Toll-like Receptor (TLR) Signaling and Differential Activation of PGC Family Genes in a Mouse Model of Staphylococcus aureus Sepsis

by

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Date: 5/20/2010
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Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Pathology in the Graduate School of Duke University

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ABSTRACT

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Abstract

Sepsis is a major cause of morbidity and mortality in the United States, and *Staphylococcus aureus* (*S. aureus*) is the bacteria most commonly cultured from septic patients [1]. In severe sepsis, the relationship between the systemic inflammatory response and the resulting mitochondrial and metabolic dysfunction is not fully understood, especially with respect to the mechanisms of mitochondrial damage resolution. The process of mitochondrial biogenesis, which leads to the restoration of metabolic and anti-oxidative functions in damaged or stressed cells and tissues, is pro-survival and is a critical protective response in sepsis. Mitochondrial biogenesis requires the coordinated expression of multiple regulatory proteins, including the PPARγ-coactivator (PGC) family of proteins. Previous work in sepsis has focused on mitochondrial biogenesis in response to late signals of mitochondrial damage; however, for acute sepsis, we have hypothesized a direct and early link between the innate immune response and the transcriptional activation of mitochondrial biogenesis. Since the Toll-like receptors (TLRs) are a major part of the innate immune response, we hypothesized that they could activate mitochondrial biogenesis in bacterial sepsis. Earlier work showed that TLR4 (which responds to components of Gram-negative bacteria) was necessary for mitochondrial biogenesis induction in response to heat-killed *E. coli* challenge. For this work, the objective was to investigate whether signaling by TLR2 (which responds to components of Gram-positive bacteria) would activate mitochondrial biogenesis in response to *S. aureus* sepsis in mice. The sepsis model was initially characterized in wild-
type (WT) mice by PCR analysis of hepatic RNA, in which the up-regulation of several regulatory proteins for mitochondrial biogenesis, including all three PGC family members, was observed. In contrast, in both TLR2<sup>-/-</sup> and TLR4<sup>-/-</sup> mice, the mitochondrial biogenesis response was deficient and delayed. In addition, PGC-1α and PGC-1β were differentially regulated in WT, TLR2<sup>-/-</sup>, and TLR4<sup>-/-</sup> mice. To identify the mechanisms involved in this induction pattern, the known TLR signaling pathways were systematically probed for activation using several strains of genetic knockout mice. These data demonstrated that the differential regulation of the PGC family is independent of the MyD88 adapter protein and is caused in part by IRF7 signaling. IRF7 is a pro-inflammatory transcription factor that is normally involved in the interferon response; in this case, IRF7 was found to be necessary but not sufficient for PGC-1α/β induction. In addition, a second level of regulation was identified in the microRNA mmu-mir-202-3p, which is inversely correlated with the expression of PGC-1α and PGC-1β mRNA in WT, TLR2<sup>-/-</sup>, and TLR4<sup>-/-</sup> mice and was shown to functionally decrease PGC-1α mRNA. If these observations are confirmed in humans, IRF7 and mir-202-3p may be potential therapeutic targets for the up-regulation of PGC-1α/β levels in the clinical setting of sepsis and impaired mitochondrial biogenesis.
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1. Introduction

1.1 Sepsis: A synopsis of the current clinical and scientific problem

*Staphylococcus aureus* (*S. aureus*) sepsis is a serious and expanding clinical problem due to the increased prevalence of virulent strains in humans and the use of indwelling devices and invasive monitoring in health care [1]. Epidemiological studies have put the incidence of *S. aureus* bacteremia at 38 episodes per 100,000 person-years, with an in-hospital mortality rate of approximately 30% [2,3,4]. Sepsis is a complex syndrome that involves a massive and sustained amplification and recruitment of the innate immune system caused by receptor activation by pathogen-associated or damage-associated molecular patterns (PAMPs, conserved pathogenic structures such as bacterial wall components, and DAMPs, conserved structures found in normal tissue but not normally found in circulation, such as basement membrane fragments, histones, or heat shock proteins). This immune activation leads to the production of high levels of pro-inflammatory cytokines, affecting multiple physiological systems with consequent derangements in coagulation, endothelial integrity, and metabolic demand [5]. This pro-inflammatory state leads to the systemic inflammatory response syndrome (SIRS), defined clinically by the presence of two or more of the following: a temperature less than 36°C or greater than 38°C; a heart rate greater than 90 bpm; tachypnea to greater than 20 breaths per minute; or a white blood cell count less than 4000 cells/mm³ or greater than 12,000 cells/mm³ (or the presence of more than 10% immature neutrophils) [6]. Sepsis is defined as the presence of SIRS in the setting of a known infection [7]. In severe cases, sepsis can lead to septic shock, resulting in multiple organ failure and death.
Septic shock is characterized by a high-output cardiac failure caused by peripheral vasodilation, capillary leak, and, in some cases, vasoplegia [8]. This is accompanied by an increase in tissue oxygen tension and yet a paradoxical decrease in tissue oxygen consumption, a state called dysoxia, cytopathic dysoxia, or cytopathic hypoxia [9,10,11]. For a simple diagram of the pathophysiology of sepsis, see Figure 1.

There are still several major gaps in our understanding of the pathophysiology of sepsis, including how the innate immune system can activate mechanisms of cellular damage recovery, what accounts for differing susceptibilities to sepsis, and a full understanding of how the multiple cytokines and chemokines produced during sepsis give rise to the observed changes in physiology.
Figure 1: A diagram of the pathophysiology of sepsis. PAMPs: pathogen-associated molecular patterns; DAMPs: damage-associated molecular patterns; TLRs: Toll-like Receptors; NLRs: NOD-like Receptors; RIG: Retinoic acid-inducible gene helicase; TNFα: Tumor necrosis factor; IFN: Interferon; ROS, RNS: Reactive oxygen/nitrogen species.
1.2 Staphylococcus aureus virulence and pathogenicity

*S. aureus* are Gram-positive, spherical bacteria that form grapelike clusters. The Gram-positive cell wall is made of polymerized cross-linked chains of peptidoglycan (PGN), which is itself composed of dimers of N-acetylglucosamine and N-acetylmuramic acid, the latter of which is bonded to a polypeptide chain of L-alanine, D-glutamine, L-lysine, and D-alanine. The peptide chain lysine is then cross-linked to a nearby D-alanine from a different monomer through a poly-glycine bridge [12]. In addition, the PGN network is anchored to the cell membrane beneath by lipoteichoic acid (LTA), which inserts into both the lipid bilayer and the PGN layer above [13].

*S. aureus* are able to bind to surfaces (both biological, such as fibrin deposition and extracellular matrix components, and non-biological, such as implanted material) and form polysaccharide biofilms. The bacteria thus produce several secretable expanded repertoire adhesive molecules (SERAMs), such as coagulase and extracellular adherence protein (Eap) [14]. In addition, *S. aureus* also produce several anchored adhesion proteins known as microbial surface components recognizing adhesive matrix molecules (MSCRAMMs), including fibronectin-binding proteins (FnBPs), collagen binding protein (Cna), clumping factors A and B (ClfA and ClfB) and Protein A, which binds the Fc (non-antigen-specific) portion of immunoglobulins [15]. Both the SERAMs and the MSCRAMMs enable *S. aureus* to both bind and invade tissues and to escape immune surveillance. In addition, *S. aureus* produce several exotoxins that mediate virulence, such as hemolysins and leukocidins, which cause pore formation and ultimately cell
death (either apoptotic or necrotic, depending on concentration), as well as superantigens, which mediate massive immune responses [16,17].

Several studies have shown *S. aureus* to be capable of cellular invasion and persistence. Though the mechanisms have yet to be fully elucidated, it is known that *S. aureus* SERAMs and MSCRAMMs such as Eap and FnBPs form a host-cell binding complex, through which the bacterium is able to invade [18]. Usually, after bacteria are phagocytosed by a target cell, the bacterial phagosome merges with a lysosome through the autophagy pathway and the contents (and bacteria) are degraded. Some bacteria, including *S. aureus*, are able to evade this autophagic defense and thus survive intracellularly [19]. *S. aureus*-derived α-hemolysin is an exotoxin that forms pores in target membranes; recent work has shown that *S. aureus* use α-hemolysin to form pores in the phagosome, disrupting acidification of the vesicle and thus delaying phagosomal maturation [20]. The phagosome thus never merges with the lysosome, and so the bacteria are able to grow and eventually to escape intracellularly [21]. Cytosolic sensors responsive to the Gram-positive cell wall (such as NOD1 and NOD2) can thus serve as important mediators of inflammation during *S. aureus* intracellular infection [22,23] in addition to the typical plasma membrane receptor families such as the TLRs. Thus, *S. aureus* virulence and pathogenicity proceed through both extracellular and intracellular routes. This dissertation will focus on the interactions of *S. aureus* with the plasma-membrane-associated Toll-like receptors.
1.3 The role of the mitochondrion in sepsis and sepsis-induced organ damage

Mitochondria normally function to produce ATP by oxidative phosphorylation, a process during which molecular O$_2$ is reduced to water in the production of the inner-membrane proton gradient. Mitochondria are thus critically important during periods of increased metabolic demand (such as during sepsis) because they allow for greater utilization of energy than anaerobic respiration. In addition, mitochondria regulate the apoptotic cascade. Both the formation of permeability transition (PT) pores through activation of the Bcl family of proteins and frank mitochondrial disruption due to oxidant damage can lead to the release of pro-apoptotic factors such as cytochrome C or apoptosis inducing factor [24,25]. Thus, mitochondrial integrity is important in sepsis both to meet increased need for ATP production and to prevent apoptosis.

During sepsis, there is both a high post-resuscitation mortality rate and a limited amount of cell death, suggesting that the metabolic derangements are due not to problems with oxygen delivery but rather due to problems with cellular oxygen utilization [8,10,26,27]. Indeed, substantial evidence has shown that primates have severe mitochondrial damage in sepsis and in septic shock, as shown by changes in mitochondrial redox status and mitochondrial ultrastructure in response to infusion of *E. coli* [28,29,30,31]. In addition, the severity of organ dysfunction correlates with mitochondrial dysfunction [32,33].

Mitochondria normally produce reactive oxygen species (ROS) and reactive nitrogen species (RNS) during aerobic respiration. Specifically, superoxide radical (O$_2^-$)
is formed by oxidation of molecular oxygen by electrons diverted from mitochondrial complex I [34]. Superoxide is normally reduced to H$_2$O$_2$ by superoxide dismutase (SOD), which is then further reduced to water by glutathione or catalase [35]. During sepsis, at least three separate mechanisms are known to cause mitochondrial damage. First, in sepsis, high levels of nitric oxide (NO) are produced by inducible nitric oxide synthase (iNOS); superoxide then reacts with NO to form the RNS peroxynitrite (ONOO$^-$). Peroxynitrite leads to direct inhibition of the respiratory chain [36]. Second, the nitration and nitrosation of OXPHOS proteins contribute to increased local ROS production and subsequent mitochondrial damage [33,37]. Third, there is a lack of new mitochondrial antioxidant protein incorporation (e.g., glutathione and superoxide dismutase-2) such that the mitochondria are unable to deal with increased H$_2$O$_2$ production, leading to the formation of hydroxyl radical (HO$^-$) via the Fenton reaction [34]. The hydroxyl radical leads to direct damage to mitochondrial membranes, protein, and DNA [38,39]. Mitochondrial damage during sepsis leads both to decreased copies of mitochondrial DNA (mtDNA) and decreased respiratory capacity of the affected mitochondria [40,41,42,43,44]. This damage can cause a sustained decrease in metabolic activity as well as an increase in apoptosis. It has been shown in mouse models of \textit{S. aureus}- and heat-killed-\textit{E. coli}-induced sepsis as well as in human septic patients that there is up-regulation of mitochondrial biogenesis in response to this mitochondrial damage [41,44,45]. This sepsis-induced mitochondrial biogenesis improves the ability of the cell to metabolize oxygen and produce ATP, which is necessary for cell survival.
1.4 Mitochondrial biogenesis

Mitochondrial biogenesis is an adaptive cellular program that maintains the capacity for mitochondrial energy production through maintenance and repair of mitochondrial components and through the synthesis of new organelles. The mitochondrial biogenesis program is under bi-genomic transcriptional control. Most of the approximately 1,000 mitochondrial proteins are encoded in the nucleus, but there are 13 hydrophobic proteins (plus tRNAs and rRNAs) encoded on the mitochondrial genome [46]. Signals from the mitochondrion, signals from different metabolic factors, and extracellular signaling pathways can all affect the initiation and rate of mitochondrial biogenesis. The nuclear respiratory factors, NRF-1 and NRF-2 (also known as GABA-α), are transcription factors that are known to activate the expression of respiratory subunits, proteins of heme synthesis, mitochondrial antioxidant proteins, mitochondrial importation proteins, and the mitochondrial transcription factors (such as mitochondrial transcription factor A, or Tfam) [47]. The nuclear-encoded mitochondrial genes are translated in the cytosol and translated with a tag for mitochondrial import known as a mitochondrial targeting signal [48]. The members of the PPARγ-co-activator (PGC) family of transcriptional co-activators (which include PGC-1α, PGC-1β, and PGC-Related Coactivator, or PRC) have been called “master regulators” of the mitochondrial biogenesis program because they are known to co-activate target genes of both NRF-1 and NRF-2 [47,49,50,51]. Both PGC-1α and PGC-1β have been shown to be capable of stimulating mitochondrial biogenesis when over-expressed [52,53]. The relationship
between the function of PGC-1α and PGC-1β and the third member, PRC, is poorly defined. A simple diagram of the main known control points is provided in Figure 2.

![Diagram showing control points for mitochondrial biogenesis](image)

**Figure 2:** Transcriptional control points for mitochondrial biogenesis.

### 1.5 The TLR family of innate immune adaptors

The Toll-like receptor (TLR) family is an innate immune system protein family that recognizes conserved pathogen-associated molecular patterns (PAMPs). The first TLR was discovered in 1997, and the family is now known to be comprised of at least 12 members in mammals (TLR1 through TLR12) [54]. Each TLR has several extracellular leucine-rich repeat (LRR) domains, a transmembrane domain, and an intracellular Toll/IL-1 receptor (TIR) domain, through which the TLR interacts with the TLR adaptor
molecules. The TLRs can either homodimerize or heterodimerize to form a signaling complex, though it is unclear whether the dimer forms before the PAMP is bound, or vice-versa [55]. In addition, several TLRs have been shown to require additional proteins in complex to effectively bind their target PAMPs and signal intracellularly. For instance, TLR4 binding to LPS requires the proteins MD2, LBP (LPS-binding protein) and CD14 [56]. TLR2 complexes with CD36 to sense Gram-positive components and to phagocytose S. aureus [57,58], and may even require LBP and CD14 for proper signaling in response to LTA [59]. The various complexes that support TLR signaling greatly expand the range of PAMPs to which the TLRs can bind. Specifically, TLR4 homodimers respond to LPS from Gram-negative bacteria, whereas TLR1/2 and TLR2/6 heterodimers respond cell wall components from Gram-positive bacteria such as PGN and LTA. Mice deficient in specific TLRs thus produce fewer cytokines in response to their TLR-specific PAMPs, and often show increased mortality when exposed to live pathogens [60].

1.6 TLRs and mitochondrial biogenesis

Mitochondrial biogenesis, along with mitophagy (the process of controlled mitochondrial degradation), is necessary to maintain mitochondrial quality control and is considered to be an important protective process during sepsis. Therefore, we hypothesized a direct link between the innate immune response to sepsis and the activation of the mitochondrial biogenesis program. The proposed link would thus separately cause activation of mitochondrial biogenesis earlier than and in addition to the
oxidant damage in sepsis. Our group has already published that inflammation-induced NF-κB up-regulates the pro-biogenesis transcription factor NRF-1 [61]. Previous work has also demonstrated that TLR4−/− mice failed to up-regulate the mitochondrial biogenesis program in response to heat-killed E. coli [41]. This dissertation thus presents our investigations, which confirm, define, and expand the role of TLR signaling in mitochondrial biogenesis in response to S. aureus sepsis.

Our initial hypothesis was that WT mice would up-regulate the mitochondrial biogenesis program in response to S. aureus sepsis and that TLR2−/− mice would exhibit a deficiency in that up-regulation due to a lack of signaling in response to S. aureus. Based on the results of the early experiments, we subsequently exposed TLR4−/− mice to the same live S. aureus sepsis model to test whether a component of the observed inflammation was being caused by a TLR4 ligand.

2. Materials and Methods

Mouse model of S. aureus sepsis.

The studies were conducted in wild-type C57Bl/6J mice obtained from Jackson Laboratories (Bar Harbor, ME). TLR2−/−, TLR4−/−, MyD88−/−, MAL−/−, TRAM−/−, and TRIF−/− mice on a C57Bl/6J background were obtained from Shizuo Akira, Japan, and bred in the Duke University vivarium. TLR2−/− X TLR4−/− mice were bred by Paul Noble at Duke University by crossing TLR2−/− with TLR4−/− mice obtained from Shizuo Akira. IRF3−/− X IRF7−/− mice on a C57Bl/6J background were obtained from Dr. Michael Diamond, Washington University, St Louis, MO. Both the p50−/− mice and the Akt1−/− mice were on
C57Bl/6J backgrounds and were obtained from Jackson Laboratories (Bar Harbor, ME). Mice of either gender weighing 20-30 grams were used for the studies. The study protocols were approved by the Duke University Institutional Animal Care and Use Committee.

Mice were anesthetized with an intraperitoneal injection of xylazine and ketamine, and the abdomen was shaved and cleaned with povidone-iodine. Midline laparotomy was performed and an infected fibrin clot was inserted in the peritoneum. The peritoneum and abdomen were then closed with proline sutures. All mice were resuscitated with 1 ml of 0.9% NaCl administered subcutaneously. Mice were sacrificed at 6, 24, 48, or 72 hours PI by overexposure to isoflurane. Healthy control (HC) mice of each strain were also sacrificed. Livers were taken immediately and either the mitochondria were isolated at once or the tissue was snap-frozen and stored at -80°C.

To prepare the fibrin clots, S. aureus ssp. aureus (no. 25923; American Type Culture Collection, Manassas, VA) was reconstituted according to the manufacturer’s specifications and steriley inoculated onto Trypticase soy agar (BD Diagnostic Systems, Sparks, MD) slants. The slants were incubated for 18 hours at 37°C to achieve adequate log-phase growth. Bacteria were harvested in 0.9% NaCl and centrifuged, and the pellets were resuspended in 0.9% NaCl. Bacterial suspensions were quantified with a spectrophotometer (550 nm) to generate a stock solution of 1x10^{10} viable colony-forming units per milliliter. Pour plates were used to confirm viability and accuracy of the calibration. Doses of 10^5, 10^6, or 10^7 cfu were then suspended in 500μl fibrin clots (500μl
of 10mg/ml bovine fibrinogen, fraction 1, plus 10μl of 1 unit/μl bovine plasma thrombin) (Sigma, St Louis, MO).

**Limulus Amebocyte Lysate (LAL) assay.**

LAL assay was performed with a GenScript Chromogenic LAL endotoxin assay kit (GenScript, Piscataway, NJ). Thrombin and fibrinogen preps were made as described above in duplicate and then tested individually.

**Cell Culture.**

Mouse AML12 hepatocytes were purchased from the American Type Culture Collection (Manassas, VA). AML12 cells were cultured in 5% CO2–95% air at 37 °C in DMEM/F12 medium (GibcoBRL, Grand Island, NY) containing l-glutamine and 2.438 g/L sodium bicarbonate. The medium was supplemented with 10% FBS, a mixture of insulin, transferrin, selenium (ITS; Sigma, St. Louis, MO) and 40 ng/ml dexamethasone.

AML12 cells were exposed to 10^7 cfu heat-killed *S. aureus* per ml (prepared from the same strain as that implanted in mice) and were harvested at different time-points. AML12 cells were also exposed to fibrin D-dimers (AbCam) at 1 μg/ml and 10μg/ml for 4 hours and then harvested. Gene expression was tested by real time RT-PCR

AML12 cells were transfected with scrambled (AllStars Negative Control siRNA, Qiagen) or miRNA mimic for mmu-mir-202-3p (Qiagen) using Lipofectamine RNAiMax. Transfection was confirmed with BlockIT fluorescent oligo (Invitrogen).
Serum starvation was achieved by replacing cell culture media with media without FBS for 4 hours. mRNA was extracted with Trizol and gene expression was tested by real time RT-PCR.

**Mitochondria and mtDNA Isolation.**

Liver mitochondria were isolated from ~2 g of fresh tissue using a modified Clark protocol [62]: the livers were hand-dounced in a 0.25 M sucrose buffer and then centrifuged at 2,000xg for 3 min at 4°C. The supernatant was then centrifuged at 12,500xg for 8 min at 4°C, and the pellet was recovered for mtDNA extraction using a mtDNA Extraction Kit (Wako Chemical, Japan) according to the manufacturer’s instructions.

**Real-Time RT-PCR.**

RNA was extracted from frozen liver with TRIzol reagent (Invitrogen, Oslo, Norway) and subjected to reverse transcription with the ImProm-II Reverse Transcription System (Promega, Madison, WI) according to the manufacturer’s instructions. Mouse-specific primers were designed or bought commercially (see Appendix C) and real-time PCR was carried out in triplicate, using 18s primers for internal controls. Reactions were carried out in TaqMan Universal PCR Master Mix (Applied Biosystems) to 40 cycles at 60°C on an ABI PRISM 7700 and analyzed with Sequence Detector Software (Applied Biosystems). The ΔΔCt method was used to quantify mRNA levels per sample, using 18s
as the endogenous control. Real-time PCR output for HC mice of each strain was set to one, and the relative quotients at later time points are shown.

Mitochondria and mtDNA were isolated as described above, and the mtDNA copy number was quantified with real-time PCR on a 7700 Sequence Detector System (Applied Biosystems). Primers were designed for cytochrome b (cyt b) with ABI Probe Design software (see Appendix C) (Applied Biosystem). Amplifications were performed on 10 ng total mtDNA using PCR primers. One copy of linearized pGEMT-cyt b vector was used as a standard for simultaneous mtDNA quantification. Serial dilutions of $10^5$–10 copies standard cyt b plasmid were prepared to establish a standard curve. Samples were analyzed in triplicate.

**MicroRNA PCR.**

MicroRNAs were prepared with an All-in-One™ miRNA qRT-PCR Detection Kit (GeneCopoeia, Rockville, MD) according to the manufacturer’s instructions. Briefly, the extracted RNA was reverse-transcribed in the presence of a Poly-A polymerase with an oligo-dT adaptor. Quantitative PCR was then carried out with SYBR green detection with a forward primer for the mature miRNA sequence and a universal adaptor reverse primer. An ABI PRISM 7700 was used for Q-PCR, and the reactions were carried out to 40 cycles at 60°C. Standardization to 18s and quantitation using the ΔΔC_t method was as described above. SYBR green melting curve analysis was performed after 40 cycles to confirm specificity.
**Nuclear Isolation.**

Nuclei were obtained by douncing fresh liver in a nuclear homogenization buffer (0.32 M sucrose, 3 mM MgCl₂, 2 mM DTT, 20 mM K-HEPES (pH 7.2), plus 1 μM Na-Ascorbate and 1:100 dilutions of Sigma Protease Inhibitor Cocktail, Sigma Phosphatase Inhibitor Cocktail 1 and Sigma Phosphatase Inhibitor Cocktail 2, added fresh) (Sigma, St Louis, MO). The homogenate was filtered through two layers and then four layers of cheese cloth, and was then centrifuged at 3,800xg for 20 min at 4°C. The supernatant was discarded and the resulting pellet was dounce-resuspended in 1 ml nuclear isolation buffer (2 M sucrose, 1 mM MgCl₂, 2 mM DTT, 5 mM K-HEPES (pH 7.2)). This suspension was then poured over another 2 ml of isolation buffer and then centrifuged at 113,000xg for 1 h at 4°C. The supernatant was carefully removed and the pellet was dounce-resuspended in a nuclear wash buffer (150 mM KCl, 5 mM MgCl₂, 5 mM K-HEPES (pH 7.2)). This suspension was spun at 500xg for 20 min at 4°C, and the resulting pellet was either fixed for ChIP analysis or frozen at -80°C in RIPA buffer (150 mM NaCl, 1 mM EDTA, 50 mM Tris-HCl (pH 7.4), 1% Igepal, 0.25% deoxycholate, plus 1:100 dilutions of Sigma Protease Inhibitor Cocktail, Sigma Phosphatase Inhibitor Cocktail 1 and Sigma Phosphatase Inhibitor Cocktail 2, and 1 mM PMSF, added fresh) (Sigma, St Louis, MO). Nuclear extractions were confirmed by immunoblots for His3 (positive) and LDH (negative) (Santa Cruz Biotechnology, Santa Cruz, CA) as described below.
Western Blots.

Whole cell extracts were used or nuclei were isolated as above and then sonicated, and protein levels were standardized using the BCA method. Proteins were resolved with sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 4-20% gels and then transferred to polyvinylidene difluoride membranes. Membranes were probed with primaries as listed in the table below. They were then exposed to the appropriate secondary antibodies (Santa Cruz Biotechnology). Membranes were developed with ECL (Santa Cruz Biotechnology) and imaged on X-ray film. Membranes were stained with Coomassie blue as a loading control. The blots were quantified with a BioRad G-710 densitometer. Images shown are representative of at least two independent experiments.

Table 1: Antibodies used for immunoblots, ChIP, and Co-IP

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**Chromatin Immunoprecipitation.**

Nuclear extracts were exposed to a final concentration of 1% formaldehyde for 15 min at 24 ºC, and the reaction was quenched at a final concentration of 0.125 M glycine for 5 min. DNA was sheared with a Branson sonicator to a range of ~200-800 bp fragments. ChIP was carried out using a ChIP-IT Express Kit (Active Motif, Carlsbad, CA) according to the manufacturer’s instructions. Antibodies used were rabbit polyclonal anti-p65, mouse monoclonal anti-IRF-7 and rabbit polyclonal anti-RNA-Polymerase-II.
(all Santa Cruz Biotechnology). Primers were designed to correspond to the sequence around the ISRE in the PGC-1α promoter (for IRF-7) and the promoter of EF1α (for Pol-II) (See Appendix C). Endpoint PCR was carried out to 40 cycles at 60°C.

**Co-immunoprecipitation.**

Approximately 50 mg of frozen WT liver was dounced in a homogenization buffer (PBS (pH 7.4) with 1% Igepal, 0.25% deoxycholate, plus 1:100 Sigma Protease Inhibitor Cocktail and 200uM PMSF added fresh). Dithiobis[succinimidyl propionate] (DSP; Thermo Scientific) was added to a final concentration of 2 mM, and the sample was rotated for 1 h at 4°C. The reaction was stopped with 50μl of 1 M Tris-HCl (pH 7.5). The suspension was centrifuged at 14,000xg for 15 min, and the supernatant was standardized with the BCA method. Magnetic Protein A Dynabeads (Invitrogen, Oslo, Norway) were pre-washed and resuspended in PBS with 0.02% Tween-20. All protein samples were added to the prewashed beads in the presence of negative rabbit IgG. After pre-clearing for 1 h at 4°C, the supernatants were taken off and split into two groups: one group was added to dynabeads pre-bound to TLR-2 antibody (See Table 1) and the other was added to dynabeads pre-bound to negative IgG. The samples were rotated overnight at 4°C. The samples were washed 4 times with PBS (pH 7.4) and the beads were boiled for 5 min in Laemmli buffer with DTT and immunoblotted as described above.
**Immunohistochemistry.**

For tissue immunohistochemistry, livers were fixed with 4% paraformaldehyde, dehydrated, paraffin-embedded, and cut on a microtome. Antigens were retrieved and then slides were stained with TLR2 primary antibody (SC-52735, mouse monoclonal, Santa Cruz Biotechnology) and fluorescently labeled secondary, and then with DAPI. Confocal images were collected in fluorescence mode followed by electronic image merging.

**In silico analyses.**

To prepare promoter maps, mouse (Mus musculus NCBI assembly m37) and human (Homo sapiens NCBI assembly GRCh37) genomes were accessed on Ensembl (www.ensembl.org), and then aligned using zPicture (zpicon.dcode.org). The alignments were then fed into rVista 2.0 (rvista.dcode.org) and analyzed for transcription factor consensus sequences according to the Transfac Professional library (v10.2) with similarities optimized for function [63].

MicroRNA binding predictions were made with TargetScan Mouse release 5.1 (www.targetscan.org/mmu_50) [64]. Specific microRNA binding patterns were then predicted on microRNA.org (www.microrna.org). mRNA folding and single-strand frequency predictions were made with mfold version 3.2 [65,66], using mRNA sequences from Ensembl.
Statistics.

For survival curve analysis, survival at 72 hours PI at the same dosage of *S. aureus* (10⁶ cfu) was compared between WT and TLR2⁻/⁻ or TLR4⁻/⁻ mice using Fisher’s exact test. All grouped data are presented as means ± SD. The n values indicated in the figure legends are for the total number of mice from each strain. Each time point in the real-time PCR experiments was compared to the healthy control (HC) of its own strain using two-sided Student’s *t*-tests. In addition, the 6 h time points between strains were compared using two-sided Student’s *t*-tests. The levels of significance (*P* values) for all tests are given in the figure legends.

3. Results

3.1 Characterization of the mouse model of *S. aureus* sepsis

We used a previously established *in vivo* model of *S. aureus* sepsis to study the effects of mitochondrial biogenesis [45]. In this model, a known number of live *S. aureus* bacteria are resuspended in a fibrin clot, which is then implanted in the peritoneum of mice (for a full description of the model, see Materials and Methods). This is a modified version of the model first published by Ahrenholz and Simmons in 1980 [67], and subsequently employed by several other groups [68,69]. The fibrin clot model of live bacterial sepsis is widely used as an *in vivo* model because of its similarity to clinical sepsis [70]. The components of the fibrin clot were tested for LPS by LAL assay, and the total dose of LPS was less than 0.04 ng (see Table 2). Previous studies have shown that this level of LPS exposure should not cause an inflammatory response [71,72]. After the
surgery, the mice are sacrificed at 6, 24, 48, or 72 hours post-implantation (PI). Healthy controls (HCs) are also taken for baseline comparisons. We examined mitochondrial biogenesis and inflammatory changes in the liver as a whole organ, because it has resident macrophages (Kupffer cells) that reflect changes in the immune system, it is a major metabolic organ that is affected during whole-body inflammation, and it receives the portal circulation and is thus highly responsive to peritoneal sepsis. In addition, immunohistochemistry for TLR2 was done in the liver in both WT healthy controls and in WT and TLR2−/− mice at 6h PI. It was shown that hepatocytes stain for TLR2, though with less intensity than do Kupffer cells, and that TLR2 expression is increased after exposure to *S. aureus* (See Figure 3). TLR4 has been previously shown to be expressed in both Kupffer cells and hepatocytes [41].

Table 2: LPS contents of the fibrin clot components as measured by LAL assay, and total LPS per clot. Each component was measured twice, and the averages of the measurements are shown.

<table>
<thead>
<tr>
<th>Clot Component</th>
<th>EU/mL</th>
<th>ml/clot</th>
<th>EU/clot</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>0.018</td>
<td>0.1</td>
<td>0.0018</td>
</tr>
<tr>
<td>Thrombin</td>
<td>1.47</td>
<td>0.01</td>
<td>0.0147</td>
</tr>
<tr>
<td>Fibrinogen</td>
<td>0.68</td>
<td>0.5</td>
<td>0.34</td>
</tr>
<tr>
<td><strong>Total:</strong></td>
<td></td>
<td></td>
<td><strong>0.36</strong></td>
</tr>
</tbody>
</table>

1EU = 0.1ng LPS | Total ng LPS per clot: | ~0.04
Figure 3: Paraffin-embedded liver sections show immunofluorescent stains for TLR2 in (A) WT HC, (B) WT 6h PI, and (C) TLR2−/− 6h PI livers. Red, TLR2. Blue, DAPI. Each picture is representative of four sections examined from two animals from each group.
Figure 4: Survival curves, *S. aureus* fibrin clots. Survival curves for WT, TLR2−/−, and TLR4−/− mice are shown. Doses of 10⁵, 10⁶, and 10⁷ are shown for TLR2−/− mice, showing dose-response effects. At 72H PI and a dose of 10⁶ cfu, both TLR2−/− and TLR4−/− mice show significantly greater mortality than WT mice (WT vs. TLR2−/−, \( P < 0.0001 \) and WT vs. TLR4−/−, \( P = 0.0023 \), Fisher’s exact tests).

Dose-response curves were generated for clots containing differing amounts of *S. aureus* (10⁵, 10⁶, or 10⁷ cfu/clot) (See Figure 4). Wild-type mice showed very low mortality in our model at 72 hours PI, in keeping with previous studies using this model [45]. TLR2−/− mice showed a high susceptibility to *S. aureus* sepsis, with mortality rates at 72 hours PI of 5% for 10⁵ cfu, 65% for 10⁶ cfu, and 100% for 10⁷ cfu. At 10⁶ cfu at 72H PI, TLR2−/− survival was significantly less than WT (WT vs. TLR2−/−, \( P < 0.0001 \), Fisher’s exact test). The dose of 10⁶ cfu was thus chosen for all subsequent studies as a model of lethal sepsis that still allowed data collection at 72 hours PI, which is approximately the
time-point at which surviving WT mice recover their mtDNA copy number [45].

Notably, when the TLR4\(^{-/-}\) mice were put through the model at 10\(^6\) cfu, they had a higher rate of mortality at 72 hours PI (100%) than WT mice at 10\(^6\) cfu (WT vs. TLR4\(^{-/-}\), \(P=0.0023\), Fisher’s exact test). Though TLR4 is usually reported as being responsive only to components from Gram-negative bacteria, it has been shown to be responsive both to infection by \emph{S. aureus} and to components from other Gram-positive bacteria (see Table 3) [73,74,75,76,77,78,79]. Notably, while there are few published studies that have examined the \textit{in vivo} response of TLR4\(^{-/-}\) mice to \emph{S. aureus}, all of them have shown a TLR4-dependent response to live \emph{S. aureus}. Moreover, published mortality studies in TLR-deficient mice in response to \emph{S. aureus} infection have been conflicting: Some have reported that TLR2\(^{-/-}\) mice have a higher mortality than WT mice in response to \emph{S. aureus} [74,80] or other Gram-positive bacteria [81,82], while some studies have reported that there is no difference in mortality rates in \emph{S. aureus} sepsis between TLR2\(^{-/-}\) mice and WT mice [83,84]. These differences can be explained by the different models and perhaps the different bacterial subtypes used in the different papers. Nevertheless, they demonstrate that the effects of TLR deficiencies in the response to sepsis \textit{in vivo} are not fully understood.

\textbf{Table 3: (next page) The response of TLR4 to Gram-positive agonists: a summary of the literature.}
<table>
<thead>
<tr>
<th>Model</th>
<th>TLR agonist</th>
<th>Findings</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary culture of peritoneal macrophages from WT, TLR2^−/−, or TLR4^−/− mice</td>
<td>S. aureus-derived LTA or PGN</td>
<td>LTA activates TLR4, PGN activates TLR2</td>
<td>[73]</td>
</tr>
<tr>
<td>Experimental brain abscess in WT, TLR2^−/−, or TLR4^−/− mice</td>
<td>Live S. aureus</td>
<td>Increased mortality in both TLR2^−/− and TLR4^−/− mice as compared to WT</td>
<td>[74]</td>
</tr>
<tr>
<td>Pretreatment with S. aureus-derived PGN before IV injection of live S. aureus in WT, TLR2^−/−, and C3H/HeJ mice</td>
<td>S. aureus-derived PGN and live S. aureus</td>
<td>Both TLR2^−/− and C3H/HeJ mice show decreased sepsis mortality after PGN pretreatment</td>
<td>[75]</td>
</tr>
<tr>
<td>In vivo intranasal LTA administered to WT, TLR2^−/−, or TLR4^−/− mice, and in vitro LTA given to TLR2- or TLR4-transfected HEK cells</td>
<td>S. aureus-derived LTA</td>
<td>In vivo, both TLR2^−/− and TLR4^−/− mice show decreased cytokine production; in vitro, only HEK-TLR2 cells respond to LTA</td>
<td>[76]</td>
</tr>
<tr>
<td>Primary culture of bone marrow-derived dendritic cells from C3H/HeN or C3H/HeJ mice</td>
<td>S. aureus-derived leukocidin F</td>
<td>C3H/HeJ BM-DCs fail to up-regulate cytokine production in response to Leukocidin F</td>
<td>[77]</td>
</tr>
<tr>
<td>In vivo intranasal PLN administered to WT, TLR2^−/−, or TLR4^−/− mice, and in vitro PLN given to TLR2- or TLR4-transfected HEK cells</td>
<td>Streptococcus pneumoniae-derived pneumolysin (PLN)</td>
<td>In vivo, both TLR2^−/− and TLR4^−/− mice show decreased cytokine production; in vitro, only HEK-TLR4 cells respond to PLN</td>
<td>[78]</td>
</tr>
<tr>
<td>WT (HeNC2) and TLR4 mutant (GG2EE, C3H/HeJ-derived) macrophage cell lines treated with or without TLR2-inhibitory mAb</td>
<td>Streptococcus pyogenes-derived LTA or LTA plus hemoglobin (Hb)</td>
<td>Both TLR2 and TLR4 are required for the response to LTA + Hb</td>
<td>[79]</td>
</tr>
</tbody>
</table>
In order to evaluate the joint effect of TLR2/TLR4 deficiency, TLR2\(^{-/-}\) X TLR4\(^{-/-}\) mice were bred and exposed to the *S. aureus* sepsis model at 10\(^6\) cfu. These mice were unable to tolerate the conditions of the model and died within two hours post-operatively in independent trials. Thus, while single deficiencies of TLR signaling are partly compensable, there is too much dysregulation in the TLR2/4 double-knockout mice to overcome the stress of this *in vivo S. aureus* sepsis model. Unfortunately, we did not have enough of these mice to perform sham surgeries or sterile clot implantations on them, so the cause of death is unknown. Still, this is indirect evidence for the idea that TLR2 and TLR4 may have broader ranges of function than specific activation by cell wall-specific bacterial components.

![Figure 5: mtDNA Copy Numbers. Absolute mtDNA content in WT and TLR2\(^{-/-}\) was measured by Q-PCR of Cyt b in comparison to known standard. TLR2\(^{-/-}\) mtDNA](image-url)

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content is lower than WT mtDNA content in S. aureus sepsis at each time point (n=4 at each time point; #, \( P=0.1; \) *, \( P<0.01 \)). Error bars show SD.

After establishing a bacterial dose, the degree of mitochondrial damage and recovery in each strain was examined by measuring the mtDNA copy number in HC mice and at 24, 48, and 72 hours PI (see Figure 5). It was found that, compared to WT mice, TLR2\(^{-/-}\) mice had lower copy numbers at 48h PI, but only trended towards a decrease at 24h PI and 72h PI (24h: \( P=0.1; \) 48h: \( P<0.01; \) 72h: \( P=0.1 \)). This indicates that the TLR2\(^{-/-}\) mice either sustained more mitochondrial damage, or had impaired biogenesis, or both. However, since mitochondrial damage due to oxidative stress is often linked to NF-\( \kappa B \) activation, and since the TLR2\(^{-/-}\) show moderately decreased NF-\( \kappa B \) activation (see Section 2.2 below), impaired mitochondrial recovery due to a deficiency in the mitochondrial biogenic response is the more likely possibility.

### 3.2 Results: Gene expression of mitochondrial biogenesis proteins in WT, TLR2\(^{-/-}\), and TLR4\(^{-/-}\) mice

In order to assay the program for mitochondrial biogenesis in response to S. aureus sepsis, the expression levels of selected marker genes were tested by quantitative real-time PCR (Q-PCR) in WT, TLR2\(^{-/-}\), and TLR4\(^{-/-}\) mice at 6h, 24h, 48h, and 72h PI, and compared to healthy controls (HCs) from the same strain (except for TLR4\(^{-/-}\) mice at 72H PI, because there was 100% mortality by that timepoint). The expression of three transcription factors, nuclear respiratory factor 1 (NRF-1), nuclear respiratory factor 2 (NRF-2), and mitochondrial transcription factor A (Tfam) were assayed first (see Figures...
6, 7, 8). No significant differences were found among strains at the same time points. TLR2−/− mice non-significantly trended towards reaching peak expression of all three genes at 48h PI (the lack of significance is due to large variability at 48h PI in those mice). WT and TLR4−/− mice showed peak expression of these genes at 24h PI. There were also small but significant increases in Tfam mRNA levels at 6h PI in both TLR2−/− and TLR4−/− mice, suggesting that early immune signals may play a role in regulating this gene. Interestingly, the patterns of expression of each of these three genes (NRF-1, NRF-2, Tfam) were qualitatively similar at later time points, suggesting the possibility of similar transcriptional control mechanisms during sepsis. In addition, since NRF-1 and NRF-2 coordinate the expression of several genes of mtDNA promotion and transcription, the delay in the upregulation of these genes may contribute to the impaired mtDNA copy number recovery in TLR2−/− mice.
Figure 6: NRF-1 mRNA levels in *S. aureus* sepsis. The mRNA levels of NRF-1 were measured in WT, TLR2−/−, and TLR4−/− mice in healthy controls (HC) and at 6h, 24h, 48h, and 72h PI. n≥3 at each time point for each strain; #, P<0.1, *, P<0.05, compared to HC of the same strain. Error bars show SD. TLR2−/− mice show a trend towards a peak induction at 48h, whereas WT and TLR4−/− mice show peak induction at 24h.
Figure 7: NRF-2 mRNA levels in *S. aureus* sepsis. The mRNA levels of NRF-2 were measured in WT, TLR2⁻/⁻, and TLR4⁻/⁻ mice in healthy controls (HC) and at 6h, 24h, 48h, and 72h PI. \( n \geq 3 \) at each time point for each strain; \( \#; P<0.1, ^*; P<0.05, ^**; P<0.01 \) compared to HC of the same strain. Error bars show SD. TLR2⁻/⁻ mice show a trend towards a peak induction at 48h, whereas TLR4⁻/⁻ mice show peak induction at 24h. WT mice do not show significant changes at any time point.
Figure 8: Tfam mRNA levels in *S. aureus* sepsis. The mRNA levels of Tfam were measured in WT, TLR2\(^{-/-}\), and TLR4\(^{-/-}\) mice in healthy controls (HC) and at 6h, 24h, 48h, and 72h PI. \(n\geq 3\) at each time point for each strain; *, \(P<0.05\), compared to HC of the same strain. Error bars show SD. Error bars show SD. TLR2\(^{-/-}\) mice show a trend towards a peak induction at 48h, whereas WT and TLR4\(^{-/-}\) mice show peak induction at 24h. TLR2\(^{-/-}\) and TLR4\(^{-/-}\) mice also show significant induction at 6h.

Next, the expression levels of mitochondrial respiratory and antioxidant genes that are downstream of the above three transcription factors were tested with Q-PCR (Figures 9, 10, 11). Cytochrome *b* (cyt *b*) is a member of electron transport chain Complex III encoded by mtDNA and transcribed by DNA polymerase-\(\gamma\) under Tfam regulation. Superoxide dismutase 2 (SOD2) reduces superoxide radicals to hydrogen peroxide, and thioredoxin reductase 2 (TXNRD2) reduces thioredoxins, which are
important for keeping peroxidases active. These are two mitochondrial antioxidant genes that are encoded in the nucleus and up-regulated in response to mitochondrial biogenesis [85,86,87]. For all three genes, WT mice showed peak expression at 24h PI (Cyt b: 2.6-fold vs. HC, \( P=0.05 \); SOD2: 8.16-fold vs. HC, \( P<0.05 \); TXNRD2: 1.67-fold vs. HC, \( P<0.05 \)). Cyt b expression showed no significant changes in TLR2\(^{-/-}\) or TLR4\(^{-/-}\) mice during \textit{S. aureus} sepsis. SOD2 expression peaked at 48h PI in TLR2\(^{-/-}\) mice (5.27-fold vs. HC, \( P<0.05 \)), while TLR4\(^{-/-}\) mice showed no significant changes in SOD2 expression. Expression of TXNRD2 was down-regulated in TLR2\(^{-/-}\) mice at 24h PI (0.22-fold vs. HC, \( P<0.01 \)), while TLR4\(^{-/-}\) mice showed down-regulation to the same level at 48h PI (.22-fold vs. HC, \( P<0.05 \)). Since Cyt b is a downstream target of Tfam [88], and SOD2 and TXNRD2 are up-regulated in mitochondrial biogenesis [85,86], trends towards changes in peak induction of the two NRF transcription factors may be responsible for the inhibited up-regulation of the genes for mitochondrial proteins. However, the fact that the two stages of mitochondrial biogenesis (i.e., up-regulation of transcription factors and then up-regulation of mitochondrial proteins) are not coordinately dys-regulated (the transcription factors trend towards a delay to up-regulation, while the mitochondrial proteins show decreases or failures to increase in TLR-deficient mice) indicate that there may be other signals that are affected by TLR deficiency that normally contribute to mitochondrial protein transcription.
Figure 9: Cyt b mRNA levels in *S. aureus* sepsis. The mRNA levels of Cyt b were measured in WT, TLR2<sup>−/−</sup>, and TLR4<sup>−/−</sup> mice in healthy controls (HC) and at 6h, 24h, 48h, and 72h PI. n≥3 at each time point for each strain; #, *P*=0.05, compared to HC of the same strain. Error bars show SD. Only WT mice show an induction of Cyt b in response to *S. aureus*. 
Figure 10: SOD2 mRNA levels in *S. aureus* sepsis. The mRNA levels of SOD2 were measured in WT, TLR2−/−, and TLR4−/− mice in healthy controls (HC) and at 6h, 24h, 48h, and 72h PI. n≥3 at each time point for each strain; *, P<0.05, compared to HC of the same strain. Error bars show SD. WT mice show a peak induction at 24h, whereas TLR2−/− mice show a peak induction at 48h. TLR4−/− mice do not show an induction of SOD2.
Figure 11: TXNRD2 mRNA levels in *S. aureus* sepsis. The mRNA levels of TXNRD2 were measured in WT, TLR2\(^{-/-}\), and TLR4\(^{-/-}\) mice in healthy controls (HC) and at 6h, 24h, 48h, and 72h PI. \(n \geq 3\) at each time point for each strain; *, \(P<0.05\), **, \(P<0.01\) compared to HC of the same strain. Error bars show SD. WT mice show an induction of TXNRD2 at 24h, whereas TLR2\(^{-/-}\), and TLR4\(^{-/-}\) mice show a decrease at 24h and 48h, respectively.

Q-PCR was also used to examine gene expression levels of the PGC family members in WT, TLR2\(^{-/-}\), and TLR4\(^{-/-}\) mice in response to *S. aureus* sepsis (Figures 12, 13, 14). PGC-1\(\alpha\), PGC-1\(\beta\), and PRC are transcriptional co-activators of NRF-1 and NRF-2 [47,49,51]. In addition, PGC-1\(\alpha\) and PGC-1\(\beta\) are independently capable of up-regulating mitochondrial biogenesis when they are overexpressed [52]. In this model, PRC was increased to a similar degree in all three mouse strains at 24 hours PI (WT: 4.4-
fold vs. HC, $P<0.05$; TLR2$^{-/-}$: 5.2-fold vs. HC, $P<0.05$; TLR4$^{-/-}$: 5.4-fold vs. HC, $P<0.05$). PGC-1$\alpha$ and PGC-1$\beta$, however, were both maximally induced in WT mice at 6 hours PI (PGC-1$\alpha$: 6.4-fold vs. HC, $P<0.01$; PGC1-$\beta$: 4.8-fold vs. HC, $P<0.05$), but failed to be induced in TLR2$^{-/-}$ mice at 6 hours PI (PGC-1$\alpha$: 1.3-fold vs. HC, $p>0.05$; PGC1-$\beta$: 1.2-fold vs. HC, $p>0.05$), and were over-expressed in TLR4$^{-/-}$ mice at 6 hours PI (PGC-1$\alpha$: 16.7-fold vs. HC, $P=0.001$; PGC1-$\beta$: 10.0-fold vs. HC, $P<0.001$).

Comparisons of PGC-1$\alpha$ and PGC-1$\beta$ mRNA between the three strains at 6h showed that each strain behaved significantly differently from the others (PGC-1$\alpha$: WT vs. TLR2$^{-/-}$, $P=0.01$; WT v. TLR4$^{-/-}$, $P=0.01$; PGC-1$\beta$: WT vs. TLR2$^{-/-}$, $P<0.05$; WT vs. TLR4$^{-/-}$, $P<0.01$). This finding shows that the PGC family members are differentially regulated, as PGC-1$\alpha$ and PGC-1$\beta$ have similar TLR-dependent responses to $S. aureus$-induced inflammation. PRC mRNA levels, however, are not affected by disruptions of TLR2 or TLR4 signaling, and show similar increases at 24h PI in all three mouse strains in response to $S. aureus$ sepsis.
Figure 12: PGC-1α mRNA levels in *S. aureus* sepsis. The mRNA levels of PGC-1α were measured in WT, TLR2−/−, and TLR4−/− mice in healthy controls (HC) and at 6h, 24h, 48h, and 72h PI. n≥3 at each time point for each strain; *, P<0.05, **, P<0.01 compared to HC of the same strain; #, P<0.05, compared to 6h time point of the other two strains (see further descriptive statistics in Section 1.5). Error bars show SD. Both WT and TLR4−/− mice and show an increase in PGC-1α at 6h PI, but TLR2−/− mice do not.
Figure 13: PGC-1β mRNA levels in *S. aureus* sepsis. The mRNA levels of PGC-1β were measured in WT, TLR2−/−, and TLR4−/− mice in healthy controls (HC) and at 6h and 24hPI. n≥3 at each time point for each strain; *, P<0.05, **, P<0.01 compared to HC of the same strain; #, P<0.05, compared to 6h time point of the other two strains (see further descriptive statistics in Section 1.5). Error bars show SD. Both WT and TLR4−/− mice and show an increase in PGC-1β at 6h PI, but TLR2−/− mice do not.
Figure 14: PRC mRNA levels in *S. aureus* sepsis. The mRNA levels of PRC were measured in WT, TLR2\(^{-/-}\), and TLR4\(^{-/-}\) mice in healthy controls (HC) and at 6h and 24h PI. \(n \geq 3\) at each time point for each strain; *, \(P < 0.05\), **, \(P < 0.01\) compared to HC of the same strain; #, \(P < 0.05\), compared to 6h time point of the other two strains (see further descriptive statistics in Section 1.5). Error bars show SD. All three strains show a significant induction of PRC at 24h PI.

To ensure that the differential regulation of PGC-1\(\alpha\) and PGC-1\(\beta\) by TLR2\(^{-/-}\) and TLR4\(^{-/-}\) were, in fact, due to the *S. aureus* and not a secondary effect, a series of model controls were run using PGC-1\(\alpha\) mRNA levels as the outcome. First, AML12 hepatocytes were exposed to heat-killed *S. aureus* (HKSA) to see if the cells responded with an increase in PGC-1\(\alpha\). The means of three time-course experiments showed significant increases in PGC-1\(\alpha\) mRNA levels at 1, 2, and 3 hours post-exposure to HKSA (Figure 40).
AML12 cells were also shown to express TLR2 by endpoint PCR. To control for the fibrin breakdown products that are present in the fibrin clot model, AML12 cells were also exposed to purified human fibrin D-dimers at two different concentrations (1 μg/ml and 10μg/ml), and no significant change in the level of PGC-1α was detected (1 μg/ml RQ: 0.94 ±0.34; 10μg/ml RQ: 0.63±0.09).

**Figure 15:** AML12 cells were exposed to heat-killed *S. aureus* (HKSA) and PGC-1α mRNA was measured by Q-PCR. N=3 independent experiments. *, P<0.05 compared to control. Error bars show SD. AML12 cells show significant induction of PGC-1α mRNA at 1h-3h after exposure to HKSA.

To control for the different components of the animal model, mice were sacrificed at 6 hours after anesthesia only, after anesthesia plus sham surgery, and after
implanting a sterile fibrin clot (prepared in the same manner as the *S. aureus* clots, but with no bacteria added). Previous work done in our lab showed that the mice implanted with sterile clots show no mortality at 7 days PI, and have much less disruption of baseline metabolic function [45]. In Figure 16, the expression level of PGC-1α at 6 hours post-challenge (compared with HCs of the same strain) is shown for WT, TLR2−/−, and TLR4−/− mice. WT mice show an increase in PGC-1α levels in response to anesthesia alone and anesthesia plus a sham surgery, but the same effect is observed in TLR2−/− mice. In other words, anesthesia, surgery and lack of oral feeding all have an effect on PGC-1α levels, but this effect is not responsible for the observed differences in PGC-1α expression in WT and TLR-deficient mice exposed to *S. aureus* sepsis, since it is the same in WT and TLR2−/− mice. WT mice exposed to a sterile clot showed only modestly increased PGC-1α, indicating that the addition of the clot itself either represses the PGC-1α gene by a new mechanism or causes a decrease in the signal activated by anesthesia. Both TLR2−/− and TLR4−/− mice also show an intermediate state of PGC-1α induction in response to implantation of a sterile clot. The addition of *S. aureus* (10⁶ cfu) to the clot produces a trend towards increases in PGC-1α in WT mice (6.7-fold vs. 3.5 fold, *P*=0.1), decreases PGC-1α expression in TLR2−/− mice (1.3-fold vs. 3.7 fold, *P*<0.05) and increases PGC-1α expression in TLR4−/− mice (16.7-fold vs. 5.7 fold, *P*<0.01). Thus, taking both the cell study findings that exposure to HKSA is sufficient to increase PGC-1α, and the model-control studies showing that *S. aureus* is responsible for modulation of PGC-1α expression, the TLR-specific PGC-1α responses were shown to result from *S. aureus* exposure both *in vitro* and *in vivo.*
PGC-1alpha RQ, Q-PCR, Model Control Mice

Fold Induction compared to HC

Figure 16: PGC-1α mRNA levels in different model control mice at 6h after intervention. #, P=0.1; *, P<0.05; **, P<0.01. Error bars show SD. Although anesthesia and sham surgery induce PGC-1α in WT mice, sham surgery also induces PGC-1α in TLR2−/− mice, indicating this effect is not responsible for the observed differences in S. aureus sepsis. The addition of S. aureus to a sterile clot causes a trend towards increased PGC-1α in WT mice and significant changes in PGC-1α in TLR2−/− and TLR4−/− mice.

The similar patterns of gene regulation of PGC-1α and PGC-1β with respect to the genetic TLR2/4 status allowed us to limit our search for the causative factors to those that could interact with both PGC-1α and PGC-1β simultaneously. To that end, the proximal promoters of each of the PGC family members were mapped and compared. To further narrow the search to those factors that are likely to be functional, the human and mouse promoters were aligned (since conserved sites are more likely to be functional) and then analyzed for transcription factor binding sites using the web-based program Rvista (see Materials and Methods). The 2000 bp proximal to the transcription start sites of the three
genes were analyzed. (A graph of the subset of transcription factor binding sites in the proximal 500 bp is shown in Appendix A.) While there were binding sites for several genes thought to be activated in early inflammation (e.g. ATF/CREB in PGC-1α and AP2/AP4 in PGC-1β), very little overlap was found between the PGC-1α and PGC-1β promoters. Of note, the proximal promoter of PRC is very poorly conserved between mouse and human. The proximal sequences in intron 1 for all three genes were next analyzed in the same manner (See Appendix B). It was found that the PRC intron 1 has multiple binding sites for c-Myc, Max, and USF, while the PGC-1β intron 1 has only one such site, and the PGC-1α intron 1 has none. Since the promoters of PGC-1α and PGC-1β showed no qualitative similarities, we proceeded deductively downstream from TLR signaling rather than backwards from a common transcription factor.

3.3 Background: the TLRs and MAL, MyD88, and NF-κB

In order to determine how TLR signaling affects the program of mitochondrial biogenesis, the downstream effectors that the TLRs activate were examined. There are four known TIR-containing signal mediators that interact with the TLRs: Myeloid Differentiation Primary Response Gene 88 (MyD88), MyD88-Adapter-Like protein (MAL, also known as TIRAP), TIR-domain-containing adapter-inducing interferon-β (TRIF, also known as TICAM-1), and TRIF-related adapter molecule (TRAM, also known as TICAM-2). These adapter molecules either heterodimerize (MAL with MyD88, and TRAM with TRIF) or bind TLRs individually [89]. Each TLR is able to activate specific pro-inflammatory signaling pathways through these adapters. All known
TLRs (except TLR3) signal through MyD88, which, in turn, activates downstream signaling through death domain interactions with the interleukin-1 receptor-associated kinases (IRAK) family of proteins [90,91]. IRAK1 or IRAK4 can be phosphorylated by MyD88; once phosphorylated, these proteins induce TRAF6 auto-ubiquitination in a manner not fully understood [92]. When TRAF6 auto-ubiquitinates, a complex is assembled that causes activation of TAK1 and, consequently, phosphorylation of the IkB kinase (IKK) [93,94]. IKKs phosphorylate IkB proteins (cytosolic chaperones of NF-κB), which are then degraded, allowing NF-κB nuclear translocation [95]. Each TLR activates NF-κB in this manner.

In addition, the TAK1 complex is also a MAPKKK (mitogen-activated protein kinase kinase kinase) [96], so that TLR signaling also activates MAP kinases (p38, JNK/SAPK, and ERK1/2) [97]. The MAPKs, in turn, activate transcription factors such as the AP-1 family. Also, a separate pathway of TRAF-dependent activation of IRF-3 and IRF-7 has been shown in response to activation of TLRs 3, 4, 7, 8, and 9 (discussed in section 2.3 below) [98]. Thus, TLR signaling can simultaneously activate multiple inflammatory pathways at once, depending on which TLRs are activated, and by which ligands. Figure 17 shows a summary diagram of the NF-κB and MAPK signaling pathways.
Figure 17: A schematic of MyD88-dependent TLR signaling to NF-κB and the MAPKs
3.4 Results: MAL, MyD88, NF-κB and PGC-1α/PGC-1β up-regulation

The finding that PGC-1α and PGC-1β are regulated differentially by TLR activation required a more detailed investigation of the activation state of NF-κB in the TLR-deficient mice and its effect on the two genes. NF-κB can be activated by both TLR2 and TLR4 in a MyD88-dependent manner. NF-κB is comprised of a family of five transcription factors: RelA (p65), RelB, c-rel, p50, and p52. The family members can either homodimerize or heterodimerize, but do not bind DNA as monomers [99]. Dimer pairs are held in the cytosol by different inhibitors of NF-κB (IkBs). When IkB is phosphorylated, it is ubiquitinated and degraded, allowing for nuclear translocation of the NF-κB dimer [100]. In the canonical or classical NF-κB pathway, the IkBs are phosphorylated by the IkB kinases (IKKs), which are themselves activated downstream of TRAF6/TAK1 signaling in the TLR pathway (see Figure 17) [93,101]. In addition to simple nuclear translocation, some NF-κB family members (especially p65) can be phosphorylated directly to modulate their activation state. Thus, appropriate assays for NF-κB activation can include phosphorylation state assays, nuclear translocation assays, and assays for gene expression of known targets of NF-κB.

Nuclei were isolated from WT, TLR2−/−, and TLR4−/− mice from HCs and from mice at 6h and 24h PI, and then assayed by immunoblot for p65, p50, and cRel (Figure 18). The levels of nuclear p65 were not significantly increased in WT mice at 6h PI, and were variably induced in TLR2−/− and TLR4−/− HCs (the variable pattern seen was identical in two separate experiments). The immunoblots also show a lack of nuclear NF-κB in the HC TLR-deficient mice. p50 was not differentially regulated among the three different
mouse strains (and its nuclear translocation is not increased at 6h PI in any of them).

Nuclear levels of cRel were increased to the greatest degree in TLR4−/− compared with TLR2−/− or WT mice. Immunoblots on whole-cell extracts from the same mice were also probed for phospho-ser276-p65. It was found that p65 is phosphorylated at 6h in WT and TLR4−/− mice, but not in TLR2−/− mice. Overall, this shows both that the pattern of p65 phosphorylation does not match the pattern of p65 nuclear translocation, and that NF-κB activation is highly variable in the TLR-deficient mice. In addition, none of the patterns of NF-κB induction (as shown by immunoblot) match the TLR-dependent pattern of PGC-1α and PGC-1β activation.

Figure 18: Immunoblots for nuclear p65, p50, and c-rel, and whole-cell phospho-p65. Immunoblots are shown for NF-κB family members in nuclear extracts and in whole-cell extracts from WT, TLR2+/−, and TLR4−/− mice at 6h PI and in HCs. The pattern of p-p65 does not match the pattern of nuclear p65, indicating some dysregulation of p65 induction. Furthermore, neither p50 nor c-rel nuclear levels correlate with PGC induction.
The levels of several NF-κB-mediated cytokines were next examined by Q-PCR in mice at 6h and 24h PI. TNFα, IL-6, and IL-10 are all activated by NF-κB, so their mRNA levels act as approximate signals of NF-κB activation. Each cytokine was measured in WT, TLR2−/−, and TLR4−/− mice (See Figures 19, 20, 21). At 6h PI, the levels in WT mice were increased as compared to HC (TNFα: 8.0-fold vs. HC, \( P<0.01 \); IL-6: 250-fold vs. HC, \( P=0.1 \); IL-10: 231-fold vs. HC, \( P=0.08 \)), but in TLR2−/− mice at 6h compared to HC they were even higher than WT (TNFα: 27.5-fold vs. HC, \( P<0.01 \); IL-6: 193-fold vs. HC, \( P=0.1 \); IL-10: 188-fold vs. HC, \( P<0.05 \)), while in TLR4−/− mice at 6h compared to HC they were lower than WT (TNFα: 0.69-fold vs. HC, \( P=\text{NS} \); IL-6: 47-fold vs. HC, \( P<0.05 \); IL-10: 127-fold vs. HC, \( P<0.001 \)). This pattern of activation in response to \( S. \text{aureus} \) is perhaps opposite of what would be anticipated from the literature, because TLR2 is primarily activated by Gram-positive bacteria. Hence, the absence of TLR2 \textit{a priori} would be expected to cause less inflammation than in WT mice. However, several previous publications have shown that TLR2−/− mice have increased bacterial loads in \( S. \text{aureus} \) sepsis as compared to WT mice (in either intraperitoneal or intravenous bacterial injection models) [75,80], so it is therefore likely that TLR2−/− in our model would show differing bacterial counts. These differing bacterial loads could account for the differing levels of cytokine production. Also, WT mice show a greater up-regulation of TLR2 at 6h PI than TLR4−/− mice, which thus will have less ability to respond to Gram-positive cell wall components (WT: 1.98±0.17-fold vs. HC at 6h PI, \( P<0.01 \); TLR4−/−: 1.30±0.19-fold vs. HC at 6h PI, \( P<0.05 \); densitometry of endpoint PCR, 65ºC, 37 cycles). In any case, the pattern of cytokine mRNA synthesis
does not match the pattern of PGC-1α/β mRNA synthesis, making it difficult to propose that NF-κB is involved in PGC-1α/β gene activation. Although there was no further inquiry into the causes of the differences in cytokine production between the different TLR-deficient mice (because it does not appear to affect PGC-1α/β gene activation), it remains an interesting immunological question and an avenue for future research.

Figure 19: TNFα mRNA levels in *S. aureus* sepsis. The mRNA levels of TNFα were measured in WT, TLR2−/−, and TLR4−/− mice in healthy controls (HC) and at 6h and 24h PI. n≥3 at each time point for each strain; *, P<0.05, compared to HC of the same strain. Error bars show SD. WT and TLR2−/− mice show significant inductions of TNFα at 6h PI, whereas TLR4−/− mice do not.
Figure 20: IL-6 mRNA levels in *S. aureus* sepsis. The mRNA levels of IL-6 were measured in WT, TLR2−/−, and TLR4−/− mice in healthy controls (HC) and at 6h and 24h PI. n≥3 at each time point for each strain; error bars show SD; *, P<0.05, compared to HC of the same strain. WT and TLR2−/− mice trend towards large inductions of IL-6 at 6h PI, whereas TLR4−/− mice show less variability and hence a significant induction at 6h PI.
Figure 21: IL-10 mRNA levels in *S. aureus* sepsis. The mRNA levels of IL-10 were measured in WT, TLR2\(^{-/-}\), and TLR4\(^{-/-}\) mice in healthy controls (HC) and at 6h and 24h PI. \(n \geq 3\) at each time point for each strain; *, \(P<0.05\), **, \(P<0.01\) compared to HC of the same strain. Error bars show SD. WT and TLR4\(^{-/-}\) mice show large inductions of IL-10 at 6h PI, whereas TLR2\(^{-/-}\) mice show greater variability and hence only a trend towards induction at 6h PI.

Differences in NF-κB activation states were observed in the different mouse strains, and so the promoters of PGC-1α and PGC-1β were examined for NF-κB binding sites. The p65 activation state most closely matched the levels of PGC-1α mRNA induction in the three different mouse strains, and so it was subject to further investigation. The consensus sequence for p65 binding is roughly 5’-GGGRNTTCC-3’
so the PGC-1α promoter was examined for that sequence. We also used Rvista to map any other kB binding sites. In considering how far back in the promoter to look, the diminishing contributions of distant regulatory elements were weighed against data showing that even far distant kB binding sites (up to 30kb from the TSS) can be relevant to gene expression levels [102]. The promoter of murine PGC-1α was thus examined 20kb back from the TSS, and several putative kB binding sites were identified. Several chromatin immunoprecipitations were performed on WT and TLR2−/− mice using a ChIP-verified p65 antibody. Primers were made for regions that had highly specific kB binding sites, with total amplicon lengths always less than 200 bp (which was well within the fragment size range of the sheared DNA). None of the putative binding sites were ever amplified by PCR after the ChIP. Positive controls with RNA polymerase II antibodies were run on both the Ef1α and GAPDH genes as well as with TNFα primers with the p65 pulldown (data not shown; see ChIP below for controls). Negative control IgG pulldowns were used to show that the ChIP setup worked properly.

The ChIP experiments for p65 binding were negative, but this cannot eliminate the possibility of p65 regulating PGC-1α and PGC-1β, since the TLR2 signaling paradigm posits that the main downstream effector is NF-κB. Two different mouse models were thus employed to examine the effects of deficient NF-κB signaling on PGC-1α levels during S. aureus sepsis. First, mice were exposed to an inhibitor of IkB-α phosphorylation, BAY-11-7082 [103] at a dose of 20 mg/kg (based on previous literature reports [104,105]), and then exposed to the S. aureus sepsis model. IkB-α preferentially binds to either the p65 homodimer or the p50-p65 heterodimer [106], so the BAY-treated
mice thus had a deficiency in p50/p65 nuclear translocation. NF-κB inhibition was assayed by testing TNFα mRNA production by Q-PCR, which showed no increase in TNFα levels at 6h PI in BAY-treated mice (WT: 8.0-fold vs. HC; BAY: 1.1-fold vs. HC; WT vs. BAY, P<0.01) (See Figure 22). p50⁻/⁻ mice are commercially available (p65 genetic deficiency is embryonic lethal) and so were obtained and exposed to S. aureus sepsis. It was found that neither BAY treatment nor p50 deficiency had an effect on PGC-1α mRNA levels at 6h PI in S. aureus sepsis. As shown in Figure 22, the effect of p50 deficiency and BAY treatment had variable effects on TNFα mRNA levels. Curiously, TNFα was non-significantly greater at 6h PI in p50⁻/⁻ mice than in WT mice; this may indicate again that p50 does not play a solely excitatory role in TNFα induction. This is further proof that cytokine production is regulated separately from PGC-1α. Thus, together with the negative ChIP experiments, it was concluded that in WT, TLR2⁻/⁻, and TLR4⁻/⁻ mice, NF-κB activation is different, but that this does not contribute to changes in PGC-1α or PGC-1β expression.
Figure 22: PGC-1α and TNFα mRNA levels in *S. aureus* sepsis. The mRNA levels of PGC-1α and TNFα at 6h PI (as compared to HCs) were measured in WT, p50⁻/⁻, and BAY-11-7082-treated mice. n=3 for each strain; *, P<0.01 compared to WT 6hPI TNFα levels. Error bars show SD. PGC-1α was not different in any of the three mouse models (WT, p50⁻/⁻, and BAY-11-7082-treated) though TNFα was variable, indicating that PGC-1α and TNFα are regulated differently.

To further confirm that NF-κB does not influence PGC-1α/β induction, MyD88⁻/⁻ and MAL⁻/⁻ mice were obtained and exposed to *S. aureus* sepsis. Every TLR except TLR3 signals through MyD88, and some TLRs (including TLR2) bind MAL as a bridging adaptor to MyD88 [107]. In the literature, MyD88⁻/⁻ mice show very low or no activation of NF-κB in response to *S. aureus* [80,108,109], because the absence of the adaptor protein prevents TLR signaling to NF-κB. When MyD88⁻/⁻ and MAL⁻/⁻ mice were exposed to *S. aureus* sepsis, it was found that the levels of PGC-1α and PGC-1β at 6h PI
(as compared to HCs) were the same as in WT mice (MyD88\(^{-/-}\): PGC-1\(\alpha\): 8.5-fold vs. HC, \(P<0.05\); PGC1-\(\beta\): 5.5-fold vs. HC, \(P<0.01\)) (MAL: PGC-1\(\alpha\): 7.6-fold vs. HC, \(P<0.01\); PGC1-\(\beta\): 4.5-fold vs. HC, \(P<0.01\)) (inter-strain differences at 6h PI were all non-significant, \(P>0.5\)) (See Figures 23, 24). Neither strain of mice was able to up-regulate TNF\(\alpha\) at 6h PI to the same degree as WT, indicating a lack of NF-\(\kappa\)B activation (See Figure 25). Thus PGC-1\(\alpha\) and PGC-1\(\beta\) are up-regulated in a MyD88-independent, MAL-independent manner, contributing the final piece of evidence that the two genes are not under transcriptional control by NF-\(\kappa\)B in \textit{S. aureus} sepsis.

**Figure 23:** PGC-1\(\alpha\) mRNA levels in \textit{S. aureus} sepsis. The mRNA levels of PGC-1\(\alpha\) were measured in WT, MyD88\(^{-/-}\), and MAL\(^{-/-}\) mice in healthy controls (HC) and at 6h and 24h PI. \(n\geq3\) at each time point for each strain; *, \(P<0.05\), compared to HC of the same strain. Error bars show SD. PGC-1\(\alpha\) is up-regulated in MyD88\(^{-/-}\), and MAL\(^{-/-}\) mice to the same extent as in WT mice.
Figure 24: PGC-1β mRNA levels in *S. aureus* sepsis. The mRNA levels of PGC-1β were measured in WT, MyD88−/−, and MAL−/− mice in healthy controls (HC) and at 6h and 24h PI. \( n \geq 3 \) at each time point for each strain; *, \( P < 0.05 \), **, \( P < 0.01 \), compared to HC of the same strain. Error bars show SD. PGC-1β is up-regulated in MyD88−/−, and MAL−/− mice to the same extent as in WT mice.
Figure 25: TNFα mRNA levels in S. aureus sepsis. The mRNA levels of TNFα at 6h PI (as compared to HCs) were measured in WT, MyD88−/−, and MAL−/− mice. n≥3 for each strain; *, P<0.05 compared to WT 6h PI TNFα levels. Error bars show SD. TNFα is significantly less induced in MyD88−/−, and MAL−/− mice than in WT mice.

3.5 Background: TRAM, TRIF, and IRF-7

The downstream signals activated by TLR signaling vary according to which TLR is activated. In particular, both TLR3 and TLR4 can activate a MyD88-independent pathway through the adaptors TRAM and TRIF, which can activate IRF-3 and IRF-7 [110]. More specifically, TLR4 can bind to TRAM, which causes endocytosis of the TLR4 complex and activation of TRIF [111]. TLR3, which is expressed in cytosolic vesicles, binds TRIF directly. When activated, TRIF can activate either TRAF6, which...
leads to NF-κB activation as described above, or TRAF3, which activates TBK1 [112,113]. TBK1 assembles a complex with IKKe, which leads to the phosphorylation of IRF-3 and IRF-7 [114,115]. IRF-3 and IRF-7, when phosphorylated, homodimerize and are translocated to the nucleus [116,117]. For a summary of the pathway, see Figure 26. IRF-3 and IRF-7 transactivate genes which contain an interferon-stimulated response element (ISRE) in their promoters, which is a consensus sequence of GAAANNGAAANN [118]. Of the two factors, IRF-7 is more permissive in terms of binding to imperfect consensus sequences [119].
Figure 26: A schematic of TRIF-dependent TLR signaling to IRF-3 and IRF-7.
3.6 Results: TRAM, TRIF, IRF-7 and PGC-1α/PGC-1β up-regulation

The TLR2-dependent up-regulation of PGC-1α and PGC-1β was found to be independent of MAL and MyD88, and so TRIF<sup>−/−</sup> and TRAM<sup>−/−</sup> mice were exposed to S. aureus sepsis. Our hypothesis was that since there are only four known signaling adaptors for the TLRs, and the TLR2<sup>−/−</sup> mice phenotype did not match that of the MAL<sup>−/−</sup> or the MyD88<sup>−/−</sup> mice, then perhaps TLR2 was signaling through TRAM and/or TRIF to cause PGC-1α/β induction. TRAM<sup>−/−</sup> mice and TRIF<sup>−/−</sup> mice were exposed to the model of S. aureus sepsis, and it was confirmed that they failed to up-regulate PGC-1α at 6h PI (TRAM<sup>−/−</sup>: 1.1-fold vs. HC, \( P<0.01 \) compared to WT 6h; TRIF<sup>−/−</sup>: 2.3-fold vs. HC, \( P<0.05 \) compared to WT 6h) (TRIF<sup>−/−</sup> vs. TRAM<sup>−/−</sup> mice at 6h PI, \( P>0.4 \)) (See Figure 27). Only TRAM<sup>−/−</sup> mice show no up-regulation of PGC-1β; TRIF<sup>−/−</sup> mice trend towards having less activation of PGC-1β than WT mice, but still significantly up-regulate the expression of PGC-1β at 6h PI (TRAM<sup>−/−</sup>: 0.7-fold vs. HC, \( P<0.05 \) compared to WT 6h; TRIF<sup>−/−</sup>: 2.8-fold vs. HC, \( P=NS \) compared to WT 6h, \( P<0.05 \) compared to HC) (TRIF<sup>−/−</sup> vs. TRAM<sup>−/−</sup> mice at 6h PI, \( P<0.05 \)) (See Figure 28). Thus, it appears that the TLR2 signal that leads to PGC-1α/β induction acts through TRAM and TRIF, since a deficiency in either gene leads to a decrease in PGC up-regulation.
Figure 27: PGC-1α mRNA levels in *S. aureus* sepsis. The mRNA levels of PGC-1α were measured in WT, TRAM⁻/⁻, and TRIF⁻/⁻ mice in healthy controls (HC) and at 6h and 24h PI. n≥3 at each time point for each strain; *, P<0.05 compared to HC of the same strain; #, P<0.05, ##, P<0.01 compared to WT 6h. Error bars show SD. Both TRAM⁻/⁻ and TRIF⁻/⁻ mice show significantly less PGC-1α mRNA at 6h PI than WT mice.
Figure 28: PGC-1β mRNA levels in *S. aureus* sepsis. The mRNA levels of PGC-1β were measured in WT, TRAM⁻/⁻, and TRIF⁻/⁻ mice in healthy controls (HC) and at 6h and 24h PI. n≥3 at each time point for each strain; *, P<0.05 compared to HC of the same strain; #, P<0.05 compared to WT and TRIF⁻/⁻ mice at 6h PI. Error bars show SD. TRAM⁻/⁻ show significantly less PGC-1α mRNA at 6h PI than both WT and TRIF⁻/⁻ mice. TRIF⁻/⁻ mice non-significantly trend towards showing less PGC-1α induction than WT mice at 6h PI.
Since the major transcriptional effectors of TRAM and TRIF are IRF-3 and IRF-7, the activation states of IRF-3 and IRF-7 were assayed in WT and TLR2\(^{\text{+/+}}\) mice. IRF-3 and IRF-7 are thought to be constitutively expressed in the cytoplasm and then translocate to the nucleus when activated [120,121], so RT-PCR for changes in IRF-3/7 expression was not a viable approach to assess acute changes in activation state. Also, antibodies for phospho-IRF3 and phospho-IRF7 are very non-specific (we observed that a p-IRF7 antibody from the lab of Michael Diamond at Washington University, St Louis, showed the exact same expression pattern as a non-phospho-IRF7 antibody; data not shown). Thus, nuclear translocation of the two proteins was assayed by immunoblot. Nuclear immunoblots showed little difference in the levels of nuclear IRF-3 between HC mice and 6hr PI mice in WT, and TLR2\(^{\text{+/+}}\) mice, and a small increase between HC mice and 6hr PI in TLR4\(^{\text{+/+}}\) mice (See Figure 29). However, nuclear immunoblots for IRF-7 at 6h PI showed moderate changes in IRF-7 in WT mice at 6h PI, while TLR2\(^{\text{+-}}\) animals showed markedly decreased IRF-7 levels and TLR4\(^{\text{-/-}}\) animals showed markedly increased IRF-7 levels at 6h PI. This result confirmed that TLR2\(^{\text{+-}}\) mice had deficiencies in IRF-7 translocation, potentially explaining the diminished PGC-1\(\alpha\) and PGC-1\(\beta\) mRNA levels in the TRAM\(^{\text{-/-}}\) and TRIF\(^{\text{-/-}}\) mice.
Figure 29: Immunoblots for nuclear IRF-3 and IRF-7. Immunoblots are shown for IRF-3 and IRF-7 in nuclear extracts from WT, TLR2-/-, and TLR4-/- mice at 6h and 24h PI and in HCs. Immunoblots shown are representative of three independent blots.
The promoters of PGC-1α and PGC-1β were examined for ISREs (GAAANNGAAANN). Several sites were found that were similar to the consensus sequence, and primers were made to amplify the promoter region at each site. Chromatin immunoprecipitations were performed for IRF-7, and PCR was used to amplify the various sites. The site at -220 bp (GAAAGAAAAGGAAA, negative strand) from the TSS was positive in WT 6h mice, but not in TLR2⁻/⁻ mice (see Figure 30). As with the previous p65 ChIP, the appropriate positive (RNA polymerase II and EF1α) and negative (negative IgG) controls were performed. We thus demonstrated IRF-7 binding to the promoter of PGC-1α.

Figure 30: Chromatin Immunoprecipitations. ChIP was performed for IRF7 binding on the PGC-1α promoter at -220 bp from TSS. Both WT and TLR2⁻/⁻ mice (HCs, 6h PI, and 24h PI) were tested. Arrow shows representative positive lane. Positive RNA polymerase II pulldowns on EF1α are shown as loading controls.
Next, the functional relationship between IRF-7 and PGC transcript levels was evaluated using IRF-7-deficient models. First, simple RNAi experiments were undertaken with siRNA directed against IRF-7 in AML12 cells. After transient siRNA transfections with four different siRNA mixes were done without evidence of down-regulation of IRF7 protein (assessed by immunoblot), we switched to a stable transfection model. Plasmids were obtained that expressed verified shRNA directed against IRF-7, and transfected into HepG2 cells with good efficiency. The transfected cells were then further selected using kanamycin, against which the plasmid had a resistance gene. However, this still did not down-regulate IRF-7 protein levels. This negative result was likely due to the fact that double-stranded shRNA may induce TLR3 activation (and hence increase IRF-7 levels) [122,123]. Thus, a different approach to IRF-7 deficiency was employed.

IRF-3\(^{-/-}\) X IRF-7\(^{-/-}\) mice on a C57bl/6J background (IRF-3/7 DKO) were obtained from Michael Diamond at Washington University, St Louis [124], and exposed to our model of \textit{S. aureus} sepsis. At 6h PI, IRF-3/7 DKO mice did not induce PGC-1\(\alpha\) to the same extent as WT, and trended towards a diminished response in PGC-1\(\beta\) (See Figure 31). This confirmed that the proposed path, TLR2 \(\rightarrow\) TRAM/TRIF \(\rightarrow\) IRF-3/7 \(\rightarrow\) PGC-1\(\alpha/\beta\), is active in response to \textit{S. aureus} sepsis in mice \textit{in vivo}. 
Figure 31: PGC-1α and PGC-1β mRNA levels in *S. aureus* sepsis. The mRNA levels of PGC-1α and PGC-1β were measured in WT and IRF3/7−/− mice in healthy controls (HC) and at 6h and 24h PI. n≥3 at each time point for each strain; *, P<0.05 compared to HC of the same strain; ##, P<0.05, #, P=0.08 compared to WT 6h. Error bars show SD. IRF3/7−/− mice did not up-regulate PGC-1α to the same extent as WT mice and trended towards a diminished increase in PGC-1β at 6h PI.

To further confirm this pathway, a rescue of the TLR2−/− PGC-1α/β phenotype was attempted in *S. aureus* sepsis. Since TLR3 is a known activator of IRF-3 and IRF-7, TLR3 was activated in TLR2−/− mice to see if PGC-1α would be up-regulated at 6h PI. WT and TLR2−/− mice were thus injected with a dose of 400 μg of polyinosinic-polycytidylic acid (polyI:C) [125], a dsRNA mimetic that is a known activator of TLR3 [126]. It was found that at 6h PI, mice injected with polyI:C only did not up-regulate PGC-1α/β (See Figure 32). However, in TLR2−/− mice infected with *S. aureus* and then
dosed with polyI:C, there was a significant up-regulation of PGC-1α at 6h. Thus, polyI:C (and hence IRF-3/7) is necessary but not sufficient for the induction of PGC-1α in this mouse model.

**Figure 32:** PGC-1α mRNA levels after PolyI:C treatment with or without *S. aureus* sepsis. The mRNA levels of PGC-1α were measured in WT and TLR2−/− mice in healthy controls (HC), in animals dosed with 400ug PolyI:C, and in animals given PolyI:C plus *S. aureus* sepsis at 6h and 24h PI. n=3 at each time point for each strain; *, P<0.05 compared to HC of the same strain. Error bars show SD. PolyI:C was necessary but not sufficient for the rescue induction of PGC-1α mRNA in TLR2−/− mice in response to *S. aureus* sepsis.

In sum, the previous data show a MyD88-independent, TLR-2 dependent up-regulation of PGC-1α/β gene expression in response to *S. aureus* sepsis via the pathway TLR2 → TRAM/TRIF → IRF-3/7 → PGC-1α/β. This pathway was confirmed by
infecting TRAM\textsuperscript{−/−}, TRIF\textsuperscript{−/−}, and IRF-3/7 DKO mice with \textit{S. aureus}, and measuring the PGC-1\textalpha{} and PGC-1\textbeta{} mRNA expression levels. Differential regulation of IRF-7 in WT, TLR2\textsuperscript{−/−}, and TLR4\textsuperscript{−/−} mice was demonstrated by nuclear immunoblots, and IRF-7 binding to the PGC-1\textalpha{} promoter in WT mice at 6h PI but not in TLR2\textsuperscript{−/−} mice was shown by ChIP assay. Finally, polyI:C was used to induce IRF-3/7 activation in WT and TLR2\textsuperscript{−/−} mice, and the studies revealed that IRF-3/7 activation is necessary but not sufficient to induce PGC-1\textalpha{}.

### 3.7 TLR2 and non-canonical TRAM/TRIF activation

TLR2 is activated upon binding to components of Gram-positive bacteria (such as \textit{S. aureus}), including PGN and lipotechoic acid (LTA). It is thought that TLR2 does not act via MyD88-independent pathways because early studies of the intermediates TRIF and TRAM showed little binding to TLR2 [127,128]. In addition, stimulation of murine macrophages, microglia, and dendritic cells with TLR2 ligands (such as MALP, PGN, or PamCSK) showed neither increased ISRE-binding activity, nor up-regulation of interferon-\textbeta{} (IFN-\textbeta{}), nor dimerization or nuclear translocation of IRF-3 [129,130,131,132]. However, one \textit{in vitro} study showed that TLR2 activates TRIF and IRF3/7 when exposed to viral particles [133]. In addition, there is evidence to suggest that TLR2 can heterodimerize with TLR4, leading to TLR2-dependent activation of the TLR4 pathway, which would include TRIF/TRAM signaling. One such study showed that TLR-integrin constructs expressed in 293T cells were capable of forming TLR2-TLR4 dimers [134]. Another study in HEK293 cells used protein complementation
assays to demonstrate TLR2-TLR4 cytoplasmic binding, which was further confirmed by co-immunoprecipitation [135]. Finally, studies conducted on macrophages have identified damage-associated ligands such as biglycan and hemoglobin that signal jointly through TLR2 and TLR4 [79,136]. (For an excellent review of the ligands that are known to signal jointly through TLR2 and TLR4, see Kawai and Akira, 2010 [137].) Thus there could be non-canonical pathways that lead to TLR2 dependent IRF activation through a TLR2-TLR4 heterodimer activation caused by a ligand that is present in vivo in S. aureus sepsis.

In addition to the possibility of a TLR2-TLR4 heterodimer, there is some evidence that TLR2 could interact directly with TRAM, albeit more transiently than the TLR4-TRAM interaction. When TRAM was discovered, Oshiumi et al. used yeast two-hybrid screens to identify which TLR bound to TRAM. They showed in a stringent screen that only TLR4 interacted with TRAM; however, in a less stringent screen, TLR2, TLR3, and TLR8 all showed minor associations with TRAM [127]. This association is usually considered minor, as a bait-and-prey study of TLR adaptor binding found no significant interaction between TLR2 and TRAM [107].

Immunoprecipitation for TLR2 was attempted multiple times (see Materials and Methods for details), but no combination of antibodies, lysis buffers, or membrane isolation techniques yielded a successful pulldown. This may be due to antigen glycosylation on the extracellular moieties of TLR2 or to differences in the protein in vivo. In future studies, in order to identify if a complex of TLR2 and TLR4 exists in the mouse, whole-cell extract and purified membrane fractions will be run on blue native
PAGE [138] and then separately immunoblotted for TLR2 and TLR4. If TLR2 and TLR4 do exist in a complex, the antibody to each should stain a band at the same high molecular weight, in addition to binding the monomeric form of each.

3.8 Background: TLRs and MAP kinase activation

The TLRs are known to activate the mitogen-activated protein kinases (MAPKs) through activation of the IKKs. The MAPKs most commonly studied in response to TLR activation are the p38 family, the c-Jun N-terminal kinases (JNKs) and the extracellular signal-regulated kinases (ERKs) 1 and 2 [139]. In general, the MAPKs are activated by phosphorylation by the MAPK kinases (MAPKKs), which are themselves phosphorylated by the MAPKK kinases (MAPKKKs). In TLR signaling, MAPKs can be activated through TAK1, which is downstream of TRAF6 and is a MAPKKK [96]. TAK1 can activate MKKs 1-4, 6, and 7 [97], which links TLR signaling to ERK1/2 (which has two isoforms, p42 and p44), p38, and JNK. Each MAPK, in turn, activates different proteins to effect various cellular changes. In particular, the AP-1 and ATF/CREB families of transcription factors are activated by transient activation of the MAPKs [140,141].
Figure 33: Immunoblots for activation state of the MAPKs. Immunoblots are shown for the phosphorylated and total forms of p38, JNK, ERK1/2, and MEK1/2 in whole-cell extracts from WT, TLR2-/-, and TLR4-/- mice at 6h PI and in HCs. ERK1/2 activation is increased in TLR4-/- mice and decreased in TLR2-/- mice at 6h PI. MEK1/2, its upstream kinase, shows the same pattern.

3.9 Results: TLRs and MAP kinase activation

The WT, TLR2-/-, and TLR4-/- mice showed different downstream gene activation profiles, and so we hypothesized that the MAPKs, as acute inflammatory mediators, might show differential activation states at 6h PI. However, since PGC-1α and PGC-1β do not have promoter binding sites for similar MAPK-activated transcription factors that are not also present in the PRC promoter (See Appendix A), we had little reason to suspect a priori that MAPK signaling would contribute to the TLR-dependent PGC-1α/β phenotype. WT, TLR2-/-, and TLR4-/- HC and 6h PI mice were assayed via immunoblot
for the phosphorylation states of p38 (thr180/tyr182), JNK1/2/3 (thr183/tyr185) and ERK1/2 (thr202/tyr204) (See Figure 33). The p38 MAPK was not phosphorylated in any of the strains at 6h PI. JNK was variably activated, with high inter-mouse differences in the same strains. Interestingly, the upper band of ERK1/2 (p44) was found to be phosphorylated at 6h PI in WT and TLR4<sup>−/−</sup> mice, but not in TLR2<sup>−/−</sup> mice, while the lower band (p42) was increased in TLR4<sup>−/−</sup> mice and decreased in TLR2<sup>−/−</sup> mice. This pattern held for its upstream kinase, MEK1/2. There are multiple downstream targets of ERK1/2, and this differential activation would be a fruitful area for further research into the differences in WT, TLR2<sup>−/−</sup>, and TLR4<sup>−/−</sup> responses to <i>S. aureus</i> sepsis. The AP-1 family (here assayed for c-jun, c-fos, fosb, fra2), which is thought to be downstream of the MAPKs (and JNK in particular), was shown to be variably translocated to the nucleus at 6h PI in WT, TLR2<sup>−/−</sup>, and TLR4<sup>−/−</sup> mice (See Figure 34). Fra2 showed the biggest differential activation (in the TLR4<sup>−/−</sup> mice), but was not followed up because the TLR2<sup>−/−</sup> mice and the WT mice showed no difference, meaning that Fra2 activation did not match the pattern of PGC-1α/β activation. Also, the fra2 levels in HC TLR4<sup>−/−</sup> mice were low, but baseline (HC) levels of PGC-1α/β are the same in all three genetic strains.
Overall, no differences were identified in the activation states of known relevant transcription factors between the different mouse strains that could contribute to differential PGC-1α/β regulation. However, downstream targets of ERK1/2 activation could prove to contribute to the differential inflammation seen in the WT, TLR2−/−, and TLR4−/− mice.

3.10 Background: TLR2 and PI3K

In addition to the canonical TLR pathways that activate NF-κB, AP-1, and IRF-3/7, there is a growing body of literature that the TLRs, and TLR2 in particular, activates the PI3K – Akt pathway [142]. TLR2 (together with TLR1, TLR3, and TLR6) has several tyrosine motifs (YXXM) through which it can bind the p85 subunit of PI3K when
phosphorylated [143,144]. However, many of the TLRs studied so far, including TLRs 1-6 and TLR9, can activate PI3K through either direct or indirect mechanisms [142]. This is because both MAL and MyD88 interact directly with p85, serving as the bridge to PI3K signaling [145,146]. In any event, when p85 binds a phosphorylated tyrosine in the TLR complex, it activates the catalytic subunit of PI3K. Catalytic PI3K, in turn, phosphorylates PIP2 to PIP3, resulting in membrane localization of PDK1 and phosphorylation of Akt [147]. Several studies have examined the effects of PI3K signaling in TLR2 activation. In general, PI3K activation is linked with anti-inflammatory effects such as decreased cytokine production (for an excellent review of PI3K signaling deficiencies in TLR signaling, see Hazeki et al., 2007). Akt phosphorylation leads to phosphorylation and inactivation of GSK3β, which may promote the anti-inflammatory effects of PI3K [148]. In addition, Akt activation has been linked to PGC-1α down-regulation through action of the transcription factor FOXO1 [149,150]. We thus sought to probe the activation state of the PI3K pathway in WT, TLR2−/−, and TLR4−/− mice in response to S. aureus sepsis.

3.11 Results: TLR2 and PI3K

Western blots were used to probe for phosphorylation of several proteins at different steps in the PI3K cascade (See Figure 35). First, phospho-p85α (Tyr 508 and Tyr 458) was probed, but no signal was obtained (although total p85 had a robust signal). However, since p85 has multiple possible tyrosine phosphorylation sites, a negative result may not indicate a definitive lack of tyrosine phosphorylation. Next, phospho-Akt (Ser
473 and Thr 308) was probed, and Akt activation was found to be higher in TLR2−/− mice at 6h PI than in either WT mice or TLR4−/− mice. This was contrary to our initial hypothesis, since the literature clearly indicates that TLR2 activates PI3K, whereas here a deficiency in TLR2 signaling led to increases in Akt phosphorylation. To check whether this pattern held downstream of Akt, phospho-GSK3β was probed, and again a pattern was found of high TLR2−/− phosphorylation at 6h PI. It thus seems that the PI3K/Akt pathway is more activated in TLR2−/− mice at 6h PI than in WT mice or TLR4−/− mice. The transcription factor FOXO1, which is a known downstream target of Akt, was shown to be present in the nucleus at similar levels in all three mouse strains; thus, the differences in Akt activation observed do not lead to differential FOXO1 activation. Next, phosphorylation of mTOR was tested as a secondary target downstream of insulin signaling and upstream of Akt phosphorylation. No phosphorylation of mTOR was found in either HC or 6h PI mice of any strain (data not shown). Finally, Akt1−/− mice were tested in the model of S. aureus sepsis. Akt1−/− mice were found to have near-normal levels of PGC-1α induction at 6h PI compared to WT (PGC-1α 6h PI: 8.6±5.0-fold vs. HC; Akt1−/− 6h PI vs. WT 6h PI: P>0.5). Although it is possible that there may be compensation for the lack of Akt1 signaling by the remaining Akt2/3 pathways, the lack of effect of Akt1 deficiency on PGC-1α induction indicates there is no functional connection between the two.
Figure 35: Immunoblots for members of the PI3K pathway. Immunoblots are shown for total p85, phospho-thr-308-Akt, phospho-ser-473 Akt, total Akt, phospho-GSK3β, and total GSK3β in whole-cell extracts, and for FOXO1 from nuclear extracts from WT, TLR2−/−, and TLR4−/− mice at 6h PI and in HCs.

The finding of increased Akt activation in TLR2−/− mice is contrary to expectations from reports of direct TLR2 → PI3K signaling in cells. However, since Akt can be activated by numerous signals, our findings support the possibility that other signals of Akt activation in vivo such as hormones (especially insulin signaling) likely play a larger role in Akt activation than does direct TLR signaling. Although Akt activation can decrease the transcription of PGC, this likely does not play a major role in our model. TLR4−/− mice show similar ratios of Akt activation to WT mice at 6h PI, but a significantly higher PGC-1α/β level. As mentioned above, the nuclear levels of FOXO1...
were not different between the three strains, suggesting that it is not differently activated. Finally, Akt1−/− mice show near-normal induction of PGC-1α at 6h PI. Thus, the evidence strongly suggests that Akt signaling is not involved in PGC regulation.

3.12 Background: miRNAs as mediators of mitochondrial biogenesis

Much of the research on the regulation of genes involved in mitochondrial biogenesis has focused on the various cytosolic kinases and transcription factors that activate and regulate the genes involved. However, it is likely that post-transcriptional mechanisms are also important. MicroRNAs (miRNAs) are a class of non-protein-coding genes that post-transcriptionally degrade mRNAs [151]. Several miRNAs have been demonstrated to target genes involved in metabolism (including PGC-1α), as well as mitochondrial-encoded genes [152,153,154]. In addition, a recent study identified a subset of microRNAs that are localized to the mitochondria (and one of these was mir-202, discussed below) [154]. Finally, miRNA have previously been reported to be differentially regulated in sepsis [44]. We thus hypothesized that microRNAs could be involved in the differential transcriptional regulation of PGC-1α and PGC-1β observed in our model.

The miRNAs are the products of non-protein-coding genes that are processed into mature 19-21 bp sequences in the nucleus. Transcriptional control of microRNAs is thought to be similar to that for protein-coding genes, with independent promoter elements distal to the gene recruiting RNA polymerase and initiating transcription [155]. Initially, a microRNA is transcribed into a pri-miRNA, which can have several stem-
loops, and is then modified with a 5’ cap and a poly-adenylated tail. In the nucleus, the
stem-loops on this modified pri-miRNA are cleaved to a pre-miRNA by the Drosha
complex [156]. Pre-miRNAs are then exported from the nucleus, and in the cytosol they
are further cleaved into 22-bp dimers by the Dicer protein [157]. The 22-bp dimers yield
either one or two functional miRNAs; in cases where both are active, the resultant
microRNAs are labeled 3p or 5p depending on whether they are closer to the 3’ or 5’ end
of the original gene. When a mature miRNA is in the cytosol, it can be taken into an
RNA-Induced Silencing Complex (RISC), which can then bind and degrade target
mRNAs [158]. For mRNA binding, the proximal 5’ 7-8 bp of the miRNA (bps 2-8 or 2-9,
the seed region) binds to a complimentary sequence in the 3’ UTR of a target mRNA;
complementarity at the seed region is more important than binding further down (in the
3’ region of the miRNA) [159]. Furthermore, an adenosine flanking the seed region has
been shown to increase miRNA functionality [64]. Binding of the target mRNA to the
specific miRNA results in either sequestration or degradation [160]. Each miRNA is
complementary to hundreds of mRNAs in silico and in microarray analyses [161], but
comparatively few matches have been shown to result in actual gene silencing.

Figure 36: In silico 3’UTR miRNA binding analyses of PGC-1α, PGC-1β, and PRC. The 3’UTRs of PGC-1α, PGC-1β, and PRC were analyzed in the mouse using TargetScan Mouse release 5.1. miRNAs with good seed-binding are shown. Purple, 8mer seed-region binding plus trailing A; Red, 8mer seed-region binding; Blue, 7mer seed-region binding plus trailing A.
3.13 Results: microRNAs and PGC-1α/PGC-1β mRNA stability

Since PGC-1α and PGC-1β are co-regulated, we hypothesized that a single microRNA might be responsible for their failure to up-regulate at 6h PI in TLR2−/− mice. Thus, the 3’UTRs of PGC-1α, PGC-1β, and PRC were compared using the online program TargetScan. The results are shown in Figure 36. A small set of predicted miRNA binding sequences was identified that were conserved in the PGC-1α and PGC-1β 3’UTRs, but not in the PRC 3’UTR. The conserved microRNAs included the let-7 family, the mir-30 family, and mir-202-3p (families of microRNAs are described as those which share 100% seed-binding region homology). The let-7 family comprises several members, as does the mir-30 family (see Tables 4 and 5). A single primer can be made to detect 8 of 9 members of the let-7 family, and so the expression of the let-7 family was determined with a let-7a primer. We started with let-7 because it is highly expressed in the liver and is well-characterized. No difference was detected in let-7 expression between the WT, TLR2−/−, and TLR4−/− mice at 6h PI. Next, mir-202-3p was tested because it was previously found that mir-202 (unspecified as to 3p/5p) is found in the mitochondria and therefore may be associated with mitochondrial biogenesis [154]. It was found that mir-202-3p is highly expressed in TLR2−/− mice at 6h PI, but not in WT or TLR4−/− mice (discussed in section 3.2 below). The mir-30 family was thus not examined because there was already proof-of-principle in the positive result for mir-202-3p.
Table 4: Sequences of the let-7 family of microRNAs. Red characters show bp differences from canonical sequence; highlighted sequences would not be detected by a primer to the canonical sequence.

<table>
<thead>
<tr>
<th>miRNA name</th>
<th>Sequence 5’-3’</th>
<th>BLAST against canonical sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mmu-let-7a</td>
<td>UGAGGUAGUAGGUUGUUGUAUAGUU</td>
<td></td>
</tr>
<tr>
<td>Mmu-let-7b</td>
<td>UGAGGUAGUAGGUUGUUGUGGUU</td>
<td>2mer different</td>
</tr>
<tr>
<td>Mmu-let-7c</td>
<td>UGAGGUAGUAGGUUGUUGUAUGGUU</td>
<td>1mer different</td>
</tr>
<tr>
<td>Mmu-let-7d</td>
<td>AGAGGUAGUAGGUUGCAUAGGUU</td>
<td>2mer different</td>
</tr>
<tr>
<td>Mmu-let-7e</td>
<td>UGAGGUAGGAGGUUGUAUAGGUU</td>
<td>1mer different</td>
</tr>
<tr>
<td>Mmu-let-7f</td>
<td>UGAGGUAGUAGAUUGUAUAGGUU</td>
<td>1mer different</td>
</tr>
<tr>
<td>Mmu-let-7g</td>
<td>UGAGGUAGUAGUUUGUACAGUU</td>
<td>2mer different</td>
</tr>
<tr>
<td>Mmu-let-7i</td>
<td>UGAGGUAGUAGUUUGUUGCUGUU</td>
<td>4mer different</td>
</tr>
<tr>
<td>mmu-miR-98</td>
<td>UGAGGUAGUAGGUUGUAUUGUU</td>
<td>2mer different</td>
</tr>
</tbody>
</table>

Table 5: Sequences of the mir-30 family of microRNAs. Red characters show bp differences from canonical sequence; highlighted sequences would not be detected by a primer to the canonical sequence.

<table>
<thead>
<tr>
<th>miRNA name</th>
<th>Sequence 5’-3’</th>
<th>BLAST against canonical sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>mmu-miR-30a</td>
<td>UGUAAACAUCUCGCAGUGAAAG</td>
<td></td>
</tr>
<tr>
<td>mmu-miR-30b</td>
<td>UGUAAACAUCUCUACACUCAGCU</td>
<td>7mer different</td>
</tr>
<tr>
<td>mmu-miR-30c</td>
<td>UGUAAACAUCUCUACACUCACG</td>
<td>6mer different</td>
</tr>
<tr>
<td>mmu-miR-30d</td>
<td>UGUAAACAUCCCCGACUGGAAG</td>
<td>1mer different</td>
</tr>
<tr>
<td>mmu-miR-30e</td>
<td>UGUAAACAUCCUUGACUGGAAG</td>
<td>1mer different</td>
</tr>
<tr>
<td>mmu-miR-384-5p</td>
<td>UGUAAACAUUCCUAGGCAUGU</td>
<td>10mer different</td>
</tr>
</tbody>
</table>

Binding sites for the mouse microRNA mmu-mir-202-3p were identified in both the PGC-1α and PGC-1β 3’UTRs. Analysis of the binding sites showed that in addition to perfect seed-region binding, the miRNA had homology to the downstream region (see Figure 37). Also, in silico analyses performed using the online program mFold showed that the PGC-1α and PGC-1β mRNAs are often in a single-strand formation in the binding site in the 3’UTR (see Figure 38). The expression of mir-202-3p was examined
by qRT-PCR in WT, TLR2\(^{-/-}\), and TLR4\(^{-/-}\) mice in HC and at 6h PI and 24h PI. It was found that mir-202-3p was significantly increased at 6h and 24h in TLR2\(^{-/-}\) mice (6h: 3.7-fold vs. HC, \(P<0.001\); 24h: 3.8-fold vs. HC, \(P<0.01\)), was unchanged at 6h, but was increased at 24h PI with borderline significance in WT mice (6h: 1.4-fold vs. HC, \(P=0.16\); 24h: 3.3-fold vs. HC, \(P=0.10\)), and was unchanged at 6h but decreased at 24h in TLR4\(^{-/-}\) mice (6h: 1.1-fold vs. HC, \(P=0.3\); 24h: 0.4-fold v. HC, \(P<0.05\)) (Figure 39A).

The miR-202-3p levels thus correlated inversely with PGC-1\(\alpha\) and PGC-1\(\beta\) mRNA levels at 6h and 24h PI. To illustrate this, the average fold-induction of PGC-1\(\alpha\) and PGC-1\(\beta\) were plotted separately against the fold-induction of mir-202-3p at the same times in the same strains. The best-fit function showed a negative exponential relationship, with \(R^2\) values of 0.56 for PGC-1\(\alpha\) and 0.83 for PGC-1\(\beta\) (Figure 39B). This thus confirmed an inverse correlation between mir-202-3p expression and PGC-1\(\alpha/\beta\) expression.

![Figure 37](image-url) Predicted binding of mir-202-3p to the PGC-1\(\alpha\) 3'UTR at 24 bp and to the PGC-1\(\beta\) UTR at 9 bp and at 16 bp.

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Figure 38: *In silico* 3’UTR mRNA folding analyses of PGC-1α and PGC-1β. The entire mRNA sequences of PGC-1α and PGC-1β were separately fed into the program *mfold*, and predictions were made on average single-strand characteristics of the mRNA. Shown are the regions surrounding the predicted mir-202-3p binding sites. The green bars show the seed-binding regions, while the red bars show the length of predicted mir-202-3p binding.
Figure 39: mir-202-3p is associated with PGC-1α and PGC-1β degradation. (A) mir-202-3p levels were measured by Q-PCR in WT, TLR2−/−, and TLR4−/− mice in healthy controls (HC) and at 6h and 24h PI. n=3 at each time point for each strain; *, P<0.05, compared to HC of the same strain. Error bars show SD. (B) mir-202-3p expression is negatively correlated with the mRNA levels of PGC-1α (R² = 0.56) and PGC-1β (R² = 0.83).
In order to confirm this correlation, a microRNA mimic of mir-202-3p (a dsRNA sequence that matches the mir-202-3p sequence) was obtained from Qiagen and transfected into AML12 cells (mouse hepatocytes). AML12 cells were chosen because they are mouse hepatocytes, and so similar to the organ being studied (mouse liver). AML12 cells are very difficult to transfect, however, and require up to 10 times the amount of transfection reagent as recommended to obtain >80% transfections rates as measured by fluorescent oligonucleotide uptake (data not shown). The AML12 cells were transfected with either mir-202-3p mimic or scrambled RNA, and were left for 24 hours. They were then serum-starved for 4 hours to induce PGC-1α mRNA (See Figure 40). In this system, PGC-1α mRNA increased approximately 7-fold on starvation, but this effect was blunted by the presence of the mir-202-3p mimic (starvation only PGC-1α: 7.1-fold vs. control, \( P < 0.01 \); mir-202-3p mimic PGC-1α: 2.1-fold vs. control, \( P \) value vs. starvation only: \( P < 0.01 \)). There was also a significant reduction from the scramble-treated cells (scramble PGC-1α: 3.3-fold vs. control, \( P \) value vs. starvation only: \( P < 0.05 \)), however this reduction was significantly less than in the mir-202-3p treatments (scramble vs. mir-202-3p mimic \( P < 0.01 \)). The significant effect of scrambled RNA on the system could be due to activation of a nonspecific dsRNA response leading to inflammation. Nevertheless, the microRNA mir-202-3p is confirmed to functionally decrease PGC-1α mRNA levels \textit{in vitro}. Further studies will focus on HepG2 (human hepatocytes) cells, which are easier to transfect, but in which mir-202-3p should still be specific for PGC-1α and PGC-1β. The decrease in transfection reagent required may decrease the nonspecific effects of the scramble controls.
4. Conclusions

4.1 Summary of the main findings

Mitochondrial biogenesis is a pro-survival response to sepsis-induced mitochondrial oxidant damage. In this work, a fibrin clot of model of S. aureus sepsis was employed to examine the effects of innate immune signaling on the up-regulation of the mitochondrial biogenic response. It was observed that, compared to WT mice, both TLR2⁻/⁻ and TLR4⁻/⁻ mice show increased mortality in response to S. aureus sepsis, and...
have dampened and delayed up-regulation of gene expression of several factors involved in mitochondrial biogenesis. These include both the biogenesis-specific transcription factors NRF-1, NRF-2, and Tfam, and the downstream targets of mitochondrial biogenesis, including SOD2, TXNRD2, and Cyt b. Most striking, though, was the difference in early induction of the transcriptional co-activators PGC-1α and PGC-1β, which were increased in WT mice at 6h PI, were not up-regulated in TLR2−/− at 6h PI, and were over-expressed in TLR4−/− mice at 6h PI. In addition, the third member of the PGC co-activator family, PRC, was not differently expressed by the three different mouse strains, and also peaked at a different time (24h PI). The co-regulation of PGC-1α and PGC-1β in acute inflammation, and their expression dependence on TLR signaling, are major novel findings.

The finding that TLR4−/− mice show increased mortality along with increased expression of PGC-1α and PGC-1β at 6h PI leads to two conclusions. First, the temporal over-expression of PGC-1α and PGC-1β is not enough to protect these mice from increased mortality. Significant increases in NRF-2 mRNA were found in TLR4−/− mice but not in WT mice, perhaps downstream of the increase in PGC-1α and PGC-1β (although they could also be jointly increased by the same signal, or even coincidentally up-regulated). Thus, the short time for which PGC-1α and PGC-1β expression is increased may not be enough for these factors to functionally increase mitochondrial biogenesis. Second, the TLR4−/− mice likely have increased mortality through other mechanisms (i.e., not having to do with mitochondrial biogenesis). Since TLR4 is important to innate immune function and may thus allow for activation of humoral
immunity, its absence may cause severe immune dysregulation (as evidenced by the attenuated expression of pro-inflammatory cytokines such as TNFα and IL-6). Thus, the increased mortality of TLR4−/− mice shows both that short peaks in PGC-1α and PGC-1β expression may not be enough to functionally increase mitochondrial biogenesis and that TLR4−/− mice show dysregulated immunity in response to S. aureus sepsis.

In order to determine the causes of the TLR-dependent differential expression of PGC-1α and PGC-1β, both transcriptional and post-transcriptional mechanisms of control were examined for the two genes. Since TLRs are known to activate NF-κB, early work focused on determining whether NF-κB might up-regulate PGC-1α and PGC-1β translation. It was determined first that, although p65 is differentially activated in the three different strains, it cannot be localized on the PGC-1α promoter by ChIP. Second, both MAL−/− and MyD88−/− mice were examined and were shown to induce PGC-1α and PGC-1β at 6h PI, even though they were deficient in NF-κB signaling. Finally, in p50−/− and BAY-11-7082-treated mice, there was no difference from WT mice in PGC-1α expression at 6h PI. It was thus concluded that NF-κB is not likely to be an important causative factor of PGC-1α and PGC-1β induction in acute S. aureus sepsis.

Since neither the MAL−/− and MyD88−/− mice reproduced the TLR2−/− phenotype (ie, low PGC-1α expression), both TRAM−/− and TRIF−/− mice (the only other TLR adaptors) were obtained and exposed to S. aureus sepsis. Both TRAM−/− and TRIF−/− mice failed to induce PGC-1α and PGC-1β at 6h PI. Since the main downstream signaling event of TRAM/TRIF activation is the phosphorylation and activation of IRF-3 and IRF-7, nuclear IRF-3/7 levels were determined by immunoblots. IRF-7 was shown to be
increased in WT and TLR4−/− mice at 6h PI, but not in TLR2−/− mice. The PGC-1α promoter was examined for ISRE sites, and, using ChIP assay, it was shown that IRF-7 binds the PCG-1α promoter. IRF-3−/− X IRF-7−/− mice were then exposed to S. aureus sepsis and showed that they do not up-regulate PGC-1α or PGC-1β at 6h PI. This confirmed IRF-7 is necessary for PGC-1α up-regulation. Finally, PolyI:C was used as a TLR3 agonist to induce IRF-7 in the setting of the model of S. aureus sepsis. WT mice dosed with PolyI:C alone did not up-regulate PGC-1α, but TLR2−/− mice given both S. aureus sepsis and injected with PolyI:C do show an increase in PGC-1α at 6h PI. Thus IRF-7 was shown to be important for the induction of PGC-1α/β. The proposed pathway TLR2⇒TRAM/TRIF⇒IRF-7⇒PGC-1α/β was thus confirmed in vivo, representing a broadening of scope of the innate immune response to S. aureus.

The cause for the lack of PGC-1α induction in response to PolyI:C is unclear. It could be that one of the several transcription factors found to be up-regulated in TLR2−/− mice in response to S. aureus (such as the AP-1 family, or STAT1 or STAT3, or an untested factor known to be involved with PGC-1α, such as CREB) could be necessary to co-activate PGC-1α with IRF7. In addition, it could be that the activation of TLR3 by polyI:C alone is not sufficient to induce IRF-3/7; some evidence exists that the activation of several TLRs at once can be dramatically different than simply the sum of their activations alone [162]. One way to test for this would be to measure IFN-α production in the polyI:C-dosed mice to ensure IRF-3/7 activation. However, previous Q-PCR for IFN-α in the livers of WT mice exposed to the model of S. aureus yielded often-undetectable
amplification at 40 cycles, indicating that this may not be a good marker of IRF-7 induction capable of inducing PGC-1α in this model.

Although IRF-7 was shown to be involved in the induction of PGC-1α and PGC-1β, we hypothesized that there could also be post-translational degradation by microRNAs that play a role in the striking lack of PGC-1α/β message seen in the TLR2−/− mice at 6h PI. The search for candidate microRNAs was greatly aided by the co-regulation of PGC-1α and PGC-1β, since the responsible factor would have to be predicted to bind the 3’UTR of both genes. The in silico analysis yielded a small number of candidate conserved microRNAs, one of which was mir-202-3p. Further in silico work showed good predicted binding characteristics of mir-202-3p to the 3’UTRs, which themselves were predicted to be in the necessary single-strand formation in about half of the predicted binding models. Quantitative real-time PCR analysis of mir-202-3p showed its up-regulation in TLR2−/− mice at 6h and 24h PI, but not in WT or TLR4−/− mice at 6h PI. A comparison of mir-202-3p expression to the gene expression of PGC-1α and PGC-1β showed an inverse correlation for both genes with significant R²-values. A cell experiment was also conducted which showed a functional decrease in PGC-1α mRNA in response to mir-202-3p. We thus identified a microRNA that is responsible, at least in part, for the degradation of PGC-1α and PGC-1β mRNA in vivo in response to acute S. aureus sepsis.

Thus, a summary of the major new findings of this work is as follows:
1. Both TLR2−/− and TLR4−/− mice show increased mortality in response to S. aureus sepsis
2. TLR2−/− mice show impaired mitochondrial biogenesis in S. aureus sepsis.
3. PGC-1α and PGC-1β are regulated separately from PRC in *S. aureus* sepsis, and PGC-1α and PGC-1β induction is influenced by TLR2 and TLR4 in *S. aureus* sepsis.

4. NF-κB signaling is not involved with this early up-regulation.

5. The pathway TLR2 → TRAM/TRIF → IRF-7 → PGC-1α/PGC-1β was confirmed. The association of TLR-2 with TRAM/TRIF and its activation of IRF-7 represents a major broadening of scope in the ability of TLR signaling to respond to Gram-positive sepsis *in vivo*.

6. miR-202-3p is inversely correlated with PGC-1α/-1β in acute *S. aureus* sepsis and functionally decreases PGC-1α *in vitro*.

### 4.2 Implications for clinical medicine

Several of the new findings of this work have important therapeutic implications. First, the observation that PGC-1α and PGC-1β are co-regulated in acute inflammation separately from PRC may indicate that PGC-1α and PGC-1β play similar roles in sepsis-induced mitochondrial biogenesis, and that PRC could control a different aspect of mitochondrial phenotype. If a suitable means of transiently increasing PGC-1α and PGC-1β (or PRC) can be found, then mitochondria can be probed for the expression of proteins with different functions (for instance, heme synthesis/breakdown, antioxidation, mitochondrial repair, glucose/fat metabolism, or mitochondrial fission/fusion). This would be a significant step in determining mitochondrial phenotypic changes in response to PGC gene induction.
Another therapeutic implication is that since PGC-1α and PGC-1β transcript levels were found to be controlled by TLR signaling, and IRF-7 was shown to be necessary for the early up-regulation of both genes in an *in vivo* model of *S. aureus* sepsis, then IRF7 up-regulation may present a means through which to increase PGC gene induction. Longer-term PGC gene induction may yield clinically significant mitochondrial biogenesis. Similarly, the microRNA mir-202-3p was found to correlate with a failure to induce PGC-1α and PGC-1β *in vivo* and to cause its decrease *in vitro*. This is the first miRNA identified that regulates both genes, and mir-202-3p is a potential therapeutic target for septic patients with respect to the stimulation of mitochondrial biogenesis. Again, this could require showing that escape from PGC gene repression induces mitochondrial biogenesis. To be useful clinically, there would need to be a means to identify patients with a poor mitochondrial biogenic response and a suitable mechanism for decreasing specific miRNA expression.

5. Future Research Directions

The work presented in this dissertation opens up several new avenues for further research. The following four areas are the most important for advancement of the field.

First, having identified two mechanisms that affect the levels of PGC-1α and PGC-1β, the effects of differing levels of expression of these two genes on downstream genes of mitochondrial biogenesis can be examined *in vivo*. Using either PolyI:C (or some other activator of IRF-7) or a mir-202-3p-specific inhibitor, one could stimulate the over-expression of PGC-1α and PGC-1β and then probe downstream targets for
activation. One could also examine the effects of either treatment on PGC levels in other organs, such as the heart, adipose tissue, and brain, where PGC-1α is a known modulator of disease. Since the duration of over-expression that is necessary to get a significant increase in mitochondrial density (and hence a difference in survival outcomes) is unknown, this would be a major goal of such research. In addition, such work would be have immediate potential translational significance, since it could provide therapies (or targets for therapies) for patients with *S. aureus* sepsis.

Second, the reasons for differential pro-inflammatory cytokine expression by WT, TLR2 deficient (TLR2^−/−^), and TLR4 deficient (TLR4^−/−^) mice in response to *S. aureus* are not clear. The main outstanding gap in our knowledge is the identification of which proteins are involved in the TLR signaling complex at the cellular surface that are activated by *S. aureus* sepsis *in vivo*. There is a significant (and frequently-reproduced) discrepancy between the *in vivo* and *in vitro* responses to *S. aureus*. Notably, each paper that has studied the effects of TLR4 in the response to *S. aureus* sepsis *in vivo* has found that TLR4-deficient mice have altered signaling patterns (See Table 2) [73,74,75,76,77,78,79]. This means that either the *in vivo* signaling complex is different from cells studied *in vitro*, or that there are *in vivo* ligands that induce a TLR2-TLR4 interaction that is not present in simple *in vitro* models. To test for the presence of a TLR2-TLR4 complex, blue native PAGE [138] will be performed to try to identify a protein complex of TLR2 and TLR4. Further probes for the presence of TRAM in a TLR2 complex would provide more evidence for TLR2-dependent TRAM-dependent signaling. Other techniques, such as FRET or confocal microscopy, can co-localize dimerized proteins (though FRET is much more specific for protein proximity).
Both techniques are more difficult in tissues than in cells but still may be technically feasible. Thus the TLR2-TLR4 \textit{in vivo} complex idea is certainly not un-testable. In addition, in this \textit{in vivo} fibrin clot model of \textit{S. aureus}, the presence of fibrin breakdown products and the physical manipulation of the gut (leading to possible LPS translocation) could both lead to the presence of additional DAMPs (such as basement membrane products) or PAMPs (such as LPS translocated from the gut). This makes the model a beneficial area for study since it more closely resemble clinical models of infection, where physical damage and the subsequent generation of fibrin frequently form a bed for infection. In order to identify which DAMPs and PAMPs are being produced, cell-surface and damage-receptor activation studies could be done in models of sterile fibrin deposition, gut manipulation, or \textit{S. aureus} injection in an attempt to tease apart the contribution of each in the generation of acute-phase cytokines. It is possible that either long basement membrane proteins (possibly bound by \textit{S. aureus} MSCRAMMs) or \textit{S. aureus} itself could be large enough and heterologous enough to bind both TLR2 and TLR4 at the same time, thus signaling as though the two proteins were in complex even though they never actually dimerize. One could test this with basement membrane-deficient mice or with infection with mutant \textit{S. aureus} that does not express certain MSCRAMMs (such as FnBPs). In addition, unc93b1-mutant (3d) mice, which are deficient in TLR3, TLR7, TLR8, and TLR9 signaling, are being obtained by the laboratory and will be exposed to the model of \textit{S. aureus} sepsis. The unc93b1 protein normally functions in ER trafficking; the 3d mutant mice are thus deficient in TLR signaling for those TLRs which are found on endosomes (TLR3, TLR7-9) [163,164].
Studying the 3d mutant mice will thus report whether those other TLRs are involved in the differential immune activation observed in response to *S. aureus* sepsis.

Third, several of the differences between WT, TLR2<sup>-/-</sup>, and TLR4<sup>-/-</sup> mice (such as Akt, ERK1/2, and Fra2 activation) were followed up only in the context of PGC up-regulation, and not cytokine generation. These signaling paths could contribute to cytokine differences between the three strains, and understanding their upstream kinases and receptors could yield clues as to the identities of the *in vivo* signaling complexes that respond to *S. aureus*. Thus, further work involving PI3k inhibition with small-molecule inhibitors (such as LY2009 or wortmannin) could be used to assess the contribution of PI3k/Akt to cytokine production. Similarly, kinase-deficient mice or transcription factor-deficient mice (such as ERK1<sup>-/-</sup> and Fra2<sup>-/-</sup>, both of which are commercially available) could be studied for varying immune activation in response to *S. aureus* sepsis. For the reasons outlined above regarding differential responses to *S. aureus*, it would be important to conduct these studies first *in vivo* and then *in vitro* in order to elucidate the cause of differential immune activation in this model.

The fourth avenue of future work is the transcriptional control of mir-202-3p. Although microRNAs not infrequently belong to families that make it difficult to distinguish which microRNA gene is actually responsible for the observed phenotype, mir-202-3p does not share seed-region homology with any other known miRNAs, and its expression is thus comparatively easy to study. We examined the proximal 1000 bp of the mir-202-3p promoter by aligning the sequences between mouse and human, and found only a very small island of conservation in the promoter region. This region showed
conserved binding sites for the CREB/ATF family of transcription factors, as well as for estrogen receptor α (ERα) and hepatic nuclear factor 4 (HNF4), all of which are known to interact with or regulate PGC-1α. It is possible that mir-202-3p expression could be one way in which those factors influence PGC-1α (and PGC-1β) expression levels. To determine which transcription factors are involved in mir-202-3p regulation, one would first perform ChIP studies proving transcription factor association with the mir-202-3p promoter, and then use RNAi to knock down the target transcription factors and assess mir-202-3p expression. Since proteins are still easier therapeutic targets than microRNAs, such work could identify an upstream target for PGC (and hence mitochondrial biogenic) transcriptional control that could be activated therapeutically.
Appendix A – PGC family Promoter Maps
Figure 41: The proximal 500 bp from the TSS in PGC-1α, PGC-1β, and PRC from both mouse and human were aligned with zPicture and then submitted to rVista, with all transcription factors searched under an optimized matrix.
Appendix B – PGC family Intron 1 Maps

PGC-1alpha, proximal 1000 bp in intron 1
Figure 42: The proximal 1000 bp in intron 1 in PGC-1α, PGC-1β, and PRC from both mouse and human were aligned with zPicture and then submitted to rVista, with all transcription factors searched under an optimized matrix.
# Appendix C – PCR Primers

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<th>Real-Time PCR Probes</th>
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References


Biography

Timothy Elisha Sweeney

Born, Ft. Lee, NJ, USA, Sept. 20, 1983

Education:

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Medical Scientist Training Program, 2005-2011

Ph.D. in Pathology, May 2010

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B.A., Chemistry, 2005

Graduated with Honors

Peer-Reviewed Publications:


Submitted, PLOS one.


Bartz RB, Suliman HB, Fu P, Welty-Wolf K, Carraway MS, MacGarvey NC, Withers CM, Sweeney TE, and Piantadosi CA. “Staphylococcus sepsis and the regulation of the OGG1 mitochondrial DNA repair enzyme.” In 1st revision, AJRCCM.

Barbas AS, Sweeney TE, Collins BH, Kuo PC, Tuttle-Newhall JE, Sudan DL, Marroquin CE. "Kidney-Pancreas transplantation in the setting of donor ethylene glycol poisoning.". Accepted, Dialysis and Transplant, 2010

Posters and Presentations:


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