contributing to the persistence phenotype seen *in vitro*. Moreover, when IRAK-1 is knocked out, we no longer observed the persistent phosphorylation of JNK. Instead JNKs activation was significantly ablated. Previous finding have also linked IRAK-1 as a critical signaling kinase required for the removal of nuclear receptors and the activation of C/ebpδ in macrophages challenged with low doses of LPS [107]. Clearly, further research is warranted to further elucidate the role of IRAK-1 in subclinical endotoxemia.

Based upon our findings and others, it is apparent that low dosages of LPS signal through a very specific pathway that is distinct from high dose LPS signaling. The exact mechanism that allows the cell to differentiate between different doses of LPS has yet to be revealed. However, it is likely due to a novel conformation of receptors (TLR4, MAC-1, and scavenger receptors) that regulate the recruitment of adaptor molecules to their intracellular domain. This could explain the small number of signaling pathways that seem to be employed upon a challenge of low doses of LPS.

This study has revealed a novel signaling pathway that may provide useful targets for therapeutic intervention of relative inflammatory diseases. Clearly this work supports claims that IRAK-1 is a high value molecular target useful in treating chronic inflammatory diseases especially since the function of IRAK-1 is not required to clear bacterial infection. In addition, specifically inhibiting or blocking JNK or C/ebpδ might also prove beneficial in preventing mild persistent inflammation due to endotoxin leakage.

Chapter 4:

MECHANISM FOR LIPOPOLYSACCARIDE MEDIATED PRIMING AND TOLERANCE OF INFLAMMATION IN MURINE MACROPHAGES

MECHANISM FOR LIPOPOLYSACCHARIDE MEDIATED PRIMING AND TOLERANCE OF INFLAMMATION IN MURINE MACROPHAGES

4.1 Abstract

The persistent and chronic expression of inflammatory mediators in response to low dosages of endotoxin is strongly correlated to many human diseases. It has been long understood that innate immune cells are capable of mounting completely difference responses depending on their prior challenges to endotoxin. In this study we examined the molecular mechanisms responsible for LPS-induced priming and tolerance. Initially we were able to show that pretreatment with low doses (50 pg/mL) of LPS causes increase in IL-6 message when compared to cells that received only a secondary high dose (100 ng/mL) treatment of LPS. Unlike the typical LPS induced activation of PI3K and subsequent phosphorylation of AKT induced by high dosages (>1 ng/mL) of LPS, AKT phosphorylation is actually suppressed at low dosages of endotoxin (50 pg/mL). AKT phosphorylation seems to be significantly ablated and delayed when BMDMS are pretreated with a low dose of LPS followed by a secondary high dose when compared to cells pretreated with a high dose. Resting levels of pAKT are also significantly higher following pretreatment with a high dose than a low dose. In addition, IRAK-1 is required for LPS-induced priming of BMDMs. Previous reports of IRAK-1's contribution in low dose endotoxemia and the existence of a mutual inhibition between IRAK-1 and PI3K suggest that cross-talk between these two kinases may contribute to LPS-induced priming and tolerance.

4.2 Introduction

Low dose endotoxin seems to be the root cause for many chronic human inflammatory diseases. Recent evidence has shown that unhealthy lifestyle habits such as heavy alcohol consumption, smoking, and high fat diets all contribute to low level leakage of endotoxin into circulation [8, 40, 55]. In addition to subclinical doses of endotoxin being linked to chronic diseases, it seems likely that the low circulating levels of LPS may also be the underlying cause of a very poor prognosis in acute diseases like sepsis and severe non-disseminated bacterial infections. More unfavorable outcomes for the patient are likely due to elevated levels of inflammation compared to health individuals experiencing the same acute disease. That is, unhealthy individuals experience a more uncontrolled inflammatory response that ultimately leads to severe tissue damage, organ dysfunction, and potentially death.

Much of the research regarding how prior exposures of endotoxin effect secondary exposures is centered, both *in vivo* and *in vitro*, with relatively high concentrations of endotoxin. Concentrations of LPS >1 ng/mL are only seen in tissue with a localized infection or in the case of sepsis where infection has become disseminated. These levels of endotoxin are not observed in otherwise healthy individuals. Collectively these studies have shown that prior exposure to high levels of LPS actually tolerizes the immune response such that a secondary exposure results in much less inflammation and often an improved prognosis. Very little is known about how clinically relevant circulating levels of LPS (pg/mL range) affect the immune response. A study by Vogel et. al showed that *in vitro* macrophages that are expose to extremely low doses of LPS first actually exhibit increased induction of pro-inflammatory mediators when challenged with a secondary dose high dose of LPS [144]. To our knowledge, this is the first report of LPS-induced priming. Another study links our hypothesis that aging can cause a priming phenotype

when exposed to a high dose of LPS. A study by Penderen et. al. showed that whole blood samples from elderly patients showed a more rapid and robust expression of TNFα, soluble TNF receptor-I (sTNFR-I), and CRP when compared to their younger counterparts[146]. Indeed, this data many be explained by the priming of the immune system because of elevated levels of circulating endotoxin as observed in other aging studies [37].

A lot is known about the signaling mechanisms governing cellular responses to high doses of endotoxin >1ng/mL. Collectively, high levels of LPS initiate a robust cytokine storm that is quickly reined in by many suppressive mechanisms. On the other hand, recent research has revealed that low dosages of LPS do not induce these suppressive mechanisms, hence allowing a mild and chronic inflammatory response to occur. Mechanistically, very little is known about how LPS-induced priming occurs. Clearly, signaling cross-talk must contribute to this phenotype. Our previous findings showing the importance of IRAK-1 in low dose signaling may offer some clues. We know that IRAK-1 is a dynamic molecule with many functions, including cross-talk with other signaling pathways. Perhaps the mutual inhibition of IRAK-1 and PI3K contributes to the priming phenotype [109, 110]. In this study we focused our efforts on understanding the involvement of IRAK-1 and PI3K in LPS-induced priming. Intriguingly, we observed that low doses of LPS actually suppress the PI3K pathway and that IRAK-1 seems to be critical for LPS-induced priming to occur *in vitro*.

4.3 Materials and Methods

4.3.1 *Reagents*

LPS (*Escherichia coli* 0111:B4) and wortmannin was obtained from Sigma Aldrich. Anti-pAKT (#4058) antibody was purchased from Cell Signaling Technology. Anti-AKT1/2 (H-136) was from Santa Cruz Biotechnology.

4.3.2 *Isolation of Bone-Marrow Derived Macrophages (BMDMs) and Cell Culture*

Wild-type C57BL/6 mice were purchased from Charles River Laboratory. IRAK-$1^{-/-}$ C57/BL/6 mice were provided by Dr. James Thomas from the University of Texas Southwest Medical School. WT and IRAK-$1^{-/-}$ mice were housed and bred in the Derring Hall Animal Facility in compliance with approved Animal Care and Use Committee protocols at Virginia Polytechnic Institute and State University. Bone-marrow derived macrophages (BMDMs) were harvested as previously described[107]. Macrophage cells were grown in DMEM (Invitrogen™, Inc., CA) supplemented with 10% FBS #SH30071.03 (Hyclone™), 1% Penicillin/Streptomycin (Invitrogen™, Inc., CA) , 1% L-Glutamine (Invitrogen™, Inc., CA), and 30% L-929 conditioned medium as a source of m-CSF at 37°C with 5% CO_2. On the third day of culture, cells were fed 25mL of fresh medium. On the seventh day, the cells were harvested for experimentation. All cells were starved overnight with DMEM containing 1% FBS before treatment.

4.3.3 *Real time RT-PCR (qRT-PCR)*

Total RNAs were harvested from BMDMs treated with or without LPS using Trizol® Reagent (Invitrogen) according to the manufacturer's protocol. cDNAs were generated using the High Capacity cDNA Reverse Transcription Kit (Applied BioSystems™, Foster, CA). Real-time PCR

analyses were performed using the iQ Sybr® Green Supermix (BioRad™ Laboratories, Hercules, CA) on an IQ5 thermocycler (BioRad). The relative levels of transcripts were calculated using the ΔΔCt method using *GAPDH* as the internal control. The relative levels of mRNA from the untreated samples were adjusted to 1 and served as the basal control value. The following primers were used perform qRT-PCR: mouse *Gapdh* forward: 5'- AAC TTT GGC ATT GTG GAA GGG CTC-3', reverse: 5'- TGG AAG AGT GGG AGT TGC TGT TGA-3'; mouse *IL-6* forward: 5'-ATC CAG TTG CCT TCT TGG GAC TGA-3', reverse: 5'- TAA GCC TCC GAC TTG TGA AGT GGT-3'.

4.3.4 *Western Blot Analysis*

Isolation of whole cell was performed as described previously [143]. Whole cell lysates were obtained using RIPA buffer. Protein samples were analyzed by SDS-PAGE and Western Blot. Images were taken with Fujifilm Multi Gauge software.

4.3.5 *Statistical Analysis*

Data significance was determined using the Student's t-test. P-values less than 0.05 were considered statistically significant as indicated by an asterisk (*).

4.4 Results

4.4.1 Low doses of LPS (50 pg/mL) are capable of causing priming; whereas, high doses of LPS (100 ng/mL) cause tolerance.

Previous reports *in vitro* have shown that when macrophages were pretreated with subclinical doses of LPS, followed by a secondary high dose challenge of LPS priming or enhanced gene expression of pro-inflammatory cytokines is observed [144]. Similar priming effect has been observed to *in vivo*. This reaction was coined the 'General Shwartzman Reaction'. To confirm these observations in murine bone-marrow derived macrophages, we setup priming and tolerance assays *in vitro*. As shown in **Figure 4.1**, pretreating BMDMs with a low dose of LPS (50 pg/mL), followed by a secondary high dose of LPS (100 ng/mL) causes a significant increase in the expression of *IL-6* when compared to cells that received no pretreatment. This phenotype is known as LPS-induced priming. When the initial pretreatment dose was high instead of a low, the expression of *IL-6* was significant suppressed (**Figure 4.1**). This is known as LPS-induced tolerance. Other studies have shown that crosstalk exists between several of the signaling pathways downstream of TLR4. Since IRAK-1 seems to be necessary for the activation of JNK upon low dosages of LPS, we hypothesized that cross-talk involving IRAK-1 somehow mediates or allows for the priming and tolerance phenotypes observed in **Figure 4.1**. Other investigators have shown IRAK-1 to be regulated at two distinct phases, one dependent on PI3K and the other independent of PI3K [110]. To this regard, we examined the effect of priming and tolerance models *in vitro* on the levels of AKT phosphorylation, a direct indicator of PI3K activation.

4.4.2 Low doses of LPS cause the suppression of PI3K activity; whereas, high doses cause its activation.

As shown in **Figure 4.2**, pAKT is suppressed upon stimulation of BMDMs with a low dose of LPS. On the other hand, pAKT is induced upon stimulation with a high dose of LPS. AKT phosphorylation is a direct indicator of PI3K activity. In addition, when BMDMs are pretreated with either a low or a high dose of LPS and then followed by a secondary challenge of a high dose of LPS, two unique patterns emerge. Following the pretreatment dose is 50 pg/mL for 4 hours, the resting levels of AKT phosphorylation are very low and over time AKT phosphorylation returns (**Figure 4.3C**). As shown in **Figure 4.3C**, after a pretreatment dose of 100 ng/mL, the levels of pAKT are significantly higher when compared to the cells that received the low dose pretreatment. Furthermore, the levels of AKT phosphorylation seem to increase much stronger and more rapidly than the cells that received the low dose pretreatment. Taken together, this data suggests that the suppressive effects on PI3K, perhaps by IRAK-1, are much stronger when the cells are exposed to a low dosage of LPS than a high dose. Further supporting the dominate suppressive role of PI3K at high doses of LPS is that when PI3K activity is blocked by wortmannin, an increase in IL-6 expression was observed (**Figure 4.4**).

4.4.3 IRAK-1 is a critical signaling kinase that is required for LPS-induced priming to occur.

To test the involvement of IRAK-1 in the priming phenotype, we used IRAK-1 deficient BMDMs to see if the priming phenotype is still observed in terms of *IL-6* transcription. As shown in **Figure 4.5**, when IRAK-1 is lost, we no longer observed priming. Instead, a LPS-

induced tolerance phenotype is seen. Collectively, IRAK-1 serves as a key kinase that is required for low dose endotoxemia signaling processes.

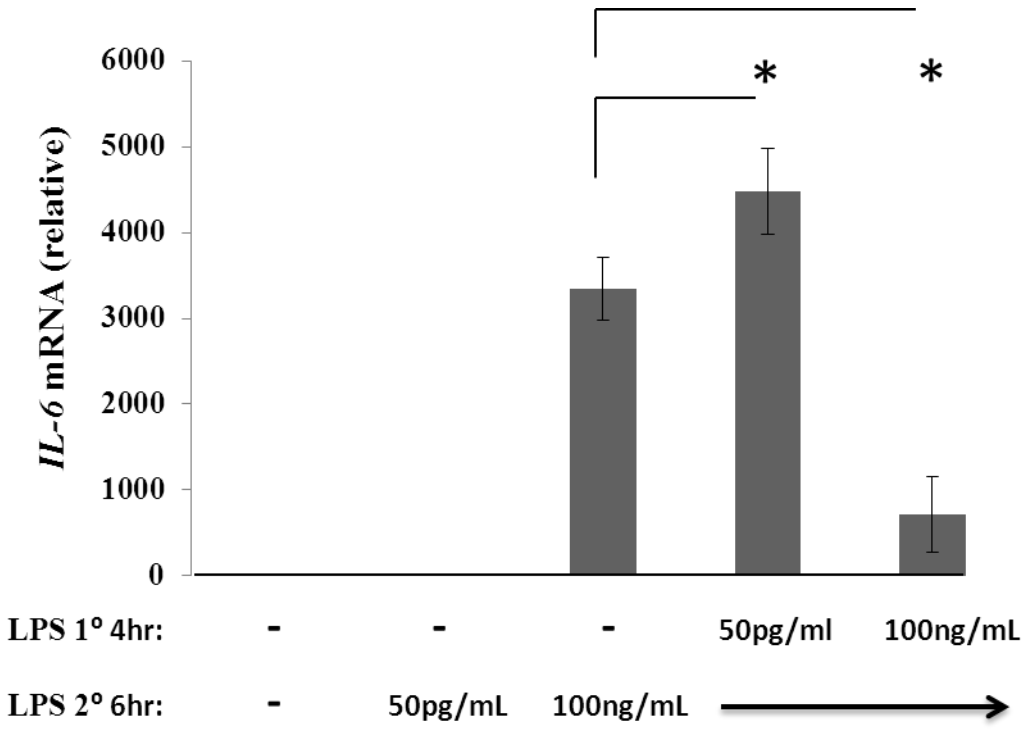

Figure 4.1: Pretreatment of BMDMs with a low dosage of LPS, followed by a secondary high dose causes priming. Pretreatment of BMDMs with a high dosage of LPS , followed by a secondary high dose causes tolerance.

All cells were starved overnight in DMEM supplemented with 1% FBS. Wild-type BMDMs were either treated with or without low (50 pg/mL) or high (100 ng/mL) dosages of LPS for 4 hours (LPS 1°). All cells were then washed twice with PBS (warmed to 37^0C) and additional pre-warmed starving media was added back to the monolayer which contained 100 ng/mL of LPS (LPS 2°). Total RNAs were harvested and subjected to real-time quantitative RT-PCR analysis to determine the relative levels of *IL-6* message compared to the untreated samples. The results are expressed as means +/- standard deviation performed in triplicate. *Statistically significant difference determined by Student's t-test ($P<.05$)

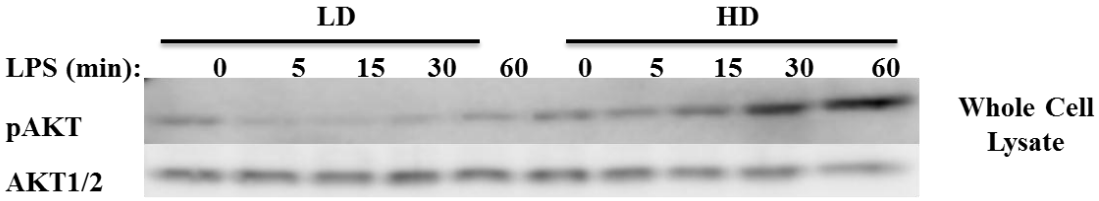

Figure 4.2: Low dosages of LPS causes a suppression of AKT phosphorylation (Serine 473) and high dosages cause a robust phosphorylation of AKT.

WT BMDMs were treated with or without low (50 pg/mL) or high (100 ng/mL) dosages of LPS for 0, 5, 15, 30 or 60 minutes. Whole cell lysate was analyzed by Western blot using an anti-pAKT antibody. Total AKT served as a loading control.

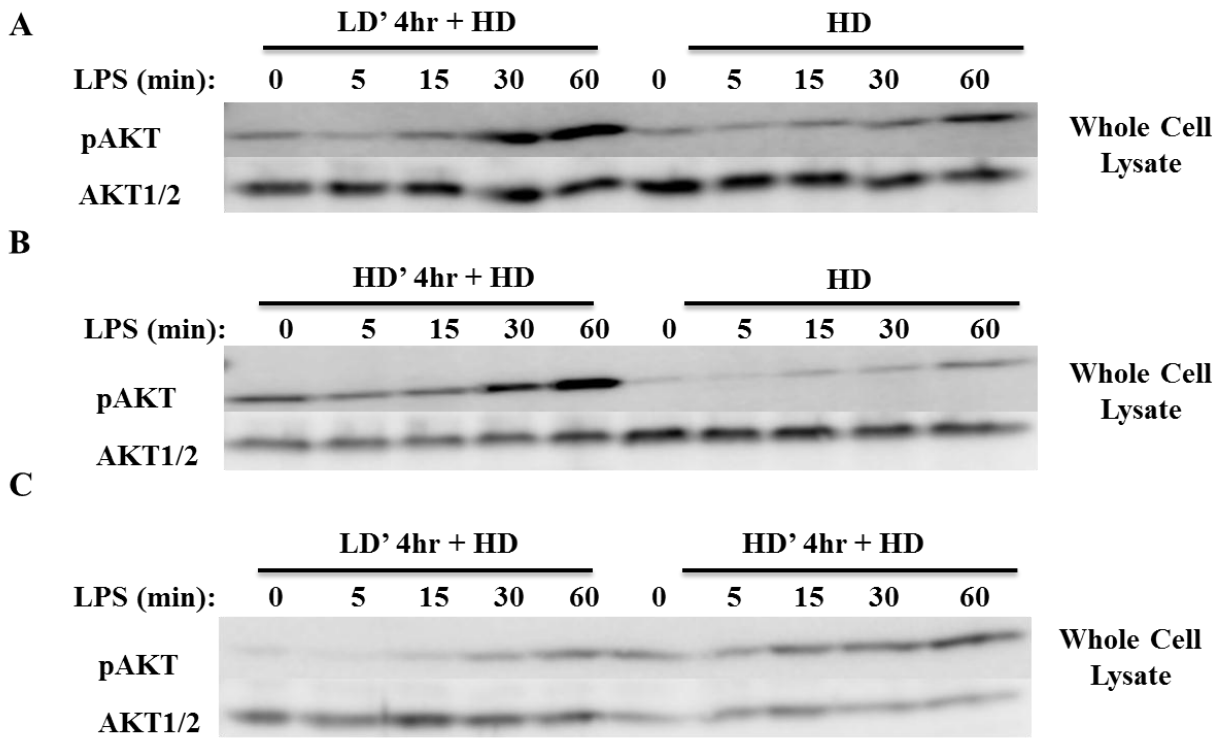

Figure 4.3: Pretreatment with low dosages of LPS, followed by a secondary high dose causes a significant decrease in AKT phosphorylation, when compared to BMDMs pretreated with a high dosages of LPS, followed by a secondary high dose.

Wild type BMDMs were pretreated with or without low (50 pg/mL) or high (100 ng/mL) dosages of LPS for 4 hours. Immediately following pretreatment BMDM monolayers were washed twice with pre-warmed PBS, and then the medium was replaced with pre-warmed DMEM supplemented with 1% FBS containing 100 ng/mL of LPS. Whole cell lysate were as a time course. Total protein lysates were analyzed by Western blot using an anti-pAKT antibody. Total AKT served as a loading control.

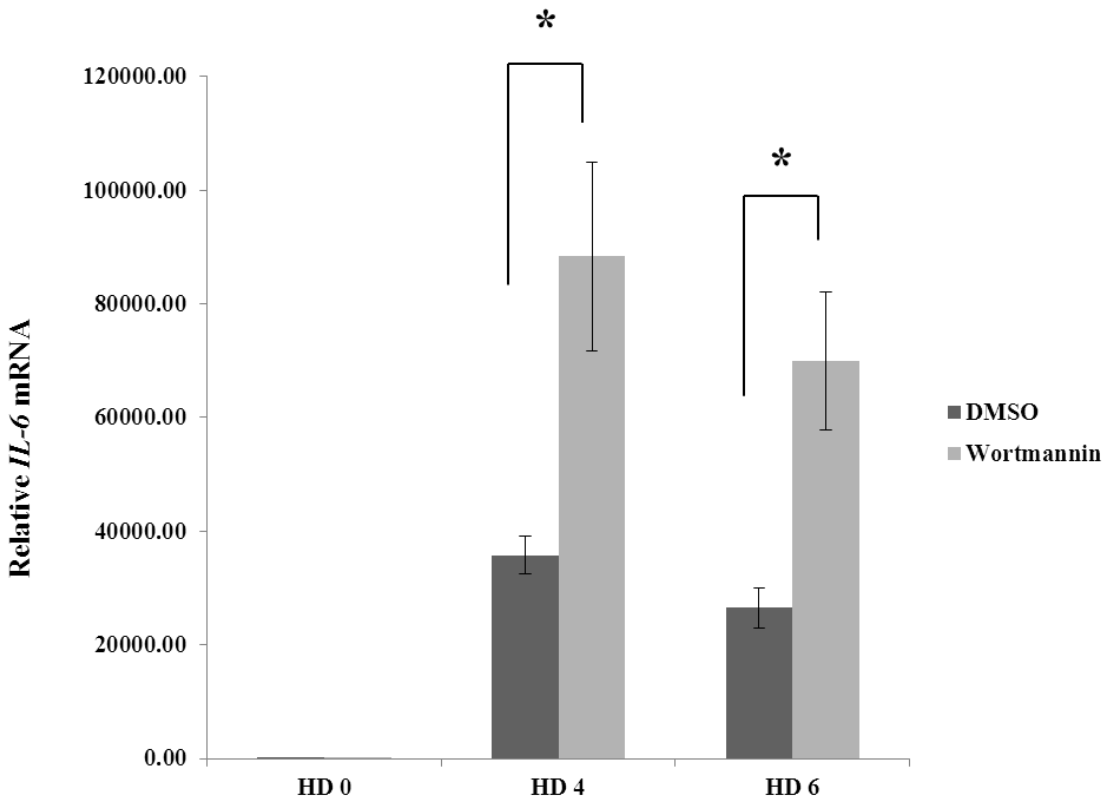

Figure 4.4: Loss of PI3K causes an increased expression of IL-6 in response to high doses of LPS.

Wild type BMDMs were pretreated with or without 0.5uM wortmannin, a known inhibitor of PI3K, for 1 hour. Following pretreatment the cells were treated with 100 ng/mL of LPS for a time course. Total RNA was extracted using Trizol. Transcripts were quantified using qRT-PCR for primers specific for IL-6 and normalized to the untreated samples. The results are expressed as means +/- standard deviation performed in triplicate. *Statistically significant difference determined by Student's t-test ($P<.05$)

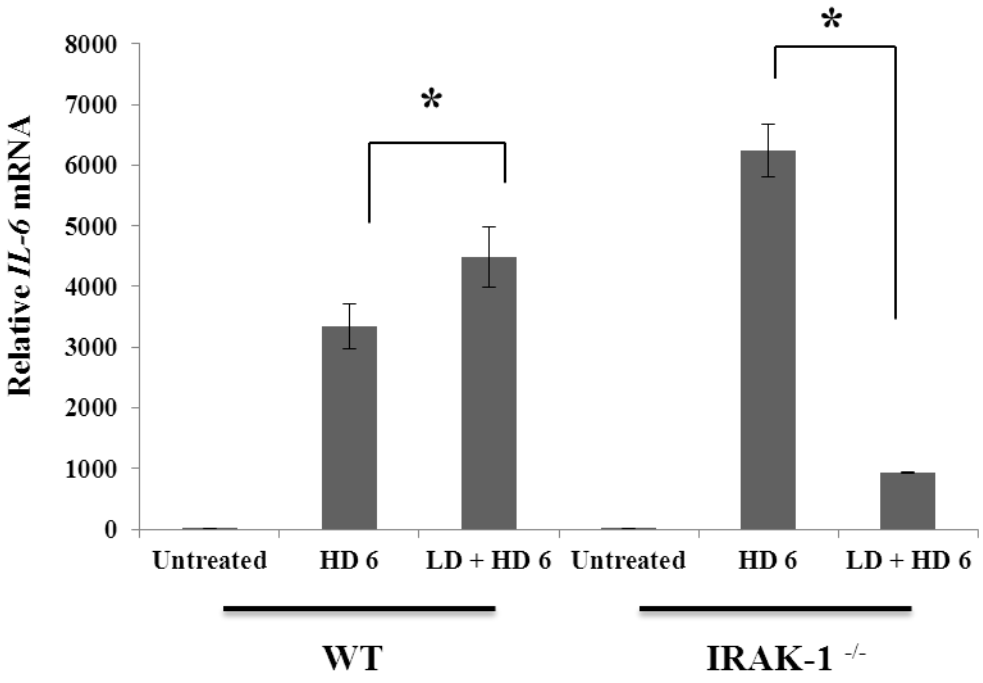

Figure 4.5: IRAK-1 is essential for low dose priming to occur.

Wild type or IRAK-1 deficient BMDMs were pretreated with or without a low (50 pg/mL) dosage of LPS for 4 hours. Following pretreatment all monolayers were washed twice with pre-warmed PBS, and then the medium was replaced with pre-warmed DMEM supplemented with 1% FBS containing 100 ng/mL of LPS. Total RNA was extracted 6 hours later. Transcripts were quantified using qRT-PCR for primers specific for IL-6 and normalized to the untreated samples. The results are expressed as means +/- standard deviation performed in triplicate. *Statistically significant difference determined by Student's t-test ($P<.05$)

4.5 Discussion

Our findings demonstrate that macrophages, which were once thought to be very generic phagocytic innate immune cells, are actually capable of mounting a precise inflammatory response that is unique to the challenge, the dose of the stimulate, as well as its prior history or memory of past challenges. As shown by us, murine BMDMs are capable of sensing subclinical doses of endotoxin. These extremely low doses of endotoxin activate a very narrow signaling pathway that is distinct from signaling that occurs in response to high doses of LPS. It has been widely known that *in vivo* and *in vitro* LPS is capable of inducing tolerance to itself. That is when an individual is challenged with a high enough dose of endotoxin a secondary challenge actually causes a decrease in the expression of inflammatory mediators [144, 147, 148]. This report clearly demonstrates that if the initial dose of endotoxin is low enough ≤ 50 pg/mL *in vitro* then a secondary challenge of LPS causes a significant increase is inflammatory mediators.

To our knowledge, this is the first report that clearly shows low doses of LPS actually cause the suppression of the PI3K signaling pathway. Others have demonstrated that high dose of LPS actually induces this pathway. This data suggests that a suppressive mechanism must be more prevalent/active at low doses and that this same mechanism must not dominate in response to high doses of LPS. This would explain the pAKT data presented in **Figure 4.2**. Further supporting this claim is that when PI3K is blocked using wortmannin, cells have a much higher induction of IL-6 when challenged with high doses of LPS. Taken together, crosstalk must exist that allows for the dose sensitive suppression of pAKT.

Our study has identified IRAK-1 as a critical signaling kinase that is required for LPS-induced priming. When IRAK-1 is lost, under conditions that normally induce priming, tolerance is observed. Previously, our lab has shown that IRAK-1 is a critical signaling kinase

that is required for low dose induced inflammation [107]. In addition, other studies have shown that a mutual inhibition exists between IRAK-1 and PI3K [109, 110]. Taken together, this data leads us to believe crosstalk between IRAK-1 and PI3K regulates the 'memory' of a prior endotoxin challenge.

Although much more research is needed to substantiate these claims, we propose the following mechanism for LPS-induced priming. Based upon our lab's data and others, it is clear that low doses of endotoxin preferentially activate inflammation using a signaling pathway that involves IRAK-1, not NFkB or PI3K. Because of this, IRAK-1 is capable of exerting its suppressive effects on PI3K before PI3K is capable of suppressing IRAK-1. Therefore, when a secondary challenge of LPS occurs, signaling that normally occurs downstream of PI3K is no longer possible. This would then block many of the suppressive effects the PI3K signaling pathway normally has, thereby causing an increase in inflammatory mediators (LPS-induced priming). On the other hand, when the initial dose of LPS is high, both IRAK-1 and PI3K are activated. However, the suppressive effects of PI3K are able to overwhelm the suppressive effects of IRAK-1. This would then effectively block inflammation induced by TLR4/IRAK-1 when a secondary high dose of endotoxin occurs, thus explaining the LPS-induced tolerance phenotype.

Through this current study, it is clear that LPS-induced priming occurs if the initial dose is low enough. Mechanistically, crosstalk between the pro and anti-inflammatory signaling pathways must exist. Our data suggests a novel role for both IRAK-1 and PI3K in LPS-induced priming. Clearly, more research is needed to better demonstrate the mechanism for the priming phenotype. This research has significant clinical implications for many human diseases. The priming effect potentially explains why unhealthy individuals with elevated levels of circulating

endotoxin fare so poorly during sepsis and acute infections compared to their health counterparts. This study offers additional evidence that IRAK-1 is a critical signaling kinase that if inhibited could significantly improve the outcome of septic individuals.

Chapter 5:

CONCLUSIONS AND FUTURE STUDIES

CONCLUSIONS

5.1 Conclusions

Low dose endotoxemia is a growing problem in today's society. The instances of obesity, heart disease, and cancer are on the rise. Understanding exactly what leads to the progression of these illnesses could provide valuable information to combat them. It is widely accepted that low grade chronic inflammation is at the root of most major human diseases. Therefore, understanding the molecular mechanisms underlying the uncontrolled or overexpression of these pro-inflammatory mediators is extremely important. Exactly how different cell types respond to different doses of endotoxin is also equally important. One has to remember that these events *in vivo* involve potentially all cell types and tissues in the body. The primary goal of this work was to determine the molecular mechanisms that govern the expression of persistent pro-inflammatory genes in response to endotoxin. The following is a summary of the progress made during my graduate studies.

5.1.1 Molecular mechanism underlying persistent induction of lipocalin 2 by lipopolysaccharide in kidney fibroblasts.

IRAK-1 is necessary for the persistent induction of the acute phase protein lipocalin 2 (LCN2) in response to 100 ng/mL of LPS in kidney fibroblasts. LCN2 transcription requires the transient binding of AP-1/cJUN and the persistent binding of C/ebpδ. Furthermore, C/ebpδ is capable of inducing its own transcription in response to LPS also in a persistent fashion. This is likely due to the ability of C/ebpδ to sustain its own activation via a positive feedback loop previously reported by Aderem's group [141]. C/ebpδ seems to be the primary player involved in the persistent induction of LCN2 since similar persistent binding patterns were not seen in the transient pro-inflammatory gene TNFα.

5.1.2 Kidney Fibroblasts to Macrophage Rational

Previous reports have shown that IRAK-1 is quickly degraded after a high dose challenge of LPS in macrophages. Interestingly, IRAK-1 degradation was not seen after kidney fibroblasts were challenged with these same high doses. Therefore, we predicted that macrophages are much more sensitive to LPS than fibroblasts and that perhaps if the LPS dose was lowered enough, we might see a similar phenotype of persistence and no IRAK-1 degradation. Due to the critical role of macrophages in the inflammatory process, we transitioned from kidney fibroblasts to bone marrow derived macrophages in Chapters 3 and 4 [149].

5.1.3 Mechanisms for persistent expression of IL-6 in macrophages

We were able to demonstrate that low doses of LPS (50 pg/mL) was sufficient to drive the induction of IL-6 and C/ebpδ, but unlike expression due to high doses of LPS, their expression was persistent in nature. Similar to the expression of LCN2 in fibroblasts, IRAK-1 was also not degraded. Low doses of LPS fail to activate the classical NFkB signaling pathway, but do selectively activate both JNK and C/ebpδ in a persistent manner. We were able to show that ATF2, an AP-1 transcription factor family member, bound transiently to the promoters of both IL-6 and C/ebpδ. Just like our findings using kidney fibroblasts, C/ebpδ was also recruited to both promoters persistently. Low dose TLR4/LPS signaling requires the adaptor protein MyD88. The induction of SOCS1, ATF3, and MKP-1, classic negative regulators of TLR4 signaling was not observed. Doses as low as 25 pg/mL were sufficient to drive the expression of IL-6 and if the cells were pretreated with LPS first, a much lower dose of LPS was required to sustain IL-6 transcription. Finally, IRAK-1 functions at a critical node for low dose endotoxin signaling.

5.1.4 Mechanism for lipopolysaccharide mediated priming and tolerance of inflammation in murine macrophages

It seems as though cells have developed a novel way to 'remember' their prior history of exposure to LPS. By mechanisms not yet known, macrophages are capable of sensing the dose of LPS and preferentially utilizing a narrow signaling circuit. If the initial dose of LPS is low enough and the cells are stimulated with a secondary high dose of LPS, an LPS-induced priming phenotype is observed. On the other hand, if the initial dose is too high then a tolerance phenotype is seen. We also identified IRAK-1 as a critical component to see LPS-induced priming. Furthermore, if PI3K activity is blocked using wortmannin, we see increased expression of IL-6, much like we observed when cells were pretreated with low doses. Taken together, we hypothesize that the priming effect involves previously observed crosstalk between IRAK-1 and PI3K [110].

5.2 Final Remarks

Our work has revealed many novel signaling pathways used during low dose endotoxemia. Although much more work is needed to clearly define the signaling dynamics that lead to the persistent induction of pro-inflammatory cytokines, the foundation has been set. The optimal place to intervene this signaling pathway to prevent chronic inflammation is not quite evident. However, the unique role of IRAK-1 in the persistence of IL-6 as investigated in Chapter 3 and in the priming phenotype summarized in Chapter 4 cannot be overlooked. Due to IRAK-1's redundant role in fighting acute infection, it may serve as a therapeutic target for chronic inflammatory diseases. More extensive research is warranted to identify IRAK-1's role in other human diseases.

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A. Appendix:

Inflammation, tissue damage and organ injury

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TABLE OF CONTENTS

A.1 Abstract

Inflammation is a highly coordinated complex molecular and cellular surveillance process essential for anti-microbial defense and wound healing. Many host cells including leukocytes, fibroblasts, endothelial cells and epithelial cells are involved in the inflammatory process. Cellular receptors, such as Toll-Like-Receptors (TLRs) and cytokine receptors, are responsible for recognizing and processing diverse foreign and host challenges. In addition, they regulate the expression of secondary inflammatory mediators such as cytokines, chemokines, complement proteins, and co-stimulatory molecules. These mediators modulate cellular responses by the activation and recruitment of immune cells mediating host cellular and tissue remodeling. Although inflammation is beneficial for host wound healing and defense toward infection, excessive or altered inflammation often leads to a wide range of tissue injuries and human diseases including cardiovascular diseases, diabetes, and multi-organ failure. This review specifically addresses the contribution of macrophages and fibroblasts to inflammation and tissue injury.

A.2 Introduction

The term inflammation was first coined by a Roman scientific writer Aulus (Aurelius) Cornelius at the turn of the first century. The four cardinal features ascribed to inflammation include swelling, redness, heat, and pain. Triggers for inflammation range from microbial infection, physical shock, chemical and biological irritants, as well as abnormal metabolites. Various germ line-coded innate receptors such as cell surface Toll-Like-Receptors (TLR), NOD-Like-Receptors (RLR), cytokine receptors, scavenger receptors, and G-protein-coupled-receptors (GPCR) can specifically recognize diverse molecular patterns embedded within various danger signals (1-3). Subsequent activation of intracellular signaling pathways eventually leads to the

activation of transcription factors such as NFkB, STATs, Smads, and NFATs which are responsible for the expression of pro- or anti-inflammatory genes.

Among the various cells involved in mediating inflammation, macrophages and fibroblasts are two types of cells universally present in almost all tissues and organs. The differential activation of macrophages and fibroblasts are involved in many facets of inflammation and tissue injury. Classically activated macrophages (M1 macrophages) express pro-inflammatory cytokines, interferon gamma (INFγ) and reactive oxygen/nitrogen species, which are involved in the phagocytosis and killing of microbes (4). During chronic inflammation, M1 macrophages lead to exacerbation of inflammation and tissue damage. Consequently, classically activated macrophages are associated with inflamed tissues during the course of diabetes, atherosclerosis, and multi-organ injury (5). On the other hand, alternatively activated macrophages (M2 macrophages) fail to express pro-inflammatory mediators, and may contribute to resolution of inflammation. Fibroblasts form the connective tissues of various organs and participate in the wound healing process. However, excessive proliferation of fibroblasts and production of extracellular matrix proteins during chronic inflammation can lead to pathological fibrosis.

A.3 Contribution of macrophages and fibroblasts during tissue damage and organ injury

A.3.1 Macrophages in inflammation and tissue injury

Macrophages are key innate immune cells capable of diverse functions including the phagocytosis of foreign cells and particulates, the expression of reactive oxygen species, the production of proteins/enzymes involved in tissue remodeling, and the expression of chemokines

and pro/anti- inflammatory cytokines. As a result, macrophages are involved in modulating the inflammatory process during the pathogenesis and resolution of tissue injury and inflammation. Despite the fact that macrophages exhibit significant plasticity and are capable of expressing mediators with distinct pro- or anti-inflammatory effects, local macrophages subjected to particular challenges often adopt unique phenotypes. The phenotypes are characterized by either preferentially secreting pro-inflammatory mediators (TNF, IL-6, iNOS) or anti-inflammatory mediators (IL-10, arginase1), which differentially modulate inflammation, tissue injury, and repair. Macrophages exposed to LPS and IFNγ are the classically activated macrophages (M1) which express pro-inflammatory cytokines that are involved in many inflammatory diseases including diabetes and insulin resistance, atherosclerosis and stroke, shock, and ischemia/reperfusion injury (6-8). In contrast, macrophages exposed to IL-4 are the alternatively activated macrophages which preferentially express arginase-1 and other anti-inflammatory mediators that counter-act the effect of pro-inflammatory mediators. M2 macrophages are critical for wound repair and resolution of inflammation (9,10). Despite the fact that M2 macrophages exhibit anti-inflammatory effects, excessive differentiation and proliferation of M2 macrophages may be exploited by tumor cells and can contribute to tumor cell proliferation (11-13).

Intra-cellular molecular signaling pathways responsible for the differentiation of M1 or M2 macrophages are not clearly understood. Synergy among TLR signaling and IFNγ mediated signaling may be required to induce the activation of NFkB and STAT1/3. Collectively, NFkB and STAT1/3 may lead to elevated expression of typical M1 genes such as TNFα, IL-6, and iNOS. Regarding M2 macrophages, IL-4 activates at least three downstream effector pathways including PI3K, RAS-MAPK, and STAT6 (14). Thus far, STAT6 has been shown to be

important for the expression of selected M2 marker genes including arginase 1, YM1, and FIZZ1 (15-17). The contribution of the PI3K and RAS pathway has not been fully studied. Intriguingly, the differentiation of M1 and M2 macrophages seems to be mutually exclusive. We recently demonstrated that LPS/IFNγ not only induces M1 differentiation, but also suppresses IL-4 mediated expression of arginase 1 (unpublished data). In contrast, IL-4 has been shown to suppress LPS-induced expression of iNOS and TNFα (18). Conceivably, yet-to-be-defined cross-talks and feedbacks exist among these pathways that contribute to the orderly macrophage differentiation.

The contribution of macrophages to the pathogenesis and resolution of many inflammatory diseases and tissue injuries has become increasingly evident. Several recent studies demonstrated that M1 macrophages are highly elevated in fat tissues from diabetic patients and animals (6, 19). Lumeng et al demonstrated that a novel F4/80+CD11c+ macrophage population exists in fat tissues harvested from obese and diabetic mice (6). In addition, these macrophages expressed elevated levels of TNFα and iNOS. A separate study by Kanda et al reported that the serum levels of chemokines (MCP-1) and cytokines (TNF, IL-6) are elevated in obese mice (20). Furthermore, elevated M1 macrophages were shown to exacerbate inflammation, and cause related complications including diabetic fibrosis, nephropathy, and retinopathy (21). In contrast, adipose tissue macrophages (ATM) found in lean and non-diabetic mice expressed genes characteristic of M2 macrophages including arginase 1 and Ym1. The Chawla group demonstrated that peroxisome proliferator-activated receptor delta (PPARδ) is at least partially required for maturation of alternatively activated macrophages (9). IL-4 can activate PPARδ and induce the expression of the alternative phenotype in Kupffer cells and adipose tissue macrophages of lean mice. Consequently, interventions that skew macrophages

into the M2 phenotype have been shown to be beneficial for the resolution of insulin resistance (9).

During the pathogenesis of atherosclerosis, activated macrophages not only exacerbate local injury by secreting pro-inflammatory cytokines and reactive oxygen species, but also by actively assimilating cholesterol and low density lipoproteins (LDL) leading to the formation of foam cells and plaques on vessel walls (22). In addition, M1 macrophages can also attract other immune cells to aggravate the pathogenesis of atherosclerosis (23). During the late phase of atherosclerosis, M1 macrophages may also contribute to the rupture of plaques by secreting proteases such as metalloproteases (MMPs) (24).

Inflammatory processes mediated by M1 macrophages not only contribute to chronic inflammatory damages and diseases, but also acute injuries. Studies demonstrated that macrophages play important roles in acute septic shock, ischemia and reperfusion injuries of multiple organs and tissues (25, 26). Elevated levels of inflammatory mediators during septic shock and ischemia injury are closely linked with the severe outcome of organ damage and mortality.

Conceivably, therapies aimed at reducing M1 macrophage populations during inflammatory responses may hold potential in treating various inflammatory diseases. Indeed, a recent study showed that the Sphingosine-1-phosphate agonist FTY720 can potentiate M2 differentiations and decrease M1 differentiation (5). FTY720 has shown promise in treating diabetes and related complications (27, 28). Several separate studies indicate that depletion of M1 macrophages attenuated ischemia/reperfusion mediated lung or kidney inflammation and damage (29, 30).

Despite recent progress, the complexity of macrophage differentiation and subsequent physiological/pathological implication are far from fully defined. A recent study suggests that intermediate macrophage phenotype may exist and correlate with the severity of diabetes and insulin resistance in humans (19). Macrophages collected from human adipose tissues express M2 genes (Arginase 1 and TNFα)□. Mosser et al reported that LPS and IgG can induce a unique macrophage phenotype expressing TNF and IL-10 (31). However, the physiological implication for this phenotype is not yet defined. Future studies are clearly warranted to explore the combinatory effects of various agents on macrophage differentiation and function.

A.3.2. Fibroblasts in inflammation and tissue injury

Fibroblasts maintain the extracellular matrix by undergoing proliferation and secreting proteins involved in the generation and remodeling of extra-cellular matrix. Proper remodeling of the extracellular matrix is required for wound healing. On the other hand, excessive proliferation or secretion of extracellular matrix proteins often leads to pathologic progression of fibrosis and tissue injury.

Pathological signals that can trigger altered expression profile of extracellular matrix molecules include paracrine signals from activated immune cells such as lymphocytes and macrophages, autocrine factors secreted by fibroblasts, and pathogen-associated molecular patterns (PAMP) produced by pathogenic organisms that interact with Toll-Like- receptors on fibroblasts. Cytokines (IL-13, IL-4, and TGFβ), chemokines, angiogenic factors (VEGF), growth factors (PDGF), and acute phase proteins (SAP) have also been identified as important inducing signals of fibrosis. Molecular pathways responsible for the fibroblast response to these signals include G-protein coupled pathway (in response to angiotensin), Smad pathway (in response to TGFβ), TLR pathway (in response to PAMP), and JAK-STAT pathway (in response to

cytokines, leptin, and others) (1, 32-34). Molecular signaling processes regarding these pathways have been extensively reviewed elsewhere, and will not be further discussed here. However, it is worth note that less focus has been paid regarding the cross-talk and integration of these pathways, which likely play a critical role in the balancing act of injury and repair.

Recent evidence increasingly indicates that cross-talks are prevalent among pathways affecting tissue injury, repair, and fibrosis. For example, TGF was shown to synergize with angiotensin to exacerbate fibrosis and tissue injury in the lung and heart (35, 36). In contrast, PPAR agonists can inhibit angiotensin-induced cardiac fibrosis (37). In addition, IL-4 can inhibit cytokine-induced expression of MMPs in fibroblasts, preventing remodeling and exacerbating fibrosis (38). The detailed molecular mechanisms responsible for the effects of cross-talks are not clearly understood. Several scenarios exist. For example, TGFβ has been shown to induce the expression of angiotensin receptor (35). Likewise, angiotensin can also induce the expression of TGFβ (39). Alternatively, the transcription factor Smad activated by TGFβ may synergize with NFkB, AP-1 or others activated by angiotensin to induce the transcription of genes involved in fibrosis (35). On the other hand, PPARα has been shown to bind with Smad, STAT, and others to alleviate the expression of collagens involved in the pathogenesis of fibrosis (40, 41).

Since many of the above-mentioned fibrotic signals are also involved in regulating inflammatory and metabolic processes, it comes with no surprise that fibrotic tissue injury is associated with diverse inflammatory and metabolic diseases including diabetes and cardiovascular diseases. For instance, kidney fibrosis is one of the most common complications of late stage diabetes (42, 43).

Undoubtedly, the fine cross-talks among various inflammatory and metabolic signaling pathways dictate the fates of macrophages as well as fibroblasts, which consequently play crucial balancing acts modulating homeostasis or tissue injury. Dissecting out these molecular cross-talks is essential for subsequent successful intervention of numerous inflammatory diseases and related tissue injuries.

A.4 Summary and Perspective

Over the past few decades, an enormous amount of information has been collected to reveal the significant roles of macrophages and fibroblasts in the process of tissue injury and inflammation. A plethora of genes are expressed and tightly regulated in macrophages and fibroblasts depending on their environments and challenges, which are involved in either tissue repair or injury. Molecular and cellular networks responsible for the complex gene expression patterns are intertwined and exhibit feed-back as well as feed-forward cross-talks. Unless we have a clear picture of these complex cross-talks, it is still a daunting task to identify viable therapeutic targets to treati tissue injuries associated with various inflammatory diseases. A combination of experimental approaches with computational simulation is needed to dissect the complex signaling networks.

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A.7 Abbreviations

FIZZ, Found In Inflammatory Zone; IgG, Immunoglobin G; IL-4, Interleukin 4; IL-6, Interleukin 6; IL-10, Interleukin 10; iNOS, inducible NO synthease; JAK, Janus activated kinase; LPS, Lipopolysaccaride; MAPK, Mitogen-Activated Protein Kinase; MCP-1, Monocyte chemoattractant protein; NFAT, Nuclear Factor of Activated T-cells; NFkB, Nuclear Factor kappa B; PDGF, Platelet-derived growth factor; PI3K, Phosphoinositide 3 Kinase; SAP, Serum Amyloid P-component Precursor; STATs, Signal Transducer and Activators of Transcription; TNF, Tumor Necrosis Factor; VEGF, Vascular endothelial growth factor.