

Circadian rhythms of cellular immunity in rheumatoid arthritis: a hypothesis-generating study

C.M. Spies¹, P. Hoff^{1,2}, J. Mazuch³, T. Gaber^{1,2,4}, B. Maier³, C. Strehl^{1,2}, M. Hahne^{1,2,5}, M. Jakstadt^{1,2,4}, D. Huscher^{1,2}, G.-R. Burmester¹, J. Detert¹, A. Kramer³, F. Buttgerit¹

¹Department of Rheumatology and Clinical Immunology, Charité-Universitätsmedizin Berlin; ²German Arthritis Research Centre (DRFZ), Berlin; ³Laboratory of Chronobiology, Institute for Medical Immunology, Charité-Universitätsmedizin Berlin; ⁴Berlin-Brandenburg Centre for Regenerative Therapies, Charité-Universitätsmedizin Berlin; ⁵Berlin-Brandenburg School for Regenerative Therapies, Charité-Universitätsmedizin Berlin, Germany.

Abstract

Objective

The circadian rhythm of clinical symptoms in rheumatoid arthritis (RA) has been primarily attributed to circadian variations in humoral factors and hormones. In this study, we investigated circadian rhythms of cellular immunity in RA (CiRA study).

Methods

Peripheral blood of female postmenopausal patients with active RA (DAS 28 \geq 4.2) (n=5) and female postmenopausal non-RA controls (n=5) was collected every 2 hours for 24 hours and analysed by flow cytometry, cytokine multiplex suspension array and quantitative RT-PCR of clock gene expression in isolated CD14⁺ monocytes. Endogenous circadian rhythms of macrophages were investigated by BMAL1-luciferase bioluminescence. Significance of circadian rhythms was tested by Cosinor analysis.

Results

We found (i) circadian rhythms in the relative frequency of peripheral blood cell populations that were present in postmenopausal non-RA controls but absent in patients with active RA, (ii) circadian rhythms that were absent in non-RA controls but present in patients with RA and (iii) circadian rhythms that were present in both groups but with differences in peak phase or amplitude or amplitude/magnitude. The circadian rhythm in expression of the clock genes PER2 and PER3 in CD14⁺ monocytes was lost in patients with RA. The amplitude of BMAL1-luciferase bioluminescence tended to be lower in patients with RA than in non-RA controls.

Conclusion

We conclude that (i) in RA some immune cell populations lose their normal circadian rhythms whereas others establish new “inflammatory” circadian rhythms and (ii) these findings provide a good basis for further identifying pathophysiological aspects of RA chronobiology with potential therapeutic implications.

Key words

circadian rhythm, rheumatoid arthritis, circadian clock, monocytes, clock genes, postmenopausal

Cornelia M. Spies, MD
 Paula Hoff, MD
 Jeannine Mazuch
 Timo Gaber, Dr. rer. nat.
 Bert Maier, MD
 Cindy Strehl, Dr. rer. nat.
 Martin Hahne, Dr. rer. nat.
 Manuela Jakstadt
 Dörte Huscher, Dipl. Math
 Gerd-Rüdiger Burmester, MD
 Jacqueline Detert, MD
 Achim Kramer, PhD
 Frank Buttgerit, MD

Please address correspondence to:
 Cornelia Spies, MD,
 Department of Rheumatology
 and Clinical Immunology,
 Charité-Universitätsmedizin Berlin,
 Charitéplatz 1,
 D-10117 Berlin, Germany.
 E-mail: cornelia.spies@charite.de
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Introduction

In rheumatoid arthritis (RA), disease-related symptoms follow a circadian rhythm with the highest severity in the night and early morning (1-5). Morning stiffness is strongly associated with pain and functional disability and predictive of shortened working ability (6). The circadian variation of symptoms in RA has so far primarily been explained by an underlying circadian variation in pro-inflammatory cytokine and hormone levels, mainly the overnight rise of interleukin (IL)-6 preceding the morning rise of cortisol (2, 7-9). These observations have led to the successful application of chronotherapy with modified-release prednisone (10). Modified-release prednisone, if taken at 10 PM, is effective at around 2 AM and prevents the nocturnal rise of IL-6 in RA patients. It was demonstrated that chronotherapy with prednisone produces a sustained reduction of morning stiffness and IL-6 levels, in addition to all known therapeutic effects of conventional prednisone (10-12).

In contrast to clinical and humoral findings, much less is known about cellular circadian rhythms in RA. Under physiological conditions in rodents and healthy humans numerous cellular immune functions and parameters have been described to be time-of-day dependent, for example lymphocyte proliferation and natural killer cell activity, as well as rhythms in absolute and relative numbers of circulating white blood cells and T cell subsets (7, 13-18). Immune cells, such as macrophages, have cellular circadian clocks that modulate inflammatory immune responses (19). We hypothesised that cellular circadian rhythms may contribute to the circadian variation in clinical symptoms in RA. Therefore, the aim of this pilot study was to investigate molecular, cellular and humoral circadian parameters of peripheral blood cells in five postmenopausal female RA patients in comparison to five age-matched postmenopausal control subjects.

Materials and methods

Study design and patients

Eligible subjects were females aged 45–75 years. The restriction to postmeno-

pausal women was to minimise the impact of hormonal factors. Participants had to have good vein conditions. Mid-sleep (midpoint between sleep onset and wake up) in Munich Chrono Type Questionnaire (MCTQ) (20) had to be at 4:00±2:00 hours. The control group (n=5) comprised age-matched non-RA postmenopausal female subjects. Patients had to have active RA (21) with a modified 28-joint disease activity score (DAS28) (22) ≥4.2 measured in the morning. Therapy with glucocorticoids (≤10 mg/d prednisolone equivalent) and/or non-steroidal anti-inflammatory drugs had to be at stable doses for ≥4 weeks prior to the study day. Disease-modifying anti-rheumatic drugs were unrestricted. Subjects were excluded if they had intra-articular glucocorticoid therapy ≤4 weeks before screening (*b.s.*), biological drugs ≤a period of 5 half-lives *b.s.*, other autoimmune diseases, chronic infections, acute infections ≤4 weeks *b.s.*, diseases leading to haematologic/serologic blood changes, diseases affecting the circadian or sleep-wake-rhythm (*e.g.* neurologic or psychiatric diseases, uncontrolled hypertension, chronic obstructive pulmonary disease, sleep apnoea, fever), opiate drug intake, alcohol/drug abuse, history of malignancy or anaemia (haemoglobin concentration ≤12.0 g/dl), and if they had flown through ≥3 time zones ≤2 weeks *b.s.* or were night shift workers. The study protocol was approved by the responsible local administrative body and ethics committee. All participants provided written informed consent before enrolment.

Study procedures

At screening (day -28 to -8) eligible participants were physically examined and standard blood and urine tests were performed, including erythrocyte sedimentation rate (ESR) and plasma concentrations of C-reactive protein (CRP). Participants rated pain and stiffness using a visual analogue scale (maximum 100 mm), estimated duration of morning stiffness (minutes) and completed different questionnaires (20, 23-27). DAS28 was calculated based on 3 variables (DAS28-3): 28-tender joint count (TJC28), 28-swollen joint

count (SJC28) and ESR; $DAS28-3 = [0.56 * \sqrt{(TJC28)} + 0.28 * \sqrt{(SJC28)} + 0.70 \ln(ESR)] 1.08 + 0.16 (22)$.

At baseline (day -7) blood was obtained for bioluminescence-analysis of the circadian clock in monocytes. In order to harmonise sleep-wake-rhythms, participants were provided with diaries and instructed to sleep for at least 6 hours between 22:00 hours and 9:00 hours on days -4 to 0, take food at regular intervals and drink sufficient fluid. On the 24-hour study day (day 1), participants arrived before 9:00 am; after a rest, permanent peripheral vein catheters were placed and blood was taken for analysis of the circadian clock in monocytes. From 10:00 am onwards, blood was taken every 2 hours until 10:00 hours the following day. DAS28 was assessed on arrival and at intervals but not during the night (10:00 hours, 14:00 hours, 18:00 hours, 22:00 hours and 10:00 hours). Participants were provided regular meals, permitted to eat snacks ad libitum, carry out passive activities and sleep during the night in the dark.

Flow cytometric analysis

Peripheral blood samples were collected in heparinised tubes. After centrifugation and plasma separation, the pellets were re-suspended and incubated in erythrocyte lysis buffer (0.01 M $KHCO_3$, 0.155 M NH_4Cl , 0.1 mM EDTA, pH 7.5) for 6 min at 4° C. Subsequently, samples were washed with PBS/BSA (137 mM NaCl+2.7 mM KCl+1.5 mM KH_2PO_4 +7.9 mM $Na_2HPO_4 \cdot xH_2O$, pH 7.2+30 mM bovine serum albumin). Obtained leukocytes were filtered through a MACS pre-separation filter (30 μ m, Miltenyi Biotec) and treated with human IgG (Flebogamma®) to block unspecific binding. Subsequently, stainings were performed with the following antibodies: anti-CD3 (UCHT1), anti-CD4 (RPA-T4), anti-CD45 (HI30), anti-CD69 (FN50), anti-CD14 (M5E2), anti-CD16 (3G8), anti-CD19 (HIB19), anti-CD20 (2H7), anti-CD56 (B159), anti-CD25 (M-A251), anti-CD127 (hIL-7R-M21), anti-CD181 (5A12), anti-CXCR4 (12G5), anti-CCR7 (3D12) (all BD Biosciences, Heidelberg, Germany); anti-CD8 (3B5), anti-CD45RA

(MEM-56), anti-CD27 (CLB-27/1) (all Caltag Laboratories, Hamburg, Germany); anti-CD62L (DREG-56) (BioLegend, Fell, Germany); anti-CD126 (B-R6) (Diaclone, Besancon Cedex, France); anti-HLADR (L243), anti-CD45RO (UCHL-1) (both DRFZ, Berlin, Germany) for 10 min at 4° C. The antibodies were conjugated to different dyes: Pacific blue, PE-Cy7, PE, PE-Cy5, APC-Cy7, APC, FITC, APC-Alexa750 and Pacific orange. Acquisition and analysis were performed using a LSR II cytometer (BD Biosciences) and FlowJo software (Tree Star, Ashland, OR, USA).

Quantification of plasma cytokine concentration

Plasma of the peripheral blood samples was immediately frozen and stored at -80° C for batch analysis. The concentrations of IL-1 β , IL-1 receptor antagonist (IL-1ra), IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12 (p70), IL-13, IL-15, IL-17A, interferon-gamma (IFN- γ), interferon-gamma induced protein 10 kD (IP-10), tumour necrosis factor-alpha (TNF- α), monocyte chemotactic protein-1 (MCP-1), macrophage inflammatory protein-1 α (MIP-1 α), MIP-1 β , regulated upon activation normal T-cell expressed and secreted (RANTES), eotaxin, basic fibroblast growth factor (FGF basic), platelet-derived growth factor (PDGF), vascular endothelial growth factor 121/165 (VEGF), granulocyte colony-stimulating factor (G-CSF), granulocyte macrophage colony-stimulating factor (GM-CSF) and macrophage migration inhibitory factor (MIF) were quantified by multiplex suspension array (Bio-Rad) according to the manufacturer's instructions. Data acquisition was conducted using the Bio-Plex suspension system.

Clock gene expression

Five ml of heparinised whole blood was taken every 2 hours from patients and controls and immediately placed on ice. CD14⁺ blood monocytes were collected by MACS sort using whole blood CD14-microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) and the Auto-MACS-Pro device. All steps

were performed at 4° C according to the manufacturer's instructions. CD14⁺ cell fraction was pelleted by centrifugation and frozen at -80° C. After completion of the entire time series, total RNA was isolated using RNeasy Mini Kit (Qiagen, Hilden, Germany) and reverse transcribed (Fermentas, St. Leon-Rot, Germany). RT-PCR was performed on the clock genes *REV-ERBa*, *PER2*, *PER3*, *BMAL1*, *CRY2* and *GAPDH* as housekeeping gene using QuantiTect Primer assay (Qiagen) and SYBR-Green MasterMix (Invitrogen, Darmstadt, Germany) on an ABI7000 System (Applied Biosystems, Darmstadt, Germany). Gene expression of individual samples were normalised to individual *GAPDH* levels and finally to the mean gene expression of control subjects.

Real-time bioluminescence recordings

Ten ml of heparinised whole blood was taken on day -8 and day 1 and mononuclear cells (PBMCs) were sorted by density gradient centrifugation (LSM 1077 Lymphocyte, PAA Laboratories, Coelbe, Germany). CD14⁺ monocytes were isolated via MACS sort using CD14-microbeads (Miltenyi) performed according to the company's protocol. Cells of each subject (1.2 x 10⁶ cells) were placed in Petri dishes and cultured in RPMI 1640 containing M-CSF. Medium was replaced every third day. After 7 days cells were transduced with a *BMAL1:luc*-reporter virus (first described by (28)), synchronised with forskolin (40 μ M, removed after synchronisation and replaced by fresh media) and cultured in DMEM supplemented with 10% FCS and 0.1 mM luciferin (Promega, Mannheim, Germany). Dishes were placed in light-tight boxes with single photomultiplier tubes (Hamamatsu Photonics, Herrsching, Germany). Bioluminescence was recorded in 5 min bins at 37° C.

Statistical analysis

Differences in baseline characteristics between the groups were analysed using the Mann-Whitney or Fisher test (IBM SPSS Statistics version 19, Armonk, NY, USA), for comparison of two different time-points the Wilcoxon test for paired samples was used. For time

series analysis, chronograms were constructed for each single raw data series in order to assess the data for patterns, trends and outliers. To determine circadian variations of the groups, the parameters were analysed using the group (population) mean analysis of the time series analysis software Cosinor 6.3 (Expert Soft Technologie, Richelieu, France). Cosinor analysis uses the least squares method to fit a sine wave to a time series. The fitted Cosinor curves were calculated with a 24 hour rhythm and are represented by the following function: $C(t) = \text{magnitude} + \text{amplitude} * \cos[(2\pi * t / 24h) - \text{rad}(\text{peak phase})]$, where magnitude (MESOR) (*i.e.* the mean of the oscillation), amplitude (*i.e.* half the difference between the highest and the lowest values), and peak phase (acrophase) (*i.e.* the timing of the cosine maximum given in degrees, where 360° corresponded to a 24-hour cycle). Group differences between the magnitudes of the time series were determined using the Brunner-Test, a non-parametric repeated measures analysis of variance (ANOVA) (SAS V. 8.2, SAS Institute, Cary, NC, USA). For analysis of cytokine concentrations, values out of range were substituted with zero. Probability values of $p < 0.05$ were considered to be statistically significant.

Results

Circadian variation of clinical parameters

The characteristics of postmenopausal RA patients and non-RA controls are summarised in Table I. The non-rheumatic medication allowed in the study comprised antihypertensive drugs, lipid-lowering drugs, antiplatelet drugs, osteoporosis medications and antidepressant drugs. In the 24-hour time course of clinical variables in patients with RA, measured at 6 time points excluding the night, stiffness was greatest at 9:00 hours, tender joint count was highest at 10:00 hours, and swollen joint count was also highest at 10:00 hours, whereas pain was most severe at 14:00 hours (data not shown). ESR did not show a clear oscillation. DAS 28-3 was stable over 24 hours. In Cosinor analysis none of these variations was significantly circadian.

Table I. Characteristics of patients with RA and non-RA controls at screening visit.

Characteristic	RA patients (n=5)	Non-RA controls (n=5)
Female	5 (100%)	5 (100%)
Postmenopausal	5 (100%)	5 (100%)
Age (years)	58.2 (6.1)	59.0 (11.1)
Mid sleep working days	2:55 h (0:00 h)*#	3:06 h (0:42 h)
Mid sleep non-working days	3:44 h (0:45 h)	3:50 h (0:58 h)
Concomitant osteoarthritis	2 (40%)	2 (40%)
Duration of RA (years)	3.3 (3.1)**	0 (0)
RF positive	5 (100%)*	0 (0%)
ACPA positive	4 (80%)*	0 (0%)
DMARDs	5 (100%)*	0 (0%)
Glucocorticoid treatment	2 (40%)	0 (0%)
Dose prednisone equivalent (mg)	2.5 (3.5)	0 (0)
NSAIDs	4 (80%)*	0 (0%)
<i>Non-rheumatic medications</i>		
Anti-hypertensive drugs	3 (60%)	1 (20%)
Lipid-lowering drugs	1 (20%)	1 (20%)
Anti-platelet drugs	0 (0%)	1 (20%)
Osteoporosis medications	2 (40%)	0 (0%)
Anti-depressant drugs	1 (20%)	0 (0%)
DAS 28-3	4.8 (0.3)**	1.6 (1.0)
TJC28	9.8 (5.9)*	1.6 (3.6)
SJC28	5.6 (2.1)**	0 (0)
ESR (mm/h)	21.0 (6.8)**	6.0 (2.8)
CRP (mg/dl) (<0.5 mg/ml)	0.14 (0.12)	0.15 (0.11)
VAS pain (mm)	47.2 (17.5)	10.0 (22.4)
VAS stiffness (mm)	41.0 (27.5)	14.0 (31.3)
Duration of morning stiffness (min)	39.0 (25.1)	9.0 (20.1)
HAQ (functional status)	1.0 (0.3)**	0.1 (0.1)
<i>SF-36 (quality of life)</i>		
Physical component summary	38.3	54.7
Mental component summary	40.9	49.4
<i>MFI-20 (fatigue)</i>		
General fatigue	12.0 (3.6)	9.0 (4.3)
Physical fatigue	13.4 (2.3)*	6.8 (3.6)
Reduced activity	11.8 (5.2)	6.8 (4.1)
Reduced motivation	11.0 (3.7)	6.6 (4.2)
Mental fatigue	10.8 (4.2)	7.2 (3.8)
PD-Q Score (neuropathic pain)	17.6 (5.0)**	2.6 (5.8)

Data are mean (SD) or number (%); p -values as determined by Mann-Whitney U test or Fisher test for frequencies; * $p < 0.05$; ** $p < 0.01$; #only one RA patient was working; ACPA: anti-citrullinated peptide antibodies; CRP: C-reactive protein; DAS28: disease activity score of 28 joints; DMARDs: disease modifying antirheumatic drugs; ESR: erythrocyte sedimentation rate; HAQ: health assessment questionnaire; MFI: multidimensional fatigue inventory; NSAIDs: non-steroidal anti-inflammatory drugs; PD-Q, painDETECT questionnaire; RA, rheumatoid arthritis; RF, rheumatoid factor; SF-36, short-form 36; SJC28: swollen joint count of 28 joints; TJC28: tender joint count of 28 joints; VAS: visual analogue scale.

Circadian rhythms of peripheral blood cell populations and cytokines

In the 24-hour analysis of 2-hourly collected blood samples we found significant circadian rhythms in the relative frequency of peripheral blood cell populations that were present in postmenopausal non-RA controls but absent in patients with RA (Fig. 1, Table II). This was the case for: effector CD4⁺ T helper cells, effector CD8⁺ cytotoxic T cells, naïve CD8⁺ T cells, CD45RA⁺ CD45RO⁻ CD4⁺ T cells, natural killer

T cells, IL-2 receptor expressing CD4⁺ T cells, IL-6 receptor expressing CD4⁺ and CD8⁺ T cells, “homing receptor” CD62L expressing CD8⁺ T cells, and the plasma concentration of the chemokine MCP-1.

We also found circadian rhythms that were absent in non-RA controls but present in patients with RA (Fig. 2, Table II). This was the case for: IL-8 receptor expressing CD14⁺ monocytes, CD20⁺ CD27⁺ memory B cells, CD20⁺ HLA-DR⁺ activated B cells, “homing recep-

Circadian rhythms that are...

... present in non-RA controls but absent in RA patients

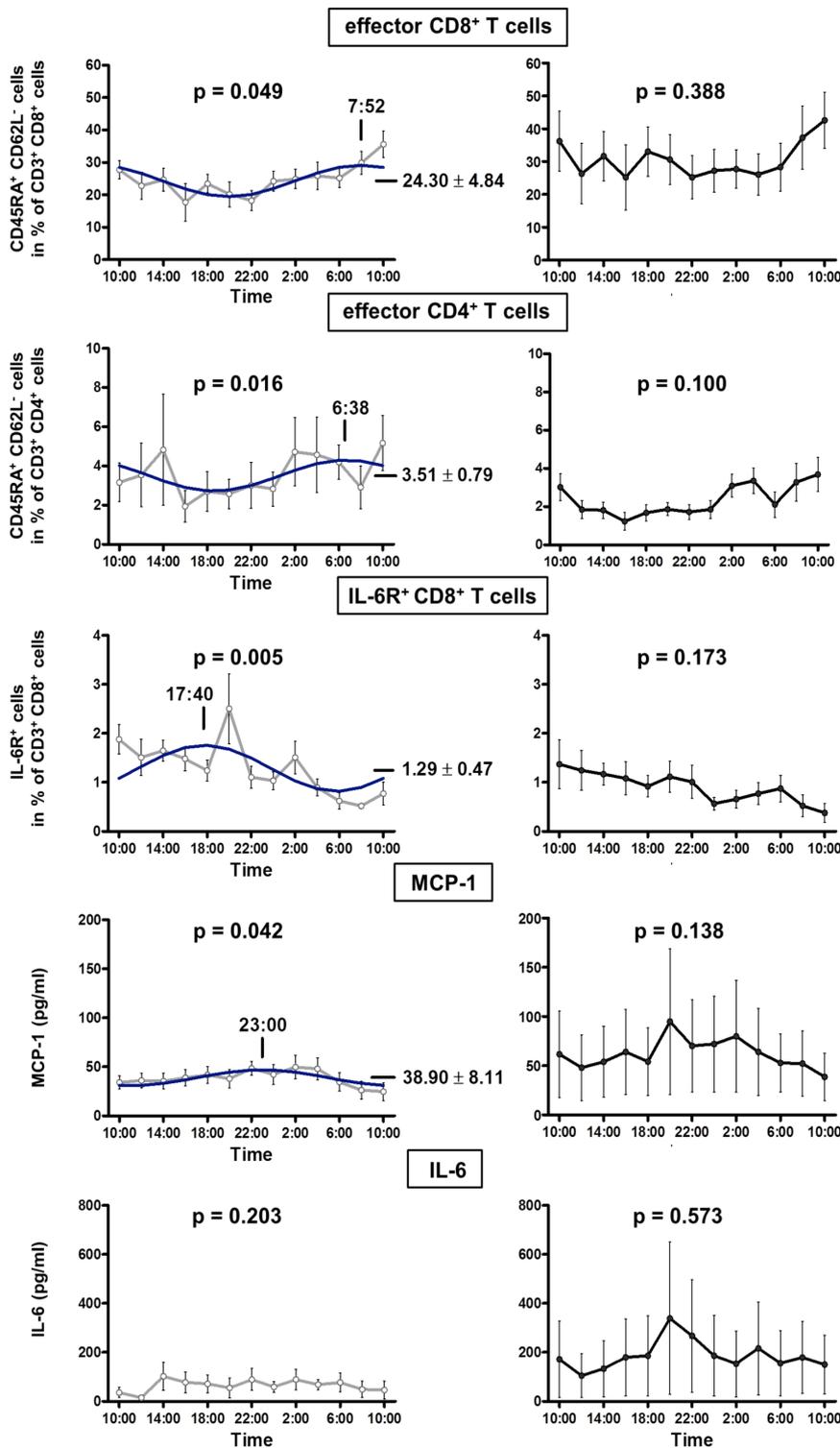


Fig. 1. Circadian rhythms of peripheral blood cell populations and cytokines that are present in postmenopausal non-RA controls but absent in RA patients. Relative frequency of effector CD8⁺ T cells, effector CD4⁺ T cells, IL-6 receptor expressing CD8⁺ T cells, and concentration of the cytokines MCP-1 and IL-6 in monocytes of postmenopausal non-RA controls (left) and RA patients (right). Shown are means/SEM (n=5) and Cosinor curves (blue), showing peak phase, magnitude±amplitude, and *p*-value of Cosinor analysis.

tor⁺ CD62L expressing CD4⁺ T cells, effector memory CD4⁺ T cells, central memory CD8⁺ T cells, CD45RO^{high} expressing CD45RA⁻ T cells, CD3⁺ T cells, and CD4⁺ T helper cells.

Finally, we found circadian rhythms that were present in both non-RA controls and in patients with RA, but with differences in either peak phase, amplitude or both amplitude and magnitude (relative difference of >10%; there were no identical circadian rhythms) (Fig. 3, Table II); in comparison to non-RA controls peak phase was earlier in RA patients in CD62L expressing B cells; amplitude was higher in RA patients in chemokine receptor CXCR4 expressing CD4⁺ T cells and CD8⁺ T cells; both magnitude and amplitude were higher in RA patients in CD45RA⁻ CD45RO⁻ CD8⁺ T cells, natural killer cells, and IL-8 receptor expressing CD4⁺ and CD8⁺ T cells.

No significant circadian rhythm in postmenopausal non-RA controls and RA patients was detectable in the following populations: lymphocytes, granulocytes, monocytes, HLA-DR⁺ monocytes, B cells, CD8⁺ T cells, CD69⁺ CD4⁺ and CD8⁺ T cells, CD45RA⁺ CD45RO⁻ naive CD4⁺ T cells, CD45RO^{dim} CD45RA⁻ CD4⁺ and CD8⁺ T cells, CD45RO^{high} CD45RA⁺ CD4⁺ and CD8⁺ T cells, CD45RO^{high} CD45RA⁻ CD8⁺ T cells, CD45RA⁺ CD62L⁺ naive CD4⁺ and CD8⁺ T cells, central memory CD4⁺ T cells, effector memory CD8⁺ T cells, CD25⁺ CD127⁻ T reg cells, IL-2 receptor expressing CD8⁺ T cells, IL-6 receptor expressing B cells and monocytes, IL-7 receptor expressing CD4⁺ and CD8⁺ T cells, IL-8 receptor expressing B cells, and CCR7⁺ CD4⁺ and CD8⁺ T cells; all cytokines/chemokines measured except for MCP-1, including IL-6 (Fig. 1, lowest row).

Circadian clock gene expression in monocytes

In order to identify underlying mechanisms, clock gene expressions were assessed in CD14⁺ monocytes isolated from peripheral blood of postmenopausal RA patients and non-RA controls. *REV-ERBA* showed a significant circadian expression pattern in both RA patients and controls (Fig.

Table II. Cosinor data for peripheral blood cell populations and cytokines.

Parameter	Cosinor non-RA controls				Cosinor RA patients				ANOVA group <i>p</i> -value
	Mag.	Ampl.	Peak Ph.	<i>p</i> -value	Mag.	Ampl.	Peak Ph.	<i>p</i> -value	
Circadian rhythm present in postmenopausal non-RA controls but absent in RA patients									
CD45RA ⁻ CD45RO ⁻ CD4 ⁺ T cells [% of CD4 ⁺ T cells]	9.71	4.11	6:16	0.036	8.85	3.70	6:04	0.051	0.730
CD45RA ⁺ CD62L ⁻ effector CD4 ⁺ T cells [% of CD4 ⁺ T cells]	3.51	0.79	6:38	0.016	2.31	0.88	6:18	0.100	0.472
CD45RA ⁺ CD45RO ⁻ naive CD8 ⁺ T cells [% of CD8 ⁺ T cells]	54.10	3.69	6:18	0.044	50.90	5.84	7:40	0.109	0.475
CD45RA ⁺ CD62L ⁻ effector CD8 ⁺ T cells [% of CD8 ⁺ T cells]	24.30	4.84	7:52	0.049	30.30	4.01	10:28	0.388	0.451
CD3 ⁺ CD56 ⁺ NK T cells [% of lymphocytes]	2.80	0.74	14:52	0.008	5.10	0.87	14:04	0.108	0.061
IL-6R ⁺ CD4 ⁺ T cells [% of CD4 ⁺ T cells]	0.85	0.35	15:12	0.008	0.55	0.25	14:52	0.136	0.037
IL-6R ⁺ CD8 ⁺ T cells [% of CD8 ⁺ T cells]	1.29	0.47	17:40	0.005	0.90	0.24	15:40	0.173	0.174
IL-2R ⁺ CD4 ⁺ T cells [% of CD4 ⁺ T cells]	3.71	1.52	20:12	0.026	4.73	1.35	17:12	0.143	0.190
CD62L ⁺ CD8 ⁺ T cells [% of CD8 ⁺ T cells]	42.30	3.24	19:40	0.014	43.00	2.68	20:12	0.197	0.794
MCP-1 [pg/ml]	38.90	8.11	23:00	0.042	63.20	15.00	22:32	0.138	0.395
Circadian rhythm absent in postmenopausal non-RA controls but present in RA patients									
CD45RA ⁻ CD62L ⁻ effector memory CD4 ⁺ T cells [% of CD4 ⁺ T cells]	18.30	2.79	5:24	0.064	18.70	2.15	1:54	0.036	0.983
CD62L ⁺ CD4 ⁺ T cells [% of CD4 ⁺ T cells]	77.00	4.79	18:36	0.058	78.70	3.73	14:48	0.005	0.980
IL-8R ⁺ monocytes [% of CD14 ⁺ monocytes]	63.30	4.85	16:52	0.384	66.60	11.40	16:12	0.016	0.678
CD45RO ^{high} CD45RA ⁻ CD4 ⁺ T cells [% of CD4 ⁺ T cells]	34.50	5.88	18:52	0.260	38.20	8.72	18:52	0.025	0.004
CD20 ⁺ CD27 ⁺ memory B cells [% of CD19 ⁺ B cells]	43.80	0.87	17:44	0.368	38.10	4.36	19:52	0.035	0.318
HLA-DR ⁺ CD20 ⁺ activated B cells [% of CD19 ⁺ B cells]	72.70	0.52	22:08	0.845	66.90	5.88	20:08	0.006	0.335
CD45RA ⁻ CD62L ⁺ central memory CD8 ⁺ T cells [% of CD8 ⁺ T cells]	14.00	1.99	21:40	0.056	17.20	1.91	21:28	0.037	0.962
CD3 ⁺ T cells [% of lymphocytes]	70.40	0.92	23:20	0.691	76.10	2.96	22:32	0.004	0.261
CD3 ⁺ CD4 ⁺ T cells [% of lymphocytes]	53.60	2.28	23:08	0.154	59.40	4.17	23:32	0.027	0.212
Circadian rhythm present in both postmenopausal non-RA controls and RA patients									
CD45RA ⁻ CD45RO ⁻ CD8 ⁺ T cells [% of CD8 ⁺ T cells]	6.37	2.95	5:39	0.046	8.97	3.92	5:48	0.017	0.319
CD3 ⁺ CD56 ⁺ NK cells [% of lymphocytes]	3.59	1.12	11:52	0.005	4.55	1.64	12:04	0.029	0.716
IL-8R ⁺ CD4 ⁺ T cells [% of CD4 ⁺ T cells]	41.10	6.41	16:24	0.013	54.70	10.40	15:00	0.012	0.002
CXCR4 ⁺ CD4 ⁺ T cells [% of CD4 ⁺ T cells]	55.90	8.04	14:48	0.046	50.20	10.30	15:00	0.012	0.522
CXCR4 ⁺ CD8 ⁺ T cells [% of CD8 ⁺ T cells]	39.90	7.22	15:36	0.001	41.20	11.10	16:00	0.020	0.792
CD62L ⁺ B cells [% of CD19 ⁺ B cells]	83.70	5.75	18:28	0.037	84.40	5.99	16:12	0.009	0.914
IL-8R ⁺ CD8 ⁺ T cells [% of CD8 ⁺ T cells]	14.60	2.96	17:04	0.024	31.70	5.81	17:12	0.045	0.000

Sorted for peak phase of RA patients; Mag: magnitude; Ampl: amplitude; Peak Ph: peak phase.

4). In contrast, the clock genes *PER2* and *PER3* were expressed in a circadian manner with significant amplitude in the control group only, indicating that the circadian rhythm in RA subjects is disrupted (Fig. 4). For the clock genes *CRY1* and *BMAL1* no significant circadian rhythm was detectable (data not shown).

BMAL1-bioluminescence recording in macrophages

The *BMAL1-luc*-bioluminescence, reflecting the endogenous circadian rhythm of the respective cells, was measured over 5 days in macrophages obtained from peripheral blood samples at day -8 and day 1, respectively. A rhythm was detectable in six of ten subjects only. The amplitude of the detected rhythms appeared to be lower and damping to be higher in the post-

menopausal RA group than in non-RA controls (mean±SD: 0.066±0.038 (n=3) vs. 0.184±0.077 (n=3), *p*=0.2 (Mann-Whitney U-test) and 34.3±11.3 vs. 19.7±2.3, *p*=0.1, respectively), whereas period and phase were not altered (23.5±0.6 vs. 24.2±2.0, *p*=1.0, and 15.5±1.0 vs. 15.5±1.0, *p*=0.7, respectively) (Fig. 4).

Discussion

It is currently thought that the circadian rhythms of clinical symptoms in RA are primarily driven by underlying variations in cytokines and hormones (2, 8, 9). In this study, we show for the first time that immune cellular rhythms are also altered in postmenopausal patients with RA, compared to postmenopausal non-RA controls. The study was conceived of as a hypothesis generating study, in order to identify previously

unknown target cell types which are especially active in RA. We were able to detect significant circadian rhythms in the relative frequency of circulating peripheral blood cell populations, although (because of the high technical effort) we examined only 5 participants per group. However, we have found no circadian rhythm of serum IL-6, though we know, that this particular rhythm exists (9). The variability in both patients and controls was substantial. Therefore, the robustness of data needs to be confirmed by examining a larger replication group of individuals. The RA group was heterogenous with regard to treatment, as glucocorticoid therapy was taken by two of the RA patients at low and stable dosages, a factor we would improve in further studies.

The circadian clock drives daily physiological rhythms that are necessary to

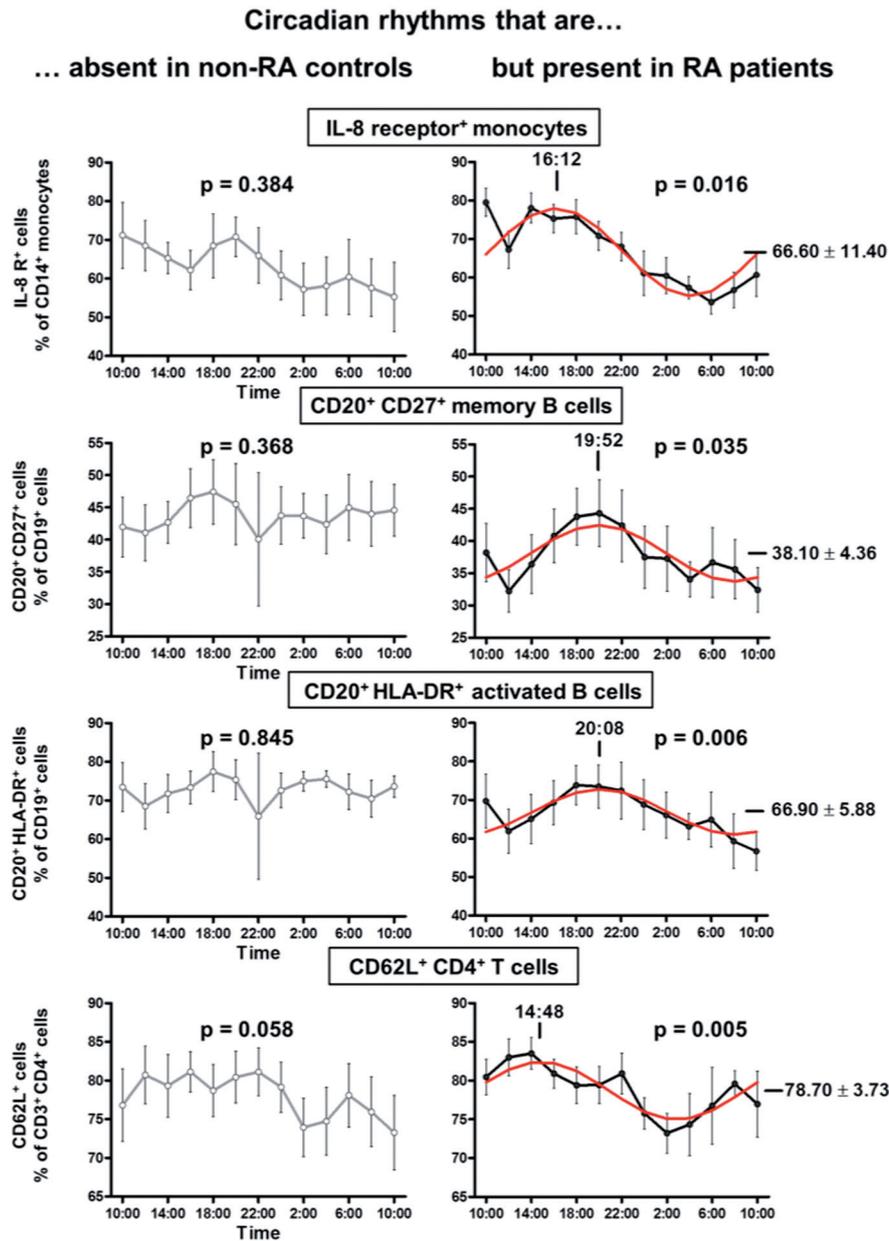


Fig. 2. Circadian rhythms of peripheral blood cell populations that are absent in postmenopausal non-RA controls but present in RA patients. Relative frequency of IL-8 receptor expressing CD14⁺ monocytes, CD20⁺ CD27⁺ B cells, CD20⁺ HLA-DR⁺ B cells, and CD62L expressing CD4⁺ T cells of postmenopausal non-RA controls (left) and RA patients (right). Shown are means/SEM (n=5) and Cosinor curves (red), showing peak phase, magnitude±amplitude, and p-value of Cosinor analysis.

synchronise the functions of the organism with the 24-hour environment (29). The major activities of the immune system occur during sleep, because during the day energy-rich fuel is allocated primarily to brain and muscles (30). In accordance with the literature, we found cells of the adaptive immune system (e.g. B cells and CD4⁺ T cells) to be most prominent in peripheral blood during the evening and night, whereas

cells of the innate immune system (e.g. NK cells and effector cells) were most prominent during morning and noon (30-32). Expression of chemokine and homing receptors peaked during the afternoon, possibly preceding redistribution of cells of the adaptive immune system to the bone marrow or inflammatory tissue. However, we also have to keep in mind that the composition and concentration of blood constituents are

dependent on body position, which can cause up to 10% changes. One cause is leakage through the vascular wall, a factor which could differ between patients with RA and controls.

Cellular immune rhythms are synchronised by the mammalian master clock located in the hypothalamic supra-chiasmatic nucleus (SCN), impacting on sympathetic nervous system and regulatory hormones, mainly the pro-inflammatory hormones growth hormone, prolactin and melatonin (peaking at night), and anti-inflammatory cortisol (peaking in the morning) (31). We found circadian rhythms present in postmenopausal non-RA controls, but absent in postmenopausal patients with RA, e.g. for several effector cell populations, e.g. effector CD4⁺ or CD8⁺ T cells and natural killer T cells, which may be relevant for ongoing inflammation in RA. Loss of rhythm may be consequence or cause of dysregulation in RA. It has previously been shown that circadian rhythms could be lost in RA. In RA patients with high disease activity, the circadian rhythm of cortisol secretion is abolished and the response of the hypothalamic-pituitary adrenal (HPA) axis is inadequately low in relation to ongoing inflammation (2, 15, 33-35). Two recent studies have demonstrated disruption or alteration, respectively, of cellular rhythms of molecular clocks in synovial fibroblast cells of patients with RA and osteoarthritis (36, 37). We also observed rhythm loss in RA at the molecular level. Expression of the clock genes *PER2* and *PER3* was disrupted in monocytes of patients with RA. *PER* is central in the transcription/translation feedback loop of the molecular machinery required to enable a cell to oscillate: the encoded proteins of the clock genes *CLOCK* and *BMAL* dimerise (*CLOCK/BMAL*) and activate transcription of *PERIOD* (*PER*), *CRYPTOCHROME* (*CRY*), *REV-ERB* and *ROR* (29). As negative feedback, the dimeric complex of *PER/CRY* inhibits *CLOCK/BMAL* transactivation (29). Degradation of *PER/CRY* during the night allows *CLOCK/BMAL* to start a new cycle of transcriptional activation (29). The disruption of *PER2* gene expression may be due to inflammatory

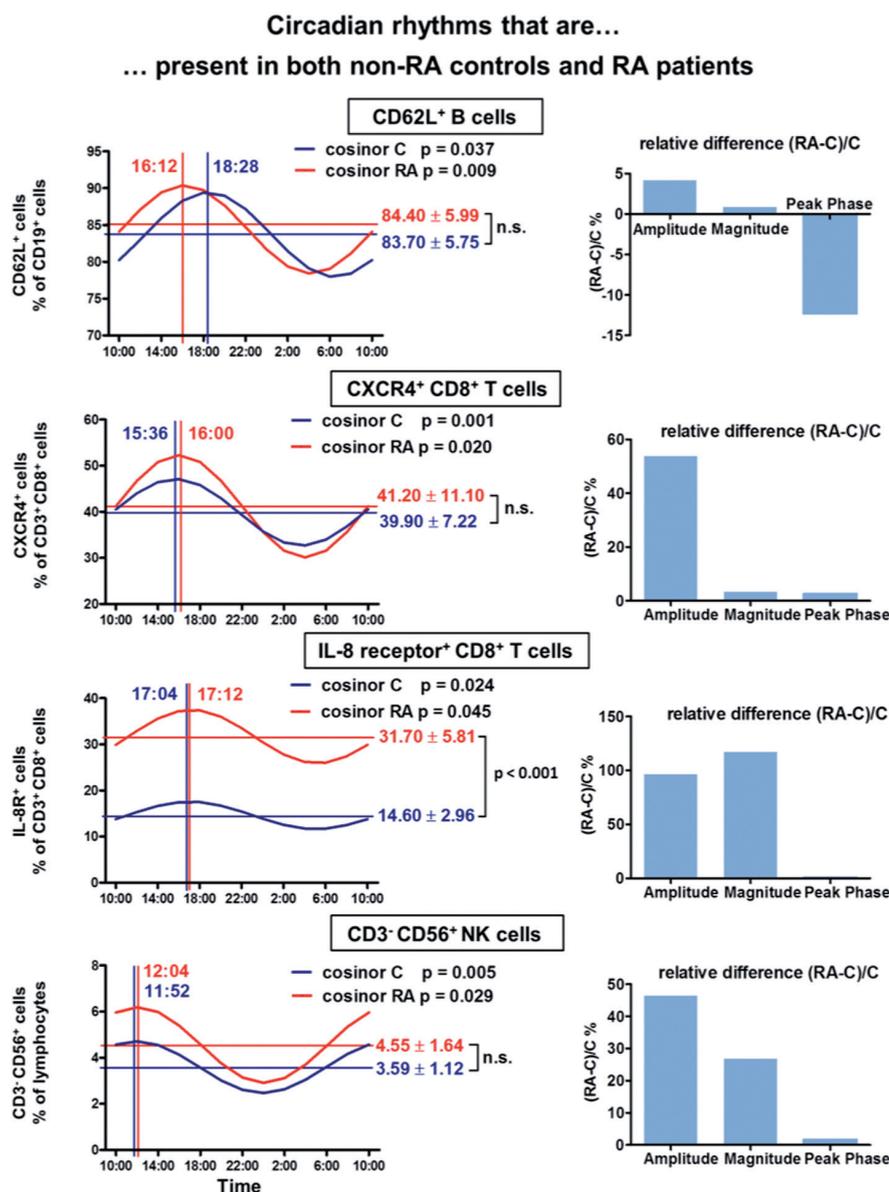


Fig. 3. Circadian rhythms that are present in both postmenopausal non-RA controls and in RA patients, but with differences in either peak phase, amplitude or both amplitude and magnitude. Left: Cosinor curves of CD62L⁺ B cells, CXCR4⁺ CD8⁺ T cells, IL-8 receptor expressing CD8⁺ T cells, and CD3⁺ CD56⁺ natural killer (NK) cells in postmenopausal non-RA controls (blue curves) and RA patients (red curves), showing peak phases, magnitudes \pm amplitudes, *p*-values of Cosinor analysis, and *p*-values of ANOVA test for difference between groups. Right: Relative differences in amplitude, magnitude and peak phase.

TNF- α expression (38). Conversely, rhythm loss can aggravate arthritis (39). We also found lower amplitudes of the endogenous circadian rhythm of *BMAL1* expression in macrophages in RA patients. This may be associated with the rhythm loss of MCP-1 in RA patients, as *BMAL1* regulates MCP-1 expression in mouse macrophages (40).

Furthermore, we identified circadian rhythms that were not detectable (*i.e.*

apparently not necessary) in postmenopausal non-RA controls but established in RA patients, for example in memory and activated B cells, and several chemokine or homing receptor expressing populations (*e.g.* receptors for IL-8 and CD62L). Gain of rhythm may facilitate inflammation. Examples for establishment of circadian rhythms are expression of IL-6 in human RA patients (8, 35) or serum-amyloid A and TNF- α after the onset of murine arthri-

tis (41, 42). IL-6 differentially regulates leucocyte recruitment, explaining why expression of many cell populations can be affected (43). It is not clear, whether the differences found suggest some mechanism related perhaps to IL-6. The changes are rather not consistent with known effects of cytokines. Recent studies of the pathogenesis of RA have revealed that both synovial fibroblasts and T cells participate in the perpetuation of joint inflammation as dynamic partners in a mutual activation feedback, via secretion of cytokines and chemokines that stimulate each other (44). We hypothesise that in RA circadian rhythms of the expression of chemokine and homing receptors (*e.g.* receptors for IL-8 and CD62L) may be established, which facilitate recruitment of inflammatory cells to the inflammatory tissue.

We have found no circadian rhythm in the variation of clinical parameters, probably because here only six time points were measured, not separated by identical intervals. The disease was moderately active in all of the RA patients despite of any glucocorticoid therapy, only taken by two of the RA patients at low and stable dosages. The DAS28 was more driven by tender and swollen joints, as ESR was only mildly increased and CRP concentrations normal. In several populations we found no circadian rhythms in our postmenopausal RA patients and controls, though rhythms are known to be present in young healthy persons (16, 45, 46).

To date there is only scattered knowledge on cellular circadian rhythms in RA (29). Previous investigations with clinical impact in RA have concentrated on cytokines and humoral factors (2, 7, 9). However, cellular circadian rhythms have the potential to represent novel therapeutic targets if there are differences in between RA patients/healthy subjects, or between patients with active/inactive disease or responders/non-responders to therapy. We think, for example, of depleting therapy at peak phases of target cells, which are especially active in RA patients. There has been limited success with a “biomarker approach” to identify factors associated with poor re-

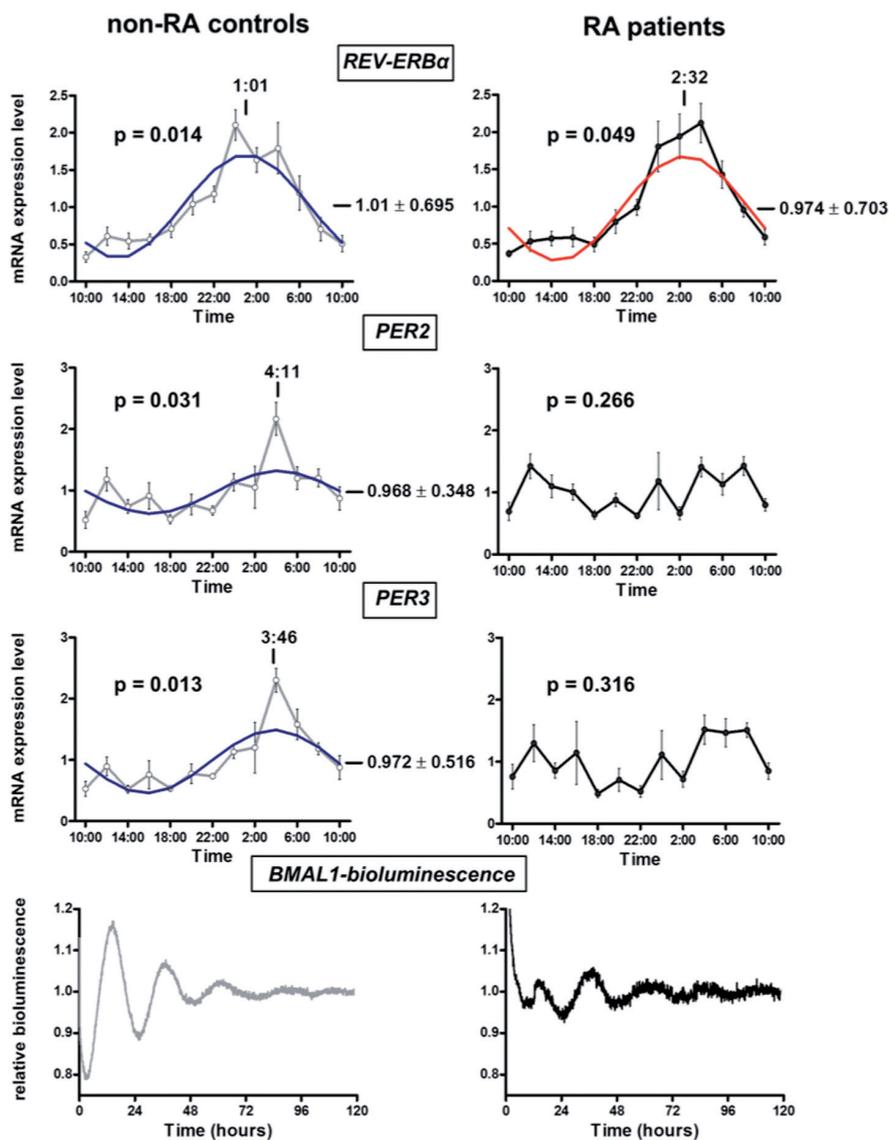


Fig. 4. Circadian clock gene expression of *REV-ERBa*, *PER2* and *PER3* in monocytes of postmenopausal non-RA controls (left) and RA patients (right). Shown are means/SEM (n=5) and Cosinor curves (blue or red), showing peak phase, magnitude ± amplitude, and p-value of Cosinor analysis. Gene expression was normalised to *GAPDH* levels. Lowest row: *BMAL1-luc*-bioluminescence in macrophages measured over 120 hours. Shown are two representative measurements.

sponse to treatment. We hypothesise that a “circadian pattern approach” could be more successful in this regard. There is a current and challenging clinical need to understand the reasons for lack of treatment efficacy, due to the fact that a considerable proportion of patients with RA are (partial) non-responders (47-50). The circadian rhythms of the humoral factors TNF-alpha and IL-6 are important, and indeed they have been successfully targeted by glucocorticoid chronotherapy (10). For this reason, we need a target search, which does not

look at humoral factors only, but also at cellular circadian rhythms. Chronotherapy with methotrexate could be a successful approach (42).

Conclusion

In summary, we conclude that immune cells in RA may lose their normal circadian rhythms and establish new “inflammatory” circadian rhythms. These findings provide new aspects of RA chronobiology which need further investigation and may have pathophysiological and therapeutic implications.

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References

- CUTOLO M, STRAUB RH, BUTTGEREIT F: Circadian rhythms of nocturnal hormones in rheumatoid arthritis: translation from bench to bedside. *Ann Rheum Dis* 2008; 67: 905-8.
- STRAUB RH, CUTOLO M: Circadian rhythms in rheumatoid arthritis: implications for pathophysiology and therapeutic management. *Arthritis Rheum* 2007; 56: 399-408.
- BELLAMY N, SOTHERN RB, CAMPBELL J, BUCHANAN WW: Circadian rhythm in pain, stiffness, and manual dexterity in rheumatoid arthritis: relation between discomfort and disability. *Ann Rheum Dis* 1991; 50: 243-8.
- HARKNESS JA, RICHTER MB, PANAYI GS *et al.*: Circadian variation in disease activity in rheumatoid arthritis. *Br Med J (Clin Res Ed)* 1982; 284: 551-4.
- KOWANKO IC, KNAPP MS, POWNALL R, SWANNELL AJ: Domiciliary self-measurement in the rheumatoid arthritis and the demonstration of circadian rhythmicity. *Ann Rheum Dis* 1982; 41: 453-5.
- WESTHOFF G, BUTTGEREIT F, GROMNICA-IHLE E, ZINK A: Morning stiffness and its influence on early retirement in patients with recent onset rheumatoid arthritis. *Rheumatology (Oxford)* 2008; 47: 980-4.
- PETROVSKY N, MCNAIR P, HARRISON LC: Diurnal rhythms of pro-inflammatory cytokines: regulation by plasma cortisol and therapeutic implications. *Cytokine* 1998; 10: 307-12.
- ARVIDSON NG, GUDBJORNSSON B, ELFMAN L, RYDEN AC, TOTTERMAN TH, HALLGREN R: Circadian rhythm of serum interleukin-6 in rheumatoid arthritis. *Ann Rheum Dis* 1994; 53: 521-4.
- 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; 68: 63-8.
- BUTTGEREIT F, DOERING G, SCHAEFFLER A *et al.*: Efficacy of modified-release versus standard prednisone to reduce duration of morning stiffness of the joints in rheumatoid arthritis (CAPRA-1): a double-blind, randomised controlled trial. *Lancet* 2008; 371: 205-14.
- BUTTGEREIT F, DOERING G, SCHAEFFLER A *et al.*: Targeting pathophysiological rhythms: prednisone chronotherapy shows sustained efficacy in rheumatoid arthritis. *Ann Rheum Dis* 2010; 69: 1275-80.
- BUTTGEREIT F, MEHTA D, KIRWAN J *et al.*: Low-dose prednisone chronotherapy for rheumatoid arthritis: a randomised clinical trial (CAPRA-2). *Ann Rheum Dis* 2013; 72: 204-10.

13. ESQUIFINO AI, SELGAS L, ARCE A, MAGGIORE VD, CARDINALI DP: Twenty-four-hour rhythms in immune responses in rat submaxillary lymph nodes and spleen: effect of cyclosporine. *Brain Behav Immun* 1996; 10: 92-102.
14. ARJONA A, SARKAR DK: Circadian oscillations of clock genes, cytolytic factors, and cytokines in rat NK cells. *J Immunol* 2005; 174: 7618-24.
15. KAWATE T, ABO T, HINUMA S, KUMAGAI K: Studies of the bioperiodicity of the immune response. II. Co-variations of murine T and B cells and a role of corticosteroid. *J Immunol* 1981; 126: 1364-7.
16. DIMITROV S, BENEDICT C, HEUTLING D, WESTERMANN J, BORN J, LANGE T: Cortisol and epinephrine control opposing circadian rhythms in T cell subsets. *Blood* 2009; 113: 5134-43.
17. MAZZOCCOLI G, DE CATAA, GRECO A *et al.*: Aging related changes of circadian rhythmicity of cytotoxic lymphocyte subpopulations. *J Circadian Rhythms* 2010; 8: 6.
18. YOUNG MR, MATTHEWS JP, KANABROCKI EL, SOTHERN RB, ROITMAN-JOHNSON B, SCHEVING LE: Circadian rhythmometry of serum interleukin-2, interleukin-10, tumor necrosis factor-alpha, and granulocyte-macrophage colony-stimulating factor in men. *Chronobiol Int* 1995; 12: 19-27.
19. KELLER M, MAZUCH J, ABRAHAM U *et al.*: A circadian clock in macrophages controls inflammatory immune responses. *Proc Natl Acad Sci USA* 2009; 106: 21407-12.
20. ROENNEBERG T, WIRZ-JUSTICE A, MERROW M: Life between clocks: daily temporal patterns of human chronotypes. *J Biol Rhythms* 2003; 18: 80-90.
21. ARNETT FC, EDWORTHY SM, BLOCH DA *et al.*: The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. *Arthritis Rheum* 1988; 31: 315-24.
22. PREVOO ML, VAN 'T HOF MA, KUPER HH, VAN LEEUWEN MA, VAN DE PUTTE LB, VAN RIEL PL: Modified disease activity scores that include twenty-eight-joint counts. Development and validation in a prospective longitudinal study of patients with rheumatoid arthritis. *Arthritis Rheum* 1995; 38: 44-8.
23. FRIES JF, SPITZ P, KRAINES RG, HOLMAN HR: Measurement of patient outcome in arthritis. *Arthritis Rheum* 1980; 23: 137-45.
24. WARE JE, KOSINSKI M, KELLER SD: SF-36 Physical and mental health summary scales: a user's manual. Boston: The Health Institute, New England Medical Center, 1994.
25. SMETS EM, GARSSEN B, BONKE B, DE HAES JC: The Multidimensional Fatigue Inventory (MFI) psychometric qualities of an instrument to assess fatigue. *J Psychosom Res* 1995; 39: 315-25.
26. SMETS EM, GARSSEN B, CULL A, DE HAES JC: Application of the multidimensional fatigue inventory (MFI-20) in cancer patients receiving radiotherapy. *Br J Cancer* 1996; 73: 241-5.
27. FREYNHAGEN R, BARON R, GOCKEL U, TOLLE TR: painDETECT: a new screening questionnaire to identify neuropathic components in patients with back pain. *Curr Med Res Opin* 2006; 22: 1911-20.
28. BROWN SA, FLEURY-OLELA F, NAGOSHI E *et al.*: The period length of fibroblast circadian gene expression varies widely among human individuals. *PLoS Biol* 2005; 3: e338.
29. GIBBS JE, RAY DW: The role of the circadian clock in rheumatoid arthritis. *Arthritis Res Ther* 2013; 15: 205.
30. STRAUB RH, CUTOLO M, BUTTGEREIT F, PONGRATZ G: Energy regulation and neuroendocrine-immune control in chronic inflammatory diseases. *J Intern Med* 2010; 267: 543-60.
31. CERMAKIAN N, LANGE T, GOLOMBEK D *et al.*: Crosstalk between the circadian clock circuitry and the immune system. *Chronobiol Int* 2013; 30: 870-88.
32. LANGE T, DIMITROV S, BORN J: Effects of sleep and circadian rhythm on the human immune system. *Ann N Y Acad Sci* 2010; 1193: 48-59.
33. DEKKERS JC, GEENEN R, GODAERT GL, VAN DOORNEN LJ, BIJLSMA JW: Diurnal rhythm of salivary cortisol levels in patients with recent-onset rheumatoid arthritis. *Arthritis Rheum* 2000; 43: 465-7.
34. NEECK G, FEDERLIN K, GRAEF V, RUSCH D, SCHMIDT KL: Adrenal secretion of cortisol in patients with rheumatoid arthritis. *J Rheumatol* 1990; 17: 24-9.
35. CROFFORD LJ, KALOGERAS KT, MASTORAKOS G *et al.*: Circadian relationships between interleukin (IL)-6 and hypothalamic-pituitary-adrenal axis hormones: failure of IL-6 to cause sustained hypercortisolism in patients with early untreated rheumatoid arthritis. *J Clin Endocrinol Metab* 1997; 82: 1279-83.
36. HAAS S, STRAUB RH: Disruption of rhythms of molecular clocks in primary synovial fibroblasts of patients with osteoarthritis and rheumatoid arthritis, role of IL-1beta/TNF. *Arthritis Res Ther* 2012; 14: R122.
37. KOURI VP, OLKKONEN J, KAIVOSOJA E *et al.*: Circadian timekeeping is disturbed in rheumatoid arthritis at molecular level. *PLoS one* 2013; 8: e54049.
38. YOSHIDA K, HASHIRAMOTO A, OKANO T, YAMANE T, SHIBANUMA N, SHIOZAWA S: TNF-alpha modulates expression of the circadian clock gene Per2 in rheumatoid synovial cells. *Scand J Rheumatol* 2013; 42: 276-80.
39. HASHIRAMOTO A, YAMANE T, TSUMIYAMA K *et al.*: Mammalian clock gene Cryptochrome regulates arthritis via proinflammatory cytokine TNF-alpha. *J Immunol* 2010; 184: 1560-5.
40. HAYASHI M, SHIMBA S, TEZUKA M: Characterization of the molecular clock in mouse peritoneal macrophages. *Biol Pharm Bull* 2007; 30: 621-6.
41. TO H, IRIE S, TOMONARI M, WATANABE Y, KITAHARA T, SASAKI H: Therapeutic index of methotrexate depends on circadian cycling of tumour necrosis factor-alpha in collagen-induced arthritic rats and mice. *J Pharm Pharmacol* 2009; 61: 1333-8.
42. TO H, YOSHIMATSU H, TOMONARI M *et al.*: Methotrexate chronotherapy is effective against rheumatoid arthritis. *Chronobiol Int* 2011; 28: 267-74.
43. JONES SA: Directing transition from innate to acquired immunity: defining a role for IL-6. *J Immunol* 2005; 175: 3463-8.
44. HWANG SY, KIM JY, KIM KW *et al.*: IL-17 induces production of IL-6 and IL-8 in rheumatoid arthritis synovial fibroblasts via NF-kappaB- and PI3-kinase/Akt-dependent pathways. *Arthritis Res Ther* 2004; 6: R120-8.
45. BOLLINGER T, BOLLINGER A, SKRUM L, DIMITROV S, LANGE T, SOLBACH W: Sleep-dependent activity of T cells and regulatory T cells. *Clin Exp Immunol* 2009; 155: 231-8.
46. NIEHAUS GD, ERVIN E, PATEL A, KHANNA K, VANEK VW, FAGAN DL: Circadian variation in cell-adhesion molecule expression by normal human leukocytes. *Can J Physiol Pharmacol* 2002; 80: 935-40.
47. MOOTS RJ, NAISBETT-GROET B: The efficacy of biologic agents in patients with rheumatoid arthritis and an inadequate response to tumour necrosis factor inhibitors: a systematic review. *Rheumatology (Oxford)* 2012; 51: 2252-61.
48. BURMESTER GR, MARIETTE X, MONTECUCO C *et al.*: Adalimumab alone and in combination with disease-modifying antirheumatic drugs for the treatment of rheumatoid arthritis in clinical practice: the Research in Active Rheumatoid Arthritis (ReAct) trial. *Ann Rheum Dis* 2007; 66: 732-9.
49. BOMBARDIERI S, RUIZ AA, FARDELLONE P *et al.*: Effectiveness of adalimumab for rheumatoid arthritis in patients with a history of TNF-antagonist therapy in clinical practice. *Rheumatology (Oxford)* 2007; 46: 1191-9.
50. BAKKER MF, JACOBS JW, WELSING PM *et al.*: Early clinical response to treatment predicts 5-year outcome in RA patients: follow-up results from the CAMERA study. *Ann Rheum Dis* 2011; 70: 1099-103.