Circadian rhythms in adaptive immunity and vaccination

Running title: Circadian rhythms in the adaptive immunity

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Abstract

Adaptive immunity allows an organism to respond in a specific manner to pathogens and other non-self agents. Also, cells of the adaptive immune system, such as T and B lymphocytes, can mediate a memory of an encounter with a pathogen, allowing a more efficient response to a future infection. As for other aspects of physiology and of the immune system, the adaptive immune system is regulated by circadian clocks. Consequently, the development, differentiation and trafficking between tissues of adaptive immune cells have been shown to display daily rhythms. Also, the response of T cells to stimuli (e.g. antigen presentation to T cells by dendritic cells) varies according to a circadian rhythm, due to T cell-intrinsic mechanisms as well as cues from other tissues. The circadian control of adaptive immune response has implications for our understanding of the fight against pathogens as well as auto-immune diseases, but also for vaccination, a preventive measure based on the development of immune memory.

Keywords: circadian clock; T lymphocyte; B lymphocyte; dendritic cell; trafficking; antigen presentation; vaccination

Introduction

The immune system consists of various mechanisms aiming at defending the organisms against various threats such as pathogens (e.g. bacteria, viruses, parasites) or cancer cells. It can be broadly divided in two main arms: the innate immune system and the adaptive immune system. Innate immunity is the first line of defense against infectious agents. It involves many different cell types (e.g. monocytes and macrophages, natural killer cells, granulocytes) which will respond to pathogens with low specificity, and on a short time scale (hours). Recognition will often involve receptors for pathogen-associated molecular patterns, that can be shared by many different pathogens of a group (e.g. bacterial cell wall molecules or viral nucleic acid). There has been numerous examples in the literature of a regulation of innate immune cells according to the time of day, based on circadian regulation within those cells, as well as rhythmic signals from other tissues and cell types [1].

This review article will rather focus on the other arm of the immune system: adaptive immunity. The adaptive immune system involves specialized cells called lymphocytes (T and B lymphocytes, also called T and B cells), as well as cells which can present antigens (i.e. immunogenic molecules from pathogens) to T cells, for example dendritic cells (Figure 1). Antigen-presenting cells display a fragment of the antigen as part of a complex with a major histocompatibility complex (MHC) molecule at the cell surface. This MHC-antigen complex is recognized by a receptor on T cells, called the T cell receptor (TCR). Genetic recombination events within developing T cells lead to a large diversity of TCRs, with each T cell having a TCR specific for a particular MHC/antigen complex.

Similarly, B cells also have a B cell receptor, with a high diversity, each being able to recognize a specific antigen. B cells can also secrete antibodies, antigen-specific molecules, an important part of humoral immunity (i.e. immunity via secreted macromolecules). T cells can be

divided in two families, based on the expression of the membrane molecules CD4 and CD8, which are co-receptors for the TCR. The activation of T and B cells based on recognition of antigens confers a very high specificity to adaptive responses.

Moreover, after a first infection, T and B cells can differentiate into memory cells, which remain in the organism for extended periods of times, ready to mediate a strong and rapid immune response upon future encounter with the pathogen, even years after the initial infection. This immune memory is harnessed for vaccination, in which an adaptive immune response is mounted against a pathogen — or antigen from the pathogen) in order to generate memory that will confer protection against future infections. Also, upon vaccination, B cells secrete antibodies that will be able to help neutralize the pathogen upon re-infection.

Although the circadian regulation (i.e. the variation across the 24-hour day) of adaptive immune responses has been less studied than for their innate counterparts, the research question has gained a lot of interest in the past few years. We now know that many aspects of the adaptive immunity, including cell trafficking, cell differentiation, and response to antigen presentation, are controlled by circadian clocks. This circadian control, which has implications for auto-immune diseases and vaccination, will be described in this review article.

Cells of the adaptive immune system display clock gene expression and cell number rhythms

Clock genes are expressed in adaptive immune cells

Circadian rhythms are produced by circadian clocks located in most cell types throughout the body. At the molecular level, these clocks consist of a set of so called *clock genes*, which interact in transcriptional-translational feedback loops, giving rise to 24 hour cycles of gene expression (see

lower left panel in Figure 1) [2]. As in many other tissues and cell types, circadian clock genes are expressed in adaptive immune cells, where they can control gene expression and various cellular processes. Clock genes (e.g. *Per2, Rev-erbα*) show rhythmic expression in mouse lymph nodes (LNs), lymphoid organs which are the site of the induction of the T and B cell response, and are composed in large proportions of these cell types [3, 4]. Additionally, clock gene transcripts show circadian variations in dendritic cells (DCs) and B cells enriched from mouse spleens [5], as well as CD4 T cells enriched from mouse LNs or spleen [6, 7] (but notably, not in regulatory T cells, except for *Rev-erbα* [8]). Using knock-in mice with a PER2::Luciferase fusion protein, persistent circadian oscillations of bioluminescence were observed for several days in LN explants [4], DCs differentiated from bone marrow [9, 10], CD8 T cells isolated from the spleen [10] and CD4 T cells from the spleen and thymus [11]. In humans, circadian rhythms of clock genes were observed in peripheral blood mononuclear cells (PBMCs) [12, 13], which are composed of 70-90% lymphocytes, as well as in CD4 T cells purified from blood [11]. The expression of circadian clock components in cells of the adaptive immunity suggests that this arm of the immune system may be controlled in a circadian fashion by local clocks.

The circadian clock is known to regulate with a 24 hour rhythm the expression of a multitude of genes, in a tissue-specific manner (Figure 1, lower right) [14]. This is the case for adaptive immunity, as genes important for the function of adaptive immune cells were shown to be expressed with a circadian rhythm. A notable example is CD8 T cells (isolated from mouse LNs), in which 6% of all protein-coding transcripts are rhythmic [10] (similar proportion as in macrophages, in which 8-15% of transcripts are rhythmic [4, 15]). The analysis of this rhythmic CD8 T cell transcriptome inferred a circadian variation in the activation of signaling pathway important for TCR signaling and CD8 T cell activation [10]. As for CD4 T cells, some factors relevant for their function (e.g. IFNγ, CD40L, NF-κB) were shown to be rhythmically expressed in purified human

CD4 T cells activated in vitro (using PMA/ionomycin) [11]. Finally, bone marrow-derived DCs showed genes (seventy) that are expressed according to the time after cell clock synchronization, the levels and time-dependence of many of these genes being altered in *Bmal1* knock-out (KO) DCs [9] (*Bmal1* is an essential clock gene, whose deletion abolishes circadian rhythmicity [2]).

Circadian regulation of the trafficking of adaptive immune cells

Lymphocytes are in continuous circulation between blood, lymph and lymphoid organs, to insure proper immune surveillance throughout the body. This trafficking of T and B cells is under circadian control. There is a pronounced variation in blood lymphocyte counts over the 24-hour day in mammals. For example, in the blood of mice and rats, lymphocyte counts peak in the light phase and have a trough in the early dark phase [16, 17].

The rhythmic pattern is inversed in humans, as is their rest-activity phase. In the human peripheral blood, total lymphocyte counts as well as naive CD4 and CD8 T cells follow a daily rhythm with a trough in the daytime and a peak at night [18-23]. These rhythms were preserved in subjects who were awake for a full 24-hour cycle, indicating that sleep-wake cycles do not directly induce the rhythmicity in circulating lymphocytes; of note though, subjects remained in bed for the period they would have normally been sleeping, so it cannot be excluded that the observed rhythms might be due to 24-hour cycles of activity-inactivity [19, 24]. Studies using for example a constant routine protocol (where environmental/behavioral cues that could impact on the measures are avoided [25]) might help address the endogenous circadian nature of the cell count rhythms. Of note, DCs also present a daily rhythm in human peripheral blood [24].

A number of recent studies have addressed the mechanisms underlying the daily rhythms in immune cell trafficking. The glucocorticoid cortisol (or corticosterone in rodents) is a hormone secreted by the adrenal glands, under the control of the circadian system. Cortisol (or

corticosterone) increases during the rest phase to reach a peak around wake, whereas, as mentioned above, blood T cell counts are generally high during the rest phase (night in humans, day in nocturnal rodents), and then decline to reach low levels in the day [17, 18, 21, 23]. This inverse correlation suggested that glucocorticoids might regulate T cell trafficking. This was supported by a disappearance of the daily rhythm in lymphocytes in the blood and spleen of adrenalectomized mice [17].

More recent studies in humans provided further ground to the hypothesis that glucocorticoids may regulate T-cell trafficking. Upon studying different subsets of CD4 and CD8 T cells in human subjects, Dimitrov and colleagues observed that all the subpopulations (naïve, central memory and effector memory) except effector CD4 T cells showed a 24 hour pattern in their blood cell counts [21]. In all cases, the peak of these rhythms was in the night and the trough in the afternoon, except for effector CD8 T cells, which showed antiphase rhythms. When cortisol was administered to subjects in the evening (when endogenous cortisol levels are low), the T cell populations, usually high at that time, decreased markedly over the next several hours. On the other hand, epinephrine administration led to a short selective increase of effector CD8 T cells [21].

Investigating the candidate chemokine receptors and ligand molecules mediating the distribution of T cells between blood and other tissues, it was found that the cell surface expression of several markers like CD62L, CD49d, CXCR1, CXCR4, and CX3CR1 was higher in the morning than the evening [21]. Incubating T cells, taken from the human participants in the evening, with cortisol, caused an increase in CXCR4 expression in both naïve and central memory CD4 and CD8 T cells, suggesting that cortisol upregulates CXCR4 expression [21]. Conversely, administration of mifepristone (a glucocorticoid receptor antagonist) or metyrapone (which suppresses endogenous cortisol levels) both attenuated the morning decline in T cell counts and the morning

increase in CXCR4 expression [26]. These data suggested a role for CXCR4 and its ligands mediating the effect of cortisol on T cell depletion from peripheral blood in the morning.

Results consistent with this were found in mice. The rhythms of lymphocyte counts in the blood, lymph nodes and spleen were lost in mice lacking the glucocorticoid receptor in T cells [27]. The glucocorticoid receptor directly activates the expression of interleukin (IL)-7 receptor (IL7R), leading to a rhythm of the levels of this receptor on T cells. This in turn leads to a rhythm in chemokine receptor CXCR4, with higher levels, and more efficient migration of the T cells towards cells expressing chemokine ligand CXCL12 in the spleen, in the nighttime. Accordingly, in T cell-specific *Cxcr4* KO mice, rhythms of T cell counts were lost, and levels were high at all times in the blood and low at all times in LNs and spleen, supporting that the rhythmic homing of T cells in lymphoid organs is affected by CXCR4 [27].

Other studies found additional potential factors affecting the migration of T and B cells in and out of the LNs. In mice, T and B cells in LNs peaked during the night, similar to noradrenaline [28, 29]. This suggested an adrenergic regulation of lymphocyte trafficking. Accordingly, the daily variation of T and B cell counts in LNs (and in the blood) was abolished in mice treated with 6-OHDA, a neurotoxic drug depleting adrenergic nerves, and in mice deficient for β2 adrenergic receptor (β2AR). The entry of lymphocytes in LNs was then blocked (using neutralizing antibodies against molecules important for this process) at either of two time points, Zeitgeber time (ZT) 1 and ZT13 (ZT0 is the time of lights on, ZT12 the time of lights off for animals on a 12 h light:12 h dark cycle). When entry in LN was blocked in the early night (ZT13), the fraction of B, CD4 and CD8 T cells remaining in the peripheral LNs was higher than when entry in LN was blocked in the early day (ZT1). This indicated that lymphocyte egress from LNs follows a circadian pattern with more egress during daytime. This daily variation was lost when β2AR-deficient bone marrow cells

or lymphocytes were transferred in WT mice, suggesting a local response to adrenergic input within T and B cells [28].

Another study also found rhythmic homing and egression of lymphocytes into and from mouse LNs and went on to investigate the migratory factors responsible for these rhythms [6]. It was found that the chemokine receptor CCR7 on T and B cells, and its ligand CCL21 on high endothelial venules showed rhythmic expression with peak around night onset (ZT13), the time when homing in LNs is highest. A pharmacological inhibition of chemokine signaling (using Pertussis toxin) suppressed the time-dependent variations in LN T and B cells. These rhythms were also attenuated when *Ccr7* KO lymphocytes were transferred in WT hosts. While CCR7 seems to act on rhythmic homing into the LNs, the daily variation of egress of the cells from the LNs depends on Sphingosine-1-phosphate receptor 1 (S1PR1), a receptor for chemoattractant S1P. Indeed, S1PR1 showed daily oscillation, with a peak in the morning (ZT1-9). Interestingly, in contrast to the other studies described above, which have highlighted mechanisms for the rhythmicity of lymphocyte trafficking that were non cell-autonomous (i.e. due to rhythms extrinsic to the T and B cells), Druzd and colleagues identified the T cell clock as being essential for the rhythm of T cell counts in LNs, and *Ccr7* and *S1pr1* transcript rhythm in CD4 T cells, as they are blunted in T cell-specific *Bmal1* KO mice [6].

Altogether, autonomic innervation, glucocorticoids and chemokine receptors/ligands, along with the lymphocyte-intrinsic clocks, seem to be regulators of lymphocyte trafficking. The respective roles and the interplay of these factors remains to be established. Of note though, other reports — also performed in mice — found an absence of rhythms in the abundance of B cells, CD4+, CD8+ T cells in LNs [3], CD8+ T cells in spleen [10], and CD8+ T cells in lungs and LNs [30]. Although the reasons for these discrepancies remains unclear, it can be noted that among these studies, only those from Fortier et al and Nobis et al studies report an assessment of cell counts

over 24 hours under constant darkness conditions, i.e. without masking effects of the light-dark cycle [3, 10].

Circadian regulation of antigen presentation, T cell activation and proliferation

Circadian regulation of T cell activation and proliferation

The previous section has described circadian variations of T cells in the blood and lymphoid organs, and possible mechanisms underlying them. But are the functions of T cells — e.g. their response to antigen presentation, their activation and proliferation, and acquisition of effector functions — controlled by circadian clocks? A number of studies have addressed this question by triggering the TCR in a non-specific manner, using lectins such as phytohemagglutinin or concanavalin A. These lectins bind sugars, such as those of glycosylated proteins at the surface of cells. One of these is the TCR: lectins bind the receptors, which are then crosslinked; this triggers the activation of signaling pathways downstream of the TCR, and thus, the activation and proliferation of the T cells. Lectin treatment of cells from human blood sampled at different times over day and night showed a daily rhythm of T cell response, independent of the rhythm of T cell counts, although the exact acrophase (time of peak) varied across studies [20, 31]. Experiments using cells from rat LNs also showed a rhythm of the proliferative response of T cells, generally with a peak in the daytime (but again with some variation across studies) [32, 33]. Another way to trigger the clustering of TCRs, and thus, activation of the cells, is by treating them with an antibody against the CD3ε chain of the TCR: when this was done on LN cells harvested from mice at different times over 24 hours, the proliferative response was stronger in the late day and the night [3].

Other groups have studied rhythms of T cell response by stimulating them with antigens. For example, the reactivity of human mixed lymphocyte population (harvested from human subjects over 24 hours) to a specific antigen (streptokinase-streptodornase) or to another donor's lymphocytes led to daily variations, with a peak in the night [34]. Another human study using tetanus toxoid as an antigen found a rhythm of T cell proliferation, but with a very low amplitude [35]. Human blood stimulated with *Staphylococcus aureus enterotoxin B* led to a higher cytokine response of CD4 T cells when the blood was harvested in the evening than in the morning [22]. Finally, after injection of mice with an ovalbumin peptide antigen (OVA) together with CpG oligodeoxynucleotides (ODN) as adjuvant, either at ZT7 (mid-day) or ZT19 (mid-night), LN cells from mice immunized in the night showed a higher antigen-induced proliferation and IFNγ production compared to those of ZT7-injected mice [36]. The authors proposed that this was attributable to a higher level at night of Toll-like receptor 9, the receptor for CpG ODNs [36].

Although these reports were interesting, it is difficult to assess from these results the source of the circadian rhythmicity. In some cases, the antigen must be taken up by antigen presenting cells, processed, and presented at the cell surface in the context of an MHC molecule, all steps that could potentially be regulated in a circadian manner. Also, the response to the immune adjuvant used in some of these protocols could be rhythmic, as the receptors for such molecules can be rhythmic (as is the case for example for CpG ODN/TLR9, described above [36]). Therefore, distinguishing a circadian regulation of T cells themselves vs. other immune cells can be challenging. More recently, a vaccination model using dendritic cells loaded with an OVA peptide (DC-OVA) was used to address the mechanisms underlying circadian rhythms of the T cell response to antigen presentation. In this model, all antigen uptake and processing steps are bypassed, allowing to focus on T cell-intrinsic mechanisms. DC-OVA were injected in mice at different times of the day or night, and the CD8 T cell response was checked in the spleen one week later (DCs used for these

immunizations were not clock-synchronized, and thus, all injected aliquots were equivalent in terms of circadian time). There were 2-3 times more OVA-specific CD8 T cells in the spleen after vaccination in the middle of the day, compared to mice vaccinated at other times [3, 10]. Of note, this rhythm was observed under constant darkness conditions, showing a role of the endogenous circadian system [10]. Accordingly, the circadian rhythm was lost in mice with a CD8 T cell-specific *Bmal1* gene deletion, showing an essential role for the clock in these cells.

As previously mentioned, naïve CD8 T cells expressed 6% of their transcriptome (796 transcripts) in a circadian fashion. Analysis of these rhythmic genes inferred an increased activation in the daytime (when vaccination leads to a stronger response) for mediators of TCR signaling following antigen presentation, such as ZAP-70, AKT and mTOR [10]. Conversely, pathways known to reduce TCR-mediated responses were predicted to be more active at night. Additional experiments showed that indeed, the TCR-associated tyrosine kinase ZAP-70 shows higher levels in the daytime in mouse LNs [3]. Moreover, spleen T cells harvested from mice in the daytime show stronger responses of AKT and mTOR to antigen presentation than cells harvested in the night [10]. Overall, there seems to be a transcriptional program in CD8 T cells which primes them for a strong response to antigen presentation in the day, and to a toned-down response at night (Figure 2).

The regulation of CD8 T cell activation is probably not only cell-autonomous. The glucocorticoid rhythm appears to have a role to play. In mice infected with bacteria *Listeria monocytogenes* expressing OVA antigen (Lm-OVA), OVA-specific CD8 T cells were higher in mice infected in the early night (ZT16) than in the early day (ZT4) [27]. This time-dependent difference was lost in mice lacking the glucocorticoid receptor in T cells. Mice in which the binding sites for the glucocorticoid receptor have been mutated within *Il7r* enhancer were also used: in these mice, glucocorticoids cannot impose a rhythm on IL7R and CXCR4 expression. The authors

noticed a loss of day-night variation in OVA-specific CD8 T cells. This suggested that the day-night difference in response to Lm-OVA is related to variation in mechanisms (involving IL7R and CXCR4) mediating changes in LN T cell numbers [27]. In another study, Lm-OVA infection of mice with transferred OT-I cells (restricted to presentation of OVA in the context of MHC class I) showed a time of day dependence, with higher IL-2 response at ZT2 (early day) than at ZT14 (early night) [7]. Of note, this time-dependence persisted when the OT-I cells were ablated for *Bmal1*, suggesting that this rhythmicity is due to factors extrinsic to the CD8 T cells. Finally, a time-dependent frequency of IFNγ-producing CD4 and CD8 T cells was found in a prior report, upon infection with *L. monocytogenes* at ZT0 vs. at ZT8 [37], which again supports a stronger response early in the daytime (although in this study, this time-dependent difference was proposed to be due to a rhythmicity of inflammatory monocytes and DCs).

Circadian regulation of the antigen-presenting cells

Besides a cell-autonomous regulation of T cell rhythmicity, an alternative regulation could be by the daily variation of the activity of antigen-presenting cells (APCs). The main professional APCs are the DCs. A possible role for the clock transcription factors REV-ERBα and β in the differentiation of DCs was uncovered using mice KO for these factors: DCs differentiated in vitro from bone marrow of either *Rev-erbα* KO or *Rev-erbβ* KO mice had higher expression of MHC class II and CD86, which are markers of mature DCs [38]. A reverse effect was observed when the cells were differentiated in the presence of a REV-ERB agonist. More work will be needed to confirm a role for REV-ERB factors in the differentiation of DCs in vivo.

In the previously described study on CD8 T cell response to OVA antigen presentation, the possible role of the clock in DCs (which present the antigen in the context of their MHC class I in this model) was investigated. When DCs prepared from *Bmal1* KO mice were used for the

immunization, a day/night difference in CD8 T cell expansion was still observed although it was attenuated [10]. *Bmal1* KO DCs had similar levels of antigen loading, activation and differentiation, suggesting that the lower activation of CD8 T cells was due to another role for *Bmal1* in DCs. Indeed, an in vivo migration assay indicated a deficit in migration of the *Bmal1* KO DCs to the spleen [10], which might explain the reduced T cell activation upon immunization in the daytime. More work will be needed to investigate how *Bmal1* in DCs regulate their migration to lymphoid organs.

A role for circadian clocks in controlling DC trafficking was also described in a recent report, which showed a circadian regulation of the migration of DCs from the skin to lymphatic vessels in mice [39]. This migration was enhanced in the skin in the day (ZT7) than in the night (ZT19). This was paralleled with higher daytime expression of pro-migratory factors (such as LYVE-1, CD99 and JAM-A) and chemokine CCL21 in lymphatic endothelial cells, and of CCL21 receptor CCR7 in skin DCs. A pharmacological manipulation of these factors blunted the day-night difference. Interestingly, deleting *Bmal1* gene in either LECs or in DCs abolished the daily variation in DC migration, suggesting a role for the circadian clock in both of these cell types [39].

The DC clock also appears to be involved in the whole animal response to infections. Hopwood and colleagues studied the parasitic worm *Trichuris muris* and found that the time of day of infection with eggs had an impact on the subsequent immune response and expulsion of the worm, which occurs about three weeks later [9]. Early day (ZT0) infection led to an earlier expulsion of the parasite than following early night infection (ZT12). This was paralleled by a bias towards Th2/IgG1 responses (which are important for fighting off this kind of parasite) in morning-infected mice. This morning-evening difference was lost in DC-specific *Bmal1* KO mice, suggesting a role for the clock in these APCs, in polarizing T cell responses.

Antigens can also be presented to T cells by other cell types. Another study found a daily variation in the expression of MHC class II in small intestine epithelial cells (SIECs) [40]. This rhythm is dependent on the food intake rhythm (via regulation of the gut microbiome) rather than the circadian clock [40]. Interestingly, SIEC MHC class II modulates the frequency, activity and time dependence of intra-epithelial CD4+ IL-10+ T cells. In turn, IL-10 secretion from these CD4+ IL-10+ T cells leads to a daily variation in the barrier function of the small intestine (which could have importance for small intestine auto-inflammatory disorders).

Circadian regulation of Th17 and Treg cells, and implications for immune-related diseases

Among CD4 T cells, several subtypes were shown to be critical to the development of autoimmune diseases, in which autoreactive T cells target self-antigens. One notable example is multiple sclerosis (MS), where IFN-γ-expressing Th1 and IL-17-expressing Th17 cells are key to the destruction of myelin covering the axons of neurons. Another type of CD4 T cells, regulatory T cells (Treg), negatively regulate effector T cells (as well as other immune cell types), and thus show an opposite action on the development of the disease. Recent research has found an implication of circadian clock components in the differentiation of CD4 T cells into Th17 and Treg cells, and accordingly, an effect on immune-related diseases.

Mechanisms underlying MS can be studied in mice using a model called experimental autoimmune encephalitis (EAE): mice are immunized with MOG_{35-55}, a peptide from a myelin protein, leading to a pathology reminiscent of human MS, including demyelination of axons. The time of day at which immunization is performed was shown to impact the disease, with higher clinical scores and more demyelination after immunization in the daytime (ZT8) than in in the nighttime (ZT20) [6, 41]. This was paralleled with higher Th17 counts in the LNs [6]. The time-dependent variation was lost in mice lacking the essential clock gene *Bmal1* specifically in T cells,

with a clinical score reduced after daytime immunization, to match lower nighttime levels [6]. These data indicate that the clock in T cells is essential for time-of-day variation in EAE pathology, and that clock regulation of Th17 cells might be among the underlying mechanisms. Of note, another study did not find any effects of T cell-specific *Bmal1* KO on EAE pathology or Th17 cell counts [7]; however, the immunization for EAE and cell sampling for T cell stimulation were done at only one time point, which was unspecified.

Several studies have directly addressed the role of clock components in Th17 biology. Yu and colleagues observed a reduced Th17 cell frequency after in vitro differentiation of CD4 T cells from *Rev-erbα* KO mice (and similar results using *Clock* gene mutant mice, which have low *Rev-erbα* expression) [42]. The master regulator of Th17 differentiation is the nuclear receptor RORγt. The link from REV-ERBα to RORγt and Th17 differentiation was proposed to be NFIL3 (which is a well-known circadian-regulated transcription factor, also known as E4BP4 [43]). Indeed, NFIL3 binds and represses *Rorγt* promoter, and RORγt levels are increased in *Nfil3* KO mice. Moreover, *Nfil3* expression is increased in *Rev-erbα* KO T cells [42]. This draws a pathway from the clock to RORγt, via the transcription repressors REV-ERBα and NFIL3, affecting Th17 cell differentiation.

However, more recent studies have suggested that additional mechanisms might be at play. In contrast to the previous study, Amir and colleagues have observed higher Th17 cell frequencies among in vitro stimulated CD4 T cells from *Rev-erbα* KO mice, compared to WT controls [44]. This was associated with increased RORγt levels, and with a stronger autoimmune phenotype in the EAE model. A REV-ERBα agonist had reversed effects. These authors proposed a more direct model for the action of REV-ERBα on RORγt: the two factors compete for the same RORE DNA elements, and thus, REV-ERBα represses RORγt-mediated T cell differentiation to Th17 [44]. Similarly, Chang and colleagues also presented data supporting a direct REV-ERBα/RORγt functional competition [45]. However, their T cell-specific *Rev-erbα/Rev-erbβ* double KO mice

had lower Th17 frequencies (like the Yu et al study) and lower EAE clinical score. Oddly, using a transgenic mouse model with induced REV-ERBα expression in MOG peptide-specific T cells, they also found reduced Th17 differentiation and reduced EAE pathogenesis. These authors suggested that the discrepancies between the studies might be due to the differences between the models (tissue location of *Rev-erbα* deletion, magnitude of REV-ERBα activation or induction), and that the system might be sensitive to subtle changes in REV-ERBα levels or activity [45]: insufficient REV-ERBα activity would lead to increased NFIL3 expression, lower RORγt expression, and thus lower Th17 levels. At very high REV-ERBα activity levels, the other model would come into play, with REV-ERBα out-competing RORγt at genes important for Th17 differentiation. Th17 would be promoted at intermediate REV-ERBα levels/activity.

As mentioned, T cell-specific KO of clock genes (*Bmal1, Rev-erbα/β*) led to changes in magnitude of the disease in the EAE model [6, 45]. This indicates a T cell-autonomous control of EAE (and of T cell function). This is in contrast to the results of Farez and colleagues, who showed an effect of melatonin (a hormone from the pineal gland in the brain, secreted with a circadian rhythm in many animals) on Th17 and Treg cells: melatonin blocks differentiation of T cells into Th17, where it enhances their differentiation into Tregs) [46]. Accordingly, Th17s and Tregs being pathogenic and protective in the EAE model, respectively, melatonin-treated mice had reduced clinical scores. However, melatonin is unlikely to be the factor underlying the circadian regulation of Th17 and EAE in laboratory mice, as many commonly used strains such as C57BL/6 mice (used in the previous studies described above) have very low or undetectable levels of melatonin [47]. However, melatonin could play a role in regulating MS in humans, including perhaps the seasonality of the disease, as suggested by Farez and colleagues [46].

Another disease where Treg cells are involved is rheumatoid arthritis (RA). In a mouse model of inflammatory arthritis (where an emulsion of collagen and complete Freund's Adjuvant are

injected intra-dermally at the base of the tail), inflammation in the limbs (paw swelling, inflammatory cytokine expression) was heightened in the day compared to the night [48]. This was not paralleled by a daily variation of macrophages or neutrophils in the inflamed joints. Instead, a daily variation of CD4 T cells (but not CD8 T cells), and in particular Tregs, was found in the joints of the arthritic mice, with more CD4 T cells or Tregs upon treatment at ZT18 vs. ZT6 [8]. This is consistent with the known protective role of Tregs in RA. Interestingly, data suggested that the rhythmicity was due to factors external to the T cells: glucocorticoids were shown to induce the chemokine receptor CXCR4 in Treg cells of arthritic mice, mirroring finding in naive T cells described earlier in this review [27]. Another source of rhythmic regulation external to the T cells could also be the fibroblast-like synoviocytes, joint cells that are important in the maintenance of joint health and play a role in the development of RA: *Bmal1* KO in the fibroblast-like synoviocytes had impacts on the pathology in experimental arthritis in mice [49]. Finally, despite the absence of rhythms in the counts of myeloid cells in the joints, Treg depletion at ZT18 activated the pro-inflammatory response of monocytes and macrophages in the inflamed limbs [8]. Hence, circadian rhythms in adaptive immune cells can modulate innate immunity across the day.

Regulation of B cell development and response by the circadian clock

Although circadian rhythms of B cells have been less studied than those of T cells, these antibody-producing lymphocytes also appear to be controlled by circadian clock components. *Bmal1* KO mice have lower B cell counts in their blood and spleen compared to WT mice [50], suggesting a defect in their development from bone marrow progenitors (although lower B cell numbers could also be due to defective survival of mature B cells). Is this role of *Bmal1* taking place within bone marrow cells or within other cells in the organism? To address this, *Bmal1* KO bone marrow cells were adoptively transferred into irradiated recipient mice (irradiation kills all

bone marrow cells): normal B cell counts were observed. Conversely, transferring WT bone marrow cells into irradiated *Bmal1* hosts recapitulated the B cell count defect, suggesting that *Bmal1* in cells of the bone marrow environment (e.g. stromal cells), and not bone marrow cells, plays a role in B cell development [50]. This is consistent with the normal B cell development in B cell-specific *Bmal1* KO mice [7].

The trafficking of B cells to and from lymphoid organs could also be the source of circadian regulation of the activity of these cells. Suzuki and colleagues [28] studied the humoral response following intra-dermal immunization in the day (ZT5) or in the night (ZT17) with 4-hydroxy-3-nitrophenyl hapten conjugated with chicken γ-globulin in the presence of adjuvant. Titers for antibodies (IgM, IgG1) specific for this hapten were higher after immunization at ZT17 than at ZT5. Follow up experiments suggested that this daily variation was abrogated in mice without β_2AR, and was probably dependent on the variations of lymphocyte numbers in the lymph nodes [28].

As for the response of B cells to a stimulus, an early study had suggested that it could be regulated in a daily fashion. Fernandes and colleagues measured the number of plaque forming cells in the spleen 3 or 4 days after immunization of mice with sheep red blood cells. plaque forming cell numbers, a measure of antibody production by B cells, varied according to the time of immunization (higher in early day) [51]. However, whether this was due to a variation of T cell numbers or activity (helping B cells for antibody production), or a variation of the activity of B cells themselves, was unclear.

A role of circadian clock components in B cell function was also uncovered in a recent study, where an auto-immune-like phenotype was observed in mice deficient for both *Cry1* and *Cry2* genes (which is an essential pair of genes in the core clock mechanism; see Figure 1) [52]. The mice had high IgG titers, and autoantibodies for many glomerular and nuclear antigens. The higher

IgG titers and Ig deposits were also seen in *Rag1* mice (i.e. mutant mice lacking T and B cells) adoptively transferred with *Cry* KO bone marrow (i.e. hematopoietic cells are all *Cry* KO). This was associated with increased bone marrow and peritoneal B cell counts, indicating an alteration of their development. Moreover, Double *Cry* KO B cells had an enrichment of expression of genes related to B cell receptor proximal signaling pathways. Accordingly, the T cell-independent B cell response to antigen stimulation was enhanced (T cell-dependent response was unchanged). These data suggest that the auto-immune phenotype of *Cry* KO mice is caused by B cell hyperactivation, and argues for a role of cryptochromes in B cell development and/or function.

Circadian regulation of the outcome of vaccination

Vaccination is a widely used strategy to prevent various infectious diseases. It consists in stimulating the immune system with attenuated pathogen or a molecule antigen from it, in combination with an adjuvant, to produce a cellular and humoral adaptive response specific to this pathogen. An immune memory then develops, which will allow, upon a subsequent encounter with the pathogen, to mount a quick and powerful response. A number of factors, for example age, sex or geographical location, can affect the efficiency of vaccines and the protection they confer [53]. Recent research has suggested that circadian rhythms and sleep might be additional factors to take into account to explain variability of the response to vaccines and to improve their efficiency.

As mentioned throughout this review article, various aspects of adaptive immunity, including T and B cell responses, display circadian rhythms. Some of the experiments we have summarized are mouse models of vaccination. For example, as described above, in a DC-based vaccination model in mice, the CD8 T cell response varied according to the time of vaccination [3, 10]. In this model, increased T cell proliferation and effector functions after vaccination in the daytime led to a better ability to fight off an infection with *Listeria monocytogenes* bacteria expressing the antigen

used for vaccination, compared to infection after nighttime vaccination [10]. Another study reported above used vaccination with CpG ODN adjuvant and OVA antigen, and showed a higher response at night [36].

Human studies on circadian rhythms and vaccination

There have been only a handful of studies so far addressing in human subjects a time of day-dependent variation in the immune response to vaccines (Table 1). One of the first reports looked at the time-of-day variation of the humoral response to anti-influenza vaccine [54]. Results from two different cohorts were presented, both using inactivated influenza virus, and with vaccination times ranging throughout the day (8:30 to17:00), and participant age around 45. In a first study (with 98 participants), where vaccination time had to be retrospectively estimated, there was a variation according to time of day, with higher antibody titers when vaccination was done around 11:00 to 13:00, although this was the case only for one of the three antigens. In the other cohort (with 707 participants, male and female), there was no significant time of day dependence.

Another study also looked at the antibody response to influenza vaccination, but in 276 older subjects (average age 71, subject of both sexes), using a randomized trial design. The subjects were vaccinated in the morning (9:00 to 11:00) or afternoon (15:00 to 17:00), with the subjects of the morning group showing a higher antibody response, independent of sex [55]. In a prior study from the same group, also with aged male and female participants (average age 73), participants were not assigned to time groups but were rather binned a posteriori in morning (8:00-11:00) or afternoon (13:00-16:00) bins based on their recorded times of vaccination. Although there was no time of day or sex main effects, there was a sex by time of day interaction, where men vaccinated in the morning showed a better response than those vaccinated in the late day, which was not the case for female subjects [56].

In the same article reporting the second influenza study on aged subjects, the authors also studied hepatitis A vaccination (Havrix) on a group of 75 young subjects (average age 23, males and females). Again, there was a sex by time of day interaction, with a better antibody response in male subjects of the morning group (vaccination 10:00-12:00) compared to the afternoon group (16:00-18:00), whereas female subjects had no time of day difference, but a tendency for a better antibody response when vaccinated in the afternoon [56]. In contrast, no time of vaccination effects were found for hepatitis B vaccination in a study where 63 young participants (average age 20.5, males and females) received the three vaccine doses either in the morning (8:00-8:30) or evening (17:30-18:00) [57].

A study looked at preterm infants receiving their first hexavalent vaccination (against diphteria, pertussis, tetanus, poliomyelitis, *Haemophilus influenzae* type B, hepatitis B and *Pneumococcus*), 60 days after their premature birth [58]. Babies were assigned to either a morning (7:00-10:00) or evening (19:00-22:00) vaccination group. Antibody titers for pertussis and *H. influenzae* were measured 3 months later: although the increase in titers appeared slightly higher after evening vaccination, there was no significant morning-evening differences. It was discussed by the authors that it is uncertain whether such young infants have developed circadian rhythmicity in their immune system, which might be a reason for the lack of effect of time [58].

Most of the human studies on the effect of time of day on vaccination have only looked at the humoral response to vaccination, i.e. titers of antibodies against the antigens within the individuals vaccinated at different time points. It would be valuable to also assess cellular responses, as was done in mouse models, and to look at the underlying mechanisms. One article addressed response to vaccination, namely BCG vaccination against tuberculosis, in much more detail [59]. In this article, de Bree and colleagues compared 18 participants (male and females) vaccinated in the evening (18:00) with 36 others vaccinated in the morning (8:00-9:00). Before the vaccination, and

2 weeks and 3 months after, PBMCs were collected, stimulated with either *Mycobacterium tuberculosis* (to test for the specific response to this bacterium) and *Staphylococcus aureus*, and cytokine secretion was measured (IL-1β, TNFα, IL-6, IFNγ). The use of the latter bacterium is because the BCG vaccine, even if it was designed to prevent tuberculosis, is known to confer some protection to other infections, due to a phenomenon called trained immunity, i.e. an induction of innate immune memory responses. In both cases, a stronger response was seen in subjects vaccinated in the morning. This effect of time of day on trained immunity (for *S. aureus*), but not the response against *M. tuberculosis*, was also replicated in a very large second cohort of 302 subjects, although the lack of effect for the response to *M. tuberculosis* might be due to the fact that only morning time points (8:00-12:00) were compared. Interestingly, the time dependency was also replicated on innate immune cells cultured ex vivo (i.e., monocytes harvested from subjects at 8:00 or 18:00, BCG-primed, and then LPS-stimulated in vitro). This showed that the effect of time was cell intrinsic. Finally, chromatin modification and accessibility was studied in PBMCs, showing differentially accessible regions in morning vs. evening, including an enrichment of components of the mTOR signaling pathway [59].

Altogether, the research reports so far are divided about the time of day-dependence of the outcome of vaccination (Table 1). Many studies had small sample sizes, hence with little statistical power, and/or very uniform, limiting generalization to other ethnical or age groups. Moreover, many studies only looked at two time bins (morning and afternoon), and times for lower or higher response might have been missed; it is understandable though, that studies are constrained by the times at which the participants are available and vaccination is feasible. Also, it is probably impossible to generalize the findings: there might be a circadian regulation of some vaccines, not of others, and the peak time for an optimal response might be different according to the distinct mechanisms of action of vaccines.

The effect of sleep and shift work on vaccination

It is known that sleep impacts immune defenses, generally in a positive way [60]. Therefore, some studies have been designed to specifically address whether sleep duration and quality could regulate the outcome of vaccination [61]. Young male subjects (27 subjects, average age 26) were vaccinated for hepatitis A and hepatitis B (3 doses, with 8 weeks between each) [62]. Subjects were distributed in two groups: in the night following each injection, subjects of one group were allowed to sleep, while those of the other group were kept awake. The cellular and humoral responses were measured over the few days after each dose. For both hepatitis A and hepatitis B, the humoral response (antibody titers) was enhanced in the sleep group compared to the subjects kept awake. Moreover, hepatitis antigen-specific T helper cells were increased in the sleep group, as well as their cytokine secretion. Interestingly, the time spent in slow wave sleep (deep, restorative sleep occurring mainly around the beginning of the night), as well as levels of slow wake activity (an EEG feature characteristic of slow wave sleep), both correlated with T cell response to hepatitis A vaccination [62]. Although the subjects in this experiment were all men, the same researchers had previously performed a similar experiments on a small group of 10 women, for hepatitis A only, and only looking at the humoral response: in that case also, subjects of the sleep group showed higher hepatitis A-specific antibody levels compared to those of the sleep deprivation group [63].

A similar study in young subjects, with acute sleep deprivation (or regular sleep) on the night following vaccination, was done for influenza A H1N1 virus vaccination [64]. Sleep-deprived male subjects displayed reduced H1N1-specific antibodies five days after vaccination, a difference not seen in female subjects. Of note though, the effect did not persist at later time points (10-52 days after vaccination).

Another report addressed hepatitis B vaccination, but in this case, not using a laboratory intervention on sleep, but rather quantifying sleep amount and quality, and correlating it with the outcome of vaccination [65]. Midlife (average age 50) male and female participants were given the three vaccine doses and sleep was assessed using sleep diaries (over 7 days surrounding each vaccination) and actigraphy (3 nights before and 3 nights after the initial immunization). Shorter sleep duration was associated with a lower antibody response after the third dose, and with a reduced likelihood of mounting a clinically protective response, independent of sex [65].

All in all, even though the literature directly addressing sleep and vaccination is scarce, sleep is likely to have an impact. Thus, sleep should be considered as a possible factor or confound in the design and interpretation of studies asking about the effect of circadian rhythms on the outcome of vaccination [61].

An implication of possible effects of sleep and circadian rhythms on vaccination is for shift workers. It was shown that night shift work alters the blood levels of different cytokines and chemokines [66], and that a night shift schedule impacts the rhythms of immune cells (including T cells) and their response to a stimulus [20]. In a recent study, night shift workers and day shift workers were vaccinated for meningitis at the same time of day, after a day off, and followed 4 and 8 weeks later [67]. The night shift workers had a lower increase in antigen-specific antibodies after vaccination compared to the day workers. They also had decreased percentage of CD4 T cells (but not CD8 T cells, and staining was not for antigen-specific T cells) and dendritic cells (even before vaccination). Caution is needed to interpret these data, as the shift workers may have altered lifestyles compared to non-shift workers, e.g. with regards to diet, meal timings, etc. Nevertheless, the results suggest that circadian disruption (and/or sleep restriction) associated with night shift work [68] may have an impact on the immune response to vaccines.

Conclusion

Since circadian rhythms have evolved as an adaptation to the recurring variations of factors in the environment, it is easy to imagine how innate immunity, the first line of defense against environmental insults such as pathogens, would benefit from a circadian regulation. On the other hand, it is more counterintuitive that adaptive immune response would display circadian rhythms: the responses often occur days or weeks after the stimulating event (e.g. meeting of the pathogen and presentation of the antigens to cells of the adaptive immune system). Yet research in the past few years has been uncovering clear rhythmicity in various aspects of the adaptive immune system. Not only the cell numbers in the blood and organs shows such rhythms, but also the functional responses of these cells.

Beyond this initial stage of the research of circadian rhythms in adaptive immunity, which has shown physiological significance, a lot of work remains necessary. Most studies so far have been rather descriptive: the presence of a rhythm is demonstrated, and in some case data is provided to support a role for circadian clocks (cell autonomously or by other clocks in the organism) in the generation of the rhythm, or the need of other external rhythmic cues. However, there is still very little information on the molecular mechanisms underlying these rhythms.

As for vaccination, the research in animal models and the few (and often small scale) studies performed in humans suggest that time of day might be a factor to consider to optimize the response. However, there is a need for more detailed and controlled studies, in larger and more diverse human populations. Also, it is likely that the optimal times of vaccination would be distinct for different vaccines, depending on the mechanism of the immunization. Researchers should take advantage of large scale vaccination drives, documenting the time of administration and relating it to the outcome of the vaccines.

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Declaration of Conflicting Interests

The authors declare that there is no conflict of interest.

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Figure legends

Figure 1. The cells of the adaptive immune system and circadian clocks. Schematic representation of the main cells involved in adaptive immune responses: the dendritic cells (and other antigen-presenting cells) present antigens from pathogens to T cells in the context of major histocompatibility complex (MHC) Class I (for presentation to CD8 T cells) or MHC Class II (for CD4 T cells). After T cell receptor (TCR) triggering, T cells become activated, they proliferate and they differentiate into effector cells. B cells can also recognize antigens via their B cell receptor (BCR), and they can produce antibodies for the neutralization of specific pathogens. All these cell types express clock genes and proteins, the molecular components of the circadian clock (lower left inset). The transcription factors CLOCK and BMAL1 activate genes *Period (Per)* genes and *Cryptochrome (Cry)* genes, whose protein products form complexes and repress CLOCK/BMAL1, and hence, their own expression. CLOCK/BMAL1 also activate *Ror* and *Rev-erb* genes, which encode nuclear receptors that can activate and repress, respectively, *Bmal1* gene expression (as well as *Clock* and *Cry1*). As a result of these transcriptional-translational feedback loops, the expression of many of the clock genes follows a circadian rhythms (levels varying over the 24 hour cycle), as exemplified by *Bmal1* and *Per2* transcript in the lower panel. Transcription factors with rhythmic transcriptional activity or abundance (such as CLOCK/BMAL1 or REV-ERBα) also regulate a large number of downstream genes, called clock-controlled genes, whose expression can therefore show rhythmicity (4-20% of the transcriptome in any given tissue). This underlies the circadian rhythms in cell functions.

Figure 2. Circadian regulation of the CD8 T cell response to antigen presentation. Upon antigen presentation by dendritic cells (DCs) to CD8 T cells, signaling pathways downstream of

the T cell receptor (TCR) become activated, resulting in the activation of the T cells, their massive proliferation over the course of several days, and their acquisition of effector functions, in particular cytotoxic activity allowing these T cells to kill target cells (e.g. cells infected with a virus, cancer cells). This activation and proliferation of CD8 T cells was shown to be stronger when mice are immunized with DCs loaded with an antigen in the day, compared to immunization in the night. This circadian regulation appears to involve the rhythmicity of 6% of the T cell transcriptome, and the activation of a molecular program that mediates an enhanced TCR-dependent response to antigen presentation in the daytime (signaling factors in blue), and a toned down response by the involvement of TCR signaling inhibitor in the nighttime (signaling factors in purple).

Table 1. Summary of the studies addressing the effect of time of day on the outcome of vaccination

Vaccine	Participants				Time points	Measures	Outcomes	Reference
	Average age	Sex	Ethnicity	Number of participants				
Influenza	45 ± 14.6	M/F (about equal numbers)	Mostly caucasian	98	8:30-17:00 (retrospectively estimated)	Antibody titers 3-4 weeks after vaccination	Stronger response for 11:00 and 13:00 vaccination (for one of the 3 antigens)	[54]
Influenza	43.9 ± 9	M/F (about equal numbers)	70% Caucasian, 20% Black, 10% Hispanic	707	9:00-15:00	Antibody titers 3-4 weeks after vaccination	No effect of time of day	[54]
Influenza	71 ± 5.5	M/F (about equal numbers)	73%/100% Caucasian in the AM/PM groups	276	morning (9:00 to 11:00) or afternoon (15:00 to 17:00); randomized	Antibody titers 1 month after vaccination	Stronger response for morning group	[55]
Influenza	73.1 ± 5.5	38 M/51 F	87 Caucasian, 1 Black, 1 other	89	binned as morning (8:00 to 11:00) or afternoon (13:00 to 16:00)	Antibody titers 1 month after vaccination	sex × time of day interaction; stronger response for men/morning	[56]

Hepatitis A	22.9 ± 3.9	34 M/41 F	89% Caucasian, 2.7% Asian, 1.3% Black, 7% other	75	morning (9:00 to 12:00) or afternoon (16:00 to 18:00); randomized	Antibody titers 1 month after vaccination	sex × time of day interaction; stronger response for men/morning	[56]
Hepatitis B	20.5	27 M/36 F	not specified	63	morning (8:00-8:30) or afternoon (17:30 to 18:00)	Antibody titers 1 month after last of 3 doses	No effect of time of day	[57]
Hexavalent vaccination	60 days after premature birth	42%/29% M in morning/evening groups	not specified	26	morning (7:00 to 10:00) or evening (19:00 to 22:00); randomized	Antibody titers for pertussis and *H. influenzae* type b at corrected age 3 months (i.e. a few months after vaccination)	No effect of time of day	[58]
BCG (antituberculosis)	26 ± 10	21 M/33 F	Western European	54	morning (8:00) or evening (18:00)	Counts for leukocyte populations, cytokines after PBMC ex vivo stimulation with *S. aureus* or *M. tueberculosis*, 2 weeks and 3 months after vaccination	Stronger cytokine response to both bacteria for the morning group (also seen upon in vitro priming and stimulation of monocytes)	[59]

BCG (antituberculosis)	26 ± 11	131 M/171 F	not specified	302	8:00 to 12:00	Counts for leukocyte populations, cytokines after PBMC ex vivo stimulation with *S. aureus* or *M. tueberculosis*, 2 weeks and 3 months after vaccination	Effect of time of day; stronger cytokine response to *S. aureus* for the early morning groups	[59]

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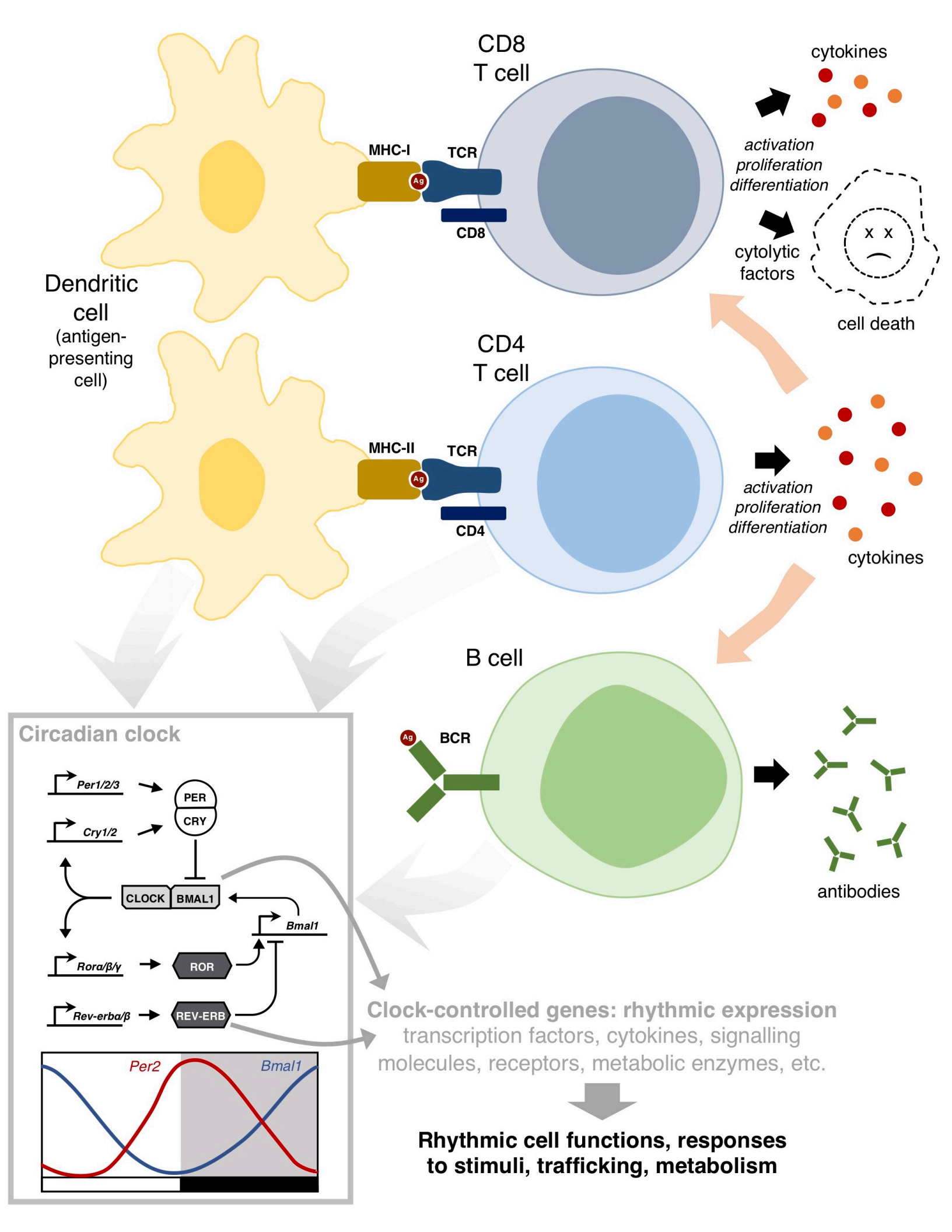
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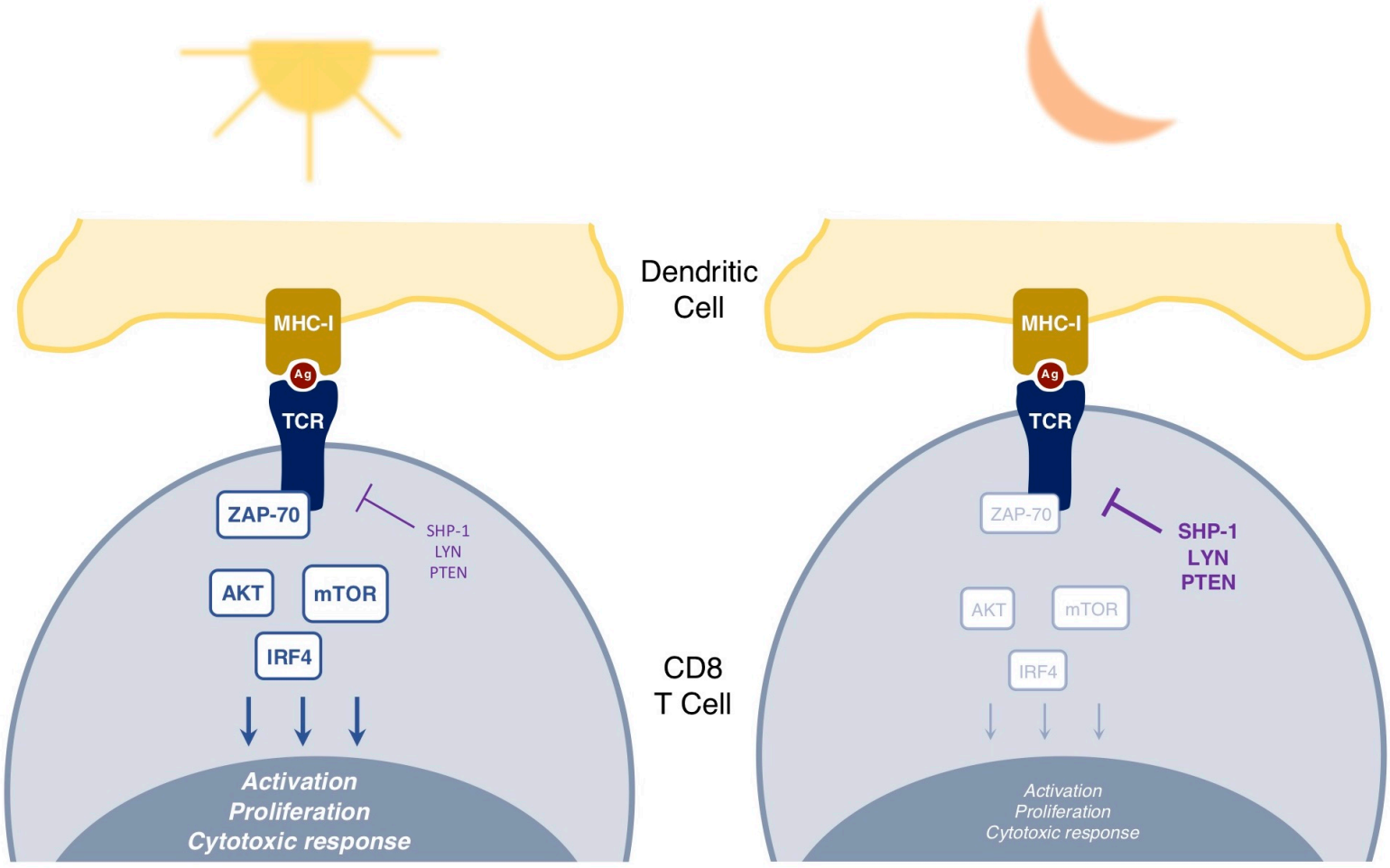
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