The Role of Toll like Receptor-4 in Exercise-induced Myokine Response and Regulation of Skeletal Muscle Metabolic Adaptation

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The Role of Toll like Receptor-4 in Exercise-induced Myokine Response and Regulation of Skeletal Muscle Metabolic Adaptation

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ABSTRACT (ACADEMIC)

Toll like receptor-4 (TLR4) is a transmembrane inflammatory receptor expressed ubiquitously on the cell surface of immune cells as well as skeletal muscle and other metabolic tissues. A compelling body of evidence shows that muscle TLR4 and the downstream cytokine signaling modulate skeletal muscle metabolism. Intriguingly, skeletal muscle has been demonstrated to gain favorable inflammatory cytokine-mediated metabolic adaptations in the context of exercise training. This paradigm suggests a role for muscle TLR4 inflammatory signaling in the regulation of exercise metabolism. As such, the question arises as to whether exercise stress response follows similar inflammatory physiological pathways to those activated by other physical and pathogenic stimuli or not. Therefore, the objective of the present study was to investigate the role of muscle TLR4 signaling in modulating skeletal muscle cytokine, also known as myokine, response and metabolic adaptations to exercise. To this end, using Cre-mediated recombination, we developed a novel muscle-specific TLR4 knockout (mTLR4^-/-) mouse model on C57BL/6J background. The differential inflammatory and metabolic responses between mTLR4^-/- mice and wild type (WT) littermates were examined following exposure to either exercise or muscle stimulus. Accordingly, different exercise and muscle contraction modalities were pursued, focusing on voluntary wheel running, forced treadmill training, and in vivo electrical muscle stimulation. Overall, this study introduces a novel muscle-specific TLR4 knockout mouse model and discloses a crucial role for mTLR4 in basal systemic cytokine homeostasis. Furthermore, our findings identify mTLR4 as a major immunomodulatory effector of exercise-induced metabolic adaptations and suggest a link between mTLR4 and physiological determinants of maximal aerobic performance.
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ABSTRACT (PUBLIC)

Exercise is an effective health care modality that exerts many physiological and metabolic benefits. Yet, the magnitude of health outcome differs between individuals, which has encouraged scientists to study biological factors responsible for variable responses to exercise. Our bodies respond to exercise as a whole, which requires harmonious communication among multiple body systems. Skeletal muscle, a major metabolic tissue mainly responsible for bodily movements, plays a key role in whole body energy balance. During exercise skeletal muscle naturally undergoes mechanical and metabolic stress with a subsequent immune reaction known as inflammation. Not only is this exercise-induced inflammatory response known to repair muscle damage, it has also been shown to modulate several of the salutary metabolic effects of exercise. The goal of the study was to better understand how exercise-induced inflammatory response and the subsequent metabolic adjustments are regulated at the level of skeletal muscle.

Our data indicate that mTLR4, an immune receptor imbedded in the surface of muscle cells, modulates the inflammatory signals initiated during exercise. Furthermore, we found that genetically modified mice lacking mTLR4 were unable to develop the normal metabolic adaptations to exercise training. Unlike wild type mice, these mTLR4 deficient mice failed to improve fat and/or glucose utilization after one month of either voluntary wheel running or controlled treadmill training. These findings suggest that defects in this immune receptor, commonly reported with obesity, may alter whole body metabolism and the health outcomes of exercise. Future studies should aim to investigate whether different exercise modalities, such as resistance training, could possibly bypass these limitations induced by mTLR4 abnormalities.
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CHAPTER I

INTRODUCTION

Exercise is a physical stressor that induces a range of physiological responses stimulated by a variety of molecular signals[1]. The strong association between exercise and the subsequent elevations in systemic inflammatory markers encouraged researchers to explore the impact of the latter on the regulation of exercise metabolism (Figure 1). In response to acute exercise, contracting muscles secrete small inflammatory proteins known as cytokines [2]. These muscle-derived cytokines, recognized as myokines, signal locally in autocrine and paracrine manners as well as systemically following an endocrine mechanism [3-5]. Circulating myokines, released in response to exercise, are believed to facilitate a crosstalk between active muscle and other body organs such as the liver and adipose tissue [4]. Secretome analysis and mechanistic studies have identified several myokines and their roles in exercise metabolism [6]. Exercise-associated myokines such as interleukin-6 (IL-6), monocyte chemoattractant protein-1 (MCP-1), interleukin-8 (IL-8), and interleukin-15 (IL-15) exhibit varied time course profiles [7, 8] suggesting involvement of different regulatory mechanisms. Several of these myokines, most notably IL-6, are demonstrated to mediate favorable metabolic responses to exercise [9, 10]. In vitro exposure to IL-6 has been shown to stimulate glycogen synthesis and glucose oxidation in human skeletal muscle [11]. It has also been observed that IL-6 infusion in humans augments lipid oxidation with increased reliance on free fatty acids as a source of energy production in skeletal muscle under basal conditions [12].
Although the release of myokines upon muscle contraction has been known for decades, the underlying mechanisms are still unclear. Several studies proposed different molecular mechanisms regulating these inflammatory cascades triggered by exercise and muscle contraction. Post-exercise muscle glycogen content is reported to be tightly related to levels of circulating cytokines and has been shown to regulate myokine gene expression [14, 15]. Another line of evidence has suggested that exercise-induced intracellular calcium flux as well as production of reactive oxygen species (ROS) activates transcriptional factors promoting myokine synthesis [16-18]. Nonetheless, research undertaken to characterize the mechanisms of exercise-induced myokine release is limited to the effect of changes in the intracellular signaling networks within the active muscle such as an altered metabolite concentration or changes in gene
expression [19]. In fact, skeletal muscle is a part of a dynamic environment rich with potent circulating signaling molecules (e.g. hormones, ROS) that prime muscle metabolism to meet the heightened energy demand during an exercise challenge [20]. Thus, to better understand the interplay between the inflammatory signals and exercise-induced metabolic responses, further research should investigate the role of inflammatory signaling pathways mediated by cell surface receptors that can sense extracellular changes in muscle microenvironment.

Toll-like receptors (TLRs) are cell surface proteins that function in the innate immune system by recognizing microbial pathogens as well as endogenous extracellular signaling molecules [21-27]. Interestingly, a class of TLRs, primarily TLR4, was found to be highly expressed in skeletal muscle and other major metabolic tissues, suggesting a role in body energy homeostasis [28]. In addition to mediating pathogen-induced cytokine production, a crucial role for TLR4 in modulating skeletal muscle metabolism has been identified [29]. Meanwhile, dysregulation of muscle TLR4 expression was found to generate metabolic perturbation in the context of inflammatory diseases such as obesity and type 2 diabetes [28, 30, 31]. These findings suggest TLR4 as a potential modulator of exercise metabolism. In concert, similar to a typical response to an endotoxin challenge, a down-regulation in TLR4 gene expression has been observed following prolonged vigorous exercise [32, 33]. Furthermore, prolonged exercise has been documented to stimulate the induction of molecules including heat shock proteins (HSPs) and none esterified fatty acids (NEFAs) that are suggested to be activators of TLR4 [34, 35]. Collectively, these results suggest that TLR4 could be a prime candidate to serve as a central effector in exercise metabolism [36]. This effect is proposed to work either directly via modulating key exercise-associated metabolic regulators or indirectly through downstream myokine-dependent mechanisms. To date however, there is no direct evidence to support this
paradigm. Therefore, the objective of the present study was to investigate the role of muscle TLR4 signaling in modulating skeletal muscle cytokine response and metabolic adaptations to exercise. In this study, muscle-specific TLR4 knockout mice and wild type littermates were utilized. The inflammatory and metabolic responses of each of the genotypes were examined following exposure to either exercise or muscle stimuli. Accordingly, different exercise and muscle contraction modalities were pursued, focusing on voluntary wheel running, forced treadmill training, and in vivo electrical muscle stimulation.

References


CHAPTER II

REVIEW OF LITERATURE

Introduction

Exercise performance is a multifactorial process that impacts multiple body systems in the acute as well as chronic settings [1-4]. Exercise has multiple systemic effects on the major metabolic tissues ranging from skeletal muscle remodeling to improved whole body energetics [5]. Skeletal muscle is the major site of glucose and fatty acids disposal as it accounts for about 40-50% of total body mass [6]. Hence, exercise is recognized as an effective tool to regulate skeletal muscle metabolism and thereby maintain whole body energy homeostasis. Exercise-induced metabolic adjustments are due to the transient nature of multiple exercise inducible factors as well as the accumulation of chronic exercise training effects. Meanwhile, the metabolic events mediated by exercise are quite specific to the intensity, duration, and frequency of a given exercise mode [7]. Exercise stimulates the secretory nature of skeletal muscle to induce marked local and systemic physiological and molecular changes including increased lactate concentration, production of hypoxia inducible factors, changes in hormone levels, synthesis of heat-shock proteins, generation of oxidative stress, and the production of inflammatory cytokines along with many other mediators related to the immune response [8-10]. Among others, these molecular changes are essential to activate cascades of signal transduction modulating skeletal muscle plasticity. It signals in a pleiotropic fashion to promote muscle contractile machinery and to regulate substrate metabolism for optimal performance during an exercise challenge [5]. The transient rises in inflammatory signals play a multidimensional role in acute exercise [11]. Exercising is known to induce acute muscle injury and tissue damage with a subsequent inflammatory response as a physiological mechanism for muscle repair and growth [12]. Furthermore, the role of muscle-
derived inflammatory mediators in eliciting several of the salutary metabolic effects of exercise has received substantial attention in the past two decades [13].

Figure 1. Exercise-inducible Factors Reported to Modulate Physiological and Metabolic Events

The current literature provides strong evidence supporting a crucial role for inflammatory cytokines in mediating many of the exercise-induced metabolic benefits. Distinguishable from adipocyte-derived inflammation characterized in disease conditions such as obesity, elevations observed in circulating cytokines are released from active muscle upon exposure to exercise stimulus. Moreover, pathological induction of inflammatory cytokines is demonstrated to be a TLR-mediated event. Toll-like receptor-4 (TLR4) is an inflammatory signaling receptor that is widely expressed in skeletal muscle [14]. Muscle TLR4 is a dual function protein linking inflammation to metabolism as it also modulates carbohydrate and lipid oxidation in basal conditions [15, 16]. On the other hand, obesity and other systemic inflammatory conditions are characterized with muscle TLR4 gene expression abnormalities often accompanied by impaired
metabolic events [17, 18]. Surprisingly, research neither aimed at understanding the role of muscle TLR4 in exercise metabolism nor was it directed towards discerning its putative contribution to the exercise-induced myokine response. Therefore, the question remains as to whether exercise stress response follows similar inflammatory physiological pathways to those activated by other physical and pathogenic stimuli or not.

This review discusses recent research identifying the major exercise-associated inflammatory mediators. It also illustrates the role of the inflammatory signals in exercise metabolism and the mechanisms by which these signals can influence skeletal muscle metabolic adaptations to exercise. In addition, it proposes possible avenues for further investigations into a possible role for muscle TLR4 in the regulation of myokine production and metabolic adjustments in response to exercise.

**Exercise as a Stimulus: Emphasis on Skeletal Muscle Cytokine Response**

Exercise is a physical stress that modulates several factors, which alter cellular homeostasis and signaling networks in a variety of tissues. Skeletal muscle is one of the major tissues impacted by exercise in a multifaceted manner. During exercise, skeletal muscle undergoes morphological adjustments as well as intracellular changes in gene expression and metabolite and electrochemical balance. In general, these responses are intrinsic to the active muscle to meet the energy demand and to sustain contractile function. Furthermore, some of these muscle inherent signaling networks are not limited to muscle autoregulation, instead it selectively reaches out to target tissues known to be significant to exercise performance. The dedicated work by Pedersen’s team and others, has laid a strong foundation for the muscle-mediated immunomodulatory effect of exercise [19-22]. Early in this millennium, a novel concept of skeletal muscle as an endocrine organ has been developed in muscle physiology research [23-26]. Bente Pedersen and colleagues
have theorized how contracting muscle fibers are able to secrete cytokines which they identified as “myokines” [27]. These muscle secretory factors were reported to carry out endocrine-like functions in distal tissues and organs, including the adipose tissue [28, 29], colon [30], liver [31], kidney [32], pancreas, and brain [33]. The mechanistic paradigm of the local and systemic actions of exercise-induced myokines is proposed to work through anti-inflammatory and metabolic pathways [13, 34, 35].

Extensive research has been invested in exploring skeletal muscle protein secretory machinery to discover the key exercise-induced myokines and their role in health and disease. To date, at least 12 myokines have been identified. Exercise has been shown to stimulate the production of a class of myokines with paracrine actions on the organ of secretion including leukemia inhibitory factor

Figure 2. Muscle as an Endocrine Organ(Adapted from BK Pedersen et al. 2012)[36]
Other myokines reported to further exhibit endocrine functions include the well-established “exercise factor” IL-6 [40], Irisin [28], calprotectin [41], myonectin [31], oncostatin M (OSM) [42] and secreted protein acidic and rich in cysteine (SPARC)[30]. Recent proteome analysis of skeletal muscle-derived myokines detected a marked increase in mRNA and plasma levels of fractalkine (CX3CL1) and monocyte chemoattractant protein-1(MCP-1) in humans in response to acute endurance exercise [43]. Furthermore, other putative myokines were discovered using the MyoMouse strategies developed by Kenneth Walsh of Boston University [44]. The MyoMouse is a genetically engineered mouse model expressing a gene (Akt1 gene) that produces proteins that mimic the effects of weight training, such as decreased insulin resistance, blood glucose, and body fat as well as improvements in other metabolic parameters, without an increase in physical activity or a loss of appetite. Using this experimental approach, Walsh and colleagues were able to identify follistatin-like 1 [45] and fibroblast growth factor 21 (FGF21) [46] as contractile-activated myokines.

**Inflammation and Exercise-induced Muscle Growth**

Unaccustomed exercise induces tissue damage and a subsequent inflammatory response. Exercise-induced muscle damage progresses into two phases (Figure 3) [47]. The primary damage phase corresponds to the mechanical strain imposed on muscle fibers during exercise, whereas the secondary phase is characterized with the delayed inflammatory response [48, 49]. A compelling body of evidence indicates that inflammatory signals are a key element in exercise-induced muscle remodeling [50]. Clinical studies reported that administering anti-inflammatory drugs (e.g. NSAIDs and Ibuprofen) compromises the post-exercise muscle microenvironment by attenuating protein synthesis and satellite cells proliferation [51, 52].
Furthermore, post-exercise recovery via cold water immersion was shown to diminish strength outcome and to reduce muscle hypertrophy compared to post-exercise active recovery methods [53]. As such, a coordinated inflammatory milieu is a necessity for proper tissue repair and restructuring. In this context, muscle-derived cytokines are a class of proteins thought to orchestrate the inflammatory signals that recruit pertinent immune cells and anabolic pathways [54]. In particular, neutrophils and macrophages are mobilized to the site of injury [55] to eliminate damaged muscle fragments via ROS- and pro-inflammatory-dependent phagocytic mechanisms [56, 57]. In succession, regeneration processes of traumatized muscle tissue are launched by localized satellite cells [58].

**Figure 3. Secondary Phase of Muscle Damage (Adapted from P Baumert et al. 2016) [47]**

Exercise type is well-reported as a significant determinant of the magnitude of muscle injury [59, 60]. Consistent data show that eccentric exercise is associated with a greater muscle damage
characterized by a higher creatine kinase activity compared with concentric exercise modalities [61]. Therefore, eccentric, concentric, and other modes of exercise tend to exhibit differential myokine response. Downhill running has been shown to cause a 135% increase in muscle interleukin-1 beta (IL-1 beta) [59], whereas the systemic levels have been reported with a marginal increase [62]. IL-1 beta promotes inflammation by increasing intracellular calcium and nitric oxide (NO), thereby increasing the expression of adhesion molecules, intercellular adhesion molecules (ICAMs) and vascular cell adhesion molecule-1 (VCAM-1) [63, 64]. Similarly, tumor necrosis factor-α (TNF-α) is expressed during the early phase of inflammatory response as its level rises locally in skeletal muscle [65-67] but with subtle systemic elevations following exercise [68]. TNF-α is also implicated in the activation of pro-inflammatory genes and the ubiquitin–proteasome pathway as means to degrade damaged cellular debris [69]. Interestingly, some cytokines such as IL-6 are better described as double inflammatory agents, where they manifest either pro- or anti-inflammatory properties depending on the physiological environment. Downhill running resulted in substantial but delayed elevations (460%) in plasma IL-6 compared to pre-exercise levels [70]. However, prolonged running is reported to induce the greatest increase in IL-6 concentration in circulation [62, 68, 71, 72]. In vivo and in vitro studies have shown that IL-6 expression plays a significant role in stimulating satellite cells proliferation in response to acute muscle-lengthening contractions [73] and in satellite cell- mediated muscle hypertrophy [74].

Inflammation and Exercise-induced Skeletal Muscle Metabolic Response

Physical exercise imposes immunological and metabolic stress on the body’s organs [75]. As a result, skeletal muscle, which is the major tissue responsible for force generation and movement, undergoes disruptions in the cytoskeleton, fiber organization, and metabolic regulation [62].
Consequently, cellular sensors transmit signal transductions to pair these homeostatic derangements with transcription of inflammatory and metabolically active proteins [76]. Previously, numerous studies were conducted in search of a contractile-inducible molecule/s, an “exercise factor” that mediates local and systemic metabolism. This led to the discovery of hormone-like cytokine families released from contracting muscles, also known as myokines [77]. These myokines have extensively been implicated in myocellular and global restitution of energy homeostasis following exercise [13]. Nonetheless, muscle is unlikely the only source of these signaling molecules, for instance adipose tissue and liver were reported to secrete metabolically active factors [78-80]. The secretory nature of skeletal muscle and its extracellular structure, equipped with cell surface receptors, produce a two-way communication channel between active muscle and other distant tissues. Herein, we will address the metabolic significance of a key inflammatory family of myokines in exercise metabolism and regulation of skeletal muscle adaptations.

**Myokines and Exercise Metabolism**

B. K. Pedersen et al. [13] suggested that “cytokines and other peptides that are produced, expressed, and released by muscle fibers and exert either paracrine or endocrine effects should be classified as myokines.” Although the list includes many protein molecules that fulfill the criteria, we will focus only on myokines presenting the utmost contribution to exercise metabolism.

**Interleukin-6**

Interleukin-6 (IL-6) is a multifunctional cytokine that plays a central role in inflammation, tissue recovery, and metabolism (Figure 4). Its effects are mediated through interaction with its
receptor complex which comprises two membrane proteins, the ligand-binding α-subunit receptor (IL-6R) and the signal transducing β-subunit, gp130 [81]. The IL-6R exists both in a membrane-bound and a soluble form (sIL-6R). IL-6 and sIL-6R form a binary complex that binds to two molecules of gp130 and leads to IL-6 transduction, which includes activation of JAK/STAT, ERK, and PI3k signal transduction pathways [82]. Notably, gp130 is expressed ubiquitously on the cell surface, thus IL-6/sIL-6R complex widens the range of cell types that may respond to IL-6 [83].

A great deal of research has been directed to delineate the molecular pathways underlying the beneficial effects of exercise. The massive release of IL-6 from the contracting skeletal muscle has been proposed as one of the molecular signals “a crucial driver” mediating the exercise-induced effects [84]. It has been reported that the release of IL-6 from active muscle is an intensity-dependent event, which is associated with increased muscle glucose uptake and arterial adrenaline concentration. To this end, IL-6 was claimed to be a carbohydrate sensor because of the inverse relationship between post-exercise muscle glycogen content and plasma IL-6 levels [85]. In addition, IL-6 was also shown to increase skeletal muscle glycogen synthesis through a PI3K-dependent mechanism; to replenish the glycogen depot and restore myocellular homeostasis [86]. Furthermore, several in vitro studies have demonstrated that IL-6 can enhance both skeletal muscles glucose uptake [86, 87] and fat oxidation [86], principally via the activation of AMP-activated protein kinase (AMPK), with the acute but not with long-term treatment. Conversely, in vivo investigations have demonstrated that irrespective of the mode of delivery, acutely or chronically, IL-6 administered to rats for 14 days improved basal insulin sensitivity and promoted glucose disposal. Moreover, while the IL-6 intervention increased circulating fatty acids, it did not increase lipid accumulation in either skeletal muscle or the liver.
which was found to be coupled with increased muscle protein content of both PPAR-α and UCP2 [88]. In accordance, IL-6 knockout mice develop late onset obesity and impaired glucose tolerance [89]. Interestingly, IL-6 offers further metabolic merits through anti-inflammatory mechanisms. IL-6 stimulates the production of anti-inflammatory cytokines and suppression of TNF-α, which has been reported to have a role in insulin resistance in humans [90]. As such muscle-derived IL-6 is likely to protect against TNF-α-induced insulin resistance as well as exercise-induced muscle damage.

![Metabolic Mechanism of IL-6](image)

**Figure 4. Metabolic Mechanism of IL-6 (Adapted from BK Pedersen et al. 2008) [72]**

On the other hand, it has been shown that insulin responsive tissues may respond differently to IL-6. While skeletal muscle appears to be the major target for the reported beneficial effects of IL-6, Weigert et al. [91] confirmed that, in the liver of mice, IL-6 activates two inhibitory mechanisms that are implicated in insulin resistance, marked induction of SOCS-3 and
phosphorylation of IRS-1. Intriguingly, IL-6 had no substantial role in these insulin desensitization mechanisms in skeletal muscle. Furthermore, skeletal muscle and adipose tissue show differential responses to IL-6 stimulation. The infusion of recombinant human IL-6 (rhIL-6) was documented to activate skeletal muscle lipolysis via STAT3-dependent mechanisms, whereas this stimulatory effect was absent in adipose tissue. These results suggest that a transient increase in IL-6 within physiological levels selectively promote fat catabolism in skeletal muscle, but not in adipose tissue [92].

In summary, although it is evident that IL-6 mediates exercise-modulated muscle metabolism, different variables should be considered while interpreting research data. IL-6 is a multifunctioning myokine with pro- and anti-inflammatory properties. As such, the action of IL-6 is specific to tissue type, IL-6 levels, time course of IL-6 exposure, causes and components of inflammatory environment. Another important point not to be overlooked in animal studies is the fact that mouse and human IL-6 share only about 42% sequence identity [93]. Thus, data discrepancies might be observed in human trials compared to mouse studies. These confounding factors, in turn, may give rise to the controversy of the role of IL-6 in modulating substrate metabolism and energy homeostasis. Therefore, the signaling potential of IL-6 in whole body physiology is incompletely understood, so research is still needed to model the IL-6 role considering the aforementioned determinants.

Conclusion

Exercise has a great therapeutic value for preventing and treating modern epidemics of obesity, type II diabetes, cardiovascular complications, and likely many other diseases. Exercise metabolism is a major entry point where systemic control of energy homeostasis is transferred from skeletal muscle to other body organs. The cytokine secretory capacity of skeletal muscle
transformed our understanding of the complex relationship between inflammation and exercise metabolism. Apparently a healthy inflammatory profile is a target in quest for flexible inflammatory and metabolic responses to exercise. That being said, differences in exercise-induced cytokine response in healthy individuals and others with inflammatory conditions (e.g. aged, obese) after exercise have not yet been characterized. Further research should study whether the altered inflammatory profile of some disease states (e.g. obesity, type II diabetes, and aging) could induce metabolic limitations attenuating exercise-concomitant benefits.

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CHAPTER III

SPECIFIC AIMS

Overview: The overall goal of this study is to investigate the significance of the skeletal muscle TLR4 (mTLR4) in modulating exercise-induced metabolic adaptations as well as post-exercise myokine production and how mTLR4 deletion influences endurance performance in mice. Towards this goal, we developed a muscle-specific TLR4 knockout mouse (mTLR4−/−) on C57BL/6J background and utilized different exercise and in-vivo muscle contraction modalities.

Working Hypothesis: We hypothesize that mTLR4 mediates key myokine responses and metabolic adaptations to exercise; and that the lack of its inflammatory signals limits endurance performance.

Specific Aim 1: To determine the role of mTLR4 as a modulator of exercise-induced metabolic adaptations

Objective 1: To examine the interaction between voluntary exercise training and the muscle-specific TLR4 deletion and their effect on the metabolic adaptations in mTLR4−/− mice versus wild type (WT) littermates

Specific Aim 2: To determine the role of mTLR4 as a determinant of exercise capacity

Objective 2: To examine the interaction between standardized exercise training and the muscle-specific TLR4 deletion and their effect on the exercise capacity and metabolic adaptations in mTLR4−/− mice versus WT littermates

Specific Aim 3: To determine the role of mTLR4 as a mediator of myokine production in response to exercise and muscle contraction
**Objective 3:** To examine the interaction between the muscle-specific TLR4 deletion and an acute bout of exercise and their effect on serum cytokine levels in mTLR4⁻/⁻ mice versus WT littermates

**Objective 4:** To examine the interaction between the muscle-specific TLR4 deletion and electrical muscle stimulation (EMS) and their effect on myokine gene expression in TLR4⁻/⁻ mice versus WT littermates
CHAPTER IV

RESEARCH APPROACH AND EXPERIMENTAL DESIGN

Study One: Differential exercise-induced metabolic adaptations between mTLR4⁻/⁻ mice and WT littermates using voluntary wheel running

Table 1. Study One Experimental Design: Voluntary Wheel Running

<table>
<thead>
<tr>
<th>Genotype</th>
<th>WT</th>
<th>mTLR4⁻/⁻</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Exercise Condition</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sedentary (n=7)</td>
<td>Sedentary (n=7)</td>
</tr>
<tr>
<td></td>
<td>Wheel Running (n=7)</td>
<td>Wheel Running (n=7)</td>
</tr>
</tbody>
</table>

Experimental Design.

Voluntary wheel running will be used where 28 mTLR4⁻/⁻ and WT mice will be randomized to either a sedentary control or exercise group with 24 hour-free access to the running wheels, Table 1. Body composition parameters will be assessed prior to and after the completion of the exercise program. Mice will be maintained on the wheel running protocol for 4 weeks. Mice will be sacrificed after 24 hours of the last exposure to the wheels. Blood samples will be collected using cardiac puncture. Skeletal muscle (gastrocnemius, quadriceps, and soleus) will be harvested for substrate oxidation (fatty acid, pyruvate, glucose) and maximal enzymatic activity assays, mitochondria bioenergetics, and gene expression analysis of key metabolic and inflammatory markers.
Figure 1. Study One Timeline: Voluntary Wheel Running

Study Two: Differential exercise-induced metabolic adaptations and aerobic capacity between mTLR4<sup>−/−</sup>mice and WT littermates using treadmill running

Table 2. Study Two Experimental Design: Chronic Treadmill Training

<table>
<thead>
<tr>
<th>Genotype</th>
<th>WT</th>
<th>mTLR4&lt;sup&gt;−/−&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exercise Condition</td>
<td>Sedentary (n=8)</td>
<td>Treadmill Training (n=8)</td>
</tr>
</tbody>
</table>

Experimental Design.

Forced treadmill running will be used where 32 mTLR4<sup>−/−</sup> and WT mice will be randomized to either a sedentary control or exercise group, Table 2. Mice will be acclimated to the treadmill for 2 days. Body composition parameters and maximal aerobic capacity will be assessed prior to and after the completion of the exercise program. Furthermore, mice will perform a moderate intensity 50-minute run 5 days/week for 4 weeks. The protocol will progress into two phases. The first two weeks mice will run at 0.30 m/sec where the second two weeks will be at 0.33
m/sec. Mice will be sacrificed 36 hours after the last bout of exercise. Skeletal muscle (gastrocnemius, quadriceps, and soleus) will be harvested for substrate oxidation (fatty acid, pyruvate, glucose) and maximal enzymatic activity assays, mitochondria bioenergetics, and gene expression analysis of key metabolic (e.g. PGC1-α, PPARr-Delta) and inflammatory markers (IL-6, MCP-1).

**Figure 2. Study Two Timeline: Chronic Treadmill Training**

**Study Three: Differential exercise-induced serum myokine response between mTLR4<sup>−/−</sup> mice and WT littermates using acute treadmill running**

**Table 3. Study Three Experimental Design: Acute Exhaustive Treadmill Running**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>WT</th>
<th>mTLR4&lt;sup&gt;−/−&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Condition (Time point)</td>
<td>Pre-exercise serum sample (n=6)</td>
<td>Pre-exercise serum sample (n=6)</td>
</tr>
<tr>
<td></td>
<td>Post-exercise serum sample (n=6)</td>
<td>Post-exercise serum sample (n=6)</td>
</tr>
</tbody>
</table>
Experimental Design.

Forced acute treadmill running will be used and 12 mTLR4\(^{-/-}\) and wild type mice will undergo an acute bout of prolonged endurance exercise where mice will run until exhaustion at 0.3/sec, table 3. Blood samples will be drawn from the saphenous vein prior to and immediately after the exercise bout. Serum cytokine profile will be analyzed to monitor levels of exercise-induced inflammatory markers (e.g. IL-6, MCP-1).

![Figure 3. Study Three Timeline: Acute Exhaustive Treadmill Running](image)

Study Four: Differential muscle contraction-induced myokine gene expression between mTLR4\(^{-/-}\) mice and WT littermates using in-vivo electrical muscle stimulation

Table 4. Study Four Experimental Design: In Vivo Electrical Muscle Stimulation

<table>
<thead>
<tr>
<th>Genotype</th>
<th>WT</th>
<th>mTLR4(^{-/-})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Condition</td>
<td>In vivo EMS (n=9)</td>
<td>Control (n=9)</td>
</tr>
<tr>
<td></td>
<td>In vivo EMS (n=9)</td>
<td>Control (n=9)</td>
</tr>
</tbody>
</table>
Experimental Design.

In this study 18 mTLR4−/− and wild type mice will undergo in vivo electrical muscle stimulation, table 4. Every mouse from each group will serve as both an experimental and control unit simultaneously. One leg will undergo in vivo electrical stimulation to induce muscle contraction whilst the other leg will serve as a non-stimulated control. Mice will be sacrificed 3 hours post-stimulation. Skeletal muscle (gastrocnemius, quadriceps, and soleus) will be harvested for gene expression assays to quantify mRNA level of key inflammatory myokines (IL-6, MCP-1).

Figure 4. Study Four Timeline: In Vivo Electrical Muscle Stimulation

Limitations, Alternative Approaches, and Future Directions:

The major limitation is the breeding rate of the mTLR4−/− mouse model, since it is domestic to our laboratory and is not commercially available. This breeding concern could hinder the ability to obtain a sample size that enables the statistical power sufficient to detect significant (P<0.05) differences between different experimental groups. Furthermore, mTLR4 deletion selectively knockout muscle TLR4 gene expression, however, many other immune and fat cells are inherent to muscle microenvironment. Contamination with non-muscle cells will likely reveal
confounding results with false positive inflammatory gene expression. A proposed alternative approach to study the role of TLR4 in skeletal muscle during exercise is using C2C12 skeletal myotubes and gene silencing techniques to obtain dysfunctional mTLR4. As well, isolating satellite cells from mTLR4<sup>−/−</sup> mice will provide high quality pure knockout muscle cells. Furthermore, electrical pulse stimulation for cultured myocytes has become a well-established technique that mimics exercise-induced muscle contraction. This study will lead to a better understating of the interplay between inflammatory signals and metabolic adaptations to exercise and whether the altered inflammatory profile some disease states (e.g. obesity, type II diabetes) could induce metabolic limitations attenuating concomitant benefits of exercise as a health tool. Future studies could aim at investigating the discrepancies in the metabolic adaptations seen potentially among lean and obese individuals in response to exercise. Furthermore, future studies could help better understand the exercise-drug interactions as related to the relevance of administering, or lack thereof, anti-inflammatory therapies during an exercise regimen.

**Extended Methods**

**Animal husbandry and generation of mTLR4<sup>−/−</sup> mice.** Animal studies will be performed under an approved protocol by the Institutional Animal Care and Use Committee (IACUC) at Virginia Tech. The muscle-specific TLR4 knockout (mTLR4<sup>−/−</sup>) mouse model on C57BL/6J background was created at the Transgenic Core at the Pennington Biomedical Research Center using the Cre-mediated recombination driven by the muscle creatine kinase promoter (Appendix A). Male mTLR4<sup>−/−</sup> and wild type (WT) littermates will be maintained on a 12-hour light/dark cycle and fed standard chow diet. All experiments will be performed following an overnight fast (10-12 hours), unless otherwise stated. To confirm muscle-specific deletion of TLR4, animals
will be sacrificed and flexor digitorum brevis (FDB) muscle will be isolated. The FDB muscle group will be used because isolation of single muscle fibers limits confounding influences of non-muscle cell types that are present in whole muscle groups (e.g. gastrocnemius and quadriceps).

**Western blot analysis.** Western blot analysis will be performed using skeletal muscle homogenates prepared in 50 mM Hepes, (pH 7.5), 15 mM NaCl, 1 mM MgCl2, 1 mM CaCl2, 2 mM EDTA, 10% glycerol, 1% Triton x-100, 10 mM NaP2O7, 100 mM NaFl, 10 mM PMSF, and 10 g/mL aprotinin. Proteins (~30 µg per sample) will be separated using a 10% Criterion-Tris-HCl gel (Bio-Rad, Hercules, CA) and subsequently transferred to PVDF membrane (Bio-Rad, Hercules, CA). PVDF membrane will be probed with primary antibody against genes of interest.

**FDB muscle fibers isolation and preparation.** Mice will be euthanized using CO2 asphyxiation followed by cervical dislocation according to Virginia Tech IACUC protocols. The hind foot will be cut off above the ankle joint. The foot will be pinned with the plantar side facing up onto a flat foam board. Skin will be reflected starting from the proximal side of the foot, at the ankle, and will be pulled upwards toward the toes. The FDB muscle bundle will be dissected by cutting the tendon at the heel and pulling it upward while detaching the muscle fibers from other adjacent tissues. The FDB muscle bundle will be removed by cutting at the insertion tendons attached to each of the four lateral toes. The FDB muscle will be immediately placed in a tube with 500 µL of dissociation buffer containing cell lysis buffer mixed with protease and phosphatase inhibitors (1/100) as well as collagenase (4 mg/mL). The tube will be rocked in a horizontal position on a shaker for approximately 90 minutes at 37°C. Digested muscle will be transferred into a 35 mm plastic dish with 500 µL of cell lysis buffer mixed with
protease and phosphatase inhibitors. Using a 1 mL micropipette, the FDB muscle bundle will be triturated against the side of the dish to break up the fibers. Trituration will be repeated until the muscle will be stripped from the surrounding white connective tissue. The FDB will be collected in a 1.5 mL microcentrifuge tube and will be washed 3 times by gently resuspending muscle fibers in 500 µL of cell lysis buffer with the protease cocktail. Each time, the tube will be centrifuged at 3000 rpm and supernatant will be discarded. Muscle fibers will be homogenized in 300 µL of cell lysis buffer, protease cocktail added, and further processed to determine protein concentration for western blotting analysis.

**Blood sampling and flow cytometry analysis.** To determine serum cytokine profile, blood samples will be drawn using Microvette CB 300 Z tubes through the lateral saphenous vein which runs dorsally and then laterally over the tarsal joint. Blood will be then centrifuged at 8000 rpm for 10 minutes at 4°C for serum separation. Serum samples will be blocked with mouse IL-6, IL-10, MCP-1, IFN-γ, and TNF-α capture beads using the commercial Cytometric Bead Array (CBA) Mouse Inflammation Kit (cat# 552364) according to the manufacturer's instructions (BD Biosciences).

**Wheel running protocol.** mTLR4−/− and WT mice will undergo 4 weeks of voluntary running on a cage-wheel protocol connected to the LabVIEW software, which monitors distance run, running rate, and running duration. Wheel rotations with a diameter of 11.5 cm are counted continuously, and the sum is sent to the software every two minutes.

**Body composition analysis.** Body mass will be determined prior to assessing body composition using a portable balance (measuring to 0.01g). The body composition of mice will be assessed
using a Bruker LF90 NMR analyzer to measure lean and fat mass for all mice prior to and post the wheel running program.

**Testing aerobic capacity.** Using incremental treadmill running, the testing protocol begins with 5 minutes warm-up at low work rate followed by stepwise increase in the power output. Mice will go through incremental running starting at 0.20 m/s and 0° inclination. The treadmill velocity will be increased by 0.03 m/s every two minutes until the mice reach exhaustion and refuse to keep running. The speed at which the mouse cannot continue running will be recorded. The testing session is to be terminated at any point at which a mouse meets either of these exhaustion criteria:

1. Spending greater than 5 consecutive seconds on the shock grid without attempting to reengage the treadmill.

2. Spending greater than 50% of its time on the shock grid.

3. The third time a mouse is willing to sustain 2 seconds or more of shock without attempting to reengage the treadmill.

**In-vivo plantar flexor torque assay.** In-vivo measures of plantar flexor torque (PFT) will be obtained with mice under isoflurane anesthesia (platform at 35°C). The mouse’s foot will be secured to a torque transducer (Aurora Scientific) and the tibial nerve will be stimulated at 2Hz for a series of contractions to fatigue (4 bouts X 8 minutes; 1 minute rest between bouts). At the end of the fatigue protocol, the leg will be aseptically cleaned with povidone-iodine solution while the mouse recovered from anesthesia (~2-5 minutes in 100% O2). Hindlimb PFT data will be analyzed using Dynamic Muscle Analysis (DMA) software versions 3.2 (Aurora Scientific).
**Skeletal muscle whole homogenate preparation.** Approximately 50 mg of fresh muscle samples will be immediately placed into 0.2 ml of a modified sucrose EDTA medium (SET) on ice containing 250 mM sucrose, 1 mM EDTA, 10 mM tris-HCl, and 1mM ATP, pH 7.4. Muscle samples will be then minced with scissors and then SET buffer will be added to a 20-fold diluted (wt:vol) suspension. The minced samples will be homogenized in a Potter-Elvehjem glass homogenizer at 12 passes across 30 seconds at 150 rpm with a motor-driven teflon pestle, and measures of fatty acid oxidation, glucose oxidation, pyruvate oxidation, and maximal enzyme activities will be performed.

**Substrate metabolism and metabolic flexibility.** Red gastrocnemius and quadriceps muscles will be collected for substrate metabolism. End products of substrate oxidation as measured by [1-14C]-palmitic acid oxidation to 14CO2, [1-14C]-pyruvic acid oxidation to 14CO2 and [U-14C]-glucose oxidation to 14CO2 will be assessed. Metabolic flexibility will be assessed by measuring [1-14C]-pyruvate oxidation +/- palmitic acid. For FAO, GO, and PDH activity assays, tissue homogenates from red skeletal muscles will be incubated in 1μCi/mL of [1-14C]-palmitic acid, 1μCi/mL of [U-14C]-glucose, and 0.35 μCi/mL of [1-14C]-pyruvic acid, respectively, for 1 hour and then acidified with 45% perchloric acid to elute gaseous CO2. 14CO2 will be trapped in 1M sodium hydroxide for 1 hour. The sodium hydroxide will be then sampled and placed in a liquid scintillation counter and quantified. Pyruvate oxidation will be used to assess the activity of pyruvate dehydrogenase (PDH), the enzyme that catalyzes the oxidation of pyruvate resulting in the provision of glucose-derived acetyl CoA to the TCA cycle. This is a direct measure of pyruvate dehydrogenase activity as the number one carbon of pyruvate is liberated as CO2 in the oxidation of pyruvate to acetyl-CoA.
Assessing glycolytic intermediates. For determination of muscle glucose and glucose 6-phosphate concentrations, 300 μl of extracted muscle sample will be added to a tube containing 1.7 mL of 0.47 M triethanolamine, 35 mM MgCl2, 25 mM EDTA, and 1.2 mM NADP. An aliquot (200 μl) will be then added in triplicate to a 96-well plate. Initial absorbance (OD1) will be recorded using a spectrophotometer (SPECTRAmax ME, Molecular Devices Corporation, Sunnyvale California) at a wavelength of 340 nm. Glucose-6-phosphate dehydrogenase (35 μl; 3.5 IU/sample) will be added to each tube and incubated for 20 min. Triplicate aliquots from each tube will be transferred to 96-well plates and absorbance will be measured again (OD2) at 340 nm. For determination of glucose, hexokinase (21 μl; 2.1 IU/sample) and ATP (41 μl; 11 mg/ml) will be added to each tube. Tubes will be allowed to incubate for 20 min before triplicate aliquots will be transferred to 96-well plates and absorbance (OD3) measured at 340 nm. Absorbance values associated with muscle glucose 6-phosphate will be calculated by subtracting OD1 from OD2. Similarly, absorbance values associated with muscle glucose will be determined by subtracting OD2 from OD3.

Phosphofructokinase (PFK) activity. PFK activity will be measured spectrophotometrically at 340 nm at 37°C. Briefly, 30 μl of sample homogenate will be pipette in duplicate (or triplicate). Then, assay buffer (12 mM MgCl2, 400 mM KCl, 2 mM AMP, 1 mM ATP, 0.17 mM NADH, 0.0025 mg/mL Antimycin 0.05 mg/mL Aldolase 0.05 mg/mL GAPDH, in100 mM Tris buffer, pH=8.2) will be added into each well. After a 2 min background reading, 3 mM fructose-6-phosphate will be added to each sample well and followed by a 7 minute kinetic reading. Changes in absorbance across time will be recorded and expressed relative to protein content. PFK activity will be calculated and reported as μmol/min/mg.
**Citrate synthase (CS) activity.** Citrate synthase catalyzes the formation of citrate and CoASH from acetyl-CoA and oxaloacetate. CoASH reduces DTNB and CS activity will be determined from the reduction of DTMB over time. Briefly, ten microliters of a 1:5 diluted muscle homogenate will be added, in duplicate, to 170μl of a solution containing Tris buffer (0.1M, pH 8.3), DNTB (1mM, in 0.1M in Tris buffer) and oxaloacetate (0.01M, in 0.1M Tris buffer). Following a 2min background reading, the spectrophotometer (SPECTRAmax ME, Molecular Devices Corporation, Sunnyvale California) will be calibrated and 30μl of 3 mM acetyl CoA will be added to initiate the reaction. Absorbance will be measured at 405nm at 37C every 12 seconds for 7 minutes. CS activity will be calculated and reported as μmol/min/mg.

**Malate dehydrogenase (MDH) activity.** MDH activity will be measured spectrophotometrically at 340nm at 37°C. Briefly, 10ul of sample will be pipetted in duplicate or triplicate in wells. Then, 290ul of reaction media (0.1 M potassium phosphate buffer, PH 7.4 plus 0.006 M oxaloacetic acid, prepared in potassium phosphate buffer plus 0.00375 M NADH, prepared in potassium phosphate buffer) will be added to the wells and samples will be read for 5 minutes at 340nm. The rate of disappearance of NADH will be analyzed and expressed relative to protein content. MDH activity will be calculated and reported as μmol/min/mg.

**β-hydroxyacyl-CoA dehydrogenase (β-HAD) activity.** For the determination of β-HAD activity, oxidation of NADH to NAD will be measured. Volume of 35 μl of whole muscle homogenate will be added to 190μl of a buffer containing 0.1M liquid triethanolamine, 5mM EDTA tetrasodium salt dihydrate, and 0.45mM NADH. The spectrophotometer (SPECTRAmax PLUS 384, Molecular Devices Corporation, Sunnyvale California) will be calibrated and 15μl of 2mM acetoacetyl CoA will be added to initiate the reaction. Absorbance will be measured at 340
nm every 12 seconds for 5 minutes at 37C. β-HAD activity will be calculated and reported as μmol/min/mg.

**Cytochrome c oxidase (COX) activity.** Cytochrome c oxidase enzyme activity will be measured spectrophotometrically at 550nm every 10 seconds for 7 minutes. Absorbance will be measured based on the oxidation of ferrocytochrome c to ferricytochrome c by cytochrome c oxidase. COX activity will be calculated and reported as μmol/min/mg.

**RNA extraction and qRT-PCR.** mRNA will be extracted using an RNeasy Mini Kit (Qiagen, Valencia, CA) and DNase I treatment (Qiagen, Valencia, CA), 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 will be used according to the manufacturer's specifications (Applied Biosystems, Foster City, CA). Target gene expression will be normalized to β-actin RNA levels. Primers and 5# FAM-labeled TaqMan probes will be purchased as prevalidated assays (Applied Biosystems, Foster City, CA).

**Statistical analysis.** Descriptive and inferential statistics methods will be employed to analyze the collection of data of the study. The descriptive statistics as measured by mean and standard error will be used to summarize the sample and to quantitatively describe the main features of the data (e.g. body mass, lean mass, fat percent... etc.). Results are expressed as mean ± standard error of the mean (SEM). As we are interested in studying the interaction effect of both exercise and genotype differences (WT vs. mTLR4−/−) on manipulating exercise-induced myokine and metabolic responses, the statistical significances of differences among control and experimental groups will be determined using the Two-Way ANOVA. If a significant F-ratio will be obtained, comparisons between groups will be made using Tukey’s post-hoc test. Furthermore, Student’s t-
test will be used to report the differences in gain scores between the control and experimental groups to better understand the genotype X exercise interaction effect. The level of significance will be established as $P < 0.05$. 
CHAPTER V

Muscle-Specific Deletion of Toll-like Receptor-4 Impairs Metabolic Adaptation to Wheel Running in Mice.

Abstract
Toll like receptor 4 (TLR4) is a transmembrane inflammatory receptor expressed ubiquitously on the cell surface of skeletal muscle and other metabolic tissues. Skeletal muscle gains favorable inflammatory-mediated metabolic adaptations from exercise training. However, overexpression as well as pathogen-stimulated activation of muscle TLR4 (mTLR4) and subsequent downstream inflammatory signaling can compromise skeletal muscle metabolism. This paradox raises a concern about the significance of mTLR4 signaling to the metabolic gains associated with exercise performance. Thus, herein, we aimed to investigate the role of mTLR4 in modulating exercise-induced metabolic adaptations. To this end, we developed a novel muscle-specific TLR4 knockout (mTLR4-/-) mouse model on C57BL/6J background. Male mTLR4-/- mice and wild type (WT) littermates maintained either regular cage activity or performed voluntary wheel running for 4 weeks. Following the training protocol, gastrocnemius and quadriceps muscles were harvested. Notably, mTLR4 deletion revealed marked reductions in downstream interleukin-1 receptor-associated kinase-4 (IRAK4) phosphorylation in muscle homogenates of mTLR4-/- mice compared to WT littermates. In addition, disruption of mTLR4 signaling completely blunted the metabolic adaptations observed in trained WT mice. In particular, trained WT mice significantly increased levels of glucose oxidation (GO) and fatty acid oxidation (FAO) coupled with a substantial increase in glycolytic substrate availability as well as improvements in pyruvate dehydrogenase (PDH) activity, β-hydroxyacyl-CoA dehydrogenase (β-HAD), and citrate synthase (CS) catalytic capacities. In contrast, no significant differences
were detected in levels of substrate metabolism or enzymatic activity in trained mTLR4−/− mice compared with their sedentary controls. Taken together, this study introduces a novel muscle-specific TLR4 knockout mouse model. Furthermore, our findings identify mTLR4 as a major immunomodulatory effector of exercise-induced metabolic adaptations in skeletal muscle.

**Key Words**: skeletal muscle, toll-like receptor-4, wheel running, glucose oxidation, fatty acid oxidation.

**Introduction**

Skeletal muscle is a major metabolic tissue that accounts for approximately 40-50 % of total body mass [1]. Toll-like receptor 4 (TLR4) is an inflammatory signaling receptor that is widely expressed in skeletal muscle and other metabolic tissues [2, 3]. TLR4 is not only known to stimulate the production of inflammatory cytokines, but also modulates carbohydrate and lipid metabolism [4, 5]. Many reports have indicated that skeletal muscle is the source of elevations observed in plasma cytokines during and after exercise [6, 7]. Muscle-derived cytokines (myokines) released in response to exercise such as interleukin-6 (IL-6), monocyte chemoattractant protein (MCP-1), interleukin 8 (IL-8), and interleukin 15 (IL-15), exhibit varied time course profiles [8, 9], suggesting involvement of different regulatory mechanisms. Several of these myokines that are produced with muscle contraction, most notably IL-6, mediate favorable metabolic responses to exercise [10, 11]. In vitro exposure to IL-6 has been shown to stimulate glycogen synthesis and glucose oxidation in human skeletal muscle [12]. It has also been observed that IL-6 infusion in humans augments lipid oxidation with increased reliance on free fatty acids as a source for energy production in skeletal muscle under basal conditions [13]. Of note, many of these exercise-responsive inflammatory mediators of metabolic events appear to be direct targets for the upstream TLR4 signals. However, to the best of our knowledge, it has
not been determined whether mTLR4 specific intracellular signaling transduction is necessarily involved in regulating the metabolic responses to exercise. Thus the role of TLR4 as a potential modulator of exercise-induced metabolic adaptations is appropriate to study.

Recent research has documented that prolonged exercise stimulates the induction of molecules including heat shock proteins (HSPs) and plasma free fatty acids (FFAs) that are suggested to be activators of certain toll-like receptors (TLRs) and the subsequent downstream cytokine production [14, 15]. Additionally, similar to an endotoxin challenge (e.g. lipopolysaccharide, LPS), a down-regulation in TLR4 gene expression has been observed following prolonged vigorous exercise [16, 17]. These intriguing results have developed a recent interest postulating a role for TLRs in modulating inflammatory events and presumably metabolic adaptations in response to exercise [18]. In line with this notion, non-esterified fatty acids (NEFA) have been suggested to activate P38 MAPK and JNK pathways via TLR4-dependent mechanisms in skeletal muscle during endurance exercise [19]. To date, however, there is no direct evidence to support this paradigm. Furthermore, the vast majority, if not all, of related studies emphasize the suppressive actions of exercise training on TLR4 abundance and activation. We adopted a novel concept hypothesizing that mTLR4 signaling is an essential contributor to the metabolic gains of long-term exercise. Therefore the objective of the present study was to investigate the role of mTLR4 signaling in modulating skeletal muscle metabolic response to exercise training.

Material and Methods

Animal husbandry and generation of mTLR4^{−/−} mice. Animal studies were performed under an approved protocol by the Institutional Animal Care and Use Committee (IACUC) at Virginia Tech. The muscle-specific TLR4 knockout (mTLR4^{−/−}) mouse model on C57BL/6J background was created at the Transgenic Core at the Pennington Biomedical Research Center using the Cre-
mediated recombination driven by the muscle creatine kinase promoter. Male mTLR4−/− and wild type (WT) littermates were maintained on a 12-hour light/dark cycle and fed a standard chow diet. All experiments were performed following an overnight fast (10-12 hours), unless otherwise stated. To confirm muscle-specific deletion of TLR4, animals were sacrificed and flexor digitorum brevis (FDB) muscle was isolated. The FDB muscle group was used because isolation of single muscle fibers limits confounding influences of non-muscle cell types that are present in whole muscle groups (e.g. gastrocnemius and quadriceps).

**FDB muscle fibers isolation and preparation.** Mice were euthanized using CO2 asphyxiation followed by cervical dislocation according to Virginia Tech IACUC protocols. The hind foot was cut off above the ankle joint. The foot was pinned with the plantar side facing up onto a flat foam board. Skin was reflected starting from the proximal side of the foot, at the ankle, and was pulled upwards toward the toes. The FDB muscle bundle was dissected by cutting the tendon at the heel and pulling it upward while detaching the muscle fibers from other adjacent tissues. The FDB muscle bundle was removed by cutting at the insertion tendons attached to each of the four lateral toes. The FDB muscle was immediately placed in a tube with 500 µL of dissociation buffer containing cell lysis buffer mixed with protease and phosphatase inhibitors (1/100) as well as collagenase (4 mg/mL). The tube was rocked in a horizontal position on a shaker for approximately 90 minutes at 37°C. Digested muscle was transferred into a 35 mm plastic dish with 500 µL of cell lysis buffer mixed with protease and phosphatase inhibitors. Using a 1 mL micropipette, the FDB muscle bundle was triturated against the side of the dish to break up the fibers. Trituration was repeated until the muscle was stripped from the surrounding white connective tissue. The FDB was collected in a 1.5 mL microcentrifuge tube and washed 3 times by gently resuspending muscle fibers in 500 µL of cell lysis buffer with the protease cocktail.
Each time, the tube was centrifuged at 3000 rpm and supernatant was discarded. Muscle fibers were homogenized in 300 µL of cell lysis buffer, protease cocktail added, and further processed to determine protein concentration for western blotting analysis.

**Western blot analysis.** Western blot analysis was performed using FDB muscle fibers. Samples were homogenized in 50 mM Hapes, (pH7.5), 15 mM NaCl, 1 mM MgCl2, 1 mM CaCl2, 2 mM EDTA, 10% glycerol, 1 % Triton x-100, 10 mM NaP2O7, 100 mM NaFl, 10 mM PMSF, and 10 g/mL aprotinin. Proteins (~30ug per sample) were separated using a 10% Criterion-Tris-HCl gel (Bio-Rad, Hercules, CA) and subsequently transferred to PVDF membrane (Bio-Rad, Hercules, CA). PVDF membrane were probed with a primary antibody against TLR4 (abcam, cat# ab13556) in a 1:200 dilution of BSA, phospho-IRAK4 (Cell Signaling, cat# 7652S) in a 1:1000 dilution of BSA.

**Wheel running protocol.** mTLR4−/− and WT mice underwent 4 weeks of voluntary running on a cage-wheel protocol connected to the LabVIEW software, which monitors distance run, running rate, and running duration. Wheel rotations with a diameter of 11.5 cm were counted continuously, and the rotations were updated by the software every two minutes.

**Body composition analysis.** Body mass was determined prior to assessing body composition using a portable balance (measuring to 0.01g). We assessed the body composition of mice using a Bruker LF90 NMR analyzer to assess lean and fat mass for all mice prior to and following the wheel running program.

**Skeletal muscle whole homogenate preparation for substrate metabolism.** Approximately 50 mg of fresh muscle samples were immediately placed into 0.2 ml of a modified sucrose EDTA medium (SET) on ice containing 250 mM sucrose, 1 mM EDTA, 10 mM tris-HCl, and 1mM
ATP, pH 7.4. Muscle samples were then minced with scissors and SET buffer was added to achieve a 20-fold diluted (wt:vol) suspension. The minced samples were homogenized in a Potter-Elvehjem glass homogenizer at 12 passes across 30 seconds at 150 rpm with a motor-driven teflon pestle, and measures of fatty acid oxidation, glucose oxidation, pyruvate oxidation, and maximal enzyme activities were performed.

**Substrate metabolism and metabolic flexibility.** Red gastrocnemius and quadriceps muscles were collected for substrate metabolism. End products of substrate oxidation as measured by [1-14C]-palmitic acid oxidation to 14CO2, [1-14C]-pyruvic acid oxidation to 14CO2 and [U-14C]-glucose oxidation to 14CO2 were assessed. Metabolic flexibility was assessed by measuring [1-14C]-pyruvate oxidation +/- 100uM palmitic acid. For FAO, GO, and PDH activity assays, tissue homogenates from red skeletal muscles were incubated in 1μCi/mL of [1-14C]-palmitic acid, 1μCi/mL of [U-14CO2]-glucose, and 0.35 μCi/mL of [1-14C]-pyruvic acid, respectively, for 1 hour and then acidified with 45% perchloric acid to elute gaseous CO2. 14CO2 was trapped in 1M sodium hydroxide for 1 hour. The sodium hydroxide was then sampled and placed in a liquid scintillation counter and quantified. Pyruvate oxidation was used to assess the activity of pyruvate dehydrogenase (PDH), the enzyme that catalyzes the oxidation of pyruvate resulting in the provision of glucose-derived acetyl CoA to the TCA cycle. This is a direct measure of pyruvate dehydrogenase activity as the number one carbon of pyruvate is liberated as CO2 in the oxidation of pyruvate to acetyl-CoA.

**Assessing glycolytic intermediates.** For determination of muscle glucose and glucose 6-phosphate concentrations, 300 μl of extracted muscle sample were added to a tube containing 1.7 mL of 0.47 M triethanolamine, 35 mM MgCl2, 25 mM EDTA, and 1.2 mM NADP. An aliquot (200 μl) was then added in triplicate to a 96-well plate. Initial absorbance (OD1) was recorded
using a spectrophotometer (SPECTRAmax ME, Molecular Devices Corporation, Sunnyvale California) at a wavelength of 340 nm. Glucose-6-phosphate dehydrogenase (35 μl; 3.5 IU/sample) was added to each tube and incubated for 20 min. Triplicate aliquots from each tube were transferred to 96-well plates and absorbance was measured again (OD2) at 340 nm. For determination of glucose, hexokinase (21 μl; 2.1 IU/sample) and ATP (41 μl; 11 mg/ml) were added to each tube. Tubes were allowed to incubate for 20 min before triplicate aliquots were transferred to 96-well plates and absorbance (OD3) measured at 340 nm. Absorbance values associated with muscle glucose 6-phosphate were calculated by subtracting OD1 from OD2. Similarly, absorbance values associated with muscle glucose were determined by subtracting OD2 from OD3.

**Citrate synthase (CS) activity.** Citrate synthase catalyzes the formation of citrate and CoASH from acetyl-CoA and oxaloacetate. CoASH reduces DTNB and CS activity was determined from the reduction of DTMB over time. Briefly, ten microliters of a 1:10 diluted muscle homogenate was added, in duplicate, to 170μl of a solution containing Tris buffer (0.1M, pH 8.3), DNTB (1mM, in 0.1M in Tris buffer) and oxaloacetate (0.01M, in 0.1M Tris buffer). Following a 2min background reading, the spectrophotometer (SPECTRAmax ME, Molecular Devices Corporation, Sunnyvale California) was calibrated and 30μl of 3 mM acetyl CoA was added to initiate the reaction. Absorbance was measured at 405nm at 37C every 12 seconds for 7 minutes. CS activity was calculated and reported as μmol/min/mg.

**β-hydroxyacyl-CoA dehydrogenase (β-HAD) activity.** For the determination of β-HAD activity, oxidation of NADH to NAD was measured. 35 μl of whole muscle homogenate was added to 190μl of a buffer containing 0.1M liquid triethanolamine, 5mM EDTA tetrasodium salt dihydrate, and 0.45mM NADH. The spectrophotometer (SPECTRAmax PLUS 384, Molecular
Devices Corporation, Sunnyvale California) was calibrated and 15μl of 2mM acetoacetyl CoA was added to initiate the reaction. Absorbance was measured at 340 nm every 12 seconds for 5 minutes at 37C. β-HAD activity was calculated and reported as μmol/min/mg.

**Phosphofructokinase (PFK) activity.** PFK activity was measured spectrophotometrically at 340nm at 37°C. Briefly, 30ul of sample homogenate were pipette in duplicate (or triplicate). Then, assay buffer (12 mM MgCl2, 400 mM KCl, 2 mM AMP, 1 mM ATP, 0.17 mM NADH, 0.0025 mg/mL, Antimycin 0.05 mg/mL Aldolase 0.05 mg/mL GAPDH, in100mM Tris buffer, pH=8.2) was added into each well. After a 2 min background reading, 3 mM fructose-6-phosphate was added to each sample well and followed by a 7 minute kinetic reading. Changes in absorbance across time were recorded and expressed relative to protein content. PFK activity was calculated and reported as μmol/min/mg.

**RNA extraction and qRT-PCR.** mRNA was extracted using an RNeasy Mini Kit (Qiagen, Valencia, CA) and DNase I treatment (Qiagen, Valencia, CA), according to the manufacturer's instructions. qRT-PCR was performed using an ABI PRISM 7900 Sequence Detection System instrument and TaqMan Universal PCR Master Mix used according to the manufacturer's specifications (Applied Biosystems, Foster City, CA). Target gene expression was normalized to β-actin RNA levels. Primers and 5# FAM-labeled TaqMan probes were purchased as prevalidated assays (Applied Biosystems, Foster City, CA).

**Statistical analysis.** Descriptive and inferential statistics methods were employed to analyze the collection of data of the study. Descriptive statistics as measured by mean and standard error were used to summarize the sample and to quantitatively describe the main features of the data (e.g. body mass, lean mass, fat percent… etc.). Results are expressed as mean ± standard error of
the mean (SEM). As we were interested in studying the interaction effect of both exercise and genotype differences (WT vs. mTLR4⁻/⁻) on manipulating exercise-induced myokine and metabolic responses, the statistical significances of differences among control and experimental groups were determined using the Two-Way ANOVA. If a significant F-ratio was obtained, comparisons between groups were made using Tukey’s post-hoc test. Furthermore, Student’s t-test was used to report the differences in gain scores between the control and experimental groups to better understand the genotype X exercise interaction effect. The level of significance was established as \( P < 0.05 \).

**Results**

**mTLR4⁻/⁻ mouse model.** mTLR4⁻/⁻ mice and their WT littermates were identified following tail biopsy genotyping procedures. mTLR4⁻/⁻ mice showed 68% lower FDB TLR4 protein content compared to the WT counterparts (Figure s 1A, B). Investigating key downstream markers mediating the TLR4 signaling transduction, mTLR4⁻/⁻ mice exhibited ~ 70% lower phosphorylation of IRAK4 (\( P < 0.05 \)) (Figures 1C, D).
Figure 1. mTLR4 protein content is decreased and is coupled with lower IRAK4 phosphorylation in mTLR4−/− mice. WT (n=6) and mTLR4−/− mice (n=6) were sacrificed after overnight fasting and muscle lysates were processed for western blotting analysis to assess muscle TLR4 protein abundance (A, B) as well as IRAK4 phosphorylation status (C, D), downstream from TLR4. Data are expressed as mean ± SEM (*P < 0.05).

Voluntary wheel running and body composition parameters. Upon the completion of the wheel running program, no differences were detected in voluntary wheel running performance (WT: 1117 ± 246 meter/day vs. mTLR4−/−: 1357 ± 417 meter/day) between WT and mTLR4−/− mice (Figure 2A). Meanwhile, wheel running was associated with significant reductions in body mass and fat mass as well as an increase in fat free mass in both WT and mTLR4−/− mice (P < 0.05) (Figures 2B-D).
Figure 2. Wheel running was associated with reductions in body mass and fat mass as well as an increase in fat free mass, however, no genotype differences were observed in wheel running performance. WT (n=6) and mTLR4−/− mice (n=7) underwent 4 weeks of voluntary running on a cage-wheel protocol connected to LabVIEW software, which monitored distance run, running rate, and running duration. Average daily wheel running performance was
determined (A). Prior to the wheel running protocol, body composition parameters (B-D) were assessed via the Bruker mini spec LF90. Independent from genotype, wheel running reduced body mass (B), fat mass (C), and fat free mass (D). Data are presented as means ± SEM (*P < 0.05).

**mTLR4 and wheel running-induced metabolic adaptations.** Data from whole red skeletal muscle homogenates indicate genotype-specific metabolic improvements in response to wheel running. Although in sedentary conditions the basal metabolic substrate oxidation was not different between WT and mTLR4/fl mice, results showed a significant genotype-wheel running interaction as manifested by a greater change in oxidative capacity of WT mice compared with mTLR4/fl littermates. Compared to respective sedentary controls, wheel running resulted in 28.3% higher fatty acid oxidation in WT mice (WT Sed: 68.4 ± 8.1 nmol/mg protein/hr vs. WT WR: 87.7 ± 5.5 nmol/mg protein/hr) and 16% lower oxidation in mTLR4/fl mice (mTLR4/fl Sed: 75 ± 4.8 nmol/mg protein/hr vs. mTLR4/fl WR: 63 ± 3.4 nmol/mg protein/hr) (Figures 3A, B; P<0.05). Moreover, compared to sedentary controls, wheel running induced 50.7% higher glucose oxidation in WT mice (WT Sed: 33 ± 2.9 nmol/mg protein/hr vs. WT WR: 49.7 ± 3.1 nmol/mg protein/hr; P<0.05) and 15.7% lower oxidation in mTLR4/fl mice (mTLR4/fl Sed: 35.5 ± 2.8 nmol/mg protein/hr vs. mTLR4/fl WR: 30.4 ± 3.2 nmol/mg protein/hr) (Figures 3C, D; P<0.05). Despite the substantial differential metabolic response to wheel running between WT and mTLR4/fl mice, differences in metabolic flexibility did not reach significant levels in trained WT and mTLR4/fl mice compared to their respective sedentary controls (P = 0.09, Figures 3E, F).
Figure 3. mTLR4 mediates wheel running-induced increases in substrate oxidation in skeletal muscle. WT (n=12) and mTLR4^{-/-} mice (n=14) were sacrificed 24 hours after the completion of the wheel running protocol and red gastrocnemius and quadriceps muscle were collected for substrate metabolism. Fatty acid oxidation (A, B), glucose oxidation (C, D), and metabolic flexibility (E, F) were assessed. To assess glucose oxidation, muscle homogenates were incubated in [U^{-14}C]-glucose at concentrations of 1µCi/mL and ^14CO2 production was
measured. For fatty acid oxidation, muscle homogenates were incubated in 1μCi/mL of [1-14C]-palmitic acid and 14CO2 production was measured. Data are presented as means ± SEM (*P < 0.05).

**mTLR4 and wheel running-induced adaptations of muscle enzymatic machinery.** Increased oxidative metabolism observed in trained WT mice was supported by functional augmentations in enzymatic activity of PDH (WT Sed: 1880.4 ± 121.5 nmol/mg protein/hr vs. WT WR: 2658.1 ± 210.5 nmol/mg protein/hr: P<0.05) (Figure 4A, B), CS (WT Sed: 409.5 ± 24 µm/mg/min vs. WT WR: 578.6 ± 28.5 µm/mg/min P<0.05) (Figure 4C, D) and β-HAD (WT Sed: 162.4 ± 9.6 µm/mg/min vs. WT WR: 213 ± 16.3 µm/mg/min: P<0.05) (Figure 4E, F). Although PFK enzymatic activity trended to an increase in WT mice and to a decrease in the mTLR4−/− littermates, these differences did not achieve statistical significance (Figure 4G, H). Meanwhile, muscle glucose content remained unchanged between sedentary control and trained groups in WT and mTLR4−/− mice (Figure 5A). However, wheel running induced a significant increase in muscle glucose-6-phosphate content in trained WT mice (WT Sed: 36.4 ± 5.3 µmol/mg vs. WT WR: 83.2 ± 5.5 µmol/mg: P<0.05) (Figure 5B). On the other hand, the mTLR4−/− littermates failed to develop any of the aforementioned oxidative adaptations displayed by WT mice in response to wheel running.
Figure 4. mTLR4 modulates the oxidative capacity of muscle mitochondrial enzymes in response to wheel running. WT (n=12) and mTLR4−/− mice (n=14) were fasted overnight and whole muscle homogenates were obtained to measure mitochondrial enzymatic activity. Pyruvate dehydrogenase activity (A, B) was measured by incubating muscle homogenates in [1-14C]-Pyruvic acid at concentrations of 0.35μCi/mL and 14CO2 production was measured. Citrate synthase (C, D), β-hydroxyacyl-CoA dehydrogenase (E, F), and phosphofructokinase-1 (G, H) activities were determined spectrophotometrically. Wheel running-induced oxidative adaptations were completely blunted in mTLR4−/− mice compared to WT littermates. Data are presented as means ± SEM (*P < 0.05).

Figure 5. mTLR4 signaling is required to enrich glucose-6-phosphate metabolite pools in response to wheel running. The glycolytic metabolites, glucose and glucose-6-phosphate, were measured spectrophotometrically in muscle extracts from WT (n=12) and mTLR4−/− mice (n=14). Across different genotypes, wheel running did not impact intracellular glucose levels (A). However, G-6-P availability was significantly enhanced in trained WT mice but not in mTLR4−/− littermates (B). Data are presented as means ± SEM (*P < 0.05).
Gene expression in response to wheel running. Wheel running did not induce significant changes in mRNA levels of PGC1-α (Figure 6A), PPAR-delta (Figure 6B), and IL-6 (Figure 6C) in WT or mTLR4^−/− mice.

Figure 6. Changes in gene expression of key metabolic and inflammatory regulators in response to wheel running. Across different genotypes, PGC1-α (A), PPAR-delta (B), and IL-6 (C) mRNA levels remained unchanged after wheel running, when comparing the sedentary WT and mTLR4^−/− mice to their respective trained groups. Data were normalized to β-Actin and presented as means ± SEM.
Discussion

Physical exercise induces multi-systemic changes that excite global mobilization of signaling molecules important for cell communication and survival. These signaling messengers are detected by target cells via membrane receptors translating external information into intercellular responses. In turn, host cells adjust to new demands imposed by this exercise stimulus. TLR4 has consistently been portrayed as a pleiotropic cellular receptor modulating multiple inflammatory and metabolic pathways [20-22]. Nonetheless, the significance of TLR4 in exercise metabolism has not been revealed. Identifying the role of TLR4 in exercise performance and energy control may delineate a mechanistic basis for the disrupted exercise-induced adaptations often observed in inflammatory conditions [23-25] characterized with pathologically altered TLR4 signaling, such as obesity and type 2 diabetes [26]. In this study, we used a wheel running exercise modality to investigate the role of skeletal muscle TLR4 in voluntary exercise performance and the subsequent metabolic adaptations. We utilized mTLR4⁻/⁻ mice and wild type littermates which underwent 4 weeks of voluntary wheel running. Following the completion of the exercise protocol, skeletal muscle was collected for oxidative metabolism measurements and assessment of enzymatic activity and gene expression of protein targets thought to be important regulators of metabolic pathways. Herein, we provide data that suggest a possible role for mTLR4 in modulating molecular events required to initiate and develop a normal metabolic adaptation in oxidative tissues in response to exercise training.

For almost two decades since the discovery of TLR4, there has been a growing interest in exploring its implications in health and disease. The loss of function approach has provided a great tool for examining the physiological role of TLR4 within different populations and conditions. Many laboratories have developed various TLR4 knockout mouse models for their
own research [27-30]. As many of the mouse models are whole body knockouts of TLR4 signaling, it is unclear which exact cell type(s) mediate the phenotypes observed in these models. Distinguishable from others, we selectively generated a targeted knockout for mTLR4 in the genome of C57BL/6J mice. Our model manifests a 70% reduction in mTLR4 protein levels. The marginal TLR4 residual protein detected in muscle homogenates from mTLR4^{-/-} mice could plausibly be attributed to other cell types inherent to muscle microenvironment such as macrophages and dendritic cells [31, 32]. To further confirm the functional inactivation of mTLR4, we subsequently analyzed the phosphorylation status of IRAK4 which has been identified as a downstream obligatory contributor to TLR-dependent immune responses [33]. Although IRAK4 activation is a target event of other upstream membrane-spanning receptors [34], IRAK4 protein phosphorylation was decreased by roughly 60% in mTLR4^{-/-} mice compared with WT littermates. As expected, TLR4 protein content and the downstream signal transductions were specifically disrupted in mTLR4^{-/-} mice. However, the placement of our gene mutagen construct under the control of the creatine kinase promoter could raise a tissue-specificity concern. The disruption of TLR4 signaling in cardiac muscle is a potential limitation to heart functionality and therefore to the exercise capacity of mTLR4^{-/-} mice. Noteworthy, previous studies have reported that C57BL/6J mice, out of 7 different inbred mouse strains, were the highest performers on wheel running [35]. We speculated that voluntary, low intensity, wheel running does not necessarily constitute a physical challenge to mTLR4^{-/-} mice on the C57BL/6J background. In concordance with this assumption, our WT and mTLR4^{-/-} littermates exhibited similar exercise patterns with no significant differences in wheel running performance.

Wheel running has repeatedly been demonstrated to improve the oxidative capacity in rodents [36-38]. Interestingly, typical exercise-induced metabolic adaptations were blocked as a result of
mTLR4 absence. Contrary to WT littermates, mTLR4−/− mice failed to enhance levels of fatty acid oxidation after four weeks of wheel running training. These metabolic ramifications of mTLR4 deficiency were further confirmed by the lack of wheel running-induced augmentations of beta oxidation as measured by β-HAD enzymatic activity thereby limiting substrate flux feeding into the tricarboxylic acid (TCA) cycle. TLR4 has previously been linked to key regulators of cellular energy homeostasis. Our results are consistent with a recent report demonstrating that exercise-induced p38 MAPK signaling and JNK activation, two crucial modulators of fat metabolism, were abolished in TLR4-deficient mice [19]. Additionally, TLR4 deletion has been shown to diminish exercise-associated inflammatory response in a tissue-specific manner, which implies impaired cytokine signaling [39]. Upon muscle contraction, IL6 release has been well documented to promote AMPK activation and the concomitant accelerations in rates of fatty acid oxidation [11, 40]. These oxidative adaptations are possibly depressed in mTLR4−/− mice. This paradigm represents a legitimate molecular basis for the role of mTLR4 in modulating fat metabolism in response to exercise training. Furthermore, our data indicate that carbohydrate utilization in response to exercise is also modulated by mTLR4 signaling. Improvements in glucose oxidation observed in WT mice were completely blunted by the mTLR4 gene disruption. Undoubtedly, TLRs, including TLR4, up-regulate glycolysis in an almost Warburg Effect fashion [41]. A primary target for TLR4-mediated elevation of glycolysis is hexokinase [42]. Consequently, inhibiting mTLR4 is very likely inhibiting its activation of glycolysis. Besides, as mentioned previously, TLR4-stimulated cytokine production includes IL-6 which has recently been shown to enhance glycolysis through hexokinase- and phosphofructokinase-dependent mechanisms [43]. In concert with these notions, levels of upstream glycolytic intermediates remained unchanged in mTLR4−/− muscle, whereas WT
littermates exhibited a substantial increase in levels of glucose-6-phosphate in response to wheel running. Increased glucose uptake and glycogen reserves potentially underlie elevations observed in substrate availability in trained WT mice as oppose to mTLR4\(^{-/-}\) counterparts. Intriguingly, although not statistically significant, wheel running tended to increase phosphofructokinase activity in WT mice, yet, trained mTLR4\(^{-/-}\) littermates barely sustained basal activity levels detected in their sedentary controls. It is important to bear in mind that amplified beta-oxidation observed in trained WT mice may expectedly increase cytosolic citrate content which could partially account for the restrained phosphofructokinase activity. These findings offer a possible mechanistic understanding to our observation of a significant suppression of PDH adaptive response in mTLR4\(^{-/-}\) mice, which failed to match PDH elevations witnessed in WT mice in response to wheel running. Indeed, impaired glycolytic flux observed in mTLR4\(^{-/-}\) mice may limit substrate availability for mitochondria, thus abrogating the need for PDH complex up-regulation. Taken together, concurrent maladaptation of key enzymes involved in both beta-oxidation and aerobic glycolysis suggests a scarcity of acetyl CoA supply. Lack of carbon substrates feeding into the TCA cycle may account for exercise-irresponsiveness of CS activity elicited in trained mTLR4\(^{-/-}\) mice compared with the corresponding WT littermates. Noteworthy, however, the metabolic superiority of WT mice to mTLR4\(^{-/-}\) littermates, as defined by enzymatic capacity and substrate oxidation, was insufficient to induce noticeable phenotypic differences in wheel running performance. Several factors can modulate maximum oxygen consumption and increased aerobic capacity associated with exercise performance. Yet, the contribution of mitochondrial enzymes to exercise-induced VO2 max adjustments is controversial. Saltin and colleagues indicated that skeletal muscle has a propensity to overbuild its enzymatic machinery to an extent that exceeds the oxygen transport capacity of the circulation.
As such, the genotype discrepancies we found in enzyme capacities may not be a vital determinant controlling submaximal exercise performance.

In conclusion, this study introduces a novel muscle-specific TLR4 knockout mouse model. In addition, the current results reveal a vital role for mTLR4 in mediating exercise-induced metabolic adaptations. Nevertheless, the absence of adaptations as observed in WT mice did not impede voluntary running wheel performance in the mTLR4\(^{-}\) mice. However, the underling mechanisms linking exercise performance to mTLR4 activation and the downstream signaling are yet to be fully elucidated. Moreover, controlled studies with known exercise doses are needed for more reliable genotype comparisons.

References


CHAPTER VI

Skeletal Muscle Toll-like Receptor-4 Deficiency Suppresses Exercise-induced Myokine Response and Diminishes Aerobic Training Adaptations in Mice.

Abstract

There is strong evidence supporting a crucial role for inflammatory cytokines in modulating aerobic capacity and metabolic adaptations to exercise. Many reports have indicated that skeletal muscle is the source of systemic elevations of cytokine levels, termed myokines, observed during and post exercise. However, the exact mechanism mediating these muscle-derived inflammatory cascades has yet to be elucidated. Pathogen-mediated activation of muscle TLR4 (mTLR4) is known to stimulate the production of pro-inflammatory myokines. However, it is not clear if mTLR4 is also implicated in the inflammatory responses associated with exercise and muscle contraction. As such, the purpose of this study was to investigate the role of mTLR4 signaling in modulating myokine production and release in active muscle and how mTLR4 deficiency (mTLR4−/−) may impact the exercise-induced aerobic and oxidative adaptations. Intriguingly, mTLR4 genetic inactivation depressed aerobic capacity improvements observed in WT mice after treadmill training. Additionally, contrary to WT littermates, mTLT4−/− mice lacked treadmill running-induced acute elevations in circulating myokines. In concert, mTLR4−/− mice also displayed attenuated myokine gene expression in response to in vivo electrical muscle stimulation (EMS) when compared with WT controls. Furthermore, mTLR4−/− mice experienced metabolic limitations in skeletal muscle adaptability to exercise where they failed to match oxidative improvements of WT littermates for glucose oxidation, fat oxidation, and mitochondrial enzymatic capacity. Overall, these data disclose a direct association between mTLR4 signaling and exercise-induced myokine production and release, a mechanism that may
account for metabolic maladaptation observed in mTLR4<sup>-/-</sup> mice. Furthermore, these data suggest a link between mTLR4 and physiological determinants of maximal aerobic performance.

**Key Words**: skeletal muscle, toll-like receptor-4, treadmill training, myokines, aerobic capacity, metabolic adaptations

**Introduction**

Skeletal muscle is a major site controlling fuel depots and energy expenditure [1]. Exercise modulates whole body energy homeostasis via mechanisms manipulating skeletal muscle phenotype. The activation of exercise-responsive intracellular signaling pathways stimulates transcriptional events that dictate skeletal muscle properties. Inflammatory signaling plays a critical role in exerting the effect of exercise locally in the active muscle and systemically through endocrine mechanisms [2, 3]. Skeletal muscle has recently been recognized as a secretory organ [4]. Myokines are a class of inflammatory proteins produced by active muscle to convey many of the exercise-induced changes in muscle mass, contractile and metabolic properties [5].

Exercise-stimulated myokine production has been established to be an intensity- and duration-dependent response [6, 7]. However, the precise molecular mechanism(s) modulating these inflammatory cascades has yet to be elucidated. Post-exercise muscle glycogen content is reported to be tightly related to levels of circulating cytokines and has been shown to regulate myokine gene expression [8, 9]. Another line of evidence has suggested that exercise-induced intracellular calcium flux and production of reactive oxygen species trigger transcriptional factors promoting IL-6 synthesis [10, 11]. Nonetheless, the question remains as to whether the
exercise stress response follows similar inflammatory physiological pathways to those activated by other physical and pathogenic stimuli.

Due to a spontaneous genetic mutation in their endotoxin response locus, C3H/HeJ mice were used to initially identify the gene target for LPS [12]. This identification led to the discovery of TLR4 which demonstrated a critical role in mediating the pro-inflammatory cytokine production in multiple tissues including skeletal muscle [13-15]. Intriguingly, in comparison to six other inbred mouse strains, C3H/HeJ mice showed the worst aerobic capacity as measured by maximal treadmill performance [16]. Moreover, contrary to respective WT controls, C3H/HeJ mice had a significant attenuation of exercise-induced IL-6 and IL-10 responses in the hypothalamus where this defect was found to abolish the exercise sensitization of insulin and leptin [17]. Together, TLR4 is likely to exert its effect through modulating the exercise-associated cytokine response, a mechanism that may link TLR4 signaling to maximal exercise performance.

Exercise promotes the induction of metabolic and muscular injury by-products that invoke consequent inflammatory events. Depending on mode and duration, exercise has been documented to induce a transient increase in extracellular concentrations of damage-associated molecular patterns (DAMPs) and non-esterified fatty acids (NEFAs) in response to trauma and heightened energy demand, respectively [18, 19]. These molecules have been shown to stimulate TLR4 signaling [20, 21]. A growing body of evidence indicates that TLR4 activation is vital for the resolution of muscle injury and long-term adaptations to chronic exercise [22, 23]. TLR4 has also been reported to modulate key cellular metabolic regulators including P38 MAPK and JNK [23]. Nonetheless, the role TLR4 as a modulator of maximal exercise performance in response to aerobic training is still unanswered. Furthermore, the role of TLR4 in the exercise-induced cytokine response and its concomitant effects remains to be investigated. Skeletal muscle serves
as the primary tissue responsible for physical activity and systemic control of energy homeostasis. As such, understanding the molecular network governing its contribution to systemic adaptability to aerobic training is of great significance to the effectiveness of exercise intervention programs.

**Material and Methods**

**Animal husbandry and mTLR4\(^{-/-}\) mice.** Animal studies were performed under an approved protocol by the Institutional Animal Care and Use Committee (IACUC) at Virginia Tech. The muscle-specific TLR4 knockout (mTLR4\(^{-/-}\)) mouse model on C57BL/6J background was created at the Transgenic Core at the Pennington Biomedical Research Center using the Cre-mediated recombination driven by the muscle creatine kinase promoter. Male mTLR4\(^{-/-}\) and wild type (WT) littermates were maintained on a 12-hour light/dark cycle and fed standard chow diet. All experiments were performed following an overnight fast (10-12 hours), unless otherwise stated.

**Acute and chronic treadmill running protocols.** Forced treadmill running was used. Thirty two mTLR4\(^{-/-}\) and WT mice were randomized to either a sedentary control or exercise group. Mice were acclimated to the treadmill for 2 days. Blood serum samples were collected and body composition parameters were assessed prior (baseline) to and after (post-training) the completion of the exercise program. Due to limitations with mTLR4\(^{-/-}\) mice available for the study, a subgroup of 12 mice, out of the 32 mice, were used to examine changes in serum cytokine levels in response to acute exhaustive treadmill running. The acute exercise protocol consists of one bout of prolonged endurance exercise where mice ran until exhaustion at 0.30 m/sec. In addition to the baseline serum samples that were collected at rest (the pre-exercise time point); another sample was collected immediately after the termination of the acute bout of treadmill running.
Allowing 3 days of recovery, mice then performed a maximal aerobic capacity test. Then mice were submitted to the treadmill training program which consists of a moderate intensity 50-minute run 5 days/week for 4 weeks. The protocol progressed into two phases. The first two weeks mice ran at 0.30 m/sec where the second two weeks were at 0.33 m/sec. After the completion of the treadmill running program mice performed a maximal aerobic capacity test. Mice were sacrificed 36 hours after testing their aerobic capacity as a wash out period avoiding the effect of last bout exercise. Skeletal muscle (gastrocnemius, quadriceps, and soleus) were harvested for processing and data collection.

Figure 1. Schematic representation of the treadmill study design

**Body composition analysis.** Body mass was determined prior to assessing body composition using a portable balance (measuring to 0.01g). We assessed the body composition of mice using a Bruker LF90 NMR analyzer to assess lean and fat mass for all mice prior to and post the wheel running program.
**Testing aerobic capacity.** Using incremental treadmill running, the testing protocol begins with 5 minutes warm-up at low work rate followed by a stepwise increase in power output. The mice went through incremental running starting at 0.20 m/sec and 0˚ inclination. The treadmill velocity was increased by 0.03 m/s every two minutes until the mice reached exhaustion and refused to continue running. Speed and total distance at which the mouse could not continue running were recorded. The testing session was to be terminated at any point at which a mouse met either of these exhaustion criteria:

1. Spending greater than 5 consecutive seconds on the shock grid without attempting to reengage the treadmill

2. Spending greater than 50% of its time on the shock grid

3. The third time a mouse was willing to sustain 2 seconds or more of shock without attempting to reengage the treadmill

**In-vivo plantar flexor torque assay.** In-vivo measures of plantar flexor torque (PFT) were obtained with mice under isoflurane anesthesia (platform at 35°C). The mouse’s foot was secured to a torque transducer (Aurora Scientific) and the tibial nerve was stimulated at 2Hz for a series of contractions to fatigue (4 bouts X 8 minutes; 1 minute rest between bouts). At the end of the fatigue protocol, the leg was aseptically cleaned with povidone-iodine solution while the mouse recovered from anesthesia (~2-5 minutes in 100% O2). Hindlimb PFT data were analyzed using Dynamic Muscle Analysis (DMA) software versions 3.2 (Aurora Scientific).

**Blood sampling and flow cytometry analysis.** To determine serum cytokine profile, blood samples were drawn using Microvette CB 300 Z tubes through the lateral saphenous vein which runs dorsally and then laterally over the tarsal joint. Blood was then centrifuged at 8000 rpm for
10 minutes at 4oC for serum separation. Serum samples were blocked with mouse IL-6, IL-10, MCP-1, IFN-γ, and TNF-α capture beads using the commercial Cytometric Bead Array (CBA) Mouse Inflammation Kit (cat# 552364) according to the manufacturer's instructions (BD Biosciences).

Skeletal muscle whole homogenate preparation for substrate metabolism. Approximately 50 mg of fresh muscle samples were immediately placed into 0.2 ml of a modified sucrose EDTA medium (SET) on ice containing 250 mM sucrose, 1 mM EDTA, 10 mM tris-HCl, and 1mM ATP, pH 7.4. Muscle samples were then minced with scissors and then SET buffer was added to a 20-fold diluted (wt:vol) suspension. The minced samples were homogenized in a Potter-Elvehjem glass homogenizer at 12 passes across 30 seconds at 150 rpm with a motor-driven teflon pestle, and measures of fatty acid oxidation, glucose oxidation, pyruvate oxidation, and maximal enzyme activities were performed.

Substrate metabolism. Red gastrocnemius and quadriceps muscles were collected for substrate metabolism. End products of substrate oxidation as measured by [1-14C]-palmitic acid oxidation to 14CO2 and [U-14C]-glucose oxidation to 14CO2 were assessed. For FAO and GO assays, tissue homogenates from red skeletal muscles were incubated in 1μCi/mL of [1-14C]-palmitic acid or 1μCi/mL of [U-14C]-glucose respectively, for 1 hour and then acidified with 45% perchloric acid to elute gaseous CO2. 14CO2 was trapped in 1M sodium hydroxide for 1 hour. The sodium hydroxide was then sampled and placed in a liquid scintillation counter and the trapped 14CO2 was quantified.

Citrate synthase (CS) activity. Citrate synthase catalyzes the formation of citrate and CoASH from acetyl-CoA and oxaloacetate. CoASH reduces DTNB and CS activity was determined
from the reduction of DTMB over time. Briefly, ten microliters of a 1:5 diluted muscle homogenate was added, in duplicate, to 170μl of a solution containing Tris buffer (0.1M, pH 8.3), DNTB (1mM, in 0.1M in Tris buffer) and oxaloacetate (0.01M, in 0.1M Tris buffer). Following a 2min background reading, the spectrophotometer (SPECTRAmax ME, Molecular Devices Corporation, Sunnyvale California) was calibrated and 30μl of 3 mM acetyl CoA was added to initiate the reaction. Absorbance was measured at 405nm at 37C every 12 seconds for 7 minutes. CS activity was calculated and reported as μmol/min/mg.

**Malate dehydrogenase (MDH) activity.** MDH activity was measured spectrophotometrically at 340nm at 37°C. Briefly, 10ul of sample were pipetted in duplicate or triplicate in wells. Then, 290ul of reaction media (0.1 M potassium phosphate buffer, PH 7.4 plus 0.006 M oxaloacetic acid, prepared in potassium phosphate buffer plus 0.00375 M NADH, prepared in potassium phosphate buffer) was added to the wells and samples were read for 5 minutes at 340nm. The rate of disappearance of NADH was analyzed and expressed relative to protein content. MDH activity was calculated and reported as μmol/min/mg.

**β-hydroxyacyl-CoA dehydrogenase (β-HAD) activity.** For the determination of β-HAD activity, oxidation of NADH to NAD⁺ was measured. Volume of 35 μl of whole muscle homogenate was added to 190μl of a buffer containing 0.1M liquid triethanolamine, 5mM EDTA tetrasodium salt dihydrate, and 0.45mM NADH. The spectrophotometer (SPECTRAmax PLUS 384, Molecular Devices Corporation, Sunnyvale California) was calibrated and 15μl of 2mM acetoacetyl CoA was added to initiate the reaction. Absorbance was measured at 340 nm every 12 seconds for 5 minutes at 37C. β-HAD activity was calculated and reported as μmol/min/mg.
Cytochrome c oxidase (COX) activity. Cytochrome c oxidase enzyme activity was measured spectrophotometrically at 550nm every 10 seconds for 7 minutes. Absorbance was measured based on the oxidation of ferrocytochrome c to ferricytochrome c by cytochrome c oxidase. COX activity was calculated and reported as μmol/min/mg.

RNA extraction and qRT-PCR. mRNA was extracted using an RNeasy Mini Kit (Qiagen, Valencia, CA) and DNase I treatment (Qiagen, Valencia, CA), according to the manufacturer's instructions. qRT-PCR was performed using an ABI PRISM 7900 Sequence Detection System instrument and TaqMan Universal PCR Master Mix used according to the manufacturer's specifications (Applied Biosystems, Foster City, CA). Target gene expression was normalized to β-actin RNA levels. Primers and 5# FAM-labeled TaqMan probes were purchased as prevalidated assays (Applied Biosystems, Foster City, CA).

Western blot analysis. Western blot analysis was performed using skeletal muscle homogenates prepared in 50 mM HEPES, (pH7.5), 15 mM NaCl, 1 mM MgCl2, 1 mM CaCl2, 2 mM EDTA, 10% glycerol, 1 % Triton x-100, 10 mM NaP2O7, 100 mM NaFL, 10 mM PMSF, and 10 g/mL aprotinin. Proteins (~30ug per sample) were separated using a 10% Criterion-Tris-HCl gel (Bio-Rad, Hercules, CA) and subsequently transferred to PVDF membrane (Bio-Rad, Hercules, CA). PVDF membrane was probed with primary antibody against GLUT4 (Cell Signaling, cat# 2213S) in a 1:1000 dilution.

Statistical analysis. Descriptive and inferential statistics methods were employed to analyze the collection of data of the study. The descriptive statistics as measured by mean and standard error were used to summarize the sample and to quantitatively describe the main features of the data (e.g. body mass, lean mass, fat percent… etc.). Results are expressed as mean ± standard error of
the mean (SEM). As we were interested in studying the interaction effect of both exercise and genotype differences (WT vs. mTLR4+/−) on manipulating exercise-induced myokine and metabolic responses, the statistical significances of differences among control and experimental groups were determined using the Two-Way ANOVA. If a significant F-ratio was obtained, comparisons between groups were made using Tukey’s post-hoc test. Furthermore, Student’s t-test was used to report the differences in gain scores between the control and experimental groups to better understand the genotype × exercise interaction effect. The level of significance was established as P < 0.05.

Results

Maximal aerobic capacity and body composition parameters. WT and mTLR4+/− littermates experienced significant reductions in body fat percent (Figure 2A) in response to treadmill training. Furthermore, data shows a main effect of the treadmill training protocol which significantly increased running capacity of WT and mTLR4+/− mice, comparing between pre and post-training protocol time points (Figure 2B). Data are presented as means ± SEM.
Figure 2. The effect of the treadmill training protocol on body fat and maximal aerobic capacity. Differences in body fat percent and aerobic capacity between WT and mTLR4−/− mice at pre and post-treadmill training time points. Treadmill training resulted in significant decreases in body fat percent (A) and increases in maximal running performance (B) in WT (n=8) and mTLR4−/−(n=8) mice. Data are presented as means ± SEM (* P < 0.05).

Force production in response to in-vivo electrical muscle stimulation. There was a significant main effect of time but no significant genotype X time interaction for the plantar flexor muscle torque (Figure 3). The force production profile showed a significant incremental decrease over 12 time points representing the time course of the EMS fatigue protocol. Data are presented as means ± SEM.
Figure 3. Assessment of force production over the course of the in-vivo electrical muscle stimulation protocol. The force production profile showed a significant incremental decrease over 12 time points representing the 32-minute duration of the EMS fatigue protocol. WT (n=7) and mTLR4−/− mice (n=9) produced similar plantar flexor muscle torque. Data are presented as means ± SEM (*P < 0.05).

Serum cytokine levels in response to acute exhaustive treadmill running. There was a significant genotype X acute running interaction for circulating cytokines. IL-6 (4A), TNF-α (4B), INF- γ (4C), but not MCP-1 (4D), levels in WT and TLR4−/− mice increased and decreased respectively post-exercise. mTLR4−/− mice lacked normal cytokine responses following acute exhaustive treadmill running. Data are presented as means ± SEM.
Figure 4. Assessment of inflammatory markers post-exhaustive treadmill running. In response to acute exhaustive treadmill running, elevations of circulating IL-6 (A), TNF-α (B), INF-γ (C), and MCP-1 (D) were suppressed in mTLR4−/− mice (n=6) compared with those observed in WT littermates (n=6). Data are presented as means ± SEM (*P < 0.05).

Myokine gene expression in response to in-vivo electrical muscle stimulation. There was a main effect of muscle stimulation and a significant genotype X muscle stimulation interaction for mRNA levels of IL-6 and MCP-1. qRT-PCR analysis showed that in-vivo electrical stimulation induced muscle contraction that resulted in significant increases in IL-6 (Figure 5A) and MCP-1(Figure 5B) gene expression in WT and mTLR4−/− mice but to a significantly lesser extent in the latter. Data are normalized to β-Actin and presented as means ± SEM.
Figure 5. Assessment of myokine gene expression in response to in-vivo electrical muscle stimulation. Compared with respective augmentations found in WT controls (n=7), in-vivo electrical muscle stimulation revealed a blunted contraction-induced mRNA increase of muscle IL-6 (A) and MCP-1 (B) in mTLR4−/− mice (n=7). Data were normalized to β-Actin and presented as means ± SEM (*P < 0.05).

Metabolic adaptations in response to chronic treadmill training. Treadmill training resulted in significant increases in glucose oxidation in WT and mTLR4−/− mice (Figure 6A), but to a significantly greater magnitude in WT mice (WT: +110% vs. mTLR4−/−: +43%: P<0.05) (Figure 6B). Although not statistically significant, there was a trend of an increase in levels of FAO in WT mice, whereas mTLR4−/− littermates showed a completely blunted response to treadmill training (Figure 6C). Furthermore, while WT mice witnessed trends of augmentations in enzymatic activities of CS (Figure 7A), MDH (Figure 7B), β-HAD (Figure 7C), and COX (Figure 7D) in response to treadmill training, mTLR4−/− mice exhibited activity levels similar to the basal rates observed in their untrained counterparts. Notably, each of the mTLR4 deletion and treadmill training independently induced main effects manifested in significant augmentations in β-HAD enzymatic activity (Figure 7C: P<0.05) Data are presented as means ± SEM.
Figure 6. Assessment of changes in oxidative metabolism in response to treadmill training.

Compared with that shown in respective WT littermates (n=8), treadmill training revealed a significantly less pronounced increase in GO (A, B) in mTLR4−/− mice (n=8). However, FAO levels (C) remained unchanged in WT and mTLR4−/− mice. Data are presented as means ± SEM (*P < 0.05).
Figure 7. Assessment of changes in mitochondrial maximal enzymatic activity in response to treadmill training. Although not statistically significant, WT mice (n=8) showed trends of increases in CS activity (A), MDH (B), β-HAD (C), and COX (D) in response to treadmill training compared with a blunted response characterized in mTLR4−/− mice (n=8). However, albeit mostly driven by WT mice, treadmill training induced a main effect of a significant increase in β-HAD activity (C) regardless of the genotype differences. Meanwhile, mTLR4 deletion appears to have a main effect of a significant increase in β-HAD activity (C) compared with WT littermates. Data are presented as means ± SEM (βP < 0.05 WT vs. mTLR4−/−, *P < 0.05 Sedentary vs. Trained).

Gene expression in response to chronic treadmill training. There was a significant genotype X treadmill training interaction for GLUT4 mRNA levels (Figure 8A) and protein content
Figure 8. Assessment of changes in gene expression of key metabolic and inflammatory genes in response to treadmill training. GLUT4 mRNA (A) increased significantly in treadmill trained WT mice (n=8), but not in trained mTLR4<sup>−/−</sup> (n=8), compared with respective sedentary
controls. PPAR-delta (B), SOD2 (C), IL-6 (D), and MCP-1 (E) mRNA remained unchanged. Data are presented as means ± SEM (*P < 0.05).

**Figure 9.** Assessment of changes in GLUT4 protein levels in response to treadmill training.

GLUT4 protein content increased significantly in treadmill trained WT mice (n=8), but not in trained mTLR4−/− (n=8), compared with respective sedentary controls. Representative western blots are included below their respective columns. Data are presented as means ± SEM (*P < 0.05).

**Discussion**

TLR4 is an exercise-responsive cellular receptor upstream from IL-6 and other inflammatory cytokines that have demonstrated critical roles in exercise adaptability. Due to a mutation in the TLR4 gene, C3H/HeJ mice are widely used in different research areas. Intriguingly, C3H/HeJ mice were shown to lack the exercise-induced hypothalamic IL-6 response and the associated insulin and leptin sensitization effect [17]. Meanwhile, Faldt and colleagues have reported reduced treadmill endurance in IL-6 deficient mice [24]. Together, these findings gained our
impetus to explore the significance of TLR4 signaling to maximal exercise performance and its concomitant myokine and metabolic responses in skeletal muscle as a master regulator of whole body energy homeostasis. Towards this goal, we used an mTLR4−/− mouse model and pursued tightly standardized exercise and muscle stimulation modalities.

The main findings of this study were 1) Skeletal muscle TLR4 gene inactivation resulted in diminished muscle contraction-induced myokine gene expression as well as attenuation in the post-exercise elevations of circulating cytokines; and 2) mTLR4 signaling appears to play a critical role in modulating metabolic adaptations in response to aerobic training. 3) Maximal exercise performance is not a function of mTLR4 signaling. Designing an exercise experiment can be pursued by multiple approaches including voluntary and forced exercise modalities. The inflammatory and metabolic signaling associated with exercise performance has repeatedly been reported to be a mode- and duration-dependent response. With this in mind, treadmill running has best served our goal where we were able to control the intensity and amount of muscle work to limit the confounding factors that may arise from receiving different doses of exercise.

In addition to controlling the exercise dose, the diffusion characteristics of the muscle cell microenvironment are of fundamental importance in gene transcription regulation of skeletal muscle [25-28]. The extracellular milieu of a myocyte contains ions, metabolic substrates, neuroactive chemicals, signaling proteins and other substances that can profoundly alter the intramuscular signaling pathways [28-31]. At the same time, the extracellular space of the muscle is a highly complex medium that can be modulated by several factors including age [32], fat mass [33] and stress perception [34] each of which is unique to each experimental animal. As such, to minimize the mouse to mouse variability errors we also utilized in-vivo electrical muscle
stimulation to induce muscle contraction in one leg while the contralateral leg was used as a negative control.

The present study is the first to specifically examine the role of mTLR4 signaling in mediating the exercise-induced myokine response and how this may modulate aerobic capacity and skeletal muscle metabolism. Our data show no differences between WT and mTLR4^−/− mice in basal (before training) aerobic capacity. Our observations are consistent with those reported by H. Zbinden and colleagues who did not detect significant differences in the maximal treadmill running velocity between WT and transgenic TLR4^−/− mice [23]. However, although not statistically significant, our model shows that mTLR4 gene deletion resulted in a trend of attenuation in the aerobic training-induced improvements in maximal treadmill performance observed in the WT littermates. Noteworthy, mouse breeding and sample size issues may generate experimental limitations impacting the power of the statistical inference tests employed in data analysis.

Myokine response to exercise has always been shown to be mediated through muscle contraction-stimulated intracellular signaling. Intramuscular exercise-sensitive components including glycogen content, calcium flux, as well as reactive oxygen species have been implicated in modulating myokine synthesis and release [9, 35]. Distinguishably, we propose that the myokine cascades associated with exercise can also be modulated, in part, by systemic factors (e.g. NEFA, HSP70) [36]that may exert their effects through cellular receptors localized in the extracellular matrix including TLR4. Herein, we demonstrate that muscle-contraction induced myokine gene expression entails mTLR4 signaling. As expected, mTLR4^−/− failed to activate IL-6 and MCP-1 gene transcription up to the levels observed in WT littermates in response to in-vivo electrical muscle stimulation. This was further supported by the lack of
normal elevations in circulating cytokines in mTLR4\(^{-/-}\) mice subjected to acute, exhaustive treadmill running, unlike the increase seen in WT littermates. In concert, it was previously reported that exercise-induced hypothalamic IL-6 and IL-10 responses were repressed in C3H/HeJ mice that possess a dysfunctional TLR4 gene.

Impaired exercise-induced myokine response in mTLR4\(^{-/-}\) mice was accompanied by marked perturbations in metabolic adaptations to exercise training. Although treadmill training contributed to an increase in levels of glucose oxidation in mTLR4\(^{-/-}\) mice, yet this increase failed to reach the magnitude of improvements achieved by WT. We have previously showed that voluntary wheel running resulted in a substantial increase in upstream glycolytic intermediates in WT mice, but not in mTLR4\(^{-/-}\), that may be facilitated, in part, by improved glucose uptake. A major target for TLR4 and its downstream myokine signaling in skeletal muscle, as an insulin-sensitive tissue, is GLUT4. There is a direct correlation between an increase in insulin sensitivity and increased cell surface GLUT4 content. Exercise training results in adaptations that facilitate insulin-mediated glucose uptake and are regulated by different mechanisms. It leads to changes in gene expression, signaling, greater blood flow and changes in GLUT4 protein exocytosis and endocytosis [37]. Our current data suggest a link between mTLR4 and GLUT4 gene reprogramming in response to exercise training. GLUT4 transcriptional adaptations following treadmill training, as measured by mRNA and protein levels, were diminished in mTLR4\(^{-/-}\) mice compared to the significant augmentations achieved by trained WT littermates. Furthermore, it has been reported that acute IL-6 exposure improves insulin-stimulated glucose disposal in humans and glucose uptake in-vitro [38]. So the fact that mTLR4 gene disruption resulted in a depressed IL-6 response may explain the alterations observed in glucose trafficking and metabolism in mTLR4\(^{-/-}\) mice [35, 38, 39]. Moreover in
response to treadmill training, WT mice had a trend of an increase in bet-oxidation which was accompanied by a trend of an increase in fatty acid utilization, though did not reach statistical significance. Collectively, these adjustments in carbohydrate metabolism in WT mice possibly increased the substrate supply to the TCA cycle but resulted only in trends of increases, with no statistical significance, in the mitochondrial oxidative enzymes citrate synthase, malate dehydrogenase, and cytochrome c oxidase, a marker for the ETC activity. This could be justified, in part, by lack of statistical power due to small sample size and difficulties with matching the different experimental groups. In contrast, mTLR4−/− mice exhibited a comprehensive inhibition to aerobic-training induced metabolic adaptations. Indeed, TLR4 signaling was shown to be essential to activate key regulators of cell energy homeostasis including MAPK [23].

Overall, our results demonstrate a critical role for mTLR4 in modulating the host adaptability to aerobic training outcomes, specifically the metabolic responses. As such, further studies are of great importance to explore a potential skeletal muscle fiber type-specific response to the interplay between the muscle specific deletion of TLR4 and different modes of exercise. This approach may lead to identifying an exercise modality that could bypass the genotype and phonotypical perturbations in mTLR4 signaling which appear to impair aerobic training outcomes in oxidative muscle. Furthermore, future studies should aim at characterizing physiological adaptations to muscle work in the mTLR4−/− mouse model including muscle and cardiac functions and properties that modulate maximal aerobic capacity.
References

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CHAPTER VII

Conclusions and Future Directions

TLR4 plays a critical role in skeletal muscle in both health and disease. The understanding of TLR4 signaling in skeletal muscle has been limited by the lack of a muscle-specific manipulation that enables the study of the functional signaling of muscle TLR4 in vivo. Our novel mouse model with the muscle-specific deletion of TLR4 bridges this research gap and methodological limitation. Our in vivo investigations suggest an essential function for muscle TLR4 in developing some of the health benefits commonly reported with exercise interventions. Findings from the current study imply that muscle TLR4 mediates the inflammatory cascades associated with exercise, thereby modulating the concomitant metabolic adaptations in oxidative tissues. TLR4 appears to control exercise-mediated oxidative metabolism by initiating signals that secure continuous supply of metabolic substrates to the working muscle as well as boosting the enzymatic machinery serving the associated metabolic pathways. These effects could impact lifestyle-related diseases.

Developed countries with a westernized diet and sedentary lifestyle suffer from chronic ailments with inflammatory etiology, including obesity and type-2 diabetes, which may relate to inappropriate TLR4 signaling. In these conditions, the power of TLR4 is often modulated by negative regulators suppressing the chronic excess of inflammatory signaling. These negative effects may compromise TLR4 signaling and abolish its mediatory role in exercise-induced metabolic adaptations and any consequent health benefits. In obese humans, TLR4 signaling is chronically activated in a pathological manner associated with a concurrent increase in the expression of suppressors of cytokine signaling (SOCS) proteins in metabolic tissues. It has been
shown that elevations in the expression of SOCSs mediate inhibitory effects on insulin signaling and glucose metabolism. In this context, the impaired inflammatory tone with chronic anti-inflammatory signaling cascades could potentially disrupt normal TLR4-associated inflammatory and metabolic mechanisms. Further studies are needed to characterize the differential physiological and metabolic response of obese and healthy lean individuals to different exercise modalities. This may delineate the adaptive processes and mechanisms that are potentially altered, which may lead to the identification of key molecular targets for therapeutic interventions.
APPENDICES

Appendix A: Generation of Skeletal Muscle-Specific TLR4 Knockout Mice

A targeting vector was generated using recombineering. Isogenic DNA containing TLR4 gene was retrieved from a clone (RP24-209H15) of the C57BL6 BAC genomic library via gap repair. The loxP-neo-loxP was inserted into intron 1 and the third loxP site into intron 2 via homologous recombination to make a targeting vector with the exon 2 flanked by the last two loxP sites. For mouse ES cell targeting, NotI-linearized TLR4 targeting vector DNA, consisting of 2 kb 5'‑arm and 8 kb 3'‑arm, was electroporated into C57BL/6J ES cell line Bruce 4 (a gift from the NCI, NIH). Correct targeting was confirmed using high fidelity PCR analyses and DNA sequencing. ES cells were injected into the blastocysts of female albino B6 strain C57BL/6J-Tyrc-2J obtained
from The Jackson Laboratory. Chimeras were then crossed with the albino B6 strain to screen for heterozygous carriers of the modified allele. The Pgk-neo gene was removed by mating heterozygotes (TLR4loxneo/+)) with B6.FVB-Tg (EIIa-cre) to generate heterozygous mice (TLR4lox/+). To generate muscle-specific KO mice for TLR4, TLR4lox/+ mice were bred to MCK-Cre recombinase mice (from Gerald Bothe, Wadsworth Center, Albany, NY) that were backcrossed for 10 generations to C57BL6 mice. Therefore, all analyses are from mice on the C57BL6 background.