Circadian Clock Proteins and Immunity

Anne M. Curtis [1*], Marina M. Bellet [2, 4], Paolo Sassone-Corsi [2] & Luke A.J. O'Neill [1]

[1] School of Biochemistry and Immunology, Trinity Biomedical Sciences Institute, Trinity College Dublin, Dublin 2, Ireland

[2] Center for Epigenetics and Metabolism, Department of Biological Chemistry, University of California, Irvine, Irvine, CA 92697, USA

[4] Present address: Department of Experimental Medicine, University of Perugia, 06132 Perugia, Italy

[*] Correspondence to Anne M. Curtis, Email: Acurtis@tcd.ie

Immune parameters change with time of day and disruption of circadian rhythms have been linked to inflammatory pathologies. A circadian clock-controlled immune system might allow an organism to anticipate daily changes in activity and feeding and the associated risk of infection or tissue damage to the host. Responses to bacteria have been shown to vary depending on time of infection, with mice being more at risk of sepsis when challenged ahead of their activity phase. Studies highlight the extent to which the molecular clock, most notably the core clock proteins BMAL1, CLOCK and REV-ERBα, control fundamental aspects of the immune response. Examples include the BMAL1:CLOCK heterodimer regulating toll-like receptor (TLR9) expression and repressing expression of the inflammatory monocyte chemokine ligand (CCL2) as well as REV-ERBα suppressing the induction of interleukin-6. Understanding the daily rhythm of the immune system could have implications for vaccinations and how we manage infectious and inflammatory diseases.

Introduction

Organisms require the ability to tell the time of day in order to anticipate and respond to changes in the external environment imposed by solar time. This need is illustrated in the physiological changes required to accommodate the daily pattern of rest, activity and feeding. Circadian rhythms are these daily changes or oscillations in physiology and are due to numerous genes whose expression peaks and troughs approximately 12 hours apart to undergo a full cycle within 24 hours. The full breadth of control by the molecular clock on the genome was revealed by Zhang et al. who harvested multiple tissues at 2 hour intervals and found that more than a third of the mammalian genome is under clock control (Zhang R., 2013).

The master clock resides in the suprachiasmatic nucleus (SCN) of the brain and is oriented to the external environment via the retinohypothalamic tract receiving light information from the eye. The clock is cell autonomous and in its simplest form, consists as a transcription-translation oscillator loop. At the core of this oscillator lies the heterodimeric partnership of the basic helix loop helix PER-ARNT-SIM (PAS) domain proteins, BMAL1 (also known as ARNTL) and CLOCK, that bind E-box sites and induce the expression of the repressors Period (PER) and Cryptochrome (CRY) which in time translocate back into the nucleus and inhibit their own expression by interfering with the BMAL:CLOCK complex (Fig.1). As PER and CRY proteins are gradually degraded, the repression on BMAL1 and CLOCK is relieved and the cycle begins afresh with another 24h cycle. This machinery lies at the heart of the circadian clock in the SCN. However, an important finding was the existence of the same clockwork machinery in peripheral cells (Schibler and Sassone-Corsi, 2002) including fibroblasts cultured *in vitro* using what is now known as the serum shock model (Balsalobre et al., 1998). The serum shock model synchronizes cells so that circadian gene expression and outputs of the clock can be analyzed within an *in vitro* system. Since then, the molecular clock has been characterized in immune cells (Boivin et al., 2003) including macrophages (Keller et al., 2009), T-cells (Bollinger et al., 2011) dendritic cells and B-cells (Silver et al., 2012a).

Here we discuss recent findings indicating that certain clock proteins, notably,

BMAL1, CLOCK and REV-ERBα can indeed impact on immune cell function, host defense and inflammation. The emerging picture is that time of day is critical to the nature of the immune response and that dysregulation may lead to inflammatory diseases or immunodeficiency. New therapeutic options are presenting themselves that might provide a new prospect for correcting pathologies associated with aberrant immunity and inflammation.

The Anatomy of the Molecular Clock

Molecularly, the core oscillator is comprised of at least three interlocking feedback loops. As stated above the central loop consists of the BMAL1:CLOCK heterodimer driving expression of the repressors, PER1-3 and CRY1-2, which feedback and limit their own expression (Fig. 1). This loop provides oscillations in *Per* and *Cry* transcription and also oscillations in the activity of the BMAL:CLOCK heterodimer. The second loop consists of the nuclear receptors, RAR-related orphan receptor (ROR) (α,β,γ) and REV-ERB (α,β□ (also known as NR1D1 and NR1D2) □which through E-box activation by BMAL1:CLOCK can then translocate back into the nucleus to bind to receptor-related orphan receptor response elements (ROREs) in the promoter of *Bmal1* (Fig. 1). RORs activate whereas REV-ERBs repress the expression of BMAL1 (Preitner et al., 2002; Sato et al., 2004). The third loop is comprised of the transcriptional activator albumin D-box binding protein (DBP), regulated through its E-box and the repressor nuclear factor interleukin 3 (NFIL3; also known as E4BP4), regulated via a RORE. These two factors then synergistically regulate the expression of D-box genes including that of *Per*. This tripartite system forms the foundation of the molecular oscillator and controls the circadian network of clock-controlled genes. In addition to this network of transcriptional control, posttranslational modifications provide a further layer of regulation onto this network with various kinases and phosphatases regulating the precision and function of the clock along with histone modifications and the epigenetic code. These areas have been reviewed extensively elsewhere (Aguilar-Arnal and Sassone-Corsi, 2013; Bellet and Sassone-Corsi, 2010; Feng and Lazar, 2012).

Although not entirely understood, the SCN clock keeps peripheral clocks in harmony

via the hypothalamus pituitary adrenal (HPA) axis and the autonomic nervous system (ANS), and their respective hormones glucocorticoids and the catecholamines (epinephrine and norepinephrine) which act as synchronizing messengers or "zeitgebers" to the peripheral clock (Curtis et al., 2007; Kalsbeek et al., 2012). Circulating glucocorticoids, catecholamines and other hormones such as prolactin, melatonin and growth hormone, all of which affect the immune system, peak at certain times of the day. The control by the SCN on these autonomic and endocrine outputs keeps peripheral clocks, including that of immune cells, in phase with each other and allows for the coordination of a temporal program of physiology across many tissues at certain times of the day (Guo et al., 2006).

The Molecular Clock Controls Anti-bacterial Host Defense, Sepsis and Inflammation

Pioneering studies in the 1960s and 70s showed a striking circadian variation in survival when mice were treated with lethal doses of bacteria or bacterial products (Halberg et al., 1960; Shackelford and Feigin, 1973). These studies showed enhanced lethality towards the end of the resting phase, approximately 2 hours before onset of activity. Mice are nocturnal species and therefore the onset of activity occurs when lights are turned off in a mouse facility. This is denoted by Zeitgeber Time (ZT) relating to the time at which the lights are turned on and off in the animal facility. ZT0 denotes lights on and start of inactive phase and ZT12 denotes lights off and start of activity phase (Fig.2). Numbers of leukocytes in the mouse circulation have a strong circadian variability and reach their highest number at around ZT5, and are recruited in greater numbers into tissues at ZT13, just after the animal's transition to activity (Scheiermann et al., 2012). These changes coincide with the sensitivity to lipopolysaccharide (LPS) in which the observed phenotype of increased cytokine release at ZT12 (Gibbs et al., 2012) could be in part due to rising leukocyte numbers at this transition point. Host defense response is also altered according to circadian time. Bellet et al. reported that, after intragastric infection of wild type mice with the pathogen *Salmonella Thyphimurium* at different circadian times, ZT4 (day) and ZT16 (night), the degree of intestinal inflammation and cytokine gene expression, as well as bacterial colonization, change depending on both time of infection and time of sacrifice (Bellet et al., 2013). The ability to clear the bacteria 72 hours post infection

(hpi) from the colon was greater in mice infected during the night (ZT16) than during the day (ZT4), however *Clock* mutant mice had lower numbers of bacteria at both time points. The authors found that the growth of *Salmonella* and its ability to compete with other microorganisms in the gut is dependent on the circadian regulation of antimicrobial peptides. They found that the antimicrobial peptide lipocalin-2, to which *Salmonella* is resistant, is higher during the day than at night. This suppresses the resident microbiota allowing for the higher outgrowth of *Salmonella* during the day versus the night.

In addition, a circadian variation has been found in the ability of mouse macrophages to ingest particles with the increase observed ahead of the transition to activity (Hayashi et al., 2007). This may also enhance bacterial clearance at the beginning of the active phase. The induction of pro-inflammatory cytokines and chemokine ligands is far greater when mice are challenged with LPS at ZT12 versus ZT0. This includes a greater induction of interleukin-6 (IL6), IL-12(p40), chemokine (C-C motif) ligand 2 (CCL2) and CCL5 from macrophages challenged at ZT12 versus ZT0 (Gibbs et al., 2012). Again these differences go some way to explaining the boost in host defense as mice transition into activity, but equally the greater risk of sepsis due to an over active response. Keller et al. harvested peritoneal macrophages from mice every 4 hours for 24 hours and performed microarray analysis. They found that 8% of the macrophage transcriptome was cycling with a circadian variation, and when they analyzed genes involved in the toll-like receptor 4 (TLR4) pathway they found circadian control on genes involved in all aspects of the TLR4 response. This indicates further that the entire TLR4 pathway in macrophages is under tight circadian control and suggests again that the circadian clock prepares the immune cell for an integrated response at times of greatest risk.

Therefore the temporal increase in chemo-attractants, leukocyte trafficking, proinflammatory cytokines and phagocytic ability in the hours approaching the commencement of activity is interpreted as being indicative of clock-controlled enhanced sensitivity and immunosurveillance ahead of activity and feeding when the risk of infection would be highest. Conversely, perturbations or desynchronization of the clock (by modulating light input) can adversely affect immune function. Under constant dark conditions (Marpegan et al., 2009), or with circadian desynchronization

by experimental jet-lag (Castanon-Cervantes et al., 2010), a challenge of LPS in mice leads to an uncoordinated and more severe inflammatory response and increased mortality. One molecular feature of circadian desynchronization by jet lag is a sustained reduction in *Bmal1* transcript (Castanon-Cervantes et al., 2010). This mirrors a ZT12 macrophage, in which the circadian phase of *Bmal1* mRNA is at its lowest (Keller et al., 2009). Macrophages subjected to jetlag and ZT12 macrophages have a heightened inflammatory response once activated by LPS, as both have low expression of BMAL1 prior to activation. This suggests that BMAL1 may be one of the central gatekeepers governing the circadian inflammatory response (Fig. 3). BMAL1 will be discussed in more detail later.

The effects of the central clock in the SCN and its rhythmic control on autonomic and endocrine regulators are important determinants of immune modulation (Logan and Sarkar, 2012). The SCN produces diffusible signals as well as axonal projections to both the paraventricular nucleus (PVN) and arcuate nucleus (ARC) in the hypothalamus of the brain. For example the PVN governs noradrenergic input to the spleen and releases norepinephrine to modulate the activity of natural killer (NK) cells (Logan et al., 2011). Also, the enhanced recruitment of neutrophils to skeletal tissues at ZT13 is dependent on circadian noradrenergic input to those skeletal tissues, producing rhythmic expression of the adhesion molecule ICAM-1 (Scheiermann et al., 2012). The glucocorticoids are under clock control. The SCN and adrenal clock drive rhythms in glucocorticoid secretion from the adrenal glands. The SCN controls the release of adrenocorticotropin (ACTH) from the pituitary gland and ACTH in turn stimulates release of glucocorticoids from the adrenal gland (Dickmeis et al., 2013). The adrenal clock then controls sensitivity of the adrenal gland to ACTH (Oster et al., 2006). Glucocorticoids exhibit broad anti-inflammatory properties and can control cytokine production and leukocyte trafficking (Coutinho and Chapman, 2011). Glucocorticoids peak as the animals transition to the active phase, and may possibly be required at that time due to the heightened risk of infection and injury. A comprehensive analysis of the role and targets of glucocorticoids in the circadian control of innate immunity has yet to be performed. Importantly, lack of endocrine rhythms does not ablate the rhythms in immune cell function as mice lacking the adrenal glands and splenocytes in culture still maintain rhythms of cytokine release over a number of days (Keller et al., 2009). This suggests that the intracellular

immune clock also plays a role in determining the circadian rhythm of immune function.

In simple terms, the circadian system may partition the immune system into two states over the circadian day (Fig. 2). One being a state of heightened alert as the animal prepares to transition to activity and the risk of infection or injury is greatest. This state would require an increase in leukocyte numbers along with increased sensitivity of immune cells to infectious agents or danger signals. The second state would occur when the animal rests and the risk of infection and injury is lessened. This state may provide an opportunity for resolution of inflammation and repair of tissues.

It is no surprise that the immune system would be under circadian control given that a clock system would enable a precise, integrated and cohesive response across a number of immune cells. The molecular clock could act as a "ground controller" for immune cells, orchestrating a response, dependent on the integration of a range of inputs, including neural, hormonal, and local factors along with the circadian phase of gene expression within the cell. There is a daily rhythm to life, and with that comes daily challenges for which an organism must prepare for and respond to. For example, the clock system prepares an organism to anticipate the onset of activity and feeding, but with that comes the increased threat of infection associated with foraging and feeding. Given the intense energy demands for mounting an immune response a clock might allow the body to utilise energy differently across tissues during periods of rest versus periods of activity. If the immune system needs to be "poised for attack" just as the animal enters the activity and feeding phase, a clock might then allow for the enhancement of immune function with resolution and repair of tissues occurring at alternative times (Fig. 2).

Specific Clock Proteins in Immune Cell Function

More recently, roles for the specific clock proteins in the immune response have been studied. Mice with clock gene manipulations have unveiled the important contribution of the core clock genes on the immune response. In this section we will discuss the

core clock components, paying particular attention to BMAL1, CLOCK, REV-ERBα and RORα and the emerging data surrounding their impact on the immune system.

BMAL1

BMAL1 is the central clock component and is the only single clock gene knockout in which the mouse looses all rhythmic behavioral activity (Bunger et al., 2000). Global deletion of *Bmal1* causes a range of underlying pathologies under steady state conditions related to accelerated aging (Kondratov et al., 2006) thus making them unsuitable for investigating immune function. Using a mouse model in which the *Bmal1* gene was selectively deleted from myeloid cells, Nguyen et al investigated the role of myeloid BMAL1 in the clearance of the gram-positive bacterium *Listeria Monocytogenes* (Nguyen et al., 2013). Ly6C^{hi} monocytes provide the first line of defense against this bacterium and the authors had detected a diurnal variation in the absolute numbers of these specific monocytes in blood and in spleen under basal conditions, and enhanced recruitment of these monocytes to an inflamed peritoneum at ZT8 versus ZT0. Improved bacterial clearance was observed at ZT8 versus ZT0 and this was likely to be due to the higher recruitment of Ly6C^{hi} monocytes at sites of infection and a higher production of the pro-inflammatory cytokines, IL-1β, IL-6, tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) and the chemokine ligand CCL2. The rhythmic oscillation in the numbers of Ly6C^{hi} monocytes in circulation across 24 hours and enhanced recruitment of these cells into inflamed tissue was entirely dependent on BMAL1 in myeloid cells as the circadian control over Ly6C^{hi} was abolished in the myeloid BMAL1-depleted mice. The authors found that BMAL1 binds to E-boxes in the promoters of *Ccl2* (encoding chemokine ligand 2; Ccl2), *Ccl8* and *S100a8* (encoding S100 calcium binding protein A8) and recruits with it members of the polycomb repressor complex (PRC2) epigenetically marking histones for repression. The authors went onto show that BMAL1 reduces *Ccl2* transcription and attenuates Ly6C^{hi} monocyte numbers and inflammation at an inflamed site.

Myeloid deletion of *Bmal1* also exacerbates metabolic disease driven by a high fat diet. The authors speculated that this could be due to BMAL1 limiting Ly6C^{hi} monocytosis and recruitment into metabolically stressed tissues such as the fat pads. The absence of BMAL1 in these cells leads to chronic inflammation, insulin

resistance and hyperglycemia.

BMAL1 therefore functions as an anti-inflammatory molecule in monocytes in part because of its repression on *Ccl2*. This anti-inflammatory effect of BMAL1 was confirmed previously in another study (Gibbs et al., 2012). Peritoneal macrophages lacking BMAL1 produced higher amounts of IL-6 in response to LPS at ZT0 in comparison to wild type peritoneal macrophages. In circulating monocytes and in peritoneal macrophages, *Bmal1* mRNA is high at ZT0 and low at ZT12 and this could impact on the response to LPS or infection at these two times. However, it is not the absolute amount of *Bmal1* mRNA but the binding of BMAL1 to gene promoters that will determine the magnitude of response. ChIP-Seq analysis conducted on mouse livers harvested every 4 hours across the 24 hours revealed that BMAL1 binding to gene promoters rises from circadian time (CT, see glossary) CT0 to CT8 and this then falls from CT12 to CT20 (Koike et al., 2012). When the animals transition to activity, the reduced binding of BMAL1 to promoters may allow for a more robust inflammatory response, with better clearance of a pathogen. However excessive inflammation leading to sepsis can occur when BMAL1 is absent in myeloid cells. Nguyen et al. demonstrated this as a non-lethal dose of *Listeria* in wild type mice caused massive lethality in mice lacking myeloid BMAL1 when challenged at both ZT0 and ZT8.

Apart from *Ccl2*, what other immune genes might be BMAL1-regulated? Silver et al. observed that the expression of TLR9, the pattern recognition receptor that recognizes bacterial and viral DNA, has a circadian pattern of expression. It is controlled directly by CLOCK:BMAL1 which binds E-boxes in the TLR9 promoter. TLR9 expression in spleen cells was far greater in mice at ZT19 versus ZT7. This daily variation in TLR9 led to a more severe response in a model of cecal ligation puncture when performed at ZT19 versus ZT7. The control of BMAL1:CLOCK on TLR9 expression has a strong impact on the adaptive response, as immunization of mice using CpG as an adjuvant showed maximum responsiveness to vaccination at ZT19 versus ZT7, coincident with enhanced clock controlled expression of TLR9 (Silver et al., 2012b). This single challenge with CpG at ZT19 resulted in a more marked response to antigen (OVA) four weeks after the initial immunization. This illustrates that the circadian control of TLR9 has long-term effects in modulating the magnitude of the

adaptive immune response, suggesting that time of vaccination could have a significant impact on efficacy. If the phase of TLR9 expression is similar in humans, the optional time to vaccinate humans may also be during the middle of the night or early morning.

CLOCK

The BMAL1 binding partner CLOCK has also been shown to impact on immune signals. Unlike transcription of *Bmal1*, *Clock* mRNA does not alter significantly throughout the day in macrophages (Keller et al., 2009). However, CLOCK has intrinsic histone acetyl transferase (HAT) activity capable of acetylating lysine residues on histones and it is this HAT activity of CLOCK that is essential for circadian gene expression (Doi et al., 2006). CLOCK can also acetylate the glucocorticoid receptor and this suppresses binding of the glucocorticoid receptor to its target genes (Nader et al., 2009). Although not yet investigated, this may have strong implications regarding the effect of CLOCK on the immune system.

The daily variations observed in lethality from sepsis correlate with activation of the NF-κ☐ transcriptional complex and CLOCK converges directly on this key immune transcription☐☐☐ CLOCK is found in protein complexes with the NF-κ☐ subunit p65 (RELA) and overexpression of CLOCK leads to enhanced phosphorylation and acetylation of p65, leading to enhanced transcriptional activity of the NF-κ☐ complex (Spengler et al., 2012). BMAL1 attenuates the effect of CLOCK on NF-κ☐ most likely by sequestering CLOCK. This is likely to be another mechanism by which BMAL1 limits inflammation. *Clock* mutant mouse embryonic fibroblasts (MEFs) (Bellet et al., 2012) and bone marrow derived macrophages (BMDMs) (Bellet et al., 2013) are less responsive to LPS or TNF-α in terms of NF-κ☐ activation, and the inflamed intestine of *Clock* mutant mice showed reduced expression of many pro-inflammatory, metabolic and circadian genes and reduced number of genes involved in the immune response oscillating in a circadian manner, compared to wild type mice (Bellet et al., 2013). Given that *Salmonella* and other pathogens exploit host defenses to their own advantage (Raffatellu et al., 2009), *Clock* mutant mice also have reduced colonization of Salmonella in the gut (Bellet et al., 2013). However the absence of CLOCK may not be beneficial against infection with more susceptible microorganisms. Therefore, conditional knockouts of CLOCK in discrete immune

compartments will provide a clearer picture on the function of CLOCK in the immune system.

The Nuclear Receptors REV-ERBα and RORα

There is also compelling evidence for REV-ERBα (also known as NR1D1) and RORα affecting immunity.

A synthetic agonist for REV-ERBα limits the release of IL-6 from macrophages (Gibbs et al., 2012). This agonist was also shown to reduce *Cxcl11*, *Ccl2, Cxcl6, Il19* mRNA but not *Il8* mRNA. The mechanism behind this pharmacological effect has not yet been described, however it may be due to the ability of REV-ERBα to recruit the repressor complex NCoR to selected pro-inflammatory genes including *Il6*. REV-ERBα also has effects on enhancer-derived RNAs (eRNAs). eRNAs are short RNA strands produced from an enhancer site and play a role in the transcription of an adjacent gene. Binding of REV-ERBα proteins that bring in histone de-acetylases repress the production of these eRNAs and thus lead to the inactivation of the adjacent gene (Lam et al., 2013). Target genes here include the metalloproteinase *Mmp9* and the chemokine receptor *Cx3cr1*, providing another mechanism for the anti-inflammatory effects of REV-ERBα activation. In a macrophage, BMAL1 induces the transcription of *Nr1d1* (Fig.1), thus suggesting that some of the anti-inflammatory effects of BMAL1 may be through its direct regulation on REV-ERBα. In support of this, a recent study showed that REV-ERBα also represses the *Ccl2* gene by binding to an RORE sequence in the promoter of *Ccl2* (Sato S, 2014). As stated above BMAL1 represses *Ccl2* by binding to an E-box in the *Ccl2* promoter (Nguyen et al., 2013). This indicates that BMAL1 and REV-ERBα, cooperate across the circadian cycle to repress *Ccl2* transcription under inflammatory conditions. The effect of REV-ERBα is possibly cell type specific. REV-ERBα has been shown to drive T_H17 cell differentiation by repression of *Nfil3* transcription which in turn will allow RORγt to induce IL17 production (Yu et al., 2013). In adaptive immunity REV-ERBα may actually promote inflammation.

BMAL1 also controls the expression of RORα, and mice deficient in RORα (the neurological mutant mouse Staggerer) are susceptible to LPS lethality (Stapleton et al., 2005). RORα has been shown to induce the transcription of Iκ□α (nuclear factor

of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha) and limit NF-κ☐ translocation to the nucleus. Therefore another mechanism whereby BMAL1 might be anti-inflammatory could be via induction of RORα which in turn will induce IκBα and limit NF-κB (Delerive et al., 2001). Therefore both nuclear receptors REV-ERBα and RORα, that are components of the molecular clock, also have strong anti-inflammatory capability.

PER and CRY

Two other clock proteins PER and CRY also modulate inflammation. The PER2 mutant leads to the loss of a daily rhythm in IFN-γ (Arjona and Sarkar, 2006) . The oscillation in mortality from LPS is abolished in PER2-deficient mice, with $Per2^{-/-}$ mice being protected against LPS induced lethality at all time points analysed (Liu et al., 2006). The serum concentrations of IFN-γ and IL-1β were lowered dramatically in the $Per2^{-/-}$ mice with LPS injection, however amounts of TNF-α, IL-6 and IL-10 were normal. The impaired IFN-γ was attributed to defective natural killer (NK) cell function. It was also observed that $Per2$ mutants have reduced amounts of $Tlr9$ mRNA in macrophages and activation of TLR9 by CpG produced less TNF-α and IL-12 from these macrophages (Silver et al., 2012b). Therefore PER2 may function to upregulate pro-inflammatory cytokines at certain stages of the circadian cycle. $Per2$ mRNA rhythms are anti-phase to $Bmal1$ and $Per2$ mRNA peaks in macrophages just after the animals enter the active phase (Keller et al., 2009). PER2 is the negative component of the feedback loop that inhibits BMAL1:CLOCK activity as the animal enters the active phase (Fig.1). Therefore, PER2 may promote inflammation in part by repressing the activity of BMAL1. PER2 abundance also inhibits the function of REV-ERBα (Preitner et al., 2002) and repressing REV-ERBα could lead also to greater inflammation from the mechanisms discussed above (Fig. 3). The other components of the negative feedback loop are the cryptochromes (Fig. 1). Absence of the cryptochrome CRY1 and CRY2 in fibroblasts and BMDMs leads to increase $Il6$, $Tnf\alpha$, $Cxcl1$ and $Inos$ mRNA at baseline. Upon LPS stimulation, IL-6 and TNF-α expression are significantly higher in the BMDMs lacking $Cry1$ and $Cry2$. In fibroblasts this was due to CRY1 binding adenylate cyclase and limiting cAMP production. In the absence of CRY1, elevated cAMP increases PKA activity, leading to enhanced phosphorylation of p65 and NF-κB activation (Narasimamurthy et al.,

2012). *Cry1^{-/-}Cry2^{-/-}* mice have heightened inflammatory joint disease and enhanced production of TNF-α in a model of collagen induced arthritis (Hashiramoto et al., 2010). Treatment of these mice with anti-TNF-α antibody reduced the severity of the disease in the *Cry1^{-/-}Cry2^{-/-}* mice and ectopic expression of *Cry1* in mouse embryonic fibroblasts from *Cry1^{-/-}Cry2^{-/-}* mice significantly reduced activation of the TNF-α promoter luciferase construct. This may again be due to the effects of *Cry1* to limit PKA induced phosphorylation of p65 and NF-κ□ activation in fibroblasts (Narasimamurthy et al., 2012).

Inflammation Disrupts the Molecular Clock

As discussed above, the clock can impact on the inflammatory process in a number of ways. In a reciprocal fashion inflammation induced by agents such as LPS, TNF-α and IFN-γ (Cavadini et al., 2007a; Kwak et al., 2008; Lundkvist et al., 2002; Marpegan et al., 2005; Okada et al., 2008) or acute bacterial infection (Bellet et al., 2013) can affect the circadian clock. The oscillations in the clock genes can be disrupted with inflammation and infection, with effects on the expression of core clock genes and clock-controlled genes, including clock controlled metabolic genes being reported (Bellet et al., 2013). Rodent studies indicate that LPS transiently suppresses clock gene expression and oscillation in the SCN (Okada et al., 2008) and phase-shift locomotor activity (Marpegan et al., 2005; Marpegan et al., 2009). One possible explanation for this is that TNF-α and IL-1β can inhibit the ability of BMAL1:CLOCK to induce activation of E-box dependent genes in the SCN and liver (Cavadini et al., 2007b). Also the NF-κB subunit RelB interacts directly with BMAL1 to repress the circadian gene *Dbp* (Bellet et al., 2012). The mRNA expression of *Bmal1* was repressed in the spleens across the full circadian day in mice subjected to collagen induced arthritis versus controls (Hashiramoto et al., 2010). The constitutively low expression of BMAL1 in this model of arthritis could promote further inflammation from immune cells by the mechanisms stated above (Fig. 3). The effect of cytokines on the circadian system is important given the increased use of biotherapeutics such as anti-TNF-α for chronic inflammatory conditions such as arthritis. Anti-TNF-α, by relieving the repression on the BMAL1:CLOCK heterodimer may restore the function of the clock heterodimer and its ability to

repress inflammatory genes (Fig. 3). This could be another mechanism of action for this biological therapy in chronic diseases such as rheumatoid arthritis (RA).

Molecular Clocks in Human Health and Disease

The recent work identifying the roles for clock proteins such as BMAL1 and REV-ERBα in immunoregulation may provide new insights into the pathogenesis of infectious and inflammatory diseases. It is well known that inflammatory diseases such as asthma (Barnes et al., 1980; Kraft et al., 1996) RA (Haus et al., 2012) and atherosclerosis (Paschos and FitzGerald, 2010) have strong circadian components with exacerbations at night and into the early morning hours. There is also an intimate link between circadian rhythms and cancer, a disease associated with aberrant inflammation (Sahar and Sassone-Corsi, 2009). In healthy humans the pro-inflammatory cytokines, TNF-α and IL-6, peak in serum at 3am and 6am respectively (Cutolo and Straub, 2008). In RA patients the peak of both cytokines shift forward to the early morning with peak amounts of IL-6 ten fold higher in the serum of patients with RA versus controls (Cutolo and Straub, 2008). In addition, oscillations in clock proteins is lost in synovial fibroblasts cultured from patients with RA (Kouri et al., 2013). These data suggest severe circadian disruption in RA. The irregularity in the amount and timing of these cytokines might be responsible for the symptoms of stiffness and pain for RA patients in the morning hours. Therefore, the underlying basis of many inflammatory conditions could depend on a perturbed clockwork system both systemically and locally. Chronic disruption of the external cues of light to the molecular clock, as imposed by shift work and airline travel, augments the inflammatory response (Castanon-Cervantes et al., 2010) and increases susceptibility to many metabolic diseases with inflammatory features, such as atherosclerosis, obesity and diabetes (Antunes et al., 2010). Our clock and output rhythms deteriorate and have a reduced amplitude with advancing age (Froy and Chapnik, 2007). Dampened circadian rhythms also occur with mice allowed *ad lib* access to high fat diet (Kohsaka et al., 2007) and coincident metabolic disease. Therefore, the modern 24-hour society, in terms of our ad hoc pattern of activity, continuous caloric consumption, and high exposure to ambient light, can run at odds with our internal

clock. It has also been concluded that 'shift work that involves circadian disruption is probably carcinogenic to humans' (Straif et al., 2007) and can lead to higher incidence of cardiovascular disease and obesity (Karlsson et al., 2001; Stevens, 2009). Such studies on shift work might however be confounded by other variables such as gender, age and weight. Mice that undergo a model of jet lag however (akin to one transatlantic flight every week for four weeks) followed by recovery for one week produce three times as much IL-6 upon challenge with LPS (Castanon-Cervantes et al., 2010). Conversely, recovery from sepsis induced in rats by caecal ligation and puncture is impaired when the regular 12:12 light:dark cycle is replaced by constant conditions (either constant light or constant darkness) after surgery (Carlson and Chiu, 2008). These observations highlight the possibility that recovery of patients from sepsis could be improved by simple measures such as reducing nocturnal light and noise and improving quality of sleep in intensive care units (ICU) units (Hrushesky and Wood, 1997) (Herdegen, 2002).

<u>Conclusions and Future Directions</u>

The picture that is fast emerging is that time of day is critical in terms of the type of immune response generated by an organism. The type of immune response can be broadly partitioned into two states, one of heightened alert and the other of rest and repair. The last decade provided a number of descriptive insights regarding the rhythmic changes that occur in immune parameters, however this decade has provided some of the molecular mechanisms underpinning these descriptions. What is intriguing is that the key components of the molecular clock, whose expression and activity will change across the circadian day, have direct relationships with important components of the immune system. We have detailed the data that we know so far connecting the core clock components, most notably, BMAL1, CLOCK and REV-ERBα and RORα, with important regulators of immune function and inflammation. BMAL1 appears to be a central mediator connecting both the clock and immune system together, its role being to limit inflammation.

However we are only at the beginning of our journey to understand the tight coupling of the circadian and immune systems, and how disturbances to this relationship can

lead to pathology and disease. There are still a number of questions to answer, amongst them:

1) What are the main signals that entrain or perturb the immune cell clock under different situations such as infection or inflammation? 2) What role do the clock proteins play in each type of immune cell? 3) What role does the clock play in the homeostatic functioning of the immune system? 4) Is the response of the human immune clock partitioned in the same way as the mouse immune clock? 5) Are inflammatory diseases in essence diseases of clock dysfunction? One question to investigate in this last regard is whether the anti-inflammatory effects of BMAL1 and REV-ERBα are somehow disrupted in diseases such as RA giving rise to an inflammatory phenotype.

Given that the molecular clock may fundamentally regulate many if not all aspects of our immune system, an understanding of how the clock and immune function intersect may reveal much needed therapeutic opportunities for some of our most common chronic diseases. These insights might also provide rationale for chronotherapy, the dosing of medications with reference to 24-hour rhythms of disease activity, for existing and upcoming treatments of immune diseases.

References

Aguilar-Arnal, L., and Sassone-Corsi, P. (2013). The circadian epigenome: how metabolism talks to chromatin remodeling. Current opinion in cell biology *25*, 170-176.
Antunes, L.C., Levandovski, R., Dantas, G., Caumo, W., and Hidalgo, M.P. (2010). Obesity and shift work: chronobiological aspects. Nutrition research reviews *23*, 155-168.
Arjona, A., and Sarkar, D.K. (2006). The circadian gene mPer2 regulates the daily rhythm of IFN-gamma. Journal of interferon & cytokine research : the official journal of the International Society for Interferon and Cytokine Research *26*, 645-649.
Balsalobre, A., Damiola, F., and Schibler, U. (1998). A serum shock induces circadian gene expression in mammalian tissue culture cells. Cell *93*, 929-937.
Barnes, P., FitzGerald, G., Brown, M., and Dollery, C. (1980). Nocturnal asthma and changes in circulating epinephrine, histamine, and cortisol. The New England journal of medicine *303*, 263-267.

Bellet, M.M., Deriu, E., Liu, J.Z., Grimaldi, B., Blaschitz, C., Zeller, M., Edwards, R.A., Sahar, S., Dandekar, S., Baldi, P., *et al.* (2013). Circadian clock regulates the host response to Salmonella. Proc Natl Acad Sci U S A *110*, 9897-9902.

Bellet, M.M., and Sassone-Corsi, P. (2010). Mammalian circadian clock and metabolism - the epigenetic link. Journal of cell science *123*, 3837-3848.

Bellet, M.M., Zocchi, L., and Sassone-Corsi, P. (2012). The RelB subunit of NFkappaB acts as a negative regulator of circadian gene expression. Cell cycle *11*, 3304-3311.

Boivin, D.B., James, F.O., Wu, A., Cho-Park, P.F., Xiong, H., and Sun, Z.S. (2003). Circadian clock genes oscillate in human peripheral blood mononuclear cells. Blood *102*, 4143-4145.

Bollinger, T., Leutz, A., Leliavski, A., Skrum, L., Kovac, J., Bonacina, L., Benedict, C., Lange, T., Westermann, J., Oster, H., and Solbach, W. (2011). Circadian clocks in mouse and human CD4+ T cells. PloS one *6*, e29801.

Bunger, M.K., Wilsbacher, L.D., Moran, S.M., Clendenin, C., Radcliffe, L.A., Hogenesch, J.B., Simon, M.C., Takahashi, J.S., and Bradfield, C.A. (2000). Mop3 is an essential component of the master circadian pacemaker in mammals. Cell *103*, 1009-1017.

Carlson, D.E., and Chiu, W.C. (2008). The absence of circadian cues during recovery from sepsis modifies pituitary-adrenocortical function and impairs survival. Shock *29*, 127-132.

Castanon-Cervantes, O., Wu, M., Ehlen, J.C., Paul, K., Gamble, K.L., Johnson, R.L., Besing, R.C., Menaker, M., Gewirtz, A.T., and Davidson, A.J. (2010). Dysregulation of inflammatory responses by chronic circadian disruption. J Immunol *185*, 5796-5805.

Cavadini, G., Petrzilka, S., Kohler, P., Jud, C., Tobler, I., Birchler, T., and Fontana, A. (2007a). TNF-alpha suppresses the expression of clock genes by interfering with E-box-mediated transcription. Proc Natl Acad Sci U S A *104*, 12843-12848.

Cavadini, G., Petrzilka, S., Kohler, P., Jud, C., Tobler, I., Birchler, T., and Fontana, A. (2007b). TNF-alpha suppresses the expression of clock genes by interfering with E-box-mediated transcription. Proc Natl Acad Sci U S A *104*, 12843-12848.

Coutinho, A.E., and Chapman, K.E. (2011). The anti-inflammatory and immunosuppressive effects of glucocorticoids, recent developments and mechanistic insights. Molecular and cellular endocrinology *335*, 2-13.

Curtis, A.M., Cheng, Y., Kapoor, S., Reilly, D., Price, T.S., and Fitzgerald, G.A. (2007). Circadian variation of blood pressure and the vascular response to asynchronous stress. Proc Natl Acad Sci U S A *104*, 3450-3455.

Cutolo, M., and Straub, R.H. (2008). Circadian rhythms in arthritis: hormonal effects on the immune/inflammatory reaction. Autoimmunity reviews *7*, 223-228.

Delerive, P., Monte, D., Dubois, G., Trottein, F., Fruchart-Najib, J., Mariani, J., Fruchart, J.C., and Staels, B. (2001). The orphan nuclear receptor ROR alpha is a negative regulator of the inflammatory response. EMBO reports *2*, 42-48.

Dickmeis, T., Weger, B.D., and Weger, M. (2013). The circadian clock and glucocorticoids--interactions across many time scales. Molecular and cellular endocrinology *380*, 2-15.

Doi, M., Hirayama, J., and Sassone-Corsi, P. (2006). Circadian regulator CLOCK is a histone acetyltransferase. Cell *125*, 497-508.

Feng, D., and Lazar, M.A. (2012). Clocks, metabolism, and the epigenome. Molecular cell *47*, 158-167.

Froy, O., and Chapnik, N. (2007). Circadian oscillation of innate immunity components in mouse small intestine. Molecular immunology *44*, 1954-1960.

Gibbs, J.E., Blaikley, J., Beesley, S., Matthews, L., Simpson, K.D., Boyce, S.H., Farrow, S.N., Else, K.J., Singh, D., Ray, D.W., and Loudon, A.S. (2012). The nuclear receptor REV-ERBalpha mediates circadian regulation of innate immunity through selective regulation of inflammatory cytokines. Proc Natl Acad Sci U S A *109*, 582-587.

Guo, H., Brewer, J.M., Lehman, M.N., and Bittman, E.L. (2006). Suprachiasmatic regulation of circadian rhythms of gene expression in hamster peripheral organs: effects of transplanting the pacemaker. The Journal of neuroscience : the official journal of the Society for Neuroscience *26*, 6406-6412.

Halberg, F., Johnson, E.A., Brown, B.W., and Bittner, J.J. (1960). Susceptibility rhythm to E. coli endotoxin and bioassay. Proc Soc Exp Biol Med *103*, 142-144.

Hashiramoto, A., Yamane, T., Tsumiyama, K., Yoshida, K., Komai, K., Yamada, H., Yamazaki, F., Doi, M., Okamura, H., and Shiozawa, S. (2010). Mammalian clock gene Cryptochrome regulates arthritis via proinflammatory cytokine TNF-alpha. J Immunol *184*, 1560-1565.

Haus, E., Sackett-Lundeen, L., and Smolensky, M.H. (2012). Rheumatoid arthritis: circadian rhythms in disease activity, signs and symptoms, and rationale for chronotherapy with corticosteroids and other medications. Bulletin of the NYU hospital for joint diseases *70 Suppl 1*, 3-10.

Hayashi, M., Shimba, S., and Tezuka, M. (2007). Characterization of the molecular clock in mouse peritoneal macrophages. Biological & pharmaceutical bulletin *30*, 621-626.

Herdegen, J.J. (2002). Intensive care unit sleep disruption: can the cycle be restored? Critical care medicine *30*, 709-710.

Hrushesky, W.J., and Wood, P.A. (1997). Circadian time structure of septic shock: timing is everything. The Journal of infectious diseases *175*, 1283-1284.

Kalsbeek, A., van der Spek, R., Lei, J., Endert, E., Buijs, R.M., and Fliers, E. (2012). Circadian rhythms in the hypothalamo-pituitary-adrenal (HPA) axis. Molecular and cellular endocrinology *349*, 20-29.

Karlsson, B., Knutsson, A., and Lindahl, B. (2001). Is there an association between shift work and having a metabolic syndrome? Results from a population based study of 27 485 people. Occupational and Environmental Medicine *58*, 747-752.

Keller, M., Mazuch, J., Abraham, U., Eom, G.D., Herzog, E.D., Volk, H.D., Kramer, A., and Maier, B. (2009). A circadian clock in macrophages controls inflammatory immune responses. Proc Natl Acad Sci U S A *106*, 21407-21412.

Kohsaka, A., Laposky, A.D., Ramsey, K.M., Estrada, C., Joshu, C., Kobayashi, Y., Turek, F.W., and Bass, J. (2007). High-fat diet disrupts behavioral and molecular circadian rhythms in mice. Cell metabolism *6*, 414-421.

Koike, N., Yoo, S.H., Huang, H.C., Kumar, V., Lee, C., Kim, T.K., and Takahashi, J.S. (2012). Transcriptional architecture and chromatin landscape of the core circadian clock in mammals. Science *338*, 349-354.

Kondratov, R.V., Kondratova, A.A., Gorbacheva, V.Y., Vykhovanets, O.V., and Antoch, M.P. (2006). Early aging and age-related pathologies in mice deficient in BMAL1, the core componentof the circadian clock. Genes & development *20*, 1868-1873.

Kouri, V.P., Olkkonen, J., Kaivosoja, E., Ainola, M., Juhila, J., Hovatta, I., Konttinen, Y.T., and Mandelin, J. (2013). Circadian timekeeping is disturbed in rheumatoid arthritis at molecular level. PloS one *8*, e54049.

Kraft, M., Djukanovic, R., Wilson, S., Holgate, S.T., and Martin, R.J. (1996). Alveolar tissue inflammation in asthma. American journal of respiratory and critical care medicine *154*, 1505-1510.

Kwak, Y., Lundkvist, G.B., Brask, J., Davidson, A., Menaker, M., Kristensson, K., and Block, G.D. (2008). Interferon-gamma alters electrical activity and clock gene expression in suprachiasmatic nucleus neurons. Journal of biological rhythms *23*, 150-159.

Lam, M.T., Cho, H., Lesch, H.P., Gosselin, D., Heinz, S., Tanaka-Oishi, Y., Benner, C., Kaikkonen, M.U., Kim, A.S., Kosaka, M.*, et al.* (2013). Rev-Erbs repress macrophage gene expression by inhibiting enhancer-directed transcription. Nature *498*, 511-515.

Liu, J., Malkani, G., Shi, X., Meyer, M., Cunningham-Runddles, S., Ma, X., and Sun, Z.S. (2006). The circadian clock Period 2 gene regulates gamma interferon production of NK cells in host response to lipopolysaccharide-induced endotoxic shock. Infect Immun *74*, 4750-4756.

Logan, R.W., Arjona, A., and Sarkar, D.K. (2011). Role of sympathetic nervous system in the entrainment of circadian natural-killer cell function. Brain, behavior, and immunity *25*, 101-109.

Logan, R.W., and Sarkar, D.K. (2012). Circadian nature of immune function. Molecular and cellular endocrinology *349*, 82-90.

Lundkvist, G.B., Hill, R.H., and Kristensson, K. (2002). Disruption of circadian rhythms in synaptic activity of the suprachiasmatic nuclei by African trypanosomes and cytokines. Neurobiology of disease *11*, 20-27.

Marpegan, L., Bekinschtein, T.A., Costas, M.A., and Golombek, D.A. (2005). Circadian responses to endotoxin treatment in mice. Journal of neuroimmunology *160*, 102-109.

Marpegan, L., Leone, M.J., Katz, M.E., Sobrero, P.M., Bekinstein, T.A., and Golombek, D.A. (2009). Diurnal variation in endotoxin-induced mortality in mice: correlation with proinflammatory factors. Chronobiology international *26*, 1430-1442.

Nader, N., Chrousos, G.P., and Kino, T. (2009). Circadian rhythm transcription factor CLOCK regulates the transcriptional activity of the glucocorticoid receptor by acetylating its hinge region lysine cluster: potential physiological implications. FASEB journal : official publication of the Federation of American Societies for Experimental Biology *23*, 1572-1583.

Narasimamurthy, R., Hatori, M., Nayak, S.K., Liu, F., Panda, S., and Verma, I.M. (2012). Circadian clock protein cryptochrome regulates the expression of proinflammatory cytokines. Proc Natl Acad Sci U S A *109*, 12662-12667.

Nguyen, K.D., Fentress, S.J., Qiu, Y., Yun, K., Cox, J.S., and Chawla, A. (2013). Circadian gene Bmal1 regulates diurnal oscillations of Ly6C(hi) inflammatory monocytes. Science *341*, 1483-1488.

Okada, K., Yano, M., Doki, Y., Azama, T., Iwanaga, H., Miki, H., Nakayama, M., Miyata, H., Takiguchi, S., Fujiwara, Y.*, et al.* (2008). Injection of LPS causes transient suppression of biological clock genes in rats. J Surg Res *145*, 5-12.

Oster, H., Damerow, S., Kiessling, S., Jakubcakova, V., Abraham, D., Tian, J., Hoffmann, M.W., and Eichele, G. (2006). The circadian rhythm of glucocorticoids is regulated by a gating mechanism residing in the adrenal cortical clock. Cell metabolism *4*, 163-173.

Paschos, G.K., and FitzGerald, G.A. (2010). Circadian clocks and vascular function. Circulation research *106*, 833-841.

Preitner, N., Damiola, F., Lopez-Molina, L., Zakany, J., Duboule, D., Albrecht, U., and Schibler, U. (2002). The orphan nuclear receptor REV-ERBalpha controls circadian transcription within the positive limb of the mammalian circadian oscillator. Cell *110*, 251-260.

Raffatellu, M., George, M.D., Akiyama, Y., Hornsby, M.J., Nuccio, S.P., Paixao, T.A., Butler, B.P., Chu, H., Santos, R.L., Berger, T.*, et al.* (2009). Lipocalin-2 resistance confers an advantage to Salmonella enterica serotype Typhimurium for growth and survival in the inflamed intestine. Cell host & microbe *5*, 476-486.

Sahar, S., and Sassone-Corsi, P. (2009). Metabolism and cancer: the circadian clock connection. Nature reviews. Cancer *9*, 886-896.

Sato S, S.T., Ogasawara J, Takahashi M, Izawa T, Imaizumi K, Taniguchi N, Ohno H, Kizaki T. (2014). A Circadian Clock Gene, Rev-erbα, Modulates the Inflammatory Function of Macrophages through the Negative Regulation of Ccl2 Expression. J Immunol. *192*, 407-417.

Sato, T.K., Panda, S., Miraglia, L.J., Reyes, T.M., Rudic, R.D., McNamara, P., Naik, K.A., FitzGerald, G.A., Kay, S.A., and Hogenesch, J.B. (2004). A functional genomics strategy reveals Rora as a component of the mammalian circadian clock. Neuron *43*, 527-537.

Scheiermann, C., Kunisaki, Y., Lucas, D., Chow, A., Jang, J.E., Zhang, D., Hashimoto, D., Merad, M., and Frenette, P.S. (2012). Adrenergic nerves govern circadian leukocyte recruitment to tissues. Immunity *37*, 290-301.

Schibler, U., and Sassone-Corsi, P. (2002). A web of circadian pacemakers. Cell *111*, 919-922.

Shackelford, P.G., and Feigin, R.D. (1973). Periodicity of susceptibility to pneumococcal infection: influence of light and adrenocortical secretions. Science *182*, 285-287.

Silver, A.C., Arjona, A., Hughes, M.E., Nitabach, M.N., and Fikrig, E. (2012a). Circadian expression of clock genes in mouse macrophages, dendritic cells, and B cells. Brain, behavior, and immunity *26*, 407-413.

Silver, A.C., Arjona, A., Walker, W.E., and Fikrig, E. (2012b). The circadian clock controls toll-like receptor 9-mediated innate and adaptive immunity. Immunity *36*, 251-261.

Spengler, M.L., Kuropatwinski, K.K., Comas, M., Gasparian, A.V., Fedtsova, N., Gleiberman, A.S., Gitlin, II, Artemicheva, N.M., Deluca, K.A., Gudkov, A.V., and Antoch, M.P. (2012). Core circadian protein CLOCK is a positive regulator of NF-kappaB-mediated transcription. Proc Natl Acad Sci U S A *109*, E2457-2465.

Stapleton, C.M., Jaradat, M., Dixon, D., Kang, H.S., Kim, S.C., Liao, G., Carey, M.A., Cristiano, J., Moorman, M.P., and Jetten, A.M. (2005). Enhanced susceptibility of staggerer (RORalphasg/sg) mice to lipopolysaccharide-induced lung inflammation. American journal of physiology. Lung cellular and molecular physiology *289*, L144-152.

Stevens, R.G. (2009). Light-at-night, circadian disruption and breast cancer: assessment of existing evidence. International Journal of Epidemiology *38*, 963-970.

Straif, K., Baan, R., Grosse, Y., Secretan, B., El Ghissassi, F., Bouvard, V., Altieri, A., Benbrahim-Tallaa, L., and Cogliano, V. (2007). Carcinogenicity of shift-work, painting, and fire-fighting. The lancet oncology *8*, 1065-1066.

Yu, X., Rollins, D., Ruhn, K.A., Stubblefield, J.J., Green, C.B., Kashiwada, M., Rothman, P.B., Takahashi, J.S., and Hooper, L.V. (2013). TH17 cell differentiation is regulated by the circadian clock. Science *342*, 727-730.

Zhang R., L.N., Ballance H.I., Hughes M.E., Hogenesch J.B., (2013). A Transcriptional Map of Mouse Circadian Time and Space. Keystone Symposia on Molecular Clockworks and the Regulation of Cardio-Metabolic Function (C9) at Snowbird, UT 2013.

Figure Legends

Figure 1. The molecular clock consists of at least three interlocking feedback loops. Loop 1 consists of the core clock proteins BMAL1 and CLOCK binding to E-box elements within the genes encoding the repressor proteins PER and CRY and REV-ERBα RORα and DBP. After a period of time, PER and CRY can translocate back into the nucleus and repress their own expression by interfering with the BMAL1:CLOCK complex on the gene promoters. The expression of these proteins is regulated by post-translational modifications such as phosphorylation of PER by CKIε, which marks it for proteosomal degradation. Loop 2 consists of the alternate regulation by REV-ERBα and RORα on RORE promoter elements, which includes *Bmal1* and *Nfil3*. Loop 3 consists of the alternate regulation by NFIL3 and DBP on D box promoter elements. The clock products from each of these loops can shuttle back to the nucleus and either repress or reactivate these loops. The transcription factors within each of these loops can also regulate clock-controlled genes (CCGs). These are genes that have a circadian profile of expression but do not feedback to affect the core molecular clock. If some of these CCGs are themselves transcription factors, they may confer a circadian profile of expression on their target genes. Therefore, the core clock components and CCGs have the capacity to regulate transcription of a wide variety of cellular programs that is independent of their function within the molecular clock.

Figure 2. Circadian effects on immune function. The data so far indicates a partitioning of the immune system into two states: 1) a state of anticipation and enhanced immune activity (red dotted line) and; 2) an opposing period which may relate to repair and regeneration of the immune system (green dotted line). Data indicates that around the time when mice transition into activity (ZT12) the immune system is poised and anticipates the higher risk of infection, however with that comes enhanced susceptibility to sepsis. This is evidenced by enhanced clearance of bacteria

at ZT8 and ZT10. This period also correlates with enhanced lethality from high doses of LPS observed at ZT10, greater induction of pro-inflammatory cytokines at ZT12, and enhanced numbers of leukocytes at ZT13. The opposing state of regeneration and repair is mainly speculative and requires further investigation. It does coincide however with the period of reduced induction of pro-inflammatory cytokines along with reduced clearance and lethality from bacteria. Whether similar processes occur in humans at the same states of transition have yet to be clearly determined.

Figure 3. BMAL1 is the central mediator on the circadian control of the immune system and promotes an anti-inflammatory state. BMAL1 directly represses *Ccl2* gene expression leading to lower numbers of the Ly6C^{hi} inflammatory monocytes in circulation and lower recruitment of these inflammatory monocytes into inflamed tissues. BMAL1 sequesters CLOCK and prevents it from acetylating and activating p65, this leads to lower amounts of transcriptionally active NF-κB leading to less induction of specific genes such as cytokines and regulators of survival and proliferation. BMAL1 drives the expression of *Nr1d1* (*Rev-Erbα*) that can inhibit *Il6* and *Ccl2* expression. BMAL1 drives the expression of *Rorα* that can increase the expression of IκB, a major negative regulator of NF-κB. This would have the effect of retarding the NF-κB complex as a non-active form in the cytoplasm preventing it from translocating into the nucleus and activating a range of genes including cytokines.

Glossary of Terms used in Circadian Biology

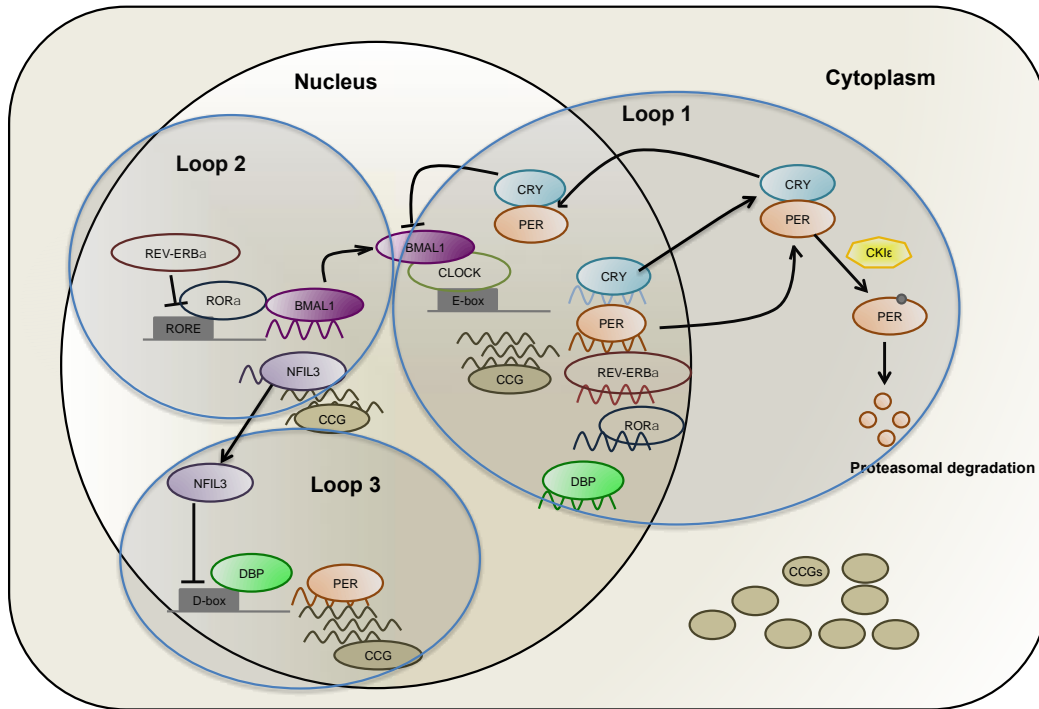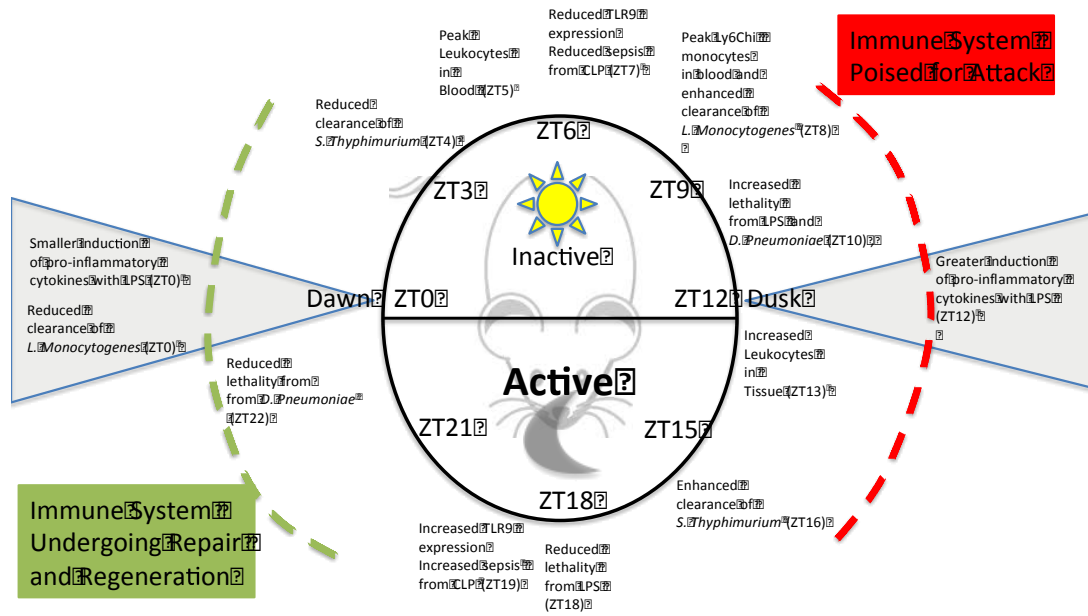|	
Zeitgeber	An environmental cue that synchronizes the endogenous rhythm of the clock in an organism with the Earths 24 hour cycle. Examples include light, food and temperature.
Zeitgeber Time (ZT)	Corresponds to the time (hours) after onset of light. In an animal facility with 12 hours of light and dark, lights go on at ZT0 and lights go off at ZT12
Circadian Time (CT)	Corresponds to time without the zeitgeber of light. The lights remain off from CT0 onwards.
Diurnal	Daily rhythms with the zeitgeber of light
Period Length	The time to complete a full cycle

Figure 1

Figure 2

Peak Leukocytes in Blood (ZT5)

Reduced TLR9 expression Reduced sepsis from CLP (ZT7)

Peak Ly6Chi monocytes in blood and enhanced clearance of *L. Monocytogenes* (ZT8)

Immune System Poised for Attack

Reduced clearance of *S. Thyphimurium* (ZT4)

ZT6

ZT3

ZT9

Inactive

Increased lethality from LPS and *D. Pneumoniae* (ZT10) ,

Smaller induction of pro-inflammatory cytokines with LPS (ZT0)

Reduced clearance of *L. Monocytogenes* (ZT0)

Dawn ZT0

ZT12 Dusk

Greater induction of pro-inflammatory cytokines with LPS (ZT12)

Active

Increased Leukocytes in Tissue (ZT13)

Reduced lethality from from *D. Pneumoniae* (ZT22)

ZT21

ZT15

Immune System Undergoing Repair and Regeneration

ZT18

Enhanced clearance of *S. Thyphimurium* (ZT16)

Increased TLR9 expression Increased sepsis from CLP (ZT19)

Reduced lethality from LPS (ZT18)

Figure 3