

# **Role of IRAK-1 in the Dynamic Regulation of Reactive Oxygen Species**

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## **Abstract**

Generation of reactive oxygen species (ROS) by mammalian host cells is a double-edged sword. ROS are clearly beneficial in directly killing pathogens and as a signaling molecule to alert macrophages and neutrophils to the site of infection. However, ROS are also capable of damaging host cells by destroying DNA, oxidizing proteins and lipids, inactivating enzymes, and eliciting apoptosis. Therefore the balance of ROS generation and clearance is essential for homeostasis. Although multiple mechanisms can contribute to the generation of ROS, NADPH oxidase (Nox) is a primary producer. In terms of clearance, several ROS scavenging enzymes are induced by Nrf2, a sensor of excessive ROS. The mechanisms behind the skewing of this balance toward prolonged accumulation of ROS under chronic inflammatory conditions are not well understood.

Lipopolysaccharide (LPS), a major component of the Gram-negative bacteria cell wall, is specifically recognized by Toll-like receptor 4 (TLR4). LPS triggers robust activation of Nox and ROS production through TLR4, while also activating Nrf2 and ROS clearance. Intracellular pathways regulating ROS generation and clearance mediated by TLR4 are not well defined. Since interleukin-1 receptor associated kinase 1 (IRAK-1) is a key downstream component of TLR4, we test the hypothesis that IRAK-1 may play a critical role in maintaining the balance of LPS triggered ROS generation and clearance.

Using wild type and IRAK-1 deficient murine embryonic fibroblasts, we tested the dynamic induction of Nox1 (a key NADPH oxidase) and Nrf2 by varying dosages of LPS. Our data confirm that high dose LPS (as seen in acute bacterial infection) induced both Nox1 and Nrf2. The generation of Nox1 is IRAK-1 dependent. Low dose LPS (as seen in chronic metabolic endotoxemia) fails to induce Nrf2 and induces mild and prolonged expression of Nox1. Cells pre-challenged with low dose LPS are primed for more robust expression of ROS following a second LPS challenge. The conclusions and implications generated by this study are that chronic low dose endotoxemia (prevalent in adverse health conditions) may skew the balance of ROS generation and clearance to favor prolonged ROS accumulation, and that IRAK-1 represents a potential therapeutic target to treat chronic inflammatory diseases.

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## Chapter 1: Introduction

The list of diseases being attributed to inflammation is growing at an astounding rate. Inflammation is now considered to be responsible for many of the detrimental effects of diabetes and obesity [1-3], cancer [4-7], atherosclerosis and stroke [1, 4, 8], and neurological disease [9, 10]. In order to respond to this growing trend more and more research is focused on harnessing immune responses to alleviate disease complications. One of the ways that the body defends itself against invading pathogens is by the production of reactive oxygen species. Reactive oxygen species (ROS) consist of a group of oxygen derived molecules that either are or can easily be converted to oxygen radicals or can act as oxidizing agents [11]. They are known to be involved in ischemic injury, destruction of DNA, lipid peroxidation [12], oxidation of proteins, apoptosis, and enzyme inactivation [12-16]. Paradoxically all of these effects are detrimental to the host but reactive oxygen species are known to play essential roles for the host defense system [12, 17, 18].

There are two different types of inflammation; acute and chronic. Acute inflammation refers to the immediate inflammatory response which involves cells that are already present within the tissue. Acute inflammation is characterized by swelling, redness, warmth, discomfort, and loss of function [19]. Blood flow is increased causing swelling. Cells responding to the inflammatory signals move into the site of injury or infection causing it to appear red. Localized increases in metabolism warm the area making it unsuitable for microbial growth. The combination of these three (swelling, redness, and heat) cause discomfort in the area of injury. If these acute inflammatory symptoms persist tissue damage can occur, which if not alleviated, can

evolve into chronic inflammation. Chronic inflammation is a sustained state of inflammation that is known to be responsible for atherosclerosis and obesity. The symptoms of chronic inflammation can last for weeks, months, and even years. Chronic inflammation can ultimately lead to scarring and tissue damage which can be permanent and can cause long term health problems.

The innate immune system is considered to be the first line of defense against foreign pathogens [20]. In order to protect the body, the innate immune system recruits a variety of cell types responsible for engulfing foreign microbes including macrophages and neutrophils [21]. These "first-responder" cells play important roles in initiating inflammation and the progression of inflammatory diseases. However, all cells in the body (myocytes, epithelial cells, endothelial cells, etc) are capable of signaling to alert immune cells that there is a problem. Cells found in injured areas will often release ROS, creating a gradient for immune cells to follow to find the area of injury. Furthermore, these cells may be capable of mounting a small scale immune response to protect themselves. One of the ways they may be able to do so is through the production of reactive oxygen species.

As part of the innate immune system, the production of reactive oxygen species is considered to play a role in the body's first line of defense against foreign pathogens [22-24]. However, the production of reactive oxygen species does not occur solely for immune reasons. Reactive oxygen species are produced by all cells in the body at all times as part of normal metabolic processes [25]. Reactive oxygen species are produced by; mitochondria, peroxisomes, lipoxygenase, and NADPH oxidases via a variety of pathways [16, 26]. The NADPH oxidase

family is made up of proteins whose primary function within cells is to produce superoxide [11, 27, 28]. Once produced, superoxide is not stable in the cell for long, and it quickly gets converted into reactive oxygen species [24]. Since ROS are produced by immune and non-immune cells alike the production of reactive oxygen species allows non-immune cells to signal traditional immune cells, while also offering some form of personal protection before the arrival of macrophages and neutrophils. In acute inflammation, the effects of reactive oxygen species can be seen immediately. ROS production is up-regulated in order to allow engulfing macrophages to kill invading pathogens [23]. Increased ROS can also be released from cells to combat invading pathogens. In chronic inflammation ROS produced to combat an infection also causes tissue damage to the host. This damage, if allowed to persist can turn into scarring and tissue destruction, a major problem in atherosclerosis, cirrhosis, neurological disease, and fibrosis.

Perhaps one of the most used models of inflammation is stimulation by lipopolysaccharide (LPS). LPS is found in the cell walls of Gram negative bacteria [29]. LPS varies between different types of bacteria, but all contain the hydrophobic domain lipid A, also referred to as endotoxin [29]. It activates inflammatory pathways, thereby inducing inflammation and complications that arise as a result of inflammation. LPS is known to activate the toll like receptor 4 (TLR4) pathway, leading to the induction of several pro-inflammatory cytokines [30]. Toll like receptors are essential to the innate immune response and are fundamental to host protection. They have been shown to be involved in the expression of chemokines, cytokines, complement factors, co-stimulatory molecules, as well as cellular proliferation and activation [4]. TLR4 is a central receptor in the innate immune system and

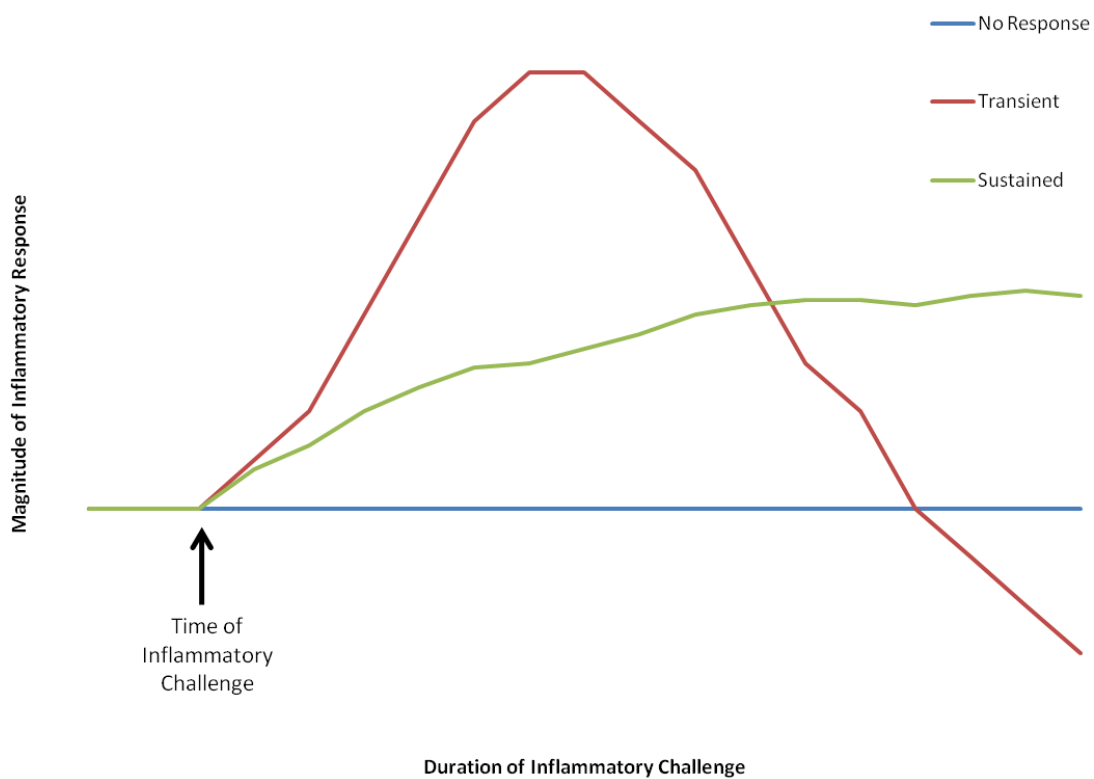
plays key roles in the inflammatory response by leading to the expression of IL-1, IL-6, and IL-8 as well as the upregulation of co-stimulatory molecules [31]. TLR4 activates two different signaling pathways, the MyD88 dependent and independent pathways [30, 32, 33]. These two pathways are known to involve different components and are able to be independently activated at the cellular receptor by LPS. The MyD88 dependent pathway involves the activation of interleukin-1 receptor associated kinase 1 (IRAK-1) which is known to regulate the innate immune response [34]. IRAK-1 has already been linked to atherosclerosis and other forms of inflammatory disease [35, 36]. The TLR4 signaling pathway has been studied extensively and is known to recruit molecules leading to a pro-inflammatory response. This pathway also interacts with other, parallel signaling pathways causing an exacerbated response. We hypothesize that the second TLR4 activated pathway, the MyD88 independent pathway is sensitive to different levels of LPS. In general, this pathways is not very well understood.

There is evidence showing that the immune system responds to an inflammatory stimulus in a dose dependent manner. The greater the dose, and the longer an inflammatory situation is allowed to continue, the more severe the inflammatory disease [21]. This can be seen with atherosclerosis, obesity, and sepsis cases that develop into septic shock. Therefore, anti-inflammatory feedback loops are important for reducing the potential for persistent chronic inflammation. Furthermore, the severity of the initial challenge can determine if these feedback loops are activated immediately, after some time, or may not be activated at all. A severe dose of endotoxin may result in the activation of an anti-inflammatory feedback loop whereas lower levels of endotoxin may not stimulate a response sufficient for crossing the threshold response necessary to activate feedback.

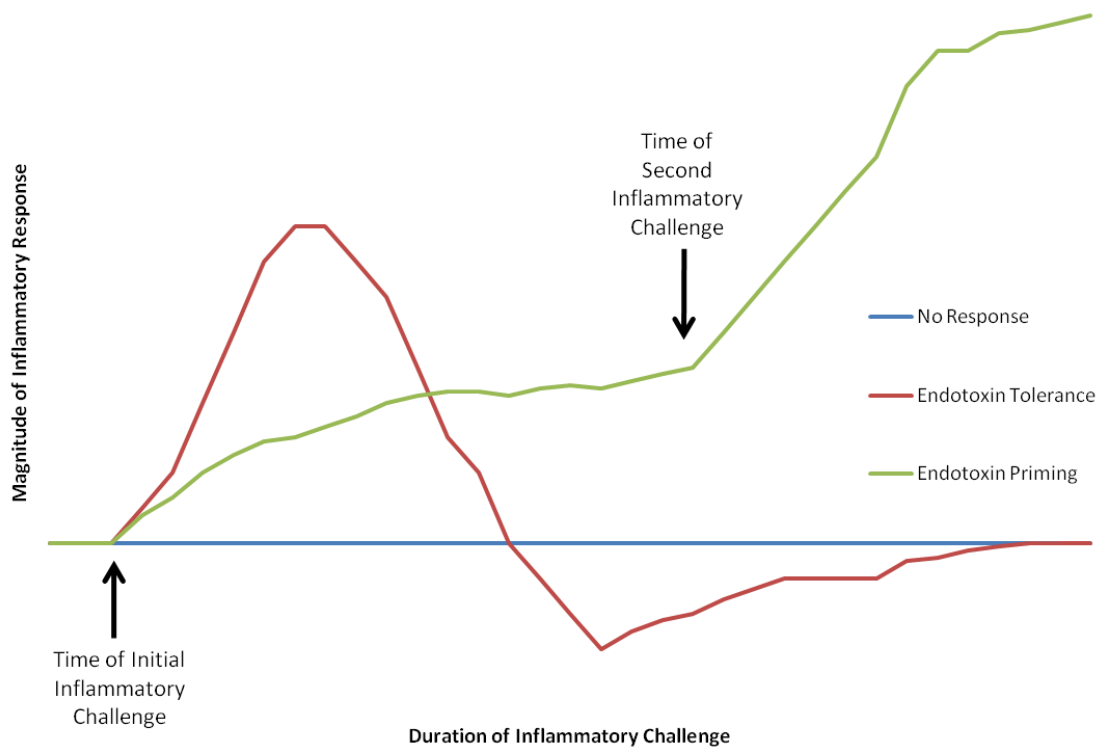
The TLR4 activation consequences involve the immediate acute response to a stimulus and more prolonged chronic inflammatory responses. Under some conditions a short but intense transient response that involves the activation of the complement system, cytokines, and ROS production is quickly extinguished once the pathogen has been eradicated (Figure 1.1). We believe this type of response requires a certain amount of time for the system to reset itself to be prepared for future attack, thus the system has become temporarily tolerant or unresponsive to a stimulus. In another case a slower more sustained activation of the system results in a mounting response that increases over time (Figure 1.1). If another challenge follows the initial challenge there are also multiple ways the system can respond. It has been suggested that extremely high doses of endotoxin cause a system to react intensely and transiently and then require a longer recovery time before the system is able to combat a future stimulus (endotoxin tolerance, Figure 1.2) [37]. However, low levels of endotoxin may 'prime' the system resulting in a heightened response when compared to the original response (endotoxin priming, Figure 1.2). Thus, it is important to focus not only on the immediate response of the TLR4 system to a challenge, but also to the future repercussions of a challenge.

The following studies were conducted to elucidate the role of IRAK-1 in the activation and induction of NADPH oxidase, while evaluating both acute and chronic models of inflammation. The overall goals of this work are to characterize the differentiating mechanisms between endotoxin priming and endotoxin tolerance by looking at the production of reactive oxygen species as a final read-out. We hypothesize that low dose endotoxin is capable of priming a system for future ROS production whereas high dose endotoxin causes the

development of tolerance. We propose that an important underlying mechanism for the differences between these two systems is differential regulation of NADPH oxidase (Nox) and antioxidant pathways that may be upregulated by the activation of Nox and increased ROS production.



**Figure 1.1.** *Comparison of transient inflammatory response to sustained inflammatory response.* Systems treated with inflammatory stimuli exhibit one of three responses: no response, a transient response, or a sustained response. When a system responds transiently, the result is a quick response of large magnitude that reaches a peak then quickly disappears. A sustained response occurs when a system responds to an inflammatory stimuli by slowly mounting a response that instead of disappearing is maintained for a long period of time.



**Figure 1.2.** Representation of endotoxin tolerance and endotoxin priming. A transient inflammatory response results in a system that needs time to recover before it is able to mount a response to a later inflammatory challenge. Systems producing a sustained inflammatory response may be able to mount an additional inflammatory response to a later challenge. This produces an inflammatory response that is significantly increased over the initial inflammatory response produced in both transient and sustained response systems.



## Chapter 2: Literature Review

### 2.1 Innate Immunity

The innate immune system is responsible for the initial recognition of foreign or abnormal conditions. This system provides for natural immunity, as it is already present and fully functional at birth. It requires no previous exposure to a pathogen in order to recognize threats to homeostasis. The innate immune system has three primary functions: recognize, attack, and activate [20]. The system must be able to recognize the presence of a pathogen or abnormal condition. Upon recognition, the innate immune response attacks the pathogen, allowing it to be contained or sometimes eliminated. Finally, the innate immune system is responsible for alerting and activating the adaptive immune system to the pathogen. The signals sent by the innate immune system stimulate the adaptive immune system to produce antibodies and enable an enhanced response to future pathogen encounters. Inhibition or elimination of various pathways of the innate immune system increases susceptibility to infection even when the adaptive immune system is functioning properly [38, 39].

The activation of the innate immune system is due to microbial substances that stimulate cells, alerting them to the presence of foreign pathogens [40]. These substances are referred to as pathogen-associated molecular patterns (PAMPs). The PAMP expressed is specific to classes of pathogens: viruses, gram-negative bacteria, gram-positive bacteria, etc [41]. The PAMP expressed by the pathogen is recognized by pattern recognition receptors (PRRs) [40] and PAMP-PRR combinations determine which signaling pathway the pathogen activates. These pattern recognition receptors are found on cell surfaces and in intracellular compartments, or they can be found extracellularly once secreted into tissue fluids and the bloodstream [42].

There are many different receptors linked to intracellular signal transduction pathways responsible for activating a variety of cellular responses, including the inflammatory response.

### **2.1.1 Toll-like receptors**

The toll-like class is a major class of receptors that has been conserved throughout evolution. Toll protein was first recognized in *Drosophila melanogaster* in 1985 [43], and in 1996 was identified as being responsible for mounting an effective immune response [44]. Soon afterwards, a mammalian homolog, toll-like receptor 4 (TLR4), was identified. Toll-like receptor 4 is known to be constitutively active and can induce the expression of pro-inflammatory genes encoding for interleukin-1, 6, and 8 as well as the upregulation of co-stimulatory molecules [31, 45]. Thirteen toll-like receptors have been identified in mammals; ten TLRs have been found in humans while all 13 have been found in mice [46].

The TLR family consists of type I transmembrane proteins that belong to the IL-1 receptor (IL-1R) family. The N-terminal region contains a leucine rich repeat domain for ligand binding and a C-terminal intracellular signaling domain [41]. The TLRs have one transmembrane domain with the N-terminal region extending externally (Figure 2.1). The C-terminal region of TLRs is homologous to the IL-1R intracellular domain and is therefore referred to as the Toll/IL-1R domain [41]. Toll-like receptors are expressed in many different cell types including hematopoietically derived cells like macrophages, neutrophils, and dendritic cells as well as nonhematopoietic epithelial and endothelial cells [41]. TLRs are ubiquitously expressed, but each cell type expresses different types and combinations of TLRs. Furthermore, the activity of TLRs is dependent upon the formation of either a homo or heterodimer formed between two TLRs [46], and these combinations are responsive to different ligands (Table 2.1)

[47]. Toll-like receptors are able to sense an extremely diverse range of structures and pathogens. The variety of expressed receptor agonists enables the relatively small family of TLRs to monitor the pathogenic universe [48].

Toll-like receptors are known to activate signal transduction through two different adaptor systems (Figure 2.1). The first is the myeloid differentiation factor 88 (MyD88, the MyD88 dependent pathway), and the second is the TIR domain-containing adaptor-inducing interferon- $\beta$  (TRIF, MyD88 independent pathway) [32, 33]. These two adaptors lead to the induction of distinct effectors of the innate immune system. There are several members of the TLR family that are known to signal through the MyD88 dependent pathway, including: TLR2, 4, 5, 7, 8, 9, and 11 [47, 49-53]. The C-terminus of MyD88 has a TIR domain capable of interacting with the TLR (or IL-1R) TIR domain [54, 55]. The N-terminus of MyD88 features a death domain that can bind with other death domains [56-58]. A protein capable of binding to the MyD88 death domain relevant to this work is interleukin-1 receptor associated kinase (IRAK) 4 [49, 59-61]. IRAK-4 then recruits and phosphorylates (thereby activating) IRAK-1 [59]. IRAK-1 phosphorylates itself, recruiting tumor necrosis factor (TNF) receptor-associated factor-6 (TRAF6) to the complex. The IRAK-1/TRAF6 complex then disassociates from the TLR/MyD88/IRAK4 complex [62]. This leads to the early phase activation of NF- $\kappa$ B which results in the production of pro-inflammatory cytokines [63].

The MyD88 independent response involves the activation of late phase NF- $\kappa$ B which can also cause the production of inflammatory cytokines. However the MyD88 independent pathway also results in the activation of interferon regulatory factor 3, and the induction of interferon- $\beta$  and interferon inducible genes [64, 65]. The most well known agonist of TLR4 is

lipopolysaccharide, however it is known to be activated by other agonists (Table 2.1), including lipoteichoic acid and, heat shock proteins especially hsp60 [66-69].

## **2.2 Interleukin-1 Receptor Associated Kinases: Mediators of Toll Like Receptor Signaling**

The interleukin-1 receptor-associated kinase (IRAK) family is made up of four intracellular kinases recruited by the TLR complex. Each IRAK protein is differentially regulated and plays distinct roles in mediating downstream signaling processes [7, 70-72]. IRAK-4 has been shown to be critical for the classical activation of the NF- $\kappa$ B pathway (MyD88 dependent pathway) [61, 73-80]. IRAK-2 also appears to be involved in the classical NF- $\kappa$ B pathway by facilitating the ubiquitination of TRAF6 [72]. IRAK-1 is selectively involved in enhancing transcriptional activities of IRF5/7, and p65/RelA by facilitating their phosphorylation [70, 81-84]. The final member of the IRAK family, IRAK-M is known to be involved in deactivation of TLR-mediated NF- $\kappa$ B activation, thereby preventing excessive expression of inflammatory mediators [78, 85]. In addition to gene transcription, the IRAK family has been implicated in the regulation of non-transcriptional cellular events such as phosphorylation of p47phox which is necessary for activation of NADPH oxidase and increased cellular ROS production in neutrophils [86].

The IRAK family has recently been implicated in non-TLR mediated signaling processes. Some IRAK members are capable of associating with proteins involved in B and T cell receptor-mediated signaling pathways [80, 87, 88]. This suggests an important role for the IRAK family not only in innate immunity but also in adaptive immune signaling [46, 87, 88]. Furthermore, IRAK proteins are believed to be involved in the regulation of multiple signaling pathways in

different cell types including; hepatocytes [89, 90], neuronal cells [91], endothelial cells [92], and epithelial cells [93, 94]. Thus the IRAK family of proteins is involved in signaling networks responsible for mediating a diverse range of physiological processes. Genetic variations in the genes encoding for various IRAK members are linked with a diverse array of diseases -- sepsis, atherosclerosis, infection, autoimmune diseases, and cancer [5, 75, 76, 95-97].

### **2.2.1 IRAK-1 Structure and Function**

IRAK-1 was initially discovered by Cao et al. as a kinase that co-immunoprecipitated with the intracellular domain of the interleukin-1 (IL-1) receptor [98]. Due to IL-1's significance as a critical inflammatory cytokine, IRAK-1 has since drawn great attention in the inflammatory field. Its potential significance was further elevated when they were found to be involved in the TLR signaling pathway [75, 97]. It has since been proposed that other pathways such as the GPCR mediated pathway [99], CD26 signaling [87, 100], and the insulin signaling pathway [100] may all share IRAK-1 as a critical signaling component.

After sequencing and cDNA library screening, a full-length cDNA clone encoding for a protein 712 amino acids long with a predicted molecular size of ~76 kDa was identified [75]. The human IRAK-1 gene was mapped to chromosome 28 (murine to Xq29.52-q29.7) [101]. IRAK-1 is expressed ubiquitously in different human tissues and cell types [75]. The IRAK-1 protein contains an N-terminal death domain, a central serine/threonine kinase domain, and a C-terminal serine/threonine rich region [75]. It contains both nuclear localization and exit sequences [91]. IRAK-1 can be modified by phosphorylation, ubiquitination, and sumoylation [75, 76, 91, 102, 103]. Depending on the modification that occurs, IRAK-1 is capable of performing distinct functions including activation of IRF5/7 [70, 82, 84], NF- $\kappa$ B [81, 104, 105]

and Stat1/3 [76, 106]. IRAK-1 deficiency has been demonstrated to significantly decrease activation of IRF5 and IRF7 [84], and has been linked with a decrease in interferon alpha 4 expression after stimulation with TLR7 and TLR9 ligands (ssRNA and viral CpG DNA respectively) [70].

In addition to being a major mediator of TLR signaling, IRAK-1 is also believed to participate in regulation of the adaptive immune response [87]. The T cell co-stimulatory molecule CD26 is found on the surface of most cell types. It can trigger the association between IRAK-1 and caveolin on antigen presenting monocytes which leads to the expression of co-stimulatory molecule CD86 [87]. The function of IRAK-1 is not limited solely to the immune system. IRAK-1 is expressed in many cell types and tissues including neuronal cells, hepatocytes, endothelial cells, and epithelial cells [30, 90, 107, 108], and probably plays crucial roles in phosphorylation and regulation of many different physiological processes.

### **2.2.2 Regulation of IRAK-1**

IRAK-1 can be regulated at multiple levels including post transcriptional and covalent modifications. Post-transcriptional modification of IRAK-1 involves differential splicing which leads to three distinct forms of IRAK-1; IRAK-1a, IRAK-1b, and IRAK-1c. Both the full length IRAK-1 and IRAK-1c are expressed abundantly in leukocytes and most tissues [91, 109]. However, IRAK-1b represents less than 1% of IRAK-1, existing in minute amounts in most human cells and tissues [91], and its function is not known. IRAK-1c has been suggested to be a negative regulator of inflammation due to its ability to block IL-1 $\beta$  induced activation of MAP kinase [109]. In young human brain tissue full length IRAK-1 is absent, and IRAK-1c is the predominant form [91, 109]. However, in aged humans IRAK-1 is equally represented by

IRAK-1c and full-length IRAK-1 in brain tissue [91]. These age-specific differences imply significant links between chronic inflammation and aging. One of the reasons that IRAK-1c may function as a negative inflammatory regulator is due to the fact that it, like IRAK-1b does not undergo covalent modification following stimulation as IRAK-1 does, without activation by phosphorylation it is unable to upregulate inflammatory pathways. It is thought that IRAK-1 can be phosphorylated by IRAK-4 [110]. Upon this initial phosphorylation IRAK-1 becomes activated and is capable of phosphorylating itself. This subsequent phosphorylation can lead to ubiquitination and degradation by proteasomes [102]. This degradation may serve as a negative feedback loop to help control excessive inflammatory signaling. IRAK-1 degradation has been shown to be correlated with reduced response to endotoxin challenge, and has been correlated with endotoxin tolerance in septic leukocytes [37, 62, 111-114]. Cells challenged with LPS or PAM<sub>3</sub>CSK<sub>4</sub> have exhibited IRAK-1 sumoylation [76, 91]. Once sumoylated, IRAK-1 can translocate into the nucleus and activate the expression of Stat3 genes [76].

### **2.2.3 Evidence for the involvement of IRAK-1 in disease**

Given the presence of IRAK-1 in multiple signaling networks, it is not surprising that variation in the IRAK-1 gene may lead to many different inflammatory diseases. Deletion of the murine IRAK-1 gene decreases the risk for experimental autoimmune encephalomyelitis (EAE) [97]. Our lab has previously identified that leukocytes from human atherosclerosis patients contain constitutively activated/sumoylated IRAK-1 which localizes to the nucleus [76]. Our lab also completed a human-population based study revealing that genetic variation in the IRAK-1 gene is correlated with severity of atherosclerosis and serum levels of C-reactive protein (CRP) [1]. A rare variant haplotype of IRAK-1 harboring three single nucleotide polymorphisms is

found in the human population. Humans harboring this form of IRAK-1 tend to have higher CRP levels and are at higher risk for hypertension and diabetes [1]. Furthermore, sepsis patients with this rare variant of IRAK-1 have an increased incidence of shock, require prolonged mechanical ventilation support, and have an increased 60 day mortality rate [96].

## **2.3 Inflammation**

Inflammation is the trademark response of the innate immune system, and it involves the recruitment of leukocytes and proteins to eliminate infectious agents [20]. Inflammation occurs in response to chemical, physical (heat, physical trauma), and biological (invading bacteria, viruses, fungi, parasites, cancer, etc) stressors [21] causing tissue injury. Substances released by the injured tissues cause dramatic changes in the surrounding tissues [21]. In response to an initial injury or invasion the production of secondary mediators such as cytokines, chemokines, complement proteins, and co-stimulatory factors that regulate various cellular and physiological responses occurs. Many substances involved in combating the injury can also harm surrounding, uninjured tissues. Thus, inflammation itself is a double edged sword. It exists primarily to heal, by removal of damaged or infected tissue to allow the area to resume normal activity [20], but it can also cause unwanted damage.

There are five trademarks of inflammation: redness, swelling, fever, pain, and eventually loss of function. Once an injury occurs, local blood vessels vasodilate increasing the blood flow and permeability of the capillaries in the area [21] causing a temperature increase. Increased capillary permeability results in a leakage of fluid into the interstitial space which causes swelling in the injured tissue [21]. Cells responding to the inflammatory signals (granulocytes and monocytes) move into the site of the injury or infection causing it to appear red [21]. These



three, swelling, redness, and fever lead to pain and discomfort in the area of injury. If inflammation persists, tissue damage can occur leading to chronic inflammation and inflammatory disease. The inflammatory response is responsible for many different types of disease including atherosclerosis, stroke, hypertension, neurological disease, respiratory disease, and autoimmune disease [46, 97, 115]. Inflammation can be classified in two different ways, acute inflammation and chronic inflammation.

### **2.3.1 Acute Inflammation**

Acute inflammation refers to the immediate response to an invading pathogen or injury as indicated by the four initial trademarks of inflammation; swelling, redness, heat, and discomfort. These responses are mediated by cells that are present in the area of injury, including primary immune cells such as macrophages and dendritic cells. However, non-immune cells in the area of injury are also capable of producing inflammatory mediators to begin the inflammatory cascade causing migration of immune cells into the area. The presence of bacteria causes production and secretion of signaling molecules including interleukin 1, tumor necrosis factor, and cytokines as well as activating the complement system. These compounds cause the area to be inundated with macrophages and neutrophils that are responsible for phagocytosing and ingesting the invading pathogen or damaged cells. Movement of the phagocytic cells to the site of injury is facilitated by the increased blood flow to the area that occurs as a result of vasodilation [21]. Increased permeability of the area allows these cells to move out of the vasculature and into the interstitial space where they are better able to come in contact with the offending pathogens [21]. Acute inflammation generally resolves within a few hours to a few days. However, if it continues, the inflammation will become chronic.

### **2.3.2 Chronic Inflammation**

Chronic inflammation is caused by persistent, unresolved acute inflammation. This can be the result of sustained pathogen levels, persistent inflammation, or autoimmune reactions. Chronic inflammation can ultimately lead to loss of function caused by tissue damage and fibrosis, and can lead to serious health problems including atherosclerosis and cardiovascular disease, autoimmune disease, obesity, arthritis, and cancer. These forms of chronic inflammation can result from inflammation that lasts months to years. Furthermore, despite their existence over an extended period of time inflammatory diseases can exhibit delayed onset so individuals may be completely unaware that they have an inflammatory disease until they have a heart attack or cancer is discovered. A sustained inflammatory response involves many different types of cells that are responsible for removing a pathogen from the system, often in a non-specific manner in which pathogens are not distinguished from host tissues. Therefore, non-specific damage to the host tissues may occur as the pathogens are attacked. If the inflammatory effects are not readily resolved, sustained chronic damage can contribute to debilitating and potentially fatal diseases.

### **2.4 Sources of Inflammation**

Inflammation will occur in response to an injury or the presence of foreign compounds within the body. The activation of the toll-like receptor (TLR) family has been shown to be involved in multiple inflammatory diseases including Crohn's disease and cancer through the activation of Nuclear Factor kappa B (NF- $\kappa$ B) [116, 117]. Therefore NF- $\kappa$ B as well as these disease complications can be regulated by the TLR family [118-120]. Furthermore, disruptions

to these pathways have been shown to reduce susceptibility to infection [121]. It is important to develop a better understanding of TLR4s involvement in inflammatory disease, as it may be a target for potential therapeutic intervention against inflammatory disease.

There are many different ligands that can trigger the activation of the TLR family (Table 2.1). TLRs are capable of recognizing pathogen-associated molecular patterns from fungi, protozoa, viruses, and bacteria [46]. These include (but are not limited to) zymosan and glycolipids from fungi and protozoa, double and single stranded RNA and CpG DNA from viruses, and flagellin, peptidoglycan, CpG DNA, lipopeptide, lipoprotein, and lipopolysaccharide from bacteria [4, 41, 47, 115]. Each ligand is capable of activating one or more toll-like receptors, which then activates inflammatory pathways leading to inflammation. Of the toll like receptors, TLR4 has been the best characterized, and is primarily activated by lipopolysaccharide. As discussed in section 2.1.1, TLR4 is one of the most important mediators of inflammation.

#### **2.4.1 Sources of Endotoxin**

Lipopolysaccharides (LPS) are found in the outer membrane of Gram-negative bacteria, and is often referred to as endotoxin. Endotoxins are referred to as such because they are portions of the bacteria (not secretions) that can cause toxicity and are capable of triggering inflammation. Lipopolysaccharides are made up of a polysaccharide chain, a core oligosaccharide, and a lipid. The poly- and oligosaccharide chains are variable amongst different types of Gram negative baceteria but the lipid is highly conserved. The lipid section of LPS is known as lipid A. While LPS is often referred to as endotoxin, the word actually refers to lipid A. It is this lipid A that is responsible for activating TLR4. Organisms are capable of producing

antibodies specific to the polysaccharide chain of endotoxin, but since this region varies between bacteria it is extremely important that TLR4 is able to respond to the lipid portion of LPS.

Through this activation, TLR4 is able to activate an inflammatory response against any form of LPS.

#### **2.4.2 Endotoxemia and Acute Inflammation**

The condition of circulating endotoxin in the blood is referred to as endotoxemia. Different levels of circulating endotoxin can cause different inflammatory responses. A study using canine models of septic shock showed a significant increase in the circulating endotoxin levels in non-surviving test subjects compared to surviving test subjects [122]. While clinical data concerning endotoxemia levels are limited, it does appear to suggest that high levels of circulating endotoxin are capable of inducing a severe inflammatory response that can lead to sepsis, which can quickly progress to septic shock and organ failure [122]. Septic shock is a condition that results in an extremely high mortality rate. The majority of septic shock cases are caused by endotoxin producing Gram negative bacteria. As discussed above, endotoxin is capable of activating TLR4 which then activates the inflammatory response. Normally, this would help to eradicate the bacterial infection. However, in situations where high levels of endotoxin are present, the problem can become so widespread that the immune response causes systemic vasodilation [21] and hypotension which leads to a decrease in cardiac contractility and widespread endothelial damage, thereby causing a systemic inflammatory response. This systemic hypoperfusion causes multi-organ failure that affects the liver, lungs, and heart [21]. Unless the inflammatory response and the causative endotoxin levels can be controlled very quickly, the patient usually dies.

### **2.4.3 Low grade endotoxemia and chronic inflammation**

Different levels of circulating endotoxin may be capable of causing different types of inflammatory responses. Septic shock occurs in response to high levels of circulating endotoxin. However, there are also severe problems associated with persistent, low grade endotoxemia. Increases in circulating endotoxin concentrations have been linked with obesity and diabetes. Circulating endotoxin is capable of triggering the production of pro-inflammatory cytokines when it binds with CD14 and TLR4 on the surface of innate immune cells [123]. Studies have reported that ‘healthy’ individuals have circulating endotoxin levels below 200pg/ml [124-127]. However, even in healthy individuals with very low baseline levels of circulating endotoxin, high fat meals have been shown to increase endotoxin concentrations to levels that may be sufficient to induce inflammatory activation [128]. In addition to foreign pathogen invasion, LPS is continually produced in the gut through turnover of Gram negative bacteria that assist in digestion [129]. This endotoxin is then transported into the bloodstream through intestinal capillaries in a TLR4 dependent manner [97]. Thus daily changes in circulating endotoxin levels may be sufficient to induce a chronic inflammatory response. While the effects of this response may be considered minute, the long term build-up of these effects can ultimately cause serious damage leading to obesity and diabetes, as well as atherosclerosis and cancer.

### **2.5 Reactive Oxygen Species**

Reactive oxygen species (ROS) are small oxygenated molecules that are highly reactive [11, 130]. They include oxygen radicals; superoxide ( $O_2^{\bullet-}$ ), hydroxyl ( $OH^{\bullet}$ ), peroxy ( $RO_2^{\bullet}$ ), and alkoxy ( $RO^{\bullet}$ ) [130]. Some ROS are non-radical oxygen species: hypochlorous acid (HOCl),

ozone (O<sub>3</sub>), singlet oxygen (O<sub>2</sub>), and hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) that can act as oxidizing agents, or are easily converted into radicals [130]. Reactive oxygen species are produced in a cascade that usually begins with the production of superoxide which is then dismutated into hydrogen peroxide [24, 131]. Once superoxide has been formed it can react in several ways to form other radicals: peroxynitrite is formed by a reaction between superoxide and nitric oxide, the Fenton reaction can generate hydroxyl radicals, or hypochlorous acid can be produced by a peroxidase catalyzed reaction of hydrogen peroxide [11]. ROS are known to interact with many different compounds including small inorganic molecules and cellular components (carbohydrates, nucleic acids, lipids, and proteins). Because ROS can cause irreversible destruction or significant functional alteration of its target, they are generally considered destructive molecules. However, this destructive behavior has been harnessed by immune cells, making ROS a positive player in host defense [22-24]. It has recently been discovered that ROS are not only involved in pathogen killing and damaging cells, but has also been linked to reversible regulatory redox processes that occur in nearly all tissues and cell types [22, 131-133].

### **2.5.1 Cellular Sources of Reactive Oxygen Species**

Some of the destructive reputation of ROS is due to the fact that it is produced as a byproduct of many normal cell processes. Some of the cellular elements responsible for producing ROS as a byproduct are mitochondria, cytochrome P-450 pathways, peroxisomes, and other cellular components [134-142]. The first system to be officially identified as producing ROS as its primary function (not a producer of ROS as a byproduct) is the phagocyte NADPH oxidase system, known as Nox2 [11]. Furthermore, Nox enzymes are ubiquitously expressed in almost all tissues and cells [28] and there are several isoforms of this ROS generating family;

they are not limited to phagocytes. The ROS produced by the Nox enzymes are superoxides [27]. Superoxide itself is thought to not be involved in the actual killing of microorganisms, as it has been argued that superoxide is not reactive enough to be a major destructive player by itself [143]. However, it has also been suggested that in specific environments (non-polar) or at low pH (4.8, where the protonated form of superoxide exists) [144] that it could in fact be a direct player in killing pathogens. Furthermore, these types of environments are found at specific locations in the cell that are more involved in or more likely to need host defensive action. Low pH is found in the phagosome [145], and a non-polar environment can be found at the cell membrane. However, despite the disagreements on superoxide's direct role in host defense, it certainly plays a very important secondary role. Superoxide dismutates into hydrogen peroxide, either spontaneously or facilitated by superoxide dismutase [23]. Hydrogen peroxide is then known to react with myeloperoxidase and can thereby be involved in the killing of microbes [144, 146]. Conversion of superoxide into hydrogen peroxide can lead to the formation of other highly reactive free radicals: hydroxyl radical, singlet oxygen, hypochlorous acid [11]. Superoxide can also react with nitric oxide (NO) to form peroxynitrite which can then be converted into any one of several reactive nitrogen species [16]. Therefore, superoxide can generate a battery of ROS for killing pathogens.

### **2.5.2 Roles of ROS**

One of the most important roles for ROS in the regulation of cell function is through oxidative inactivation of cysteine residues. Redox-sensitive cysteine residues can be found on protein tyrosine phosphatases [104, 105] which are responsible for controlling the phosphorylation state of many proteins involved in the regulation of cellular differentiation,

proliferation, metabolism, motility, and survival [147]. ROS have been shown to regulate tyrosine phosphorylation in many different cell types [148-151]. ROS decrease the phosphatase activity of some protein tyrosine phosphatases by oxidation of cysteine residues [148]. Since these phosphatases are no longer de-phosphorylating tyrosine, tyrosine phosphorylation is increased. Depending on the protein this may either enhance or decrease activation thereby regulating signal transduction.

Furthermore, in addition to acting on protein tyrosine phosphatases, ROS have also been shown to be involved in the activation of kinases as hydrogen peroxide leads to activation via phosphorylation of MAP kinase in human endothelial cells [133]. There are numerous documented cases of activation of the MAP kinase system by NADPH oxidase (Nox) [108-115]. How this activation occurs is unknown, but it has been suggested that it may involve the activation of pathways upstream of ERK [131], or possibly be due to inhibition of phosphatase activity by ROS [152] as described above.

ROS are known to be involved in the regulation of both intracellular [153] and plasma [154] membrane channels. This regulation could be a direct result of interaction with ROS, or possibly an indirect result through ROS-sensitive signaling systems. ROS from Nox have been suggested to play a role in the regulation of potassium and calcium channels [155-159]. With respect to neuronal firing rates., increases in ROS cause ion channel leakage, changing the potential of cells causing increases in the switch from resting potential to action potential and thereby increasing neuronal firing.

Reactive oxygen species have also been shown to induce gene expression. Nox-dependent ROS generation induces the expression of TGF-beta1 [160], TNF-alpha [161], angiotensin II [160], plasminogen activator inhibitor-1 [160], and monocyte chemoattractant



protein-1 [160]. It has been suggested that the major cause of mRNA upregulation in response to ROS is through activation of MAP kinase [162] or through transcription factors like NF- $\kappa$ B, AP-1, and p53 [163]. All of these methods of mRNA upregulation are redox-sensitive. Both NF- $\kappa$ B [26, 164-166] and AP-1 [167, 168] effect gene expression in response to reactive oxygen species derived from Nox. It has also been suggested that not only is Nox-derived ROS altering gene expression through the above systems, but that the ROS themselves may actually be involved in altering the stability of mRNA [169, 170].

Reactive oxygen species are known to trigger apoptosis and cell death directly by activation of the MAPK proapoptotic signaling pathway [171], and it can indirectly cause apoptosis by causing damage to lipids, proteins, and DNA [16]. However, it has also been suggested the reactive oxygen species derived from Nox can have a prosurvival effect. ROS have been shown to activate NF- $\kappa$ B [172] and the Akt/ASK1 pathway [173], both of which are believed to be involved in anti-apoptotic signaling. Furthermore, superoxide is known to be a natural inhibitor of cell death as mediated by Fas [174]. Thus, there are several different ways that ROS can be manipulated to be either pro or anti-apoptotic. The first factor that must be taken into consideration is the magnitude of ROS being produced, and the duration of the signal. Secondly, the localization within (or outside) the cell must be considered. Next, what redox-sensitive targets are present within the proximity of the ROS being produced [17, 175]. Finally, the metabolism of superoxide (potentially anti-apoptotic) versus that of hydrogen peroxide (pro-apoptotic) must be considered. Similar to the anti vs pro-apoptotic role for ROS discussed above, there are also other potentially contradictory functions of ROS. ROS has been suggested to be important in the aging process as it is thought that oxidative stress caused by the presence of ROS can accelerate cellular senescence [16, 176, 177]. However, both superoxide and

hydrogen peroxide are known to increase growth by stimulating the expression of early growth genes, resulting in increases rather than decreases in cell growth and differentiation [132].

#### **2.5.4 Oxidative Stress and Disease**

Oxidative stress occurs when more reactive oxygen species are being produced than can be processed by the body's antioxidant mechanisms [178, 179]. Oxidative stress and sustained oxidative damage is considered to be a primary cause of a variety of diseases including atherosclerosis, hypertension, and Parkinson's disease, as well being thought to contribute to some cases of diabetes and cancer [130, 172]. Oxidative stress is also thought to be a primary cause in radiation induced damage [130]. However, the greatest danger in oxidative stress may not be as a primary contributor to disease formation, but rather as a contributor to disease pathology. In addition to being implicated in the initiation of the above diseases, ROS are also thought to contribute to significant pathology in a plethora of diseases including atherosclerosis, hypertension, diabetes, rheumatoid arthritis, autoimmune diseases, inflammatory bowel disease, cystic fibrosis, cancer, stroke, Parkinson's disease, Alzheimer's disease, multiple sclerosis, Huntington's disease, Friedreich's ataxia, and HIV [130, 148, 172]. The secondary contribution of ROS in these diseases is long term damage of attributed to an extended inflammatory response.

#### **2.6 NADPH Oxidase: a family of ROS producers**

NADPH oxidase 1 is a member of the Nox family. The Nox family is made up of seven members, Nox1 – 5 and DUOX1 and 2 [11]. All members of the family are transmembrane

proteins [11]. They are able to transport electrons across a membrane thereby reducing oxygen and generating superoxide [11]. The superoxide produced by the Nox family can then be converted into hydrogen peroxide ( $H_2O_2$ ), and eventually to other reactive oxygen species. Before the Nox family was identified, the production of reactive oxygen species was described as a respiratory burst [11, 180, 181]. This burst has been associated with damage in biological organisms, signaling processes, and the immune response.

### **2.6.1 Structure and Activation of Nox**

The Nox family is grouped together because of several conserved structures; an NADPH-binding site, a FAD-binding region, at least six transmembrane domains, and four heme-binding histidines [11]. The first Nox protein to be discovered was Nox2 [182], is expressed primarily neutrophils and often referred to as the phagocyte oxidase [183, 184]. The activation of Nox2 occurs through a complex series of protein-protein interactions. Nox2 is constitutively associated with p22phox and it has been suggested that inactive Nox2 is stabilized by p22phox [185-187]. Stimulation then elicits the translocation and binding of other cytosolic factors to the complex [133]. The other members of the activated Nox2 complex (Figure 2.2) are p47phox, p67phox, p40phox, and Rac [181]. When p47phox is phosphorylated, a conformational change occurs which then allows it to interact with p22phox[24] p47phox has been designated the “organizer subunit” because it is considered to be responsible for the organization of the translocation of the other cytosolic subunits [11, 24]. As seen in neutrophils from patients lacking the p47subunit, all the rest of the cytosolic subunits required for the activation of the Nox2 complex (p67phox, p40phox, and Rac) failed to translocate to the membrane [188, 189]. Once p47phox has been localized to the membrane, it comes into contact with p67phox, the

“activator subunit.” This brings the activator subunit into contact with Nox2 [190], while also bringing p40phox into the complex. Finally, Rac, a small GTPase, interacts with Nox2 [22] as well as p67phox [191, 192]. Upon assembly the complete complex is active and can transfer an electron from NADPH to oxygen, creating superoxide [11].

Nox1 was the second NADPH oxidase to be discovered [193, 194]. Nox1 has ~60% sequence identity to Nox2 [193, 194]. The gene for Nox1 is located on the X chromosome in both humans and mice [193]. Nox1 is between 55 – 60 kDa in size and can be induced under many different situations [195-197]. It has been shown to be induced in vascular smooth muscle by platelet derived growth factor, prostaglandin  $F_{2\alpha}$ , and angiotensin II [194, 198-200], as well as by injury [201]. The human Nox1 gene contains binding elements for signal transducers and activators of transcription (STATs), interferon regulatory factor (IRF), AP-1, NFkappaB, CREB, CBP/p300 elements [202], and GATA factors [28]. Nox1, like Nox2, has been shown to be selective for NADPH over NADH as a substrate [203].

Also like Nox2, the generation of superoxide by Nox1 is dependent on cytosolic subunits (Figure 2.2). The Nox1 subunits were first discovered in the colon [204-208]. Nox organizer 1 (NOXO1) is a homolog to the Nox2 organizer protein p47phox [204, 208]. The gene for NOXO1 is located on chromosome 16 [11] and has a molecular mass of 41kDa. NOXO1 and p47phox share ~25% sequence identity, but their functional domains are highly conserved [204, 208]. Both have a phox domain that interacts with membrane phospholipids (allowing the proteins to become anchored to the membrane), and two SRC homology 3 (SH3) domains that can interact with p22phox in the proline rich region of the COOH terminus [208, 209]. The organizer designation can also be seen in the physical attributes of the organizer proteins: the phox homology domain interacts with membrane phospholipids, SH3 domains allow for

interaction with p22phox, and a proline rich region allows for interaction with p67phox. All of these are structures also found in NOXO1. p47phox has an autoinhibitory region that is not found in NOXO1 [11]. It has been shown that LPS is capable of activating transcription of the NOXO1 protein in guinea pig gastric mucosa [210]. In transfected HEK293 cells, NOXO1 is found to localize at the cell membrane with Nox1 and p22phox [205]. As mentioned below, constitutive activation of the Nox1 complex has been shown in mice, but not in humans.

The Nox activator 1 (NoxA1) is a homolog to the Nox2 activator protein p67phox. It is found on chromosome 9 and has a molecular mass of 51 kDa [11]. Like NoxO1 and p47phox, NoxA1 shows low (~28%) sequence identity to p67phox. However, also like NOXO1 and p47phox, NOXA1 and p67phox have a very similar domain structure[204, 208]. Both are cytoplasmic proteins containing an NH2 terminal tetratricopeptide repeat, a highly conserved activation domain, a Phox and Bem 1 domain, and a COOH-terminal SH3 domain [11]. Like NoxO1, it is likely that NoxA1 is constitutively associate with the plasma membrane, however this has not been experimentally demonstrated. NoxA1 is able to associate with Nox1 through the SH3 domain in the COOH terminus [24, 208, 211]. It is also assumed that like p67phox, NoxA1 is also able to interact directly with Nox1 through the activation domain [190, 204, 208, 212, 213]. Rac is able to interact with NoxA1 through the tetratricopeptide repeats [191, 192, 208, 214, 215]. However, unlike p67phox, NoxA1 is unable to interact with p40phox [208], thus p40phox is considered to be unnecessary for the activation of the Nox1 complex.

In transfected cells it has been observed that Nox1 can use the p47phox and p67phox to assemble an activated complex. This indicates that the cytosolic subunits may not be specific to a single Nox protein [204]. Unlike the Nox2 system, expression systems using mouse proteins suggest that the Nox1 system is constitutively active [27, 204]. However, studies using human

proteins have shown only a weak constitutive activity but gaining full activation using PMA (a PKC activator) [207, 208]. Furthermore, there are significant differences between the human and mouse proteins in the region of the phox homology domain [11].

Along with the dependence for the cytosolic units, Nox1 is capable of utilizing p22phox (the membrane subunit) [197, 216]. However, p22phox may not be required for the activation of Nox1 as it has been shown to be for Nox2 (and Nox3) [215]. It has been suggested that p22phox is responsible for binding to the Nox protein, and that the resulting heterodimer is stable [208, 217, 218]. Based on FRET and immunoprecipitation studies, p22phox has been shown to interact with Nox1 [197]. Furthermore, downregulation of p22phox by siRNA mediation leads to a decrease in Nox1 function [216, 219]. In addition to binding to the Nox protein itself, p22phox also binds to the organizer subunit, allowing for the Nox/activator/organizer complex to be assembled. p22phox contains a proline rich region within its COOH terminus [181, 209] making it possible for it to interact with the SH3 domains of NoxO1. Without this region, a loss in Nox1 activation has been observed [216, 220].

There is evidence for the involvement of Rac in the regulation of Nox1 activity [208, 210, 215, 221-224]. Rac is known to bind to NOXA1 [208, 221, 224], but it is possible that like the Nox2 system, it may directly bind to NOX1 as well as the activator protein [11]. Rac may also be involved in the regulation of the production of ROS through non-Nox dependent methods, most significantly the production of ROS by mitochondria [225, 226].

Initial studies have shown a role for Nox1 in cell proliferation and differentiation and therefore it has sometimes been referred to as mitogenic oxidase 1 (MOX-1) [194]. It has even been suggested that hydrogen peroxide is the reactive oxygen species responsible for the mediation of transformation caused by Nox1 [227]. This may be a cell type specific effect as

other studies in fibroblasts reported that Nox expression failed to produce cell transformation [17].

## **2.7 Antioxidant Regulation of Reactive Oxygen Species**

Cells utilize a variety of mechanisms to reduce the destruction caused by ROS. The most obvious way to control ROS damage is to control the amount of ROS produced. There are many different compounds capable of doing so including mitochondrial uncoupling proteins, haem oxygenases, albumin, and transferrins [130]. However, once ROS have been produced, they must be controlled by antioxidants capable of inactivating ROS. These include enzymes such as superoxide dismutase, catalase, and peroxidase [23]. Additionally, cells may offer up 'sacrificial agents' (eg glutathione, bilirubin, and albumin) that are oxidized in order to preserve essential molecules [228]. There are multiple different cellular pathways activated by the presence and production of ROS that are mediated via the antioxidant response element (ARE).

### **2.7.1 Antioxidant Response Element Mediated Regulation of Reactive Oxygen Species**

The antioxidant response element (ARE) was initially found in the promoter region of glutathione S-transferase and NADPH quinone oxidoreductase 1, two enzymes involved in detoxification of ROS [229-232]. It has since been found in the promoter sequence of many different antioxidant genes [233]. ARE is activated in response to hydrogen peroxide as well as other compounds that can be transformed into reactive intermediates [234, 235]. Furthermore, it is also activated by changes in the cellular redox status [235]. Thus elevations of ROS, reduced antioxidant capacity (change in levels of the 'sacrificial agents' mentioned above), and increased

electrophilic species can activate ARE. The genes induced by ARE include a broad range of genes involved in protective responses including the direct production of antioxidants [234, 236]. Since ROS are a byproduct of ongoing aerobic metabolism, ARE plays an important role in maintaining redox homeostasis under stressed and un-stressed conditions [237].

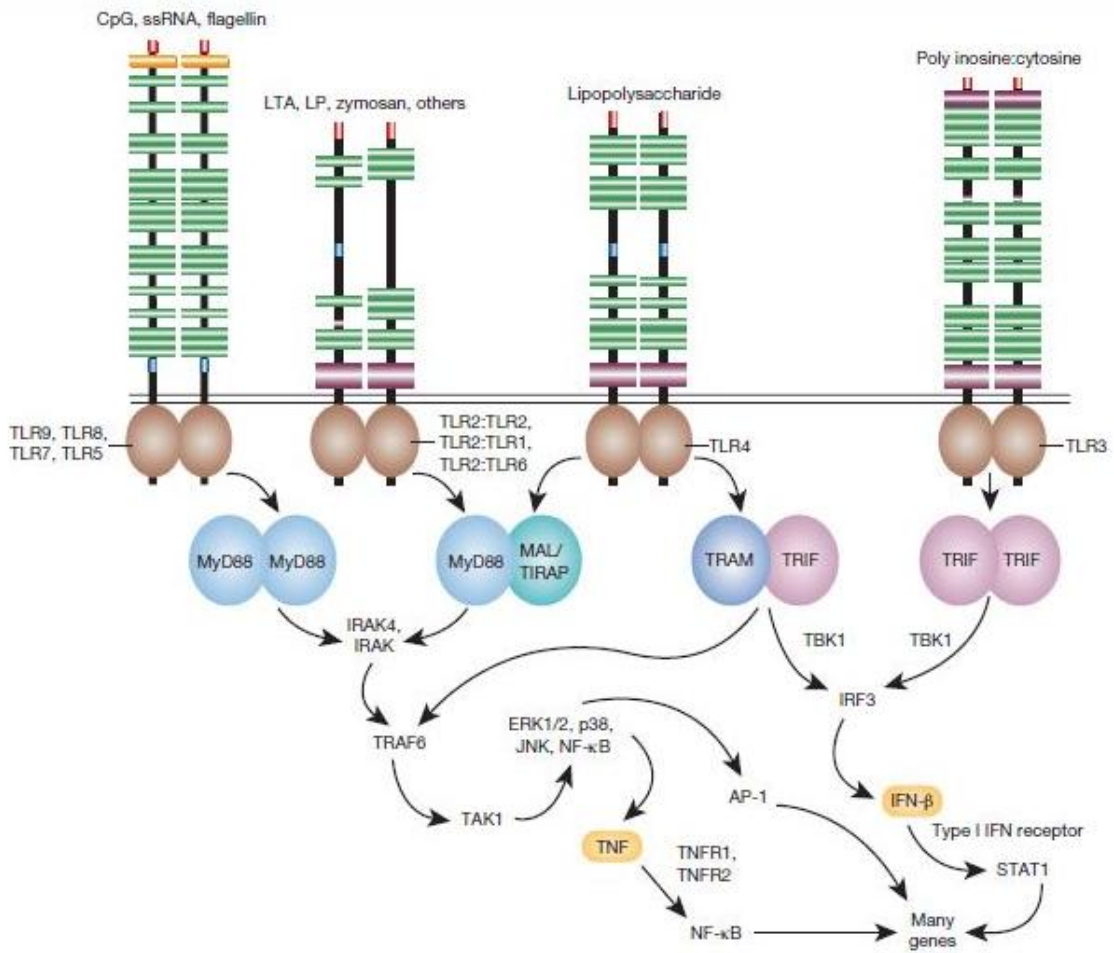
The activation of gene transcription through the ARE is primarily mediated by nuclear factor E2-related factor 2 (Nrf2). Nrf2 was originally identified as a transcription factor that binds to the ARE of human NAD(P)H quinone oxidoreductase [238]. It has since been found that Nrf2 also binds to the ARE of many different genes [239]. Furthermore, Nrf2 knockout mice showed significant impairment in the expression of several ARE-dependent genes [240, 241]. Due to the necessity of Nrf2 in the activation of ARE dependent genes, it has become known as a master regulator of the antioxidant response [234, 237, 242-245].

## **2.8 Nuclear factor-E2-related factor 2: a method of antioxidant protection**

Under unstressed conditions, Nrf2 is found in the cytoplasm [233]. The activity of Nrf2 is tightly controlled by Kelch-like ECH associated protein 1 (Keap1). Keap1 is a cysteine-rich protein, made up of 626 amino acids (human Keap1) of these, 27 are cysteines [233]. All known ARE inducers are known to react with the cysteine residues of Keap1. It is believed that a single molecule of Nrf2 is bound by two Keap1 molecules. The bonds holding Nrf2 and Keap1 together have different affinities [246]. When cells are not under oxidative stress, Keap1 facilitates ubiquitination of Nrf2 leading to proteasomal degradation [233]. When cells are stressed some of the cysteines making up Keap1 become oxidatively modified, which leads to conformational changes in Keap1 that releases the hold of the weaker Keap1 molecule on Nrf2 [233]. The higher affinity bond between Nrf2 and the second Keap1 is maintained [246].



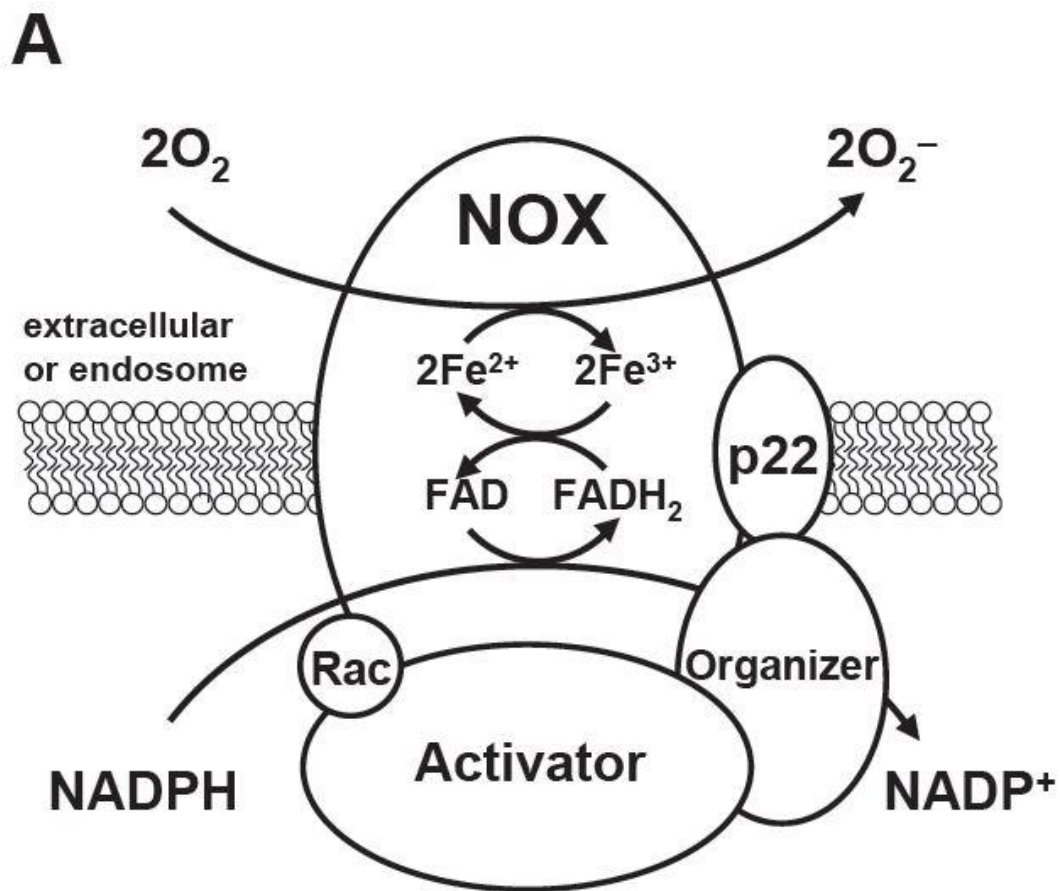
Despite the remaining association between Nrf2 and Keap1, the loss of the low affinity bond prevents ubiquitination of Nrf2 thereby preventing its degradation [246]. The sequestration of Nrf2 within the cytosol has also been linked to the activity of several kinases including PI3 kinase, ERK, p38 MAPK, PKC, and PERK [236, 247, 248]. It has been suggested that these kinases (and possibly others as well) are involved in regulating the stability and localization of Nrf2 via protein phosphorylation [249]. Once the low affinity bond between Nrf2 and a molecule of Keap1 is modified, Nrf2 then accumulates in the cytoplasm until the amount accumulated surpasses the ability of the Keap1 present to sequester it in the cytoplasm. At this point, Nrf2 is translocated into the nucleus, binds to the ARE and elicits the transcription of downstream protective genes including heme oxygenase-1, catalase, superoxide dismutase, glutathione S-transferase, and NAD(P)H:quinone oxidoreductase (Figure 2.3) [234, 236, 244].



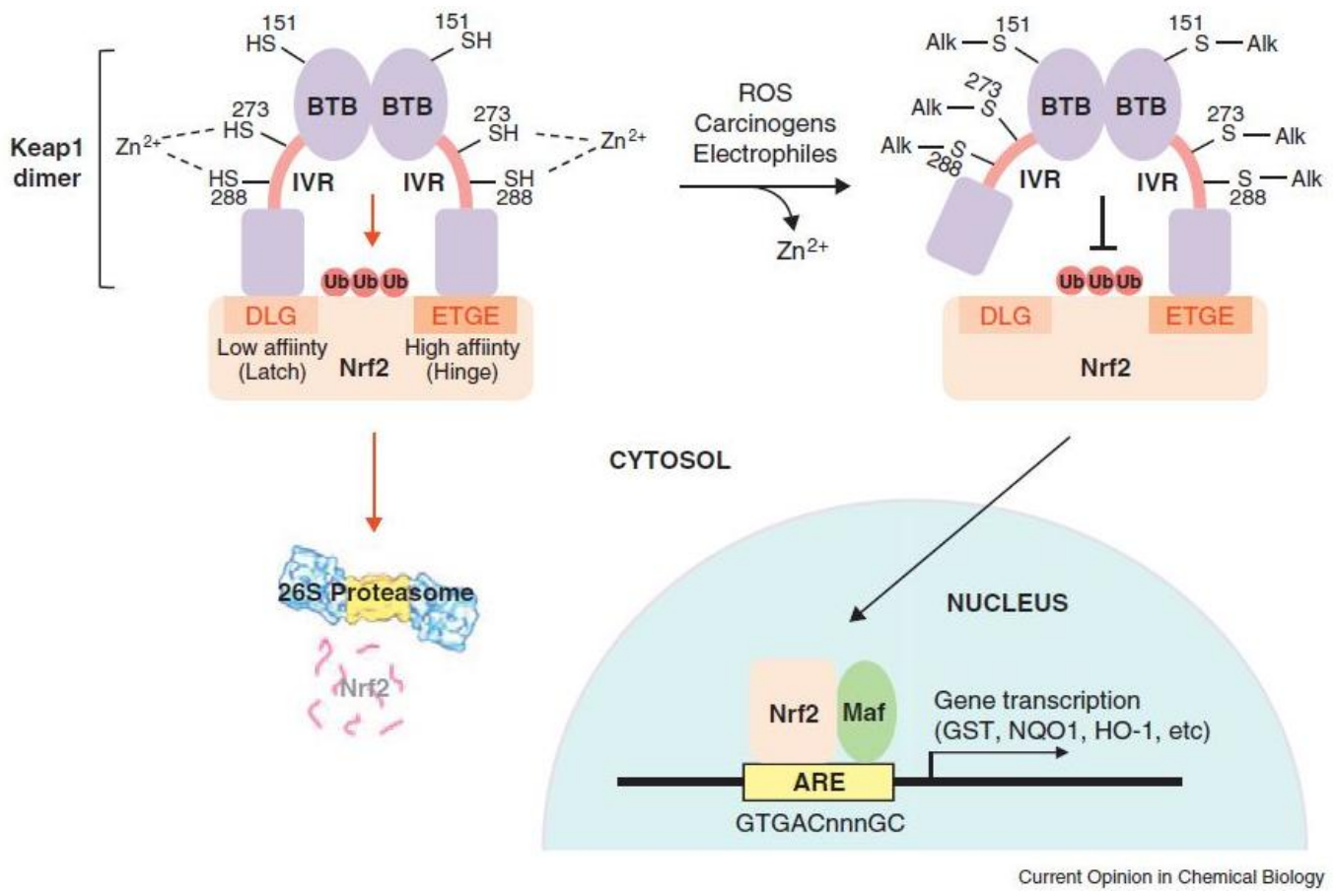
**Figure 2.1.** *Toll-like receptor signaling.* TLRs are capable of activating multiple signaling networks via several different adaptor proteins. Reprinted by permission from Macmillan Publishers Ltd: Nature [47], copyright 2005.

Receptor	Ligand	Location	Receptor	Ligand	Location	
TLR1	Tri-acyl peptides Lipoarabinomannan	Cell Surface	TLR4	Lipopolysaccharide Heat shock proteins Fibronectin Taxol Lipoteichoic acid Saturated fatty acids Heparin sulfate	Cell Surface	
TLR2	Peptidoglycan Lipoteichoic acid Zymosan HSP70 Glycolipids Lipopeptides/lipoproteins Poryphyromonas gingivalis LPS Mannuronic acid polymers Lipoarabinomannan  Outer membrane proteins HSP60	Cell Surface		TLR5	Flagellin	Cell Surface
				TLR6	di-acyl lipopeptides Zymosan Lipoteichoic acid	Cell Surface
				TLR7/8	Imidazquinoline Single Stranded RNA	Cell Compartment
TLR3	Viral RNA Poly I:C	Cell Compartment	TLR9	CpG DA	Cell Compartment	
			TLR10/12/13			
			TLR11	Profilin	Cell Surface	

**Table 2.1.** *Toll-like receptor ligands* Each TLR is sensitive to different ligands. TLRs are capable of recognizing a vast array of agonists, allowing them to successfully monitor the homeostatic state of the surrounding cells and tissues. Table adapted from den Dekker and Li [4, 115]



**Figure 2.2.** *Activated NADPH oxidase complex.* In order for Nox to successfully produce superoxide, the full complex must be assembled across a membrane. The activated complex consists of the Nox protein, the Nox activator protein (NOXA1 for Nox1 or p67phox for Nox2), the Nox organizer protein (NOXO1 for Nox1 or p47phox for Nox2), p22phox (may not be required for the activation of Nox1), and Rac. The activated complex transfers electrons from NADPH to oxygen producing superoxide. Permission to use figure [12].



Current Opinion in Chemical Biology

**Figure 2.3.** *Activation of Nrf2.* Under unstressed conditions Nrf2 is bound to two molecules of Keap1 in the cytosol. Oxidative stress causes modifications in the weaker bond between Nrf2 and one molecule of Keap1. Without both bonds, Nrf2 is no longer marked for degradation and builds up in the cytosol until the amount of Keap1 available to bond is overwhelmed by Nrf2 levels. Nrf2 can then translocate into the nucleus, bind to the antioxidant response element and increase transcription of antioxidant genes. Reprinted from Current Opinion in Chemical Biology, Volume 15/Issue 1, Wooyoung Hur and Nathanael Gray, Small molecule modulators of antioxidant response pathway, Pages 162-173., Copyright 2011, with permission from Elsevier [233].

### **Chapter 3: Specific Aims**

The production of reactive oxygen species (ROS) is a significant part of both normal and stressed cellular processes. ROS are produced by a cascade that generally starts with superoxide which is then dismutated to hydrogen peroxide [24, 131]. Normal cellular processes that produce superoxide as a byproduct include: mitochondria, cytochrome P450, and peroxisomes [134-142]. Another important producer of ROS is the NADPH oxidase family whose primary purpose is to produce superoxide. Once produced, ROS are able to oxidize inactive cysteine residues which control the phosphorylation state of proteins involved in regulating cell differentiation, metabolism, motility, and proliferation. However, ROS are also capable of doing considerable damage to proteins, lipids, and DNA, and sometimes apoptosis. These same ROS pathways are also major players in the immune response, acutely damaging cells significantly enough to cause apoptosis. Therefore, ROS are used to combat invading pathogens but if not adequately controlled, damage to host cells and tissues can contribute to chronic inflammation and inflammatory disease. Thus the host must be able to balance the amount of ROS produced in order to achieve the desired defense without causing the host significant harm.

Multiple different inflammatory stimuli have been shown to cause an increase in reactive oxygen species production. Our lab (and others) has demonstrated an increase in ROS production in response to the inflammatory stimulant lipopolysaccharide (LPS), which is a component of Gram negative bacterial cell walls. It activates toll-like receptor 4 (TLR4) and thereby is able to activate pro-inflammatory mediators including IL-1, IL-6, TNF-alpha, and ROS. Activation of TLR4 involves a signaling molecule already known to be critically involved in the regulation of inflammatory signaling, interleukin-1 receptor associated kinase 1 (IRAK-1).

Previously in our lab we have seen that cells cultured from IRAK-1 deficient mice produce fewer pro-inflammatory mediators compared to wild type cells. Therefore, we hypothesize that IRAK-1 may be involved in modulating the activation and regulation of the pro-inflammatory, ROS producing Nox family. Thus the objective of the first part of this work is to determine the role of IRAK-1 in the activation of Nox1.

In addition to the short term effects of LPS derived endotoxin on the acute activation and induction of Nox1 in IRAK-1 deficient cells compared to wild type cells, it is also important to examine the long term effects of acute inflammation that can lead to chronic inflammation. Therefore, the effects that sustained exposure to endotoxin will have on Nox1 were examined in these studies. Furthermore, it has been shown that increasing amounts of inflammatory agonist are able to induce a more severe inflammatory response. We propose that these inflammatory responses are regulated differentially by anti-inflammatory feedback mechanisms. Therefore, we hypothesize that sustained endotoxin exposure will cause the system to become tolerant by differentially regulating NADPH oxidase and anti-inflammatory feedback. We define a system to be tolerant to a later challenge if the measured inflammatory response (ROS production, Nox1 mRNA levels, and Nox1 protein levels) is less than the inflammatory response to the initial stimulus.

Activation of inflammation is known to happen in two different ways, transiently or sustainably. In transient responses, changes occur rapidly but are also resolved rapidly. However, in sustained responses a challenge will cause the inflammatory response to increase gradually and continually over time. We propose that these different inflammatory responses are

related to different severities of the initial challenge to the system. Therefore, we hypothesize that a low dose endotoxin exposure will prime a system for future LPS challenge producing more reactive oxygen species than an unprimed system.

The model proposed by this work is as follows (Figure 3.1). LPS activates TLR4, which recruits IRAK-1 thereby activating NF- $\kappa$ B which is capable of translocating into the nucleus and binding to the promoter region of Nox1 increasing the production of Nox1. Activation of TLR4 also activates basal levels of Nox1 to produce ROS. The ROS produced in response to high doses of LPS crosses the threshold necessary to activate Nrf2, however low levels of LPS do not induce enough ROS production to activate this pathway. Upon activation Nrf2 accumulates in the cytoplasm until it eventually translocates into the nucleus binding to the ARE in the promoter region of Nrf2 and other antioxidant genes thereby increasing antioxidant levels within cells. Nrf2 activation inhibits not only current ROS production, but future ROS production by inhibiting the transcription of Nox1. Cells treated with low dose LPS do see the initial activation of Nox1, as well as an increase in Nox1 transcription, however the amount of ROS produced is not enough to activate Nrf2. Thus, after longer challenge with low dose LPS more Nox1 accumulates in cells. When a second challenge is presented cells initially treated with high dose LPS will not have cleared the excess Nrf2 and antioxidants preventing a second inflammatory response, thereby exhibiting tolerance. However, cells treated initially with low dose LPS will have accumulated excess Nox1 not excess Nrf2 and will be able to respond to a second challenge with an increased inflammatory response thereby exhibiting a priming response. Therefore, the specific aims of this project were:



**Aim 1: To define the role of IRAK-1 in the activation of Nox1**

Hypothesis: IRAK-1 is responsible for LPS induced activation of Nox1.

Objective: Wild type and IRAK-1 deficient mouse embryonic fibroblasts were used to determine if IRAK-1 is involved in increased cellular levels of Nox1 mRNA and protein. DCFDA staining was used to characterize the production of ROS in response to LPS.

**Aim 2: To examine the role tolerance plays in LPS induced production of Nox1**

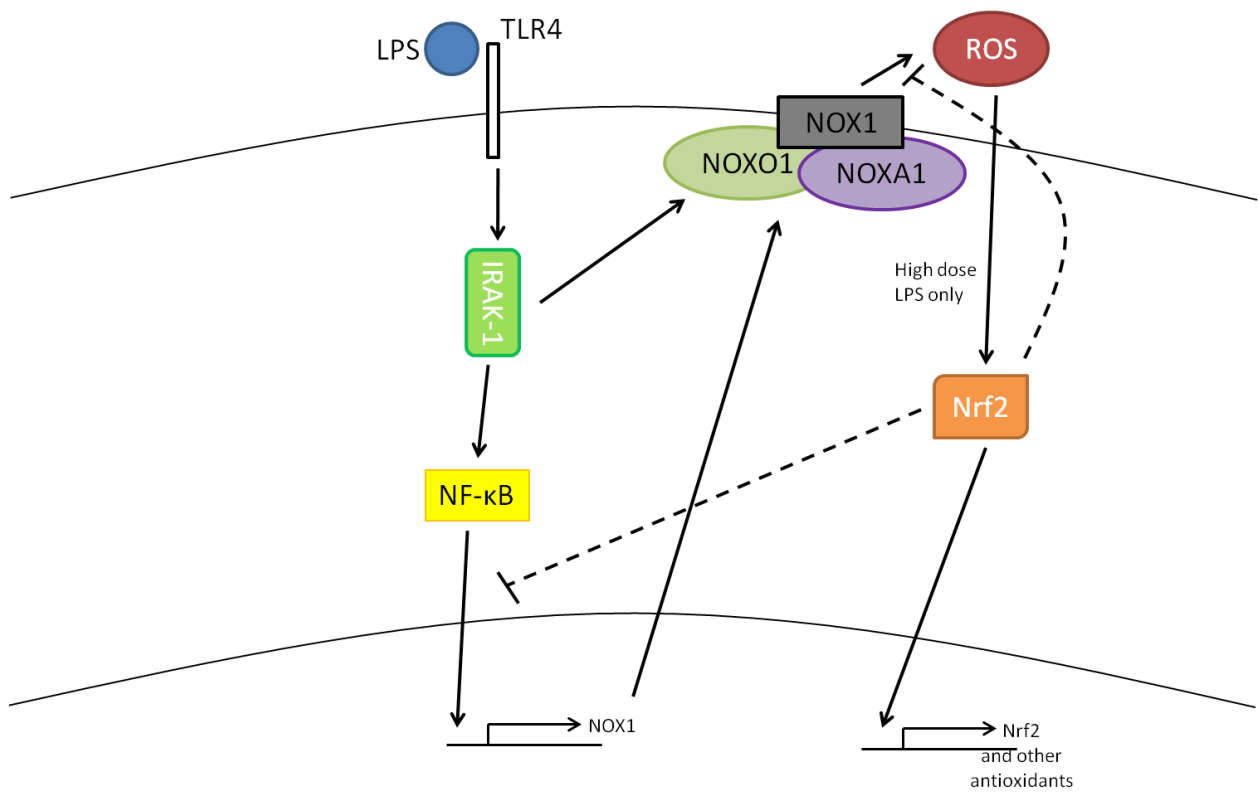
Hypothesis: A high initial dose of endotoxin causes the development of tolerance via differential regulation of NADPH oxidase 1 and Nrf2.

Objective: Mouse embryonic fibroblasts were used to evaluate the effects of LPS tolerance on cellular levels of Nox1 mRNA and protein.

**Aim 3: To examine the role that TLR4 priming has on the production of reactive oxygen species**

Hypothesis: Low dose endotoxin primes the system for future LPS challenges, so more reactive oxygen species will be produced in response to subsequent challenges than by the unprimed system.

Objective: Mouse embryonic fibroblasts were used to examine the effects of LPS priming on ROS production using DCFDA fluorescent staining for reactive oxygen species.



**Figure 3.1.** *Representation of Hypothesis.* Simplified signaling diagram representing the LS dose dependence responsible for differential regulation of ROS production in acute and chronic inflammation.

## Chapter 4: Materials and Methods

### Experimental Design

In order to explore the hypothesis that IRAK-1 is required for LPS induced activation of Nox1, the following experiments were performed. Dose response studies were performed in wild type (WT) and IRAK-1 deficient mouse embryonic fibroblasts (MEF) to determine if ROS were produced in response to LPS. Measurements of ROS production were made using the fluorescent dye DCFDA to see immediate changes in ROS production in response to LPS. Whole cell protein samples were isolated from WT and IRAK-1 deficient MEF to examine the change in Nox1 protein levels in response to different concentrations of LPS in these cells. Finally, mRNA was isolated from WT and IRAK-1 deficient MEF to examine the change in Nox1 mRNA levels in response to different doses of LPS.

Once it was determined that IRAK-1 is necessary for Nox1 dependent increases in ROS, we examined the effects of high and low dose LPS on a later inflammatory challenge. For these experiments, WT MEF were treated with high (100ng/ml) and low (200pg/ml) concentration of LPS for 2 to 18 hours. After LPS challenge, mRNA was collected from these cells to examine the change in Nox1 mRNA, and the change in Nrf2 mRNA (an antioxidant) in response to extended exposure to LPS. DCFDA fluorescent staining was used to measure ROS production in these cells. Finally, a PI3 kinase inhibitor (wortmannin) was used to inhibit the activation of Nrf2 (PI3 kinase activation is upstream of Nrf2 activation). After treatment with wortmannin +/- LPS, whole cell protein samples and mRNA samples were taken from these cells to examine the change in Nox1 and Nrf2 mRNA and protein levels.

After looking at the long term effects of high and low dose LPS on ROS production, protein, and mRNA levels we examined the role of different concentrations of LPS on future inflammatory stimuli. WT MEF were treated with high (100ng/ml) and low (200pg/ml) doses of LPS for 2 hours. After 2 hours, the media (containing excess LPS) was removed from and replaced with normal media (containing no LPS). These MEF were allowed to rest for up 16 hours before being treated with a second dose of either high (100ng/ml) or low (200pg/ml) dose LPS. ROS production rates by these cells were assessed using DCFDA fluorescent dye, and mRNA samples were isolated to examine mRNA levels of Nox1 in these cells.

## **Analytical Methods**

### *Cells*

Mouse embryonic fibroblasts (MEF) were isolated from wild type and IRAK-1 deficient C57BL/6 mice as previously described [250]. Briefly, embryos were harvested after 16 days of gestation. The head and all internal organs were removed from the embryos leaving only the carcasses. These were washed with PBS, cut into small pieces with scissors, and incubated in 0.25% trypsin at 4°C overnight. The embryos were then incubated for 20 minutes at 37°C in order to activate the trypsin. DMEM (Invitrogen) containing 10% heat-inactivated FBS and supplemented with penicillin and streptomycin (Invitrogen, 100 U/ml) was added to stop trypsin action. The cells were dissociated by pipetting several times, and the remaining cell solution was centrifuged at 1000rpm for 6 minutes. The pellet was resuspended in growth media (DMEM supplemented with 10% FBS, 100 U/ml penicillin-streptomycin, and 1X non-essential amino acids) and cultured in 5% CO<sub>2</sub> at 37°C until they reached confluence. Cells were split as needed.

The cultured MEF were detached from culture dishes by incubating them with 1% trypsin in PBS for 5 minutes at 37°C in 5% CO<sub>2</sub>, removed from the plate, suspended in 10% FBS DMEM, and centrifuged for 5 minutes at 800rpm. The pellet was then resuspended in 10% FBS DMEM and plated at the proper cell density. The MEF were allowed to attach to the plates for 8 hours, and then rested in 1% FBS DMEM for 4 hours prior to treatment.

#### *Reactive Oxygen Species Detection*

To examine the effects of LPS on the production of reactive oxygen species, 4x10<sup>4</sup> MEF/well were cultured in 96 well culture treated plates. ROS production was detected by oxidation of DCFDA[23]. Fifty micrograms of the fluorescent probe chloromethyl-2',7'-dichlorofluorescein diacetate (Molecular Probes, CM-DCFDA) were dissolved in 8.65uL DMSO (10mM). The DMSO/DCFDA solution was then diluted in 8.65mL of Normal Ringers (140mM NaCl, 4mM KCl, 1.8mM CaCl<sub>2</sub>, 1mM MgSO<sub>4</sub>, 10mM HEPES, 10mM glucose, 5mM NaHCO<sub>3</sub>). Cells were incubated in 10μM CM-DCFDA for 30 minutes, then gently washed with 100uL Normal Ringers 4 times, and 100ul Normal Ringers were added to each well of cells. A basal fluorescence reading was taken using an excitation wavelength of 488nm and an emission wavelength of 535nm. Cells were then treated with the proper concentration of lipopolysaccharide (LPS) (0.05 – 100ng/ml) (R&D systems) and readings were taken over 30 minutes. Fluorescent emission curves were then plotted and the change of fluorescence (e.g. slope of the lines) was determined.

### *Gene Expression Studies: Real-Time Quantitative PCR*

To examine the effects of LPS on mRNA expression of Nox1, Nrf2, and GAPDH,  $2 \times 10^6$  cells were cultured in the absence or presence of increasing concentrations of LPS (0.2 – 100ng/ml) for 0 – 18 hours. The total cellular RNA was isolated from cells using Trizol reagent (Invitrogen) according to the manufacturer's protocol. 1.5 ug RNA was used to synthesize cDNA using the High Capacity cDNA reverse transcription kit protocol (Applied Biosystems). An iQ SYBR Green Supermix (Bio-Rad) was used for real-time PCR, and the amplifications were performed on an iQ5 Multicolor Real-Time PCR detection system (Bio-Rad). mRNA levels of NADPH oxidase 1 (Nox1) and nuclear factor (erythroid-derived 2)-like 2 (Nrf2) were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Table 4.1 contains the sequence of the primer sets used for each gene.

### *Protein Expression Studies: Whole Cell Isolation*

Proteins were extracted from whole cell extracts of MEF to examine the relative amounts of Nox1, Nrf2, and GAPDH found in each sample. MEF were plated at a density of  $3 \times 10^6$ /plate on 60mm plates. Cells were allowed to rest in 1% FBS DMEM for 4 hours, and then treated with different concentrations of LPS for 0, 15, and 30 minutes, and 1, 2, 4, 6, 8 hours. After treatment the cells were washed once with PBS (Sigma, Dulbeccos Phosphate Buffered Saline) and incubated 150 $\mu$ L lysis buffer (50mM HEPES pH 7.6, 150mM NaCl, 1mM EDTA, 0.5% NP-40, 1X Phosphatase Inhibitor 1, 1X Phosphatase Inhibitor 2, 1X Protease Inhibitor) for 20 minutes. Samples were then centrifuged for 10 minutes at 4°C at 3000rpm and the supernatant was removed and stored at -20°C. Total protein sample concentrations were determined using a kit for Bradford protein assay (Bio-Rad). Supernatant samples were subsequently used for Western

blotting or immunoprecipitation studies. Samples used for total protein Western blot were boiled at 100°C with 5uL 5X SDS sample buffer for 10 minutes.

### *Immunoprecipitation*

The Nrf2 antibody used for immunoprecipitation was a rabbit polyclonal antibody raised against a section of the human Nrf2 N-terminus, obtained from Santa Cruz biotechnology. The protein lysate samples (500ug) were mixed with antibodies (2ug), and then incubated for 1 hour at 4°C. After incubation, the lysate/antibody solution was treated with Protein A/G Plus Agarose beads (Santa Cruz Biotechnology), and was incubated overnight at 4°C while rotated constantly. The samples were washed 3 times with lysis buffer, treated with 5X laemmli buffer and boiled for 10 minutes to separate the protein from the beads. After boiling, samples were centrifuged, and the supernatants were used for Western blotting.

### *Western Blot*

Protein samples were loaded onto a protein minigel (10% SDS gel, as outlined below), and run in 1X SDS gel running buffer at 80V for 20 minutes, and then 120V until dye 90 minutes. The gels were then transferred to a PVDF for 2 hours at 110V in transfer buffer with ice surrounding the apparatus. After transfer the membrane was blocked for 2 hours at room temperature in 5% skim milk prepared in TBS-T. Membranes were then exposed to the primary antibody diluted in 5% skim milk to antibody specifications overnight at 4°C on a rocker. The Nrf2 antibody was a rabbit polyclonal antibody raised against a section of the N-terminus of Nrf2 of human origin. The GAPDH (control) antibody was a rabbit polyclonal antibody raised against the full length human GAPDH. The Nox1 antibody was obtained from Dr. J.B. Lambeth at Emory School of

Medicine. After incubation, the membranes were washed five times in TBS-T on a shaker at room temperature for 10 minutes each. Membranes were then incubated in the secondary antibody for 1 hour at room temperature. The rabbit secondary antibody (Jackson ImmunoResearch Laboratories) was a peroxidase conjugated monoclonal mouse anti-rabbit IgG antibody. After incubation, the membranes were again washed (5X with TBS-T, 10 minutes per wash) and then developed by chemiluminescence detection using the ECL kit from Amersham.

<b>Protein Minigel Recipe:</b>	<b>Separating Gel (10%)</b>	<b>Stacking Gel</b>
30% acrylamide	3.33 ml	650 $\mu$ l
10% SDS	100 $\mu$ l	50 $\mu$ l
1M Tris-HCl	3.75 ml (pH 8.8)	630 $\mu$ l (pH 6.8)
H <sub>2</sub> O	2.92 ml	3.64 ml
10% APS	50 $\mu$ l	25 $\mu$ l
TEMED	10 $\mu$ l	5 $\mu$ l

**10X SDS Gel Running Buffer (1L):**

Tris Base	30 g
Glycine	144 g
SDS	10 g
H <sub>2</sub> O	1 L

**Western Blot Transfer Buffer (1L):**

Tris Base	2.9 g
Glycine	14.5 g
Methanol	200 ml
H <sub>2</sub> O	800 ml

**1X TBS-T**

NaCl	150 mM
Tris-HCl, pH 8.0	10 mM
Tween 20	0.05%



<i>Gene</i>	<i>Accession Number</i>	<i>Size</i>	<i>Sequence</i>
IL-6	NM_031168	1,087 bp	Forward: 5' ATCCAGTTGCCTTCTTGGGACTGA 3' Reverse: 5' TAAGCCTCCGACTTGTGAAGTGGT 3'
MMP3	NM_010809	1,858 bp	Forward: 5' TGGAACAGTCTTGGCTCATGCCTA 3' Reverse: 5' TGGGTACATCAGAGCTCAGCCTT 3'
Nox1	NM_172203.1	1,692 bp	Forward: 5' TCCATTCCTTCCTGGAGTGGCAT 3' Reverse: 5'GGCATTGGTGAGTGCTGTTGTTCA 3'
Nrf2	NM_010902	2,469 bp	Forward: 5' TAAAGCTTTC AACCCGAAGCACGC 3' Reverse: 5' TCCATTCCGAGTCACTGAACCCA 3'
GAPDH	NM_008084	1,254 bp	Forward: 5' ACTTTGGCATTGTGGAAGGGCTC 3' Reverse: 5' TGGAAGAGTGGGAGTTGCTGTTGA 3'

**Table 4.1.** *Primer Sequences used for Real-Time PCR*

## **Chapter 5: The Role of Interleukin 1 Receptor Associated Kinase 1 (IRAK-1) in the Induction and Activation of NADPH Oxidase 1 (Nox1)**

### **Abstract**

Bacterial endotoxins (lipopolysaccharide (LPS)) are known to cause tissue damage and injury. One of the mechanisms responsible for this damage and injury is the production of reactive oxygen species (ROS). In inflammation, ROS are produced as part of defensive immune responses, as they are capable of destroying invading pathogens. However, the targets of ROS are not specific so while they can be produced to extirpate pathogens, they can also damage host tissues. It is widely accepted that ROS production occurs in an LPS dependent manner due to the activation of NADPH oxidases (Nox). However, the molecular mechanisms involved in LPS dependent ROS production by Nox are not clearly understood. In this study the role of interleukin-1 receptor associated kinase 1 (IRAK-1) in the production of reactive oxygen species by Nox1 in fibroblasts was examined. We show here that IRAK-1 is necessary for LPS induced ROS production. IRAK-1 deficient fibroblasts showed significantly less ROS production in response to LPS than wild type fibroblasts. Furthermore, we have shown that ROS production in wild type cells occurs in a dose dependent manner, but a dose dependent response in IRAK-1 deficient cells was not observed. We hypothesize that LPS is activating Nox1 through toll-like receptor 4 (TLR4). One of the pathways activated through TLR4 is IRAK-1 dependent, and it is this pathway that we believe is responsible for activating Nox1. In accordance with this idea, we have seen that high doses of LPS are capable of increasing cellular levels of Nox1 mRNA (as measured by real time PCR) and protein (measured by western blot) in wild type cells, but not in IRAK-1 deficient MEF. Low dose LPS is capable of increasing Nox1,

but the response takes much longer and is not as IRAK-1 dependent. This suggests the potential for two different pathways that can be activated in a dose dependent manner.

## **Introduction**

Reactive oxygen species (ROS) are oxygen derived small molecules that either are, or can be easily converted into oxygen radicals [11, 130]. They are known to play several different roles in cells amongst which are the oxidation of proteins and lipids. ROS activity eventually leads to tissue damage and as a result, chronic disease and organ failure. The generation of ROS is regulated by oxidative enzymes that promote the production of ROS and by antioxidant enzymes (including superoxide dismutase, catalase, and glutathione peroxidase). ROS are known to be produced by cytochrome P450, by mitochondria, within peroxisomes, and by NADPH oxidase [134-142]. Some of these sources produce ROS as part of normal cellular functions, (eg metabolic respiration and catabolism of fatty acids), however other sources are known to produce ROS in response to certain stimulants. Lipopolysaccharide is found in the cell wall of Gram-negative bacteria. It is known to induce the expression of oxidative enzymes while decreasing the expression of antioxidative enzymes [251, 252]. As part of the normal inflammatory response to LPS, especially the associated endotoxin, cells produce ROS and contribute to the pathogenesis of several different inflammatory diseases.

The NADPH oxidase family is the only family of proteins whose primary purpose is the production of ROS. The first member of the NADPH oxidase family to be discovered was Nox2 [182]. Much of the work published on Nox is focused on the activation and regulation of Nox2, the phagocyte Nox. The activation of Nox2 occurs through a complex series of protein-protein interactions. Nox2 is constitutively associated with p22phox and it has been suggested

that Nox2 is unstable in the absence of p22phox [185-187]. The Nox2/p22phox complex is found in membranes, requiring other cytosolic factors to translocate to the complex. The other members of the activated Nox2 complex are p47phox, p67phox, p40phox, and Rac. The p47phox protein is phosphorylated, causing a conformational change which then allows it to interact with p22phox. The "organizer subunit," p47phox is considered to be responsible for the organization of the translocation of the cytosolic subunits. Once p47phox has been localized to the membrane, it comes into contact with p67phox, the "activator subunit." This brings the activator subunit into contact with Nox2 [190], while also bringing p40phox into the complex. Finally, Rac, a small GTPase interacts with Nox2 [22] as well as p67phox [191, 192]. Upon assembly the complex is active and can transfer an electron from NADPH to oxygen, creating superoxide. It is already known that stimulation with LPS causes translocation and the subsequent activation of Nox2 [183]. The second member of the Nox family to be discovered was Nox1 [193, 194]. It can be transcriptionally induced by LPS as well as be post-transcriptionally activated. However, the mechanisms of this induction and activation are poorly understood.

The pathway activated by LPS is the toll-like receptor 4 (TLR4) pathway. One of the downstream components of the TLR4 pathway is interleukin-1 receptor associated kinase-1 (IRAK-1) [4, 253, 254]. IRAK-1 is known to be involved in the activation of NFκB, STAT1/3, and IRF5/7, while being involved in negatively regulating NFAT and nuclear receptors [70, 76, 81, 104, 106, 255]. However despite the importance of IRAK-1 in the TLR4 signaling pathway, its involvement in the LPS dependent production of ROS has not been characterized.

It has been hypothesized that IRAK-1 is necessary for the production of reactive oxygen species in response to LPS due to its prominent role in the TLR4 signaling pathway. In this

study, the production of reactive oxygen species in response to LPS was examined as well as the levels of Nox1 mRNA and protein.

## Materials and Methods

*Reagents:* LPS (*Escherichia coli* O111:B4) was obtained from Sigma. The antibody against Nox1 was obtained from Dr. J.B. Lambeth at Emory School of Medicine. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used to normalize Nox1 levels, the antibody against GAPDH was obtained from Santa Cruz. Both are rabbit polyclonal antibodies. The secondary antibody used was a HRP conjugated monoclonal mouse anti-rabbit IgG antibody from Jackson ImmunoResearch Laboratories. Primer sets were obtained from IDT, the sequences are as follows: *IL-6*(+), 5'-ATCCAGTTGCCTTCTTGGGACTGA-3', and *IL-6*(-), 5'-TAAGCCTCCGACTTGTGAAGTGGT-3'; *MMP3*(+), 5'-TGGAACAGTCTTGGCTCATGCCTA-3', and *MMP3*(-), 5'-TGGGTACATCAGAGCTCAGCCTT-3' *Nox1*(+), 5'-TCCATTTCCCTTCCTGAGTGGCAT-3', and *Nox1*(-), 5'-GGCATTGGTGAGTGCTGTTGTTCA-3'; *GAPDH*(+), 5'-ACTTTGGCATTGTGGAAGGG CTC-3', and *GAPDH*(-), 5'-TGGAAGAGTGGGAGTTGCTGTTGA-3'.

*Mice and Murine Cells:* Wild type C57BL/6 mice were purchased from Charles River Laboratories. IRAK-1<sup>-/-</sup> mice with C57BL/6 background were provided by Dr. James Thomas of University of Texas Southwestern Medical School. All mice were housed and bred in Derring Hall Animal Facility in compliance with approved Animal Care and Use Committee protocols at Virginia Polytechnic Institute and State University. Mouse embryonic fibroblasts (MEF) were isolated and cultured as previously described [250]. MEF were allowed to rest in 1% FBS

DMEM for 4 hours prior to treatment with LPS. The results presented are based on 2-4 experiments of 2-3 replicates each.

*Protein Analyses:* Whole cell lysates were isolated as described earlier and Western blot technique was used to quantify the expression of Nox1 protein. Treated and untreated MEF were rinsed in PBS and lysed on ice in lysis buffer (50mM HEPES pH 7.6, 150mM NaCl, 1mM EDTA, 0.5% NP-40, 1X Phosphatase Inhibitor 1, 1X Phosphatase Inhibitor 2, 1X Protease Inhibitor). Total protein concentrations were determined using by Bradford protein assay (kit obtained from Bio-Rad). Thirty micrograms of protein were used for western blot analysis, performed as previously described. Immunoblots were developed using the ECL Plus chemiluminescent detection system kit by Amersham Biosciences (GE Healthcare). The intensities of the bands were quantified using the Fujifilm MultiGauge software and normalized to GAPDH levels.

*Nox1 Gene Expression:* Nox1 gene expression was evaluated in LPS treated and untreated wild type and IRAK-1 deficient MEF using real-time RT-PCR. Total RNA was extracted from the cells using TRIzol (Invitrogen, according to manufacturers protocol), and reverse-transcription was accomplished us the high capacity cDNA reverse transcription kit (Applied Biosystems). Real-time PCR analyses were performed using SYBR green supermix on an IQ5 thermocycler (Bio-Rad). Relative transcript levels were normalized using GAPDH and were analyzed using the  $\Delta\Delta C_t$  method.

*Intracellular ROS Measurement:* The fluorescent probe chloromethyl-2',7'-dichlorofluorescein diacetate (Molecular Probes) was used to monitor intracellular ROS accumulation. DCFDA is deacetylated and reacts with intracellular ROS which converts it to its fluorescent form, dichlorofluorescein which is retained within the cells. WT and IRAK1<sup>-/-</sup> MEF (4x10<sup>4</sup> cells/well in 96 well plates) were treated with 10µM chloromethyl-2',7'-dichlorofluorescein diacetate (DCFDA) for 30 minutes at 37°C. The cells were then washed with PBS containing calcium and magnesium. Cells were then allowed to rest for 20 minutes and treated with a range of LPS concentrations (0.05 – 100 ng/ml). Readings were taken 15 minutes prior to the start of the experiment, immediately before LPS exposure, and after 15 minutes of LPS exposure. The treatments response slopes (slope from the beginning of exposure to 15 minutes after exposure) were corrected for the pre-treatment response slopes (slope from 15 minutes prior to treatment to beginning of treatment).

*Statistical Analyses:* Statistical significance was determined using the paired two-tailed Student's t-test. Data was considered to be statistically significant if the p value was less than 0.05.

## **Comprehensive Optimization of Experimental System**

Mouse embryonic fibroblasts are often used for cell signaling studies. In vivo, fibroblasts are responsible for laying down extracellular matrix and synthesizing collagen. However, despite having a defined function, these cells can also be differentiated into other cell types. In order to ensure that MEF being used for these studies maintain the ability to respond to inflammatory stimuli in both an inflammatory and an anti-inflammatory manner, wild type MEF were challenged with LPS for 2 hours. At the end of LPS challenge, total RNA was collected

and the levels of interleukin 6 (IL-6) and matrix metalloproteinase-3 (MMP3) were measured by real time PCR. Interleukin 6 is a pro-inflammatory cytokine. Administration of IL-6 causes fever, inflammation, tissue destruction, and in severe cases can cause shock or even death. The MEF used in these studies were capable of increasing IL-6 mRNA in response to LPS challenge (Figure 5.1). Matrix metalloproteinase-3 is a member of the matrix metalloproteinase family responsible for breaking down extracellular matrix during wound healing. Increased levels of MMP3 represent an anti-inflammatory phenotype. Wild type MEF exhibited increased levels of MMP3 mRNA after LPS challenge.

The ability of MEF to respond to an inflammatory challenge in both a pro- and anti-inflammatory manner illustrates that the cells used in this study were not previously committed to either phenotype. Furthermore, the same samples were used to measure both IL-6 and MMP3 mRNA levels indicating that even after challenge these cells maintained both anti and pro-inflammatory phenotypes. Finally, these data demonstrate that the MEF used in these studies were capable of responding to LPS challenge.

## **Results**

*LPS induces the production of reactive oxygen species in a dose and IRAK-1 dependent manner:* In order to determine whether or not LPS induces intracellular ROS production, ROS levels were measured in wild type MEF. Prior to LPS treatment, MEF were stained with DCFDA which fluoresces following interaction with reactive oxygen species. Fluorescence intensities were measured using a fluorescent plate reader. Increasing amounts of LPS induced an increase in ROS production in wild type MEF (Figure 5.3). The linear regression model for ROS production in response to LPS in wild type MEF has an  $R^2$  value of 0.9257, indicating a



high positive linear relationship between the concentration of the LPS challenge and the amount of ROS produced.

In order to determine the dependence of IRAK-1 in LPS induction of ROS production, ROS levels were measured in wild type and IRAK-1 deficient MEF. Wild type MEF clearly exhibited an increase in ROS production as the concentration of LPS challenge increased. However, in IRAK-1 deficient cells, once the concentration of the LPS treatment reached 200pg/ml (and above) the rate of ROS production was significantly less in response to LPS in IRAK-1 deficient MEF when compared to wild type MEF (Figure 5.4). LPS concentrations below 200pg/ml showed little increase in ROS production in either wild type or IRAK-1 deficient cells. Furthermore, the ROS produced by IRAK-1 deficient cells did not consistently increase as the concentration of LPS used to treat the cells increased. Therefore, LPS was able to induce significantly more ROS production in wild type MEF than in IRAK-1 deficient MEF. In wild type cells, the amount of ROS produced was dose dependent, whereas in the IRAK-1 deficient cells, there was significantly less dose dependent response.

*LPS causes an increase in Nox1 mRNA in a dose dependent manner in wild type, but not IRAK-1 deficient MEF:* Previous work has shown that LPS is capable of inducing the expression of Nox1 [256]; however the mechanisms responsible for this induction are not very well understood. Since we have seen an increase in ROS, which is known to be produced by Nox1, in wild type but not IRAK-1 deficient MEF it would follow that IRAK-1 may be involved in the induction of Nox1. Therefore, wild type and IRAK-1 deficient MEF were treated with a range of concentrations of LPS for 2 hours. Total RNA was harvested and the amount of Nox1 mRNA present was measured by real time RT-PCR using these samples. For both low (200pg/ml) and

high (100ng/ml) doses of LPS, there were significant differences between the amount of Nox1 message present in wild type (Figure 5.5) and IRAK-1 deficient MEF (Figure 5.6). LPS actually appears to decrease the amount of Nox1 mRNA present in IRAK-1 deficient cells (Figure 5.6). However, the wild type cells showed a definite dose response to LPS exhibiting a positive correlation ( $R^2$  value of 0.9541) between the increase in LPS concentration and Nox1 mRNA levels (Figure 5.5). The levels of Nox1 were down-regulated after 2 hours of exposure to very low levels of LPS (200pg/ml and 1ng/ml). However, in the higher dose (10ng/ml, 50ng/ml, and 100ng/ml) treatments the levels of Nox1 mRNA were significantly elevated in wild type MEF (Figure 5.5). Thus, it appears that in agreement with ROS production, wild type MEF respond to LPS with more Nox1 mRNA in a dose dependent manner whereas the amount of Nox1 mRNA present in IRAK-1 is decreased in response to LPS stimulation.

*IRAK-1 is necessary for LPS induced expression of Nox1.* Since we have seen increased production of ROS and increases in Nox1 mRNA in response to LPS in wild type MEF but not IRAK-1 deficient MEF, we would expect to see a similar increase in the amount of Nox1 protein present in wild type MEF but not in IRAK-1 deficient MEF. Both types of cells (wild type and IRAK-1 deficient) were treated with LPS for 2 to 6 hours. Total protein lysates were collected and equal amounts of protein were used to conduct western blot analyses (Figure 5.7). Interestingly both wild type and IRAK-1 deficient cells exhibit similar basal expression levels of Nox1 protein (Figure 5.7). In agreement with the levels of Nox1 mRNA, an increase in wild type Nox1 protein levels was observed after 2 hours of stimulation with 100ng/ml LPS (Figure 5.7). This increase in Nox1 protein was transient, increasing sharply at 2 hours then declining over the remaining time points. In contrast, there were no increases in the amount of Nox1

protein present in IRAK-1 deficient cells, and in fact LPS exposures caused a decrease in the amount of Nox1 protein present in these cells, in accordance with the IRAK-1 deficient mRNA data. Thus, IRAK-1 is required for high dose (100ng/ml) LPS induced increase in Nox1 protein levels.

*Low dose LPS causes very little change in Nox1 protein levels in either wild type or IRAK-1 deficient MEF.* Wild type and IRAK-1 deficient MEF treated with low dose (200pg/ml) LPS for up to 6 hours showed no significant change in Nox1 protein levels. Whole cell protein extracts were collected from wild type and IRAK-1 deficient MEF after low dose LPS stimulation. Equal amounts of total protein were analyzed by western blot technique as described above. Wild type MEF exhibit a potential cycling of Nox1 protein, decreasing after 2 hours of LPS challenge, returning to basal levels after 4 hours of challenge, and decreasing again after 6 hours of challenge (Figure 5.8). However the differences between the band intensities is not significant. IRAK-1 deficient MEF also showed very little change in Nox1 protein levels in response to low dose LPS stimulation. There is a slight increase in band intensity after 2 hours of LPS challenge, which appears to decrease over time (Figure 5.4). However the differences in band intensity are hard to detect. Thus, low dose LPS does not significantly affect levels of Nox1 protein in either wild type or IRAK-1 deficient MEF.

*High dose LPS causes a transient increase in Nox1 mRNA in wild type, but not IRAK-1 deficient MEF.* After observing an increase in protein and ROS production in response to LPS in wild type MEF but not IRAK-1 deficient MEF, we would expect to see a similar trend in Nox1 mRNA levels in wild type and IRAK-1 deficient MEF. Wild type and IRAK-1 deficient MEF

were treated with LPS for 2-18 hours. Total RNA were collected from these cells and equal amounts of RNA were used to perform reverse transcription and real time PCR (Figure 5.9). In agreement with Nox1 protein levels, we see a quick increase in the Nox1 mRNA levels in wild type MEF. However, the increase is transient, after 4 hours of high dose (100ng/ml) LPS challenge, levels of Nox1 mRNA are rapidly decreasing. After 8 hours of high dose LPS stimulation, Nox1 mRNA levels drop below basal levels and are maintained well below basal levels for the duration of the experiment. Also in agreement with Nox1 protein levels are Nox1 mRNA levels in IRAK-1 deficient MEF. After LPS stimulation Nox1 mRNA levels decrease and are maintained below baseline levels for the duration of the experiment. Therefore, it can be concluded that IRAK-1 is necessary for the increase in Nox1 mRNA exhibited in response to high dose (100ng/ml) LPS.

*Low dose LPS induces a slower, more sustained increase in Nox1 mRNA than high dose LPS.* After observing a fast, transient increase of Nox1 mRNA in response to high doses of LPS, we then investigated the effects of a subclinical dose of LPS on the levels of Nox1 mRNA over a longer period of time. Upon treatment with 200pg/ml LPS, the immediate cellular responses of wild type MEF were reduce levels of Nox1 mRNA (Figure 5.10). However, within 6 hours of stimulation, the levels of Nox1 mRNA return to basal levels. Furthermore, after 10 hours of low dose LPS treatment, the levels of Nox1 mRNA present in these cells were increased above the basal level. Once the levels of Nox1 mRNA increase above basal levels we see a cyclic dampening of these levels. This may suggest that Nox1 activity in response to low dose LPS activates a feedback loop, shutting off Nox1 transcription or increasing Nox1 degradation. Once levels Nox1 mRNA levels return to basal levels, Nox1 mRNA levels are allowed to rise again

until they cross a threshold activating this feedback loop. This would appear to suggest that longer low dose LPS will eventually elicit an increase in Nox1 mRNA in wild type MEF and then a dampened increase/decrease cycle until levels return to normal (Figure 5.10). However, for IRAK-1 deficient MEF, it appears that the longer the system is treated with low dose LPS, the less the amount of Nox1 mRNA is allowed to accumulate in the cells (Figure 5.10). This would suggest that IRAK-1 is necessary for the intracellular build-up of Nox1 mRNA in response to low dose LPS. There were no data collected for IRAK-1 deficient MEF after 12 hours of stimulation. After 12 hours of LPS challenge, IRAK-1 deficient cells appeared to be significantly unhealthy: cells were detached from culture plates and clumped together, and it was determined that these samples were not fit for analysis. Furthermore, this may suggest that there is also an IRAK-1 independent feed-back loop responsible for controlling the amount of Nox1 mRNA present in MEF. Thus by activating the feedback loop and not allowing for the IRAK-1 dependent increase in Nox1 the amount of Nox1 mRNA in the cells continually decreases.

## **Discussion**

In this study, we have examined the role that IRAK-1 plays in the production of reactive oxygen species as well as in the accumulation of Nox1 protein and mRNA levels within mouse embryonic fibroblasts. We have begun to define a mechanism by which the induction and potentially the activation of Nox1 is IRAK-1 dependent in MEF. It has been identified that IRAK-1 is vital to LPS induced production of reactive oxygen species. Furthermore, we have shown that IRAK-1 is also critical for increases in Nox1 mRNA and the accumulation of Nox1 proteins in MEF. Collectively, IRAK-1 deficient MEF showed less ROS production in response to LPS, as well as less accumulation of Nox1 in cells. The LPS exposure levels were responsible

for differential regulation of ROS production as well as differentially regulating the accumulation of Nox1 within MEF.

These data indicated that subclinical concentrations of LPS stimulation caused increased ROS production compared to untreated samples. With acute exposures to high dose LPS (100ng/ml), ROS production rates were higher than either untreated MEF or those exposed to low doses. Considering potential sources of intracellular ROS production, we hypothesized that the reason for this increase in ROS is the activation of basal levels of Nox1. Our experiments have shown that both wild type and IRAK-1 deficient MEF contain basal levels of Nox1 protein. Thus we believe that LPS is able to activate basal levels of Nox1, thereby immediately increasing the rate of ROS production within MEF. Furthermore, despite having comparable basal levels of Nox1, ROS were produced in response to LPS at a higher rate in wild type cells than in IRAK-1 deficient cells. Similarly, an increase in the levels of Nox1 protein in wild type MEF in response to LPS was observed, whereas the levels of Nox1 protein in IRAK-1 deficient cells did not increase and actually decreased in response to LPS.

Furthermore, a significant difference between the amount of Nox1 protein and mRNA present in different lines of MEF was also observed. Wild type cells treated with 100ng/ml LPS showed an increase in the amount of Nox1 mRNA and protein after 2 hours of LPS challenge. After 6 hours of 100ng/ml LPS challenge, Nox1 protein levels remain increased above basal levels corresponding with increased levels of Nox1 mRNA after 6 hours of high dose LPS (100ng/ml) challenge. In contrast, after 8 hours of high dose LPS treatment, cellular levels of Nox1 mRNA dropped to half the basal level, and these decreased levels of mRNA were maintained for the remainder of the experiment (18 hours). This suggests that after high dose LPS challenge it takes a significant amount of time (greater than 18 hours) for the system to reset

itself as would be seen by a return to basal levels in Nox1 mRNA levels. In concurrence with the decrease in Nox1 protein found in IRAK-1 deficient MEF after 100ng/ml LPS challenge, cellular Nox1 mRNA levels are decreased and maintained below basal levels for the duration of the experiment. This indicates that high doses of LPS are capable of inhibiting Nox1 production after longer stimulations in both IRAK-1 deficient and wild type cells. Thus, IRAK-1 is necessary for upregulation of Nox1 protein in MEF.

MEF treated with low doses of LPS for an extended period of time showed a completely different response to high dose LPS. The initial response of the cells to low dose LPS treatment was a decrease in the amount of Nox1 mRNA. However, longer stimulation with low dose LPS led to an increase in the amount of Nox1 present in these cells, followed by a cyclic decrease in the level of Nox1 mRNA, reaching basal levels after 16-18 hours of low dose LPS challenge. IRAK-1 deficient cells treated with low dose LPS showed a small initial increase in Nox1 mRNA levels (not seen in wild type cells) that was quickly followed by a drop in Nox1 levels. These reduced levels were sustained longer than that of wild type cells. Once Nox1 mRNA levels started increasing, expression levels did not get as high as in wild type cells, but was maintained close to basal levels between 10 and 12 hours. Therefore, this indicates that IRAK-1 may be involved in the delayed, but increased expression of Nox1 in response to low dose LPS; however it is not required in order to recover basal Nox1 mRNA levels.

The differences in expression of Nox1 in response to low and high doses of LPS, as well as the differences between wild type and IRAK-1 deficient MEF, suggest that high doses of LPS are capable of activating Nox1 which results in increased ROS levels, as well as a brief increase in the expression of Nox1 in wild type cells. The transient nature of this response also suggest activation of an antioxidative (anti-Nox1) pathway that is then able to inhibit the expression of

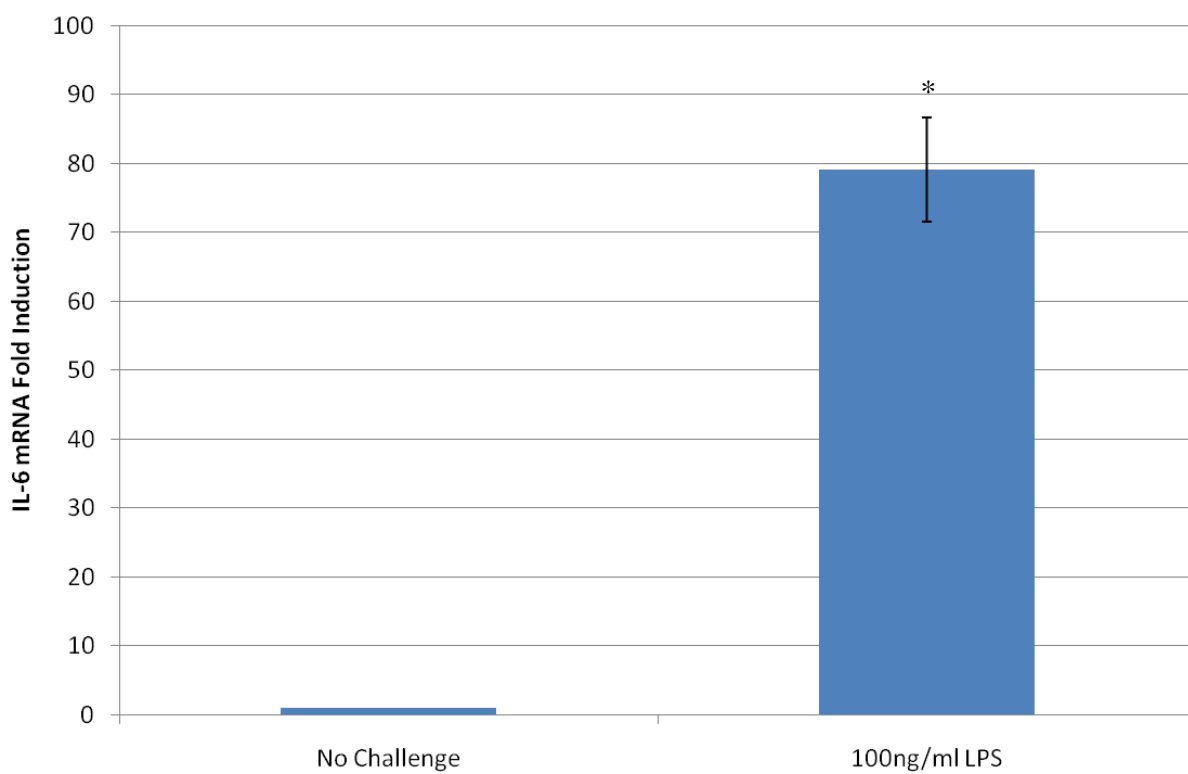
Nox1 resulting in lower than baseline levels of Nox1 protein and mRNA. We hypothesize that eventually this antioxidative feedback loop is shut off and will allow Nox1 to return to basal levels. However, this return to basal levels takes more than 18 hours as Nox1 mRNA levels remained below basal levels after 18 hours of 100ng/ml LPS challenge. Furthermore, IRAK-1 is required for the initial activation and increase of Nox1 whereas it does not or plays a less vital role in the negative feedback loop regulating Nox1. Low doses of LPS resulted in initial activation of Nox1 and then the subsequent increase in the rate of production of ROS. This increase was enough to initiate the antioxidative feedback loop for a short period of time and to a lesser degree than high dose LPS. After initial activation, this feedback loop caused a decrease in Nox1 mRNA expression (and presumably in a decrease in ROS production rates). Over a period of 6-8 hours, the system returned to normal Nox1 mRNA levels, and finally increased above basal levels (after 10 hours of LPS challenge). At this point, the antioxidative feedback loop gets turned on and Nox1 mRNA levels decrease. This cycle continues for at least 18 hours exhibiting a sinusoidal dampening in the expression of Nox1 mRNA until reaching true baseline levels when this antioxidative feedback loop is no longer activated. In IRAK-1 deficient MEF the feedback loop was activated after a short delay, so Nox1 mRNA increased after 2 hours of 200pg/ml LPS challenge, but then decreased after 4 hours of LPS challenge. Furthermore, the system takes longer to recover, not rising above basal levels of Nox1 mRNA expression until after 10 hours of LPS challenge. Finally, instead of the increase and subsequent decrease observed in wild type cells, IRAK-1 deficient cells maintain Nox1 mRNA expression close to basal levels. Thus we believe that increased expression of Nox1 in response to LPS is dependent on IRAK-1. However there are other pathways involved in maintaining basal levels of Nox1 as



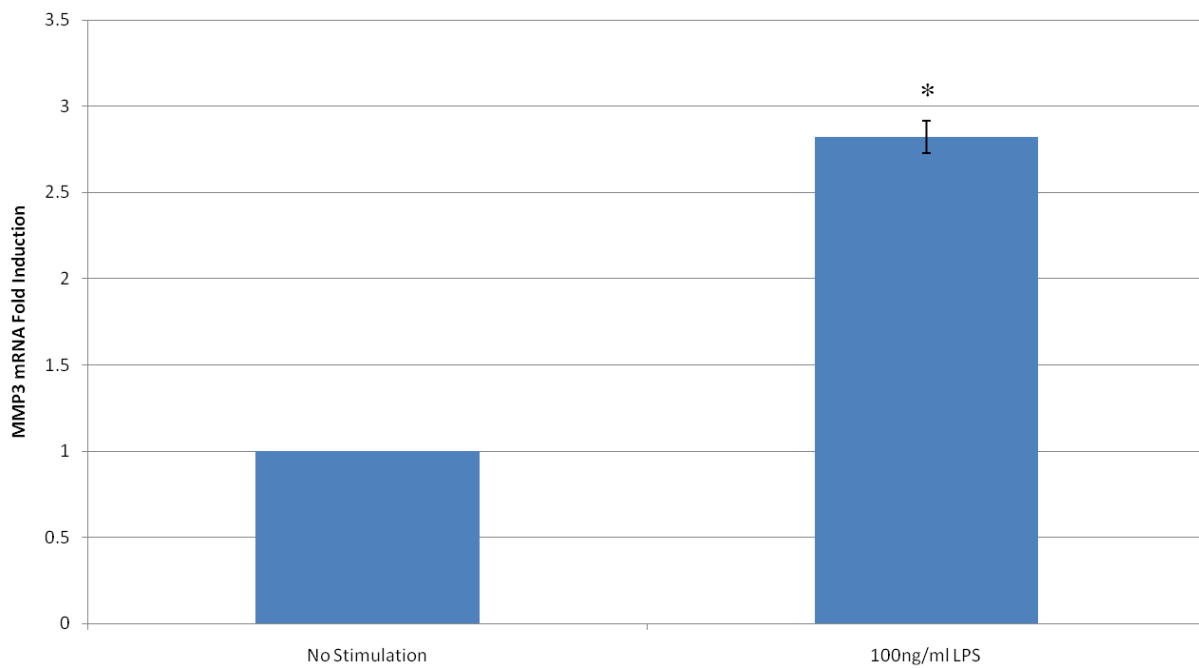
shown by the presence of Nox1 protein in IRAK-1 deficient MEF without LPS stimulation and by the increase in Nox1 mRNA back to basal levels.

Questions remain concerning the activation and regulation of the negative feedback loop activated by high doses of LPS. Previous work has shown that the presence of reactive oxygen species is capable of activating proteins critical to anti-oxidative feedback loops. One of these proteins, Nrf2, is capable of increasing transcription of antioxidative genes as well as increasing transcription of itself. This allows the initial activation by ROS to increase activation and transcription of Nrf2, further enhancing the transcription of anti-oxidative genes to inhibit the further production of ROS.

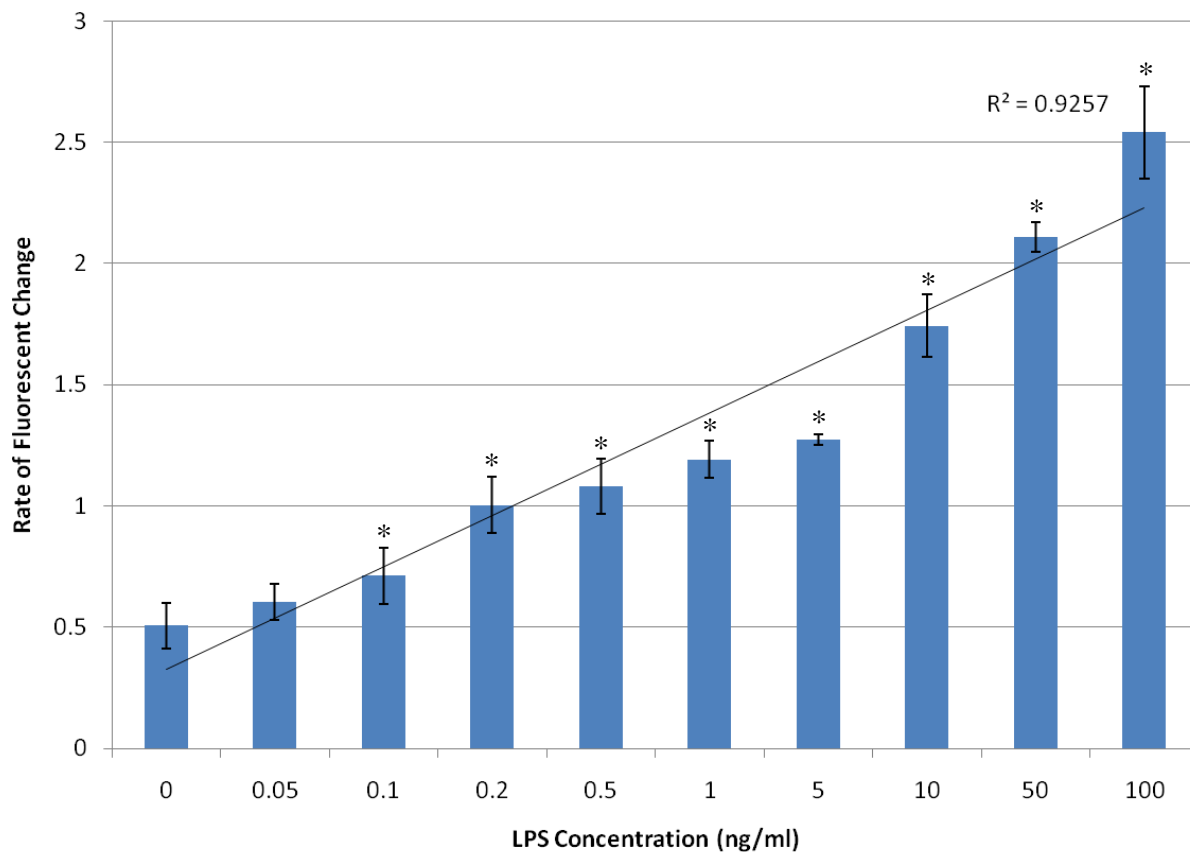
Our current work has revealed a new role for IRAK-1 as a potential regulator of the production of reactive oxygen species by Nox1 by regulating Nox1 expression. We have also shown that LPS is able to cause a decrease in Nox1 expression in an IRAK-1 independent manner, indicating the potential existence and activation of an IRAK-1 independent anti-oxidative feedback loop.



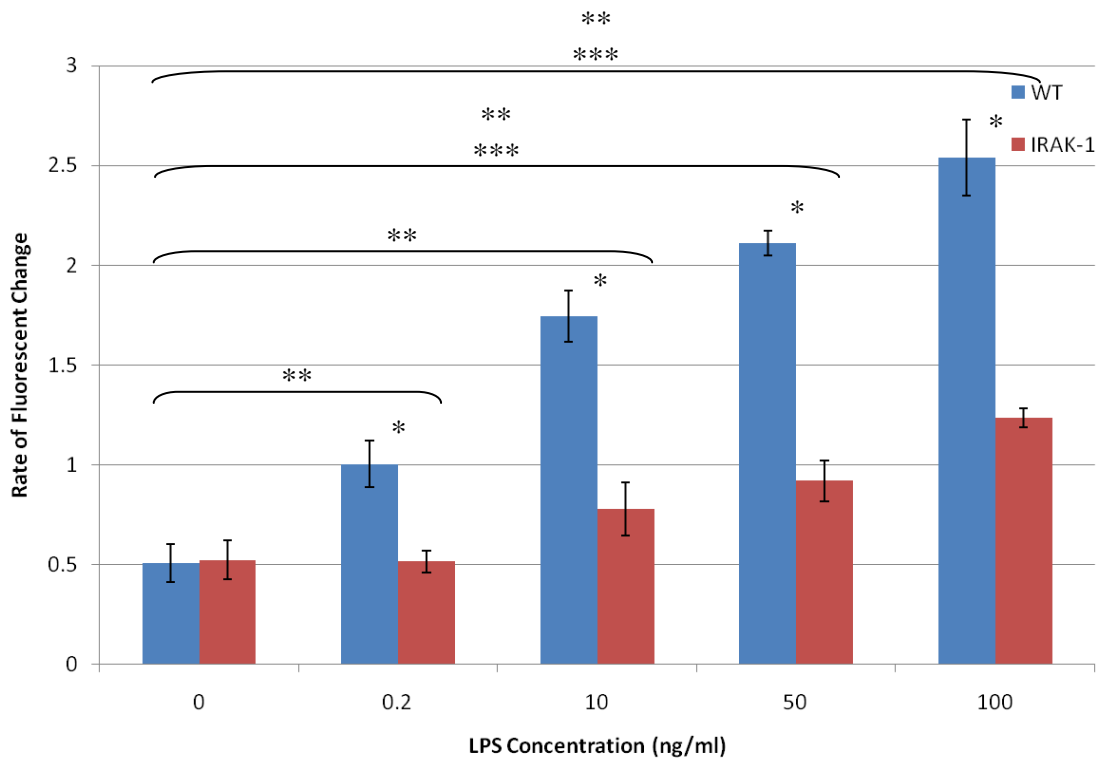
**Figure 5.1.** *LPS induces the production of pro-inflammatory mediators in mouse embryonic fibroblasts.* MEF were treated with 100ng/ml LPS for 2 hours. RNA was isolated and levels of the inflammatory cytokine IL-6 mRNA were measured by real time PCR. This plot represents an average of three independent experiments. The data presented are means +/- standard deviations. \*  $p < 0.01$ .



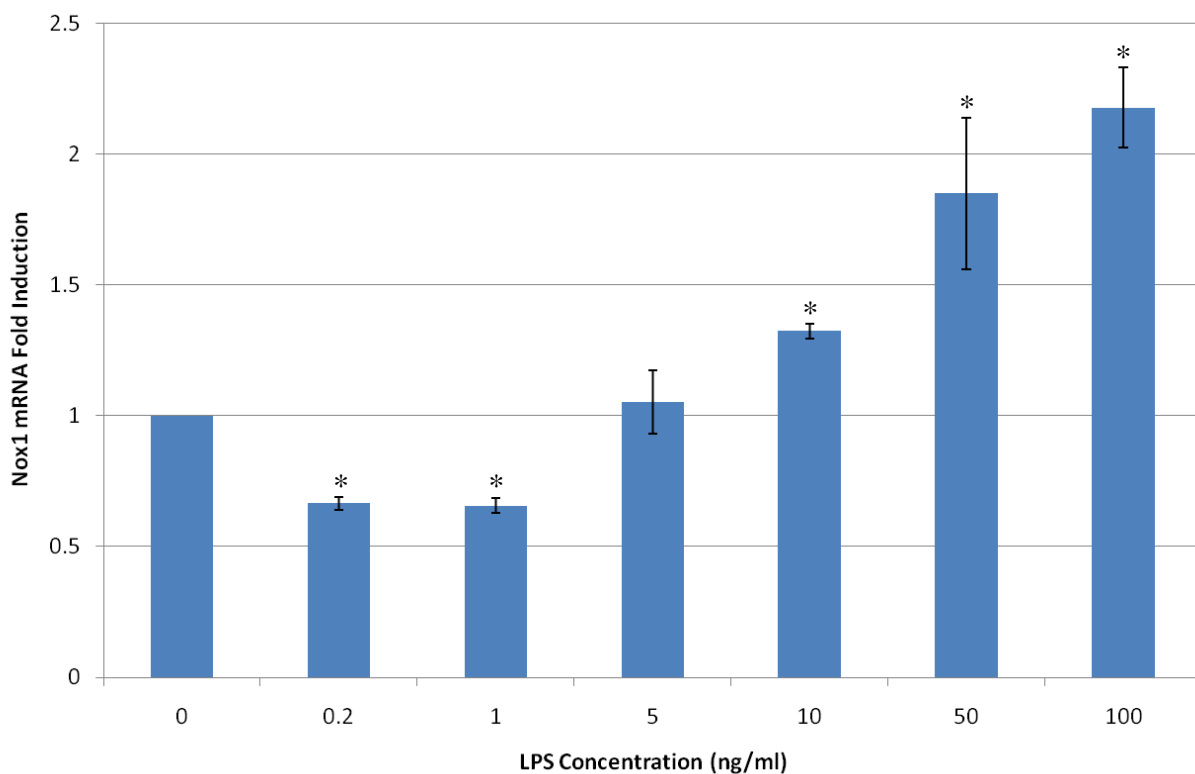
**Figure 5.2.** *LPS induces the production of anti-inflammatory mediators in mouse embryonic fibroblasts.* MEF were treated with LPS for 2 hours. Total RNA was isolated and the levels of matrix metalloproteinase 3 were measured by real time PCR. This plot represents an average of three independent experiments. \*  $p < 0.01$ .



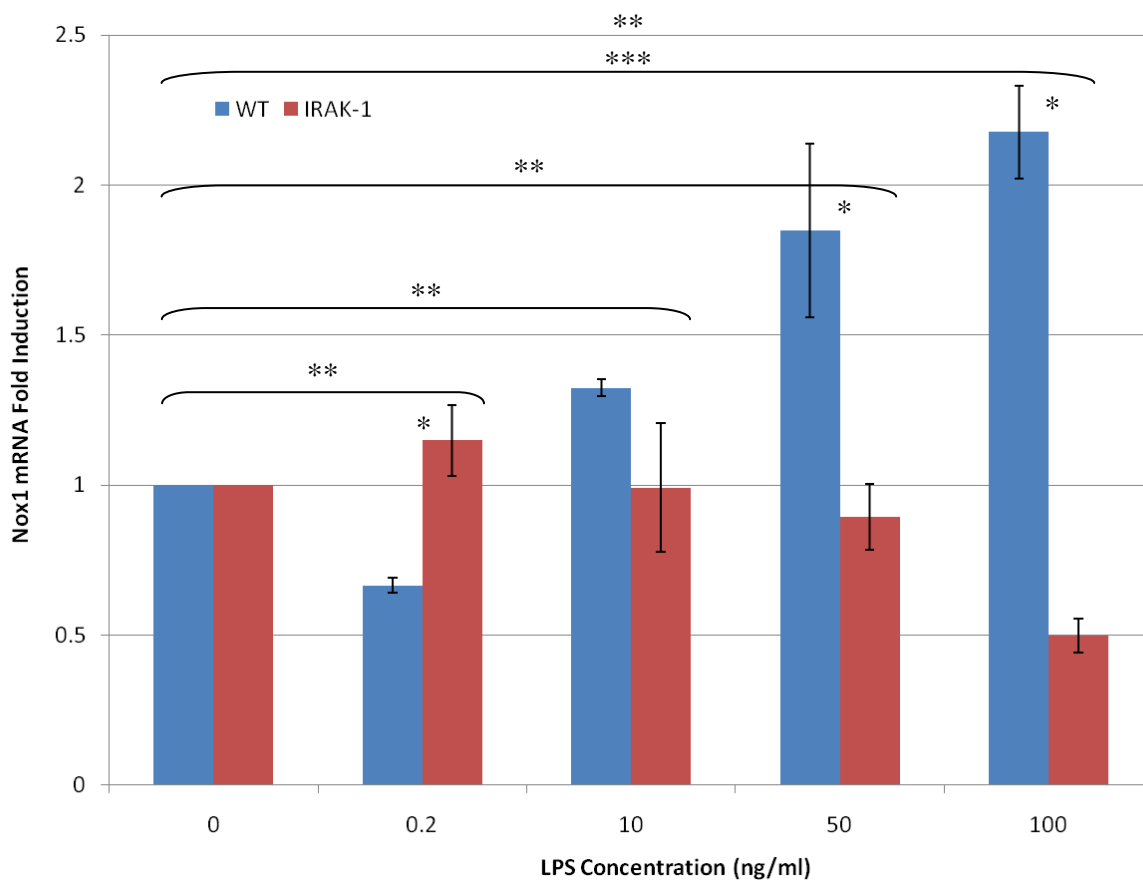
**Figure 5.3.** *LPS increases the rate of production of reactive oxygen species in a dose dependent manner.* Mouse embryonic fibroblasts were treated with LPS for 15 minutes. The rate of reactive oxygen species production was measured using DCFDA staining. These data represent 3 independent experiments performed with 2-3 replicates each. The data presented are means +/- standard deviations. \*  $p < 0.05$  when compared to baseline rate of ROS production.



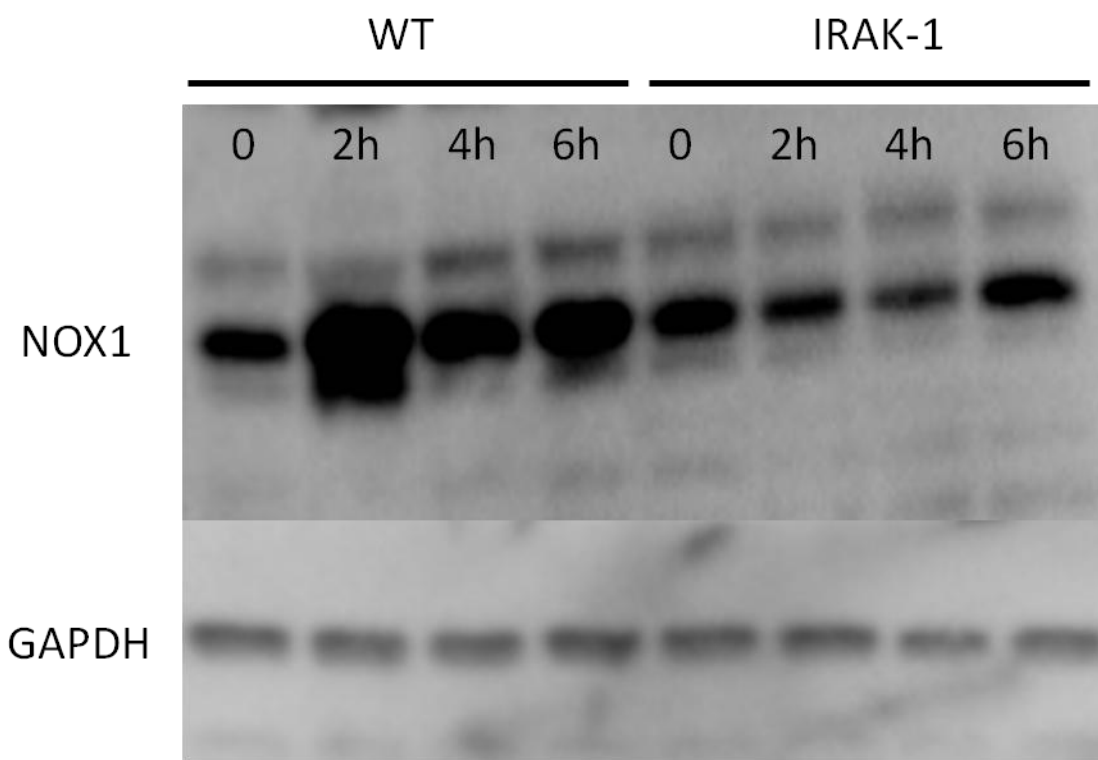
**Figure 5.4.** *LPS increases ROS production in a dose dependent and IRAK-1 dependent manner.* Wild type and IRAK-1 deficient MEF were treated with LPS for 15 minutes. The rate of ROS production was measured by staining with the fluorescent probe DCFDA. These data represent 3 independent experiments performed with 2-3 replicates each. The data presented are means  $\pm$  standard deviations. \*  $p < 0.05$  between WT and IRAK-1 deficient samples treated with the same concentration of LPS. \*\*  $p < 0.05$  for WT samples when compared to unstimulated WT cells. \*\*\*  $p < 0.05$  for IRAK-1 deficient samples when compared to unstimulated IRAK-1 deficient cells.



**Figure 5.5.** *LPS causes an increase in Nox1 mRNA in a dose dependent manner* Wild type mouse embryonic fibroblasts were treated with LPS for 2 hours. mRNA was isolated and levels of Nox1 mRNA were measured by RT-PCR. The results are fold induction relative to baseline samples of each cell type. These baseline samples were comparable between the two different groups. The results presented here are based on 3 independent experiments performed with 2-3 replicates per experiment. \*  $p < 0.05$  when compared to baseline rate of ROS production.

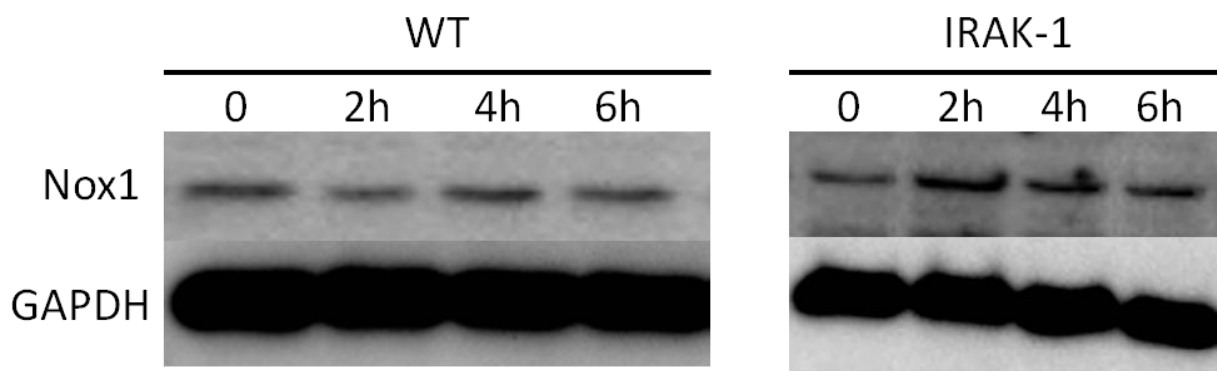


**Figure 5.6.** *IRAK-1 is necessary for LPS induced increase in Nox1 mRNA.* Wild type and IRAK-1 deficient MEF were treated with LPS for 2 hours. mRNA was isolated and levels of Nox1 mRNA were measured by RT-PCR. The results are fold induction relative to baseline samples of each cell type. These baseline samples were comparable between the two different groups. The results presented here are based on 3 independent experiments performed with 2-3 replicates per experiment. \*  $p < 0.05$  between WT and IRAK-1 deficient samples treated with the same concentration of LPS. \*\*  $p < 0.05$  for WT samples when compared to unstimulated WT cells. \*\*\*  $p < 0.05$  for IRAK-1 deficient samples when compared to unstimulated IRAK-1 deficient cells.

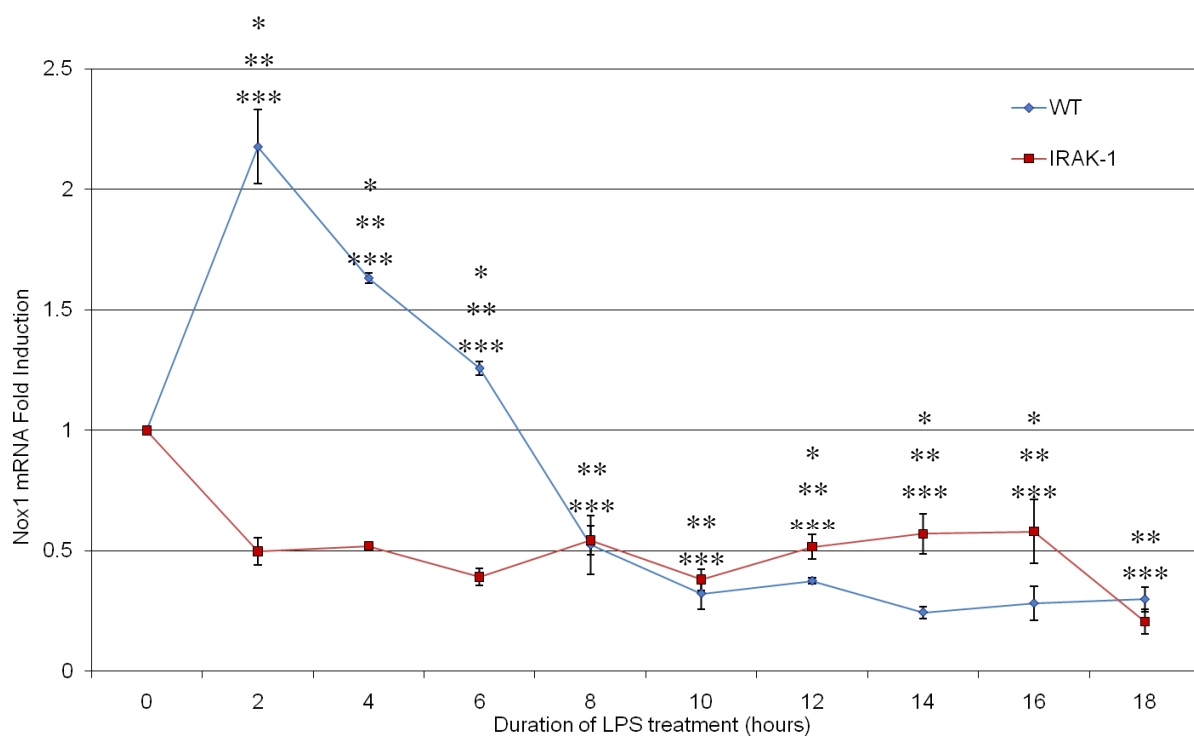


**Figure 5.7.** *IRAK-1 is necessary for LPS induced expression of Nox1.* Wild type and IRAK-1 deficient mouse embryonic fibroblasts were treated with 100ng/ml LPS for 0, 2 hours, 4 hours, and 6 hours. Whole cell extracts were collected and analyzed by western blot using an anti-Nox1 antibody. The same blots were probed with GAPDH as a loading control. This western blot is indicative of 4 different blots from 4 separate experiments.

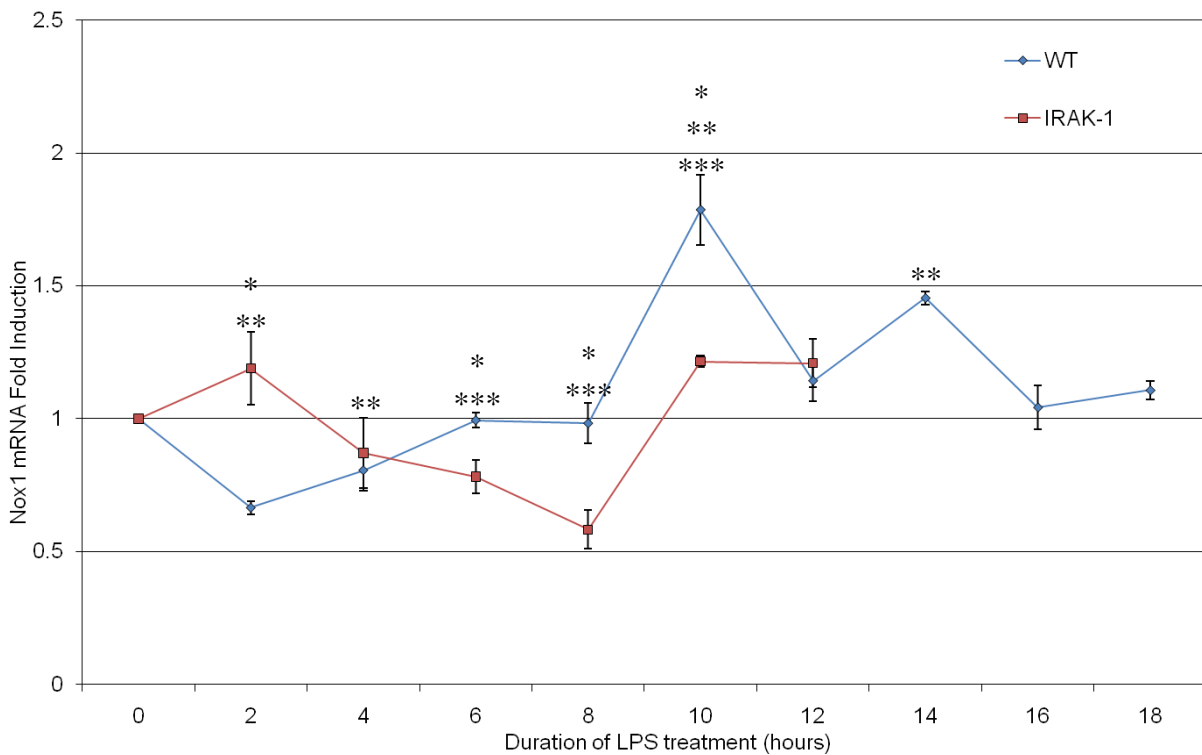




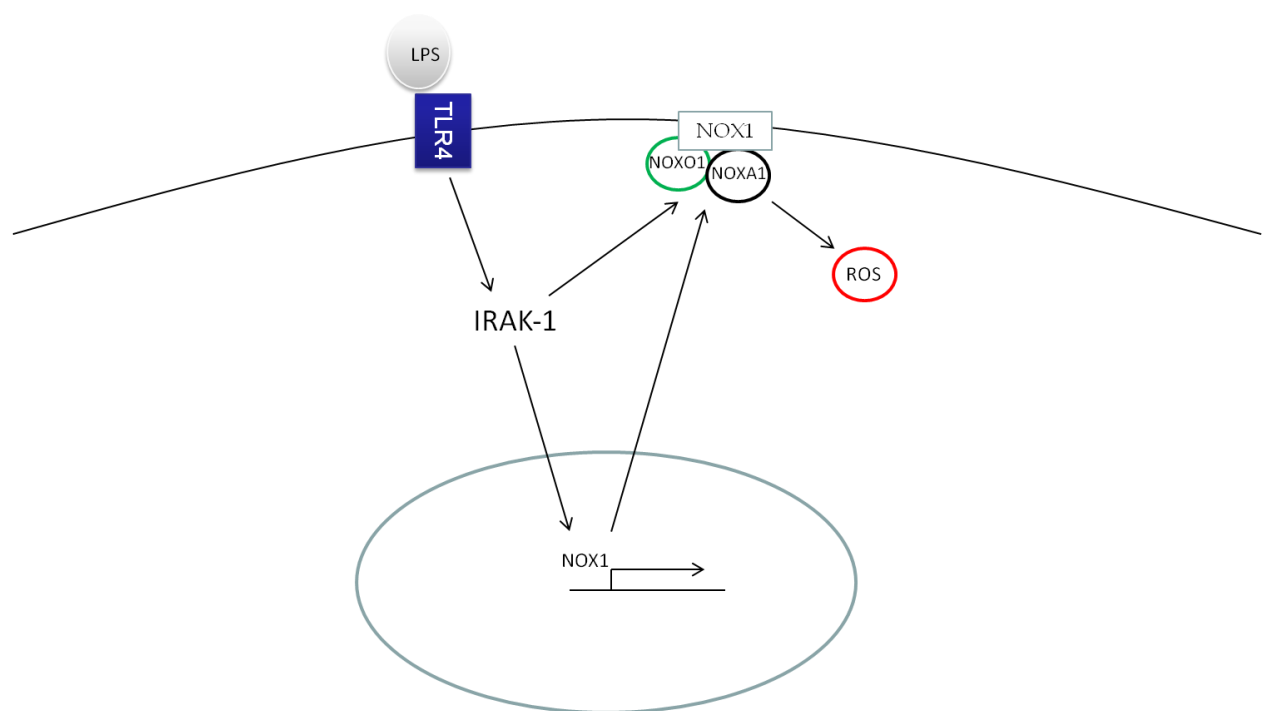
**Figure 5.8.** *Low dose LPS causes very little change in Nox1 protein levels in either wild type or IRAK-1 deficient MEF.* Wild type and IRAK-1 deficient MEF were treated with 200pg/ml LPS for 0, 2, 4, and 6 hours. Whole cell extracts were collected and analyzed by western blot analysis using an antibody against Nox1. The same PVDF membranes were then probed for GAPDH as a loading control. These western blots are representative of 3 separate experiments.



**Figure 5.9.** High dose LPS causes a transient increase in *Nox1* mRNA in wild type, but not *IRAK-1* deficient MEF. Wild type and *IRAK-1* deficient mouse embryonic fibroblasts were treated with 100ng/ml LPS for 2 hours to 18 hours. The levels of *Nox1* message were measured by RT-PCR. *Nox1* mRNA levels were normalized using *GAPDH* mRNA levels. These data represent 3 independent experiments performed with 2-3 replicates each. The data presented are means +/- standard deviations. \* p < 0.05 between WT and *IRAK-1* deficient samples treated with the same concentration of LPS. \*\* p < 0.05 for WT samples when compared to unstimulated WT cells. \*\*\* p < 0.05 for *IRAK-1* deficient samples when compared to unstimulated *IRAK-1* deficient cells.



**Figure 5.10.** *Low dose LPS induces a slower, more sustained increase in Nox1 mRNA than high dose LPS.* Wild type and IRAK-1 deficient MEF were treated with 200pg/ml LPS for 2 to 18 hours. The levels of Nox1 mRNA were measured by RT-PCR. These data represent 3 independent experiments performed with 2-3 replicates each. The data presented are means +/- standard deviations. \*  $p < 0.05$  between WT and IRAK-1 deficient samples treated with the same concentration of LPS. \*\*  $p < 0.05$  for WT samples when compared to unstimulated WT cells. \*\*\*  $p < 0.05$  for IRAK-1 deficient samples when compared to unstimulated IRAK-1 deficient cells.



**Figure 5.11.** Schematic diagram showing the role of IRAK-1 in the activation and induction of *Nox1*.

## **Chapter 6: Low Dose Endotoxin Primes While High Dose Endotoxin Tolerizes Mouse Embryonic Fibroblasts and their Ability to Produce Reactive Oxygen Species**

### **Abstract**

One of the ways cells and tissues combat inflammation and disease is via the production of reactive oxygen species. Unfortunately, reactive oxygen species are not specific; they act on both host tissues and invading pathogens. In order to control ROS, the body is also able to produce antioxidants. The understanding of how these products are activated and regulated is an important potential therapeutic target [45]. We believe that the regulation of ROS production and anti-oxidant production in response to endotoxin is dependent on the concentration of endotoxin. We therefore hypothesized that high doses of endotoxin are capable of causing the production of ROS, while also triggering the initiation of antioxidant cascades to control future ROS production. Furthermore, we have seen that low doses of endotoxin can induce ROS production, but we hypothesized that subclinical doses of endotoxin are not capable of activating the ant-oxidants. To test these hypotheses mouse embryonic fibroblasts were treated with high and low doses of LPS to look at the regulation of a major antioxidant regulator, Nrf2, by measuring protein levels by western blot analyses and RNA levels by using real-time PCR. Furthermore, we also looked at the low dose priming response of these cells by measuring ROS production via DCFDA staining. We found that high dose LPS induces an increase of Nrf2 that corresponds with a decrease in the superoxide producer Nox1, whereas low doses of LPS did not induce an increase in Nrf2. In addition, MEF primed with low dose LPS produced more ROS after a later high dose LPS challenge than unprimed cells. We conclude that the activation of antioxidants does occur in a dose dependent manner, and that low dose LPS is able to

successfully prime this system because the dose is not high enough to activate antioxidative feedback.

## **Introduction**

It has previously been demonstrated that lipopolysaccharide (LPS) can induce the production of reactive oxygen species (ROS) in mouse embryonic fibroblasts (MEF). Furthermore, we also noticed a marked decrease in the presence of a ROS producing protein, NADPH oxidase 1 (Nox1), after prolonged LPS treatment. Therefore, we propose that LPS may be inducing not only the production of reactive oxygen species, but an anti-oxidative feedback loop to control the amount of ROS being produced. Oxidative stress has been linked to many diseases, including cancer, inflammation, neurological diseases, renal disease, and atherosclerosis. Thus any protein capable of regulating and preventing the exacerbation of oxidative stress represents an important potential therapeutic tool and warrants a better understanding of how these proteins are activated and controlled.

The protein nuclear factor (erythroid-derived 2)-like 2 (Nrf2) is known to be a master regulator of oxidative stress. Once activated it is able to cause the induction of genes known to combat oxidative stress including; NAD(P)H quinone oxidoreductase, glutamate-cysteine ligase, heme oxygenase-1, and glutathione s-transferase. These genes all act in different ways to combat the production of reactive oxygen species. Unactivated Nrf2 remains in the cytosol, bound to the protein Keap1. Nrf2 can be activated either directly or indirectly by reactive oxygen species. Indirect activation occurs as a result of the activation of PI3 kinase which can occur because of ROS production. Once activated, Nrf2 becomes separated from Keap1 and translocates into the nucleus, binding to the antioxidant response element found in the promoter

region and induces transcription of a variety of anti-oxidative genes as well as causing an increase in Nrf2 transcription. Cellular increases in Nrf2 provide a positive feedback loop increasing the presence of Nrf2 and other anti-oxidant genes.

It has previously been shown that high doses of LPS increase the production of reactive oxygen species [256]. As described above, we have also seen a quick, transient increase in the presence of the superoxide producer Nox1 in response to high doses of LPS. We are therefore interested to see if there is any relationship between the rapid decrease in the presence of Nox1 (after the initial increase) and the activation of Nrf2. Furthermore, since an increase in ROS production in response to subclinical doses of LPS has been observed, we will determine if the activation of Nrf2 is dose dependent.

There are many different inflammatory diseases that are affected by ROS production and/or can be triggered by the presence of LPS. Metabolic endotoxemia is characterized by circulation of plasma endotoxin. It is known to be a precursor for metabolic disorders and can result in obesity and the development of diabetes. Much work has been conducted in which cellular responses to high doses of LPS has been evaluated, however little work has been conducted to determine the prolonged effects of subclinical doses of LPS. In addition to looking at the relationship between Nox1 and Nrf2, we have also begun looking at how initial low doses of LPS are capable of priming mouse embryonic fibroblasts, causing increased production of reactive oxygen species after a later LPS challenge.

## **Materials and Methods**

*Reagents:* LPS (*Escherichia coli* O111:B4) was obtained from Sigma. Wortmannin was obtained from Sigma (W1628). The antibodies against GAPDH and Nrf2 were obtained from

Santa Cruz. Primer sets were obtained from IDT, the sequences are as follows: *Nox1*(+), 5'-TCCATTTTCCTTCCTGGAGTGGCAT-3', and *Nox1*(-), 5'-GGCATTGGTGAGTGCTGTTGTTCA-3'; *GAPDH*(+), 5'-ACTTTGGCATTGTGGAAGGGCTC-3', and *GAPDH*(-), 5'-TGGAAGAGTGGGAGTTGCTGTTGA-3'; and *Nrf2*(+), 5'-TAAAGCTTTCAACCCGAAGCACGC-3', and *Nrf2*(-), 5'-TCCATTTCCGAGTCACTGAACCCA-3'.

*Mice and Murine Cells:* Wild type C57BL/6 mice were purchased from Charles River Laboratories. *IRAK-1*<sup>-/-</sup> mice with C57BL/6 background were provided by Dr. James Thomas of University of Texas Southwestern Medical School. All mice were housed and bred in Derring Hall Animal Facility in compliance with approved Animal Care and Use Committee protocols at Virginia Tech University. Mouse embryonic fibroblasts (MEF) were isolated and cultured as previously described [250]. MEF were allowed to rest in 1% FBS DMEM for 4 hours prior to treatment.

*Gene Expression Analysis:* Gene expression was evaluated in LPS treated, untreated, +/- wortmannin, and primed (2 hours) wild type MEF using real-time RT-PCR. Total RNA was extracted from the cells using TRIzol (Invitrogen, according to manufacturers protocol), and reverse-transcription was accomplished using the high capacity cDNA reverse transcription kit (Applied Biosystems). Real-time PCR analyses were performed using SYBR green supermix on an IQ5 thermocycler (Bio-Rad). Relative transcript levels of *Nox1* and *Nrf2* mRNA were normalized using *GAPDH* and were analyzed using the  $\Delta\Delta C_t$  method.



*Intracellular ROS Measurement:* The fluorescent probe chloromethyl-2',7'-dichlorofluorescein diacetate (Molecular Probes) was used to monitor intracellular ROS accumulation. DCFDA is deacetylated and reacts with intracellular ROS which converts it to its fluorescent form, dichlorofluorescein which is retained within the cells. Primed WT MEF ( $4 \times 10^4$  cells/well in 96 well plates) were pretreated with wortmannin (0 or 100nM) for 30 minutes, then treated with LPS for 2 hours, media was then changed and cells were maintained in 1% FBS DMEM until 1 hour prior to the second LPS treatment when they were treated with  $10 \mu\text{M}$  chloromethyl-2',7'-dichlorofluorescein diacetate (DCFDA) for 30 minutes at  $37^\circ\text{C}$ . The cells were then washed with PBS containing calcium and magnesium. Cells were then allowed to rest for 20 minutes and treated with a second dose of LPS (100 ng/ml). Readings were taken 15 minutes prior to the start of the experiment, immediately before LPS exposure, and after 15 minutes of LPS exposure. The treatments response slopes (slope from the beginning of exposure to 15 minutes after exposure) were corrected for the pre-treatment response slopes (slope from 15 minutes prior to treatment to beginning of treatment).

*Protein Expression Analysis:* Immunoprecipitation and Western blotting techniques were used to characterize the level of Nrf2 protein.

*Immunoprecipitation:*  $1 \times 10^7$  MEF were used for each timepoint. MEF treated with LPS (100ng/ml or 200pg/ml) +/- wortmannin (100nM) were rinsed in PBS and lysed on ice in lysis buffer (50mM HEPES pH 7.6, 150mM NaCl, 1mM EDTA, 0.5% NP-40, 1X Phosphatase Inhibitor 1, 1X Phosphatase Inhibitor 2, 1X Protease Inhibitor). Then 500ug of protein was treated with 2ug Nrf2 antibody, that was incubated for 1 hour at  $4^\circ\text{C}$ . After incubation, the lysate/antibody solution was treated with Protein A/G Plus Agarose beads (Santa Cruz

Biotechnology), and was incubated overnight at 4°C while being rotated constantly. The samples were washed 3 times with lysis buffer and treated with 5X laemli buffer and boiled for 10 minutes to separate the protein from the beads. After boiling, samples were centrifuged and the supernatants were used for Western blotting.

*Western Blot:* Western blot analysis was performed as previously described. Immunoblots were developed using the ECL Plus chemiluminescent detection system kit by Amersham Biosciences (GE Healthcare). The intensities of the bands was quantified using the Fujifilm MultiGauge software and normalized to GAPDH levels.

*Statistical Analyses:* Statistical significance was determined using the paired two-tailed Student's t-test. Data was considered to be statistically significant if the p value was less than 0.05.

## **Results**

*High dose LPS induced a sustained increase in Nrf2 mRNA, and a transient increase in Nox1 mRNA.* We previously demonstrated a transient increase in Nox1 mRNA in response to high doses of LPS. Due to this transient increase we hypothesized that high dose LPS is activating a negative feedback loop, that involves Nrf2 and is responsible for the eventual decrease in Nox1 expression. Analyses of mRNA samples from MEF treated with 100ng/ml LPS for up to 18 hours, indicated a transient increase in the levels of Nrf2 as well as Nox1 present in these cells (Figure 6.1). We saw a phase shift between the two increases, 2 hours after Nox1 increased the levels of Nrf2 increased significantly. The levels of Nrf2 then rapidly decreased, but the levels of Nrf2 expression in these cells remained above that of untreated cells over the entire 18 hour timecourse. Therefore, while Nox1 expression levels quickly increased

and then decreased below basal levels, Nrf2 expression levels also increased but remained above basal levels throughout the experiment. Thus, elevated Nrf2 levels corresponded with reduced levels of Nox1 expression that were maintained below baseline levels.

*Low dose LPS did not cause an increase in Nrf2 mRNA, even though there was a slow, sustained increase in Nox1 mRNA.* After examining the effects on Nrf2 expression in response to high dose LPS, the effects of low dose LPS on Nrf2 levels were then evaluated. When MEF were treated with subclinical (200pg/ml) doses of LPS, there is little to no change in the expression of Nrf2 mRNA within the cells (Figure 6.2). Furthermore, the same cells showed a gradual increase in the amount of Nox1 mRNA maintained within these cells. Therefore, we conclude that low doses of LPS are not sufficient enough to cause an increase in Nrf2 mRNA. When taken with the above data showing that high dose LPS caused an increase in Nrf2 mRNA and a decrease in Nox1 mRNA, this would suggest that without the activation of Nrf2, intracellular Nox1 levels would continue to increase within the cells.

*Low dose LPS was able to prime MEF for ROS production, whereas high dose LPS induces tolerance in the system.* After examining the expression of Nox1 and Nrf2 in response to LPS, we then evaluated the effects of these expression patterns on the future activation of Nox1 and the resulting production of ROS. Our group has previously demonstrated that MEF treated with LPS produce more ROS than untreated cells. MEF were treated with LPS for 2 hours (priming) and then allowed to rest for 2, 4, 6, 8, or 16 hours before being treated with LPS again (figure 6.3). Cells primed with 200pg/ml LPS and then treated with 100ng/ml LPS after resting produced more ROS than cells treated with only 100ng/ml LPS (Figure 6.4).

Furthermore, the longer the cells were allowed to rest after the initial stimulus the higher the rate of ROS production in response to the second stimulus (Figure 6.5)

However, cells primed with 100ng/ml LPS and then treated with a second dose of 100ng/ml LPS after the rest period develop tolerance to the inflammatory stimulus and do not produce more ROS in response to the second dose (Figure 6.4). These cells appear to require a longer recovery time, requiring at least 4 hours of rest before the system is able to produce the same response as unprimed cells (Figure 6.6). In addition, these cells needed at least 6 hours of rest before they were able to begin exhibiting the priming response seen with little rest in the MEF primed with 200pg/ml LPS. Thus, low dose LPS is able to prime MEF for the later production of ROS, however high dose LPS tolerizes the system requiring much more recovery time than the low dose samples.

*High dose LPS increased the amount of the Nrf2 mRNA and protein which can be inhibited by wortmannin.* One of the ways that Nrf2 is activated is through the PI3 kinase. Therefore, by inhibiting this kinase, a reduction in the amount of Nrf2 present in these cells would be expected. After treatment with wortmannin (a PI3 kinase inhibitor) cells treated with LPS expressed less Nrf2 than cells that did not receive the wortmannin pretreatment (Figure 6.7). This is further confirmed by looking at the levels of Nrf2 protein present in wortmannin pretreated and unpretreated MEF (Figure 6.8). After both low (200pg/ml) and high (100ng/ml) LPS treatment, MEF showed an increase in the amount of Nrf 2 protein present in the cells. Furthermore, Nrf2 levels in cells treated with LPS were reduced with wortmannin pretreatment. Therefore, pretreatment with wortmannin reduces the amount of Nrf2 message and the amount of Nrf2 protein present in MEF, indicating the important role of PI3 kinase in Nrf2 expression.

*MEF pretreated with wortmannin expressed more Nox1 in response to LPS stimulation.*

After establishing that wortmannin reduces the amount of Nrf2 present in MEF, changes in Nox1 levels after pretreatment with wortmannin and LPS treatment were evaluated. Overall, cells treated with wortmannin contained more Nox1 mRNA than untreated cells (Figure 6.9).

Furthermore, cells that received both wortmannin pretreatment and LPS treatment contained more Nox1 mRNA than untreated cells and cells that received only the LPS treatment.

Wortmannin is capable of inhibiting multiple enzymes. The data provided for Nox1 and Nrf2 suggest that Nrf2 expression is inhibited by wortmannin but Nox1 expression is not inhibited, and in fact Nox1 levels of wortmannin treated cells were higher. Therefore a negative correlation between Nrf2 expression and Nox1 expression was again observed. This would appear to indicate that PI3 kinase and therefore Nrf2 are involved in reduced expression of Nox1 within MEF. Reduced levels of Nox1 and Nox1 activation were associated with inhibition of reactive oxygen species production. Thus, by activating Nrf2 an anti-oxidative feedback loop is turned on, thereby decreasing the amount and activation of Nox1.

## **Discussion**

In this study we have begun to define a mechanism for inhibiting the LPS induced production of reactive oxygen species by Nox1 in fibroblasts. The primary activator of this inhibitor is the oxidative stress regulator Nrf2. The activation of Nrf2 as an inhibitor was minimal in response to subclinical doses of LPS. However, high dose LPS significantly increased the amount of Nrf2 present in MEF. Due to this lack of activation at low doses, low dose LPS was used to successfully prime for ROS production in MEF. Collectively, low dose

LPS did not stimulate the activation and increase in Nrf2 in fibroblasts, but high dose LPS did activate this pathway.

Our data indicate that high doses of LPS are capable of activating and increasing the amount of Nox1 build up in MEF. However, high dose LPS is also able to turn on the pathway activating Nrf2. The increase in Nrf2 is slightly delayed compared to that of Nox1, but is accompanied by a significant decrease in the presence of Nox1. The activation of Nox1 causes an increase in the production of ROS. This increase in ROS is then capable of activating PI3 kinase which can activate Nrf2. Nrf2 can also be directly activated by ROS. Once activated, Nrf2 is released from Keap1, which is responsible for maintaining limited levels of Nrf2 in the cytoplasm. Without the interaction between Nrf2 and Keap1, Nrf2 can then translocate into the nucleus and induce the transcription of anti-oxidative genes as well as causing increased transcription of Nrf2. As Nrf2 builds up in cells, it is able to overwhelm the cytoplasmic Keap1 and accelerate this loop. This loop is able to significantly increase the production of ROS by inhibiting Nox1. Further work needs to be done to determine exactly how Nox1 is shut down.

Cells treated with low doses of LPS showed a delayed increase in Nox1, and a small delayed increase in Nrf2. Furthermore, instead of maintaining cellular levels of Nrf2 above baseline (as is seen in the MEF treated with high dose LPS), after a delayed increase, Nrf2 levels appear to drop below baseline and were maintained there for the duration of our studies. Our group has previously shown that low dose LPS is able to increase ROS in fibroblasts. It appears that the ROS increase induced by low dose LPS is not sufficient to cause an immediate increase in Nox1 or Nrf2. Low dose LPS is sufficient to increase Nox1 in fibroblasts, but is not sufficient to increase Nrf2 levels. Therefore, over time Nox1 levels are maintained above basal levels, eventually produces enough ROS to activate Nrf2. This would explain the delayed increase in

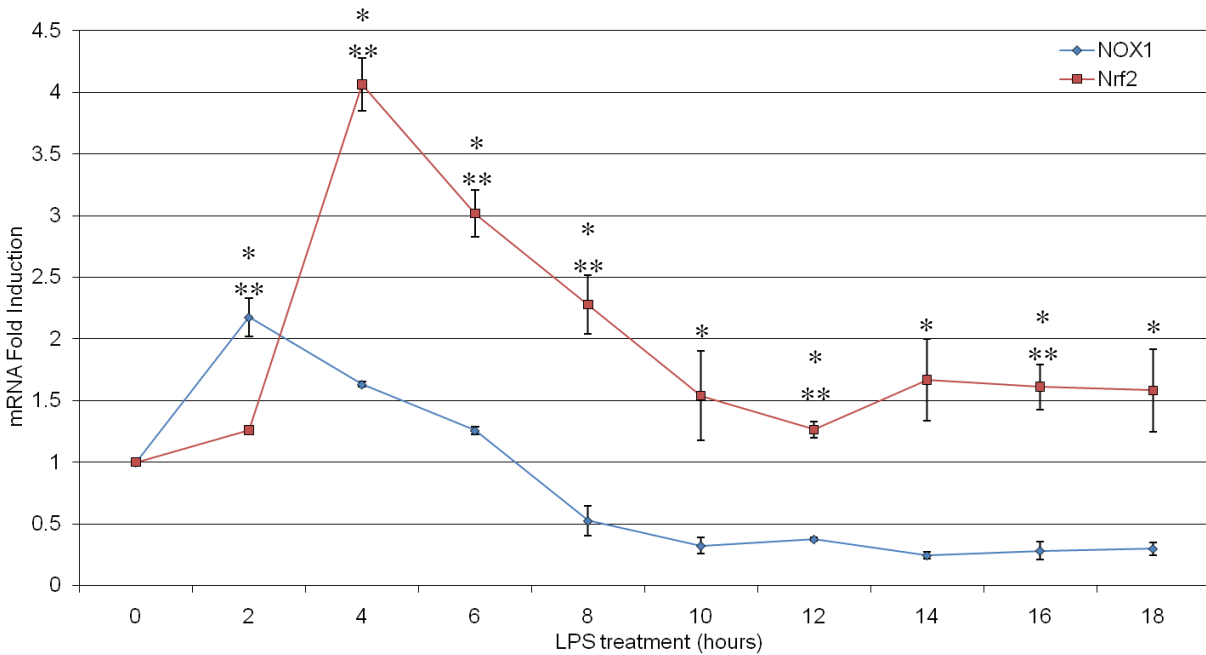
Nrf2 levels as well as the decrease in Nox1 levels that follow. Furthermore, we believe that this also explains the priming phenomenon seen after low dose LPS challenge. Fibroblasts treated with low dose LPS for two hours show an increase in Nox1 without the activation of Nrf2. Then, when the system is challenged a second time with high dose LPS, significantly more ROS are produced than in unprimed fibroblasts. This response was not observed when fibroblasts were primed with the high dose LPS. This suggests that instead of preparing the system to respond more to a later challenge (as the low dose does), Nrf2 inhibits the buildup of Nox1 in the cells. Furthermore, when the cells are primed with low dose LPS and challenged later with low dose LPS, a small increase in ROS production over unprimed cells was observed (data not shown), but the difference between the two was not significant.

Fibroblasts treated with wortmannin (a PI3 kinase inhibitor) and LPS show significantly more Nox1 after both 2 hours and 4 hours of LPS treatment than fibroblasts that did not receive the wortmannin treatment. One of the things that wortmannin is capable of doing through its inhibition of PI3 kinase is to inhibit Nrf2 activation. Without the Nrf2 activation, more Nox1 is accumulated in cells. In agreement with this, when we look at Nrf2 levels in the same cells, wortmannin (and LPS) caused a significant decrease in Nrf2 expression when compared to cells that received only high dose LPS treatment. The gene expression results are also reflected in similar changes in the protein levels. Both high dose and low dose LPS caused an increase in the amount of Nrf2 immunoprecipitated from fibroblasts when compared to untreated cells. Furthermore, when these cells were treated with wortmannin and LPS, the cells that received the wortmannin (and LPS) treatment showed levels of Nrf2 only slightly increased over the control and significantly less than cells that received only LPS treatment.

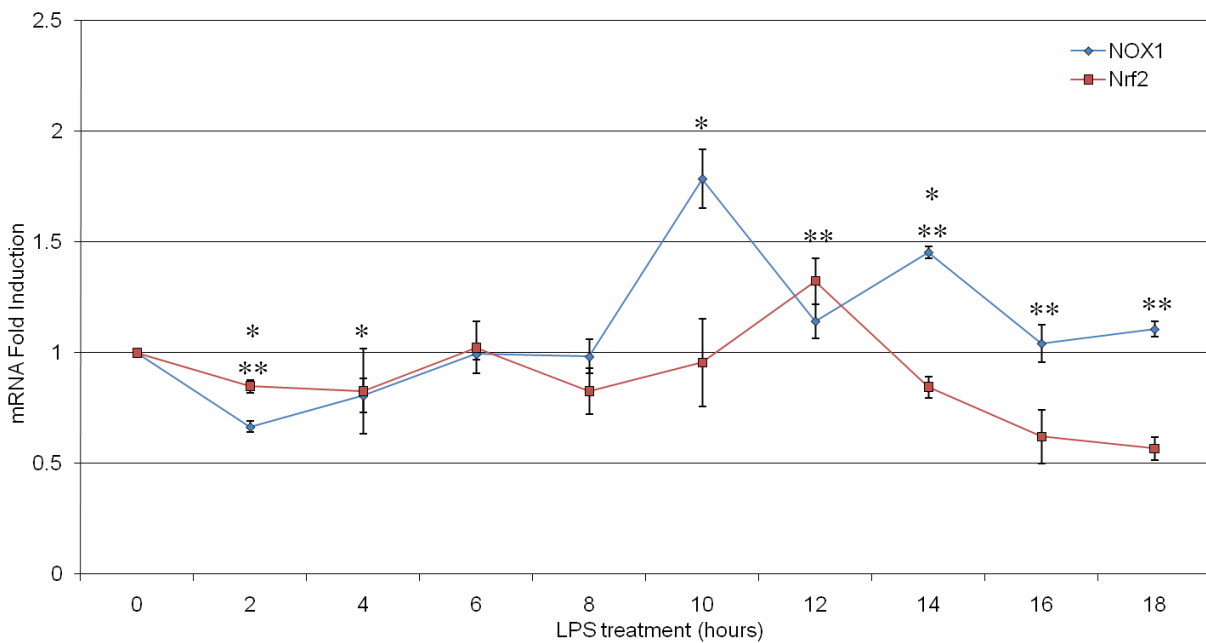
We thereby propose that low dose LPS is capable of causing a slower Nox1 increase than high dose LPS. The initial reaction of the fibroblasts to the low dose LPS does not produce enough ROS to significantly activate Nrf2. This allows Nox1 levels in the cell to increase so that when treated with high dose LPS at a later time, there is significantly more Nox1 present and able to produce ROS than in cells that do not receive the priming dose. This results in much more ROS produced in response to the second challenge of high dose LPS. This large increase in ROS is sufficient to activate Nrf2 (through PI3 kinase or directly). Nrf2 is then able to inhibit ROS production and Nox1 increase. This causes Nox1 levels to drop below basal levels so that when cells are challenged with LPS later, they are unable to mount a response via Nox1 dependent ROS production.

Our data provide compelling evidence that fibroblasts react differently to different levels of LPS stimulation. This relates to not only the immediate cellular response, but to future cellular response as well. Future work needs to be conducted to evaluate how Nrf2 is involved in the decrease of Nox1 levels, as well as the role that inhibition of Nrf2 may have in the priming response of fibroblasts and in their ability to respond to LPS.



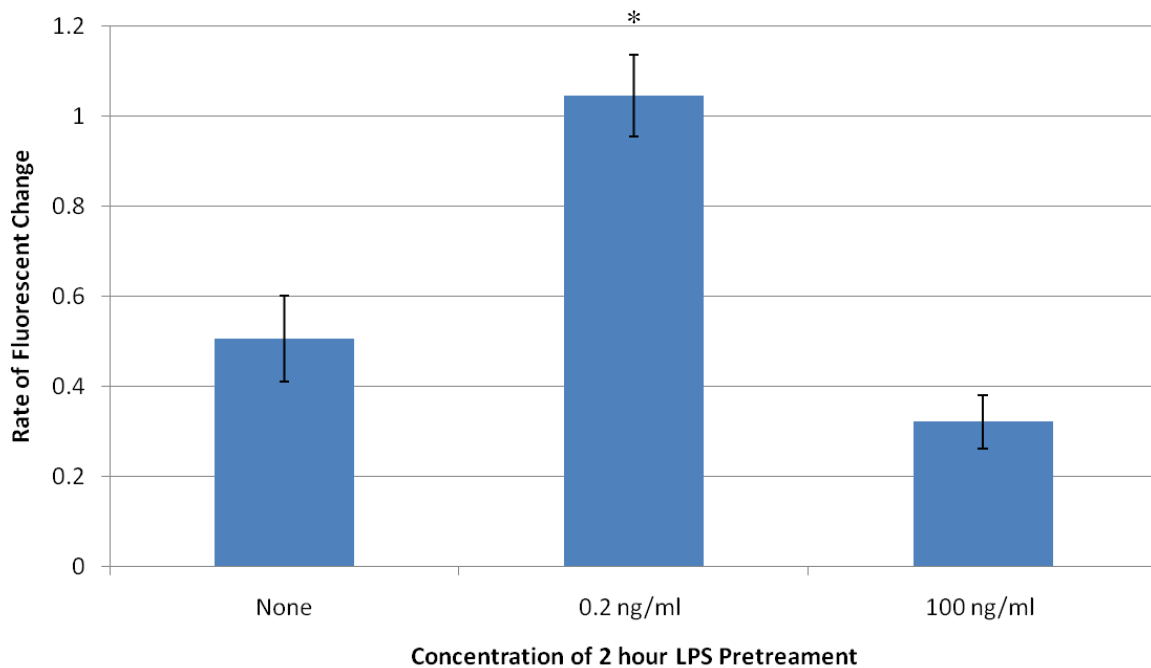


**Figure 6.1.** High dose LPS was able to induce a sustained increase in *Nrf2* mRNA, and a transient increase in *Nox1* mRNA. Wild type mouse embryonic fibroblasts were treated with 100ng/ml LPS for 0 to 18 hours. mRNA was isolated from cell lysates and *Nox1* and *Nrf2* gene expression levels were measured using RT-PCR. Results presented here are based on three separate experiments consisting of 2-3 replicates per time point per experiment. \*  $p < 0.05$  for *Nox1* samples when compared to unstimulated cells. \*\*  $p < 0.05$  for *Nrf2* samples when compared to unstimulated cells.

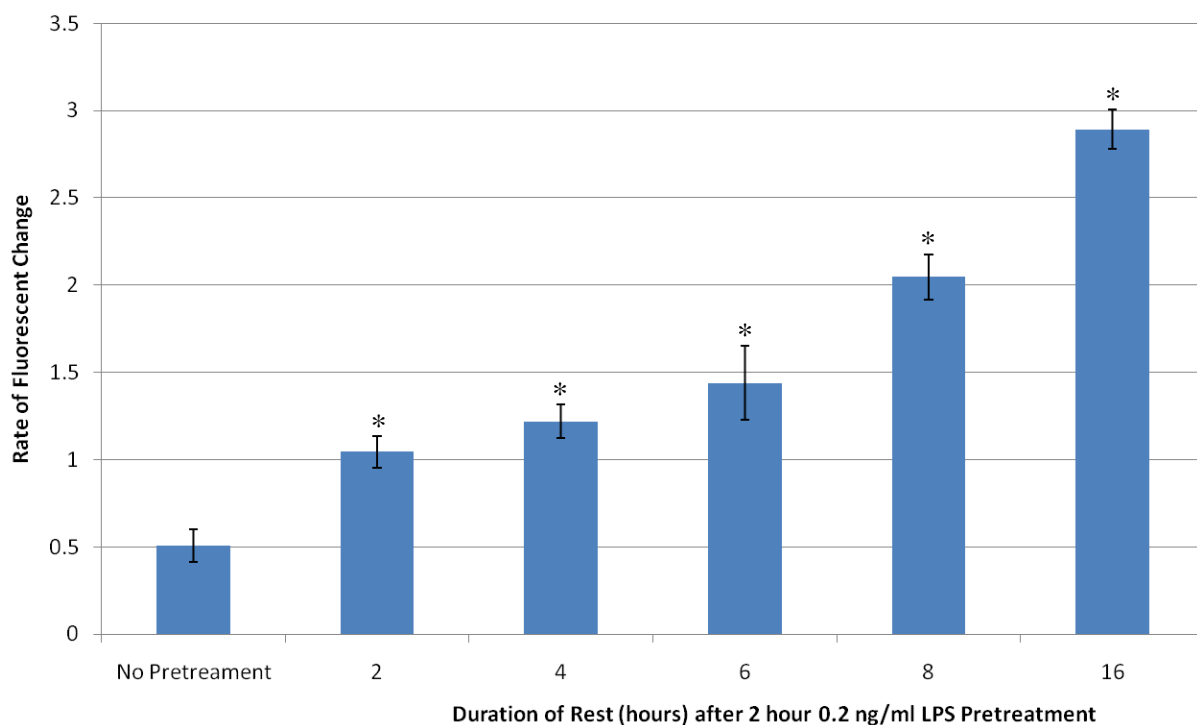


**Figure 6.2.** *Low dose LPS did not cause an increase in Nrf2 mRNA, while it does cause a slow, sustained increase in Nox1 mRNA.* Wild type mouse embryonic fibroblasts were treated with 200pg/ml LPS for 0 to 18 hours. mRNA was isolated from cell lysates and Nox1 and Nrf2 gene expression levels were measured using RT-PCR. Results presented represent data from 3 separate experiments, each time point is made up of 2-3 replicates per experiment. \*  $p < 0.05$  for Nox1 samples when compared to unstimulated cells. \*\*  $p < 0.05$  for Nrf2 samples when compared to unstimulated cells.

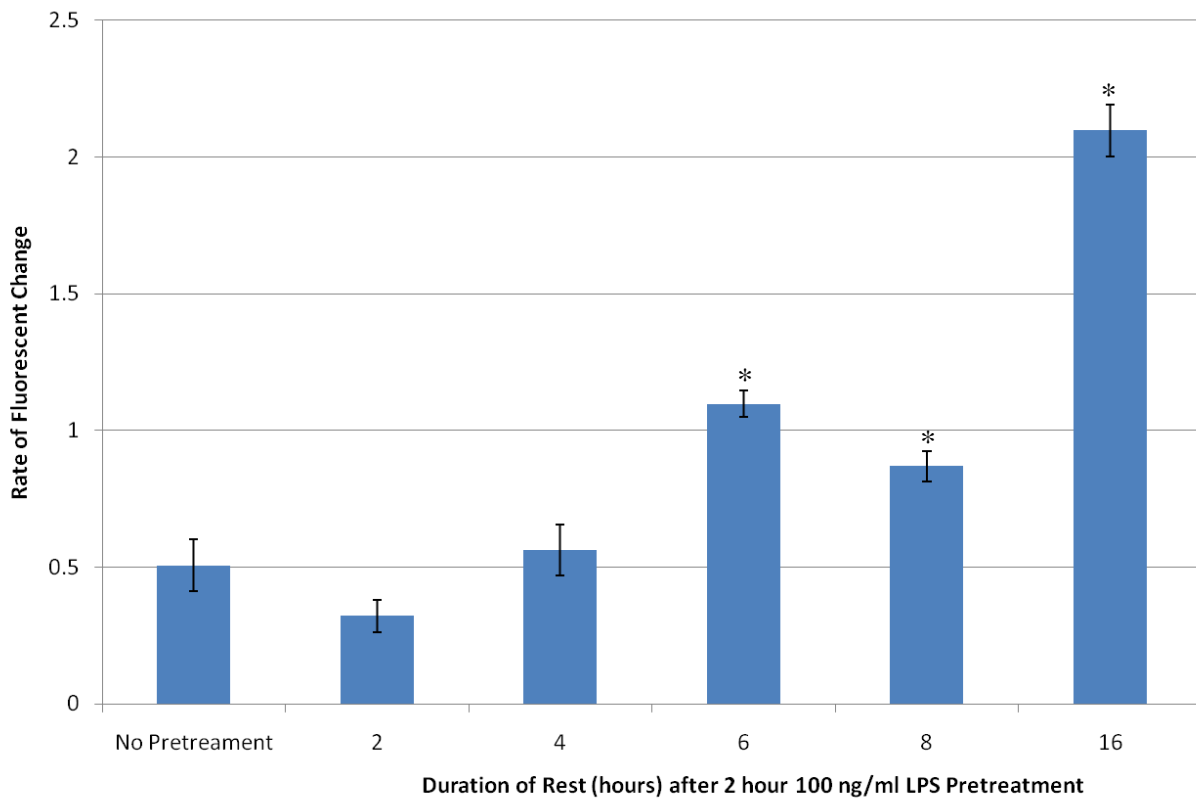




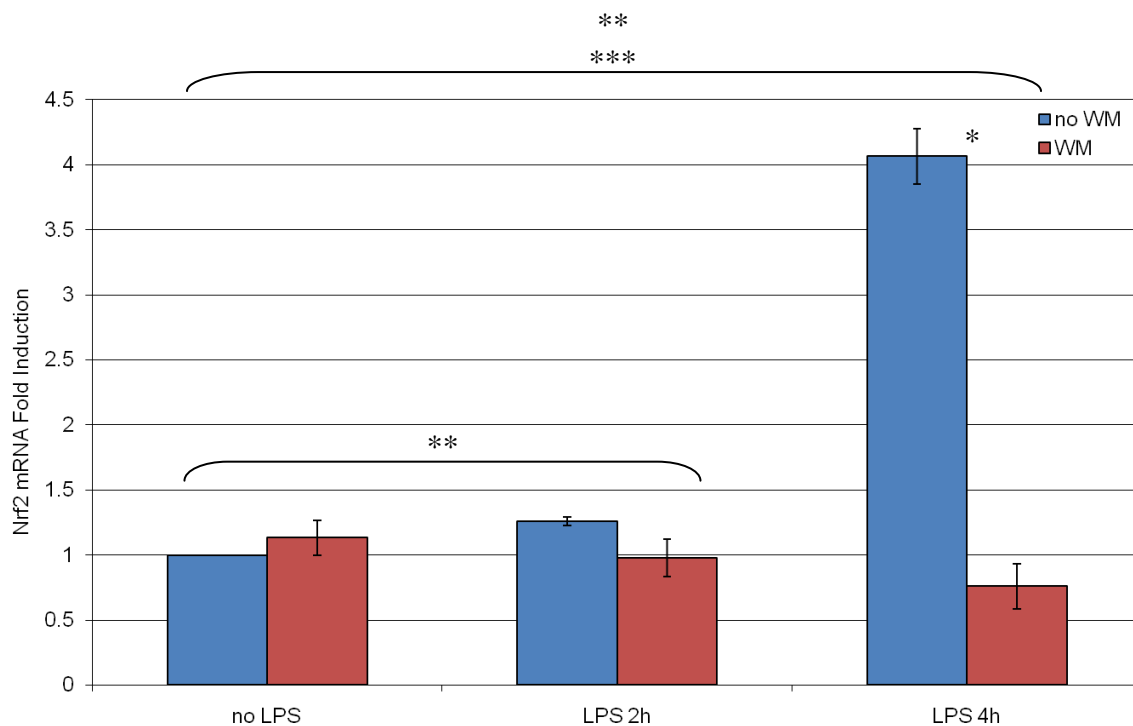
**Figure 6.4.** *Low dose LPS was able to prime for ROS production, whereas high dose LPS tolerizes the system.* MEF were pretreated with high (100ng/ml) or low (200pg/ml) LPS for 2 hours. The LPS was then removed and the cells were allowed to rest for 2 to 16 hours. The cells were then treated with 100ng/ml LPS and the rate of reactive oxygen species production was measured by DCFDA fluorescence. Data represented here come from 3 separate experiments of 3 replicates per experiment. \*  $p < 0.05$



**Figure 6.5.** *Low dose LPS primed MEF for a later LPS challenge.* MEF were treated with 0.2ng/ml LPS for 2 hours. The media was then removed and cells were allowed to rest for 2 hours to 16 hours. At the end of the rest period, MEF were challenged a second time with 100ng/ml LPS and DCFDA readings were taken to determine the rate of ROS production. Data represented here come from 3 separate experiments of 3 replicates per experiment. \*  $p < 0.01$



**Figure 6.6.** *High dose LPS tolerizes MEF against a later LPS challenge.* MEF were treated with 100ng/ml LPS for 2 hours. The media was then removed and cells were allowed to rest for 2 hours to 16 hours. At the end of the rest period, MEF were challenged a second time with 100ng/ml LPS and DCFDA readings were taken to determine the rate of ROS production. Data represented here come from 3 separate experiments of 3 replicates per experiment. \*  $p < 0.01$

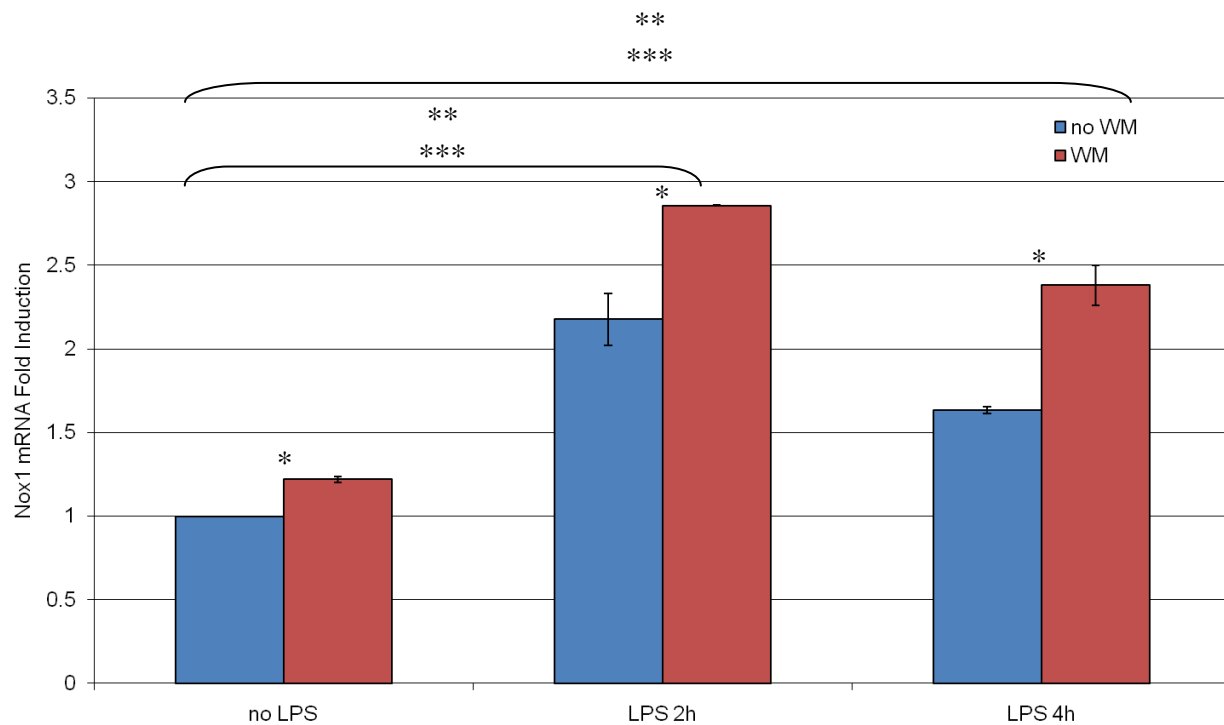


**Figure 6.7.** High dose LPS increased the amount of the Nrf2 mRNA which can be inhibited by wortmannin. MEF were treated with 100nM wortmannin for 30 minutes then treated with 100ng/ml LPS for 2 or 4 hours. The cells were then lysed and total RNA was collected. Expression of Nrf2 was measured by real time RT-PCR, and was normalized to GAPDH. Data represent three separate experiments. \*  $p < 0.05$  between samples treated with the same concentration of LPS. \*\*  $p < 0.05$  for samples stimulated with only LPS when compared to unstimulated cells. \*\*\*  $p < 0.05$  for samples stimulated with wortmannin plus LPS when compared to samples stimulated with only wortmannin.



**Figure 6.8.** *High dose LPS increased Nrf2 protein levels, wortmannin inhibited this increase.* MEF were treated with 100nM wortmannin for 30 minutes, and then treated with LPS (100ng/ml or 200pg/ml) for 2 hours. Whole cell lysates were collected and Nrf2 was immunoprecipitated from them. These samples were western blot. An example of a corresponding western blot is provided. This blot is representative of 3 separate experiments.





**Figure 6.9.** *MEF pretreated with wortmannin expressed more Nox1 in response to LPS stimulation.* MEF were pretreated with 50nM wortmannin for 30 minutes before being treated with 100ng/ml LPS for 2 and 4 hours. mRNA was isolated from the cell lysates and used to perform real time RT-PCR to evaluate expression of Nox1. Nox1 levels were normalized to GAPDH levels. The data represented in this figure are made up of 3 separate experiments. \* p < 0.05 between samples treated with the same concentration of LPS. \*\* p < 0.05 for samples stimulated with only LPS when compared to unstimulated cells. \*\*\* p < 0.05 for samples stimulated with wortmannin plus LPS when compared to samples stimulated with only wortmannin.

## Chapter 7: Conclusions and Future Directions

The goal of this project was to examine the role that IRAK-1 plays in LPS induced ROS production. In addition a mechanism was determined explaining the differences between high and low dose LPS stimulation and how that dosage plays a role in MEF developing tolerance to LPS or being primed for future LPS challenge. MEF were used to examine not only the change in rate of ROS production, but also the changes in Nox1 (a protein responsible for ROS production), and Nrf2 (an antioxidant regulator) protein and mRNA levels in response to different concentrations of LPS.

Toll-like receptor 4 mediates two separate inflammatory pathways. The classical pathway is dependent on IRAK-1, and based on the data shown here, is necessary for both high and low dose LPS induced production of ROS. We have shown that IRAK-1 is necessary for increasing Nox1 protein and mRNA levels as well as increasing Nox1 dependent ROS production. Wild type MEF produced ROS in a dose dependent manner in response to LPS, exhibiting increased rate of ROS production in response to higher concentrations of LPS. IRAK-1 deficient MEF did exhibit an increase in rate of ROS production in response to LPS, but did not exhibit the dose dependency effect demonstrated by wild type MEF. This same effect was displayed when looking at Nox1 mRNA levels in response to increasing LPS concentrations. In MEF treated with high dose (100ng/ml) LPS, wild type MEF exhibited a transient increase in protein and mRNA levels whereas IRAK-1 deficient MEF showed a decrease in Nox1 mRNA and protein levels. Low dose (200pg/ml) LPS induced a slow increase in Nox1 mRNA but almost no change in Nox1 protein levels in wild type MEF. The same treatment exhibited a decrease, and return to baseline levels of Nox1 mRNA and protein levels in IRAK-1 deficient

MEF. Thus we determined that IRAK-1 is required to increase Nox1 mRNA and protein, and that IRAK-1 is required for Nox1 mediated increases in rates of ROS production. However, we noted that based on the transient nature of the increase in Nox1 mRNA and protein to high dose LPS, and the sinusoidal increase and decrease in Nox1 mRNA levels in response to low dose LPS, that an anti-oxidative feedback is being activated in order to control Nox1 expression, thereby controlling a major contributor to ROS production.

Upon determining that a feedback loop existed we began examining possible mediators of antioxidant activity. Nuclear factor (erythroid-derived 2)-like 2 (Nrf2) is a known master regulator of oxidative stress, capable of upregulating itself as well as increasing transcription of multiple genes responsible for combating oxidative stress. Nrf2 is known to be activated by ROS. Pilot experiments showed no differences in Nrf2 expression between wild type and IRAK-1 deficient MEF. Wild type MEF were utilized to determine the effects of high and low dose LPS on Nrf2 expression. High dose LPS induced an increase in Nrf2 expression that corresponded with the decrease in Nox1 mRNA levels. This decrease in Nox1 was reduced with pretreatment of the PI3 kinase inhibitor wortmannin. PI3 kinase is upstream of Nrf2 activation, therefore we believe that wortmannin was able to inhibit Nrf2 activation maintaining the LPS induced increase in Nox1. Furthermore, low levels of LPS failed to induce increases in Nrf2 mRNA levels. Therefore we believe that high dose LPS activated Nox1, increasing ROS production enough to activate Nrf2 and turning on a Nrf2 dependent antioxidative state inhibiting further ROS production and increases in Nox1. Low doses of LPS were capable of increasing ROS and slowing increasing Nox1, but we believe this activation was not sufficient to activate Nrf2 until significantly later in the experimental time course.

The final aim of this project was to examine the effects of an initial inflammatory challenge on the inflammatory response to a later challenge. The data demonstrate that low dose LPS was able to 'prime' the system for a future inflammatory challenge. We believe the delayed activation of Nrf2 allowed Nox1 to accumulate within the MEF so that the second challenge activated Nox1 (substantially increased when compared to basal levels), and produced significantly more ROS. However, high dose LPS activated Nrf2 after the initial challenge inhibiting Nox1 accumulation and causing Nox1 levels to fall well below basal levels. Thus, when these MEF were challenged for a second time the amount of Nox1 in cells was less than that available for initial activation, resulting in decreased production of ROS. We have termed these cells to be in a tolerant state. Thus we demonstrated that low dose LPS is capable of priming MEF for a future LPS challenge, but that high dose LPS causes MEF to become tolerant and incapable of reacting to a later challenge.

Oxidative stress and oxidative damage are considered to be primary causes in atherosclerosis, hypertension, Parkinson's disease and are believed to contribute to some cases of cancer and diabetes. Furthermore, ROS are believed to be contributors to the pathology of rheumatoid arthritis, autoimmune diseases, stroke, cystic fibrosis, multiple sclerosis, Alzheimer's disease, and HIV. Obviously ROS and oxidative stress are believed to be significant contributors to many major health concerns. Therefore, it is important to understand the underlying molecular and cellular mechanisms responsible for the major inflammatory responses leading to these diseases and the role that ROS plays in their pathology. We have identified Nox1 as being a major contributor to LPS induced ROS production in MEF. Furthermore, we have shown that

this response as well as the cellular capability to control this response varies with the severity of the challenge. This work suggests that LPS is capable of activating not only ROS production but also antioxidative feedback by activation of Nrf2. We have also shown that while ROS production is IRAK-1 dependent that activation of antioxidative feedback is not. This may lead to potential therapeutic methods for antioxidative upregulation without activation of ROS production, thereby more effectively controlling inflammation and the damage it performs.

While this work has begun to explain the mechanism by which Nox1 dependent ROS production is regulated, future studies should be done to examine the role of other cellular sources of ROS under inflammatory stress. Furthermore, more work needs to be done to determine the antioxidants responsible for the down regulation of Nox1. Nrf2 is considered to be a major antioxidative regulator and we believe is a key player in this project, but it is responsible for the activation of other antioxidants and the activation and actions of these antioxidants needs to be further clarified. Learning to control the interactions between the inflammatory response and mechanisms of inflammatory regulation would be an extremely useful tool in developing strategies to combat many of the diseases listed above.

In the last several years the field of immunology and the understanding of disease pathology and progression has come a long way. Discoveries of key receptors, agonists, mediators, transcription factors, signaling pathways, and pathway interactions involved in the development of the immune system continue to open up new avenues to be discovered. Our findings have added to this body of knowledge, allowing for better understanding of the regulation and control of the inflammatory pathways of our immune system.

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