The involvement of host circadian clocks in the regulation of the immune response to parasitic infections in mammals

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The authors declare that there is no conflict of interest

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Abstract

Circadian rhythms are recurring variations of physiology with a period of ~24 hours, generated by circadian clocks located throughout the body. Studies have shown a circadian regulation of many aspects of immunity. Immune cells have intrinsic clock mechanisms, and innate and adaptive immune responses – such as leukocyte migration, magnitude of inflammation, cytokine production and cell differentiation – are under circadian control. This circadian regulation has consequences for infections including parasitic infections. In the context of *Leishmania* infection, the circadian clock within host immune cells modulates the magnitude of the infection and the inflammatory response triggered by the parasite. As for malaria, rhythms within the immune system were shown to impact the developmental cycles of *Plasmodium* parasites within red blood cells. Further, host circadian rhythms impact infections by multicellular parasites; for example, infection with helminth *Trichuris muris* shows different kinetics of worm expulsion depending on time of day of infection, a variation that depends on the dendritic cell clock. Although the research on the circadian control of immunity in the context of parasitic infections is in its infancy, the research reviewed here suggests a crucial involvement of host circadian rhythms in immunity on the development and progression of parasitic infections.

Keywords: Circadian rhythms, immune cells, parasite, mammalian host, *Leishmania*, *Plasmodium*, helminths

Introduction: circadian rhythms in the immune system

Living organisms are subjected to various environmental fluctuations including 24-hour cycles due to the rotation of the Earth on its axis, e.g. day-night cycles. Being able to predict such recurring variations, rather than passively responding to them, has conferred evolutionary adaptation to the organisms. This has been possible thanks to biological clocks, which are found in almost all phyla. In addition to anticipating environmental variations, internal clocks enable the partitioning of the organism's physiology into different temporal niches (for example to avoid two concurrent opposing phenomena). Circadian rhythmicity of immune responses and reaction to environmental pathogens is one important aspect to this adaptation to the environmental rhythms. In a host-parasite interaction, the host and the parasite are under constant process of co-evolution. Using their internal clocks, hosts try to anticipate the time of infection and to optimize their immune response accordingly, while on the other hand, parasites can take advantage of the rhythmic circulation of host's immune cells and other physiological rhythms (Figure 1). This review will focus on how circadian rhythms within the immune system of mammalian hosts can affect the defense against parasites and the outcome of the infection (Figure 2).

In mammals, circadian clocks can be found throughout the body, in most organs and cell types [1,2]. The suprachiasmatic nucleus (SCN), located in the hypothalamus in the brain, is the master circadian clock, which plays a key role in coordinating the clocks located in other tissues[3]. As described below, this includes cells of the immune system. The molecular basis of the clock is based on circadian clock genes [1,4]. These genes participate in transcriptional/translational autoregulatory feedback loops. The transcription factors CLOCK (Circadian locomotor output cycles kaput) and BMAL1 (brain and muscle ARNT-like protein 1, also called ARNTL) form a heterodimer and bind promoters at E-box sequence elements, thereby activating the transcription of *Period* (*Per*1 and *Per*2), *Cryptochrome* (*Cry*1 and *Cry*2) genes, as well as genes encoding nuclear receptors of the REV-ERB and ROR families. PER and CRY slowly accumulate to negatively feedback and inhibit CLOCK/BMAL1

activity, and thus, their own transcription, whereas REV-ERBα/β represses *Bmal1* transcription (and also *Clock* and *Cry1*) and RORα/β/γ activates it. These positive and negative feedback loops act temporally in such a manner that the transcripts and proteins encoded by many of the clock genes show a ~24 hour variation in their abundance. The timing and robustness of this circadian clockwork is aided by post-transcriptional and post-translational modifications mediated by many enzymes such as kinases, phosphatases, acetyl transferases, ubiquitin ligases, etc. [1,5].

The circadian clockwork regulates various cellular and physiological processes via the control of the expression of clock-controlled genes (CCGs). Transcription factors such as CLOCK/BMAL1 or REV-ERB*α* bind numerous genes besides their targets within the clock. Since the abundance and/or activity of these transcription factors varies over 24 hours, their target genes have the potential to show a circadian pattern of expression. More than half of all genes are predicted to be rhythmic in at least one location in the body, and any organ has 4% to 20% of its transcriptome rhythmically expressed[6]. Recent advances in proteomic approaches have also highlighted the diurnal oscillations of protein abundance in SCN and peripheral organs, and the role of posttranscriptional and posttranslational modifications [7,8].

Such a circadian control of molecular and cellular processes is pervasive throughout the immune system. Although the reader is referred to recent reviews for a comprehensive description of circadian rhythms in the immune system [9-12], we will overview some main principles, which are relevant to the immune response against parasites. Circadian rhythms in immunity have been particularly well studied for cells of the innate immune system, which are central in parasitic infections. Innate immunity acts as a first line of defense against pathogens. This response occurs via several mechanisms such as phagocytosis, production of antimicrobial agents, and cytotoxicity (induction of cell death) [9]. Innate immune mechanisms are not specific for a particular pathogen. Instead, pathogen detection is broader, often via the recognition of motifs shared by pathogens of broad families, called PAMPs (pathogen-

associated molecular patterns), via PRRs (pattern-recognition receptor) present on the innate immune cells [9].

Innate immune cells, such as macrophages[13-15], neutrophils[16], eosinophils, mast cells[17], dendritic cells (DCs)[13] or natural killer (NK) cells[18,19] express clock genes. As other cell types, they also express a large number of CCGs. Based on this, many functions of innate immune system — e.g. cell recruitment to target tissues, phagocytosis, PRR expression and signalling, cytolytic activity, release of cytokines — are clock-controlled [9,13-15,20-22]

For example, about 8% of genes in peritoneal macrophages are expressed in a circadian manner. These CCGs include genes playing a role in phagocytosis, stress response, immune regulation, metabolism and wound healing [15,23]. Recent work showed that rhythmic protein expression also plays a major role in regulating the clock output in macrophages[23]. Therefore, it is not surprising that many studies have uncovered circadian rhythms in various aspects of macrophage or monocyte functions, including:

1) *The response of these cells to PAMPs via different PRRs.* For example, secretion of cytokines and chemokines in response to lipopolysaccharide (LPS) depends on the time this bacterial wall component is injected in mice[24,25]or added to macrophages in vitro[14,15,26]. This was dependent on a clock within myeloid cells, and the rhythmic transcription factor REV-ERBα seems to have a role in this rhythmicity[20].

2) *Phagocytosis.* This process, by which cells engulf particles and pathogens, shows diurnal variation in macrophages in vitro and is regulated by the circadian clock[14,16,23,26]. *Bmal1* gene deletion enhances bacterial phagocytosis by macrophages in vitro[22]. A recent study, however, has questioned the clock-dependent regulation of phagocytosis [27]

3) *Mitochondrial dynamics, metabolism and oxidative stress.* About 29% of the proteome in macrophages is under circadian control, out of which are proteins with roles in the electron transport chain, glycolysis and TCA cycle. Hence, the basal respiratory rate in macrophagic mitochondria also

shows diurnal variation [23]. This circadian regulation of macrophage metabolism might be connected to the rhythmicity found in mitochondrial dynamics and oxidative stress pathways in these cells[26,28].

4) *Monocyte trafficking across tissues.* The total numbers of monocytes in the blood, spleen and bone marrow show daily variations and the recruitment of inflammatory monocytes to the site of inflammation is regulated via an action of the circadian clock on chemokines [29].

Other innate immune cells also display daily rhythms in their functions. Of particular relevance for parasitic infections, this is the case of neutrophils and NK cells. Neutrophils are key cells of the innate immune system, as they are often the first cells to be recruited to the site of infection. For example, upon nasal administration of LPS, which triggers lung inflammation, more neutrophils are attracted to the lungs upon treatment of mice in the morning than at other times of the day. This is due to a clock in lung epithelial cells, which regulates neutrophil-attracting chemokines[30,31]. The population of neutrophils is maintained in the bone marrow and released into the blood in a temporally-gated manner[32,33].Various pathways oscillate in a daily manner within neutrophils, and BMAL1, together with chemokine receptors CXCR2 and CXCR4, act as a timer to regulate the aging of the neutrophils across the day, with impact on anti-microbial activity and cardiovascular health[34]. NK cells are key to the fight against infected cells (e.g. with viruses or parasites) and cancer cells. NK cell numbers vary across the day in rodents [18,19].Cytolytic factors, such as granzyme B and perforin, are expressed by NK cells with a circadian rhythm, and consequently, the cell-killing function of these cells is time of day-dependent[18,19]. In the past few years, evidence have shown diurnal expression of clock genes in innate lymphoid cells (ILCs), which are cells of lymphoid origin but with no antigen-specific receptors[35-37]. In addition to local circadian clocks, adrenergic innervation and factors like hypoxia inducible factor-1 α have been shown to play a role in regulating circadian trafficking of leukocytes[38,39].

The adaptive immune system consists of two kinds of lymphocytes — T lymphocytes and B lymphocytes (also called T and B cells), along with antigen presenting cells (APCs), which are often

cells of the adaptive immune system, such as DCs. T and B cells mature in the thymus and bone marrow respectively, from where they migrate to secondary lymphoid organs such as lymph nodes (LNs) and the spleen. DCs (and other APCs) present antigens (associated to major histocompatibility complex [MHC] molecules) to the naive T cells, which will then proliferate and differentiate into effector T cells. CD4+ T cells act mainly via secretion of cytokines to modulate the activity of other immune cells (e.g. macrophages, B cells) which will then act on the invading pathogen; CD8+ T cells, on the other hand, secrete cytolytic factors to kill target cells, e.g. cancer cells, virus-infected cells, or, or particular interest for this review, parasite-infected cells. B cells are also involved in humoral immunity, as they produce pathogen-specific antibodies, which can neutralize the infectious agents. Circadian clock genes are expressed in adaptive immune cells such as CD4+ and CD8+ T cells, and B cells [13,15,40-42].

There have been extensive studies on the circadian rhythms of lymphocyte trafficking. The circulating lymphocyte counts in mice follow a circadian rhythm with a peak in the light phase and trough in the early dark phase [43-45]. The rhythm is inverted in the human blood, with peak counts of CD4+ and CD8+ T cells at night [43,46-48].Various regulators were shown to play a role in lymphocyte trafficking between blood and other tissues: chemokine-receptor pairs [47,49,50], glucocorticoids [47,49,50]and input from adrenergic nerves[45,47]. The circadian regulation of adaptive immunity also extends to the functional responses of lymphocytes. In particular, the response of T cells to antigen presentation by APCs is regulated by their own intrinsic clock [40,41].The development and differentiation of different subpopulations of T cells depends on the time of day and on circadian clocks. This is the case for the differentiation of Th17 and Treg cell populations, which are important for the development of auto-immune diseases such as rheumatoid arthritis and multiple sclerosis (MS), and for immunity as mucosal sites, e.g. in the intestine[51-53]. In contrast though, one study did not find an effect of T cell-specific clock inactivation on Th17 counts or in an experimental model of MS[54].

In studying the role of clock in host-parasite interactions, one must investigate the dynamics between several clocks (Figure 1): 1) The clocks located in host's tissues including in cells of the immune system, which will be the main focus of this review; 2) The clocks in the vectors (insect vectors that transmit parasites such as *Plasmodium* or *Leishmania*), which play an important role in carrying the parasites to their hosts. Timing of their traits like locomotor activity, blood feeding, mating and egg laying determines transmission of the parasite[55-58]; 3) Clocks within the parasites themselves, as even unicellular eukaryotic parasites such as *Trypanosoma* and *Plasmodium* were shown to display endogenous circadian rhythms[59-61]. Adding to the complexity, parasitic infections are known to modify and subvert host's cellular processes to their own advantage, and this is the case for circadian rhythms. For example, infecting mice with *Trypanosoma brucei,* which causes sleeping sickness, resulted in a disruption of the activity-rest rhythms, and alterations in the phase and period of the circadian rhythms, effects that seem to rely on direct effect on tissue clocks [62]. Additionally, *Plasmodium chabaudi* infection in mice resulted in a transient disruption of activity and temperature rhythms, but no effects on circadian period [62,63]. Thus, in the latter case, the changes in activity and temperature might be mediated by the systemic inflammation caused by the infection[64].

Leishmania Parasitic Infection in the context of host's circadian rhythms

Leishmaniasis is a tropical and subtropical disease – which affects 12-15 million people in the world[65]. It is caused by the intracellular parasite *Leishmania*. There are several clinical manifestations which range from less severe (cutaneous lesions) to more severe (fatal visceral infections) leishmaniasis [65]. *Leishmania* parasites are transmitted to humans through the bite of a sandfly, which injects infectious promastigotes into the host. The *Leishmania* promastigotes are then phagocytosed by innate immune cells such as macrophages, neutrophils, and dendritic cells (DCs) [66]. Neutrophils are the first and most rapidly recruited immune cells to the site of *Leishmania* infection[67]. *Leishmania* parasites are able to survive within the neutrophils and extend the neutrophil lifespan[68]. Neutrophils are thus

believed to act as transient shelter to *Leishmania* parasites, and once the neutrophils become apoptotic the parasites will be taken up by their long-term hosts macrophages[69]. Macrophages (and to a lesser extent DCs) are the primary host cells at the site of infection, and they will lead to the production of key cytokines — interferon (IFN) γ[70], tumor necrosis factor (TNF) α[71,72], and IL-12[73]. Natural killer (NK) cells will then be activated, and they can lyse the *Leishmania*-infected macrophages and DCs [74].

The adaptive immune response plays an important role in the protection, and immune response against *Leishmania* parasites in later stages of the disease. The adaptive immune response to *Leishmania* infection is driven primarily by T cells[74-77]. The Th1 immune response to *Leishmania* was shown to be protective, as C57BL/6 mice which produce mainly Th1 when infected with *Leishmania* have a self-limiting cutaneous infection[78]. On the other hand, BALB/c mice infected with *L. major* will develop more severe pathology, as they produce a Th2 immune cell response when infected[75,78,79]. CD8+ T cells are important components for host defence against *Leishmania*, as they target and kill infected cells[76,80,81]. Additionally, CD8+ T cells are important during reinfection, as they will expand 50-fold and are associated with an increased production of IFNγ, thus promoting the Th1 response and parasite clearance[80,82].

All these immune cells involved in *Leishmania* infection were shown to have a circadian clock, and to be regulated by circadian rhythms. Thus, it is of interest to ask whether *Leishmania* infection and the immune response to it might be regulated by host circadian clocks. Recent research, which will be described thereafter, has suggested that this is indeed the case.

Time of day of infection and progression of the disease

It is known that sandflies – the primary insect vector of *Leishmania* – exhibit circadian behaviour, as they tend to bite at night [83-87]. The *Leishmania* parasite will then cause a strong inflammatory response within the host. Is the time of highest likelihood of infection also a time of highest host sensitivity and response? Two studies have addressed this question by looking at parasite

load and footpad swelling (a measure of the local inflammation at the site of infection, following parasite injection in the hind paw).

A study investigated the *Leishmania* infection intensity by injecting *Leishmania major* parasites (which cause cutaneous leishmaniasis) into the footpad of C57BL/6J mice, at different circadian times (CT) under constant darkness conditions (to reveal the endogenous nature of any time-dependent variations). Slower and limited footpad swelling was observed in mice infected at the beginning of the subjective day (CT3), whereas the mice infected at other times showed faster and increased swelling [88]. This was paralleled by the parasitic load – measured by assessing parasite DNA by PCR in the footpad and the draining popliteal lymph node: parasitic burden was lowest in the mice infected at CT3, and highest in those infected at night [88].

Another group infected hamsters with *Leishmania amazonensis* (which also causes cutaneous leishmaniasis) either in the light phase (Zeitgeber Time [ZT] 8, i.e. 8 hours after lights on) or dark phase (ZT22, i.e. 8 hours after lights off). Hamsters infected at night presented reduced footpad swelling compared to those infected in the daytime, 14 days post infection. Consistently, parasite load was lower in dark-infected hamsters [89]. The apparent discrepancies between the studies in the time of greatest inflammation and parasite development might be due to the different host species, and by different times selected for the infections. However, these data point to a circadian regulation of the course of experimental leishmaniasis in mice.

Innate immune cell rhythm in response to Leishmania infection

The above-described results indicate a time of day-dependent regulation of the inflammation in response to *Leishmania* infection, which might underlie the course of the disease. Since immune cells express the clock machinery, and display circadian rhythms in cell counts and functional responses, it is likely that the circadian rhythms in *Leishmania* infection are due to rhythms in cells of the initial innate immune response to the infection.

Bone marrow-derived macrophages (BMDMs) were used to assess the attachment and internalization of *L. major* parasites over time. Cellular clocks were synchronized, and parasites were added at different times over 24 hours. Attachment of the parasite at the macrophage's surface was assessed one hour after infection, whereas its internalization inside the cells was assessed after 6 hours. In both cases, there was a variation depending on the time of infection [88]. Moreover, the 24-hour variation was abolished when BMDMs derived from mice knockout for the essential clock gene *Bmal1* were used, confirming the involvement of the macrophage clock in the time-dependent variation. *Leishmania* attaches to the macrophage's membrane via receptors. Two of these were tested, complement receptor 2 (CD11b) and mannose receptor (CD206): they were rhythmically expressed at the surface of non-infected and infected BMDMs. However, it remains to be determined whether these receptor rhythms underlie the circadian variation of attachment[88].

In the same study, the authors infected mice by injecting *L. major* within the peritoneal cavity. Immune cell frequencies were evaluated 3 and 6 hours post injection [88]. Consistent with the effect of time of day on inflammation after footpad injection, the lowest parasite load was observed after infection at CT3 (early subjective day), whereas highest parasite load occurred after infection at CT9/CT15 (late day, early night). Interestingly, recruitment of innate immune cells (neutrophils, macrophages) to the site of infection also presented a rhythm with highest levels following infection at CT9/CT15. In mice where clock function is abolished in immune cells (by irradiation followed by graft of bone marrow from *Bmal1* KO mice), the morning-evening difference in parasite load and cell recruitment was lost. Similar results were found for cytokine and chemokine expression in peritoneal cells, showing higher levels in the evening, but not in mice lacking BMAL1 within immune cells. This indicates that the clock in immune cells is driving this rhythm of parasitic infection and inflammatory response [88].

Adaptive immune cell rhythm in Leishmania parasitic infections

As described above, the adaptive immune response plays an important role in the protection and immune response against *Leishmania* parasites. Yet, very few studies have looked at circadian rhythms of adaptive immune cells in the context of *Leishmania* infection. In the Kiessling et al. study described above, CD4+ T cells in the peritoneum had circadian rhythms, which was lost in mice infected with *L. major*[88]. CD8+ and CD4+ T cells frequency was increased 6 hours post-infection, in particular after infection at CT9. However, since T cells are mainly important at later stages during the course of infection with *Leishmania*, more work will be needed to address the possible role of circadian rhythms in T cells in the infection with this parasite.

Another study looked at the antibody production in dogs infected with *Leishmania infantum* (which causes visceral leishmaniasis). Serum and saliva were sampled over 2 days, in dogs 67 days after infection. Anti-*Leishmania* antibodies were quantified. IgG2 and IgA levels did not vary within the serum and saliva over the 2-day period. This suggests that antibody levels do not vary according to the time of day in dogs infected with *Leishmania*[90]. However, the study was done in only 6 dogs, and sampling was done only over a period of 16 hours each day (no samples between midnight and 8:00AM). Moreover, it does not exclude a possible circadian regulation of B cell functions in the context of *Leishmania* infection.

Possible impact of melatonin on Leishmania infection

Melatonin is a hormone synthesized by the pineal gland during the dark phase. Additionally, the progression of bacterial, viral and parasitic infections has been shown to be modulated by melatonin[91-94]. To address a possible impact of this circadian hormone in leishmaniasis, Laranjeira-Silva et al. injected *L. amazonensis* in the footpad of hamsters in the dark phase and in the light phase [89]. As mentioned above, night-infected hamsters had a lower footpad swelling. However, hamsters treated with a non-specific antagonist of melatonin receptors (luzindole) at the beginning of the night (ZT13) and infected later in the night (ZT22) developed increased lesions, similar to hamster infected during

the light phase (ZT8). Conversely, hamsters treated with melatonin in the early day, prior to *Leishmania* infection later in that day, developed smaller lesions, similar to those infected during the dark phase. This suggests that endogenous nightly melatonin production attenuates the infection in the animals infected at night. To address a possible cellular mechanism, the authors treated mouse peritoneal macrophages with melatonin or luzindole and then infected them with *Leishmania*. Melatonin and luzindole respectively reduced and increased, by about two-fold, the infection of cells by the parasites. These results suggest that the progression of leishmaniasis may be in part controlled by circadian hormones of the host.

Rhythms in *Plasmodium* intraerythrocytic developmental cycle and inflammation

Besides leishmaniasis, another example of a parasitic disease influenced by host circadian rhythms is malaria. Malaria is a life-threatening infectious disease caused by the protozoan *Plasmodium spp.* and transmitted by *Anopheles* mosquito's bite[95,96]. Inoculation of parasite's sporozoite stage form, present in the mosquito's saliva, precedes its migration to the liver and the infection of hepatocytes[96]. Within the hepatocyte, the parasite differentiates into the merozoite form[95,96]. Under this form the parasite is released into the bloodstream to invade and asexually replicate (consequently generating the trophozoite and gametocyte forms) within their final target cells, erythrocytes (red blood cells)[95,96]. The release of gametocytes to the bloodstream is crucial for closing the parasite's life cycle, as this is the infective form for *Anopheles* mosquitos[95,96].

The release of parasites upon erythrocyte lysis enables them to infect other erythrocytes and to cause the inflammatory component of the disease and its paroxysms. Malaria's paroxysms are characterized by fever, shaking chills, muscle aches and other symptoms [95], which are the result of the activation of innate immune cells of the myeloid lineage, mainly macrophages. Strong activation of macrophages leads to the secretion of pro-inflammatory cytokines, which are the main factors for the disease severity outcomes, including enhancement of adhesion molecules expression, sequestration of

infected red blood cells in lungs and brain, renal impairment, and disruption of the blood brain barrier (cerebral malaria)[97,98].

Notably, the developmental cycle of the parasite within red blood cells (intraerythrocytic developmental cycle [IDC]) is highly synchronized within an organism. Moreover, the rupture of schizont-infected erythrocytes occurs concomitantly with the disease paroxysms and the high levels of pro-inflammatory cytokines in the bloodstream, every 24, 48 or 72 hours (depending on the parasite species) [99,100]. These rhythms with periods that are multiples of 24 hours suggested a relationship between the synchronized parasite developmental cycle and the host's circadian rhythms, more specifically rhythms in immune responses. One aspect of the inflammatory response that was proposed as a possible synchronizing signal was temperature. Increased body temperature, as in episodes of malaria fevers, is a body defense mechanism against pathogens. To address a possible impact of body temperature on the IDC, Kwiatkowki et al. cultured *Plasmodium falciparum* in two different temperature cycles: 37°C to simulate normal host temperature, and 40°C, to mimic a "febrile" state. As expected in the study, parasite growth in "febrile" conditions was impaired in comparison to normal conditions. The proportion of each parasite stage-form (more specifically rings and trophozoites) was drastically altered in this context, suggesting that temperature can indeed influence the parasite IDC[101].

Another aspect of the inflammatory response to *Plasmodium* infection is the release of pro-inflammatory cytokines (during the paroxysms). Whether cytokines might regulate the IDC was addressed using mice infected with *Plasmodium chabaudi.* It was shown that IFNγ and TNFα signaling is essential to control the IDC and synchrony of the parasite stages within red blood cells, probably via a regulation of blood glucose levels [102]. In the same study, *Rag1*$^{-/-}$ mice (mutant mice that lack both T and B lymphocytes, key players of adaptive immunity) had an impaired synchronization of the IDC whereas ablation of neutrophils did not affect IDC rhythms[102]. This was evidence that the host's adaptive immune system can influence *Plasmodium* developmental stages. In contrast, data from another group have suggested that rhythms in inflammatory response do not time the IDC. Instead, although cytokines

such as TNFα and IFNγ simply followed a phase similar as the IDC, both seemed to be controlled by other host rhythms, including the feeding behavior [103].

Another way to address the question of the host-based regulation of the IDC is to ask whether there is an impact of the host's circadian clocks. O'Donnell and colleagues provided a first indication that this might be the case: they induced a mismatch between the rhythms of the mouse host and the parasite (*P. chabaudi*), by housing the host mice and those providing the infected red blood cells on different light-dark cycles. They found that there was a fitness cost of the phase mismatch on parasite replication and transmission[104]. The same authors more directly asked the implication of the host circadian clock by comparing WT mice with mice KO for clock genes *Per1* and *Per2* (mice with no clock function), either on ad libitum feeding or on time-restricted feeding (food access 10 hours/day), all under constant darkness: the parasite synchrony and IDC rhythms depended on feeding rhythm, and not on the *Per1/Per2* genotype, suggesting that the host's clocks are not setting the IDC rhythmicity, but that the time of food availability does[105].

However, a subsequent report by Rijo-Ferreira and colleagues suggested that there must be other factors besides feeding rhythms: *P. chabaudi* IDC rhythm persisted even when feeding of the mice was evenly spread over 24 hours: therefore, feeding rhythms are not required for IDC rhythms[60]. Further, the same authors provided evidence of a role of host clocks: they used host mice with a period longer than WT mice (*Fbxl3* mutant mice), and showed that the IDC duration matched with the (longer) host period. Another study also showed that the IDC is sensitive to the phase of the host rhythms [61]. Notably, although this was observed for *P. chabaudi* (which has an IDC duration of 24h), it is not the case of *P. berghei* ANKA (whose IDC has a duration of 21-23 hours, not a multiple of 24 hours, and is not synchronized): this parasite species is resistant to host rhythms (in terms of IDC synchronization or parasite fitness)[106].

Overall, the nature of the host factors involved in the synchronization of the IDC, and the exact requirement of host clocks, are still unclear. In particular, it is still unknown whether host's immune cell clocks, or rhythms in immunity, are involved in any way. Also, most of the literature so far has focused

on the regulation of the IDC by host signals. What about other aspects of the malaria pathology and course of the *Plasmodium* infection?

The recent discovery of endogenous circadian rhythms in *Plasmodium* has added another layer of complexity. Two studies have used cultures of red blood cells infected with human parasite *P. falciparum*, whose WT strains has an IDC duration of 48 hours. The use of such cultures allows to study rhythms in isolation, without host influences that are present in experiments done in mice. Smith and colleagues studied four strains with different periods deviating from the WT period, and ranging from 35 to 60 hours. Looking at the parasite transcriptome over 60-70 hours, they found numerous genes with rhythmic expression, with a period very similar to the respective periods of the *P. falciparum* strains[107]. Similarly, Subudhi and colleagues cultured red blood cells infected with *P. falciparum*, which have a 48-hour IDC period, but in studying rhythmically expressed genes, they discovered that although some transcripts had period matching the IDC period, hundreds of them had a period of 24 hours[61]. This suggested the presence of endogenous circadian oscillations within the parasites. This was supported by experiments of Rijo-Ferreira and colleagues using *P. chabaudi*-infected mice: even when using mouse hosts without clock function (*Cry1/Cry2* KO mice), there were still thousands of transcripts with significant circadian rhythms[60].

How are host circadian rhythms, environmental cues and parasite rhythms connected? And how do parasites benefit from this balance? More importantly, how do we break this interplay to achieve therapeutic success? These are all urgent questions that must be addressed while millions of people suffer from *Plasmodium* infection.

Influence of the host circadian rhythms on the response to helminths

The periodicity of multicellular parasitic organisms, such as worms, has been reported decades ago. This was the case, for example, in infection with microfilariae, an early stage in the development of certain parasitic nematodes. Many reports showed daily variations of microfilariae in the blood of

human subjects and dogs [108,109]. Cutaneous abundance of the microfilariae was also shown to vary over the day in dogs[110,111]. Daily variations of these parasites appear to be due to a 24-hour rhythm in parasite migration between the blood and tissues[109,112]. Microfilariae are the form of the parasite that are transferred to the mosquito vector. In this respect, it was interesting that there was a coincidence in the times of highest numbers of microfilariae in the blood and the biting activity of the vectors, suggesting an evolutionary adaptation favoring transmission[109,113]. Despite these interesting data, very little is known about what drives the in vivo rhythmicity of parasitic worms, and whether other host circadian clocks, including immune cells clock, could be playing a role.

A recent report has addressed this question in the context of infection of mice with the intestinal parasitic helminth *Trichuris muris* [114]. In this model, the worms establish in the mice over the first weeks, before their expulsion about three weeks after infection. Mice were infected with worm eggs either in the early day (ZT0) or early night (ZT12). Expulsion of the parasite was delayed in mice infected at ZT12, with higher remaining worm burden in the gut after 21 days. This was paralleled by a bias towards a Th2 response in the mice injected at ZT0, which is consistent with a more efficient clearance of the parasite. These morning-evening differences in worm expulsion and immune response were lost in mice lacking clock gene *Bmal1* specifically in DCs. This suggested that the clock in DCs is required for the time of day-dependent variation in response to *T. muris*. The role of the circadian clock in DCs has been relatively understudied. The authors addressed this by comparing the transcriptome of WT and *Bmal1* KO DCs, with or without treatment with *T. muris* excretion-secretion (ES) antigen. Different pathways were affected by *Bmal1* deletion or by ES antigen exposure, including pathways dependent on IL-12 and IL-27, cytokines that promote Th1 responses (and thus, failure to expel *Trichuris*)[114].

For intestinal worms, the population of immune cells residing at the mucosal tissue is crucial in providing a controlled environment while they need to efficiently respond to intestinal parasites. In this regard, it is interesting that recent studies have uncovered a circadian regulation of the migration of

DCs across tissues. For example, *Bmal1* KO DCs injected in mice were shown to migrate less efficiently to the spleen[40]. Also, the trafficking of DCs through lymphatic vessels has a daily variation, which appears to be abrogated in mice lacking *Bmal1* in DCs[115]. Other studies have also uncovered a circadian regulation of immunity in the gut, with time-dependent regulation of antigen presentation and cytokine secretion, including a regulation of DCs (and other myeloid cells), innate lymphoid cells 3 and intra-epithelial T cells [36,37,116,117]. It would be interesting to test whether such a clock regulation of cell migration and antigen presentation at mucosae might contribute to the time-dependent effects in *T. muris* infection. Finally, given that helminths are animals, they are likely to have their own circadian system, and evidence of endogenous circadian timing mechanisms has actually been found in worms such as the nematode *Caenorhabditis elegans*[118-120]. Therefore, as discussed above for the protozoan parasites, the interplay between the host's clocks and those of the helminths will have to be considered.

Conclusions

Although research at the intersection of chronobiology and parasitology started decades ago, it is only recently that possible molecular and physiological mechanisms have started to be addressed. Here we have focused on the involvement of host circadian clocks in the parasite-mammalian host interactions, in particular via the circadian regulation of the immune response to these infections (Figure 2). Circadian rhythms in the host's immune regulation have a direct implication on the transmission, disease progression and intensity of the disease. However, most of the literature addressing this host circadian rhythms/immunity/parasite axis comes from research on only a few parasitic diseases, such as leishmaniasis, worm infection, and to a lesser extent, malaria. More research will be needed, for these diseases, and for infection with many other parasites, to fully grasp the breadth of the host immune rhythm impacts on parasitic infections, and the various mechanisms by which this can happen.

Research will also have to address the interplay between these host rhythms (including in the immune system) and the endogenous and environment-driven rhythmicity displayed by the insect vectors and by the parasite themselves (Figure 1), as several parasites (e.g. *Trypanosoma, Plasmodium*) were shown to harbour endogenous circadian rhythmicity.

Another topic of interest is whether and how parasites can act on the host's immune circadian rhythms to either tone down the protective immune response or to divert the response to their own advantage and maximize the outcome of the infection and transmission. Such knowledge could lead to strategies aiming at reversing such an effect of parasites on their hosts, by acting on circadian clocks or immune cells.

More generally, a more in-depth knowledge of the interplay between the circadian regulation of the host's immune system and the parasites will be important to design new strategies for the prevention, control and treatment of various vector-transmitted parasitic infections affecting millions of people worldwide.

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Figure legends

Figure 1. The dynamics between host, vector and parasite clocks. A schematic summary is provided to illustrate how environmental fluctuations and vector-host-parasite interactions impact each other. The legends on the arrow indicate the relationship between the two ends of the arrow. Although in the figure we depicted some specific insect vector, parasites and immune cells for illustration purposes, the principles represented in this figure could apply to other immune groups and species. See text for details. Created with Biorender.com.

Figure 2. Host immune circadian rhythms and their involvement in parasitic diseases. Host immune cells can modulate different aspects of disease progression and parasite development in a clock-dependent manner. For each parasitic disease (leishmaniasis, malaria or worm infection) immune components are cited along with the effects observed (right side of the arrow) on the parasite or infection. Detailed information for each of the studies referred to in this figure can be found in the text. Created with Biorender.com.

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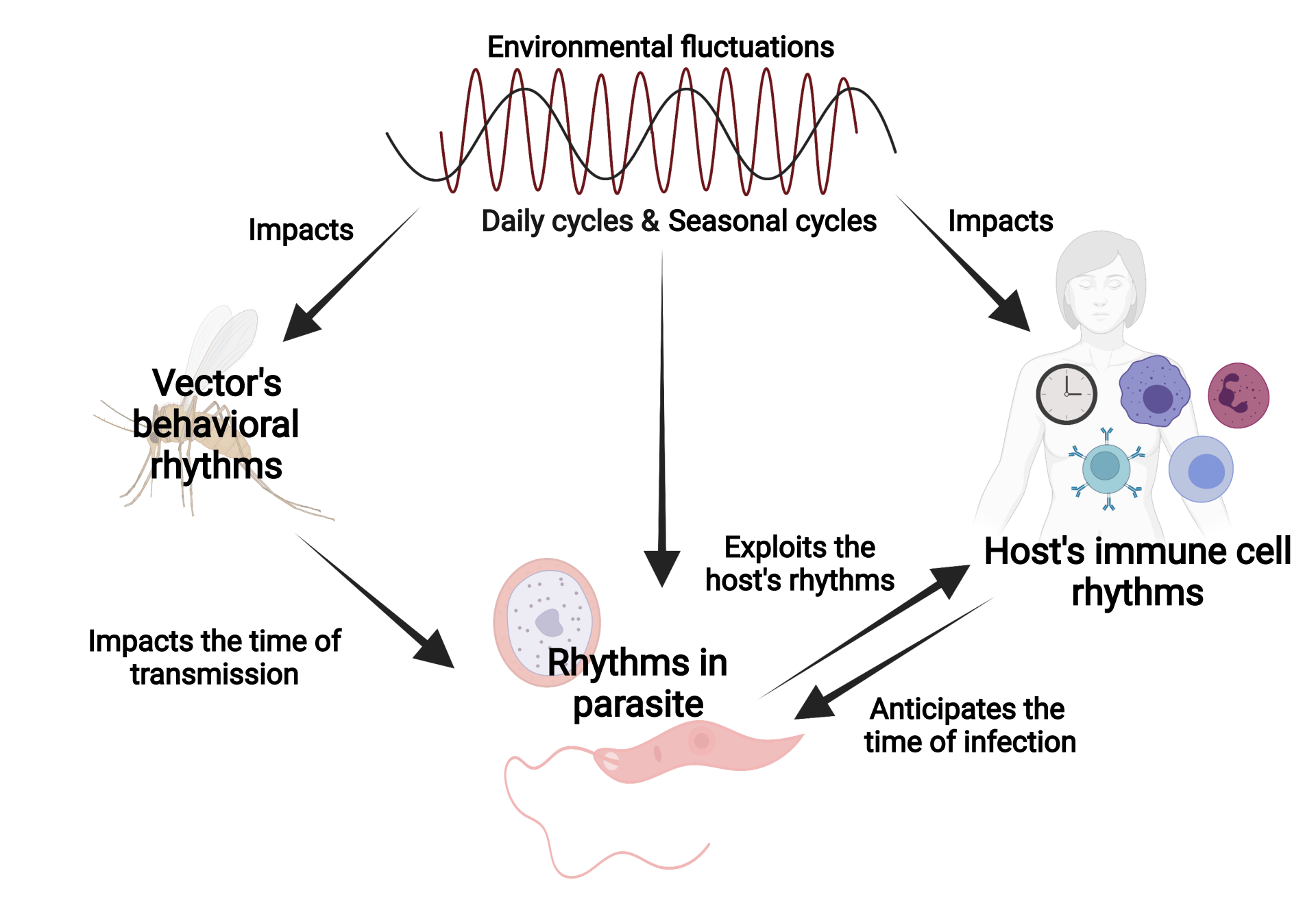
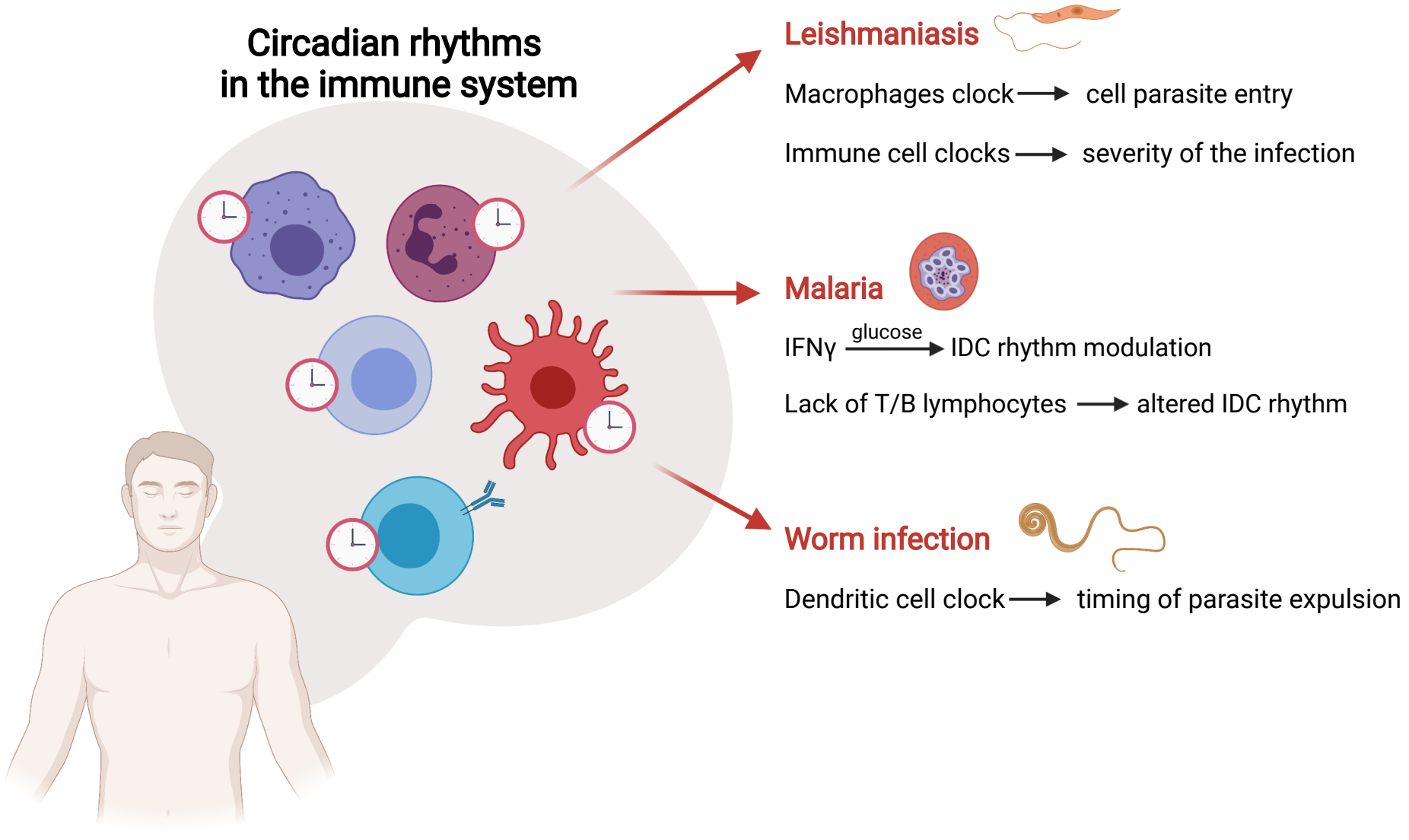

Environmental fluctuations
Daily cycles & Seasonal cycles
Impacts
Impacts
Vector's behavioral rhythms
Host's immune cell rhythms
Exploits the host's rhythms
Impacts the time of transmission
Rhythms in parasite
Anticipates the time of infection


Circadian rhythms
in the immune system
Leishmaniasis
Macrophages clock → cell parasite entry
Immune cell clocks → severity of the infection
Malaria
IFNγ glucose → IDC rhythm modulation
Lack of T/B lymphocytes → altered IDC rhythm
Worm infection
Dendritic cell clock → timing of parasite expulsion