THE EFFECTS OF CIRCADIAN RHYTHM ON INFLAMMATORY RESPONSE AND CYTOKINE LEVELS IN HEPATIC MACROPHAGES

by
Christina Orcino

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Director of Thesis/Dissertation: Dr. Michael Wheeler

Major Department: Exercise and Sport Science (Exercise Physiology)

ABSTRACT

In mammals, many aspects of daily behavior and physiology such as the sleep-wake cycle, body temperature, and liver metabolism are regulated by endogenous circadian mechanisms. Despite tissue-specific physiological differences, these circadian rhythms share a highly conserved negative feedback mechanism, consisting of transcriptional activators and repressors. This feedback loop system is critical for normal physiology and behavior, and its disruption can lead to sleep disorders, metabolic syndrome, cancer, and a whole host of other diseases, including immune related dysfunction. In this study, the loss of Bmal, a key transcriptional activator, and its ability to regulate a macrophage response was evaluated. To do this, a loxP-cre system was used to create mice with a macrophage-specific deletion of Bmal (lysZ-Bmal -/-). This was the first look at the role of macrophage-specific circadian gene expression in regulation of hepatic immune response and its influence on macrophage differentiation and inflammatory liver disease *in vivo*. Cre-negative littermate controls and lysZ-Bmal -/- mice were stimulated with the endotoxin lipopolysaccharide (LPS) to provoke a pro-inflammatory response. In lysZ-Bmal -/- mice, cells stimulated with LPS had an exacerbated pro-inflammatory response but a blunted anti-inflammatory response compared to cells from cre-negative littermates. LysZ-Bmal -/- mice also had larger liver to bodyweight ratios as well as

increased relative mRNA levels of critical pro-inflammatory cytokines and serum ALT. In summary, this study suggests that circadian regulation in macrophages may be important for lipid control and that Bmal regulates a pro-inflammatory response in hepatic macrophages.

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Christina Michelle Orcino

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Christina Orcino

APPROVED BY:

DIRECTOR OF
DISSERTATION/THESIS: _____
Dr. Michael Wheeler, PhD

COMMITTEE MEMBER: _____
Dr. Ian Hines, PhD

COMMITTEE MEMBER: _____
Dr. Joseph Houmard, PhD

COMMITTEE MEMBER: _____
Dr. Hu Huang, PhD

COMMITTEE MEMBER: _____
Dr. Katsuhiko Funai, PhD

CHAIR OF THE DEPARTMENT
OF KINESIOLOGY: _____
Dr. Stacey Altman, JD

DEAN OF THE GRADUATE SCHOOL: _____
Dr. Paul J. Gemperline, PhD

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INTRODUCTION

Circadian rhythms are daily oscillations of multiple biological processes driven by endogenous clocks (Liu J., 2006). The circadian molecular clock mechanism is a transcription-translation-based negative feedback loop comprised of core clock genes Per, Cry, Clock and Bmal1 (Silver A.C., 2012). There is an ever-increasing body of literature that describes the influence of daily rhythms on immune function (Silver A.C., 2012). However, due to the absence of an appropriate model with which to examine circadian immunoregulation directly, the molecular mechanisms of action have yet to be determined (Liu J., 2006).

Over the past decade, research has verified that sleep is necessary for physical health and can be disrupted during infection. Sleep loss has been shown to impair immune function (Imeri L., 2009) and the natural, protective inflammatory and anti-inflammatory cues of the body. While external cues such as changes in temperature, light intensity and food play a role in waking us up and putting us to sleep, we, as mammals, have a circadian rhythm, or an "internal clock", that regulates a number of physiological processes on a 24-hour cycle. This clock is driven by rhythmic feedback loops as well as activator and repressor genes. Recent evidence shows that also peripheral organs such as liver, heart, kidney, skin and even cultured cell lines contain circadian oscillators (Keller M., 2009). The master clock, the SCN, is located in the hypothalamus and synchronizes autonomous peripheral oscillators located in other organs (liver, spleen). The two main transcription factors that regulate circadian rhythm are Bmal and Clock. This heterodimer drives the expression of repressor genes, Per and Cry. Together, these positive and negative factors orchestrate nearly every physiological bodily process. Bmal:Clock positively regulate circadian dependent genes while Per-2 and Cry antagonize. Specifically, Per activates a group of signal transducer proteins that determines the inflammatory response a cell

has when injured. These proteins have a direct influence on determining the fate of a macrophage; whether it will become classically activated (M1) or alternatively activated (M2).

This particular field is of great interest because modern medicine is linking more inflammatory diseases to the regulation of circadian rhythm genes, but the functional significance of this response is largely unknown. The role of macrophages in host defense and tissue injury is well established, specifically in the liver. Liver macrophages (Kupffer cells) release a number of different immunoregulatory and inflammatory cytokines (Wang Y., 2010), however, the mechanisms governing macrophage polarization are unclear (Wang Y., 2010).

The internal circadian clock system evolved as an adaptation to this predictable day/night geophysical pattern, and the biological consequences of living at odds with the normal environmental day/night cycle are just beginning to be appreciated (Castanon-Cervantes O., 2010). Exposure to nontraditional work schedules has been linked with increased risks of colorectal (Lu Y., 2008), breast (Imeri L., 2009), lymphatic (Schibler U., 2007), and prostate (Costa N., 2012) cancers, as well as with gastric ulcers (Wang K., 2000), obesity (Kornmann B., 2007), diabetes (Balsalobre A., 2000), stroke (Ralph M.R., 2012), coronary heart disease, atherosclerosis, and heart attack (Lange T., 2012). The mechanisms for these correlations between shift work exposure and disease are unknown, however it is important to note that one common risk factor shared by many of these pathologies is inflammation (Castanon-Cervantes O., 2010).

The overall goals of the following aims outlined are to test the central hypothesis that circadian factors regulate macrophage polarization. Macrophage polarization is the process of whether a macrophage elicits a M1 or M2 inflammatory response. This study will specifically focus on the role of Bmal in macrophage polarization. Bmal1 runs near antiphase with Per genes

and Clock and has been shown to oscillate in phase with Bmal1 in the heart (Hayashi M., 2006) and in the liver (Arjona A., 2005). Macrophage polarization toward M1 or M2 phenotypes is driven by cues in the tissue microenvironment, which can include cytokines, growth factors and microorganism-associated molecular patterns (i.e., gut-derived LPS). These signals dictate a transcriptional response that shapes the phenotype and function of the macrophages on the basis of the physiological or pathophysiological context. Circulating naïve T-cells and production of pro-inflammatory cytokines (M1) peak during nighttime, whereas anti-inflammatory cytokines (M2) peak during daytime (Lange T., 2012). While pilot experiments have relied heavily on Per2-null mice, this experiment will focus on the role of Bmal, the transcriptional regulator of Per-2, as a regulator of hepatic M1:M2 phenotype balance. These experiments will utilize a conditional mutant where the *bmal* allele has been floxed, which will allow for cell specific deletion of Bmal when combined with transgenic expression of cre recombinase. Since Bmal1 and Clock are metabolically more stable than Cry and Per proteins, their abundance varies only slightly throughout the day (Keller M., 2009). This approach will allow us to test address the macrophage-specific role of circadian gene expression and test the specific hypothesis that Bmal is a regulator of hepatic M1:M2 phenotype balance. To date, Bmal1 is the only known clock gene whose inactivation immediately leads to arrhythmicity of behavior and to the ablation of mPer1 and mPer2 mRNA accumulation cycles in the SCN (Kornmann B., 2007). Bmal was chosen to be deleted because Bmal1 is a constituent of the molecular oscillator whose loss of function immediately results in the abolishment of all manifestations of circadian physiology and gene expression (Albrecht U., 2008). Bmal1 knock out mice develop progressive corneal inflammation and exhibit decreased numbers of lymphocytes (Castanon-Cervantes O., 2010).

The current aims of this study are to determine if 1) Bmal regulates an M1:M2 response in hepatic macrophages and 2) circadian regulation in macrophages is important for lipid control.

In a recent pilot study conducted by Wheeler et al., macrophage-specific Bmal-null mice were developed to address macrophage-specific circadian function. Transgenic mice carrying floxed alleles of Bmal were crossed with transgene mice expressing cre recombinase under the control of the lysozyme Z promoter elements. The resulting LysZ cre+ x Bmal.f/f mice (lysZ-Bmal) and cre-negative littermate controls were exposed to vehicle or LPS (1mg/kg, ip). Per2-/- mice were also given LPS. LPS induced liver injury was significantly blunted in Per2-/- mice and lysZ-Bmal mice compared to controls. Hepatic mRNA was evaluated by qPCR for the expression of M1 and M2 cytokine and gene expression. Importantly, pro-inflammatory cytokines (e.g., TNF-**α**, CCL-2, iNOS) were markedly blunted in lysZ-Bmal mice compared to controls. Conversely, M2 cytokines were not different or slightly increased in lysZ-Bmal -/- mice compared to wild type. These interesting observations suggest a clear role of circadian regulation of hepatic macrophage response and are instructive for the hypothesis that circadian function is critical for hepatic innate immune response. The purpose of this project will be to investigate the effect circadian rhythm has on inflammatory response and cytokine levels in hepatic macrophages.

IDENTIFY/STATE SPECIFIC RESEARCH PROBLEM

Conditions such as obesity, diabetes mellitus and cardiovascular disease are pathologies that are characterized, in part, by the inflammatory process (Imeri L., 2009). Recent developments have closely tied circadian rhythm to immune function. However, the molecular details of the circadian rhythms in macrophages are still poorly understood (Hayashi M., 2006).

The significant scientific advance to the field would be a mechanistic link between circadian regulation and innate immune response.

HYPOTHESIS

Still, one of the greatest limitations in the understanding (and in our models) of inflammatory liver disease is our lack of understanding of the regulation of the hepatic immune response. This project will specifically focus on the role of Bmal in macrophage polarization. Macrophage polarization toward M1 or M2 phenotypes is driven by cues in the tissue microenvironment, which can include cytokines, growth factors and microorganism-associated molecular patterns (i.e., gut-derived LPS). These signals dictate a transcriptional response that shapes the phenotype and function of the macrophages on the basis of the physiological or pathophysiological context. While our pilot experiments have relied heavily on Per-2-null mice, this experiment will focus on the role of Bmal, the transcriptional regulator of Per-2, as a regulator of hepatic M1:M2 phenotype balance. These experiments will utilize a conditional mutant where the *bmal* allele has been flanked with flox P sites, which allow for cell specific deletion of Bmal when combined with transgenic expression of cre recombinase. This approach will allow us to test address the macrophage-specific role of circadian gene expression and test the specific hypothesis that Bmal is a regulator of hepatic M1:M2 phenotype balance. Ultimately, we will test the hypothesis that macrophage-specific Bmal expression is critical for an M1 response in respect to liver injury and that in Bmal-null mice, cells stimulated with the endotoxin LPS will have an exacerbated M1 response but a blunted M2 response.

DELIMITATIONS

In this study, there will be two groups of mice: cre-negative littermates and macrophage-specific Bmal-null mice generated by the mac-cre transgenic system (C57Bl/6j (Jackson

Laboratories). To our knowledge, this will be the first attempt using this innovative approach to characterize the role of circadian rhythm in regulation of hepatic immune response. The direct interaction Bmal has on a M1/M2 inflammatory response will be demonstrated.

LIMITATIONS

Some limitations that could be encountered are errors in the transcribing of the mice. It will be necessary to verify the functional deletion of Bmal via gene expression. In addition, cells have the ability to up-regulate compensatory mechanisms when components of pathways are knocked out. Gene expression must be measured using other circadian components to ensure that Clock or Per-2 will not overcompensate for Bmal.

SELECTED ABBREVIATIONS AND ACRONYMS

ALT: Alanine aminotransferase

Bmal: Brain and muscle aryl hydrocarbon receptor nuclear translocator (ARNT)-like

Cry: Cryptochrome

LPS: Lipopolysaccharide

SCN: Suprachiasmatic nucleus

Per: Period

qPCR: quantitative Polymerase Chain Reaction

DEFINITIONS OF TERMS

Cytokine: A substance secreted by specific cells of the immune system which carry signals locally between cells.

Kupffer cell: Specialized macrophages located in the liver that play a critical role in directing immune response.

Transcription factor: A protein that binds to specific DNA sequences and controls the flow of genetic information from DNA to mRNA.

Macrophage: A white blood cell within a tissue produced by a monocyte; acts as a control switch of the immune system and provides a balance between an M1 and M2 response.

M1 macrophage: Classically activated, pro-inflammatory macrophages that destroy invading pathogens, tumor cells, and foreign materials (i.e. IL-6, IL-12 and TNF-a).

M2 macrophage: Regulatory, anti-inflammatory macrophages that are alternatively activated to promote tissue repair and angiogenesis (i.e. IL-4, IL-10 and TGF-b).

Microenvironment: The environment of a very small, specific area, distinguished from its immediate surroundings by such factors as the amount of incident light, the degree of moisture, and the range of temperatures; heavily influences the polarization of macrophages.

mRNA: Messenger Ribonucleic Acid; A large family of RNA molecules that convey genetic information from DNA to the ribosome, where they specify the amino acid sequence of the protein products of gene expression.

Phenotype: The composite of an organism's observable characteristics or traits based on heritable traits or environmental factors.

TNF-α: Tumor Necrosis Factor-alpha; A pro-inflammatory cytokine involved in systemic inflammation and is a member of a group of cytokines that stimulate the acute phase reaction.

Transgenic: A gene or genetic material that has been transferred naturally or by any of a number of genetic engineering techniques from one organism to another.

REVIEW OF THE LITERATURE

The purpose of this project is to investigate the effect circadian rhythm has on cytokine levels and inflammatory response in hepatic macrophages. The following chapter is an attempt to demonstrate that peripheral tissues display molecular oscillations and clock genetics regulate an inflammatory immune response.

Peripheral Tissues Display Molecular Oscillations

In humans, blood cell compartmentalization, such as with peripheral cell counts of neutrophils, T-lymphocyte subsets, B lymphocytes, monocytes and natural killer cell, displays a circadian fluctuation across the day (Born J., 1997). A similar oscillation has also been observed in rodents (Yamazaki S., 2000). Thus, the circadian immunological parameters, which affect activity both in humans and rodents, are well conserved under baseline physiological conditions, indicating parallel clock control mechanisms for the human and mouse immune systems (Liu J., 2006). Since it is considered unethical to intentionally infect humans with disease or perform endotoxic shock on them, the rodent model is that basis of much of this study. In mice, genes are knocked out in order to observe certain effects or traits the gene might display. One of the first ever studies that provided a direct link between the circadian clock system and innate immune response was done by Liu et al. in 2006. In this study, Per-2 was knocked out in mice. LPS was administered to the animals and cytokine levels were measured. The results showed that a deficiency in Per-2 rendered the animals resistant to endotoxic shock and levels of pro-inflammatory cytokines (M1) were dramatically reduced in LPS-treated, Per-2 knockouts. Techniques such as ELISA (enzyme-linked immunosorbent assay), fluorescence-assisted cell sorting (FACS) and intracellular cytokine staining were used after obtaining splenocytes from the animals post mortem. All of these tests are used to detect the presence of certain antigens in

cells. These data demonstrate that there is a circadian clock influence on host resistance to LPS-induced inflammatory death and that this rhythm is disrupted in Per-2 knockout mice.

A study conducted by Silver et al. in 2012 aimed to investigate the existence of molecular clock mechanisms in immune cells revealed the existence of such mechanisms in splenic macrophages. This study measured the circadian changes in gene expression of clock genes (Per1, Per2, Bmal1 and Clock) and clock-controlled transcription factors (Rev-erba and Dbp) in splenic enriched macrophages in both mice entrained to a light-dark cycle and under constant environmental conditions. Mice were fed a standard rodent diet and entrained to a 12 hour light/12 hour dark cycle for two weeks before tissue collection. After the animals were euthanized, spleens were collected, homogenized, filtered and spun down before RNA isolation. PCR analysis was performed to examine whether there were functional clocks in splenic B-cells by examining in vivo mRNA levels of canonical clock genes over the daily LD cycle and under constant conditions (DD). The results showed that macrophages, dendritic cells and B-cells enriched from mouse spleen possess functional molecular clocks as demonstrated by the daily oscillation in clock gene expression. Functional clocks have now been established to be present in the spleen and are able to generate functional outputs without the influence of the SCN.

For the first time, the expression of canonical components of the cellular clock in NK (Natural Killer) cells (M1) as well as their circadian oscillators in NK cells enriched from the rat spleen were demonstrated by Arjona et al. in 2005. Circadian changes in the expression of clock genes were measured as well as cytokines and cytolytic factors in NK cells from the rat spleen. NK cells produce pro-inflammatory cytokines such as IFN-y and TNF-**α** (Yoo S., 2003). After rats were maintained under constant environmental conditions for two weeks, the animals were euthanized and spleens were individually processed post mortem. RNA extraction was

performed and run through RT-PCR and Western blot analysis. RT-PCR analysis showed that both negative and positive components of the molecular clock were expressed in enriched NK cells. mRNA oscillations of Bmal and Clock were similar, showing two maxima at ZT3 and ZT7 and low levels from ZT11 to ZT23. This pattern corresponds directly to light and dark conditions, which further proves that peripheral tissues contain independent molecular oscillators. Cytokines peaked between ZT4 and ZT8 and steadily declined after ZT12. Particularly, TNF-**α** and IFN-y demonstrated circadian expression, which emphasizes the circadian nature of NK cell function. Further study is needed in the area of Kupffer cells for the reason that the liver is the only organ that is incapable of producing their own macrophages. Arguably the most important pro-inflammatory cytokine, TNF-**α**, is a crucial mediator of acute phase reactions and cell-mediated host defense against bacteria, parasites and tumor cells (Hrushesky W., 1994). The toxicity of many standard cytotoxins in both animals and humans can be reduced and dose intensity increased by appropriate circadian timing (Shi S., 2010). The therapeutic responses in animals and humans, however, have usually been achieved at doses of TNF-**α** approaching those associated with endotoxin-induced shock (Lu Y., 2008). It is important to understand the relationship between cytokines and circadian rhythm in the medical setting. It is interesting to note that hospital deaths do not occur randomly throughout the day but are more frequent between the hours of 2am and 6am. This time of day corresponds to the time in the circadian cycle when necessary M1 cytokines are each most frequently lethal (Hrushesky W., 1994). This study could potentially have the ability to develop methods in treating certain liver diseases based upon the evidence that cytokine expression is time of day dependent. The major scientific advance would be the observation that intrinsic circadian components regulate macrophage function through direct interaction with key inflammatory signaling pathways.

There is literature proving that mice with a conditionally active liver clock possess system-driven and oscillator-dependent circadian transcription (Kornmann B., 2007). Kornmann et al. wished to engineer a mouse strain with conditionally active circadian oscillators specifically in hepatocytes, in order to examine the contribution of local clocks and systematic Zeitgeber cues to rhythmic liver gene expression. As previously discussed, Rev-erba strongly represses Bmal1 transcription, so the goal of this study was to create a strain that crossed Rev-erba with an epitope-tagged version of Rev-erba to express a tetracycline-dependent transactivator specifically in hepatocytes (Wang Y., 2010). Verified by Western blot experiments and RT-PCR assays, the TRE-Rev-erba transgene remained silent and circadian oscillator function was not perturbed in liver cells in the presence of Dox (Kornmann B., 2007). Dox (doxycycline) is a tetracycline analog commonly used to treat bacterial infections. The animals were given a diet with and without Dox in it to measure the expression of the Bmal1 and Per genes. The cyclic expression of most rhythmically active genes appeared to depend on an intact hepatocyte oscillator, as the amplitude of circadian accumulation was greatly affected in animals not receiving Dox-supplemented food. The presence or absence of Dox was confirmed by Northern blot hybridization. This experiment did experience a phase delay which was assumed to reflect the time period required for the full decay of the HA-Rev-erba mRNA protein and for the consecutive accumulation of Bmal1 to levels compatible with circadian generation (Kornmann B., 2007). Heat shock proteins were also measured via culture-dish heating devices. Systematically driven circadian genes were unaffected by Rev-erba overexpression. This data revealed 350 transcripts with robust circadian accumulation independent of regulation from the SCN, suggesting that the cyclic transcription of most circadian genes is influenced by local oscillators (Kornmann B., 2007).

Clock Genes Regulate an Inflammatory Immune Response

A circadian clock in macrophages controls the immune response (Keller M., 2009). Specifically, the strength of pro-inflammatory cytokine production (M1) of macrophages in response to bacterial endotoxin is determined by the circadian phase of the macrophage clock rather than by systemic circadian modulators such as rhythmic cortisol levels (Balsalobre A., 2000). Using systematic transcriptome analysis of peritoneal macrophages, we uncover multiple possible control points in the LPS response pathway that link the macrophage-intrinsic circadian clock with crucial immunological effector functions (Keller M., 2009). In a study done by Keller et al., isolated spleen cells were stimulated with the bacterial endotoxin (LPS) at different circadian times to display circadian rhythm in pro-inflammatory cytokines. Following the popular study design of past experiments, mice were entrained to a 12 hour light/12 hour dark (LD) cycle for two weeks before being transferred to constant conditions (DD). It was found that total splenocytes were rhythmic in a circadian manor and that cytokine excretion is regulated by a circadian clock within these cells. TNF-**α** and IL-6 were measured through ELISA and showed that the ratio of monocyte and macrophage excretion correlates with a present circadian rhythm.

Studies using knockout mice have revealed important roles for adaptors in TLR4 signaling (Lu Y., 2008). The stimulation of Toll-like receptor 4 (TLR4) by lipopolysaccharide (LPS) induces the release of critical pro-inflammatory cytokines that are necessary to activate potent immune responses (Lu Y., 2008). TLRs are receptors expressed by cells in the immune system. In this study, LPS will be injected into both groups of mice (cre-negative littermate controls and Bmal knockout) in order to see a M1:M2 phenotypic response. LPS is one of the best studied immunostimulatory components of bacteria and can induce systemic inflammation and sepsis if excessive signals occur (Yamazaki S., 2000). It is an important structural

component of the outer membrane of Gram-negative bacteria and can stimulate mammalian cells through a series of interactions with several proteins. LPS induces a strong antigenic response such as up regulation of pro-inflammatory cytokines (Imeri L., 2009) and in response to LPS, mouse macrophages undergo a major change in gene expression, in particular inducing the expression and release of numerous biologically active cytokines that orchestrate the inflammatory response (Nilsson R., 2006). However, responses in animals may differ according to the dose, time of injection and the general state and history of the animal (Marpegan L., 2005), so the manner of administration of LPS in any experiment is crucial. The mortality upon LPS-induced endotoxic shock in mice depends highly on the time of day when LPS is administered, suggesting a circadian clock regulation of macrophage-dependent cytokine (Keller M., 2009). In a 2008 study done by Lu et al, MyD88 mice were used to measure the activation of pro-inflammatory cytokines via two pathways. The results ultimately yielded that MyD88-deficient macrophages failed to produce pro-inflammatory cytokines after LPS stimulation. The two pathways that were examined were the MyD88-dependent (M1) and MyD88-independent (M2). The MyD88-dependent pathway was shown to be responsible for pro-inflammatory cytokine expression, while the MyD88-independent pathway mediates the induction of Type 1 interferons (Lu Y., 2008). This data supports the hypothesis that when an important transcription factor involved in immune response is deleted from an organism, it will not produce the necessary inflammatory factors needed during tissue damage or injury response upon being stimulated by LPS injection.

Genetic manipulations of circadian timing can modulate innate immunity (Castanon-Cervantes O., 2010). In a 2010 study done on the dysfunction of inflammatory responses by chronic circadian disruption by Castanon-Cervantes et al., Per-2 knock in mice were expose to

phase shifts and LPS injections which were designed to throw off Per-2 and Bmal1 and stimulate an inflammatory response. Mice exposed to four phase shifts and LPS showed a decrease in core body temperature, which led to a decrease in survival rate. Mice exposed to LPS and only one phase shift also showed the same results which leads to the conclusion that regardless of how often circadian disruption occurs, there are drastic negative effects on the body. This data further indicates that peritoneal macrophages also exhibit significant changes in at least one important circadian clock gene: Bmal1. Relative abundance of Bmal1 was rhythmic in control mice, but constitutively low in shifted mice. Because the liver contains the largest pool of macrophages in the body, it is responsible for clearance of endotoxin and is a major source of inflammatory mediators during the early stages of inflammation. Thus, the liver may be an important target for jet lag-related morbidity and further investigation into the health effects of circadian disruption is clearly warranted.

A potential problem in this study is that either Clock or Bmal2 could overcompensate for Bmal1 when it is knocked out in mice. Ectopic expression of Bmal2 rescues metabolic phenotypes that are associated with the knockout of Bmal1 (Shi S., 2010). Shuqun et al. concluded that Bmal1 is not absolutely necessary for circadian rhythms of loco motor activity or metabolic phenotypes, but that it may functionally be replaced by expression of its paralog Bmal2 from a constitutively expressed promoter (Shi S., 2010). Since this study showed that Bmal2 has the adaptive tendency to rescue Bmal1 in knockout mice, measures would have to be taken to ensure that no compensatory mechanisms were formed. Bmal2 has been shown to play a minimal role in the clock system compared to its paralog, Bmal1, but nonetheless will be monitored.

There is an overwhelming amount of literature that supports the previously stated hypothesis and techniques that will be useful in gauging an inflammatory response in cytokines. Learning about circadian immune regulation should not only have a strong impact on the understanding of the pathophysiology of inflammatory responses but also on anti-inflammatory drug strategies (Keller M., 2009). An overwhelming amount of diseases are heavily characterized by the inflammatory process (i.e. cardiovascular, diabetes, obesity). The magnitude of the public health burden imposed by these and other diseases underscores the importance of relationships between sleep and immune function and our efforts to understand them (Imeri L., 2009). More questions are being raised more now than ever in regards to whether cytokines mediate infectious-induced alterations in sleep. As the overall health of the nation slowly declines, we are starting to incorporate the need for good sleep into diagnostic medicine and finally putting an emphasis on the risk factors associated with lack of sufficient sleep. According to recent reports, as little as 4 hours of sleep loss in a controlled laboratory setting increases the production of IL-6 and TNF by monocytes (Shi S., 2010). These modern advances in circadian rhythm and immune response have shown a promising future for medicine and inflammatory liver disease in particular.

METHODS

Over the course of this study, it is expected to demonstrate that expression of Bmal in hepatic macrophages is an important factor in the regulation of macrophage polarization, and that macrophage specific Bmal expression is necessary for a pro-inflammatory response to LPS. These observations would support the general hypothesis that intrinsic circadian components regulate hepatic macrophage polarization and are critical to inflammatory mediated liver injury. A key advance will be the use of a novel macrophage-specific Bmal-null mice generated by the Mac-cre transgenic system. As far as we know, this will be the first attempt using this innovative approach to characterize the role of circadian rhythm in regulation of hepatic immune response.

Subjects and Study Design

Mice lacking *bmal* were selected for this set of experiment for a number of reasons. First, Bmal is the master regulator of circadian gene expression and is required for Per2 expression. Second, unlike other molecular regulators of circadian rhythm, *bmal* allele targeted by floxed sites allows it to be deleted in a tissue specific manner. Thus, mice with the macrophage-specific deletion of the core circadian transcription factor Bmal were generated. The experiments performed here used the flox-cre system to generate macrophage-specific Bmal deficient mice (LysZ cre+ x Bmal.f/f) in order to test the hypothesis that Bmal function in macrophages is essential for the development of inflammatory liver injury. C57Bl/6j mice homozygous for the floxed *bmal* allele (bmal.f/f) were crossed to homozygous cre recombinase transgenic mice, where *cre* expression is under the control of a cell-specific promoter system. For the macrophage-specific deletion of Bmal, cre recombinase will be driven by lysozyme z promoter (lysz-cre). This strain has become a standard cre transgenic for targeting differentiated macrophage populations. A breeding line of mice bmal.f/f mice heterozygous for cre transgene

(bmal.f/f x cre+/) generated both lysozyme-cre positive macrophage-specific bmal-null (lysZ-Bmal-/-) and cre positive littermates (lysZ-cre-). Littermate cre-negative mice were used as controls. Genotypes were characterized by standard four-primer genomic DNA PCR approach. All procedures have been approved by the East Carolina University Institutional Animal Care and Use Committee.

To determine whether Bmal is required for hepatic macrophage response to LPS, LysZ-Bmal mice and cre-negative control littermates were given LPS (1.0 mg/kg) or saline. Groups were devised so that mice were sacrificed at 0, 3, and 24 hours after the injection of LPS or saline. The time course post-LPS administration is important since cytokines exhibit different expression kinetics.

Measurement Protocol and Equipment

First, liver injury was assessed using routine parameters (e.g., liver to body weight ratio, serum ALT, histology, gene expression). Serum ALT was assessed by standard biochemical photometric analyses. Liver sections were stained with hematoxylin/eosin (H&E) for pathological assessment.

The following protocol was used for H&E staining (steps are shown in order they were done): sections were submerged in Xylene for 6 minutes, 100% EtOH for 4 minutes, 95% EtOH solution for 4 minutes, 70% EtOH solution for 4 minutes, deionized water for 3 minutes, Harris Hemotoxin for 5 minutes. Sections were thoroughly rinsed in tap water and dipped into acid OH. Sections were then submerged back into 70% EtOH solution for 4 minutes, Eosin for 2 minutes, 100% EtOH for 6 minutes and Xylene for 4 minutes. Sections were mounted onto slides after removal from Xylene.

Secondly, to characterize the changes in cytokine gene expression in the absence of macrophage Bmal, subsets of LysZ cre+ x Bmal.f/f mice (lysZ-Bmal) and cre- littermates were sacrificed at 0, 3 and 24 hours. mRNA was harvested from whole liver and used to assess the expression of both M1 and M2 cytokine and chemokine response by real-time qPCR.

Whole liver mRNA isolation was performed according to the following procedure. Tissue samples were homogenized in Trizol phenol extraction buffer (500 μL). After samples were completely emulsified, another equal volume of Trizol (500 μL) was added. Samples incubated at room temperature for 5 minutes before chloroform (200 μL) were added. Samples were then mixed and then centrifuged (12,000 RPM) for 15 minutes at 4° C. Following centrifugation, the top layer of the solution was collected. Isopropanol (500 μL) was added and gently mixed. Samples were incubated for 10 minutes at room temperature. RNA was pelleted by centrifugation (12,000 RPM) for 10 minutes. The mRNA pellet was re suspended in 75% EtOH (500 μL) to wash. The mRNA was pelleted again at 8,500 RPM for 5 minutes. The remaining EtOH was removed, and the pellet was air dried for 10-15 min and suspended in 100 μL molecular grade RNAase-free water. Lastly, the samples were heated at 57° for 10 minutes before storage.

Total RNA (1 μg) was reverse transcribed to the first strand cDNA using high-capacity MMLv reverse transcriptase (Invitrogen Life Technologies). Relative quantitation of mRNA levels was performed by real-time qPCR (SYBR Green Assay; Ap- plied Biosystems) using an ABI prism 7700 Sequence Detector. Expression of core circadian, circadian-related transcription or regulatory factors and metabolic genes was be determined. A list of primer sequences that were used can be found in Table 1.

Statistical Analyses

Two-way ANOVA as well as a post-Hoc test will be performed after all experiments have been done to assess quality of data collection.

Expectations

Per-2, the downstream product of Bmal, acts as a transcriptional repressor of multiple nuclear receptors. Based on observations that loss of Per-2 blunted innate immune cytokine response to LPS, we expect a similar phenotype in the macrophage-specific Bmal-null mice. According to our hypothesis that Bmal is a regulator of macrophage polarization, we expect to demonstrate that the absence of Bmal in macrophages exacerbates LPS-induced pro-inflammatory cytokine response *in vivo*.

We expect that the deletion of Bmal specifically in hepatic macrophages to have no effect on systemic circadian behavior or function. Even though lysozymeZ cre expression is variable across all CD11b macrophage lineages, it is likely that other tissue macrophages such as splenic macrophages or microglia express cre. Since the total Bmal knockout has no overt phenotypic changes under normal conditions, we expect the same of the macrophage specific Bmal null strain. We do not expect that the macrophage specific Bmal null strain to have an altered circadian phenotype but only exhibit innate immune cell phenotypic changes.

Limitations and Future Experiments

It is difficult to predict the outcome of the deleting *bmal* in a cell-specific manor. Given the complexity and redundancies in the circadian mechanism, an alternative approach will be the use of the floxed *clock* gene transgenic mouse, which is also available at Jackson Laboratory.

It is not clear whether Kupffer cells are as “plastic” as non-resident macrophages. It is also difficult to distinguish is great number the difference between resident Kupffer cell and

infiltrating macrophages in liver. Thus, we hope to perform in parallel to Kupffer cells using M-CSF differentiated bone marrow derived macrophages (BMDMs) *in vitro* from cre-negative littermate controls and lysZ-Bmal mice in the future. The use of naïve BMDMs in addition to hepatic macrophages will allow us to interrogate polarization conditions without the interference of the hepatic microenvironment.

It would also be beneficial to stain for F4/80 in addition to H&E. F4/80 is a more thorough stain and will reveal more or less active Kupffer cells. Generally, the more inflammation there is, the more Kupffer cells will be present.

RESULTS

Effect of loss of Bmal on macrophage gene expression

To verify that Bmal gene expression was absent in macrophages isolated from untreated lysZ-Bmal -/- mice, mRNA levels of Bmal were analyzed (Figure 1). Compared to the untreated cre-negative littermate controls, Bmal mRNA levels in the lysZ-Bmal -/- mice were significantly reduced (Figure 1A). These data suggest that the knockout was effective.

To observe any alterations in circadian genes as a response to the loss of Bmal, mRNA levels of Per-2, Cry and Clock were measured (Fig. 1A). mRNA levels of Per-2, the antagonist to Bmal, were expressed in larger than normal quantities in the lysZ-Bmal -/- mice along with Clock and Cry, which were expressed in even larger amounts. Relative mRNA levels for all circadian genes remained the same in the cre-negative littermate controls. This data confirmed that when Bmal is deleted, the other circadian genes over express themselves in order to compensate for the loss of Bmal.

Effect of loss of Bmal on lipid metabolism genes in macrophages

To observe any changes in known circadian rhythm transcriptional regulators in untreated cre-negative littermate controls and lysZ-Bmal -/- mice, we isolated Kupffer cells and analyzed three circadian rhythm genes via RT-qPCR (Figure 1B). mRNA expression of Rev-erb, RORa and PPARg significantly decreased in the lysZ-Bmal -/- mice, showing less transcription activity. Gene expression remained unchanged in the cre-negative littermate controls. We also wanted to observe any changes in fatty acid transcription factors, which could be linked with the ability to store excess lipids. Expression of CD36 and FABP5 increased in the lysZ-Bmal -/- mice but remained the same in the cre-negative littermate controls. This data leads us to believe that mice lacking the Bmal gene will be more prone to store excess lipids in the liver.

Effect of loss of Bmal on macrophage pro-inflammatory cytokine production

To observe a pro-inflammatory cytokine response in untreated cre-negative littermate controls and lysZ-Bmal -/- mice, we isolated Kupffer cells and analyzed pro-inflammatory cytokines via RT, qPCR (Figure 1C). Levels for all pro-inflammatory cytokines remained unchanged in the cre-negative littermate controls. However, TNF-a levels increased along with IL-6 and IL-12 in the lysZ-Bmal -/- mice. gIFN levels in the lysZ-Bmal -/- were about the same as the levels in the cre-negative littermate controls.

Effect of macrophage-specific deletion of Bmal on hepatic function

To investigate the role of Bmal in cre-negative littermate controls and mice with a macrophage-specific deletion of Bmal were created and used for this experiment. In order to evaluate how the macrophage-specific deletion of Bmal impacted the expression on untreated liver pathology, liver to body weight ratio was measured (Figure 2A). Liver to body weight ratio is a measure of lipid accumulation typically as a consequence of liver dysfunction. Ten-month old, untreated cre-negative littermate controls and lysZ-Bmal -/- mice were sacrificed. Livers and blood samples from the portal vein were extracted after the animals were euthanized. Liver to body weight ratio was increased in untreated lysZ-Bmal -/- mice and decreased in the untreated cre-negative littermate controls. This data indicates that the macrophage-specific loss of Bmal is associated with a higher liver weight to body weight ratio.

Effect of macrophage-specific deletion of Bmal on liver injury

To assess general liver injury and inflammation in untreated mice, Hematoxylin and Eosin (H&E) staining was performed. Sections of liver were cut, mounted onto slides and stained to examine liver pathology (Figure 3). Liver histology appeared relatively normal with mild, micro fat accumulation in untreated cre-negative littermate controls. However, in untreated lysZ-

Bmal -/- mice, liver injury and fat accumulation drastically increased. This data revealed that the macrophage-specific loss of Bmal is associated with an elevated inflammatory response and increased liver injury.

Effect of macrophage-specific deletion of Bmal on LPS-induced liver injury

To investigate the role of Bmal in hepatic macrophages, mice with a macrophage-specific deletion of Bmal (lysZ-Bmal -/-) were created. In order to evaluate how the macrophage-specific deletion of Bmal impacted the expression on LPS-induced liver pathology, liver to body weight ratio was measured (Figure 2B). Liver to body weight ratio is a measure of lipid accumulation typically as a consequence of liver dysfunction. Three-month-old cre-negative littermate controls and lysZ-Bmal -/- mice were treated with LPS (1.0 mg/kg, intraperitineal) and sacrificed 3 hours later. Livers of the lysZ-Bmal -/- mice had apparent injury indicated by white, necrotic tissue but appeared normal in the cre-negative littermate controls. Livers and blood samples from the portal vein were extracted after the animals were euthanized. Liver to body weight ratio in the cre-negative littermate controls was increased minimally in LPS-treated cre-negative littermate controls. In untreated lysZ-Bmal -/- mice, the liver to body weight ratio was increased as the ratio in untreated cre-negative littermate controls decreased. However, LPS increased liver to body weight ratio compared to LPS-treated, cre-negative littermate controls. This data shows that LPS induced a minor, but statistically insignificant, increase in liver to body weight ratio. Importantly, the loss of Bmal in macrophages in itself led to a slight increase in liver to body weight ratio.

Effect of loss of Bmal on serum ALT values in LPS-treated mice

To assess the degree of liver injury, we collected serum ALT (Figure 4). ALT levels were relatively low in the cre-negative littermate controls after both 3 and 24 hours. Serum ALT

values in the lysZ-Bmal -/- mice remained low after only 3 hours but quadrupled compared to the cre-negative littermate controls after 24 hours, indicating that there is a higher rate of injury when the Bmal gene is not present for at least 24 hours. Serum ALT was not measured in the untreated group.

Effect of macrophage-specific deletion of Bmal on liver injury histology in LPS-treated mice

To assess general liver injury and inflammation in mice treated with LPS, Hematoxylin and Eosin (H&E) staining was performed (Figure 5). Sections of liver were cut, mounted onto slides and stained to examine liver pathology. Liver histology appeared abnormal with mild, micro fat accumulation in untreated cre-negative littermate controls. LPS administration led to an increase in liver injury in lysZ-Bmal -/- mice. In untreated lysZ-Bmal -/- mice, liver injury and fat accumulation drastically increased as untreated cre-negative littermate controls decreased. However, LPS increased the rate of inflammatory response compared to cre-negative littermate controls treated with LPS. This data showed that the macrophage-specific loss of Bmal is associated with an elevated inflammatory response and increased liver injury.

Effect of macrophage-specific loss of Bmal on hepatic gene expression following LPS exposure

Cytokines, along with certain circadian genes, were selected based on their pro-inflammatory or anti-inflammatory characteristics. Cytokine and gene expression were analyzed by RT, qPCR and normalized to the housekeeping gene 18s rRNA (Figure 6).

Pro-inflammatory cytokines

TNF-a relative mRNA levels started low in the lysZ-Bmal -/- mice but increased considerably after 3 hours. At 24 hours, expression of TNF-a declined in both the lysZ-Bmal -/-

and cre-negative littermate controls. In the cre-negative littermate controls, TNF-a relative mRNA levels were higher originally than the lysZ-Bmal -/- mice and steadily increased over 3 and 24 hours (Figure 6).

CCL-2 relative mRNA levels were very low at 0 hours in both the cre-negative littermate controls and lysZ-Bmal -/- mice but showed significant increases in the lysZ-Bmal -/- mice after 3 hours. There were minor increases from 0 to 3 hours in the cre-negative littermate controls. After 24 hours, CCL-2 expression significantly decreased in both groups (Figure 6).

iNOS expression was about the same in both groups at 0 hours and increased in both groups over 3 hours, more so in the lysZ-Bmal -/- mice. After 24 hours, mRNA levels declined in both groups to similar levels (Figure 6).

IL-12 expression was slightly higher in the lysZ-Bmal -/- mice than the cre-negative littermate controls at 0 hours. After 3 hours, IL-12 relative mRNA levels increased significantly in both groups, but were higher in the cre-negative littermate controls than lysZ-Bmal -/- mice for the first time. After 24 hours, IL-12 expression decreased about the same in both groups but remained slightly higher in the cre-negative littermate controls (Figure 6).

IL-6 relative mRNA levels were observed to be about the same at 0 hours in both groups and increased at 3 hours, more so in the lysZ-Bmal -/- mice. IL-6 expression decreased after 24 hours in both groups but remained slightly more elevated in the cre-negative littermate controls (Figure 6).

IRF-5 expression was higher in the lysZ-Bmal -/- mice than the cre-negative littermate controls at 0 hours and continued to rise to higher levels than the cre-negative littermate controls mice after 3 hours. IRF-5 mRNA levels decreased after 24 hours but remained higher in the cre-negative littermate controls (Figure 6). IRF-5 is a member of the interferon regulatory factor

(IRF) family, a group of transcription factors with diverse roles, including virus-mediated activation of interferon, and modulation of cell growth, differentiation, apoptosis, and immune system activity. IRF-5 acts as a molecular switch that controls whether macrophages will promote or inhibit inflammation.

Anti-inflammatory cytokines

IL-10 expression was much higher in the lysZ-Bmal -/- mice than the cre-negative littermate controls mice at 0 hours and increased after 3 hours in both groups, more so in the lysZ-Bmal -/- mice. IL-10 mRNA levels decreased in both groups after 24 hours but remained higher in the cre-negative littermate controls (Figure 7).

IRF-4 mRNA expression was also higher at 0 hours in the lysZ-Bmal -/- mice than the cre-negative littermate controls. After 3 hours, mRNA levels increased about the same in both groups and decreased after 24 hours. IRF-4 expression remained slightly higher in the cre-negative littermate controls than the lysZ-Bmal -/- mice after 24 hours (Figure 7). IRF-4 is a member of the interferon regulatory factor (IRF) family and is associated with an M2 inflammatory response.

Circadian genes

Per-2 relative mRNA expression was found to be significantly higher in the cre-negative littermate controls at both 0 and 24 hours than the lysZ-Bmal -/- mice. There was little to no expression for Per-2 in the lysZ-Bmal -/- mice after 24 hours (Figure 8).

Clock relative mRNA levels were higher in the lysZ-Bmal -/- mice at both 0 and 24 hours. Clock expression was significantly higher at 0 hours in the lysZ-Bmal -/- mice but was only slightly more elevated at 24 hours in the lysZ-Bmal -/- than the cre-negative littermate controls (Figure 8).

Rev-erb expression was significantly higher in the lysZ-Bmal -/- mice than the cre-negative littermate controls at both 0 and 24-hour time points (Figure 8).

DISCUSSION

In mammals, many aspects of daily behavior and physiology such as the sleep-wake cycle, body temperature, and liver metabolism are regulated by endogenous circadian clocks (Chidambaram V., 2011). Inflammatory diseases in particular exhibit strong time-of-day symptoms (Gibbs M., 2012). The purpose of this project was to demonstrate that this circadian clock and the genes primarily associated with it have a profound effect on the development of fatty liver disease and regulate an innate immune response. The main objective was to directly observe and provide supporting evidence that intrinsic circadian components regulate hepatic macrophage polarization and are critical to inflammatory mediated liver injury. It was hypothesized that in lysZ-Bmal -/- mice, cells stimulated with LPS will have an exacerbated pro-inflammatory response and a blunted anti-inflammatory response. We hoped to gain a better understanding of how certain physiology is regulated by circadian rhythm.

Unlike any other molecular regulator, the Bmal allele targeted by floxed sites allows it to be deleted in a tissue specific manner, meaning it can be removed exclusively in the macrophage. Using a macrophage specific model was important because to see how circadian rhythm works explicitly in the macrophage, not in the systemic circulation. More specifically, this was the first look at the role of macrophage-specific circadian gene expression in regulation of hepatic immune response and its influence on macrophage differentiation and inflammatory liver disease *in vivo*. This model proved effective in helping to provide evidence that Bmal regulates a macrophage response.

Per-2, the downstream product of Bmal, acts as a transcriptional repressor of multiple nuclear receptors. An experiment done by Wheeler et al. showed that an increase in Per-2 is consistent with the ablation of Bmal (unpublished data). To investigate the role of circadian Per-

2 expression in macrophage polarization, bone marrow cells were isolated and treated with M-CSF to rapidly proliferate and exhibited macrophage characteristics within 7 days. M-CSF derived BMDMs were largely F480+. F480- cells expressed CD11c, which is a predominant dendritic cell marker. Nearly 98% of F480+ BMDMs were CD11b+Gr1+, indicative of macrophage maturation. BMDMs exhibit a robust cytokine response to LPS. To characterize the "polarization" of BMDMs, cells were pretreated for 24h prior to LPS with Cx3Cl1, IL-12, or IL-4. Expression of genes associated with M1- and M2- like responses were quantified by RT-qPCR. It was apparent that IL-12 and Cx3CL exacerbated M1-like characteristics; albeit CX3Cl1 to a larger extent than IL-12. It was also observed that IL-4 strongly shifted the LPS response of macrophages toward an M2-like response. IL-4 alone was also a potent inducer of an M2-like response. BMDMs from wild type and Per-2 -/- mice were stimulated with LPS, in the presence of Cx3Cl1, IL-12, or IL-4. Per-2 -/- BMDMs expressed less M1-like genes following LPS exposure in comparison to wild type cells. Notably, M1 cytokines and iNOS expression was blunted, whereas M2 cytokines and arginase 1 was significantly enhanced, in Per-2 -/- cells compared to wild type cells. IL-4 caused a robust M2 polarization of Per-2 -/- cells compared to wild type cells. These exciting data suggest support the hypothesis that Per-2 is essential for M1 polarization of macrophages.

In a choline deficient diet model of fatty liver disease, Per-2-/- mice were resistant to fatty liver (Wheeler M.D., unpublished data). Compared to wild type mice fed choline deficient diet, Per-2-/- mice also had blunted serum ALT levels and hepatic TNF-α mRNA levels. Interestingly, Per-2 -/- mice had elevated lipolysis gene expression in liver, suggesting that elevated Per-2 suppresses lipid metabolism. Based on our observations that loss of Per-2 blunted innate immune cytokine response to LPS, we expected a similar phenotype in the macrophage

specific Bmal-null mice. According to our hypothesis that Bmal is a regulator of macrophage polarization, we expected to demonstrate that the absence of Bmal in macrophages exacerbates LPS-induced pro-inflammatory M1 cytokine response *in vivo*.

Macrophage polarization toward M1 or M2 phenotypes is driven by cues in the tissue microenvironment, which can include cytokines, growth factors and microorganism-associated molecular patterns (i.e. gut-derived LPS). These signals dictate a transcriptional response that shapes the phenotype and function of the macrophages on the basis of the physiological or pathophysiological context. Data revealed that macrophages from untreated mice lacking the Bmal gene had significant increased production of pro-inflammatory cytokines as well as a change in circadian response. TNF-α, IL-6 and IL-12 relative mRNA levels increased while untreated wild type levels remained unchanged. Per-2, Clock and Cry levels significantly increased potentially as an overcompensation mechanism in response to the loss of Bmal. Decreases were also seen in key circadian rhythm transcription factors such as Rev-erb, RORα and PPARγ. Not only did circadian rhythm genes decrease as a result of the loss of Bmal, but an increase in the expression of fatty acid transporters was also observed. FABP5 and CD36, well known fatty acid transporters, were increased in untreated lysZ-Bmal -/- mice but remained the same in wild type mice. This data leads to believe that mice lacking Bmal will have a greater ability to mobilize lipids, which is an important pathway in a pro-inflammatory response.

In mice, significant temporal dependence of LPS-induced endotoxic shock has been reported (Halberg J., 2009), and circadian disruption mimicking jetlag can greatly magnify LPS response (Castanon-Cervantes O., 2010). Many facets of immune function show diurnal variation, and recent studies have revealed that macrophages, important regulators of innate immune response, exhibit robust circadian oscillators in gene expression, including genes

responsible for pathogen recognition and cytokine excretion (Hayashi M., 2006). In a study done by Gibbs et al., circadian variation in endotoxin response was used to define key components involved in clock gating of the innate immune response. Using IL-6 as a primary M1 biomarker, Gibbs et al. showed that temporal dependence of murine responses to endotoxin challenge is abolished when the clock is disrupted in macrophages. Similarly to our findings, Bmal deletion caused constitutive nonrhythmic expression of Per-2 and Cry as well as suppressed transcripts for Rev-erb. Using a near identical mouse model to our study, Gibbs et al. also showed robust increases in relative mRNA levels of pro-inflammatory cytokines after being stimulated with LPS. Since LPS is not a strong inducer of M2 cytokines, an increase in M1 cytokine levels was expected along with a minimal change in M2 levels.

Pro-inflammatory cytokines are critical for lipid control and can disrupt lipid mobilization. Decreased hepatic ability to burn fat is another result of uncontrolled fat content in the liver. Our data revealed a chronic pro-inflammatory response, which could be a contributing factor to the significant lipid accumulation seen in the aged, untreated lysZ-Bmal -/- mice. In addition, substantial amounts of scarring and inflammation that indicate hepatic injury were observed in the lysZ-Bmal -/- mice treated with LPS. Elevated liver to body weight ratios were also seen in both the untreated and LPS-treated lysZ-Bmal -/- mice, which could also suggest higher lipid content in the livers lacking Bmal. This leads us to further believe that circadian regulation in macrophages plays an important role in lipid control and the development of fatty liver disease.

It was no surprise that a dramatic increase in serum ALT levels was seen after 24 hours in lysZ-Bmal -/- mice. Since serum ALT levels rise in disease states that cause hepatocellular injury, serum ALT levels can effectively identify an ongoing liver disease process as well as

correlate with the severity of nonalcoholic fatty liver disease (Kim J., 2011). These data further suggest that circadian rhythm directly influences liver injury.

In addition to glucoregulatory pathways, the circadian system also regulates lipid homeostasis and adipose tissue metabolism (Huang H., 2012). Both intestinal lipid transport and de novo lipid synthesis exhibit circadian variation (Hussain M.M., 2009), as do levels of adipose tissue hormones such as adiponectin (Gomez-Abellan P., 2010) and leptin (Licinio J., 1998). Hypertriglyceridemia is evident in *Clock* mutant mice (Turek F.W., 2005), likely due to both intestinal over absorption and hepatic overproduction (Pax D., 2007). One node of coupling circadian and lipogenic pathways involves REV-ERBα (Duez H., 2008). REV-ERBα controls SREBP signaling and bile acid homeostasis, both of which are essential for lipid metabolism (Le Martelot G., 2009). Furthermore, BMAL1 is necessary for adipogenesis, as embryonic fibroblasts from *Bmal1* knockout mice fail to differentiate into adipocytes (Shimba S., 2005). Clock genes may also indirectly regulate adipogenesis via PPARs. Finally, the SCN has also been shown to be critical for regulation of the diurnality of leptin release (Kalsbeek A., 2001, Ahima R.S., 1998). Further studies are necessary to assess the potential impact of clock genes on additional functions of the adipocytes, including thermogenesis and lipokine secretion.

With regards to the field of exercise physiology, this study could have an influence on how the body recovers post exercise. Getting adequate sleep is essential when it comes to maximizing anabolic hormone production, both testosterone and human growth hormone (HGH). Both of these hormones are regulated by circadian rhythm, with HGH being produced in waves during deep, REM sleep (Van Cauter E., 2005), and testosterone production growing during sleep and peaking around dawn. HGH, a key hormone that increases muscle mass, is both anabolic (promotes protein synthesis) and lipolytic (promotes fat burning) (Bidlingmaier N.,

2004). With nearly 70% of HGH production occurring during sleep, any circadian disruption could be detrimental to skeletal muscle regeneration/repair and physical performance. While men obviously produce more testosterone than women, it's contribution to protein synthesis and anabolic drive is well known. Thus, sufficient sleep is a must for optimal daytime performance and HGH production.

In this particular field, there seem to be more questions than answers regarding macrophage polarization and circadian rhythm. The liver has a remarkable capacity to regenerate after injury; yet, the role of macrophages in this process remains controversial mainly due to difficulties of distinguishing between different macrophage subsets (Zigmond B., 2001). It is not clear whether Kupffer cells are as "plastic" as non-resident macrophages. It is also difficult to distinguish a difference between resident Kupffer cell and infiltrating macrophages in the liver. Thus, a future experiment to perform in parallel would be to use M-CSF differentiated bone marrow derived macrophages (BMDMs) from cre-negative littermate controls and lysZ-Bmal mice. The use of naïve BMDMs in addition to hepatic macrophages will allow us to interrogate polarization conditions without the interference of the hepatic microenvironment. The molecular mechanism which couple immune function to the circadian clockwork remains largely unknown, however, these exciting data propose that circadian gene expression regulates a macrophage response and have a profound effect on the progression of fatty liver disease.

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Primer	Sequence
18s	5’-CTTAGAGGGACAAGTGGCG-3’ (forward) 5’-ACGCTGAGCCAGTCAGTGTA-3’ (reverse)
Bmal	5′-AGTACGTTTCTCGACACGCAATAG-3′ 5′-TGTGGTAGATACGCCAAAATAGCT-3′
CCL-2	5’-TTCACAGTTGCCGGCTGG-3’ 5’-TGAATGAGTAGCAGCAGGTGAGTG-3’
CD36	5′-CAGCTCATACATTGCTGTTTATGCATG-3’ 5’-GGTACAATCACAGTGTTTTCTACGTGG-3’
Clock	5’-TCTGGTGAAGGAAATGCGTAAA-3’ 5’-GCAGCCTTGGAATGTCTTTCTC-3’
Cry	5′-CGGTTGCCTGTTTCCTGACT-3′ 5′-GCTCCAATCTGCATCAAGCA-3′
FABP5	5’-TTCGATGAAATCACC-3’ 5’-GGTCGACTTTCCATC-3’
IFN-g	5’-AGCTCTTCCTCATGGCTGTT-3’ 5’-TTTGCCAGTTCCTCCAGATA-3’
IL-6	5’-GAGGATACCACTCCCAACAGACC-3’ 5’-AAGTGCATCATCGTTGTTCATACA-3’
IL-10	5’-GGTTGCCAAGCCTTATCGGA-3’ 5’-ACCTGCTCCACTGCCTTGCT-3’
IL-12	5’-CTCACCTGTGACACGCCTGA-3’ 5’-CAGGACACTGAATACTTCTC-3’
iNOS	5’-GAGATTGGAGGCCTTGTG-3’ 5’-TCAAGCACCTCCAGGAACGT-3’
IRF-4	5’-GACCAGTCACACCCAGAAATCCC-3’ 5’-GTTCCTGTCACCTGGCAAC-3’
IRF-5	5’-TGACGTCACTGGAGTTGTACGG-3’ 5’-GGTTCATGTCATGGATGGTGC-3’
Per-2	5’-TTGGGTGGATGCTCACACTG-3’ 5’-TTGCCCATGCAGATTCCC-5’
PPAR-g	5’-CTGTTTTATGCTGTTATGGGTGAAA-3’ 5’-GCACCATGCTCTGGGTCAA-3’
Rev-erb	5’-CAGCTGATGGTCCCAGTGAA-3’ 5’-TTCCTTGACCTTATTCTCCACGAT-3’
RORa	5′-ATGGAGCTGTGTCAAAATGATCA-3′ 5′-AGGCACGGCACATCCTAATAA-3′
Srebp1c	5’-GGAGCCATGGATTGC-3’ 5’-GGAAGTCACTGTCTT-3’
TNF-a	5'-AGCCCACGTAGCAAACCACCAA-3' 5’-ACACCCATTCCCTTCACAGAGCAAT-3’

Table 1. List of used PCR primers with corresponding forward and reverse transcription sequences.

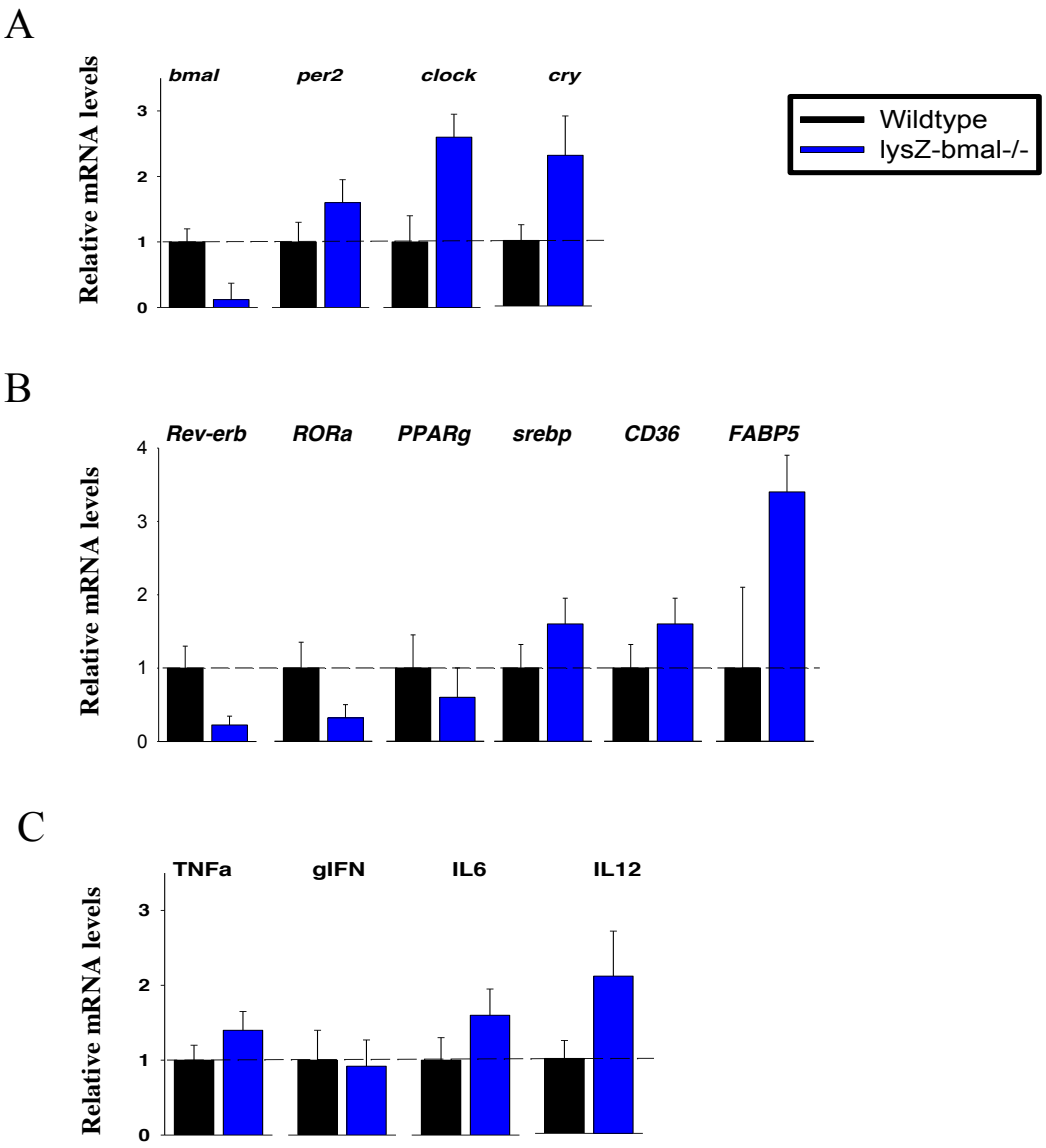

Figure 1. Effect of loss of Bmal on hepatic macrophage gene expression. Hepatic macrophages from untreated control cre- mice and Lyso-Bmal-/- mice were isolated. Cytokine and gene expression was analyzed by qPCR and normalized to the housekeeping gene 18s rRNA. *(A)* Effect of loss of Bmal on macrophage gene expression. *(B)* Effect of loss of Bmal on lipid metabolism genes in macrophages. *(C)* Effect of loss of Bmal on macrophage pro-inflammatory cytokine production.

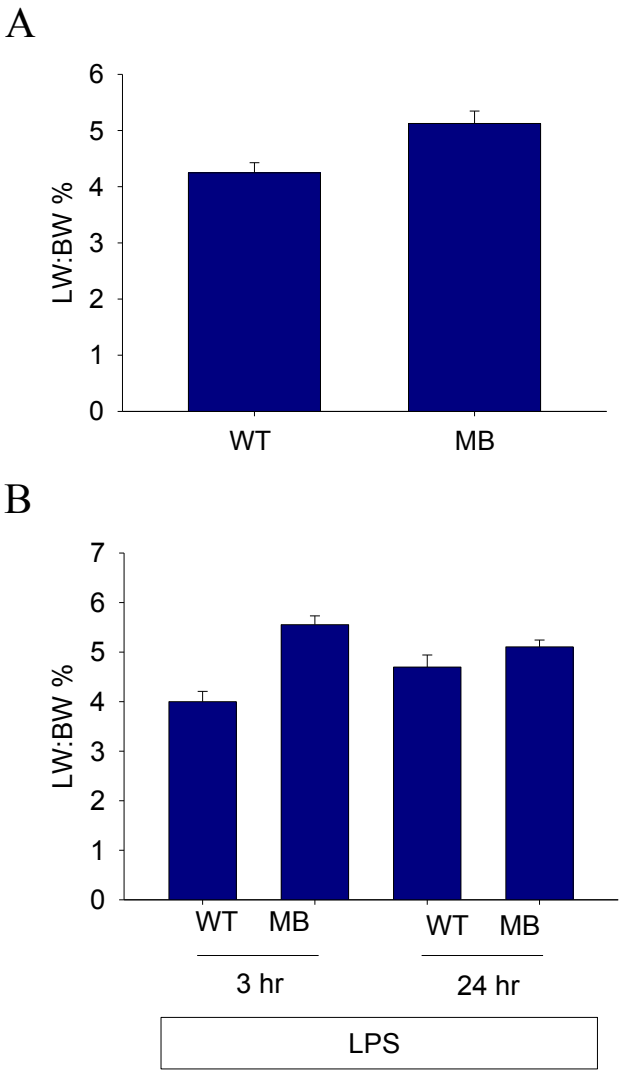

Figure 2. Effect of deletion of Bmal on hepatic lipid accumulation. (A) Liver to body weight was determined for both control cre- mice (WT) and LysZ-Bmal-/- (MB) in at 10 months of age. *(B)* Effect of macrophage-specific loss of Bmal on LPS-induced liver injury and lipid accumulation. Livers were weighed post extraction and measured in comparison to total relative body weight. WT represents cre-negative littermate controls. Data are expressed as mean ±SEM and are representative of 4-6 mice per group.

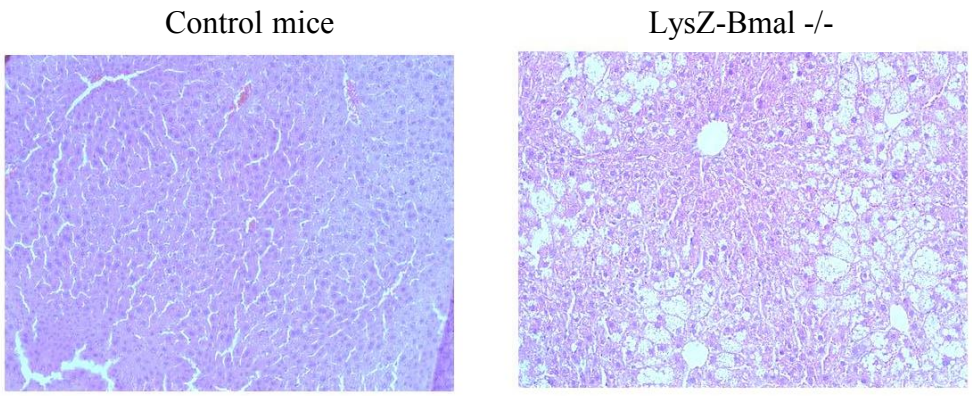

Figure 3. Effect of macrophage-specific loss of Bmal on liver histology. Photomicrographs (10X) of H&E histochemical staining of livers from untreated cre-negative littermate control mice and LysZ-Bmal -/- mice at 10 months of age. Photomicrographs are representative of 4-6 mice per group.

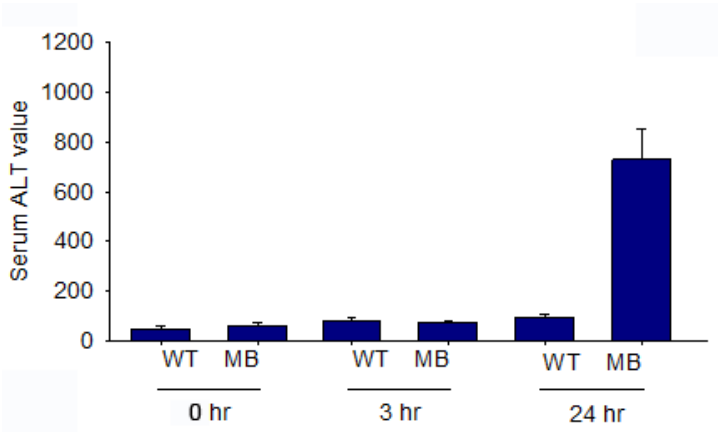

Figure 4. Effect of macrophage-specific loss of Bmal on LPS-induced liver injury. Serum ALT levels were determined from serum harvested from control cre- littermates (WT) and LysZ-Bmal-/- (MB) mice at 0, 3 and 24 after LPS (1mg/kg, ip) exposure. Data are expressed as mean ±SEM and are representative of 4-6 mice per group.

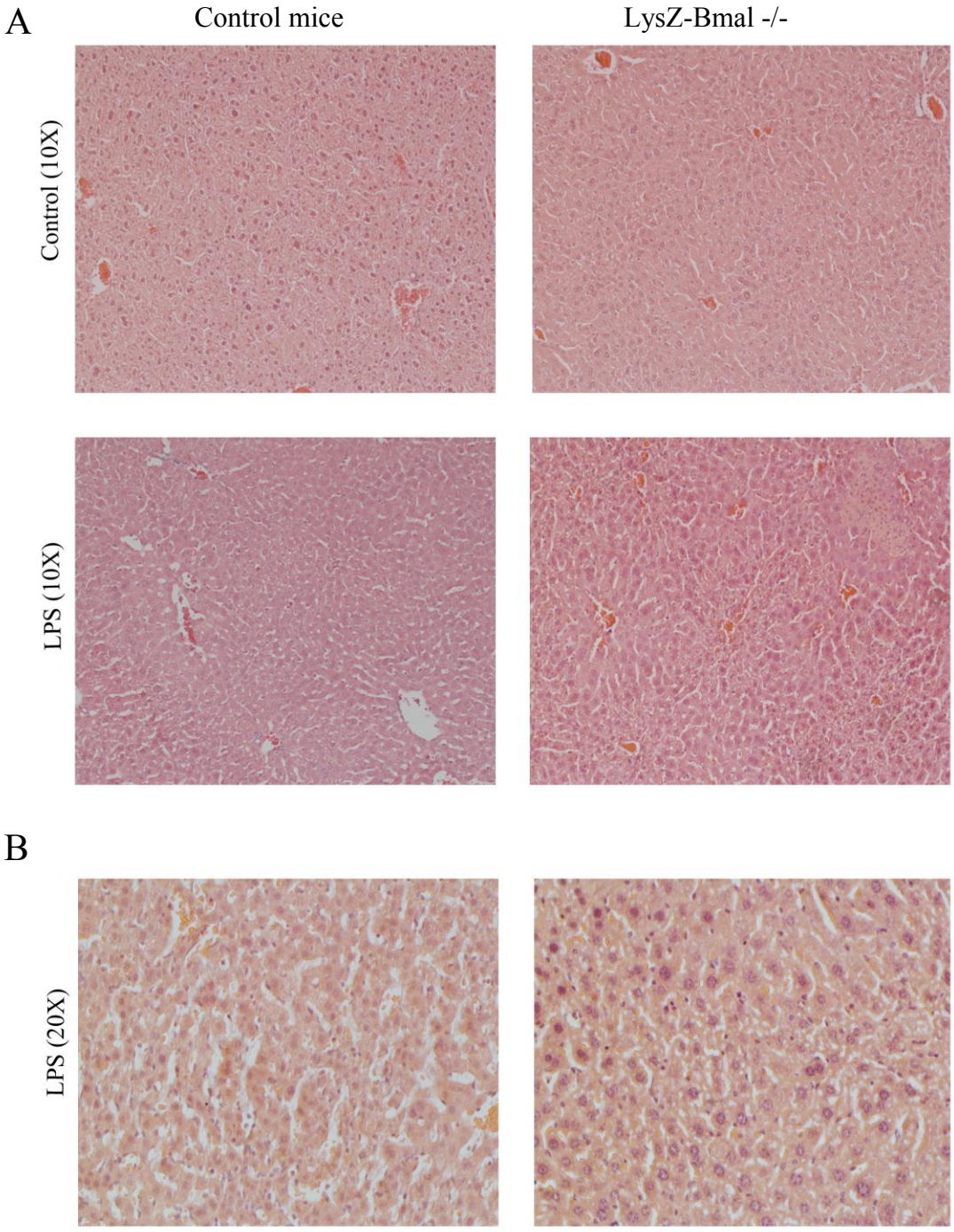

Figure 5. Effect of loss of Bmal on liver pathology in LPS-treated mice. (A) Photomicrographs (10X) of H&E histochemical staining of livers from cre-negative littermate control mice and LysZ-Bmal -/- mice treated with vehicle (control) or LPS (1mg/kg, ip) after 24 hours. (B) Photomicrographs (20X) of control and LysZ-Bmal-/- treated with LPS (1mg/kg, ip) after 24 hours. Photomicrographs are representative of 4-6 mice per group.

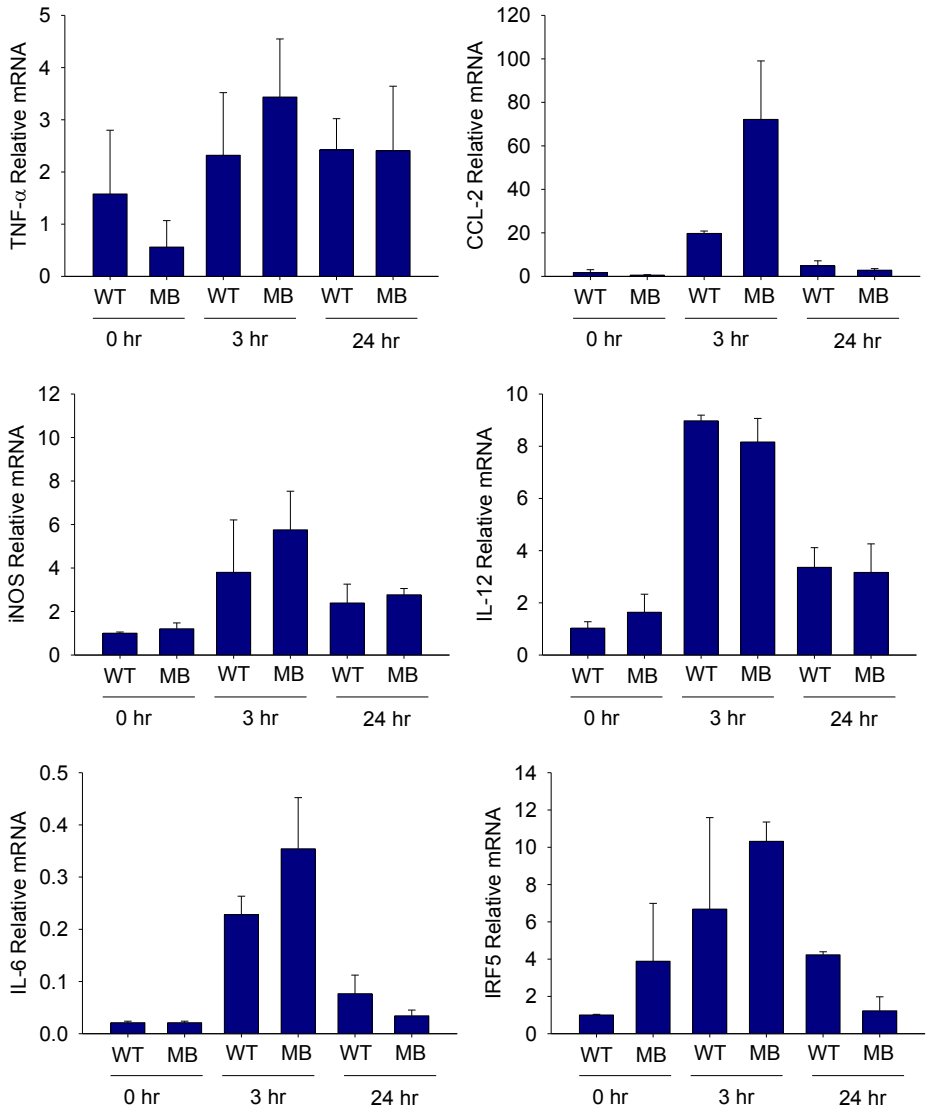

Figure 6. Effect of macrophage-specific loss of Bmal on hepatic pro-inflammatory related gene expression following LPS exposure. Whole liver mRNA was harvested from control cre-littermates (WT) and LysZ-Bmal-/- (MB) mice at 0, 3 and 24 hours after LPS (1mg/kg, ip) exposure. Relative mRNA levels of pro-inflammatory genes TNFα, CCL2, iNOS, IL-12, IL-6 and IRF5 was analyzed by real-time qPCR and normalized to the housekeeping gene 18s rRNAl -/- mice. Data are expressed as mean ±SEM and are representative of 4-6 mice per group.

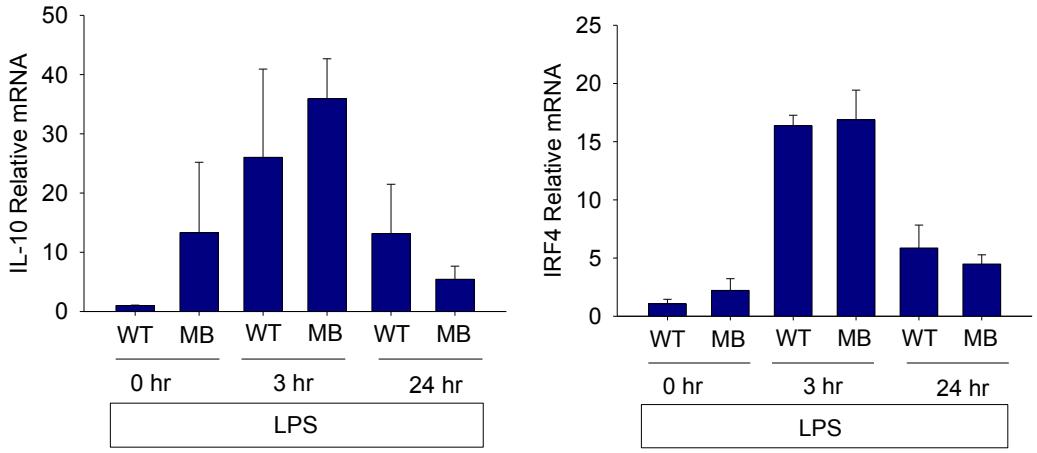

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Figure 7. Effect of macrophage-specific loss of Bmal on hepatic anti-inflammatory related gene expression following LPS exposure. Whole liver mRNA was harvested from control cre-littermates (WT) and LysZ-Bmal-/- (MB) mice at 0, 3 and 24 hours after LPS (1mg/kg, ip) exposure. Relative mRNA levels of anti-inflammatory/regulator genes IL-10 and IRF4 was analyzed by real-time qPCR and normalized to the housekeeping gene 18s rRNAl -/- mice. Data are expressed as mean ±SEM and are representative of 4-6 mice per group.

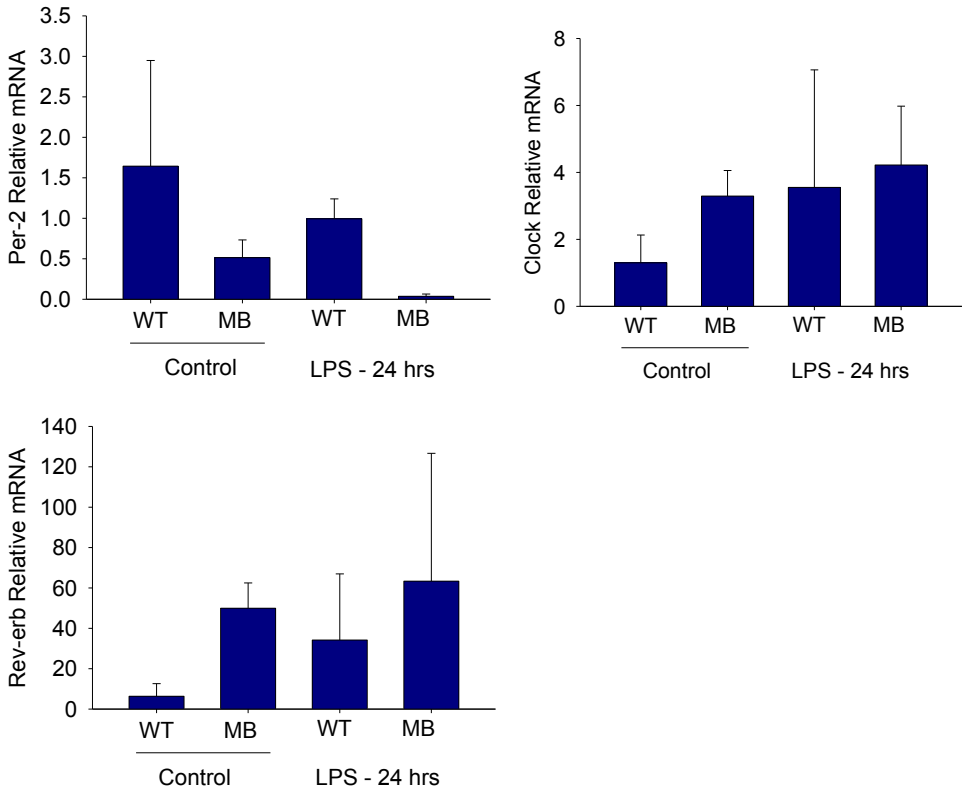

Figure 8. Effect of macrophage-specific loss of Bmal on hepatic circadian related gene expression following LPS exposure. Whole liver mRNA was harvested from control cre-littermates (WT) and LysZ-Bmal-/- (MB) mice 24 hours after vehicle (control) or LPS (1mg/kg, ip) exposure. Relative mRNA levels of core circadian genes per-2, clock and rev-erb was analyzed by real-time qPCR and normalized to the housekeeping gene 18s rRNAl -/- mice. Data are expressed as mean ±SEM and are representative of 4-6 mice per group.

APPENDEX A: IACUC Approval Letter

East Carolina University

Animal Care and Use Commitee
212 Ed Warren Life Sciences Building
East Carolina University
Greenville, NC 27834

252-744-2436 office
252-744-2355 fax

July 18, 2012

Michael Wheeler, Ph.D.
Department of Nutrition
Rivers Building
East Carolina University

Dear Dr. Wheeler:

Your Animal Use Protocol entitled, "Hepatic Immunity and Chronic Liver Disease" (AUP #P075) was reviewed by this institution's Animal Care and Use Committee on 7/18/12. The following action was taken by the Committee:

"Approved as submitted"

Please contact Dale Aycock at 744-2997 prior to hazard use

A copy is enclosed for your laboratory files. Please be reminded that all animal procedures must be conducted as described in the approved Animal Use Protocol. Modifications of these procedures cannot be performed without prior approval of the ACUC. The Animal Welfare Act and Public Health Service Guidelines require the ACUC to suspend activities not in accordance with approved procedures and report such activities to the responsible University Official (Vice Chancellor for Health Sciences or Vice Chancellor for Academic Affairs) and appropriate federal Agencies.

Sincerely yours,

Susan McRae, Ph.D.
Chair, Animal Care and Use Committee

SM/jd

enclosure