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Peripheral Neuroimmunoendocrine Interactions: Contribution of TNFRp55 to the Circadian Synchronization of Progesterone and Cytokine Production in Joints of Mice in Late Pregnancy

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Circadian rhythm · Cytokine · Pregnancy · Joint · TNFRp55

Abstract
Objective: Circadian rhythms are generated by the suprachiasmatic nucleus of the hypothalamus and involve rhythmic expression of clock genes and proteins. This rhythmicity is transferred to peripheral tissues by neural and hormonal signals. Late pregnancy is considered a state of inflammation which impacts on peripheral tissues such as joints. Tumor necrosis factor (TNF) mediates inflammatory and circadian responses through its p55 receptor (TNFRp55). Neuroimmunoendocrine interactions in joints have not been studied completely. The purpose of this study was to analyze these interactions, investigating the circadian rhythms of progesterone (Pg) and pro- and anti-inflammatory cytokines in the joints at the end of pregnancy (gestational day 18). Moreover, the impact of TNFRp55 deficiency on these temporal oscillations was explored. ***Methods:*** Wild-type and TNFRp55-deficient (KO) C57BL/6 mice were kept under constant darkness in order to study their endogenous circadian rhythms. The expression of the clock genes *Bmal1* and *Per1* at circadian time 7 was studied by reverse transcription polymerase chain reaction in the ankle joints of nonpregnant and pregnant (gestational day 18) mice. In late pregnancy, Pg and the cytokines interleukin 17 (IL-17), IL-6, and IL-10 were measured in the joints throughout a 24-h period by radioimmunoassay and enzyme-linked immunosorbent assay, respectively. ***Results:*** A significant increase in *Bmal1* and *Per1* mRNA expression was detected in the joints of pregnant KO mice. Furthermore, KO mice displayed a desynchronization of articular Pg and cytokine production. ***Conclusions:*** Our results show that TNF, via TNFRp55 signaling, modulates articular Pg and cytokine circadian rhythms in late pregnancy. These findings suggest a temporal neuroimmunoendocrine association in peripheral tissues in late pregnancy.

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Introduction

Neuroimmunoendocrine interactions have been demonstrated at both the systemic and local levels, including the synovial tissue [1]. These interactions are dynamic and governed by a complex network determined by a crosstalk among circadian rhythms, cellular processes, and molecular signaling [2]. Daily rhythms have been observed in different models of inflammation, including animal models of arthritis [3]. Particularly, clinical signs and symptoms, such as swelling, pain, stiffness, and functional ability, are known to fluctuate during the day in rheumatoid arthritis (RA). These observations may suggest involvement of the circadian clock in the joints of RA patients [4].

In mammals, circadian (from Latin: *circa* [close] and *diem* [day]) rhythms, i.e., oscillations with a period close to 24 h, are generated by a central clock in the suprachiasmatic nucleus (SCN) of the hypothalamus. These rhythms are transferred to peripheral body cells and tissues by neural and hormonal signals [5]. Thus, most mammalian tissues show circadian oscillations and have their own cellular clock machinery [6, 7].

In cells, the circadian rhythmicity is achieved by the oscillating expression of the heterodimerized transcription factor BMAL1:CLOCK, which activates the transcription of period (*Per1*, *Per2*, and *Per3*) and cryptochrome (*Cry1* and *Cry2*) genes by binding to E-box (CACGTG or CANNTG) motifs in their promoters. PER and CRY proteins undergo various posttranslational modifications and translocate to the nucleus where they act as cyclic repressors of their own and of other clock-controlled gene transcriptions by interfering with the binding of BMAL1:CLOCK complex to DNA [8–10]. Other components that complete the molecular clock machinery are the transcription factors retinoic acid receptor-related orphan receptor (ROR) and REV-ERB acting in concert to activate or to repress the *Bmal1* gene transcription, respectively [11].

Keller et al. [12] showed that mouse spleen, lymph nodes, and peritoneal macrophages contain intrinsic circadian clocks which operate autonomously, even ex vivo. These clocks regulate the circadian rhythms of inflammatory innate immune functions [13, 14]. Recently, Haas and Straub [15] described daily *Bmal1* and *Per1* rhythms in synovial fibroblasts of healthy subjects. Expression of clock proteins and their transcripts such as *Bmal1*, *Clock*, *Per1*, and *Per2* has also been reported in synovial cells and tissues of patients with osteoarthritis and RA [16].

A close relationship between the molecular clock and tumor necrosis factor (TNF), a multifunctional cytokine, has been investigated in several cells and tissues, including the SCN [17, 18]. There is evidence that the mammalian clock gene *Cry* regulates arthritis via the proinflammatory cytokine TNF [19]. Arjona and Sarkar [20] demonstrated strong circadian variations of cytokines and cytolytic factors in enriched natural killer cells from rat spleen, which suggests that these functions may be subjected to circadian regulation. Particularly, increased production of proinflammatory cytokines (i.e., interleukin 6 [IL-6], IL-17, TNF) has been well documented during synovitis and correlates with the worsening of clinical symptoms at the beginning of the day in patients with RA [21]. Petrzilka et al. [22] reported that TNF reduces the expression of E-box-driven clock genes in cultured fibroblasts. Yoshida et al. [16] recently demonstrated that TNF enhances *Bmal1* and *Cry1* expression, but reduces *Per2* and *Dbp* mRNA levels in synovial cells. As can be seen, TNF modulates circadian rhythms in joints by regulating the expression of clock genes. Duhart et al. [23] showed that TNF induces phase shifts in the SCN PER2 rhythms when administered intracerebroventricular, provoking changes in behavioral circadian rhythms and SCN activation in wild-type (WT), but not in TNFRp55-deficient (KO) mice. This observation indicates that TNFRp55 might mediate the circadian modulator role of TNF.

In late pregnancy, virtually all cytokines increase significantly, suggesting the occurrence of a physiological inflammatory status [24]. The impact of this low-grade inflammatory response, involving both pro- and anti-inflammatory cytokines, on joints remains unclear. The effect of fluctuations of pregnancy-related steroid hormones over local production of cytokines (i.e., TNF) is not completely understood either [24].

De Man et al. [25] observed an attenuation in RA symptoms in pregnant women. However, it has also been reported that there is an increased risk of developing RA or an exacerbation of an already established RA particularly in the first 3–12 months after delivery [26]. Additionally, Guthrie et al. [27] also found that a shorter time interval from the last childbirth is strongly and significantly correlated with a reduced RA risk.

Progesterone (Pg), the main hormone controlling pregnancy, is produced by the corpus luteum in the ovary and the placenta. Its serum levels rise 5- to 10-fold higher during pregnancy, sustaining receptivity, decidualization, and immune privilege during gestation [28]. This hormone plays a key role in pregnancy-related immunoregulation and is increased in the synovial fluid of RA patients [29, 30].

Taking into account all the above observations, the purpose of this work was to explore neuroimmunoendocrine interactions in joints of mice at the end of pregnancy. We observed that expression of clock genes increases significantly in the joints of late-pregnant KO mice. Hence, we analyzed the temporal variation of Pg in serum and joints as well as the circadian oscillations of pro- and anti-inflammatory cytokines in the joints of mice at the end of pregnancy. We also evaluated the effects of a lack of TNFRp55 on the temporal patterns of Pg as well as pro- and anti-inflammatory cytokines.

Materials and Methods

Animals and Housing

Eight-week-old female mice of the inbreed strain C57BL/6 (WT and KO) were used in this study. KO mice were obtained from the Max von Pettenkofer Institute, Munich, Germany. C57BL/6 WT mice were purchased from the National University of La Plata (Argentina). Breeding colonies of both groups of mice were established at the animal facility of the National University of San Luis (Argentina). The mice were housed in order to synchronize their estrous cycle and maintained on a 12 h light:dark schedule (lights on at 7 a.m.), with irradiated food and sterile water available ad libitum at a constant temperature (24 ± 2 °C). In order to study endogenous circadian rhythms, WT and KO pregnant mice were kept from gestational day 12 under constant darkness. On gestational day 18 (late pregnancy), 4 or 5 animals per group were euthanized by cervical dislocation, every 6 h, beginning at circadian time 2 (CT2) (with CT0 established as the beginning of the subjective day). Such frequency of sampling has been used by other authors to study circadian and diurnal changes of immunoendocrine mediators [31, 32]. Joints from hind limbs were rapidly removed and frozen at –80 °C.

Expression of Clock Genes in Joints

On gestational day 18 at CT7 (middle of rest period), ankle joints were excised as previously described in Di Genaro et al. [33]. The frozen tissue was placed in 1 mL of TRIzol reagent (Invitrogen, San Diego, CA, USA) and RNA extraction was performed as described by the manufacturer. Each total RNA sample was treated with the RQ1 RNase-free DNase according to the manufacturer's instructions (Promega, Madison, WI, USA). RNA from individual joint samples was analyzed. Synthesis of cDNA was performed using 2 μg of total RNA and the ProtoScript M-MuLV First Strand cDNA synthesis kit (New England Biolab, Ipswich, MA, USA) according to the manufacturer's instructions. Polymerase chain reaction was performed on cDNA for a total of 35 cycles at 94 °C for 30 s, 60 °C (for *β-actin* and *Bmal1*) or 58 °C (for *Per1*) for 45 s, and 72 °C for 60 s. Primer sequences were as follows: *β-actin* forward, 5′-CGT TGA CAT CCG TAA AGA CCT-3′; *β-actin* reverse, 5′-CTT GAT CTT CAT GGT GCT AGG AG-3′; *Bmal1* forward, 5′-CGG TCA CAT CCT ACG ACA AAC-3′; *Bmal1* reverse, 5′-CAG AAG CAA ACT ACA AGC CAA C-3′; *Per1* forward, 5′-CAA GTG GCA ATG AGT CCA ACG-3′; *Per1* reverse, 5′-CGA AGT TTG AGC TCC CGA AGT-3′. The polymerase chain reaction products were analyzed by agarose gel electrophoresis and visualized by Gel Red staining (Biotum Inc., Fremont, CA, USA). Semiquantitative mRNA levels were determined by analyzing the intensity of each band using the program ImageJ (version 1.34s). Values were expressed in arbitrary units as the ratio of mRNA to the corresponding *β-actin* mRNA level.

Cytokine Measurements

Homogenates of ankle joints were obtained as previously described by Eliçabe et al. [34]. Mouse IL-17, TNF, IL-6, and IL-10 were quantified in clarified joint homogenates by using capture enzyme-linked immunosorbent assay kits (eBioscience, San Diego, CA, USA) following the manufacturer's instructions. Data were expressed as pg/μg of total protein in the extracts. The sensitivity of the assays was 4 pg/mL for IL-17, 8 pg/mL for TNF, 4 pg/mL for IL-6, and 30 pg/mL for IL-10.

Pg Assessment

Serum and tissue Pg was measured by radioimmunoassay using an antiserum kindly provided by Dr. Ricardo P. Deis (Laboratory of Reproduction and Lactation, Mendoza, Argentina) as previously described in Vallcaneras et al. [35]. The sensitivity was <5 ng/mL and the inter- and intra-assay coefficients of variation were <10%. This assay has been validated by Bussmann and Deis [36].

Statistical Analysis

Time point data were expressed as mean ± standard error of the samples. Pertinent curves were drawn. Time series from each group were analyzed by one-way ANOVA followed by Tukey post hoc test for specific comparisons. A p value ≤0.05 was considered significant. In addition, chronobiological statistics were used to validate temporal changes as rhythms. Each series was analyzed using the Chronos-fit 1.06 software [37]. A p value ≤0.05 was taken as indicative of the presence of a rhythm with the 24-h (anticipated) period. A Cosinor analysis was also performed using the Cosinor 2.4 software (S.E.P.T.M.R., Physiology Software Editor). This analysis generated the rhythm descriptors: mesor (circadian rhythm-adjusted mean), amplitude, and acrophase (time of the peak of a rhythm) [38]. Differences in theses rhythm characteristics between pregnant WT and KO mice were analyzed by Student's t test. Data were expressed as mean ± standard error and p values ≤0.05 were considered to be statistically significant.

Results

Expression of Clock Genes in Joints

Since pregnancy status might reduce mouse locomotor activity, we selected the middle point of the rest period (CT7) to analyze the molecular clock in the joints of non-pregnant and pregnant groups under constant darkness. We evaluated the mRNA expression levels of *Bmal1* and *Per1* in the mice's joints. We found a significantly higher mRNA expression (almost a three-fold increase) of *Bmal1* (Fig. 1a, b) and *Per1* (Fig. 1a, c) in the joints of pregnant KO mice compared with their counterpart WT mice. Furthermore, *Per1* mRNA reached a significantly greater expression as caused by pregnancy under TNFRp55 deficiency (Fig. 1c).

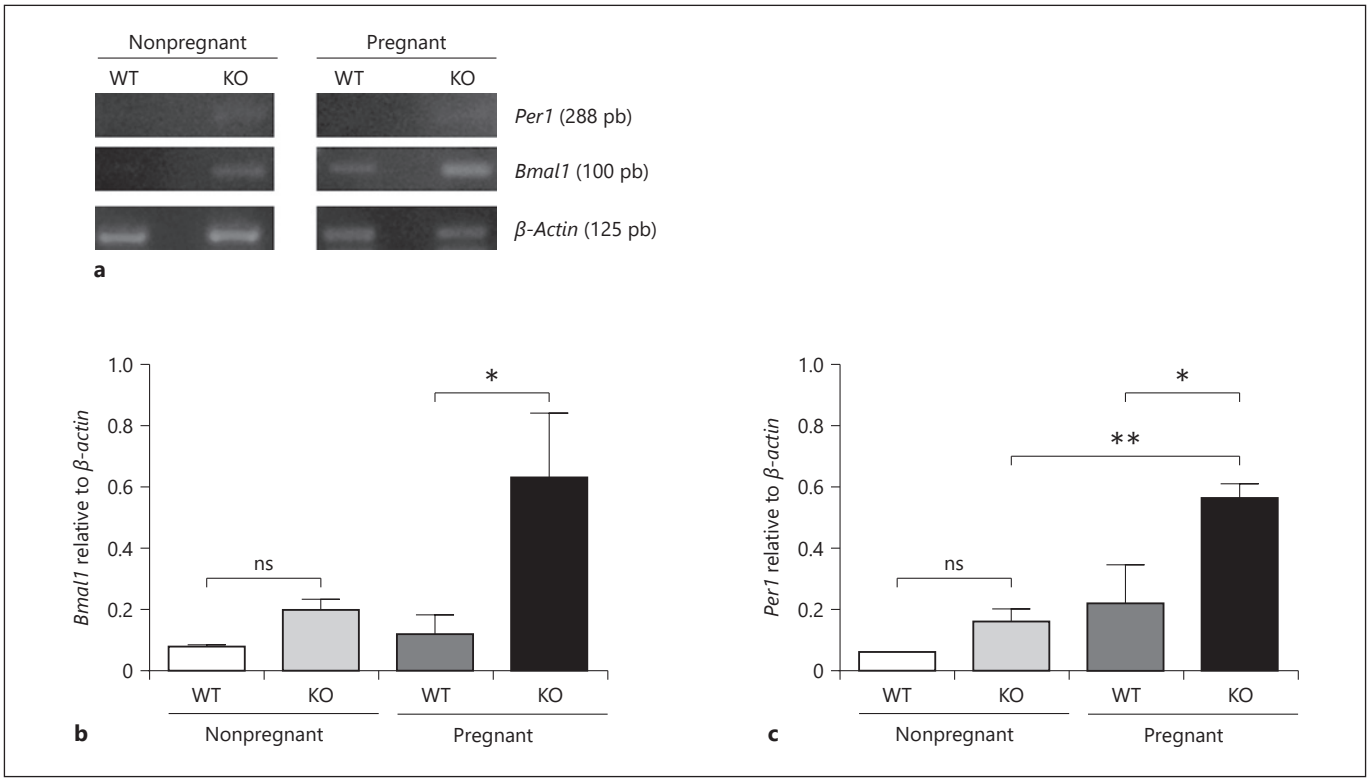

Fig. 1. Expression of clock genes in the ankle joints of the mice. **a** Transcript levels of *Bmal1* and *Per1* genes in joints of nonpregnant and pregnant (gestational day 18) WT and KO mice, analyzed by RT-PCR at CT7 (middle of the rest time) in constant darkness. Representative PCR products are shown. **b**, **c** Semiquantitative measurements of mRNA levels of *Bmal1* (**b**) and *Per1* (**c**) were analyzed using the program Image J, normalized to nonoscillating *β-actin* expression levels. Results are presented as mean ± standard error ($n = 4$). Representative values from two independent experiments are shown. * $p < 0.05$, ** $p < 0.01$. CT, circadian time; KO, TNFRp55-deficient; ns, not significant; PCR, polymerase chain reaction; RT-PCR, reverse transcription polymerase chain reaction; WT, wild-type.

Pg Rhythms

Taking into account that Pg is the main hormone regulating pregnancy, we continued analyzing its circadian variation in serum and joints of mice in late pregnancy. No Pg rhythmicity was found in the serum of WT animals (Fig. 2a). However, we observed that the Pg levels varied significantly throughout a 24-h period ($p < 0.01$) in articular tissues. Our analysis revealed that such variation is rhythmic, circadian, and endogenous-driven in these tissues (Cronos-fit $p < 0.01$, %rhythm 63.42) with an acrophase at CT 19.23 ± 0.86 (Fig. 2b; Table 1). The Pg circadian oscillating pattern was significantly modified in the joints of KO mice, showing phase advance in the acrophase when compared to WT mice (CT 19.23 ± 0.86 in WT mice vs. CT 10.68 ± 0.85 in KO mice, $p < 0.05$) (Fig. 2b; Table 1).

Cytokine Rhythms

The circadian patterns of articular pro- and anti-inflammatory cytokines were analyzed on gestational day 18. The resultant circadian characteristics (mesor, amplitude, and acrophase) for the cytokines are presented in Table 1. Interestingly, synovitis-associated cytokines (IL-17 and IL-6) as well as an anti-inflammatory cytokine involved in immunoregulation (IL-10) varied significantly throughout the day (by ANOVA $p < 0.05$, $p < 0.05$, and $p < 0.01$, respectively) and displayed similar patterns of endogenously driven circadian rhythms in the joints of pregnant WT mice (Fig. 3a–c). Thus, our chronobiological analysis revealed that the circadian rhythms of IL-17 (by Cosinor analysis Chronos-fit $p < 0.01$, %rhythm 59.25), IL-6 (Chronos-fit $p < 0.05$, %rhythm 46.54), and IL-10 (Chronos-fit $p < 0.01$, %rhythm 66.45) were in phase, peaking at CT 17 ± 1.06, 20.85 ± 0.45, and 19.5 ± 0.72, respectively. In contrast, the circadian variation of

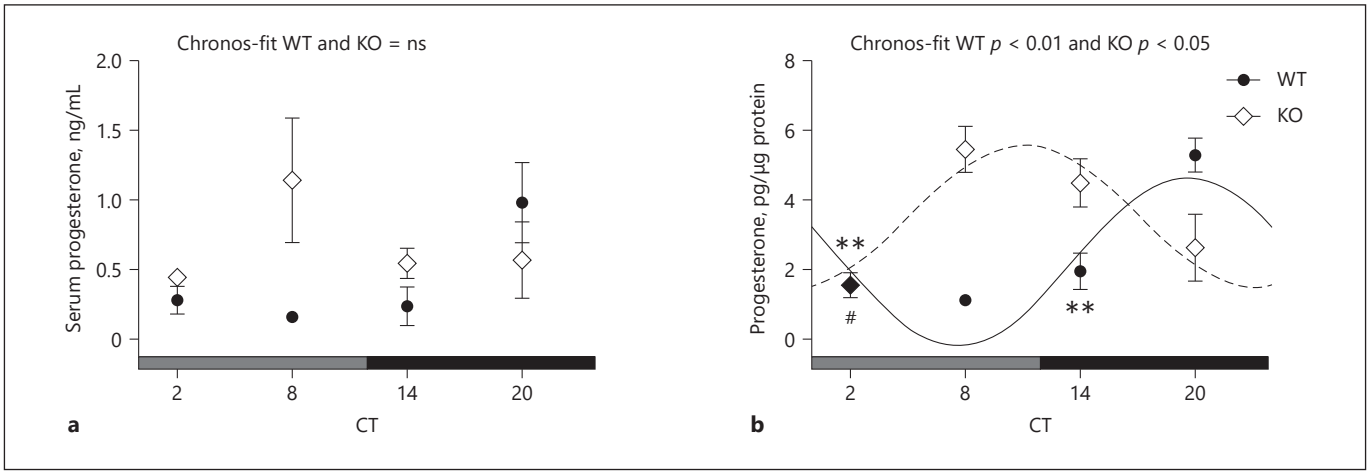

Fig. 2. Circadian rhythms of Pg in the serum and ankle joints of pregnant mice. Results are presented as mean ± standard error of serum (**a**) or joint (**b**) samples (n = 5) at a given CT. Horizontal bars just over the x axis represent the distribution of subjective day (gray) and night (black) phases of the light cycle over a 24-h period that the animals synchronized before the transition to constant darkness. The circadian rhythm was evaluated by statistical analysis of the Pg levels at each CT using one-way ANOVA followed by Tukey multiple comparison test. Asterisks (for WT mice) or hash symbols (for KO mice) indicate the CT that showed the greatest difference compared to the CT with the highest Pg level in each animal group ($^{\#}p < 0.05$, $^{**}p < 0.01$). In addition, the circadian rhythm was also examined by Chronos-fit analysis, displayed on top of each graphic ($p \leq 0.05$ indicated circadian rhythm). CT, circadian time; KO, TNFRp55-deficient; ns, not significant; Pg, progesterone; WT, wild-type.

the pivotal cytokine TNF showed a marked phase shift when compared to the other cytokines, reaching its highest level at CT 0.26 ± 1.28 (Fig. 3d).

To investigate whether TNF influences circadian rhythms of local cytokines via its TNFRp55 receptor, we further analyzed the articular circadian rhythms of IL-17, IL-6, and IL-10 as well as TNF in late pregnancy of KO mice. The lack of the TNF receptor impacted significantly on the temporal profiles of the cytokine in the joints. Indeed, it produced significant phase delays in the circadian rhythms of TNF, IL-6, and IL-10, with their acrophases changing from CT 0.26 ± 1.28 to CT 5.92 ± 1.05 ($p < 0.05$) for TNF, from CT 20.85 ± 0.45 to CT 23.96 ± 1.23 ($p < 0.05$) for IL-6, and from CT 19.5 ± 0.72 to CT 0.02 ± 1.16 ($p < 0.05$) for IL-10 when the highest level of each cytokine in WT vs. KO mice was compared (Fig. 3). The amplitude of TNF and mesor of IL-6 showed a trend for variation in KO mice compared with WT mice (Table 1). However, the TNFRp55 deficiency decreased the mesor of the TNF circadian rhythm (0.43 ± 0.07 in WT mice vs. 0.10 ± 0.01 in KO mice, $p < 0.01$) (Fig. 3d), IL-10 mesor and amplitude (mesor: 5.61 ± 0.60 in WT mice vs. 1.9 ± 0.13 in KO mice, $p < 0.01$; amplitude: 6 ± 0.34 in WT mice vs. 1.08 ± 0.32 in KO mice, $p < 0.001$) (Fig. 3c), and abolished the circadian oscillation of IL-17 in the joints of late-pregnant mice (Fig. 3a; Table 1).

Table 1. Circadian rhythm descriptors: mesor (circadian rhythm-adjusted mean), amplitude, and acrophase (time of the peak of a rhythm) derived from a Cosinor analysis by Cosinor 2.4 software of Pg and cytokine levels in ankle joints of pregnant WT and KO mice

Cytokine/ hormone		WT	KO	p value
IL-17	mesor	1.85±0.08	NA	–
	amplitude	0.64±0.11	NA	–
	acrophase	17±1.06	NA	–
IL-6	mesor	0.43±0.03	0.25±0.02	0.057
	amplitude	0.17±0.07	0.10±0.03	ns
	acrophase	20.85±0.45	23.96±1.23	<0.05
IL-10	mesor	5.61±0.60	1.9±0.13	<0.01
	amplitude	6±0.34	1.08±0.32	<0.001
	acrophase	19.5±0.72	0.02±1.16	<0.05
TNF	mesor	0.43±0.07	0.10±0.01	<0.01
	amplitude	0.38±0.09	0.16±0.02	0.065
	acrophase	0.26±1.28	5.92±1.05	<0.05
Pg	mesor	2.33±0.17	3.63±1.46	ns
	amplitude	2.12±0.28	1.89±0.39	ns
	acrophase	19.23±0.86	10.68±0.85	<0.05

IL, interleukin; KO, TNFRp55-deficient; NA, not applicable; ns, not significant; Pg, progesterone; TNF, tumor necrosis factor; WT, wild-type.

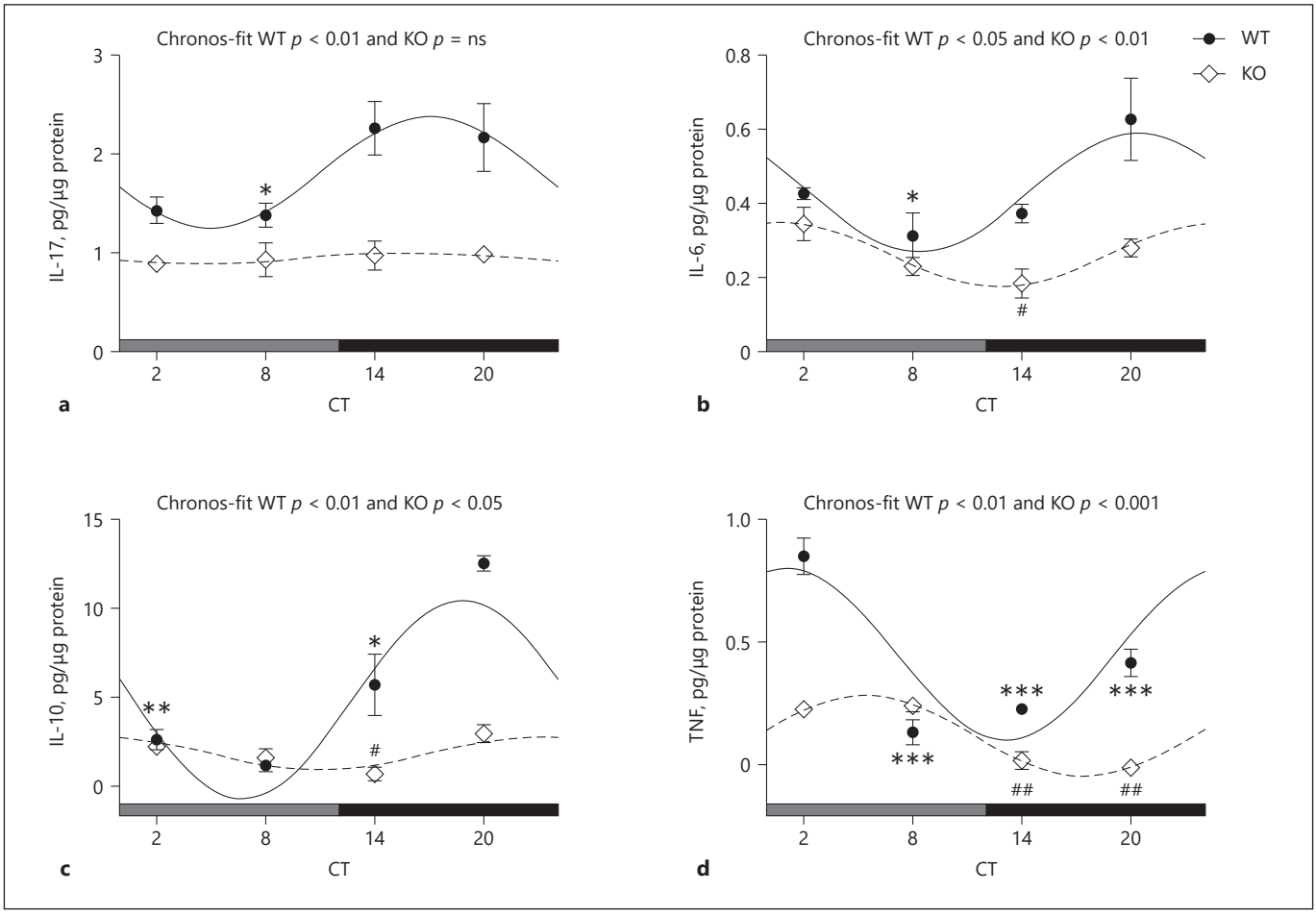

Fig. 3. Circadian rhythms of the cytokines IL-17 (**a**), IL-6 (**b**), IL-10 (**c**), and TNF (**d**) in the ankle joints of pregnant mice. Results are presented as mean ± standard error of joint samples ($n = 5$) at a given CT. Horizontal bars just over the x axis represent the distribution of subjective day (gray) and night (black) phases of the light cycle over a 24-h period that the animals synchronized before the transition to constant darkness. The circadian rhythm was evaluated by statistical analysis of the cytokine levels at each CT using one-way ANOVA followed by Tukey multiple comparison test. Asterisks (for WT mice) or hash symbols (for KO mice) indicate the CT that showed the greatest difference compared with the CT with the highest cytokine levels in each animal group ($^{*,\#}p < 0.05$, $^{**,\#\#}p < 0.01$, $^{***}p < 0.001$). In addition, the circadian rhythm was also examined by Chronos-fit analysis, displayed on top of each graphic ($p \leq 0.05$ indicated circadian rhythm). CT, circadian time; IL, interleukin; KO, TNFRp55-deficient; ns, not significant; TNF, tumor necrosis factor; WT, wild-type.

Discussion

To our knowledge, this is the first study showing that Pg and cytokines display circadian endogenously driven rhythms in the joints of late-pregnant mice. In addition, we found that TNFRp55 plays a role in the modulation of articular Pg and cytokine circadian rhythms.

Robust circadian rhythms in clock genes have been detected in peripheral tissues [39, 40]. Mice are a nocturnal species, and therefore their locomotor activity occurs when lights are turned off in a mouse facility. In the present work, we evaluated the mRNA expression of the clock genes *Bmal1* and *Per1* at CT7 on gestational day 18. Our results show that the diurnal expression of *Bmal1* and *Per1* is higher in the joints of pregnant KO mice. This observation would indicate a role for TNF, via its p55 receptor, in the modulation of clock gene transcription. An effect of TNF on clock gene expression in murine and human cells has been previously observed. Our results are, somehow, consistent with those reported by Cavadini et al. [41], who showed that TNF inhibited the expression of *Per1*/*Per2*/*Per3*; however, this cytokine did not affect

Bmal1 transcript levels in NIH3T3 mouse fibroblast cells. The effect of TNF on *Per1* gene expression was dependent on p38, mitogen-activated protein kinase, and/or calcium signaling [22]. On the contrary, TNF enhanced the expression of BMAL1 and did not affect, or slightly enhanced, the expression of PER1 in human monocytic THP-1 cells [16, 42–44]. In addition, previous studies have also reported an association between TNFRp55 and clock genes. The in vivo administration of TNF resulted in a significant delay in locomotor activity circadian rhythms, whereas no phase shifts were detected in KO mice [23]. All of the above observations would indicate differential cell-specific effects of the TNF signal. Particularly, our results suggest that TNF may negatively modulate, through TNFRp55 signaling, the expression of clock *Bmal1* and *Per1* genes in the joints of pregnant-mice.

Having observed that TNF regulates the expression of clock genes in joints during pregnancy and taking into account that the Pg hormone contributes significantly to immune regulation during pregnancy [28], we continued analyzing the temporal variation of Pg levels in the serum and joints of our animals. Circadian rhythms of serum Pg have been reported during pregnancy in humans [45]. However, our chronobiological analysis revealed that circulating Pg does not vary rhythmically on a circadian basis in pregnant mice on gestational day 18. Here, we show that Pg levels display a circadian rhythm in the joints of late-pregnant mice. Pg oscillation was in phase with IL-17, IL-6, and IL-10 rhythms, but in antiphase with the TNF circadian fluctuation (Fig. 2, 3). This means that Pg acrophase concurs with IL-17, IL-6, and IL-10 peaks in the same temporal window. These results are consistent with previous reports showing that Pg alters the systemic profile of cytokine secretion, resulting in increased production of anti-inflammatory cytokines such as IL-10 and in a reduced production of proinflammatory cytokines such as TNF [28]. Thus, our results indicate a positive association between Pg and IL circadian rhythms.

The earlier peak of articular TNF may suggest a hierarchical and pivotal role of this cytokine. To address this possibility, we investigated whether TNF could modulate cytokine and hormone circadian variations in the joint by using a KO mouse model. Previous studies reported that these mice are fertile and exhibit normal organ sizes of lymphatic tissues and lymphocyte populations similar to the ones in WT mice [46]. Furthermore, we observed that birth and the newborn KO mice were normal (macroscopic observation). Unexpectedly, we observed the TNFRp55 deficiency advances the Pg acrophase. This result could point out to TNFRp55 as a mediator of the circadian organization of Pg during late pregnancy in the mouse joint.

It is known that levels of proinflammatory cytokines, such as IL-1β, IL-6, IL-8, and TNF, are elevated in amniotic fluid, uterine tissues, fetal membranes, and maternal serum during pregnancy when parturition is imminent [47–49]. Even though variations in cytokine levels have been observed at different stages of pregnancy, an increased production of proinflammatory cytokines such as TNF and IL-17 has been shown to be a hallmark of late pregnancy [28, 50]. We observed that IL-17, IL-6, and IL-10 circadian rhythms are in phase in the mouse joints at the end of pregnancy, with peaks occurring in the second half of the subjective night, suggesting a consistent and synchronized regulation of both pro- and anti-inflammatory cytokines within the joints. These observations are in agreement with previous reports on day-night variations of inflammatory mediators in immune cells [17, 51]. Indeed, it has been reported that around the time when mice transit into activity, the immune system anticipates the higher risk of infection, inducing proinflammatory cytokines [52]. Whether a proinflammatory cytokine triggers the increase in other ones is unknown. Our findings show that TNF levels display a different pattern of circadian rhythm, with acrophase occurring at the beginning of the subjective day and preceding the Pg and IL-6, IL-10, and IL-17 peaks. Our data support the participation of TNFRp55 in maintaining a temporal organization of immune and endocrine factors in the joints of pregnant mice. In accordance, there is evidence that TNF regulates clock gene expression through its receptor p55, indicating a pivotal role of this cytokine in the bidirectional circadian-immune connection [41].

Desynchronization was observed in the circadian rhythms of articular IL-17, IL-6, IL-10, and TNF in KO mice. The role of IL-17 in the pathogenesis of synovitis in RA has been well documented [53]. Additionally, we have previously demonstrated that IL-17 mediates articular inflammation in reactive arthritis [34]. In the present study, we found that the articular IL-17 circadian rhythm was abolished in the KO mice, suggesting a role for TNF in the circadian regulation of IL-17.

On the other hand, IL-10 is an important regulatory cytokine for pregnancy maintenance and immune tolerance [7]. This cytokine rises in serum immediately after midpregnancy to counterbalance the parallel increase in TNF, IFN-γ, and IL-6 [28]. In the present work we show that the IL-10 peak concurs with IL-6 acrophase and precedes TNF increase in the joint of late-pregnant mice. In turn, since TNFRp55 deficiency reduced the IL-10 circa-

dian amplitude significantly and advanced the IL-10 acrophase, we demonstrate that TNF is necessary for shaping temporal patterns of IL-10 circadian rhythmicity in the joints of pregnant mice. Thaxton and Sharma [54] reported that IL-10 is a multifaceted agent of pregnancy. In this regard, we recently found that galectin-1, an immunoregulatory lectin which promotes immune tolerance during pregnancy by inducing IL-10 production, can also regulate murine circadian rhythmicity [55, 56]. It remains to be elucidated whether TNF signaling triggers galectin-1 expression to regulate the rhythm of pregnancy-associated cytokines.

Furthermore, we also observed that the lack of TNFRp55 receptor modified the IL-6 circadian oscillation at the end of pregnancy, reducing the mesor and delaying the acrophase. This would be relevant given that IL-6 has been established as an essential determinant of on-time parturition in mice [57]. In addition, IL-6 is an IL-17-inducing cytokine secreted by synovial fibroblasts of RA patients [53]. Therefore, our results suggest the participation of TNFRp55 in the articular circadian rhythm of a cytokine, with immune-endocrine functions as a potent inductor of IL-17 and a trigger of labor.

In conclusion, the sequence of Pg and cytokine peaks occurring during the night (activity period) in the joints of mice would depend on an intact TNFRp55 signaling which modulates the expression of clock genes. Therefore, the reported bidirectional interaction between the immune and the circadian system [58] is confirmed by the present study, contributing to the understanding of the interplay between immune, endocrine, and chronobiological parameters in the joint under a physiological inflammatory condition. Since women have a high risk of developing inflammatory disorders after pregnancy and studies on therapies that target TNF for pregnant women are still quite limited [59], our study provides data as well as a temporal basis to be considered in the use of these therapies.

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Statement of Ethics

All animal procedures were performed according to the National Institute of Health (USA) standards for the use of laboratory animals. Experimental protocols were approved by the Animal Care and Use Committee of the National University of San Luis (protocol number B 226/16). All efforts were made to minimize the number of mice and their discomfort.

Disclosure Statement

The authors declare that they have not conflicts of interest.

References

1 Cutolo M. Chronobiology and the treatment of rheumatoid arthritis. Curr Opin Rheumatol. 2012 May;24(3):312–8.
2 Cutolo M, Straub RH, Bijlsma JW. Neuroendocrine-immune interactions in synovitis. Nat Clin Pract Rheumatol. 2007 Nov;3(11):627–34.
3 Soliman KF, Soliman MR, Owasoyo JO, Walker CA. Diurnal variation in the phlogogenic response of rats to inflammatory agents. J Pharm Pharmacol. 1983 Jun;35(6):388–9.
4 Gibbs JE, Ray DW. The role of the circadian clock in rheumatoid arthritis. Arthritis Res Ther. 2013 Feb;15(1):205.
5 Dibner C, Schibler U, Albrecht U. The mammalian circadian timing system: organization and coordination of central and peripheral clocks. Annu Rev Physiol. 2010;72(1):517–49.
6 Balsalobre A, Damiola F, Schibler U. A serum shock induces circadian gene expression in mammalian tissue culture cells. Cell. 1998 Jun;93(6):929–37.
7 Stratmann M, Schibler U. Properties, entrainment, and physiological functions of mammalian peripheral oscillators. J Biol Rhythms. 2006 Dec;21(6):494–506.
8 Koike N, Yoo SH, Huang HC, Kumar V, Lee C, Kim TK, et al. Transcriptional architecture and chromatin landscape of the core circadian clock in mammals. Science. 2012 Oct;338(6105):349–54.
9 Sellix MT, Menaker M. Circadian clocks in mammalian reproductive physiology: effects of the "other" biological clock on fertility. Discov Med. 2011 Apr;11(59):273–81.
10 Takahashi JS. Finding new clock components: past and future. J Biol Rhythms. 2004 Oct;19(5):339–47.
11 Guillaumond F, Dardente H, Giguère V, Cermakian N. Differential control of Bmal1 circadian transcription by REV-ERB and ROR nuclear receptors. J Biol Rhythms. 2005 Oct;20(5):391–403.

12 Keller M, Mazuch J, Abraham U, Eom GD, Herzog ED, Volk HD, et al. A circadian clock in macrophages controls inflammatory immune responses. Proc Natl Acad Sci USA. 2009 Dec;106(50):21407–12.
13 Arjona A, Sarkar DK. The circadian gene mPer2 regulates the daily rhythm of IFN-gamma. J Interferon Cytokine Res. 2006 Sep; 26(9):645–9.
14 Tsoumtsa LL, Torre C, Ghigo E: Circadian control of antibacterial immunity: findings from animal models. Front Cell Infect Microbiol. 2016 May;6:54.
15 Haas S, Straub RH. Disruption of rhythms of molecular clocks in primary synovial fibroblasts of patients with osteoarthritis and rheumatoid arthritis, role of IL-1β/TNF. Arthritis Res Ther. 2012 May;14(3):R122.
16 Yoshida K, Hashiramoto A, Okano T, Yamane T, Shibanuma N, Shiozawa S. TNF-α modulates expression of the circadian clock gene Per2 in rheumatoid synovial cells. Scand J Rheumatol. 2013;42(4):276–80.
17 Logan RW, Arjona A, Sarkar DK. Role of sympathetic nervous system in the entrainment of circadian natural-killer cell function. Brain Behav Immun. 2011 Jan;25(1):101–9.
18 Paladino N, Mul Fedele ML, Duhart JM, Marpegan L, Golombek DA. Modulation of mammalian circadian rhythms by tumor necrosis factor-α. Chronobiol Int. 2014 Jun;31(5): 668–79.
19 Hashiramoto A, Yamane T, Tsumiyama K, Yoshida K, Komai K, Yamada H, et al. Mammalian clock gene Cryptochrome regulates arthritis via proinflammatory cytokine TNF-alpha. J Immunol. 2010 Feb;184(3):1560–5.
20 Arjona A, Sarkar DK. Evidence supporting a circadian control of natural killer cell function. Brain Behav Immun. 2006 Sep;20(5): 469–76.
21 Perry MG, Kirwan JR, Jessop DS, Hunt LP. Overnight variations in cortisol, interleukin 6, tumour necrosis factor alpha and other cytokines in people with rheumatoid arthritis. Ann Rheum Dis. 2009 Jan;68(1):63–8.
22 Petrzilka S, Taraborrelli C, Cavadini G, Fontana A, Birchler T. Clock gene modulation by TNF-alpha depends on calcium and p38 MAP kinase signaling. J Biol Rhythms. 2009 Aug; 24(4):283–94.
23 Duhart JM, Leone MJ, Paladino N, Evans JA, Castanon-Cervantes O, Davidson AJ, et al. Suprachiasmatic astrocytes modulate the circadian clock in response to TNF-α. J Immunol. 2013 Nov;191(9):4656–64.
24 De Castro J, Sevillano J, Marciniak J, Rodriguez R, González-Martín C, Viana M, et al. Implication of low level inflammation in the insulin resistance of adipose tissue at late pregnancy. Endocrinology. 2011 Nov;152 (11):4094–105.
25 De Man YA, Dolhain RJ, van de Geijn FE, Willemsen SP, Hazes JM. Disease activity of rheumatoid arthritis during pregnancy: results from a nationwide prospective study. Arthritis Rheum. 2008 Sep;59(9):1241–8.
26 Barbhaiya M, Bermas BL. Evaluation and management of systemic lupus erythematosus and rheumatoid arthritis during pregnancy. Clin Immunol. 2013 Nov;149(2):225–35.
27 Guthrie KA, Dugowson CE, Voigt LF, Koepsell TD, Nelson JL. Does pregnancy provide vaccine-like protection against rheumatoid arthritis? Arthritis Rheum. 2010 Jul;62(7): 1842–8.
28 Orsi NM, Gopichandran N, Ekbote UV, Walker JJ. Murine serum cytokines throughout the estrous cycle, pregnancy and post partum period. Anim Reprod Sci. 2006 Nov; 96(1–2):54–65.
29 Druckmann R, Druckmann MA. Progesterone and the immunology of pregnancy. J Steroid Biochem Mol Biol. 2005 Dec;97(5):389–96.
30 Rovensky J, Kvetnansky R, Radikova Z, Imrich R, Greguska O, Vigas M, et al. Hormone concentrations in synovial fluid of patients with rheumatoid arthritis. Clin Exp Rheumatol. 2005 May–Jun;23(3):292–6.
31 Eriksson E, Royo F, Lyberg K, Carlsson HE, Hau J. Effect of metabolic cage housing on immunoglobulin A and corticosterone excretion in faeces and urine of young male rats. Exp Physiol. 2004 Jul;89(4):427–33.
32 Gibbs J, Ince L, Matthews L, Mei J, Bell T, Yang N, et al. An epithelial circadian clock controls pulmonary inflammation and glucocorticoid action. Nat Med. 2014 Aug;20(8): 919–26.
33 Di Genaro MS, Cargnelutti DE, Castro DO, Eliçabe RJ, Gutiérrez JV, Correa SG, et al. Yersinia-triggered arthritis in IL-12p40-deficient mice: relevant antigens and local expression of Toll-like receptor mRNA. Scand J Rheumatol. 2007 Jan–Feb;36(1):28–35.
34 Eliçabe RJ, Cargnelutti E, Serer MI, Stege PW, Valdez SR, Toscano MA, et al. Lack of TNFR p55 results in heightened expression of IFN-γ and IL-17 during the development of reactive arthritis. J Immunol. 2010 Oct;185(7):4485–95.
35 Vallcaneras SS, Delgado SM, Motta A, Telleria CM, Rastrilla AM, Casais M. Effect of prolactin acting on the coeliac ganglion via the superior ovarian nerve on ovarian function in the postpartum lactating and non-lactating rat. Gen Comp Endocrinol. 2013 Apr;184:1–8.
36 Bussmann LE, Deis RP. Studies concerning the hormonal induction of lactogenesis by prostaglandin F2 alpha in pregnant rats. J Steroid Biochem. 1979 Oct;11(4):1485–9.
37 Paul T, Lemmer B. Disturbance of circadian rhythms in analgosedated intensive care unit patients with and without craniocerebral injury. Chronobiol Int. 2007;24(1):45–61.
38 Wharfe MD, Mark PJ, Wyrwoll CS, Smith JT, Yap C, Clarke MW, et al. Pregnancy-induced adaptations of the central circadian clock and maternal glucocorticoids. J Endocrinol. 2016 Mar;228(3):135–47.
39 Dudek M, Meng QJ. Running on time: the role of circadian clocks in the musculoskeletal system. Biochem J. 2014 Oct;463(1):1–8.
40 Schiaffino S, Blaauw B, Dyar KA. The functional significance of the skeletal muscle clock: lessons from Bmal1 knockout models. Skelet Muscle. 2016 Oct;6(1):33.
41 Cavadini G, Petrzilka S, Kohler P, Jud C, Tobler I, Birchler T, et al. TNF-alpha suppresses the expression of clock genes by interfering with E-box-mediated transcription. Proc Natl Acad Sci USA. 2007 Jul;104(31):12843–8.
42 Kouri VP, Olkkonen J, Kaivosoja E, Ainola M, Juhila J, Hovatta I, Konttinen YT, Mandelin J: Circadian time keeping is disturbed in rheumatoid arthritis at molecular level. PLoS One. 2013; 8: ID e54049.
43 Perez-Aso M, Feig JL, Mediero A, Cronstein BN. Adenosine A2A receptor and TNF-α regulate the circadian machinery of the human monocytic THP-1 cells. Inflammation. 2013 Feb;36(1):152–62.
44 Yoshida K, Nakai A, Kaneshiro K, Hashimoto N, Suzuki K, Uchida K, et al. TNF-α induces expression of the circadian clock gene Bmal1 via dual calcium-dependent pathways in rheumatoid synovial cells. Biochem Biophys Res Commun. 2018 Jan;495(2):1675–80.
45 Junkermann H, Mangold H, Vecsei P, Runnebaum B. Circadian rhythm of serum progesterone levels in human pregnancy and its relation to the rhythm of cortisol. Acta Endocrinol (Copenh). 1982 Sep;101(1):98–104.
46 Pfeffer K, Matsuyama T, Kündig TM, Wakeham A, Kishihara K, Shahinian A, et al. Mice deficient for the 55 kd tumor necrosis factor receptor are resistant to endotoxic shock, yet succumb to L. monocytogenes infection. Cell. 1993 May;73(3):457–67.
47 Christiaens I, Zaragoza DB, Guilbert L, Robertson SA, Mitchell BF, Olson DM. Inflammatory processes in preterm and term parturition. J Reprod Immunol. 2008 Oct;79(1): 50–7.
48 Osman I, Young A, Ledingham MA, Thomson AJ, Jordan F, Greer IA, et al. Leukocyte density and pro-inflammatory cytokine expression in human fetal membranes, decidua, cervix and myometrium before and during labour at term. Mol Hum Reprod. 2003 Jan; 9(1):41–5.
49 Romero R, Avila C, Santhanam U, Sehgal PB. Amniotic fluid interleukin 6 in preterm labor. Association with infection. J Clin Invest. 1990 May;85(5):1392–400.

50 Ostojic S, Dubanchet S, Chaouat G, Abdelkarim M, Truyens C, Capron F. Demonstration of the presence of IL-16, IL-17 and IL-18 at the murine fetomaternal interface during murine pregnancy. Am J Reprod Immunol. 2003 Feb;49(2):101–12.
51 Gibbs JE, Blaikley J, Beesley S, Matthews L, Simpson KD, Boyce SH, et al. The nuclear receptor REV-ERBα mediates circadian regulation of innate immunity through selective regulation of inflammatory cytokines. Proc Natl Acad Sci USA. 2012 Jan;109(2):582–7.
52 Curtis AM, Bellet MM, Sassone-Corsi P, O'Neill LA. Circadian clock proteins and immunity. Immunity. 2014 Feb;40(2):178–86.
53 Lubberts E. Th17 cytokines and arthritis. Semin Immunopathol. 2010 Mar;32(1):43–53.
54 Thaxton JE, Sharma S. Interleukin-10: a multi-faceted agent of pregnancy. Am J Reprod Immunol. 2010 Jun;63(6):482–91.
55 Casiraghi LP, Croci DO, Poirier F, Rabinovich GA, Golombek DA. "Time sweet time": circadian characterization of galectin-1 null mice. J Circadian Rhythms. 2010 Apr;8(0):4.
56 Ramhorst RE, Giribaldi L, Fraccaroli L, Toscano MA, Stupirski JC, Romero MD, et al. Galectin-1 confers immune privilege to human trophoblast: implications in recurrent fetal loss. Glycobiology. 2012 Oct;22(10):1374–86.
57 Robertson SA, Christiaens I, Dorian CL, Zaragoza DB, Care AS, Banks AM, et al. Interleukin-6 is an essential determinant of on-time parturition in the mouse. Endocrinology. 2010 Aug;151(8):3996–4006.
58 Guissoni Campos LM, Buchaim RL, da Silva NC, Spilla CS, Hataka A, Pinato L. Suprachiasmatic nucleus and subordinate brain oscillators: clock gene desynchronization by neuroinflammation. Neuroimmunomodulation. 2017;24(4–5):231–41.
59 Hazes JM, Coulie PG, Geenen V, Vermeire S, Carbonnel F, Louis E, et al. Rheumatoid arthritis and pregnancy: evolution of disease activity and pathophysiological considerations for drug use. Rheumatology (Oxford). 2011 Nov;50(11):1955–68.