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Immunometabolism: Is it under the eye of the clock?

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ABSTRACT

Molecular clocks allow an organism to track time of day, providing the means to anticipate and respond to the daily changes within the environment. In mammals the molecular clock consists of a network of proteins that form auto-regulatory feedback loops that drive rhythms in physiology and behavior. In recent times the extent to which the molecular clock controls key metabolic and immune pathways has begun to emerge. For example, the main clock protein BMAL1 has been linked to mitochondrial metabolism, mitochondrial dynamics and various host defense pathways. The molecular clock may function to integrate daily metabolic changes driven by feeding-fasting to immune function and output. Understanding how the clock intersects with metabolic pathways within immune cells to affect immune phenotypes will have broad implications for the management of metabolic, inflammatory and infectious diseases.

Contents

1. Introduction

Life on earth follows a daily 24 hour (hr) rhythm due to the rotation of the earth on its axis and the daily cycle of light and temperature. The sleep-wake cycle in humans provides a clear example of our daily rhythmicity and connection to our 24 hr environment. Alterations in physiology that peak and trough in a 24 hr timescale are classified as circadian and are caused by oscillations in the proteins of the molecular circadian clock (Fig. 1). The word circadian comes from the two Latin words, circa (about) and dian (day) (Table 1). The molecular clock exhibits profound control over the genome with at least 10% of all transcripts being regulated in a circadian manner in most tissue types [1,2]. In this review we will investigate current literature linking the circadian clock and its associated proteins to immune and metabolic functions. We will detail how the clock alters immune and metabolic parameters and how disruption of the molecular clock can lead to a range of metabolic and immunological pathologies.

2. The history of clocks

Nearly all organisms contain some form of molecular time keeping system, even down to unicellular organisms such as the model

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Table 1
A glossary of circadian terms and definitions.

Term	Acronym	Description
Circadian		An event that peaks and troughs within a 24hr time scale.
Diurnal		Any pattern that repeats on a daily basis.
Zeitgeber	ZT	An environmental cue that entrains the circadian clock in an organism. ZT refers to Zeitgeber time, which corresponds to time after the onset of light. ZT0 is lights on and ZT12 is lights off in an animal facility
Circadian time	CT	An estimation of an organism's subjective time, with CT0 being the beginning of a subjective day and CT12 being the beginning of a subjective night. CT0 would be the beginning of a diurnal organisms activity.
Brain and muscle ARNT-like-protein-1	BMAL1	A bHLH-PAS domain transcription factor capable of binding to CLOCK and NPAS2 to bind E-boxes in gene promoters.
Circadian locomotor Output cycles kaput	CLOCK	A bHLH-PAS domain transcription factor that can dimerise with BMAL1 to induce circadian gene expression.
Neuronal PAS domain protein 2	NPAS2	Transcription factor which is a paralogue of CLOCK.
Cryptochrome	CRY	Transcriptional repressors that bind with PER to inhibit BMAL1:CLOCK mediated gene transcription. Functions as a light-responsive receptor in drosophila and plants.
Period	PER	A PAS domain containing protein that dimerises with CRY to inhibit BMAL1:CLOCK mediated gene expression.
Rev-erb	NR1D	Consists of two members alpha and beta (NR1D1/2). Act as transcriptional repressors, that switch off *Bmal*1 transcription.
Retinoid related orphan receptors	ROR	Consists of three members, alpha, beta and gamma. Act as transcriptional activators, that switch on *Bmal1* transcription.
Suprachiasmatic Nucleus	SCN	A region located in the anterior hypothalamus that is responsible for receiving and processing light signals and synchronizing rhythmicity in all tissues.

cyanobacteria species, *Synechococcus elongates* [3]. Evidence of circadian rhythms in nature appeared as early as the time of Alexander the Great, with daily leaf movement having been noted in the leaves of the tamarind tree [4]. The first recorded observations of endogenous circadian oscillations were recorded in 1729 by French scientist Jean-Jacques d'Ortous de Mairan [5]. He noted that leaves of the *Mimosa pudica* opened in the morning and closed in the evening and that this 24hr cyclical movement continued when the plants were kept in the dark. This illustrated the presence of an endogenous clock separate from the external stimulus of light. The term circadian was first coined in the 1950′s by Franz Halberg [6] who provided some of the first evidence of circadian control of immunity [7]. Although clock mechanisms differ between organisms, conservation of a timer system throughout the evolutionary tree exemplifies its importance. In recent years significant advances have been made in our understanding of how the circadian cycle is controlled, and how this can, in turn, regulate many aspects of our physiology.

2.1. The clockwork machinery

Circadian rhythms are governed by a "master clock", which resides within the cells of the suprachiasmatic nucleus (SCN) in mammals, located in the anterior region of the hypothalamus [8]. Photosensitive melanopsin ganglion cells within the retina relay light input from the external world to the SCN. The neurons of the SCN are capable of firing with 24 hr rhythmicity, driven by a network of interlocking positive and negative feedback loops. The loops that drive 24 hr-rhythms, or circadian rhythms comprise the molecular clock. Importantly, the clockwork machinery is present in virtually all cell types [9] including those of the immune system [10]. The master clock of the SCN relays signals which then synchronise molecular clocks found within central and peripheral tissues [11]. While not entirely understood, synchronisation of peripheral clocks by the SCN appears to be largely orchestrated via the hypothalamus pituitary adrenal (HPA) axis and the autonomic nervous system (ANS) [12]. Evidence of this axis is observed

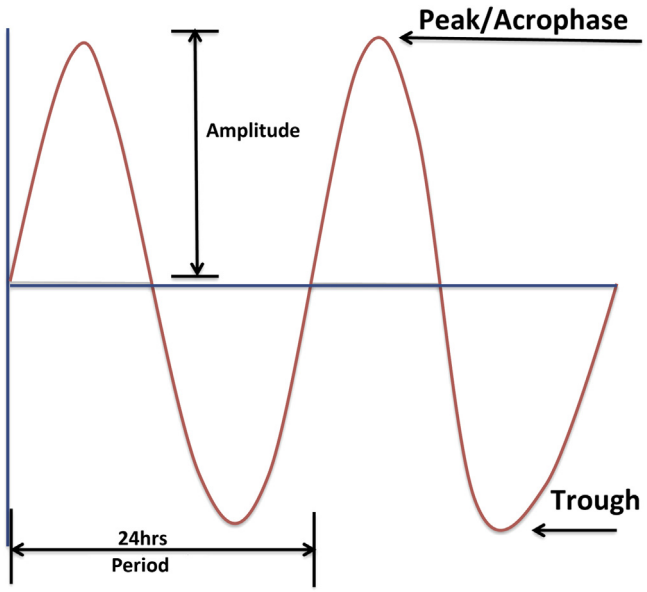

Fig. 1. Properties of a circadian gene.
For a gene to be considered circadian, it must peak and trough within a 24hr timescale.

in the temporal release of corticosterone, which displays a robust circadian rhythm [13]. The time dependent/temporal release of hormonal and neuronal signals act as synchronizers and messengers of the circadian clock [14]. The SCN's control over endocrine and autonomic outputs keeps the peripheral cells in synchrony with each other, allowing for strict coordination of tissue functions over the course of the day [15,16].

In mammals the molecular clock is powered by rhythmic activity of a number of clock proteins (Fig. 2). At the center of the machinery lies the heterodimeric partnership of two transcription factors, Brain Muscle Arnt-Like Protein 1 (BMAL1) and Clock

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Locomotor Output Kaput (CLOCK). In their cooperative partnership, these proteins can bind to E-box sites in the genome to induce gene expression [17]. Neuronal PAS Domain Protein 2 (NPAS2), which is expressed highly in the brain, is also capable of dimerising to BMAL1 to induce expression of E-box controlled genes [18]. The presence of E-boxes within the promoters of the clock repressors, period (PER) and cryptochrome (CRY), generate a negative feedback loop. Following translation, PER and CRY cooperatively translocate back to the nucleus, inhibiting their own transcription by disrupting the BMAL1:CLOCK complex [19]. The nuclear receptors RAR-related orphan receptor (ROR) (α, β, γ) and REV-ERB (α, β) are also induced via E-box sites in their promoters and translocate back into the nucleus to bind receptor-related orphan receptor response elements (ROREs) in the promoter of *Bmal1* [16]. REV-ERBs are capable of repressing *Bmal1* transcription [20], whereas RORs cause transcriptional activation of *Bmal1* [21]. It is believed that post transcriptional modifications, including control by miRNAs [22], provide another layer of organisation to this tightly regulated system.

The BMAL1:CLOCK heterodimer can bind thousands of sites in the genome and it is the oscillation of this binding that leads to circadian expression of clock-controlled genes. However, mRNA expression is just one clock output. Protein modifications including epigenetics [23], subcellular localization [24], rates of translation [25,26] reduction-oxidation (REDOX) [27] and ion concentrations [28] are some of the cellular functions that have been identified as clock regulated.

The molecular clock exists within immune cells [9], including B cells [29], T cells, [30] and macrophages [31], and appears to play a key role in each of these cell types. The presence of circadian rhythms in metabolic pathways has also been described [32]. In recent years, realisation that the metabolic state of immune cells can contribute to their immune phenotype has lead to the emergence of a new field called immunometabolism [33]. Therefore the clock may intersect with immunity and metabolism via the axis of immunometabolism, possibly opening up a new body of study, circadian immunometabolism.

2.2. Circadian disruption and disease

To appreciate the relationships between the environment, clocks and physiology, one must only observe the consequences of perturbations in the sleep-wake cycle. Modern life and technology has seen us adopting lifestyles that remove us from the solar world in which we have evolved. Rather than rising upon first light and retiring upon its disappearance, light has become omnipresent in our lives, leading to circadian disturbances and alterations in our physiological responses. For example, over 15 percent of the working population of the United States are engaged in some sort of shift work [34]. Living at odds with our internal pacemaker may be a contributing factor to the current epidemic of obesity, and consequentially obesity related disorders such as diabetes, cancer and cardiovascular disease [35]. Social jetlag is a concept to describe and quantify circadian misalignment due to individuals living out of synchrony with their body clocks. Increased levels of social jetlag are associated with increased body mass index (BMI) [36]. Alternating feeding patterns in mice such as restricted feeding to certain times of the day causes uncoupling of the SCN from the peripheral clocks in the kidney, heart and pancreas [37], suggesting a role for nutritional regulation in circadian rhythms in peripheral tissues. What we eat and when we eat it could impact on social jetlag [38]. Mice fed a high fat diet during their inactive phase gained significantly more weight than mice fed during their active phase [39]. A similar phenomenon is observed in humans with people who preferentially eat their main meal later in the day losing less weight than those who eat at earlier times [40]. Interestingly, the tendency to eat later in the day in humans correlated with a higher frequency of a *Clock* gene mutation [40]. Indeed single nucleotide polymorphisms in the *Clock* gene have previously been associated with obesity disorders [41]. In mice, mutations in *Clock* lead to obesity and metabolic disturbances [41].

Non-traditional working hours has been linked to several metabolic and immune related disorders, including obesity [42], diabetes [43,44] stroke [44], atherosclerosis[45] and coronary heart disease [46]. Various cancers have been linked to disrupted circadian cycles, such as lymphatic [47], prostate [48,49] and breast cancer [50]. In 2007 the International Agency for Research on Cancer (IARC) claimed that shift work with circadian disruption is a highly probable human carcinogen [51]. This interconnection between disrupted clocks and cancer was demonstrated recently in an adenocarcinoma mouse model, whereby the tumours impacted on clock-control of liver lipid metabolism [52]. In a mouse model of acute myeloid leukemia (AML), knockdown of *Bmal1* and *Clock* in mouse leukemia cells depleted the pool of leukemic stem cells and reduced proliferation [53]. A REV-ERBα and REV-ERBβ agonist repressed *Bmal1* and inhibited viability of both mouse and human AML cells. These studies suggest that different cancers have a differential reliance on clock activity for progression. The mechanisms linking circadian disruption to these varied cancers are not yet fully known, but it is worth noting that altered metabolism and immune function are common features shared by many of these conditions.

Immune function is highly rhythmic in both mice and humans (Fig. 3A and B-red shading). In the 1960′s, Halberg was the first to observe a circadian variation in the susceptibility of mice to lipopolysaccharide (LPS)-induced death. Mice injected with LPS, a component of gram negative bacteria, towards the end of the resting phase displayed greater lethality in comparison to any other time of day [7]. A day-night difference in response to LPS induced endotoxaemia has been described in humans. Subjects injected with low dose LPS at noon displayed higher levels of the anti-inflammatory cytokine interleukin (IL)-10. However, when treated at midnight, levels of tumour necrosis factor (TNF) α and IL-6 were elevated [54]. Circadian disruption by acute jet-lag caused an exacerbated response to LPS induced death in mice [55]. Mice subjected to 6 hr phase advances of the light/dark schedule over the course of 4 consecutive weeks displayed 81% mortality versus 21% mortality in control mice. The jet-lagged mice also produced significantly higher levels of pro-inflammatory cytokines. Altered expression of the core clock genes in the jet-lag mice was evident in the liver, thymus and peritoneal macrophages, however no loss in sleep or altered stress-response was evident. Therefore, circadian disruption, and not sleep loss leads to an increase in the innate immune response following experimentally induced jet-lag in mice[55], which has relevance for the increased inflammatory conditions observed with shift work. Stimulating night shift conditions disrupts the rhythm of immune function in humans [56]. Following night shift conditions the peak level of cytokines from peripheral blood mononuclear cells (PBMCs) treated with LPS was advanced by 4.5–6 hrs.

Immune cell number trafficking and cytokine levels appear to alter with time of day [57]. The number of haematopoeietic stem and progenitor cells (HSPCs) and mature leukocytes peak in the blood during the resting phase in mice [58], while levels of pro-inflammatory cytokines including IL-1β and TNF peak during the active phase [57,59]. In mice leukocytes reach their highest numbers in blood at zeitgeber time (ZT) 5 but their greatest recruitment to tissues is at ZT13 [60]. Such rhythms are also evident in humans. Chemokines and cytokines such as GM-CSF [61], IL-1β [62] and IL-6 [62,63] have a circadian rhythm in humans. Circulating levels of neutrophils and lymphocytes appear to be higher between the hours of 16:00 to 24:00 while reaching a trough in levels between 04:00 to 12:00 [61]. It appears these differences in cel-

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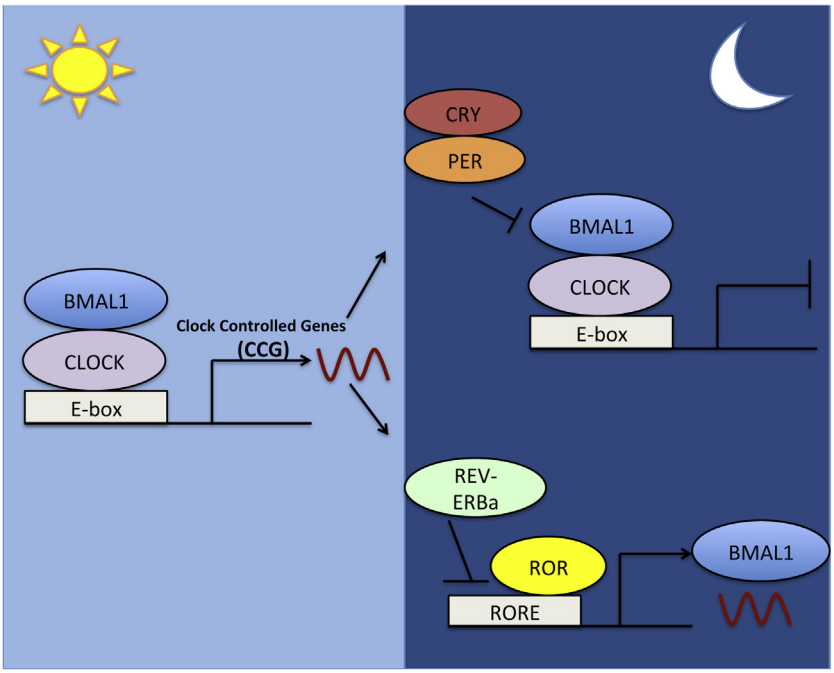

Fig. 2. The oscillation in levels of circadian clock proteins.
During the day, levels of BMAL1 are high. BMAL1 binds to CLOCK and induces gene expression of clock-controlled genes (CCG). These include the repressors period (PER) and cryptochrome (CRY), which peak during the night, translocate back to the nucleus and disrupt the BMAL1:CLOCK heterodimer. Similarly REV-ERB and ROR reach high levels during the night with REV-ERB transcriptionally repressing *Bmal1* and ROR transcriptionally activating *Bmal1*. The dynamic nature of these genes facilitates the rhythmic binding of BMAL1:CLOCK to E-box sites generating circadian rhythms in CCGs.

lular and cytokine levels may not just fluctuate on a daily basis, but may also display seasonal variation. In humans white blood cell numbers peak in the winter months, correlating with a profound pro-inflammatory transcriptomic profile [64]. C-reactive protein peaks during the winter, which is a biomarker for inflammatory diseases [64]. Seasonality in clock gene expression is also observed, including *Bmal1*, which was shown to peak during the summer and trough during the winter [64]. Alterations in clock genes expression over the course of the year could play a role in the observed seasonal risk of certain inflammatory diseases including rheumatoid arthritis and type 1 diabetes [64].

Circadian control of immunity is also apparent with host defense. Mice infected with *Salmonella typhimurium* (*S. typhimurium*) at ZT16 display a greater ability to clear the bacteria from the colon 72 hr post infections versus mice treated at ZT4 [65]. This demonstrates that during the active phase, the innate immune system is primed to mount an anti-bacterial response. This may allow for more effective protection at a time when mice are at greater risk of encountering pathogens. Macrophage phagocytosis is also heightened at the transition into the active phase in mice [66]. Using the serum shock model, a cell culture technique to synchronise clocks in vitro[67], circadian rhythms in phagocytosis of *S. typhimurium* and bactericidal activity were observed [68]. Bacterial uptake and killing in murine macrophages was greatest at 16 hr post serum shock.

2.3. Clocks and metabolism

There are many examples by which the clock controls metabolism within various cells (Fig. 3A and B-blue shading). Metabolomics indicate that approximately 20% of metabolites fluctuate with a circadian variation [69–71]. Blood glucose levels undergo time-of-day variation in humans and rodent models [72]. These fluctuations continue to occur under fasting conditions [73] and are ablated in animals lacking a SCN [73,74]. Genetic manipulation of the molecular clock in specific cell types demonstrates the impact of the clock on metabolism at the cellular level. The clock within beta cells is required for the rhythmic secretion of insulin and mice lacking BMAL1 exhibit beta cell dysfunction and diabetes due to a loss of glucose-stimulated insulin secretion (GSIS) [75]. BMAL1 deletion in the beta cell causes reactive oxygen species (ROS) induced oxidative damage and subsequent mitochondrial uncoupling, leading to the loss of GSIS. Other tissue specific clock disruptions lead to metabolic pathologies, with loss of clock function in the liver being shown to induce hypoglycemia during the fasting phase, implicating the circadian system in buffering levels of circulating glucose [76]. Further investigations with tissue specific clock deletion will be required to fully elucidate how the molecular clock is selectively controlling metabolism in discrete tissue and cellular compartments.

2.4. Specific clock proteins and their role in immunity and metabolism

Given the evidence for circadian influence on the functionality of metabolism and the immune response, determining the specific functions that clock proteins play in these processes is important. The impact of the clock proteins on metabolic and immune control, and the intersection between both these systems will be discussed below (Table 2).

2.5. BMAL1-a master regulator

BMAL1 is a basic-helix-loop-helix (bHLH), PER-ARNT-SIM (PAS) domain protein that functions as a major transcription factor, binding to over 6000 E-box sites in the genome with the aid of CLOCK [77,78]. The levels of *Bmal1* mRNA and protein cycle over 24 hr, with mRNA levels of the gene being highest at circadian time (CT) 0 and lowest at CT12 in multiple tissues in mice [2]. Chromatin Immunoprecipitation-sequencing (ChIP-seq) analysis in mouse liv-

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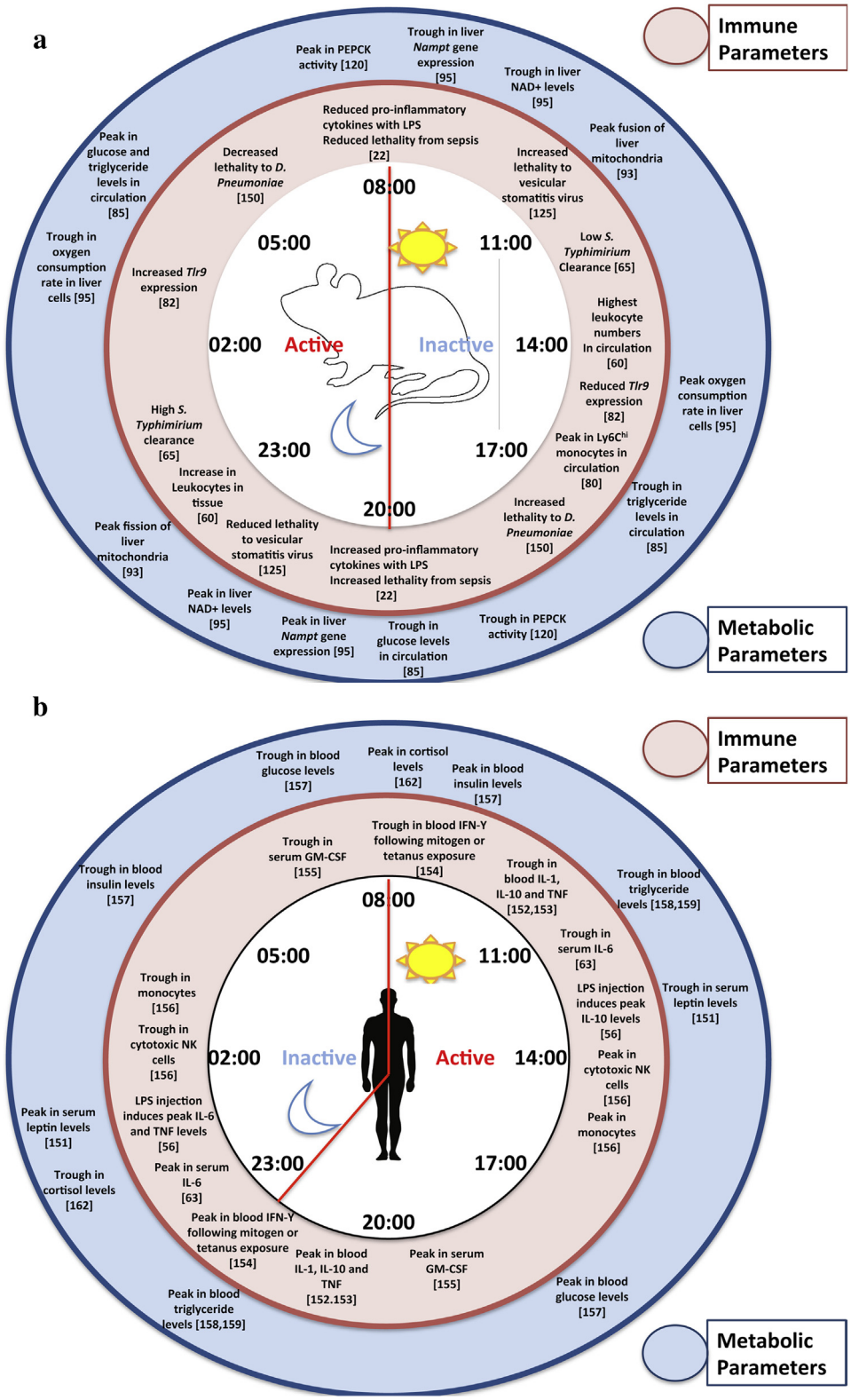

Fig. 3. Alterations in immune and metabolic parameters in mice and humans across the circadian day [150–159,162].
(a) Refers to mice. (b) Refers to humans.
Immune parameters are depicted in the red circle and metabolic parameters are depicted in the blue circle. The numbers refer to time of day based on a 24hr clock.

ers harvested every 4 hr across 24 hr demonstrated BMAL1 binding to gene promoters is increased at CT0 up to CT8 and then begins to fall from CT12-CT20 [77]. BMAL1 is the main orchestrator of the molecular clock, as it is the only single clock gene deletion that leads to complete ablation of the molecular clock, resulting in a loss of all rhythmic behavioural activity [79].

We have shown that the time of day dependency to LPS-induced lethality observed by Halberg is dependent on BMAL1 in

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Table 2
A table of circadian gene deletions, depicting their metabolic and immune phenotypes.

Gene	Deletion	Metabolic phenotype	Immune phenotype
Bmal1	Global	• Diabetes [84] • Reduced lifespan and premature decrease in organ size [84] • Oxidative damage and ROS release [84]	• Two-fold number of neutrophils and monocytes [84] • Progressive cornea inflammation [84]
	Liver	• Increased mitochondrial ROS [93] • Decreased NAD+ and ATP. Decreased triglycerides [95]	
	Pancreas	• Diabetes due to loss of glucose induced insulin secretion [76] • Increased ROS [76]	
	Myeloid	• 30% increased weight gain on high fat diet [80]	• Increased susceptibility to sepsis with LPS or Listeria [22,65] • Greater IL-6, TNFα [22] • Lower IL-10 [22]
Clock	Global	• Partial diabetes [160]	• Reduced NF-κB activation in MEFs and BMDMs. [66,116]
Clock Δ19	Global	• Obese and display impaired glucose sensitivity with insulin resistance and reduced islet size [112,113] • Increased plasma triglyceride and glucose levels [85,114]	• Increased fibrotic damage in model of pulmonary fibrosis [87] • Impaired anti-oxidant defense [87]
Cry1/Cry2	Global	• Increased NAD+ and ATP. Decreased lactate and triglycerides in MEFs [95]	• Greater inflammatory damage in CIA model [146] • Increased *Cxcl1*. *Il-6* and *Tnfα* RNA. Lower NF-κB activation [147]
Cry1 (AP-Tg)	Global	• Elevated glucose levels in serum and urine [161] • Common symptoms of diabetes mellitus, polydipsia and polyuria [161]	
Per2	Global	• Reduced levels of triacylglycerol and non- sterified fatty acids [139] • Enhanced adipocyte differentiation [139] • Increased plasma insulin and impaired gluconeogenesis [142,143]	• Protected from sepsis-induced death [144] • Lower IL-1β and IFNϒ in serum [144] • Lower *Tlr9* expression [82]
Per3	Global	• Enhanced adipogenesis [139] • Increased adipose tissue and decreased muscle tissue mass [139]	
Rev-erbα	Global	• Increased adiposity and mild hyperglycemia [119] • Altered lipid and glucose metabolism [119] • Increase in G6Pase and PEPCK [120]	• Increased IL-6 in serum [124]
RORα sg/sg	Global	• Reduced adiposity and protection from obesity [128] • Lower serum and liver triglyceride levels [128]	• Impaired Th17 cell development [133] • Highly susceptible to LPS induced sepsis [136]
RORγ	Global	• Enhanced production of insulin sensitive adipocytes. [131]	• Impaired Th17 cell development [132]
RORα/γ	Global		• No Th17 cell populations [133] • Resistant to EAE [134]

the myeloid lineage [22]. With myeloid lineage specific deletion of *Bmal1*, the time of day variation in response to bacteria and LPS induced lethality is lost [22,80]. Monocytes and macrophages lacking *Bmal1* produce greater levels of pro-inflammatory cytokines and chemokines while producing reduced levels of the anti-inflammatory cytokine IL-10 (Fig. 4A). *Bmal1* itself is regulated by the innate immune response, as activation of macrophages induces the expression of the microRNA miR-155 that targets *Bmal1* mRNA for degradation [14]. Similarly, TNFα suppresses clock gene expression by interfering with E-box mediated transcription [81]. The inflammatory response may in fact slow down or stop the clock. This may benefit the host by facilitating a rapid response and clearance of the infection. However, the consequence of chronic inflammation or autoimmunity on circadian function has yet to be fully determined.

BMAL1:CLOCK controls expression of the pattern recognition receptor (PRR) TLR9, that recognises bacterial and viral DNA. Peak expression of *Tlr9* occurs at ZT19 in splenocytes [82]. Mice immunized with CpG, a TLR9 ligand, at ZT19 display an increased lymphocyte proliferative response and heightened IFNγ [82]. Human data indicates that the efficiency of certain serotypes of the influenza vaccine in elderly subjects might be modulated by time-of-day administration [83]. Antibody titres against the vaccine were greatest in subjects vaccinated in the morning versus the afternoon. Therefore, a greater understanding of the mechanisms by which the molecular clock intersects with the immune system may provide novel strategies in maximising vaccination efficiency across a range of different vaccines in different patient populations.

Global deletion of *Bmal1* in mice leads to a plethora of pathologies including metabolic-circadian uncoupling, immune

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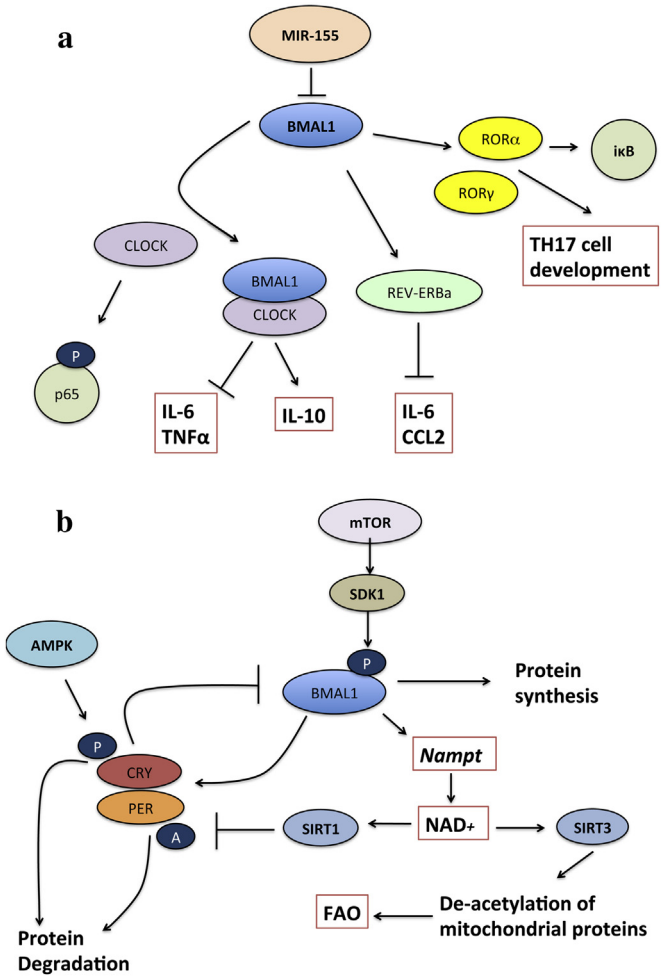

Fig. 4. Bmal1 in immunity and cellular metabolism.
(a) The pro-inflammatory miRNA miR-155 targets *Bmal1* for degradation. Clock can phosphorylate the p65 subunit of NF-κB. BMAL1 can sequester CLOCK from driving inflammation. BMAL1 can also drive production of the anti-inflammatory cytokine IL-10 and suppress pro-inflammatory cytokines. BMAL1 can act through REV-ERBα, which is capable of repressing IL-6 and CCL2. BMAL1 can also act through RORα to promote IκB production and can increase RORα and RORγ, which are both crucial for Th17 cell development.
(b) The mTOR pathway acts through SDK1 to phosphorylate BMAL1, driving protein synthesis. BMAL1 drives expression of *Nampt*, the rate-limiting enzyme in NAD+ synthesis, the cofactor for sirtuin de-acetylases. SIRT3 activity leads to de-acetylation of multiple mitochondrial proteins resulting in increased fatty acid oxidation (FAO). SIRT1 can feedback to promote BMAL1 activity by de-acetylating PER, reducing its ability to repress BMAL1:CLOCK. AMPK can similarly boost the activity of BMAL1 by phosphorylating CRY, inhibiting its activity.

deregulation and accelerated ageing [84,85]. While glucose and triglyceride levels are known to oscillate in WT mice, this pattern is lost with global *Bmal1* deletion and these mice are further prone to insulin induced hypoglycemia, and display impaired gluconeogenesis [85]. Global deletion of *Bmal1* causes nearly two fold higher neutrophils and monocytes in comparison to WT mice, while lymphocytes were decreased. At forty weeks of age >50% of the *Bmal1*-/- mice exhibited progressive cornea inflammation with massive neovascularization and subsequent lymphoid cell infiltration [84]. The ageing phenotype correlated positively with greater reactive oxygen species (ROS) levels. Supplementing the diet of *Bmal1*-/- mice with N-acetyl L cysteine (NAC), a potent antioxidant, extended their lifespan. While the global deletion of *Bmal1* results in a wide array of phenotypes, implicating it in a number of physiological processes, it is important to note that the timing of this deletion has major ramifications. A recent study has made use of tamoxifen inducible mice to remove BMAL1 protein during adult life [86]. These mice exhibited many of the phenotypes described by Kondratov et al. [84], including ocular abnormalities and brain astrogliosis, however, lifespan, blood glucose levels, fertility etc. were largely unaffected [86]. This study highlights a possible role of BMAL1 and the molecular clock in developmental stages, but it also highlights the need for re-evaluation of the consequences of disruption of the molecular clock and the accuracy of global deletion knockouts [86].

To coincide with evidence of oxidative stress with deletion of *Bmal1* [84], the BMAL1:CLOCK heterodimer has been demonstrated to be important in regulating anti-oxidant defense in the lung via rhythmic transcriptional activation of a key antioxidant transcription factor Nuclear Factor (erythroid-derived 2)-like 2 (*Nrf2*). Disruption of the clock leads to a loss of rhythmic transcription and expression of *Nrf2* mRNA and protein, reduced levels of glutathione and increased oxidative damage to the lungs by pulmonary fibrosis [87]. ROS, such as superoxide and hydrogen peroxide are products of metabolism that increase during pathological conditions and are capable of inducing inflammation, either via non-specific damage of proteins or lipids, or by acting as signaling molecules [88,89]. ROS production can depend on NADPH oxidases or enzymes with oxidase activity [90], however, it can also be generated via mitochondrial oxidative-phosphorylation, either basally or due to metabolic dysfunction [91]. Mitochondria themselves are dynamically changing via fission, fusion and mitophagy, collectively termed "mitochondrial dynamics", allowing them to respond to changes in metabolic inputs and energy demands [92]. Hepatic BMAL1 has been linked to the control of mitochondrial dynamics and production of mitochondrial ROS from mitochondrial oxidative phosphorylation [93]. In liver specific *Bmal1*-/- mice, a circadian rhythm in mitochondrial dynamics was discovered [93]. Many of the genes involved in mitochondrial dynamics such as mitochondrial fission protein1 (*Fis1*), BCL2/adenovirus E1B 19 kd-interacting protein 3 (*Bnip3*), and PTEN-induced putative kinase 1 (*Pink1*) display a diurnal rhythm in WT mice that was ablated in the liver specific *Bmal1*-/- mice [93]. In WT mice increased fusion was observed during the resting period, followed by subsequent fission and mitophagy during the active phase. Liver cells lacking *Bmal1* displayed no circadian variations in mitochondrial morphology/mitophagy at any time point and were enlarged, malformed and produced greater ROS. This study implicates a major role for *Bmal1* and the molecular clock in mitochondrial function, energy metabolism and mitochondrial ROS. Circadian variations in mitochondrial function have been demonstrated in immune cells [68]. Synchronized murine peritoneal macrophages at the beginning of serum shock exhibit fragmented, fissioned mitochondria, whereas 12–16 hrs post serum shock the mitochondria had undergone fusion forming mitochondrial networks. Mitochondrial membrane potential was similarly shown to follow a circadian pattern, with increase in membrane potential beginning at 4 hrs and peaking at 12 hrs post synchronisation. The impact of mitochondria in innate immune signaling has been described. For example mitochondria have been linked to regulation of NOD-Like-Receptor family member X1 (NLRX1), TNF receptor associated factor (TRAF6) and NOD-Like-Receptor protein 3 (NLRP3) [94]. Similarly to the role of BMAL1 described in the liver [93], BMAL1 in macrophages may affect mitochondrial health and bioenergetics which may in turn lead to alterations in pathogen sensing and host response.

BMAL1 also impacts on cellular metabolism (Fig. 4B). Mouse embryonic fibroblast (MEF) and liver cells lacking *Bmal1* display a pro-glycolytic phenotype, evidenced by lower mitochondrial ATP production and higher production of lactate [95]. Levels of the oxioreductase factor nicotinamide adenine dinucleotide (NAD+) and fatty acid oxidation (FAO), which display a circadian rhythm in WT mice, were diminished in cells lacking *Bmal1* leading to increased triglycerides. Deletion of the repressors *Cry1* and *Cry2*

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reversed the results, highlighting opposing effects of the forward and reverse limbs of the clock. A key mechanism modulating these changes is the circadian control of nicotinamide phosphoribosyltransferase (*Nampt*), the rate-limiting enzyme for NAD+ biosynthesis [96]. NAD+ is a co-factor for sirtuins, including SIRT1 and SIRT3, a family of proteins capable of removing acetyl groups from proteins. SIRT1 is capable of regulating BMAL1:CLOCK and PER2, demonstrating a feedback loop between metabolism and the circadian cycle [96–98]. SIRT3 is crucial for regulation of FAO during fasting as it deacetylates and boosts the activity of enzymes involved in FAO such as long-chain acyl-CoA dehydrogenase [99,100]. In the absence of *Bmal1*, *Nampt* gene expression is diminished, resulting in a decrease in overall NAD+ biosynthesis, mitochondrial protein deacetylation and FAO. Triglycerides increase which can act as pro-inflammatory signals in diseases such as atherosclerosis [101] and diabetes [102]. Other feedback loops between metabolism and the clock have been described in recent literature. AMP-activated protein kinase (AMPK) is a metabolic sensor known to transmit energy-dependent signals to the clock [103]. AMPK is capable of phosphorylating and destabilizing CRY and PER, promoting activity of BMAL1, while AMPK sub-cellular localization, substrate phosphorylation and subunit composition are dependent on clock time [103]. The mTOR-effector kinase ribosomal S6 protein kinase 1 (SDK1), a regulator of protein translation, phosphorylates BMAL1, facilitating its association with the translational machinery to stimulate protein synthesis [104].

The field of immunometabolism has grown exponentially in the last 5 years. For example, it is now clear that the shift from mitochondrial oxidative phosphorylation to glycolysis in innate immune cells is crucial in providing the energy and signals for producing pro-inflammatory cytokines and other host defense proteins during infection [105]. Metabolites such as succinate and citrate have a direct effect on the functioning of immune cells such as macrophages and dendritic cells [106]. Mitochondrial dynamics has been implicated as a major determinant in T-cell fate [107]. BMAL1 and the molecular clock are major players in immunity, metabolic reprogramming and mitochondrial homeostasis. Therefore understanding the role of BMAL1 and the molecular clock in the control of immunometabolism will be of immense interest.

3. CLOCK

CLOCK, like BMAL1, is also a bHLH PAS domain protein. However, unlike *Bmal1*, *Clock* transcript levels do not alter significantly across the day in tissues and cells including macrophages [31]. CLOCK has important functions outside of its heterodimeric partnership with BMAL1. CLOCK also has histone acetyl transferase (HAT) activity, meaning it can add acetyl groups to certain lysine residues, including those in histone proteins, altering DNA confirmation and facilitating the transcription of a given gene [108]. Global deletion of CLOCK does not lead to loss of behavioural rhythmicity, with the mutant displaying only a mild impaired response to light [109]. This is due to the CLOCK paralog, NPAS2. A CLOCK/NPAS2 double knockout does display the expected arrhythmia [18]. CLOCK was first identified using ENU, a mutagenic technique, which produced the *Clock Δ19* mouse [110]. These mice harbor a 51-amino acid deletion in the transactivation domain of *Clock*, disrupting its ability to bind to other proteins including BMAL1 [111], leading to a range of metabolic dysfunctions. *Clock Δ19* mice become obese on a high fat diet [112], insulin resistant [113], and display altered plasma triglyceride [114] and glucose levels [85].

Activation of the subunit of nuclear factor kappa-light-chain-enhancer of activated B-cells (NF-κB) correlates positively with the circadian variation in lethality to LPS [115]. CLOCK complexes with p65 causing increased acetylation and phosphorylation of p65 and enhancing the activity of the NF-κB complex [115]. Both CLOCK mutant MEFs [116] and bone marrow derived macrophages (BMDMs) lacking CLOCK, are less responsive to LPS, and TNFα induced NF-κB activation [65]. Therefore CLOCK may drive, whereas BMAL1 may limit, pro-inflammatory responses in macrophages. In agreement with this, CLOCK is unaffected in macrophages stimulated with LPS whereas BMAL1 is repressed via targeting by miR-155 [22]. Peritoneal macrophages lacking *Bmal1* show an increase in phosphorylated p65 [22]. Therefore BMAL1 may exhibit some of its anti-inflammatory properties by sequestering CLOCK and preventing CLOCK-mediated activation of p65. Isolating the role of CLOCK from BMAL1 is difficult, however, the above research indicates that CLOCK serves as a critical regulator of both inflammation and metabolism, possibly independent of BMAL1 in certain cases.

3.1. The nuclear receptors, REV-ERBα and RORα

REV-ERBα and ROR belong to a family of nuclear receptors and control an important negative and positive feedback loop of *Bmal1* [20]. REV-ERBα recruits the nuclear receptor compressor-histone deacetylase 3 (NCoR-HDAC3) directly to the *Bmal1* gene, decreasing histone acetylation on the *Bmal1* promoter and blocking transcription [117]. REV-ERBα is a potent transcriptional repressor both by recruitment of the NCoR-HDAC3 complex but also via modulation of enhancer RNAs [118]. Global deletion of *Rev-erbα* leads to a metabolic phenotype [119]. Under a chow-fed diet, *Rev-erbα-/-* mice exhibit a 2.5 fold increase in adiposity and mild hyperglycemia due to altered lipid and glucose metabolism. These metabolic disturbances are further exacerbated with a high fat diet. Lipoprotein lipase (*lpl*), a gene important in lipid utilization and storage, is constitutively up-regulated in the muscle and adipose tissue of *Rev-erbα-/-* mice, contributing to increased fat [119]. In the liver, *Rev-erbα* represses expression of the gluconeogenic genes glucose-6-phosphatase (*G6Pase*) and phosphoenol pyruvate carboxykinase (*PEPCK*) [120]. Heme reversibly binds to REV-ERBα, increasing interaction with the NCoR complex. Binding of heme suppresses hepatic gluconeogenic gene expression and glucose output [120]. GSK4112, a synthetic agonist of REV-ERBα, mimics the effect of heme [121], while the opposite is seen with use of SR8278, a REV-ERBα antagonist [122]. Heme concentrations have been demonstrated to oscillate in a circadian manner [123] and thus, REV-ERBα may utilize heme as a signal for coordination of the cellular clock as well as for glucose homeostasis and energy metabolism [120], demonstrating the importance of clock driven metabolic synchrony in energy balance [119].

REV-ERBα also impacts on the inflammatory response. WT mice treated with LPS at CT12 display greater levels of serum IL-6 versus mice treated at CT0. Deletion of *Rev-erbα* results in heightened levels of IL-6 at both time points [124]. THP-1 cells treated with GSK4112 prior to LPS, decreased a range of pro-inflammatory cytokines and chemokines, including *Ccl2*. Interestingly, the repression of *Ccl2* by REV-ERBα is important in the onset, progression and resolution of vesicular stomatitis virus (VSV)-induced encephalitis [125]. Mice infected at ZT0 with VSV had a significant decrease in survival rate versus those infected at ZT12. This increase in lethality at ZT0 was associated with higher levels of circulating CCL2, a greater number of peripherally derived immune cells accumulating in the olfactory bulb, and greater levels of pro-inflammatory cytokine production. Levels of REV-ERBα in the olfactory bulb correlated with increased survival and blocking REV-ERBα activity prior to administration of VSV caused an increase in circulating CCL2 and a decrease in survival. Overall a picture is emerging of REV-ERBα's role in metabolic homeostasis and inflammatory suppression.

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The retinoic-acid-receptor-related orphan receptors (RORs) consist of 3 members RORα, β and γ. RORs function as transcriptional activators of *Bmal1* transcription, competing with REV-ERBα for a shared binding site in the promoter of the *Bmal1* gene [126]. Disruption of the *Rorα* gene via introduction of a premature stop codon in the natural mouse mutant staggerer (*Rorα sg/sg*) causes shortened period length when mice are in constant dark conditions [127]. These mice exhibit metabolic deregulation including reduced adiposity and protection from diet induced obesity [128]. On a normal diet *Rorα sg/sg* mice display vastly lower levels of lipid metabolism regulators such as, apolipoprotein A1 (APOA1), A2 (APOA2), C3 (APOC3) and high-density lipoprotein (HDL). Subsequently, lower levels of serum and liver triglycerides were observed in these mice due to direct regulation by RORα of sterol regulatory element binding protein-1c (SREBP-1c) and fatty acid synthase (FAS) [128,129]. RORα may also play a role in gluconeogenesis as the co-activators Peroxisome proliferator-activated receptor gamma coactivator 1-alpha and beta (PGC1α and PGC1β), proteins involved in mitochondrial metabolism and gluconeogenesis, are increased in *Rorα sg/sg* mice[130]. RORγ appears to play a role in metabolism through negative regulation of adipogenesis and insulin sensitivity. When overexpressed, RORγ leads to a decrease in the number of differentiated adipocytes in-vitro [131]. In *Rorγ*-/- mice, adipocyte generation is enhanced, but these cells are insulin sensitive, protecting the mice from obesity-induced hyperglycemia [131]. With RORα and RORγ being crucial for lipid metabolism and gluconeogenesis, a role for these proteins in the development of diabetes and obesity-induced insulin resistance seems plausible.

RORα and RORγ's role in the immune response has also been elucidated. Of particular note is their role in the differentiation of mature T helper17 (Th17) cells. These cells provide defense against extracellular and intracellular pathogens, however, they are also implicated as mediators of advancing autoimmune pathologies including experimental autoimmune encephalomyelitis (EAE), a model with features similar to multiple sclerosis, and collagen induced arthritis (CIA). Over-expression of RORγt (a RORγ isoform) drives the development of Th17 cells, where as *Rorγt* knockout mice display impaired Th17 development [132]. Double knockout of RORα and RORγ leads to complete ablation of Th17 cells, making these mice resistant to EAE [133,134]. The transcription factor Nuclear Factor Interleukin 3 regulated (NFIL3) was found to suppress Th17 cell development by binding to and repressing the *Rorγt* promoter [135]. Further linking the clock to this process is that *Nfil3* is directly suppressed by REV-ERBα. *Rev-erbα* -/- mice displayed greater NFIL3, lower RORγt and subsequently a decreased capacity for Th17 cell development [135].

The ROR proteins are also important in innate immune system as *Rorα sg/sg* mice are highly susceptible to LPS induced lethality [136]. This could be in part due to less transcriptional activation of *Bmal1*, or via RORα's ability to induce transcription of nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha (IκBα), limiting translocation of NF-κB to the nucleus [137]. Once again we see the dynamic nature and multiple targets of the clock proteins and the importance of this system for metabolism and immunity.

3.2. *The clock repressors:* Period *and* Cryptochrome

The period genes along with the cryptochromes genes form a repressor complex that inhibits transcriptional activity of the BMAL1:CLOCK complex. The Period genes, *Per* 1, 2 and 3 have been linked to metabolism, immunity and cancer. Global deletion of each of these genes leads to some form of circadian disruption, generally a shortened circadian period and impaired light response, while the double knock out of *Per1* and *Per2* causes arrhythmia [138]. Of these 3 proteins, PER2 has been the most studied in terms of metabolism and immunity. *Per2*-/- mice show a reduction in levels of total triacylglycerol and non-sterified fatty acids [139]. *Per2* deletion also leads to enhanced adipocyte differentiation in cultured fibroblasts. This was due to PER2 mediated suppression of Peroxisome proliferator-activated receptor gamma (PPARγ), a nuclear receptor that is crucial in adipogenesis [139]. *Per3* deletion also leads to enhanced adipogenesis while ectopic expression of PER3 inhibits it [140]. Global *Per3* knock out mice exhibit increased deposits of adipose tissue and reduced muscle tissue in comparison to wild-type mice [140]. PER proteins have also been linked to glucose storage with *Per2*-/- mice exhibiting a loss of rhythmic glycogen accumulation in the liver, impaired gluconeogenesis and elevated plasma insulin levels [141–143].

PER2 has also been linked to the inflammatory response. Global *Per2*-/- mice exhibit the opposite effect to LPS induced endotoxic shock than the effects seen in myeloid-*Bmal1* deletion. *Per2*-/- mice are resistant to LPS-induced lethality at all time points analysed [144]. Serum levels of IL-1β and IFNγ were significantly lower in PER2-/- mice following LPS injection, although IL-6, TNFα and IL-10 levels were comparable to controls [144]. Increased levels of IFNγ were attributed to Natural Killer (NK) cell function, providing a role for PER2 in NK cells. *Tlr9* mRNA levels were diminished in the macrophages of *Per2*-/- mice and displayed a blunted response to CpG stimulation [82]. It is possible that PER2 may drive inflammation by repressing BMAL1.

Two variants of cryptochrome exist in mammals, *Cry1* and *Cry2*. In insects and plants CRY1 regulates the circadian clock in a light-dependent manner, in mammals CRY1 and CRY2 functions in a light-independent fashion [145]. *Cry1/Cry2* double knock out mice exhibit arrhythmia in free running conditions. As discussed earlier however, CRY1 and CRY2 can impact on cellular metabolism with the double knockout MEFs exhibiting increased mitochondrial ATP, NAD+, FAO and decreased levels of glycolytic lactate [95]. It may be that the cryptochrome proteins promote a more glycolytic phenotype through negative regulation of the BMAL1:CLOCK heterodimer.

Similar to that of *Bmal1*, complete clock disruption with *Cry1/Cry2* double knockout leads to enhanced inflammation. In a model of collagen induced arthritis (CIA), mice lacking CRY1 and CRY2 display greater levels of TNFα and have heightened inflammation in their joints versus wild-type mice [146]. By ectopically expressing CRY1 in MEFs isolated from *Cry1/Cry2* –/- mice, activation of a TNFα luciferase promoter was vastly decreased. This could possibly be due to CRY1 limiting NF-κB activation as has previously been demonstrated [147]. Absence of CRY1 and CRY2 in fibroblasts and BMDMs leads to a basal increase in mRNA levels of *Cxcl1, Il-6 and Tnfα*. CRY1 is capable of binding to adenylate cyclase, subsequently limiting cyclic adenosine-monophosphate (cAMP) production, decreasing protein kinase A (PKA) activity, culminating in lower levels of p65 phosphorylation and NF-κB activation. With an emerging role of CRY in regulating NF-κB, a recently discovered activator of CRY could have potential in the treatment of inflammatory conditions revolving around the innate immune response. KL001 is capable of preventing ubiquitination of both CRY1 and CRY2, resulting in lengthening of the circadian period [148]. This compound has already been shown to have metabolic effects leading to inhibition of glucagon-induced gluconeogenesis in primary hepatocytes. It has also been demonstrated to have an anti-inflammatory role in fibroblast-like synoviocytes (FLSs), a potential candidate for producing rhythmic inflammatory signals during collagen induced arthritis[149]. KL001 and other clock compounds not only provide a tool for dissection of the molecular clock, but may also aid in the development of circadian based treatment for inflammatory and metabolic diseases [148].

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4. Final remarks

The molecular clock regulates many aspects of our metabolic and immune systems and perturbation of our clocks impacts metabolism and augments the inflammatory response. Clock disturbance appears to impact on many if not all of our chronic diseases in which inflammation is an underlying factor. At each phase of the molecular clock there is some form of interplay between the individual clock proteins and metabolic and immune signaling. The clock gives rise to anticipatory immunity, the concept that the clock is allowing an organism to anticipate and prepare for upcoming challenges in its environment, such as the onset of activity and feeding. In addition, daily changes in immune function may also be a direct consequence of clock-controlled immunometabolism, in which the daily rhythm in feeding, nutrition and fuel utilization is impacting on the immune response. Such knowledge may have relevance to combat obesity and inflammatory diseases in shift workers and other populations with clock disturbances. Work done in the area of chronotherapy may prove that the clock is an ideal target to improve the efficacy of immune targeted therapies including vaccinations. In the last two decades we have made significant advances in understanding how the clock ticks and what is under the control of the clock. However there are still many unanswered questions. A comprehensive understanding of how the clock is controlling metabolism within immune cells and what impact this has on immune function is still largely unknown. Bringing together the two fields of circadian biology and immunometabolism may provide some answers as to why and how the immune system and molecular clock are so intricately linked.

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