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Full-length Article

# Circadian regulation of human peripheral neutrophils

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ABSTRACT

Neutrophils are the most abundant leukocytes in human blood. Beside being essential responders in bacterial and fungal infections, they also contribute to tissue reactions in many autoimmune and inflammatory diseases. Although several immune responses linked to neutrophil functions have been described to be rhythmic, the mechanism of the circadian regulation of these cells is still not understood. Characterization of the time-of-day-specific control of neutrophil responsiveness could help to better understand the pathomechanism of these inflammatory responses and design effective chronotherapy. Here we report that the time-dependent expression of core clock components in human neutrophils characteristically differs from that in mononuclear cells. Both the low expression and the reduced nuclear accumulation of the essential clock protein BMAL1 suggest that the molecular oscillator is down-regulated in neutrophils. By following the expression of the maturation marker *Cxcr4* and morphological attributes (side-scattering properties and nuclear segmentation), we found that the distribution of young and aged cells within the peripheral neutrophil pool displays a daily rhythm. In addition, we detected synchronous fluctuations in the plasma level of the CXCR4 ligand CXCL12, an important regulator of cell trafficking within the bone marrow. We found that expression of another maturation marker, the core component of the superoxide generating NADPH oxidase, and parallelly, the superoxide producing capacity of neutrophils were also dependent on the time of the day. In line with this, number of opsonized bacteria engulfed by neutrophils also showed time-dependent differences, supporting that clearance of pathogens shows a daily rhythm. We suggest that maturation-dependent changes in neutrophil responsiveness rather than the cellular autonomous clock are involved in the daily regulation of human neutrophil functions.



## 1. Introduction

Circadian clocks have a great impact on the daily organization of the physiology and behavior of light-sensitive organisms. The circadian time-keeping system enables the organism to anticipate environmental changes and therefore is a crucial factor of adaptation. The mammalian time-measuring system has a central pacemaker in the suprachiasmatic nucleus (SCN), which may coordinate and synchronize the peripheral oscillators present in other tissues. The endogenous rhythm is generated at the cellular level by a mechanism based on the action of interconnected transcription/translation feedback loops (recently reviewed in (Mohawk et al., 2012)). The central feedback loop consists of the transcriptional activators CLOCK and BMAL1 and the negative factors CRYPTOCHROMEs (CRYs) and PERIODs (PERs) as core components. The CLOCK-BMAL1 complex supports transcription of PERs and CRYs that accumulate and form a complex in the cytosol. This complex enters the nucleus, where it inhibits the positive factor complex, and thereby its own expression. When level of the PER-CRY complex declines in the nucleus, repression of the CLOCK-BMAL1 complex is relieved, and a new cycle is initiated. Both kinases (e.g. casein kinases, glycogen synthase kinase 3β) (Lee et al., 2001; Sahar et al., 2010) and phosphatases (e.g. protein phosphatase 1 and 5) (Partch et al., 2006; Schmutz et al., 2011) are involved in the regulation of stability and/or subcellular localization of different clock components and are therefore also essential parts of the molecular clock. Phosphorylation of PER by casein kinase 1δ and ε (CK1δ/ε) have been characterized in most detail, and is suggested to play a central role in setting the period and phase of the circadian clock. Phosphorylation affects PER's nuclear entry, interaction with other clock components and timely decay of the protein (Akashi et al., 2002; Keesler et al., 2000; Lee et al., 2011; Vielhaber et al., 2000). Via activation of the expression of the retinoic-acid-receptor-related orphan nuclear receptors, REV-ERBs and RORs, the CLOCK-BMAL1 complex induces an additional stabilizing loop that affects transcription of *Bmal1* and results in oscillation of BMAL1 protein levels in antiphase to that of PERs (Preitner et al., 2002; Ueda et al., 2002). Further feedback loops are also controlled by the CLOCK-BMAL1 complex; they involve

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e.g. the transcription factors DBP (albumin D-site-binding protein), HLF (hepatic leukemia factor), DEC1 (differentiated embryo chondrocyte protein) and DEC2 (Gachon, 2007; Lowrey and Takahashi, 2004; Takahashi et al., 2008).

The CLOCK/BMAL1 complex can also induce the transcription of different clock-controlled genes, which are involved in the rhythmic operation of various physiological and biochemical processes (Hatanaka et al., 2010; Koike et al., 2012; Rey et al., 2011). These classical clock mechanisms described here have been characterized in both the SCN and some peripheral cells. However, whether this general model fits all other cell types and moreover, how peripheral oscillations are related to and synchronized with the rhythm of other tissues, is still not well understood.

Disturbance of the circadian rhythm results in vulnerability of the organism (McEwen and Karatsoreos, 2015), and increases the incidence of various disorders, like diabetes (Kalsbeek et al., 2014), obesity (Roenneberg et al., 2012), breast cancer (Haus and Smolensky, 2013) or cardiovascular diseases (Ha and Park, 2005). Significant interconnections between circadian regulation and the immune system are underscored by both experimental and clinical data (Druzd et al., 2014; Fortier et al., 2011; Hashiramoto et al., 2010; Sato et al., 2014; Scheiermann et al., 2013). Neutrophilic granulocytes, the most abundant leukocytes in human blood constitute an essential part of the primary defense system against bacterial and fungal infections. The cell surface receptors of neutrophils recognize pathogens and trigger the activation of intracellular events leading to engulfment of microorganisms. Phagocytosed particles are then destroyed by the action of different reactive oxygen species and lytic enzymes. While reduced neutrophil activity often results in severe infectious diseases (Ottonello et al., 1995), inappropriate activation of neutrophil functions contributes to the development of various autoimmune (Nemeth and Mocsai, 2012; Wright et al., 2014) and inflammatory diseases (Alavi et al., 2014).

Accumulating evidence suggests time-of-day-dependent changes in neutrophil responses. In mice, neutrophil recruitment to tissue upon tissue damage or endotoxin challenge depends on the time of the day and is in synchrony with oscillation of the endothelial expression of adhesion molecules (Scheiermann et al., 2012). Clock-controlled expression of the epithelium-derived chemokine CXCL5 was shown to drive circadian neutrophil responses during inflammatory reaction of the lung (Gibbs et al., 2014). In addition, diurnal variations in the expression of cell adhesion molecules and phagocytic ability of neutrophils were also demonstrated (Hriscu, 2005; Melchart et al., 1992; Niehaus et al., 2002), indicating again that cell recruitment and reactivity may be controlled by the circadian clock. Moreover, neutrophil levels show low-amplitude oscillation throughout the day (Jilma et al., 1999; Sennels et al., 2011), and in a recent study, rhythmic clearance of aged murine neutrophils from blood has been shown (Casanova-Acebes et al., 2013). However, molecular clock mechanisms in neutrophils have not been characterized in detail. Although *Per1* expression in human neutrophils and mononuclear cells seems to have similar profiles (Kusanagi et al., 2004) and expression of different clock genes was found to be sensitive to endotoxin administration in neutrophils (Haimovich et al., 2010), operation of a peripheral clock in resting neutrophils has not been thoroughly characterized yet.

The goal of this study was to analyze time-keeping mechanisms controlling the responsiveness of human peripheral neutrophils. We show that expression of several clock genes is significantly different in neutrophils compared to other leukocytes. The level of the essential clock protein BMAL1 is very low and its nuclear accumulation is reduced. Our data on differentiating PLB-985 cells indicate that the molecular clock undergoes characteristic changes during myeloid maturation. We demonstrate that the human peripheral neutrophil pool displays daily oscillation in its age composition. Furthermore, levels of $Gp91^{phox}$ (component of the NADPH oxidase) that show maturation dependent expression display daily oscillation in correlation with time-dependent changes in superoxide production and phagocytosis of bacteria. Both our molecular and functional data suggest that systemic mechanisms involving timely controlled heterogeneity of the cell pool play important role in the regulation of the responsiveness of circulating neutrophils.

## 2. Material and methods

### 2.1. Subjects and study design

Healthy volunteers (both men and women), aged 20–35 years, were involved in this study. All participants signed an informed written consent and filled in the Hungarian version of the Munich ChronoType Questionnaire (MCTQ) (Haraszti et al., 2014; Roenneberg et al., 2012; Wittmann et al., 2006). All participants reported both regular work schedule and sleep-wake pattern, and an average sleep duration (7 ± 0.9 h on workdays and 9 ± 0.95 h on free days) in the MCTQ. Exclusion criteria were extreme chronotype (midpoint of sleep on free days corrected for sleep deficits accumulated during the workweek ($MSF_{sc}$) earlier than 3 am or later than 7 am), chronic diseases or an acute disease, shift work or jet lag in the preceding month and regular medication. For long-term sampling, subjects arrived at 9 am in the laboratory and left it at 8 am on the next day. Blood samples were collected at 10 am, 1 pm, 4 pm, 7 pm, 10 pm, 1 am, 4 am and 7 am. During the day, volunteers were allowed to go about their daily routines (e.g. learning, working at the computer, walking outside), except for sleeping in the institute. They were allowed to freely choose their sleep times between 10 pm and 7 am. All subjects took food three times on the day of the experiment: around 7 am, 12 am and 7 pm. Blood collections at night (after 10 pm) were carried out at low light intensities and with minimal disturbance of the subjects. The study was approved by the Scientific and Research Committee of the Medical Research Council (Hungary) (Ethical approvals 10895-0/2011-EKU and 1563/2015).

### 2.2. Cell isolation

Venous blood samples were obtained from each subject using Vacutainer® CPT™ tubes (BD) (8 ml/ sampling) and immediately processed for cell isolation. Mononuclear cells and neutrophils were separated by gradient centrifugation according to the manufacturer's instructions. Briefly, samples were centrifuged at room temperature in a horizontal rotor for 20 min at 1800 *g*. Plasma was separated and stored at −80 °C for further use. Mononuclear cells were isolated from the whitish layer under the plasma layer, whereas neutrophils were separated from the layer above the erythrocytes. The remaining red blood cells in the neutrophil samples were eliminated with ammonium-chloride-potassium lysing buffer (150 mM $NH_4Cl$, 10 mM $KHCO_3$, 0.1 mM $Na_2EDTA$, pH 7.4). Preparations were used in the experiments when purity obtained by nuclear staining (methylen-blue) was higher than 97%. Isolated cells were immediately processed for the next experimental steps.

### 2.3. Measurement of plasma cortisol levels

After centrifugation of the blood samples, plasma samples were collected and were stored at −80 °C. Cortisol levels in blood plasma samples were measured using an electrochemiluminescence immunoassay (Elecsys, Roche) according to the manufacturer's instructions. Data were normalized to the daily mean cortisol concentration measured in the samples of the particular subject.