

**Title:** Glucocorticoid circadian rhythms in immune function

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## **Abstract**

Adrenal glucocorticoid (GC) hormones are important regulators of energy metabolism, brain functions, and the immune system. Their release follows a robust diurnal rhythms and GCs themselves serve as entrainment signals for circadian clocks in various tissues. In the clinics, synthetic GC analogues are widely used as immunosuppressive drugs. GC inhibitory effects on the immune system are well documented and include suppression of cytokines and increased immune cell death. However, the circadian dynamics of GC action are often neglected. Synthetic GC medications fail to mimic complex GC natural rhythms. Several recent publications have shown that endogenous GCs and their daily concentration rhythms prepare the immune system to face anticipated environmental threats. That includes migration patterns that direct specific cell population to organs and tissues best exemplified by the rhythmic expression of chemoattractants and their receptors. On the other hand, chronotherapeutic approaches may benefit the treatment of immunological diseases such as asthma. In this review we summarize our current knowledge on the circadian regulation of GCs, their role in innate and adaptive immune functions and the implications for the clinics.

**Key words:** Glucocorticoid rhythms; circadian clock; adaptive immunity; innate immunity

## Main text

### Introduction

Organismal physiology is intimately linked to the environment and benefits from anticipating recurring changes. In almost all living creatures the existence of the 24-hour day-night cycle has led to the evolution of an internal timing system, the circadian clock. It generates a 24-hour oscillation, and drives feeding, activity, hormonal, and metabolic rhythms. On a molecular level, the clock consists of a transcription-translation feedback loop. The dimer of BMAL1 and CLOCK binds to *E-box* promotor elements to initiate transcription of its targets. Those include *Period* (*Per1*, 2 and 3), *Cryptochrome* (*Cry1* and 2), *Rev-Erba/β* (*Nr1d1* and 2), *RORα-γ* (*Rora*, *b* and *c*), *Nuclear factor, interleukin 3, regulated* (*Nfil3*), and *Albumin D-box binding protein* (*Dbp*). PER-CRY heterodimers translocate to the nucleus to repress BMAL1/CLOCK driven transcription. A second loop controls, among others, the levels of BMAL1. REV-ERBα suppresses, while RORα stimulates *Bmal1* transcription via *ROR* elements. In addition, DBP and NFIL3 can bind *D-box* elements in the promoter of *Period*, activating and inhibiting the transcription, respectively [1].

The main pacemaker of the circadian system resides in the suprachiasmatic nuclei (SCN) of the hypothalamus, but almost all tissues have self-sustained clocks, including liver, adrenal gland, immune cells, and others. The SCN receives entrainment signals, notably from the retina, and can synchronise downstream clocks in the brain and the periphery. However, peripheral clocks can entrain independently of the SCN and provide feedback to the main pacemaker [2]. An important entrainment signal for the peripheral tissues are glucocorticoids (GCs) – mainly cortisol in humans and corticosterone in nocturnal rodents. They are produced in the cortex of the adrenal gland under the control of the hypothalamus-pituitary-adrenal (HPA) stress axis. Under homeostatic conditions its rhythmic activity is controlled by the SCN, peaking at the beginning of the activity phase, but it is also acutely induced by stress. The HPA axis originates in the hypothalamic paraventricular nucleus (PVN). Upon activation, the PVN releases corticotrophic hormone (CRH) into the median eminence, stimulating the pituitary to release adrenocorticotropin (ACTH) into the bloodstream. When ACTH reaches the adrenal gland, it activates GC biosynthesis from cholesterol and its release into the blood (**Figure 1A**).

GCs are recognized by mineralocorticoid (MRs) or glucocorticoid receptors (GRs). MR binds GCs with higher affinity than GR, leading to tonic activation already at low to medium GC levels. MR is less selective, though, as it also binds other steroids such as aldosterone and progesterone. GRs, in contrast, are only transiently activated at peak GC levels, making them the prime mediators of transient GC signalling. They are expressed in almost every cell of the body – although there are marked exceptions such as the SCN. Therefore, the central clock may be isolated from direct effects of varying GC concentrations, with indirect feedback potentially being communicated by the raphe nuclei [3]. Consequently, pharmacological treatment with GCs will have a limited impact on the main pacemaker. Under homeostatic conditions, GCs will mostly interact with MR and only occupy the GR at the peak of the circadian phase or under acute stress conditions [4].

GR is a nuclear receptor. When bound to GCs, it can modulate gene transcription *via* GR response elements (*GREs*, positive regulation) or *nGREs* (negative regulation). Additionally, GR is capable of influencing gene expression through protein-protein interactions with transcription factors without binding of the DNA (*tethering*) [5-6] however the existence of multiple regulatory mechanisms of GR-mediated gene expression has also been postulated [7]. GC-GR interaction is responsible for a negative feedback of GCs on HPA axis activity at the level of the pituitary and the hypothalamus. Upon HPA axis activation GC levels rise within minutes, reaching a peak at about 30 min after stress induction. Suppression of ACTH production through GR-mediated inhibition of its precursor gene, pro-opiomelanocortin (*Pomc*), leads to subsequent downregulation of cortisol levels. Under baseline conditions this feedback results in a pulsatile cortisol secretion pattern with a period of around 90 min. Besides this ultradian pattern GC levels are further regulated in a circadian manner and, in females, along the oestrous cycle. During the day, GC levels peak at the beginning of the active phase, *i.e.*, the morning in humans and the evening in nocturnal rodents. The SCN influences HPA axis activity through innervation of the PVN and release of arginine vasopressin. Light exposure can further directly induce GC secretion through autonomic activation of the adrenal independent of the HPA axis. Regarding the oestrus cycle of rodents, GC levels show anti-cyclic regulation to oestrogen with low GC peak levels

during oestrous, *i.e.*, at the stage of highest oestrogen levels [8-9]. Similarly, through the menstrual cycle higher cortisol levels are recorded in the follicular than in luteal phase [10] (**Figure 1B**). The crosstalk between the HPA and the hypothalamus-pituitary-gonad axis that underlines sexual dimorphism of the GC rhythms has been reviewed elsewhere [11].

In addition to receptor distribution and accessibility, GC function is further regulated by its biological availability. The hydroxylase 11 $\beta$ -HSD2 (hydroxysteroid 11-beta dehydrogenase 2; HSD11b2) inactivates corticosterone. In their inactive form, 11-dehydroxycorticosterone (DHC), GCs cannot bind to GR. Reconversion to corticosterone is catalysed by 11 $\beta$ -HSD1 (HSD11b1). In blood and tissues, levels of GC are generally higher than of DHC, but this balance is gender and age dependent [12].

GCs are important synchronisers of peripheral tissue circadian clocks [13], but they are also regulators of immunity, metabolism, and the central nervous system (CNS). GCs maintain energy homeostasis and, under stress conditions, keep blood glucose levels high to facilitate fight-or-flight responses [14-15]. They promote gluconeogenesis and glycogen storage in the liver, inhibit uptake of glucose by skeletal muscle and adipose tissue and influence pancreatic alpha cells to release glucagon, overall promoting hyperglycaemia and insulin resistance. Consequently, chronically high GC levels, *e.g.*, during GC supplementation, favour the development of metabolic disease. In the CNS, GCs modulate a variety of functions, including learning and memory consolidation [16], mood and several psychiatric disorders [17-20].

In medical practice GCs are predominantly used as immunosuppressive drugs, *e.g.*, to suppress rejection after organ transplantation or to treat autoimmune or allergic conditions such as rheumatoid arthritis, inflammatory bowel disease, multiple sclerosis, psoriasis, or asthma [21-22]. Not surprisingly, when considering the broad biological action profile of GCs, those treatments carry a burden of side effects. It is therefore of interest to better understand how GCs regulate immunity, which could lead to development of new generation of immunosuppressive drugs with fewer adverse reactions.

### **GC-mediated modulation of immunity**

The landmark of GC immunosuppressive action is their effect on cytokine secretion. GCs, under different paradigms, have been reported to inhibit interleukins (IL-1 $\alpha$ , IL-1 $\beta$ , IL-2, IL-3, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12, IL-13), tumour necrosis factor alpha (TNF $\alpha$ ), and interferons (IFN $\alpha$ ,  $\beta$  and  $\gamma$ ) [23-27]. Those effects are often facilitated by GR-mediated inhibition of the proinflammatory transcription factors nuclear factor kappa-b (NF- $\kappa$ B) and activator protein-1 (AP-1) *via* tethering [5-6]) or transactivation (*i.e.*, induction of gene expression) of downstream targets such as GC-induced leucine zipper protein (GILZ) or inhibitor of nuclear factor kappa B alpha (I $\kappa$ B $\alpha$ ) [28]. Recently, however, a more nuanced image of GC action in immunity emerges. Interestingly, synthetic GCs have stronger immunosuppressive effects due to prolonged half-life, less binding to serum proteins [29] and reduced susceptibility to 11 $\beta$ -HSD inactivation [30]. By contrast, endogenous GCs are characterised by their pulsative release rhythm, leading to similar pulses of gene transcription; such effects are not replicated when administering synthetic GC treatment [22, 31].

Combined with the circadian rhythm of GCs, it seems that in homeostasis GCs play an immunomodulatory role, rather than being simply immunosuppressive. In fact, several studies show a potential of GCs to stimulate the immune response. For example, macrophage colony-stimulating factor (M-CSF) is not inhibited by dexamethasone (DEX) treatment of human lung fibroblasts, unlike granulocyte-macrophage colony-stimulating factor (GM-CSF), or IL-6 and IL-8 [32]. IL-4 has been reported to increase after DEX in primary cell cultures [33]. TNF $\beta$  activation, but not expression, is induced by DEX in human osteoblast-like cells [34]. While the induction of cytokines by GCs is not commonly observed, the corresponding cytokine receptors are often upregulated. In human immune cells after DEX treatment, expression of receptors for IL-8, IL-1, IL-6, interferon alpha and gamma (IFN $\alpha$ , IFN $\gamma$ ), and TNF were strongly upregulated [27]. In different cell types, receptors for IL-1, IL-2, IL-4, IL-6, IFN $\gamma$ , GM-CSF and TNF were also reported as upregulated. This may suggest that endogenous GCs optimise rather than simply repress the immune response [35-36].

Some GC effects are dependent on dose or time, underlining their bimodal action. Dose-dependent effects include the increase of nitric oxide (NO), TNF $\alpha$ , IL-1 $\beta$ , IL-6, IL-12, and chemokine (C-X-C motif) ligand 1 and 10 (CXCL1 and CXCL10) expression after stimulation of peritoneal macrophages with lipopolysaccharide (LPS) combined with low DEX concentrations. The opposite applies for high concentrations of DEX, where its habitual immunosuppressive effects become apparent. Both effects are dependent on the presence of GR [37]. Administering corticosterone either 2 h or 24 h before an LPS challenge potentiates the immune response, *e.g.*, through IL-1 $\beta$ , TNF $\alpha$  and IL-6, when compared with GC administration after LPS [38]. Another timing-related effect comes from studying high-intensity exercise effects on *Herpes simplex* virus (HSV) 2 infection clearance in mice. Forced treadmill running at 17 h after HSV-2 injection causes less clearance, while the same effort 8 h after injection has a positive effect. This is associated with higher numbers of circulating plasmacytoid dendritic cells, whose tissue homing is regulated by GR *via* CXCL12-CXCR4 interaction [39].

### **GC rhythms in innate immunity**

Circadian GC rhythms have an impact on immune responses throughout the body (overview in Figure 2). One prominent example is the homing of neutrophils. After LPS treatment of wild-type (WT) mice the number of neutrophils in the lung varies depending on the circadian time, with a peak at circadian time 0 (CT, *i.e.*, the beginning of the rest phase). That accumulation is followed by the highest cytokine production at CT6. This effect is lost after bronchiolar cell-specific knock-out of *Bmal1*. In addition, this genetic manipulation abolishes the rhythm of CXCL5, a neutrophil chemoattractant, which normally peaks at the beginning of the rest phase in synchrony with neutrophil lung homing. Adrenalectomy similarly eliminates rhythmic CXCL5 expression, neutrophil migration and, consequently, the rhythmic inflammatory response. This effect is GR-dependent, with GR occupancy at the *Cxcl5* promotor showing a circadian pattern in WT (high binding at CT12), but not in *Bmal1* deficient mice [40]. A subsequent study has shown that the mechanism is not completely dependent on GR, as a deletion of *Gr* in bronchial epithelium does not abolish the rhythmic neutrophil migration, even if it eliminates the CXCL5 rhythm. Sustained migration rhythm in such condition may be driven by changing neutrophil blood concentrations [41]. Another neutrophil chemoattractant seems to be

regulated by GCs. C-C motif chemokine ligand 20 (CCL20) is a homing signal for T-helper (Th) 17 lymphocytes, dendritic cells, and neutrophils. In bronchial cells of the lung, GCs increase CCL20 levels [42]. In human keratocytes, DEX similarly induces CCL20. Mice show a 2.5fold increase in CCL20 and a decrease in IL-1 $\beta$  after application of the GC analogue, halometasone, to the skin of the ear. A direct binding of GR to the *Ccl20* promoter seems to be responsible [43]. Such bidirectional effects can explain why GC therapies are not successful in all cases of asthma. Apart from their effects on neutrophil migration, endogenous glucocorticoids also promote neutrophil maturation and release into the bloodstream [44]. Finally, GCs promote phagocytosis of apoptotic neutrophils in the resolution phase of inflammation [45-46].

Macrophages defend the organism from foreign intruders by phagocytosis, but they themselves contain an intrinsic circadian clock and secrete cytokines in a rhythmic manner with a peak TNF $\alpha$  and IL-6 secretion after LPS challenge at CT8 [47]. Macrophage phagocytosis is also circadian, an effect mediated by clock control of the cytoskeleton [48]. GCs exert their immunosuppressive effects *via* GR, which in turn suppresses the p38 mitogen-activated protein kinase (MAPK), resulting in the inhibition of TNF $\alpha$ , IL-6 and cyclooxygenase 2 (COX2) [49]. GCs are also involved in macrophage movement as they upregulate dipeptidyl-peptidase 4 (DPP4; *via* direct GR transactivation) to induce migration [50]. Involvement of the intrinsic clock is also of note, as *Bmal1* suppresses mobility, as demonstrated by a macrophage specific deletion in vitro [48]. Hematopoietic deletion of the clock gene *Rev-Erba*, which is in antiphase of *Bmal1*, leads to an increased expression of CX3C chemokine receptor 1 (CX3CR1) [51]. REV-ERB $\alpha$  has been demonstrated to be important for macrophage function in another study. It was observed that the cytokine production, including IL-6, IL-12, CCL5, CXCL1, CCL2 by macrophages is clock-gated when LPS is administered, with lower response at CT0 compared with CT12. This effect is not reproduced when either *Bmal1* or *Rev-Erba* are deleted in murine macrophages. Interestingly, in *Rev-Erba* knock-out animals, the circadian clock itself is largely unaffected. At the same time, REV-ERB $\alpha$  inhibits cytokine production from macrophages [52] but has no effect on phagocytosis [48].

Another migration effect involves monocytes, where *Bmal1* regulates the diurnal migration to organs and infection sites. Monocytes highest blood concentration falls on the rest phase, and their homing to infections sites is more pronounced at *zeitgeber* time (ZT) 0 than ZT8. As a result, mice injected with *Listeria monocytogenes* at ZT0 survive longer than those exposed at ZT8. In myeloid cell knock-out of *Bmal1* in mice such migration rhythm is lost [53]. An additional regulator of monocyte migration, CXCR4, is upregulated upon DEX treatment [54]. The monocyte state of activation can determine either an increase or a decrease of inflammation. As such, GCs promote anti-inflammatory monocytes and induce phagocytosis of proinflammatory agents [55].

Natural killer (NK) cells are derived from lymphocytes, but they constitute a part of innate immune response. GCs suppress splenic NK cell numbers in mice [56] and promote more immature NK cell populations [57]. These effects are likely to be GR-dependent, as its deletion in innate lymphoid cells (which comprise NK cells) leads to higher IFN $\gamma$  production and limits the development of endotoxin tolerance [58]. Consequently, GR antagonization improves NK cell activation and proliferation and can promote tumour killing [59]. A possible mechanism of this was recently described where GCs in tandem with IL-12, IL-15 and IL-18 cause an induction of programmed cell death protein 1 (PD-1, an immune checkpoint) on NK cell surface, thereby promoting tolerance [60]. This cell type is also regulated by the circadian clock. Their secretions (granzyme B, perforin, IFN $\gamma$ , and TNF $\alpha$ ) are rhythmic and they express clock genes, both, in LD and DD [61-62]. Their activity peaks at wakefulness when the organism is most at risk of exposure to pathogens [63]. In *mPer2<sup>Brdm1</sup>* mice (*Per2* mutant strain), IFN $\gamma$  losses its rhythmic expression [61], while a knock down of *Per2* causes a decrease in granzyme B and perforin levels [62]. Furthermore, *Per1* knock-out animals present with disturbed rhythms of IFN $\gamma$ , perforin and granzyme B in splenic NK cells [64]. However, a direct link between rhythmic GCs and NK functions has not been demonstrated yet.

At the border between innate and adaptive immunity, dendritic cells (DCs) phagocytose and present antigens to lymphocytes. Those too can have different phenotypes, either promoting immune response or tolerance. In line with their immunosuppressive action, GCs counteract DC function by limiting



viability and reducing cytokine expression [65]. In addition to this, the stimulation with DEX during DC differentiation leads to more tolerogenic phenotypes [66]. This property was recently utilised in a murine encephalomyelitis model of multiple sclerosis. Animals were treated with an immuno-conjugate of DEX and a peptide antigen that can be processed by DCs, forcing co-stimulation. As a result, the disease symptoms diminished when compared to mice where DEX and antigen were delivered unconjugated [67]. DCs also respond to endogenous GCs. The DC-specific deletion of *Gr* leads to higher expression levels of IL-1 $\beta$ , TNF $\alpha$  and IL-12 – with the suppression of latter by GCs being likely responsible for the tolerogenic action [68]. Finally, the circadian clock can also fine tune the adaptive immune response *via* its effects on DCs. Indeed, integrin alpha X (CD11c)-driven knock-out of *Bmal1* in DCs influences the Th1/2 differentiation balance *via* regulation of IL-12 [69].

### **GC rhythms in adaptive immunity**

GCs and the circadian clock have an important function in migration and lymph node homing of lymphocytes, but their role is noticeable even at earlier stages of lymphocyte life, *i.e.*, during selection and differentiation (Figure 3). While the exact action of GCs on thymopoiesis is far from clear, they have been reported to promote apoptosis of thymocytes and thymic epithelial cells [70-71]. Consequently, adrenalectomy causes a drop in thymocyte cell death and thymic hyperplasia [72]. Those actions of GCs seem to be GR mediated, as the deletion of *Gr* in pre-selection thymocytes and subsequent improper selection leaves the mice carrying such mutation immunocompromised and unable to properly respond to antigens [73]. Overall, the mutual antagonism theory, first proposed by Zacharchuk *et al.* [74], postulates that the selection process of thymocytes depends on an interplay of T-cell receptor (TCR) and GR signalling, which establishes the threshold for positive and negative selection. While the GC effect on T-cells differentiation is a net negative – the suppression of the Th2 and regulatory T-cells (Tregs) is less efficient than Th1 cells, thereby shifting the differentiation in the direction of the humoral immune response. They also seem to be permissive of the Th17 phenotype [75-76]. Such mechanisms possibly protect the organism from uncontrollable immune responses. Infection associated stress would restrict Th1-mediated immunity and, by proxy, the excessive production of inflammatory cytokines. On a molecular level, GCs inhibit the production of IL-12, INF $\gamma$

and TNF $\alpha$  (Th1 promoting) by antigen-presenting cells, while simultaneously upregulating the expression of IL-4, IL-10, and IL-13 (Th2 promoting) [75]. For example, *ex-vivo* cultivated rat T-cells when activated in the presence of DEX produce more IL-4, IL-10 and IL-13 and downregulate INF $\gamma$  and TNF $\alpha$  upon secondary stimulation, leading to the promotion of Th2 action [77]. The circadian clock may also play a role in this process, as NFIL3 seems to induce Th2-promoting cytokines, and *Nfil3* is a target for repression by REVERBs. Clock regulated *Nfil3* is highly expressed in Th2, but not in Th1. Also, Th2 cells that are deficient in *Nfil3* upregulate IL-13 and IL-5, while downregulating IL-4. In control cells *Nfil3* suppresses IL-13 *via* direct promotor binding [78]. Lastly, the Treg fate promoting properties of GCs may involve the intermediary GILZ. In mice with GILZ overexpression, this T-cell type is overrepresented. Molecularly, GILZ interacts with SMAD family member 2 (SMAD2) to enhance TGF $\beta$  signalling. This increases GR binding to the promotor of the transcription factor *Forkhead P3* (*Foxp3*; a factor determining Treg differentiation of naïve T-cells) and its expression [79].

T-cells migrate between blood and lymph nodes (LNs) in search of antigen presenting cells (APCs). This trafficking is characterised by a circadian oscillation, with the peak blood concentration in the early rest phase, and the lowest during the active phase. Consequently, T-cells accumulate in the LN at the beginning of active phase [80-81]. The accumulation of T-cells in LNs during the active phase prepares the immune system for a potential contact with foreign antigens. The lymphoid organs are CXCL12 positive and attract lymphocytes expressing CXCR4 [76, 81]. Naïve T-cells express CXCR4 in a circadian fashion, with a raise of expression at the beginning of active phase, and this phenomenon is under GC control. Human subjects treated with mifepristone (a GR antagonist) or metyrapone (a suppressor of steroidogenesis) show an attenuated or blocked morning increase of CXCR4 expression on the surface of lymphocytes [80]. It is known that CXCR4 is induced by IL-7 [82]. This provides a mechanistic link to GCs, as in human blood T-cells, DEX can induce expression of the  $\alpha$  chain of IL-7R [83]. This receptor is important for the development of lymphocytes [84] and in its proximal enhancer conserved non-coding sequence 1 (*CNS1*) harbours two functioning *GREs*, which, when mutated specifically in T-cells, result in a loss of T-cell migratory oscillations, rhythmic IL-7R and CXCR4 [81, 85]. In control animals, IL-7R and CXCR4 expression is high during the active phase

(with a peak at ZT16) and induced by DEX. Consequently, the deletion of GR in T-cells abolishes the rhythmic expression of IL-7R and CXCR4, as well as T-cell migration to CXCL12 positive tissues. The deletion of CXCR4 in T-cell also abolishes migration rhythms [81]. Lymphocytes (both T- and B-cells) also have a functioning intrinsic clock that is involved in trafficking. Mice with a deletion of *Bmal1* in either T- or B-cells lose their blood/LN daily oscillation. They also no longer show rhythmic expression of C-C motif chemokine receptor 7 (CCR7) on their surface. CCL21 expressed in the LN also oscillates on mRNA level. CCL21 high expression at the beginning of the active phase roughly coincides with CCR7 expression on lymphocytes and their homing to LN. Lymphocytes that do not express the CCR7 receptor no longer migrate to the LN in a diurnal fashion and overall exhibit lower LN level. In addition to timing the CCL21-CCR7 interaction, the circadian clock (more specifically BMAL1 and CLOCK) also controls the expression of *Sphingosine 1 phosphate receptor 1* (*Slpr1*), a receptor regulating lymph node egress, which peaks at ZT5 (middle of the rest phase). Haplo-insufficient heterozygous *Slpr1* deletion abolishes diurnal lymphocyte egress. Rhythmic T-cell homing translates into divergent immune response depending on the time of antigen presence. In a mouse autoimmunity model immunisation at ZT8, as opposed to ZT20, leads to faster disease progression [86].

The relationship of B-cell function with the GCs and circadian clocks is less well studied, but an oscillatory pattern of trafficking has been described also for those cell types. In murine B-cells, GCs upregulate the CXCR4 expression analogously to T-cells, macrophages, and eosinophils. This chemokine receptor is important for the maturation of B-cell function. In mice lacking *Gr* expression in B-cells, CXCR4 levels are disturbed, resulting in impaired homing to bone marrow, without an effect on other lymphoid tissues. This specific outcome contributes to a diminished response to certain types of antigens [87]. Antibody responses are also sensitive to GCs. They have been reported to induce (in synergy with IL-4) isotype switching to promote immunoglobulin E (IgE) production [88] and, under restraint stress conditions, to negatively affect differentiation [89]. Under physiological conditions, GCs appear to promote immune response. A lack of endogenous GCs (in adrenalectomized rats) reduces antibody responses [90] and, when immunised with keyhole limpet hemocyanin, such animals have reduced IgM and IgG responses (the former can be partly recovered with corticosterone injection) [91].

## **Clinical implications**

Glucocorticoids are very widely used in clinical medicine, with an estimated 1% of the population receiving regular treatment. There is evidence that the timing of therapeutic glucocorticoids affects the disease responses, but surprisingly this information has not resulted in circadian medicine [92]. As an example, bronchial asthma is prevalent, and despite effective treatment kills thousands annually. Nocturnal symptoms wake 74% of patients, at least weekly [93]. In addition, a causative role for circadian disruption on development of asthma is suggested by the increased risk of disease seen in nightshift workers, people who are subjected to circadian misalignment [94]. The lung is a highly circadian tissue, with important clock functions located in the airway epithelium [40]. In addition, many components of the inflammatory/immune systems are themselves circadian rhythmic, including mast cells, basophils, and eosinophils [95-97].

In the clinical diagnosis of asthma variability in airflow obstruction by time-of-day results in peak symptoms during the night, affecting sleep. In addition, markers of airway inflammation show strong diurnal variability, with implications for diagnosis, and for disease activity monitoring [98]. One striking finding is that dosing of oral prednisolone, a synthetic glucocorticoid, was found to be affected by time of administration, with peak benefit obtained at 3pm, at which time there was also a marked drop in markers of airway inflammation. In contrast administration at 8am, or at 8pm lacked a significant impact [99]. These data are perhaps the most persuasive in arguing that embedding circadian clock logic in drug development, in clinical trials, and in clinical guidelines is likely to offer significant benefit for human populations.

Other autoimmune diseases also present with a daily variation in symptoms. In rheumatoid arthritis stiffness, joint pain and the resulting functional disability are most acute in the morning, which correlates with high IL-6 serum concentrations [100]. GCs are routinely administered upon awakening (between 6 and 8 AM) when it is already too late to significantly limit inflammation initiated during the night [101]. Patients that instead took prednisolone at 2 AM experienced greater improvement of their

symptoms compared with administration at 7:30 AM [102]. To combine beneficial effects of night-time GC treatment with patient overall comfort, a modified release prednisone was introduced with 4-hour delay in drug release. Such medication, taken at bedtime, shortens the duration of morning stiffness by 30 min [103]. Preliminary studies indicate a circadian pattern on symptoms in other rheumatic diseases such as polymyalgia rheumatica and ankylosing spondylitis [104]. In addition, in multiple sclerosis night-time administration of GCs leads to lower matrix-metalloproteinase 9 levels (a disease marker) when compared with day-time intake [105].

The benefits of chronopharmacological approaches to GC therapy should be assessed within each disease indication. It is important to keep in mind that the standard time of administration in the early morning, in phase with the endogenous GC peak, reduces the risk of developing tertiary adrenal insufficiency. This condition develops in patients under long-term treatment, since exogenous GCs suppress the HPA axis and ACTH levels. This predisposes to adverse outcomes in response to stress or trauma and it can lead to a life threatening adrenal crisis [106].

## **Conclusion**

The general importance of GCs in regulating innate as well as adaptive immune functions has long been acknowledged and synthetic GC analogues are amongst the most widely prescribed anti-inflammatory agents worldwide. On the other hand, the need for more individualized therapeutic strategies is gaining more and more attention – and GCs may provide an interesting showcase for such approaches because of their interaction with the circadian clock system. Endogenous GCs show prominent circadian rhythmicity while GCs, in turn, act as *zeitgebers* for circadian tissue clocks. Circadian rhythms differ in their expression from patient to patient and, likewise, does the optimal time for a specific treatment. On the background of recent developments in chronobiology this may just be the right time to start implementing circadian logic into clinical medicine, and GCs might be a good first target.

### Figure captions

**Figure 1:** A) GCs are produced by the adrenal glands upon activation of the HPA axis. The PVN region of the hypothalamus produces CRH which stimulates the pituitary gland to release ACTH. Once the hormone reaches the adrenal gland *via* the bloodstream it initiates GC synthesis and release. The axis is further regulated by negative feedback of GCs on the biosynthesis and release of ACTH and CRH. B) GCs are rhythmic in multiple dimensions. The pulsatile, ultradian rhythm of around 90 min is a consequence of the negative feedback of GCs on their upstream activator ACTH. The circadian rhythm is mainly driven by the SCN's innervation of the PVN, which results in a circadian control of CRH and downstream HPA hormone levels. Throughout the oestrus cycle, GC rhythm amplitudes vary roughly in antiphase to oestrogen (E2) concentrations.

**Figure 2:** Different aspects of innate immunity are regulated in a circadian fashion through GCs. Neutrophils migrate to the lung rhythmically in response to GR-driven *Cxcl5* expression. Monocytes also show an oscillation in their migration to infection sites, with higher monocyte levels at ZT0 when compared to ZT8. They also initiate *Cxcr4* expression when stimulated by GCs. In macrophages, GCs regulate rhythms of cytokine production. Finally, GCs promote tolerogenic dendritic cells (DCs).

**Figure 3:** GCs modulate the rhythms of adaptive immunity mainly through their effects on cell migration. In naïve T-cells, GCs induce the expression of the  $\alpha$  chain of IL-7R, which drives oscillatory lymph node (LN) homing. As LNs are CXCL12 and CCL21 positive, they attract cells expressing CXCR4 and CCR7, respectively. Those receptors are rhythmically expressed in lymphocytes, with peak expression at the beginning of the active phase. Consequently, lymphocytes accumulate in the LN at the same time. CXCR4 is induced by GCs, whose circadian levels also peak at the beginning of the active phase. In addition to regulating LN homing, GCs modulate cytokine productions and, thus, promote T-helper cell (Th2) differentiation.

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**Conflict of interest**

The authors report no conflicts of interest.

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