Cellular Reprogramming in Skeletal Muscle after Repeated Exposures to Endotoxin

Laura M. Denko

Thesis submitted to the faculty of Virginia Polytechnic Institute and State University in partial fulfillment of the requirements for the degree of

Master of Science

in

Human Nutrition, Foods, and Exercise

Matthew W. Hulver, Chair

Madlyn I. Frisard

Kevin P. Davy

Liwu Li

June 21, 2012

Blacksburg, VA

Key Words: TLR4, LPS, endotoxin, skeletal muscle

Cellular Reprogramming in Skeletal Muscle after Repeated Exposures to Endotoxin

Laura M. Denko

Abstract

Obesity-related metabolic derangements have been linked to toll-like receptor 4 (TLR4), an innate immune system receptor, due to its role in proinflammatory pathways. Lipopolysaccharide (LPS), a gram-negative bacteria cell wall component, is the ligand for TLR4, and has been shown to be elevated in states of metabolic disease. Heightened levels of circulating endotoxin is termed metabolic endotoxemia and has been linked to systemic inflammation which is associated with obesity, type 2 diabetes mellitus (T2DM), and cardiovascular disease (CVD). Immune cells exhibit a protective ability to develop endotoxin tolerance. The objective of this study was to determine if endotoxin tolerance exists in skeletal muscle cells, and if a condition that mimics a state of over nutrition, such as elevated levels of fatty acids, affect this tolerance. To this end, L6 skeletal muscle cells were treated with low (50 pg/mL)- and high (500 ng/mL)-doses of LPS, with and without the presence of free fatty acids (FFAs). Tolerance was assessed by measuring: 1) changes in mRNA expression of interleukin-6 (IL-6) and monocyte chemoattractant-1 (MCP-1) as markers of a pro-inflammatory response; and 2) mRNA levels of peroxisome proliferator-activated receptor gamma coactivator-1 alpha (PGC1-α) and mitochondrial oxidative capacity via an XF24 Flux Analyzer (Seahorse Bioscience) as measures of the metabolic response. Tolerance to LPS was observed in response to low- and high-doses with MCP-1 mRNA transcription but not IL-6. Changes in PGC1-α and mitochondrial OCR exhibited a tolerant effect in response to the high dose of LPS but not the low dose. The addition of free fatty acids to LPS treatments did not prevent the tolerant effects

under any conditions. In conclusion, LPS tolerance exists in skeletal muscle cells but appears to differ depending on pro-inflammatory target and LPS concentration. Additionally, fatty acids, in the current model, have no effect on LPS tolerance.

Acknowledgements

This thesis could not have been written without Dr. Matthew Hulver, who not only served as my supervisor, but also as a mentor, encouraging me and challenging me throughout my academic program, accepting nothing but my best work. I would also like to thank all of my committee members, Dr. Madlyn Frisard, for her support with the Seahorse experiments, Dr. Kevin Davy, for allowing me to assist with his clinical study, and Dr. Liwu Li, for teaching me everything I know about molecular medicine and immunology. I would also like to thank everyone in the Hulver and Frisard labs for all of their assistance and support, especially Dr. Ryan McMillan, Dr. Yaru Wu, Dr. Kevin Voelker, Kristin Wahlberg, and Josh Pittman. Lastly, I would like to thank my parents for supporting me throughout my academic career, and my sister, Jessica, for her encouraging words.

Table of Contents

List of Figures

List of Tables

List of Definitions

1. **CVD- Cardiovascular Disease**
2. **IL-6- Interleukin-6**
3. **LPS- Lipopolysaccharide**
4. **MCP-1- Monocyte chemotactic protein-1**
5. **MyD88- Myeloid differentiation 88**
6. **Nf-κB- Nuclear factor-kappa B**
7. **PAMP- Pathogen-associated molecular pattern**
8. **PGC1-α- Peroxisome proliferator-activated receptor-γ coactivator 1α**
9. **PRR- Pattern recognition receptor**
10. **T2DM- Type 2 Diabetes Mellitus**
11. **TBK1- TANK-binding kinase 1**
12. **TGFβ- Transforming growth factor, beta**
13. **TLR4- Toll-like receptor 4**

Chapter 1: Introduction

Introduction

Obesity, a major health disorder, is defined as having a body mass index (BMI) of greater than 30 kg/m^2, and is associated with abnormal inflammation and dysregulated skeletal muscle metabolism (79). These pathological states resulting from obesity can lead to insulin resistance, dyslipidemia, and type 2 diabetes mellitus (T2DM). Obesity-related metabolic derangements have been linked to Toll-like receptor 4 (TLR4), an innate immune system receptor, due to its role in proinflammatory pathways (82).

Lipopolysaccharide (LPS), a gram-negative bacteria cell wall component, is the primary ligand for TLR4. It enters the bloodstream via chylomicron translocation from the small intestine and colon (31). LPS binding to TLR4 initiates a proinflammatory response by the innate immune system. If this response is left uncontrolled, exacerbated inflammation may lead to tissue damage and chronic disease. Endotoxin tolerance, or desensitization, is a type of cellular reprogramming involving the mechanistic immune response to subsequent lipopolysaccharide (LPS) exposures that permits the host to defend itself from an exacerbated inflammatory state (8, 61, 87). It also enables the host to maintain cellular functions and homeostasis (15, 27, 33).

Chronic stimulation of TLR4 by low levels of LPS (~50pg/ml) has been the basis for the coined term, metabolic endotoxemia. This is defined as low levels of circulating endotoxin within the blood of humans and rodents that may be the foundation of systemic inflammation, often seen in those who are obese or have T2DM, cardiovascular disease (CVD), atherosclerosis, or chronically consume a high-fat diet (16, 27, 86).

Our laboratory has recently shown that TLR4 signaling modulates skeletal muscle substrate preference in a manner that favors the oxidation of glucose and pyruvate over that of

fatty acids, a characteristic that has been observed in skeletal muscle of obese humans (46). Based on these observations, in conjunction with the newly identified condition that is associated with obesity termed metabolic endotoxemia, a central objective of our laboratory is to ascertain the role of metabolic endotoxemia in the pathogenesis of metabolic diseases such as obesity and type 2 diabetes. As metabolic endotoxemia is thought to be a chronic condition in the obese state (15), whether or not skeletal muscle cells can develop a tolerance to endotoxin, like that of immune cells, is an important question, which to date has yet to be answered. The purpose of the studies described herein was to determine if endotoxin tolerance exists in skeletal muscle cells, and if so, does a condition that mimics a state of over nutrition, e.g., elevated levels of fatty acids, affect this tolerance.

Statement of Problem

The immune response to a foreign invader gives rise to inflammation that can lead to cellular damage and apoptosis if uncontrolled. During metabolic endotoxemia, patients are exposed to persistently low-doses of LPS, which is a common characteristic of chronic disease. Host immune cells can attenuate this inflammation through various cellular mechanisms and cross-talk between cells of the innate immune system, however, whether skeletal muscle cells also exhibit endotoxin tolerance is unknown.

Significance of the Study

LPS tolerance is a protective mechanism utilized by the host to protect itself from inflammatory damage. By investigating whether or not LPS tolerance exists in skeletal muscle, we can determine if skeletal muscle has protective mechanisms to manage chronic inflammation. With a greater understanding in this area, treatment and management of chronic inflammatory conditions characterized by metabolic endotoxemia may improve. Specifically, obese and T2DM patients may be unable to become tolerant to chronic endotoxin exposure and could experience significant inflammation that may otherwise be avoided in a healthy individual.

Specific aims

1. To determine if skeletal muscle cells exhibit endotoxin tolerance.
2. To determine if the presence of free fatty acids have an effect on endotoxin tolerance in skeletal muscle cells.

Main Hypotheses

1. Skeletal muscle cells will become tolerant to intermittent and continuous endotoxin exposure.
2. Skeletal muscle cells will not become tolerant to intermittent and continuous endotoxin exposure in the presence of free fatty acids.

Basic assumptions

1. The *in vivo* physiological characteristics of skeletal muscle are retained in skeletal muscle cell cultures.
2. The skeletal muscle cell lines used in these studies possess normal inflammatory response and metabolic function.
3. Skeletal muscle cells will respond to LPS exposure.

Limitations

1. L6 skeletal muscle cells derived exclusively from one tissue.
2. All experiments were performed from cultured cells as opposed to an in vivo research model.
3. Palmitate and oleate, albeit the most abundant fatty acids in circulation, are only two of the many fatty acids in circulation.
4. The multiple exposure model allotted only 3 hours for each "rest period", whereas the time between human meal consumption may in fact be longer.
5. The total treatment time for each timeline was 12 hours, whereas a longer timeline may have been a more accurate depiction of chronic metabolic endotoxemia.

Chapter 2: Review of the Literature

Toll-like receptors and the innate immune system

Inflammation is a pathophysiological state that results from the host body defending itself against a foreign invader, primarily through the innate immune system. The innate immune system is responsible for early detection of microbes and is stimulated by the activation of pattern recognition receptors (PRRs), specifically toll-like receptors (TLRs) (64). TLRs have the ability to identify specific microbial components known as pathogen-associated molecular patterns (PAMPs) (14). These are composed of conserved RNA and DNA sequence motifs, lipids, lipoproteins, and proteins of viruses, bacteria, and fungi (1, 66). Different PAMPs bind to different TLRs and initiate signaling pathways that lead to an immune response (1, 45). The Toll gene was first discovered during genetic studies in the fruit fly, drosophila, which then lead to the search for the mammalian homologue (4, 59). To date, 12 mammalian Toll receptors have been identified, the first being toll-like receptor 4 (TLR4) (68). Of these TLRs, only some are expressed in skeletal muscle, including TLR2-5, TLR7, and TLR9 (13, 70, 81).

TLR4 is located primarily in immune cells, but is also found in adipose, liver, and skeletal muscle tissues (28, 78). TLR4 is activated by a cellular wall component of gram-negative bacteria known as lipopolysaccharide (LPS). Upon LPS binding to TLR4, adaptor molecules are recruited to the cytoplasmic domain along the plasma membrane of a cell. Initially, TLR4 associates with myeloid differentiation factor-2 (MD-2) and then TLR4/MD-2 binds to the co-receptor, CD14. This complex binds to LPS along with the LPS's binding protein, LBP (34, 49). There are two pathways associated with LPS/TLR4 binding: the MyD88-dependent pathway and the MyD88-indendent pathway (also known as the TRIF pathway) (17).

Current literature supports the notion that early and late stages of NF-κB activation exists (44). The early-stage activation of NF-κB is associated with the MyD88-dependent pathway that is stimulated when TLR4 interacts with LPS on the cellular membrane and recruits TIRAP/Mal to the action site. MyD88 then recruits IL-1 receptor-associated kinase 4 (IRAK4) to TLR4 which leads to the phosphorylation of IRAK1. The cytokine signaling mediator, tumor necrosis factor receptor-associated factor (TRAF), is activated by IRAK1. IRAK1 becomes phosphorylated and ubiquinated, allowing for stimulation of TAB (TAK-1 binding protein-1) and subsequent NF-κB activation. Mitogen-activated protein kinases (MAPKs) can also be activated by TAK1 following phosphorylation. MAPKs act to phosphorylate proteins in order to continue signaling from extracellular stimuli.

Late-stage activation of NF-κB is characterized by the endocytosis of TLR4 into the cytoplasm of the cell (41). This cytosolic TLR4 endosome activates the TRIF pathway. TLR4 forms a complex with TRAM and TRIF that signals Tank Binding Kinase 1 (TBK1) to phosphorylate IKKε or activate interferon regulatory factor 3 (IRF-3) (25, 40, 43, 44, 71). TRIF may also signal through TRAF6 to activate TAK1. IRF-3 activates the proinflammatory cytokine, IFN-β, and the STAT1 pathway, facilitating the activation of the adaptive immune system.

Both pathways lead to the dissociation of NF-κB from its inhibitor protein, IkBα, which upon phosphorylation, is ubiquitinated, allowing translocation of nuclear factor kappa B (NF-κB) into the nucleus (35). Subsequently, NF-κB activates the gene expression of proinflammatory cytokines and chemokines, also known as regulatory peptides, which function to maintain communication between other cells in the body (11).

High levels of cytokine secretion have earned the name "cytokine storm", which facilitates the inflammatory response through NF-κB, caused by LPS. While this inflammatory response is essential for clearance of pathogens, excessive inflammation may cause tissue and cellular damage to the host cells. The extent of the damage to the host may depend on the concentration and length of LPS exposure, as well as the ability of the host to initiate protective mechanisms putting exacerbated inflammation to a halt.

LPS and the initiation of cellular reprogramming

LPS, the cell wall component of Gram-negative bacteria, can circulate throughout the blood and initiate various immune responses. Low concentrations (<50 pg/ml) of LPS in the bloodstream have been associated with endotoxemia and chronic inflammatory conditions. Some of these include type 2 diabetes mellitus (T2DM), atherosclerosis, obesity, and insulin resistance (36, 47, 50). This chronically low concentration of LPS has been shown to contribute to mild elevation of inflammatory mediators, or cytokines, by removing suppressive nuclear receptors on the promoter regions of their respective genes (55). Higher concentrations of circulating LPS (50-500 ng/ml) are seen during cases of sepsis, systemic inflammatory response syndrome (SIRS), and endotoxin shock; all of these conditions are commonly seen in hospitalized patients.

When the host cell initially encounters an antigen, in this case LPS, a primary immune response occurs, of which is called "priming" (61). Priming occurs when the cells react to an antigen and send signals to activate the innate and adaptive immune systems, secreting proinflammatory cytokines and chemokines. Priming is necessary to prepare the host for additional encounters with the same antigen, by allowing it to adapt and change the existing signaling pathways.

LPS tolerance, or cellular reprogramming, occurs after the host has been primed with LPS. Beeson first documented this phenomenon when he demonstrated that repeated injections of a typhoid vaccine in rabbits reduced their fevers initially caused by the vaccine (6, 7). Tolerance is observed when the host can no longer initiate a robust reaction to subsequent LPS exposures and thus, the immune response is blunted (8, 61). This response is characterized by the inhibition of proinflammatory markers, including the cytokines TNF-α, IL-6, and MCP-1. In addition, activation of anti-inflammatory markers IL-10 and TGF-β may be up-regulated, and act to suppress further inflammatory signals, as seen in tolerant macrophages (69). However, the changes in signaling pathways that mediate cellular reprogramming are not quite that simple.

Cellular reprogramming can be used as a protective mechanism allowing the host to maintain homeostasis. Characteristics of cellular reprogramming vary between species, cell types, and within signaling mechanisms. Several protective mechanisms utilized by host cells in response to LPS exposure have been identified and include: down regulation of TLRs, negative regulators of TLR signaling, deficient recruitment of adaptor proteins, and variations in NF-κB subunits (64). In macrophages, TLR4 expression was down-regulated after a LPS pretreatment (63). Negative regulators of TLR signaling include proteins such as SHIP, IL-1R-associated kinase-M, and more recently, microRNAs (5, 65, 83). In humans, similar mechanisms contributing to cellular reprogramming were found including decreased TLR4 expression on the cell membrane, blunted MyD88 recruitment to TLR4, and deficient IRAK1 activation (32, 58). IRAK1 has gained attention in recent years and found to be essential for mediating the proinflammatory effect of low-dose LPS (55). IRAK1-deficient mice had reduced cytokine signaling when compared to their wild-type controls, however IRAK4 and other IRAK family members may be up-regulated to take the place of IRAK1 (42, 76, 80).

Intolerance to LPS exposures

Intolerance to LPS, or in inability to maintain homeostatic conditions with the host, can lead to a robust inflammatory reaction. High levels of circulating LPS may result in sepsis, SIRS, and chronic inflammatory conditions (51, 56). This uncontrolled inflammation can lead to tissue damage and apoptosis due to the increased levels of circulating proinflammatory cytokines (85). This may affect global cellular function via signaling through TLR's.

Role of TLRs in chronic diseases and metabolic regulation

TLR2 recognizes several PAMPs that include lipopeptides, peptidoglycan, and lipoteichoic acid from Gram-positive bacteria (19, 44). It plays a cell type-specific role in recognition of different ligands to initiate an immune response. In rat and mouse myocytes, TLR2 activated NF-κB through the MyD88-dependent pathway (29). Several studies have also found that cross-talk can occur between TLRs. TLR4 can activate TLR2 and consequently its downstream signaling pathways (38). TLR2 and TLR4 signaling was found to be linked with the metabolic markers, peroxisome proliferator-activated receptor-γ coactivator 1 (PGC1), α and β, during sepsis within the liver, suggesting that inflammation can affect mitochondrial biogenesis (77). These markers regulate genes involved in fatty acid oxidation (FAO), electron transport, and oxidative phosphorylation (72). TLR2 and TLR4 are significant receptors of innate immunity and metabolism have dual roles in response to LPS.

Skeletal muscle has been shown to secrete tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), interleukin 1-β (IL-1 β), and monocyte chemotactic protein-1 (MCP-1) in response to NF-κB activation (11, 27, 29, 67). These peptides vary depending on their autocrine, paracrine, or endocrine functions. They may signal the innate or adaptive immune system or act

on nearby cells to illicit an immune response. Without the characteristic cellular reprogramming seen in immune cells in response to LPS, muscle cells may experience exacerbated inflammation, with metabolic implications such as altered substrate utilization and decreased insulin sensitivity (60). Metabolic endotoxemia can alter the host's metabolic functions, including altered mitochondrial respiration as seen in rats, and by contributing to insulin resistance (27, 73, 84). In cell culture, non-septic doses of LPS decrease fatty acid oxidation and increase glucose oxidation in C2C12 cells (27). Chronic activation of TLR4 by LPS has induced increased muscle proteolysis, contributing to cachexic wasting (22). TLR4-stimulated NF-kB activation has been shown to be involved in cachexic muscle wasting by increasing expression of IL-6 and TNF-α, and by activating skeletal muscle proteolysis (9, 10). This complication is often accompanied by chronic inflammatory states such as chronic heart failure (CHF) and diabetes (22). In addition, increased serum levels of LPS have been measured in patients suffering from CHF, T2DM, and even cancer (2, 33, 62). Taken together, these pathological states of metabolic endotoxemia may have negative impacts on muscle metabolism and contribute to a cachexic state.

Markers of metabolism in skeletal muscle are negatively impacted during inflammatory conditions. Mice injected with LPS have increased TNF-α, MCP-1, and IL-6 mRNA expression (72). TNF-α stimulated reactive oxygen species (ROS) production as well as the ubiquitin conjugating pathway lead to mitochondrial damage and proteolysis, respectively (52). Interestingly, mice injected with LPS showed decreased production of PGC1-α and PGC1-β, suggesting that chronic, low-doses of LPS may lead to mitochondrial dysfunction or a reduction in number (72).

Summary

Endotoxin tolerance is a protective mechanism utilized by the host to avoid uncontrolled inflammation and apoptosis. Immune cells, including macrophages and dendritic cells, exhibit tolerance to repeated endotoxin exposures, through multiple mechanisms, including decreased expression of TLR4 and decreased recruitment of adaptor molecules to the binding site. Chronic signaling through TLR4 can lead to low-grade inflammation as evidenced by the production of proinflammatory cytokines. These inflammatory states may play a role in alterations in skeletal muscle metabolism, as well as cachexic muscle wasting, common in chronic inflammatory diseases such as CHF, cancer, and T2DM.

Chapter 3: Methods

Cell Culture Studies

Rat L6 skeletal myoblasts were used for all experiments. This cell line provides a widely used model to observe skeletal muscle function (39). They were grown to confluence (80-90%) in Dubelcco's modified eagles medium (DMEM) containing 10% fetal bovine serum, 50U/ml penicillin, and 50ug/ml streptomycin (Invitrogen, Carlsbad CA). Cells were maintained in either 6- or 12-well plates. Once confluent, cells differentiated in DMEM containing 2% horse serum, 50U/ml penicillin, and 50ug/ml streptomycin for 4-7 days. The media was changed every other day and experiments were conducted following overnight serum-free starvation media.

Treatments

Multiple plates of cells were grown to characterize the effects of different number of exposures of the cells to low- (50 pg/ml) and high-dose (500 ng/ml) LPS. The cell culture model followed the below timeline (Table 1) to simulate the typical meal patterns of humans and sequential rise and fall of endotoxins in the blood stream. In between treatments, cells were subjected to a 3hr "rest" period in which starvation DMEM was used. After each treatment, cells were washed once with PBS, and following their final treatment, washed three times, immediately before collection for gene expression analysis. To distinguish the difference between typical meal-induced endotoxemia and chronic, low-grade inflammation, a second timeline was utilized in this cell culture model (Table 2). Cells were continuously exposed to a low dose (50pg/mL) of LPS for 4, 8, or 12 hours followed by collection. This approach was an attempt to mimic metabolic endotoxemia (low-dose LPS) in muscle cells while multiple collection points aided in determining if endotoxin tolerance exists in muscle cells. To distinguish the FFA effect from the LPS effect, cells were exposed to BSA, BSA + LPS, BSA + fatty acids, and BSA + fatty acids + LPS. Lipid-containing media was prepared by conjugating

the free fatty acids, palmitate (16:0) and oleate (18:1), with fatty acid- and endotoxin-free bovine serum albumin (BSA). The LPS treatments consisted of low- (50 pg/ml) and high-dose (500 ng/ml) LPS. Endotoxin-free BSA was used in order to negate the possibility of additional endotoxins interfering with the treatments. BSA was prepared and combined with a ratio of 2:1 palmitate to oleate to mimic a high saturated fat environmental condition (600µM FFA: 200µM BSA). Oleate (200µM) was combined with palmitate (400µM) in order to prevent the apoptotic effects that palmitate alone can initiate (54). The addition of free fatty acids to the media not only generated a more physiological setting for the cells, but also helped us determine their role in endotoxin tolerance.

TABLE 1: Intermittent endotoxin treatment timeline

Plate:	Treatment:
1	2 h LPS → Rest → 2 h LPS → Rest → 2 h LPS → Collect
2	2 h LPS → Rest → 2 h LPS → Collect
3	2 h LPS → Collect

TABLE 2: Continuous endotoxin treatment timeline

Plate:	Treatment:
1	---------- 12 h LPS ----------| Collect
2	---------- 8 h LPS ----------| Collect
3	--------- 4 h LPS ---------| Collect

Quantitative Real-Time PCR Analysis

Following treatment, cells were scraped and collected in 1ml TRIzol (Invitrogen, Carslbad, CA). Total cell mRNA was extracted using an RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. qRT-PCR will be performed using an ABI PRISM 7900 Sequence Detection System instrument and TaqMan Universal PCR Master Mix used according to manufacturer's specifications (Applied Biosystems, Inc., Foster City, CA). IL-6, MCP-1, and PGC1-α expression was normalized to β-actin mRNA levels. Primers and 5# FAM-labeled TaqMan probes were purchased as pre-validated assays (Applied Biosystems, Inc., Foster City, CA). Relative quantification of target genes were calculated using the 2-ΔCT method, which are validated for each primer/probe set.

Mitochondrial Respiration

Mitochondrial respiration of cultured L6 cells was performed using an XF24 extracellular flux analyzer (Seahorse Bioscience, North Billerica, MA) as described by Choi et al with modifications (30). Immediately after protein quantification, cells were seeded into 24 well XF24 V7 cell culture microplates at a density of 5,000 cells per well. Cells were grown unto 80% confluence and differentiated into fully differentiated myotubes. Experiments were conducted in serum-free media between days 5 and 7 of differentiation. Immediately following treatments, cells were loaded into the XF24 analyzer. Experiments consisted of 3 minute mixing, 2 minute wait, and 2 minute measurement cycles, unless otherwise stated. Oxygen consumption was measured under basal conditions, in the presence of the mitochondrial inhibitors oligomycin (0.5 uM) or rotenone (0.25 uM), or in the presence of the mitochondrial uncoupler carbonyl cyanide-

p-trifluoromethoxyphenylhydrazone (FCCP) (0.3 uM) to assess maximal oxidative capacity. The RCR was calculated as the ratio of state 3/state 4 respiration. All experiments were performed at 37°C.

Statistics

A one-way ANOVA with Tukey post-hoc analysis was used to compare results from L6 cells in response to LPS. A two-way ANOVA with Tukeys post-hoc analysis was used to compare the interaction between the fatty acid treatments and the LPS exposures. All cell culture studies were repeated at least twice to establish enough replication to observe potential significant differences. T-tests are two-tailed and the level of significance is set at $P<0.05$.

Chapter 4: Results

Cell Culture Results

Intermittent LPS exposures in skeletal muscle myotubes elicits different transcriptional response for IL6 and MCP-1.

Recent studies have investigated the effects of LPS exposure on immune cells, as they are the primary acting cells in host defense systems. A previous study by Albrecht and his colleagues demonstrated using human dendritic cells, a time-dependent decrease in cytokine mRNA

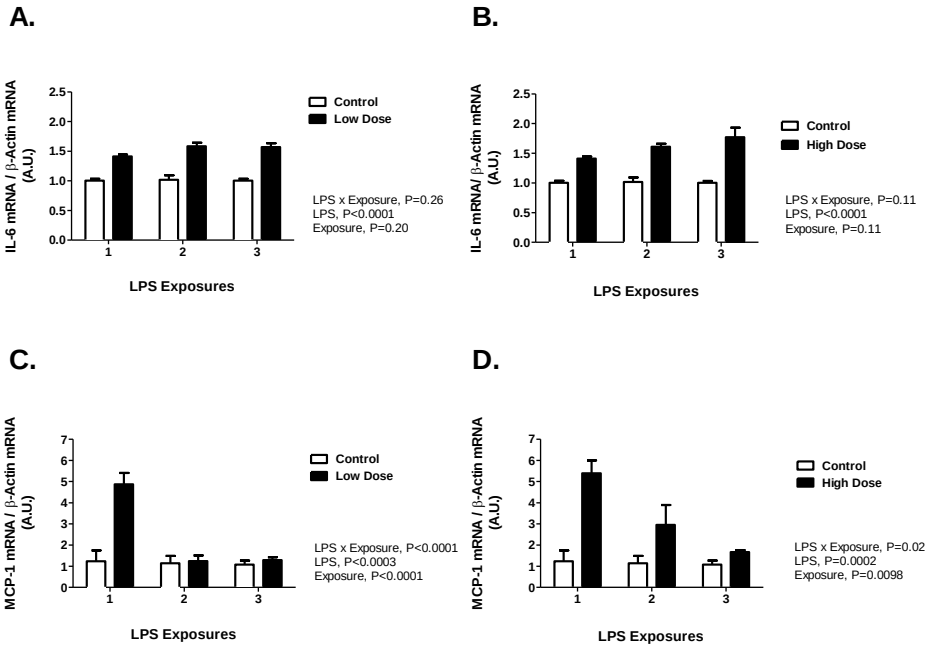

Figure 1: The effects of intermittent LPS exposures on proinflammatory cytokines. Cells were treated with control media, low-, and high-doses of LPS (50 pg/ml or 500 ng/ml, respectively) for 2 h (1 LPS Exposure), 4h (2 LPS Exposures), or 6h (3 LPS Exposures) with a 3h "rest" period between exposures, in which the cells were treated with serum-free media. There was a significant LPS effect on IL-6 expression (Figures 1 A & B) independent of concentration, with no significant LPS x exposure interaction or added effect of repeated LPS exposures. The LPS x exposure interaction, LPS effects, and repeated exposure effects were all significant for MCP-1 with both low (Figure 1C) and high (Figure 1D) doses of LPS.

expression in response to endotoxin exposure (3). In order to mimic similar conditions in our laboratory, we exposed skeletal muscle cells to either low- or high-doses of LPS for 2h (1 LPS exposure), 4h (2 LPS exposures) or 6h (3 LPS exposures). IL-6 mRNA expression increased in response to low and high doses of LPS (Figures 1 A&B) with no added or diminished effects

with multiple exposures. Both low and high doses of LPS elicited a significant induction of MCP-1 mRNA (Figures 1 C&D) with the 1st exposure of LPS, which was diminished to control levels after the 3rd exposure. These data are indicative of a tolerant effect in skeletal muscle cells to LPS with transcription of MCP-1 but not IL6.

PGC1-α transcription develops a tolerant effect to multiple LPS exposures.

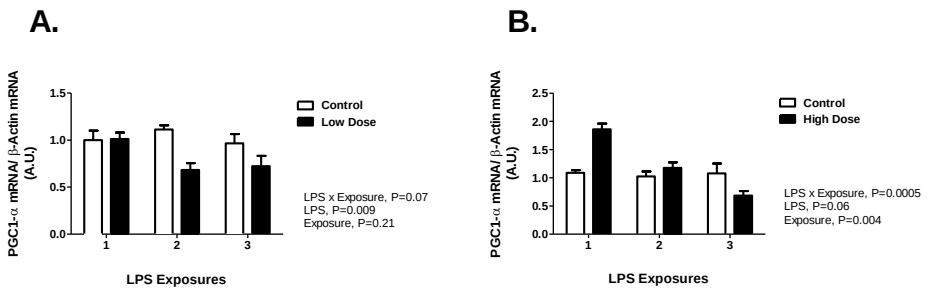

Figure 2: The effects of intermittent LPS exposures on PGC1-α mRNA. Cells were treated with control media, low-, and high-doses of LPS (50 pg/ml or 500 ng/ml, respectively) for 2 h (1 LPS Exposure), 4h (2 LPS Exposures), or 6h (3 LPS Exposures) with a 3h "rest" period between exposures, in which the cells were treated with serum-free media. With low dose LPS treatment (Figure 2A), the LPS x exposure interaction trended toward significance, which was driven by a significant LPS-induced reduction in PGC1-α mRNA following the 2nd and 3rd LPS exposures. Conversely, the high dose LPS treatment (Figure 2B) resulted in an increase in PGC1-α mRNA following one exposure., with a tolerant effect following the 2nd and 3rd exposures.

To determine the effects of multiple LPS exposures on metabolic function in skeletal muscle myotubes, we measured PGC1-α mRNA expression. Low and high doses of LPS resulted in different PGC1-α transcriptional responses with no effect of low dose LPS after one exposure with subsequent decreases with the 2nd and 3rd exposures (Figure 2 A&B). The high dose of LPS caused a significant increase in PGC1-α with the 1st exposure with reductions below control levels after the 3rd exposure. These data support an LPS tolerance effect in skeletal muscle cells with PGC1-α expression but more so with high dose LPS than low.

LPS reduces the oxygen consumption rate, which also exhibts a tolerant effect with repeated exposures.

To determine the effects of multiple LPS exposures on maximum respiration in skeletal muscle myotubes, we quantified the oxygen consumption rate (OCR) utilizing an XF24 extracellular flux analyzer. Cells were exposed to low- or high-doses of LPS for 1, 2, or 3 exposures. Both the low and high doses of LPS elicited a significant reduction in maximally stimulated oxygen consumption rate (Figure 3 A&B), which was more robust with high dose than with the low dose. This effect was absent after the 3rd exposure of LPS with both low and high doses.

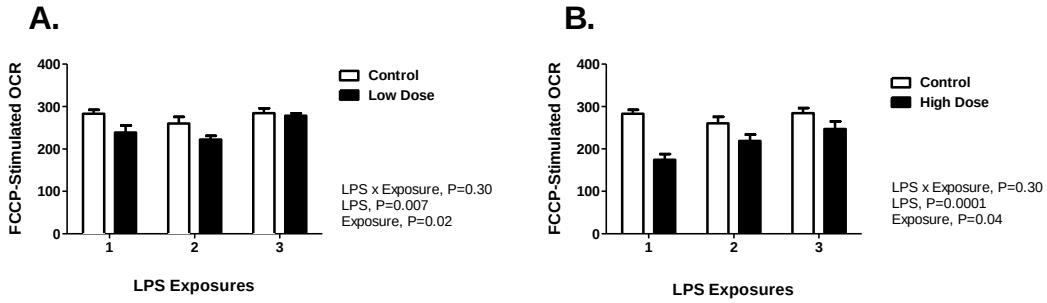

Figure 3: The effects of intermittent LPS exposures on maximum oxygen consumption. Cells were treated with control media, low-, and high-doses of LPS (50 pg/ml or 500 ng/ml, respectively) for 2 h (1 LPS Exposure), 4h (2 LPS Exposures), or 6h (3 LPS Exposures) with a 3h "rest" period between exposures, in which the cells were treated with serum-free media. With low dose LPS treatment, there was a significant reduction in maximally stimulated OCR following the 1st and 2nd LPS exposure, which was absent after the 3rd exposure (Figure 3A). The high dose LPS treatment exhibited a more robust reduction in maximally stimulated OCR (Figure 3B). This effect was abrogated in a step-wise fashion with the 2nd and 3rd LPS exposures, which is indicative of a LPS tolerance effect.

Continuous LPS exposure leads to LPS tolerance as evidenced by blunted MCP-1 but not IL-6 mRNA expression

To test the hypothesis that skeletal muscle cells would become tolerant to continuous endotoxin exposures, we exposed cells to low-doses (50 pg/ml) of continuous LPS treatments for 4, 8, or 12 h (Figure 4). IL-6 mRNA expression was significantly increased in response to LPS after 4, 8, and 12 hours of LPS exposure with the most robust effect at 8 hours. The LPS-induced IL-6 expression was most modest at the 12 hour time point, which may be indicative of a tolerance effect. MCP-1 mRNA levels were also significantly increased at all time points in response to LPS with the most robust effect being at the 4 hour time point. The LPS effect on MCP-1 mRNA was blunted at 8 and 12 hours, which again, may be indicative of LPS tolerance.

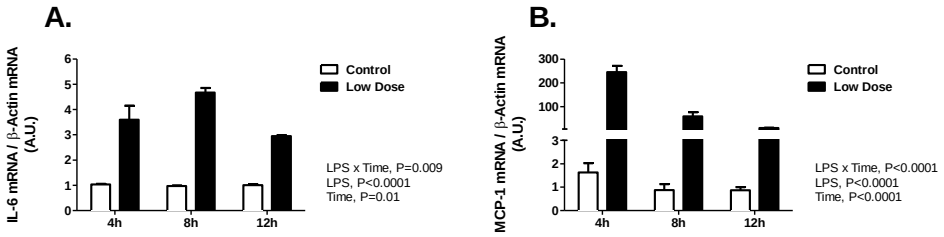

Figure 4: The effects of continuous LPS exposure on proinflammatory cytokine expression. Cells were exposed to low-dose LPS (50 pg/ml) for 4h, 8h, or 12h then collected for RNA extraction. IL-6 and MCP-1 mRNA expression was significantly increased in response to LPS (Figures 4 A & B). There was a also a significant LPS x time interaction with both IL6 and MCP-1, which is indicative of a tolerant effect, although modest.

Free fatty acids do not affect IL6, MCP-1, and PGC1-α mRNA in response to intermittent LPS exposures in skeletal muscle myotubes.

To determine if free fatty acids would prevent the observed tolerance in response to repeated LPS exposures, a 2:1 ratio of palmitate to oleate was added to the media (400µM palmitate, 200µM oleate). The addition of the free fatty acids to the LPS treatments had no affect on IL6,

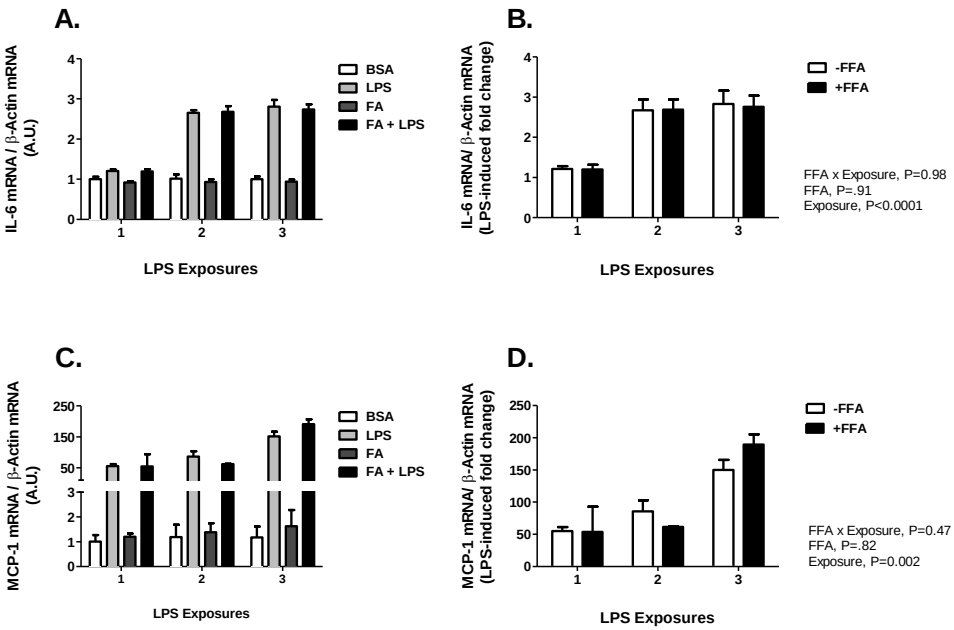

Figure 5: The effects of intermittent high-dose LPS exposure with the addition of free fatty acids on pro-inflammatory cytokine expression. Cells were treated with control media or high dose LPS +/- 200 µM FFAs (2:1 ratio of 16:0:18:1) for 2 h (1 LPS Exposure), 4h (2 LPS Exposures), or 6h (3 LPS Exposures) with a 3h "rest" period between exposures, in which the cells were treated with serum-free media. There was a significant increase IL6 mRNA expression with LPS treatment with all three exposures but more robust with the 2nd and 3rd (Figure 5A). MCP-1 mRNA levels were also increased with LPS treatment with the most robust effects following the 3rd exposure (Figure 5C). The addition of FFAs had no effect on LPS-induced cytokine expression at any time point (Figures B & D).

MCP-1, or PGC1-α expression at any time point (Figure 5 & 6). It is also noteworthy that the tolerant effect to intermittent LPS exposures observed previously with MCP-1 (Figure 1C) was not observed in these experiments. Bovine serum albumin (BSA) was added to the control media as it served as a carrier for the fatty acid treatments. As such, we speculate that the BSA may have had a confounding influence in these studies, which warrants further research.

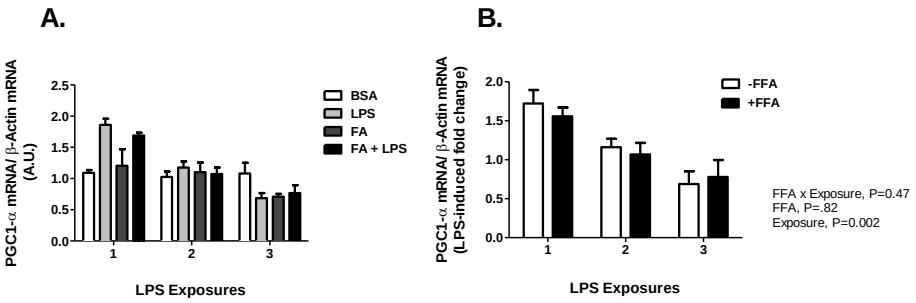

Figure 6: The effects of intermittent high-dose LPS exposure with the addition of free fatty acids on PGC1-α mRNA. Cells were treated with control media or high dose LPS +/- 200 μM FFAs (2:1 ratio of 16:0:18:1) for 2 h (1 LPS Exposure), 4h (2 LPS Exposures), or 6h (3 LPS Exposures) with a 3h "rest" period between exposures, in which the cells were treated with serum-free media. LPS treatment caused a significant increase in PGC1-α mRNA after one exposure which was absent following the 2nd exposure, and reduced following the 3rd (Figure 6A). These effects were not affected by the addition of fatty acids (Figure 6B).

The addition of FFA to continuous LPS treatments alters the effects on IL-6 and MCP-1 expression.

The addition of FFA to the LPS treatments, surprisingly, abrogated the LPS-induced IL-6 effect at 4, 8 and 12 hours of treatment, with the abrogation becoming more modest with time (Figure 7 A&B). The addition of FFA had no effect in LPS-induced MCP-1 expression at any time point (Figure 7 C&D). However, FFA alone increased MCP-1 expression at 4 hours, which was no longer evident at 8 and 12 hours.

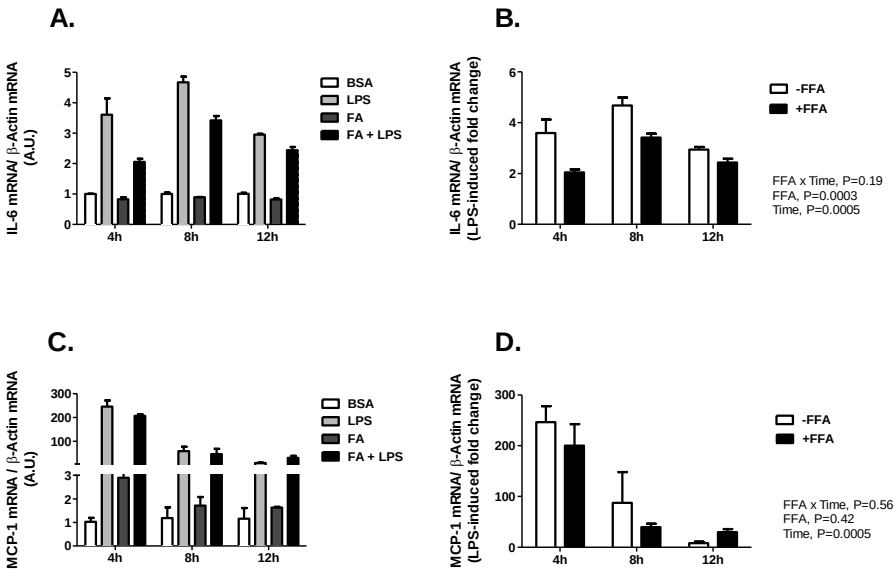

Figure 7. The effects of continuous low-dose LPS exposure with the addition of free fatty acids on pro-inflammatory cytokine expression. Cells were exposed to low-dose LPS (50 pg/ml) +/- 200 μM FFAs (2:1 ratio of 16:0:18:1) for 4h, 8h, or 12h and then collected for RNA extraction and qRT-PCR. LPS treatment increased IL6 mRNA levels at all time points with a modest tolerant effect by the 12th hour of treatment (Figure 7A). The LPS effect was abrogated by FFAs at 4 and 8h (Figure 7B). LPS-induced MCP-1 expression was significant at all time points (Figure 7C) with no effect of the addition of FFAs (Figure 7D).

Chapter 5: Discussion

The present study examined the effects of intermittent and continuous LPS treatments on skeletal muscle cells, with the hypothesis that these cells would develop an observable LPS tolerance. The use of low- and high-doses of LPS mimicked metabolic endotoxemic and septic conditions, respectively, and have been previously used in both in vitro and in vivo models (27, 74, 75). Metabolic endotoxemia, a chronic, systemic inflammatory response, has been implicated in the development of chronic inflammatory diseases, such as obesity, T2DM, and CVD (15). The action molecule in the initiation of an immune response, LPS, binds to TLR4, which sequentially signals the innate and adaptive immune systems. TLR4 is abundant in skeletal muscle, and therefore may play an important role in the development of inflammation in this tissue (27). When this inflammatory response is uncontrolled, exacerbated inflammation may lead to tissue damage, apoptosis, and chronic disease. In order to maintain cellular homeostasis, the host promotes endotoxin tolerance, a method of cellular reprogramming geared at down-regulating proinflammatory pathways and promoting anti-inflammatory pathways (8, 15). This phenomenon has been observed in animals and humans, although the precise mechanisms utilized by the host to implement endotoxin tolerance may vary among species (18, 20). While there is a significant amount of research examining LPS tolerance in immune cells, whether this tolerant effect exists in skeletal muscle is not known.

Major Findings

The major findings of these studies are: (1) Priming skeletal muscle myotubes with LPS leads to differential cytokine responses to intermittent LPS exposures; (2) PGC1-α mRNA expression is affected differently by high- vs. low-doses of LPS; (3) LPS reduces the oxygen consumption rate, which is abrogated after repeated LPS exposure; (4) free fatty acids do not prevent LPS tolerance in skeletal muscle myotubes but abrogate LPS-induced effect on IL6.

LPS Effects on mRNA Expression of Proinflammatory Cytokines

LPS signaling via TLR4 induces the translocation of transcription factor, Nf-κβ, and subsequent activation of proinflammatory cytokines: TNF-α, IL-6, and MCP-1. Studies have previously shown decreased activation of these cytokines as a tolerance mechanism to prevent an exacerbated immune response (26, 57). Our data suggest that IL6 and MCP-1 are transcriptionally regulated differently from one another. Perhaps pathways downstream of TLR4 signaling are resistant to tolerance (e.g., IL6 transcription) and others are not (e.g., MCP-1 transcription). Negative regulators of downstream signaling pathways may dictate which cytokines are transcribed by only inhibiting certain proteins. Kobayashi K et al. showed that IRAK-M acts to suppress the release of IRAK-4 and IRAK-1 from MyD88 thereby preventing continued signaling (48). Studies have shown similar results when comparing cytokine secretion from healthy verses diseased cells, and found that diseased cells may express significantly higher levels of proinflammatory cytokines than their non-diseased counterparts, suggesting a differential cytokine response depending on the mechanisms in place within those cells (38).

LPS Exposure Effects on a Key Metabolic Regulator and Maximum Respiration

Skeletal muscle is an important tissue for substrate utilization and disposal, which may be affected by frequent exposure to LPS. Responsible for transcriptional activation of genes controlling oxidative phosphorylation, mitochondrial biogenesis, and fatty acid oxidation, PGC1-α plays significant roles in skeletal muscle (23). Studies have shown that when PGC1-α gene expression is disrupted in response to an LPS challenge, mitochondrial dysfunction occurs along with a sequential accumulation of lipids (72). In our studies, PGC1-α mRNA expression

significantly decreased in response to low- and high-doses of LPS after multiple exposures, suggesting that in response to sepsis and endotoxemia, metabolism is significantly affected.

Previous studies have shown impaired respiration in sepsis models (12) and increased maximum respiration during metabolic endotoxemia (84). Herein, our results mimic those previous findings. The FCCP-stimulated OCR was decreased in response to 1 LPS exposure but this effect diminished after repeated exposures. This data reveals that mitochondrial respiration can recover from both septic and endotoxemic conditions in skeletal muscle. We have previously shown a switch from fatty acid β-oxidation (FAO) to glucose oxidation in response to low- and high-dose LPS for 2, 6, 12, and 24 hours, suggesting that these effects are independent suppressions in PGC1-α mRNA (27).

Free Fatty Acids do Not Prevent Tolerance in Skeletal Muscle Cells

Chronic inflammatory diseases, such as obesity and T2DM, are often marked by common characteristics, such as insulin resistance and hyperlipidemia. These characteristics may be a result of excess intramyocellular lipid accumulation and a switch from FAO to glucose oxidation during inflammatory states (21, 37). Skeletal muscle, a highly oxidative tissue, will normally draw FFAs from circulation for FAO, however, in times of stress and inflammation, FAO decreases, creating an abundance of FFAs that can potentially prevent LPS tolerance. Contrary to our hypothesis, the data showed that FFAs did not prevent tolerance from occurring within the skeletal muscle cells. In respect to the intermittent treatments, there was no FFA effect on IL6, MCP-1, or PGC1-α mRNA expression. Interestingly, the addition of FFAs decreased LPS-induced IL6 mRNA expression after continuous treatment for 4 and 8 hours but MCP-1 expression was unchanged. FFAs may not be a critical factor affecting LPS tolerance in healthy

skeletal muscle cells during a 12-hour period, but may be influential over a longer treatment. In addition, our 2:1 ratio of palmitate to oleate mimics a high saturated-fat diet, which in a physiological in vivo setting, may only be one of many factors contributing and participating in the inflammatory response to LPS.

Summary

Endotoxin tolerance, a host mechanism necessary for maintaining homeostatic cellular processes, is also not only present in immune cells but also skeletal muscle cells. This process varies according to the tissue in observation, and may utilize multiple signaling pathways and mechanisms to combat inflammation. Recent studies have thoroughly explored these signaling pathways and their roles in the innate and adaptive immune systems; however, until recently, skeletal muscle has largely been overlooked. Skeletal muscle, with its vital role in substrate utilization and metabolism, may be a pivotal topic of interest in the development of endotoxin tolerance, as it is associated with chronic inflammatory diseases that largely affect this tissue. Herein we show that skeletal muscle tissue may become tolerant to multiple LPS exposures, however, this response is complex and dependent upon the dose of LPS and the time course of interaction with the antigen. We have briefly scratched the surface of this new topic of interest, primarily investigating the role of the TLR4 pathway in skeletal muscle response to LPS. The presence of FFAs may alter this immune response but our data shows otherwise. FFAs are abundant in circulation in chronic inflammatory disease states, though their role may not be pertinent in the development of endotoxin tolerance.

Future Directions

The magnitude of effects that endotoxins have on cellular functions have been studied for decades. Researchers have sought answers as to how the body responds to LPS challenges and what the resulting effects are. We know that LPS induces an inflammatory response from the immune system and skeletal muscle cells, but there are still many questions unanswered, such as which signaling pathways are affected, what would happen in an in vivo situation, and what the roles of epigenetics play in skeletal muscle tissue. Apart from the items listed above, the most obvious next step in this work is to assess the targets examined herein at the protein level. Knowing the how the protein concentrations were affected under the same conditions would provide us with a better snapshot of cytokine and signaling pathway activity within the cells after each LPS exposure.

There are many interesting targets that could help distinguish the pathways of which are utilized when skeletal muscle becomes tolerant to endotoxins. Downstream of TLR4 exists the kinases, IRAK-4 and TBK1. These kinases, when measured via gene expression analysis or western blots, could help determine which pathway downstream of TLR4 is impacted after multiple LPS exposures in skeletal muscle, specifically the MyD88-dependent (upstream from IRAK-4) and TRIF (upstream from TBK1) pathways (8). Interestingly, there is a generous amount of data regarding the MyD88-dependent pathway and a modest amount of TRIF data. Additionally, other cytokines could be measured to include the proinflammatory cytokines IL-1β and IL-12, and the anti-inflammatory cytokines, TGFβ and IL-1RA. These cytokines are activated by different pairs of Nf-κβ heterodimers, and can provide additional information as to the particular mechanism(s) underlying endotoxin tolerance.

A major limitation of this study was the fact that all experiments were performed in cultured cells, whereas an in vivo model would provide researchers additional information, such as how other factors, such as hormones and immune cells, could impact the effects of LPS and progression of endotoxin tolerance. Mice could be injected with low doses of LPS under low- or high-fat fed conditions to mimic a more physiologically relevant situation. In addition, the time-course of injections and diet could be expanded to weeks or even months instead of a short, 12 hour study.

The growing area of epigenetics, also studied primarily in immune cells, may dig deeper into the roles and complexity of endotoxin tolerance in skeletal muscle tissue. In a recent study, Liu TF et al concluded that prolonged systemic TLR4 activation by LPS would result in epigenetic reprogramming in monocytes and leukocytes (53). Epigenetic reprogramming is a complex paradigm characterized by several phases: an initiation phase (i.e. LPS binding to TLR4, followed by activation of proinflammatory cytokines), an evolving phase (proinflammatory genes are turned off via transcriptional silencing and other genes, such as genes for anti-inflammatory cytokines, are turned on), and finally a resolving phase, in which the previous gene changes are reversed (24). These processes can be modified by microRNAs, conserved non-encoding RNAs that impact the magnitude of change by controlling translation of mRNA.

In conjunction with previous findings from immune cell research, these areas of scientific research may uncover the depth and magnitude of endotoxin tolerance in skeletal muscle tissue. Endotoxin tolerance is multidimensional, highly regulated, and a highly evolving multi-cellular mechanism to protect the host against uncontrollable inflammatory responses.

References

References

1. **Akira S, Uematsu S, and Takeuchi O**. Pathogen recognition and innate immunity. *Cell* 124: 783-801, 2006.
2. **Al-Attas OS, Al-Daghri NM, Al-Rubeaan K, da Silva NF, Sabico SL, Kumar S, McTernan PG, and Harte AL**. Changes in endotoxin levels in T2DM subjects on anti-diabetic therapies. *Cardiovascular diabetology* 8: 20, 2009.
3. **Albrecht V, Hofer TP, Foxwell B, Frankenberger M, and Ziegler-Heitbrock L**. Tolerance induced via TLR2 and TLR4 in human dendritic cells: role of IRAK-1. *BMC Immunol* 9: 69, 2008.
4. **Anderson KV, Bokla L, and Nusslein-Volhard C**. Establishment of dorsal-ventral polarity in the Drosophila embryo: the induction of polarity by the Toll gene product. *Cell* 42: 791-798, 1985.
5. **Bazzoni F, Rossato M, Fabbri M, Gaudiosi D, Mirolo M, Mori L, Tamassia N, Mantovani A, Cassatella MA, and Locati M**. Induction and regulatory function of miR-9 in human monocytes and neutrophils exposed to proinflammatory signals. *Proceedings of the National Academy of Sciences of the United States of America* 106: 5282-5287, 2009.
6. **Beeson PB, and With the Technical Assistance of Elizabeth R**. Tolerance to Bacterial Pyrogens : I. Factors Influencing Its Development. *The Journal of experimental medicine* 86: 29-38, 1947.
7. **Beeson PB, and With the Technical Assistance of Elizabeth R**. Tolerance to Bacterial Pyrogens : Ii. Role of the Reticulo-Endothelial System. *The Journal of experimental medicine* 86: 39-44, 1947.
8. **Biswas SK, and Lopez-Collazo E**. Endotoxin tolerance: new mechanisms, molecules and clinical significance. *Trends in immunology* 30: 475-487, 2009.
9. **Bodine SC, Stitt TN, Gonzalez M, Kline WO, Stover GL, Bauerlein R, Zlotchenko E, Scrimgeour A, Lawrence JC, Glass DJ, and Yancopoulos GD**. Akt/mTOR pathway is a crucial regulator of skeletal muscle hypertrophy and can prevent muscle atrophy in vivo. *Nature cell biology* 3: 1014-1019, 2001.
10. **Bonetto A, Aydogdu T, Kunzevitzky N, Guttridge DC, Khuri S, Koniaris LG, and Zimmers TA**. STAT3 activation in skeletal muscle links muscle wasting and the acute phase response in cancer cachexia. *PloS one* 6: e22538, 2011.
11. **Borge BA, Kalland KH, Olsen S, Bletsa A, Berggreen E, and Wiig H**. Cytokines are produced locally by myocytes in rat skeletal muscle during endotoxemia. *American journal of physiology Heart and circulatory physiology* 296: H735-744, 2009.
12. **Boveris A, Alvarez S, and Navarro A**. The role of mitochondrial nitric oxide synthase in inflammation and septic shock. *Free radical biology & medicine* 33: 1186-1193, 2002.
13. **Boyd JH, Divangahi M, Yahiaoui L, Gvozdic D, Qureshi S, and Petrof BJ**. Toll-like receptors differentially regulate CC and CXC chemokines in skeletal muscle via NF-kappaB and calcineurin. *Infection and immunity* 74: 6829-6838, 2006.
14. **Brown J, Wang H, Hajishengallis GN, and Martin M**. TLR-signaling networks: an integration of adaptor molecules, kinases, and cross-talk. *Journal of dental research* 90: 417-427, 2011.
15. **Cani PD, Amar J, Iglesias MA, Poggi M, Knauf C, Bastelica D, Neyrinck AM, Fava F, Tuohy KM, Chabo C, Waget A, Delmee E, Cousin B, Sulpice T, Chamontin B, Ferrieres J, Tanti JF, Gibson GR, Casteilla L, Delzenne NM, Alessi MC, and Burcelin R**. Metabolic endotoxemia initiates obesity and insulin resistance. *Diabetes* 56: 1761-1772, 2007.
16. **Cani PD, Bibiloni R, Knauf C, Waget A, Neyrinck AM, Delzenne NM, and Burcelin R**. Changes in gut microbiota control metabolic endotoxemia-induced inflammation in high-fat diet-induced obesity and diabetes in mice. *Diabetes* 57: 1470-1481, 2008.
17. **Chiang CY, Veckman V, Limmer K, and David M**. Phospholipase Cgamma-2 and intracellular calcium are required for lipopolysaccharide-induced Toll-like receptor 4 (TLR4) endocytosis and interferon regulatory factor 3 (IRF3) activation. *The Journal of biological chemistry* 287: 3704-3709, 2012.

18. **del Campo R, Martinez E, del Fresno C, Alenda R, Gomez-Pina V, Fernandez-Ruiz I, Siliceo M, Jurado T, Toledano V, Arnalich F, Garcia-Rio F, and Lopez-Collazo E**. Translocated LPS might cause endotoxin tolerance in circulating monocytes of cystic fibrosis patients. *PloS one* 6: e29577, 2011.
19. **Dillon S, Agrawal A, Van Dyke T, Landreth G, McCauley L, Koh A, Maliszewski C, Akira S, and Pulendran B**. A Toll-like receptor 2 ligand stimulates Th2 responses in vivo, via induction of extracellular signal-regulated kinase mitogen-activated protein kinase and c-Fos in dendritic cells. *Journal of immunology* 172: 4733-4743, 2004.
20. **Dobrovolskaia MA, Medvedev AE, Thomas KE, Cuesta N, Toshchakov V, Ren T, Cody MJ, Michalek SM, Rice NR, and Vogel SN**. Induction of in vitro reprogramming by Toll-like receptor (TLR)2 and TLR4 agonists in murine macrophages: effects of TLR "homotolerance" versus "heterotolerance" on NF-kappa B signaling pathway components. *Journal of immunology* 170: 508-519, 2003.
21. **Dohm GL, Tapscott EB, Pories WJ, Dabbs DJ, Flickinger EG, Meelheim D, Fushiki T, Atkinson SM, Elton CW, and Caro JF**. An in vitro human muscle preparation suitable for metabolic studies. Decreased insulin stimulation of glucose transport in muscle from morbidly obese and diabetic subjects. *The Journal of clinical investigation* 82: 486-494, 1988.
22. **Doyle A, Zhang G, Abdel Fattah EA, Eissa NT, and Li YP**. Toll-like receptor 4 mediates lipopolysaccharide-induced muscle catabolism via coordinate activation of ubiquitin-proteasome and autophagy-lysosome pathways. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* 25: 99-110, 2011.
23. **Eivers SS, McGivney BA, Gu J, MacHugh DE, Katz LM, and Hill EW**. PGC-1alpha encoded by the PPARGC1A gene regulates oxidative energy metabolism in equine skeletal muscle during exercise. *Animal genetics* 43: 153-162, 2012.
24. **El Gazzar M, and McCall CE**. MicroRNAs distinguish translational from transcriptional silencing during endotoxin tolerance. *The Journal of biological chemistry* 285: 20940-20951, 2010.
25. **Fitzgerald KA, Palsson-McDermott EM, Bowie AG, Jefferies CA, Mansell AS, Brady G, Brint E, Dunne A, Gray P, Harte MT, McMurray D, Smith DE, Sims JE, Bird TA, and O'Neill LA**. Mal (MyD88-adapter-like) is required for Toll-like receptor-4 signal transduction. *Nature* 413: 78-83, 2001.
26. **Foster SL, Hargreaves DC, and Medzhitov R**. Gene-specific control of inflammation by TLR-induced chromatin modifications. *Nature* 447: 972-978, 2007.
27. **Frisard MI, McMillan RP, Marchand J, Wahlberg KA, Wu Y, Voelker KA, Heilbronn L, Haynie K, Muoio B, Li L, and Hulver MW**. Toll-like receptor 4 modulates skeletal muscle substrate metabolism. *American journal of physiology Endocrinology and metabolism* 298: E988-998, 2010.
28. **Frost RA, Nystrom GJ, and Lang CH**. Lipopolysaccharide regulates proinflammatory cytokine expression in mouse myoblasts and skeletal muscle. *American journal of physiology Regulatory, integrative and comparative physiology* 283: R698-709, 2002.
29. **Frost RA, Nystrom GJ, and Lang CH**. Multiple Toll-like receptor ligands induce an IL-6 transcriptional response in skeletal myocytes. *American journal of physiology Regulatory, integrative and comparative physiology* 290: R773-784, 2006.
30. **Gerencser AA, Neilson A, Choi SW, Edman U, Yadava N, Oh RJ, Ferrick DA, Nicholls DG, and Brand MD**. Quantitative microplate-based respirometry with correction for oxygen diffusion. *Analytical chemistry* 81: 6868-6878, 2009.
31. **Ghoshal S, Witta J, Zhong J, de Villiers W, and Eckhardt E**. Chylomicrons promote intestinal absorption of lipopolysaccharides. *Journal of lipid research* 50: 90-97, 2009.
32. **Gottipati S, Rao NL, and Fung-Leung WP**. IRAK1: a critical signaling mediator of innate immunity. *Cellular signalling* 20: 269-276, 2008.
33. **Han DM, Zhang YQ, Bai QX, and Chen XQ**. Assay of AVP, CRP, and LPS in leukemia. *International journal of laboratory hematology* 29: 185-189, 2007.

34. **Haziot A, Ferrero E, Kontgen F, Hijiya N, Yamamoto S, Silver J, Stewart CL, and Goyert SM**. Resistance to endotoxin shock and reduced dissemination of gram-negative bacteria in CD14-deficient mice. *Immunity* 4: 407-414, 1996.
35. **Hoffmann A, Levchenko A, Scott ML, and Baltimore D**. The IkappaB-NF-kappaB signaling module: temporal control and selective gene activation. *Science* 298: 1241-1245, 2002.
36. **Hotamisligil GS**. Inflammation and metabolic disorders. *Nature* 444: 860-867, 2006.
37. **Hulver MW, Berggren JR, Cortright RN, Dudek RW, Thompson RP, Pories WJ, MacDonald KG, Cline GW, Shulman GI, Dohm GL, and Houmard JA**. Skeletal muscle lipid metabolism with obesity. *American journal of physiology Endocrinology and metabolism* 284: E741-747, 2003.
38. **Jagannathan M, Hasturk H, Liang Y, Shin H, Hetzel JT, Kantarci A, Rubin D, McDonnell ME, Van Dyke TE, Ganley-Leal LM, and Nikolajczyk BS**. TLR cross-talk specifically regulates cytokine production by B cells from chronic inflammatory disease patients. *Journal of immunology* 183: 7461-7470, 2009.
39. **Jove M, Laguna JC, and Vazquez-Carrera M**. Agonist-induced activation releases peroxisome proliferator-activated receptor beta/delta from its inhibition by palmitate-induced nuclear factor-kappaB in skeletal muscle cells. *Biochimica et biophysica acta* 1734: 52-61, 2005.
40. **Kagan JC, and Medzhitov R**. Phosphoinositide-mediated adaptor recruitment controls Toll-like receptor signaling. *Cell* 125: 943-955, 2006.
41. **Kagan JC, Su T, Horng T, Chow A, Akira S, and Medzhitov R**. TRAM couples endocytosis of Toll-like receptor 4 to the induction of interferon-beta. *Nature immunology* 9: 361-368, 2008.
42. **Kawagoe T, Sato S, Matsushita K, Kato H, Matsui K, Kumagai Y, Saitoh T, Kawai T, Takeuchi O, and Akira S**. Sequential control of Toll-like receptor-dependent responses by IRAK1 and IRAK2. *Nature immunology* 9: 684-691, 2008.
43. **Kawai T, Adachi O, Ogawa T, Takeda K, and Akira S**. Unresponsiveness of MyD88-deficient mice to endotoxin. *Immunity* 11: 115-122, 1999.
44. **Kawai T, and Akira S**. The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. *Nature immunology* 11: 373-384, 2010.
45. **Kawai T, and Akira S**. Toll-like receptors and their crosstalk with other innate receptors in infection and immunity. *Immunity* 34: 637-650, 2011.
46. **Kelley DE, Goodpaster B, Wing RR, and Simoneau JA**. Skeletal muscle fatty acid metabolism in association with insulin resistance, obesity, and weight loss. *The American journal of physiology* 277: E1130-1141, 1999.
47. **Kern PA, Ranganathan S, Li C, Wood L, and Ranganathan G**. Adipose tissue tumor necrosis factor and interleukin-6 expression in human obesity and insulin resistance. *American journal of physiology Endocrinology and metabolism* 280: E745-751, 2001.
48. **Kobayashi K, Hernandez LD, Galan JE, Janeway CA, Jr., Medzhitov R, and Flavell RA**. IRAK-M is a negative regulator of Toll-like receptor signaling. *Cell* 110: 191-202, 2002.
49. **Kobayashi M, Saitoh S, Tanimura N, Takahashi K, Kawasaki K, Nishijima M, Fujimoto Y, Fukase K, Akashi-Takamura S, and Miyake K**. Regulatory roles for MD-2 and TLR4 in ligand-induced receptor clustering. *Journal of immunology* 176: 6211-6218, 2006.
50. **Kriketos AD, Greenfield JR, Peake PW, Furler SM, Denyer GS, Charlesworth JA, and Campbell LV**. Inflammation, insulin resistance, and adiposity: a study of first-degree relatives of type 2 diabetic subjects. *Diabetes care* 27: 2033-2040, 2004.
51. **Li B, Zhang R, Li J, Zhang L, Ding G, Luo P, He S, Dong Y, Jiang W, Lu Y, Cao H, Zheng J, and Zhou H**. Antimalarial artesunate protects sepsis model mice against heat-killed Escherichia coli challenge by decreasing TLR4, TLR9 mRNA expressions and transcription factor NF-kappa B activation. *International immunopharmacology* 8: 379-389, 2008.

52. **Li YP, Chen Y, John J, Moylan J, Jin B, Mann DL, and Reid MB**. TNF-alpha acts via p38 MAPK to stimulate expression of the ubiquitin ligase atrogin1/MAFbx in skeletal muscle. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* 19: 362-370, 2005.
53. **Liu TF, Yoza BK, El Gazzar M, Vachharajani VT, and McCall CE**. NAD+-dependent SIRT1 deacetylase participates in epigenetic reprogramming during endotoxin tolerance. *The Journal of biological chemistry* 286: 9856-9864, 2011.
54. **Maedler K, Oberholzer J, Bucher P, Spinas GA, and Donath MY**. Monounsaturated fatty acids prevent the deleterious effects of palmitate and high glucose on human pancreatic beta-cell turnover and function. *Diabetes* 52: 726-733, 2003.
55. **Maitra U, Gan L, Chang S, and Li L**. Low-dose endotoxin induces inflammation by selectively removing nuclear receptors and activating CCAAT/enhancer-binding protein delta. *Journal of immunology* 186: 4467-4473, 2011.
56. **McGhan LJ, and Jaroszewski DE**. The role of toll-like receptor-4 in the development of multi-organ failure following traumatic haemorrhagic shock and resuscitation. *Injury* 43: 129-136, 2012.
57. **Medvedev AE, Kopydlowski KM, and Vogel SN**. Inhibition of lipopolysaccharide-induced signal transduction in endotoxin-tolerized mouse macrophages: dysregulation of cytokine, chemokine, and toll-like receptor 2 and 4 gene expression. *Journal of immunology* 164: 5564-5574, 2000.
58. **Medvedev AE, Lentschat A, Wahl LM, Golenbock DT, and Vogel SN**. Dysregulation of LPS-induced Toll-like receptor 4-MyD88 complex formation and IL-1 receptor-associated kinase 1 activation in endotoxin-tolerant cells. *Journal of immunology* 169: 5209-5216, 2002.
59. **Medzhitov R, Preston-Hurlburt P, and Janeway CA, Jr.** A human homologue of the Drosophila Toll protein signals activation of adaptive immunity. *Nature* 388: 394-397, 1997.
60. **Mehta NN, McGillicuddy FC, Anderson PD, Hinkle CC, Shah R, Pruscino L, Tabita-Martinez J, Sellers KF, Rickels MR, and Reilly MP**. Experimental endotoxemia induces adipose inflammation and insulin resistance in humans. *Diabetes* 59: 172-181, 2010.
61. **Morris M, and Li L**. Molecular mechanisms and pathological consequences of endotoxin tolerance and priming. *Archivum immunologiae et therapiae experimentalis* 60: 13-18, 2012.
62. **Niebauer J, Volk HD, Kemp M, Dominguez M, Schumann RR, Rauchhaus M, Poole-Wilson PA, Coats AJ, and Anker SD**. Endotoxin and immune activation in chronic heart failure: a prospective cohort study. *Lancet* 353: 1838-1842, 1999.
63. **Nomura F, Akashi S, Sakao Y, Sato S, Kawai T, Matsumoto M, Nakanishi K, Kimoto M, Miyake K, Takeda K, and Akira S**. Cutting edge: endotoxin tolerance in mouse peritoneal macrophages correlates with down-regulation of surface toll-like receptor 4 expression. *Journal of immunology* 164: 3476-3479, 2000.
64. **Noreen M, Shah MA, Mall SM, Choudhary S, Hussain T, Ahmed I, Jalil SF, and Raza MI**. TLR4 polymorphisms and disease susceptibility. *Inflammation research : official journal of the European Histamine Research Society [et al]* 61: 177-188, 2012.
65. **O'Connell RM, Rao DS, Chaudhuri AA, Boldin MP, Taganov KD, Nicoll J, Paquette RL, and Baltimore D**. Sustained expression of microRNA-155 in hematopoietic stem cells causes a myeloproliferative disorder. *The Journal of experimental medicine* 205: 585-594, 2008.
66. **O'Neill LA, and Bowie AG**. The family of five: TIR-domain-containing adaptors in Toll-like receptor signalling. *Nature reviews Immunology* 7: 353-364, 2007.
67. **Pedersen BK, and Febbraio MA**. Muscle as an endocrine organ: focus on muscle-derived interleukin-6. *Physiological reviews* 88: 1379-1406, 2008.
68. **Poltorak A, Smirnova I, He X, Liu MY, Van Huffel C, McNally O, Birdwell D, Alejos E, Silva M, Du X, Thompson P, Chan EK, Ledesma J, Roe B, Clifton S, Vogel SN, and Beutler B**. Genetic and physical mapping of the Lps locus: identification of the toll-4 receptor as a candidate gene in the critical region. *Blood cells, molecules & diseases* 24: 340-355, 1998.

69. **Randow F, Syrbe U, Meisel C, Krausch D, Zuckermann H, Platzer C, and Volk HD**. Mechanism of endotoxin desensitization: involvement of interleukin 10 and transforming growth factor beta. *The Journal of experimental medicine* 181: 1887-1892, 1995.
70. **Reyna SM, Ghosh S, Tantiwong P, Meka CS, Eagan P, Jenkinson CP, Cersosimo E, Defronzo RA, Coletta DK, Sriwijitkamol A, and Musi N**. Elevated toll-like receptor 4 expression and signaling in muscle from insulin-resistant subjects. *Diabetes* 57: 2595-2602, 2008.
71. **Sato S, Sugiyama M, Yamamoto M, Watanabe Y, Kawai T, Takeda K, and Akira S**. Toll/IL-1 receptor domain-containing adaptor inducing IFN-beta (TRIF) associates with TNF receptor-associated factor 6 and TANK-binding kinase 1, and activates two distinct transcription factors, NF-kappa B and IFN-regulatory factor-3, in the Toll-like receptor signaling. *Journal of immunology* 171: 4304-4310, 2003.
72. **Schilling J, Lai L, Sambandam N, Dey CE, Leone TC, and Kelly DP**. Toll-like receptor-mediated inflammatory signaling reprograms cardiac energy metabolism by repressing peroxisome proliferator-activated receptor gamma coactivator-1 signaling. *Circulation Heart failure* 4: 474-482, 2011.
73. **Schuster DP, Brody SL, Zhou Z, Bernstein M, Arch R, Link D, and Mueckler M**. Regulation of lipopolysaccharide-induced increases in neutrophil glucose uptake. *Am J Physiol Lung Cell Mol Physiol* 292: L845-851, 2007.
74. **Shih MF, Chen LY, Tsai PJ, and Cherng JY**. In vitro and in vivo therapeutics of beta-thujaplicin on LPS-induced inflammation in macrophages and septic shock in mice. *International journal of immunopathology and pharmacology* 25: 39-48, 2012.
75. **Stahl O, Loffler B, Haier J, Mardin WA, and Mees ST**. Mimicry of human sepsis in a rat model-Prospects and limitations. *The Journal of surgical research* 2012.
76. **Suzuki N, Suzuki S, Duncan GS, Millar DG, Wada T, Mirtsos C, Takada H, Wakeham A, Itie A, Li S, Penninger JM, Wesche H, Ohashi PS, Mak TW, and Yeh WC**. Severe impairment of interleukin-1 and Toll-like receptor signalling in mice lacking IRAK-4. *Nature* 416: 750-756, 2002.
77. **Sweeney TE, Suliman HB, Hollingsworth JW, Welty-Wolf KE, and Piantadosi CA**. A toll-like receptor 2 pathway regulates the Ppargc1a/b metabolic co-activators in mice with Staphylococcal aureus sepsis. *PloS one* 6: e25249, 2011.
78. **Takeda K, and Akira S**. TLR signaling pathways. *Seminars in immunology* 16: 3-9, 2004.
79. **Taube A, Schlich R, Sell H, Eckardt K, and Eckel J**. Inflammation and Metabolic Dysfunction: Links to Cardiovascular Disease. *American journal of physiology Heart and circulatory physiology* 2012.
80. **Thomas JA, Allen JL, Tsen M, Dubnicoff T, Danao J, Liao XC, Cao Z, and Wasserman SA**. Impaired cytokine signaling in mice lacking the IL-1 receptor-associated kinase. *Journal of immunology* 163: 978-984, 1999.
81. **Tournadre A, Lenief V, and Miossec P**. Expression of Toll-like receptor 3 and Toll-like receptor 7 in muscle is characteristic of inflammatory myopathy and is differentially regulated by Th1 and Th17 cytokines. *Arthritis and rheumatism* 62: 2144-2151, 2010.
82. **Tsukumo DM, Carvalho-Filho MA, Carvalheira JB, Prada PO, Hirabara SM, Schenka AA, Araujo EP, Vassallo J, Curi R, Velloso LA, and Saad MJ**. Loss-of-function mutation in Toll-like receptor 4 prevents diet-induced obesity and insulin resistance. *Diabetes* 56: 1986-1998, 2007.
83. **van 't Veer C, van den Pangaart PS, van Zoelen MA, de Kruif M, Birjmohun RS, Stroes ES, de Vos AF, and van der Poll T**. Induction of IRAK-M is associated with lipopolysaccharide tolerance in a human endotoxemia model. *Journal of immunology* 179: 7110-7120, 2007.
84. **Vanasco V, Cimolai MC, Evelson P, and Alvarez S**. The oxidative stress and the mitochondrial dysfunction caused by endotoxemia are prevented by alpha-lipoic acid. *Free radical research* 42: 815-823, 2008.
85. **Vincent JL, Sun Q, and Dubois MJ**. Clinical trials of immunomodulatory therapies in severe sepsis and septic shock. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America* 34: 1084-1093, 2002.

86. **Wang W, Deng M, Liu X, Ai W, Tang Q, and Hu J**. TLR4 activation induces nontolerant inflammatory response in endothelial cells. *Inflammation* 34: 509-518, 2011.
87. **Wheeler DS, Lahni PM, Denenberg AG, Poynter SE, Wong HR, Cook JA, and Zingarelli B**. Induction of endotoxin tolerance enhances bacterial clearance and survival in murine polymicrobial sepsis. *Shock* 30: 267-273, 2008.