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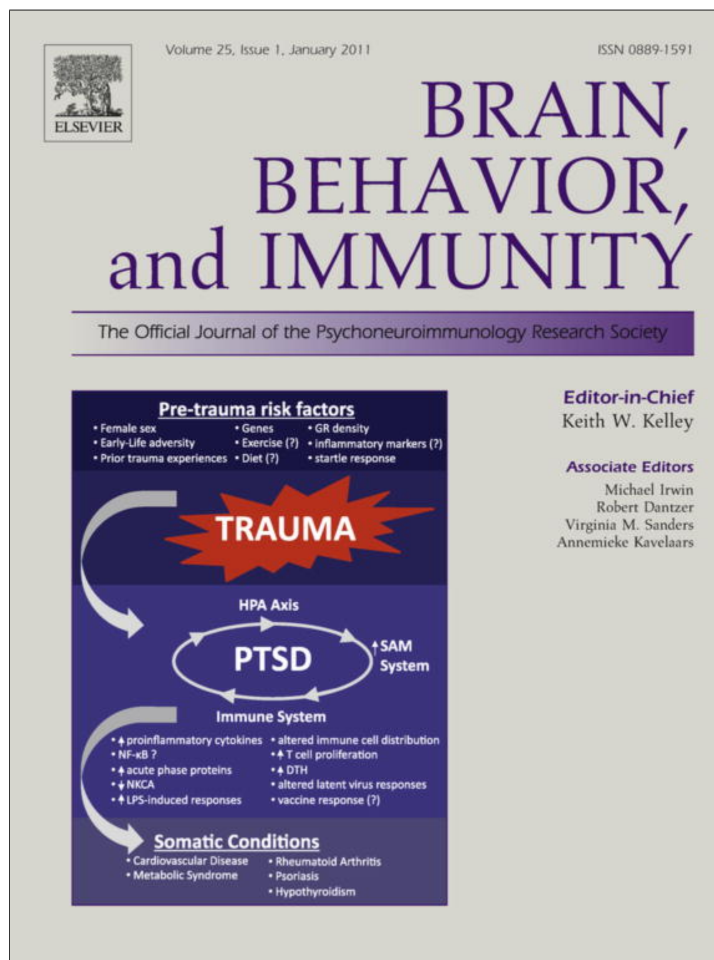

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A circadian clock in murine bone marrow-derived mast cells modulates IgE-dependent activation *in vitro*

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ABSTRACT

Circadian rhythm is expressed in most organisms, and many functions and parameters in the immune system are associated with time-of-day. However, it is largely unknown if local circadian clocks in immune cells directly control physiological outcomes. We hypothesized that a circadian clock in murine bone marrow derived mast cells (BMMCs) modulates IgE-dependent activation *in vitro*. Mature BMMCs, grown from bone marrow of C57BL/6 mice, were synchronized with serum rich media (50% horse serum). Total RNA was harvested from BMMCs at 4 h intervals for up to 72 h following synchronization and expression of circadian genes (*mPer1*, *mPer2*, *Bmal1*, *Rev-erbα*, and *Dbp*) was measured by quantitative PCR. Serum shock synchronized expression of circadian genes (*mPer2*, *Bmal1*, *Rev-erbα*, and *Dbp*) in BMMCs. Synchronized BMMCs stimulated via the high affinity IgE receptor (FcεRI) at different time intervals display circadian rhythms in IL-13 and IL-6 mRNA expression. The expression of *fcer1a* gene and FcεRIα protein displayed a circadian pattern following serum shock, with mean periods of 18.9 and 28.6 h, respectively. These results demonstrate that synchronized BMMCs provide an *in vitro* model to study circadian mechanism(s) associated with allergic disease and that circadian oscillation of cytokine production following IgE-dependent activation is at least in part due to circadian oscillation of FcεRIα.
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1. Introduction

Circadian rhythm is a conserved feature of organisms ranging from cyanobacteria to humans (Dunlap, 1999) that is manifested as oscillations of biological processes with a periodicity of approximately 24 h. The circadian system consists of three components: sensors, pacemakers, and effectors. Sensors include both photic and non-photic receptors that entrain the central clock to environmental cues. Afferent pathways convey information from the sensors to pacemakers that establish the rhythm, and efferent pathways alter effector organ function according to the established rhythm. In mammals, a central circadian pacemaker located in the suprachiasmatic nucleus (SCN) of the ventral hypothalamus (Gekakis et al., 1998) coordinates oscillators in peripheral organs (Reppert and Weaver, 2002).

The core circadian molecular clock in mammals consists of a series of transcriptional and translational loops involving *Period1–3*, *Clock*, *Bmal1*, and *Cry1–2* genes (Takahashi et al., 2008). CLOCK and BMAL1 heterodimers translocate into the nucleus, bind to E-box motifs (CACGTG), and activate transcription of *Per* and *Cry* genes (Gekakis et al., 1998). PER and CRY proteins synthesized in the cytoplasm accumulate to a critical level and bind to CKIε/δ kinase. The phosphorylated heterotrimer translocates into the nucleus and inhibits CLOCK–BMAL1 heterodimer, thereby inhibiting transcription of their own genes and other clock-controlled genes (CCGs), such as *Dbp* (Kume et al., 1999). Another feedback loop directs alternating activation and repression of BMAL1 expression by the nuclear receptors RORα and REV-ERBα (Etchegaray et al., 2003), respectively (Sato et al., 2004; Emery and Reppert, 2004).

In vivo, peripheral tissues and cells are synchronized by the SCN. However, *in vitro*, cycling of circadian oscillations in individual cells that are not entrained to an external signal is uncoordinated, and therefore, self-sustained oscillation in populations of cells is unsynchronized. Detuned circadian oscillators observed in cell lines (Nagoshi et al., 2004) and some primary cells (Keller et al., 2009) can be synchronized by a serum shock (Durgan et al., 2005; Nagoshi et al., 2004; Balsalobre et al., 1998) or pharmacological substances (Yagita et al., 2010; Huang et al., 2009; Wu et al., 2008, 2007; Balsalobre et al., 2000a).

Many diseases, including immune mediated diseases such as allergic asthma, exhibit circadian variation of symptoms. Nocturnal symptoms of asthma are common and include bronchoconstriction, airway inflammation and hyperreactivity, dyspnea, cough, and apnea during the night (Smolensky et al., 2007; Kelly et al., 2004;

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Irvin et al., 2000; Kraft et al., 1999; Ballard et al., 1989). Mast cells play a central role in allergic diseases (Brown et al., 2008); and while circadian rhythmicity is an established feature of many immune cells, including peripheral blood mononuclear cells (PBMCs) (Born et al., 1997; Murphy et al., 2007), natural killer (NK) cells (Arjona and Sarkar, 2006), and peritoneal macrophages (Keller et al., 2009), it is unknown if or how circadian rhythms may influence mast cell function. While it remains unknown how circadian variation may directly influence mast cell function, it has been reported that plasma histamine levels in patients with mastocytosis exhibit a circadian variation (Friedman et al., 1989), and circadian variation in mast cell number has been observed in thyroid gland (Catini et al., 1994), ovaries, (Gaytan et al., 1991), tongue, pinna, and dorsal skin (Chen and He, 1989). Despite this evidence, it remains unknown whether circadian clock genes are expressed in mast cells and whether these genes influence mast cell function.

Crosslinking of FcεRI (high-affinity IgE receptor) on the surface of mast cells by allergen bound IgE results in immediate release of preformed inflammatory mediators including histamine and proteases, that initiate an immediate hypersensitivity reaction (Metcalfe et al., 2009). In addition, activation of mast cells stimulates production of cytokines and chemokines, including IL-6, IL-13, CXCL8, and CCL3, which promote the late-phase inflammatory reactions.

The present study was conducted to test the hypothesis that a circadian clock expressed in murine bone marrow derived mast cells (BMMCs) modulates IgE-dependent activation *in vitro*. BMMCs were serum shocked with a high concentration of horse serum, and expression of circadian clock genes (*mPer1, mPer2, Bmal1, Rev-erbα,* and *Dbp*) were monitored for up to 72 h. Inflammatory cytokines were measured following FcεRI stimulation to examine the influence of circadian rhythm on mast cell activation. Lastly, rhythmic expression of FcεRI was evaluated by both quantitative real-time PCR and flow cytometry.

2. Materials and methods

2.1. Animals

Four week-old C57BL/6 mice were obtained from The Jackson Laboratories (Bar Harbor, ME). Mice were euthanized by CO_2 asphyxiation followed by collection of femoral bone marrow for generation of BMMCs. All animal protocols were approved by the Institutional Animal Care and Use Committee of East Carolina University.

2.2. Cell culture

Mouse BMMCs were cultured from femoral bone marrow of C57BL/6 mice. Bone marrow was collected from 3 to 4 mice per BMMC culture and all experiments were repeated 6 times with 6 different cultures of BMMCs. Cells were cultured in RPMI 1640 supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 25 mM Hepes, 1 mM sodium pyruvate, nonessential amino acids (Sigma–Aldrich, St. Louis, MO), 0.0035% 2-mercaptoethanol, and 300 ng/ml recombinant murine IL-3 (PeproTech, Rocky Hill, NJ). BMMCs were used after 4–6 weeks of culture, a time at which >95% of the cells are mast cells as determined by granule content and high surface expression of FcεRI.

2.3. Synchronization of BMMC by serum shock

Bone marrow-derived mast cells were plated in 25 cm^2 cell culture flask. At time $t = 0$, the medium was exchanged with serum rich medium (RPMI 1640 supplemented with 50% horse serum (GIBCO), 100 U/ml penicillin, 100 μg/ml streptomycin, 25 mM Hepes, 1 mM sodium pyruvate, nonessential amino acids, 0.0035% 2-mercaptoethanol, and 300 ng/ml recombinant murine IL-3), and after 2 h this medium was replaced with 1% FBS RPMI with IL-3 (Balsalobre et al., 1998). At the time points indicated in Fig. 1A, BMMCs were collected for subsequent analysis as described below.

2.4. Quantitative real-time PCR

Total RNA from BMMCs was isolated every 4 h after serum shock using a Qiagen RNeasy Mini Kit (Qiagen, Valencia, CA). Concentrations of RNA for each sample were determined using the NanoDrop 2000 (Thermo Fisher Scientific, Wilmington, DE). RNA (0.5 μg) was reverse transcribed to obtain cDNA using a QuantiTect reverse transcription kit (Qiagen). Quantitative real-time PCR was performed using QuantiTect primer assays and SYBR green master mix (Qiagen). A Bio-Rad iCycler was used to obtain cycle threshold (Ct) values for target and internal reference cDNA levels. Target cDNA levels were normalized to non-oscillated Gapdh (Fig. S1), an internal reference using the equation $2^{-[\Delta Ct]}$, where ΔCt is defined as $Ct_{target} - Ct_{internal\ reference}$. Values shown are the average of 6 independent experiments conducted with 6 different batches of BMMCs.

2.5. FcεRI-mediated BMMC activation

Mouse BMMCs were seeded at 1×10^6 cells/well in a 6-well plate and sensitized 1 h with 1 μg/ml mouse IgE anti-DNP (Sigma–Aldrich) before each given time point (0, 8, 24, 40, 56, and 64 h) following serum shock. Activation of BMMCs was performed at time points based upon surface expression of FcεRIα with peak expression at 0, 24, and 56 h; and nadir at 8 and 40 h. These times were therefore used for analysis of cytokine mRNA and release. Following 1 h sensitization, cells were washed with PBS and 100 ng/ml DNP–HSA was added to the cells (Sigma–Aldrich) (see Fig. 1B for time course).

Total RNA from BMMCs was isolated 30 min following addition of DNP using a Qiagen RNeasy Mini Kit. Supernatants of BMMCs that had been incubated with IgE and stimulated with DNP for 4 h were analyzed for IL-6, IL-13, MIP-1α, and TNF-α using a mouse Duo-Set ELISA system (R&D Systems, Minneapolis, MN).

In another set of cells after 30 min incubation with DNP–HSA at 37 °C, *p*-nitrophenyl-*N*-acetyl-β-D-glucopyranoside was added to cell supernatants and lysates (generated by addition of 0.1% Triton X-100) for 90 min as a chromogenic substrate for *N*-acetyl-β-D-hexosaminidase. The reaction was stopped with 0.2 M glycine. Optical density was measured at 405 nm using a Synergy HT microplate reader (BioTek, Winooski, VT). β-Hexosaminidase release was expressed as the percentage of total cell content after subtracting background release from unstimulated cells.

In addition to FcεRI-mediated activation, BMMCs were seeded at 1×10^6 cells/well and stimulated with 0.5 μm ionomycin (Sigma–Aldrich) at each given time point following serum shock (Fig. 1B). Total RNA and supernatants from BMMC cultures were collected following 30 min or 4 h incubation with ionomycin as described above.

2.6. Flow cytometry

For measurement of FcεR1α, BMMC were harvested at indicated time points after serum shock and incubated with 2 μg/ml phycoerythrin (PE)-conjugated anti-mouse FcεR1α (MAR-1, eBioscience) at room temperature for 30 min. PE-labeled Armenian Hamster IgG (eBioscience) was used as isotype control. Flow cytometric analysis of the stained cells was performed with a FACScan flow cytometer (Becton, Dickinson and Company) equipped with

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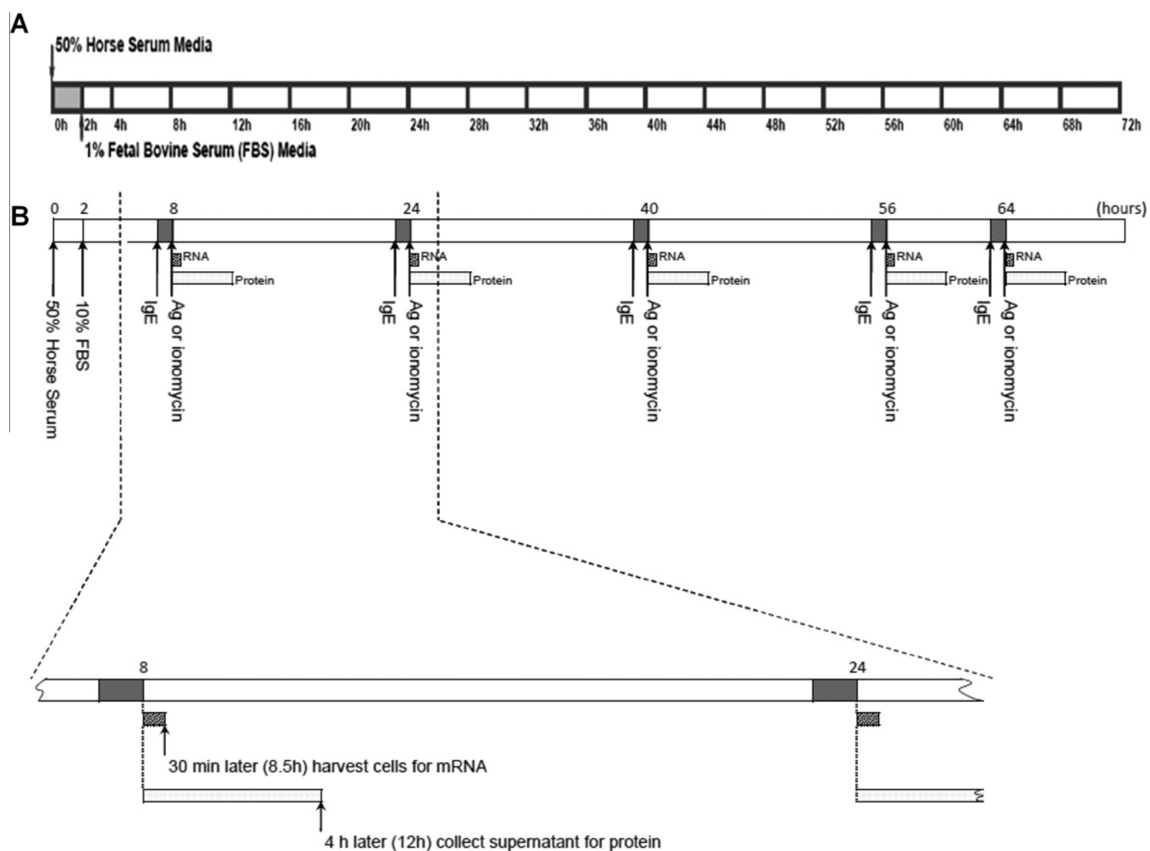

Fig. 1. Protocols. Serum shock synchronization protocol (A) and IgE/Ag or ionomycin stimulation protocol (B). Samples were collected at each time point indicated.

CELLQUEST software. 10,000 BMMCs per sample were analyzed and mean fluorescence intensity (MFI) values at each time point are reported.

2.7. Periodicity analysis

The time series data of circadian gene expression, cytokine expression, fcer1a gene expression, and FcεR1α mean fluorescence intensity (MFI) were analyzed for circadian rhythmicity by the cosinor method (Bingham et al., 1982; Nelson et al., 1979). The time series of the parameters of the cosinor, i.e. amplitude (half the difference between the minimum and maximum of the fitted cosine function), MESOR (middle value of the fitted cosine curve representing the rhythm adjusted mean), and acrophase (time of peak value of the fitted cosine function), were tested by the cosinor parameters test designed by Bingham et al. (1982).

2.8. Statistical analysis

Variation in cytokine expression and circadian gene expression in activated BMMC data were analyzed by one-way ANOVA or two-way (time × treatment) repeated-measures ANOVA using SPSS software (SPSS, Inc., Chicago, IL) followed by Bonferroni post hoc tests where appropriate. Values are reported as means ± Standard Error. A value of $p < 0.05$ was considered significant.

3. Results

3.1. Expression of circadian clock genes in serum synchronized BMMCs

Expression of *mPer2*, *Bmal1*, *Rev-erbα*, and *Dbp* exhibited a robust oscillation over a 72 h period following 2 h synchronization with serum rich media (50% horse serum) (Fig. 2). *mPer2* and *Rev-erbα*, which are negative regulatory arms of the circadian transcriptional complex, displayed peak expression levels at 19.8 and 18.5 h following synchronization. In contrast, *Bmal1*, a positive regulatory arm of the circadian transcriptional complex, exhibited peak phase at 5.9 h after synchronization (Table 1). The peak expression of *Bmal1* was 14 h in advance of the peak phase of *mPer2*. Overall, serum shock synchronized an oscillating expression profile of circadian gene mRNAs as a function of time in BMMCs.

3.2. Cytokine production in serum synchronized BMMCs following IgE-dependent and IgE-independent activation

Since we demonstrated circadian clock genes are rhythmically expressed in BMMCs, we next determined if they influence activation of BMMCs. To address this question, cytokine mRNA expression profiles were investigated following either IgE sensitization and Ag challenge or ionomycin stimulation in synchronized BMMCs at the indicated time points (0, 8, 24, 40, 56, and 64 h). IL-13 mRNA expression levels peaked at 0 and 56 h following FcεRI stimulation, while expression was lowest at 8 h ($p = 0.034$: 0 vs. 8 h; $p = 0.037$: 8 vs. 56 h; Fig. 3A). While IL-13 mRNA levels suggested a pattern of circadian oscillation and individual comparison between times was significant, analysis by fitting cosinor curve did not reach significance ($p = 0.0536$). Interestingly, IL-6 mRNA expression levels displayed the same pattern following IgE/Ag stimulation as IL-13 mRNA expression following serum shock (Fig. 3B). However, the peak and nadir of IL-6 mRNA expression levels in FcεRI stimulated BMMCs did not reach statistical significance. In synchronized BMMCs, ionomycin stimulated IL-13 mRNA production did not display any circadian oscillation ($p = 0.1337$, Fig. 3C). Likewise, IL-6 mRNA expression levels in ionomycin

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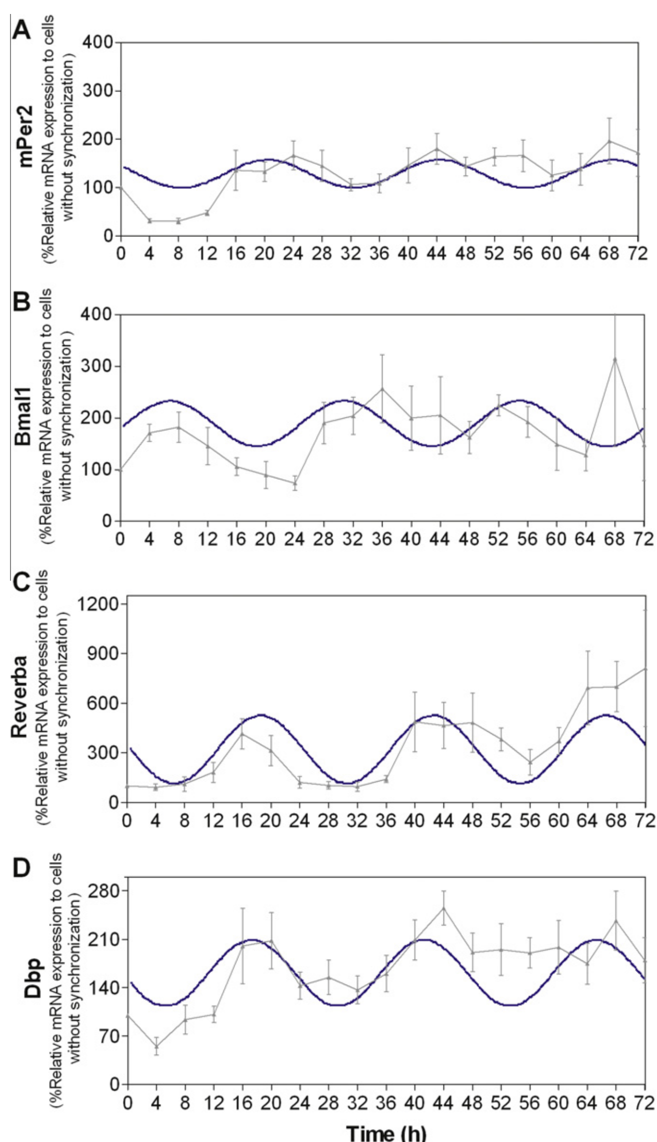

Fig. 2. Circadian clock gene expression in BMMCs following serum shock. mRNA expression of circadian clock genes (A) *mPer2*, (B) *Bmal1*, (C) *Rev-erbα*, and (D) *Dbp*. mRNA expression was normalized to non-oscillating Gapdh expression levels and expressed as a percentage of mRNA expression in non-serum shocked cells. Gray lines represent mean ± SEM for expression data. The blue line displays best-fitting 24 h cosine analysis predicted by Time Series Analysis-Single Cosinor v. 6.3 software. n = 6 independent experiments. (For interpretation of the references in colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
Peak phases and amplitudes of circadian clock gene expression in BMMCs.

Circadian gene	Peak-phase (h)	Amplitude[a]	*p* Value[b]
mPer2	19.8	33.1	0.0056
Bmal1	5.93	85.30	0.0098
Rev-erbα	18.53	206.00	0.0001
Dbp	17.27	48.00	0.0002
mPer1	2.43	19.70	0.0590

n = 6 independent experiments.
[a] Amplitudes (H0: Amplitude = 0) have been calculated by Time Series Analysis-Single Cosinor v. 6.3 software (Expert Soft Technologie).
[b] *p* Values (H0: Amplitude = 0) have been calculated by Time Series Analysis-Single Cosinor v. 6.3 software (Expert Soft Technologie).

treated synchronized BMMCs displayed no oscillation over 72 h (Fig. 3D). In synchronized BMMCs, TNFα, and MIP1α mRNA expression following activation by IgE/Ag or ionomycin did not result in circadian oscillation (data not shown).

In contrast to mRNA expression levels, IL-13, IL-6, TNFα, and MIP1α protein secretion from FcεRI or ionomycin stimulated synchronized BMMCs as measured by ELISA did not display any circadian oscillation (data not shown). Lastly, we measured β-hexosaminidase release as an indicator of mast cell degranulation, however, we did not observe any circadian oscillation in β-hexosaminidase release (data not shown).

3.3. Effects of FcεRI-mediated BMMC activation on mPer2 expression

To test whether FcεRI stimulation influences the oscillating expression of the circadian gene *mPer2*, mRNA expression profiles were investigated in 3 treatment groups: serum shocked BMMCs, FcεRI activated BMMCs synchronized by serum shock, and FcεRI activated BMMCs in the absence of serum shock. FcεRI stimulation had little effect on *mPer2* expression over 72 h in unsynchronized cells (Fig. 4). Serum shock alone, induced/unmasked a significant circadian oscillation in mPer2 levels ($F(5, 90) = 3.760$, $p = 0.004$). However, FcεRI activation in combination with serum shock did not affect the amplitude or phase of *mPer2* mRNA oscillation.

3.4. Circadian expression of FcεRIα in serum synchronized BMMCs

FcεR1α expression displays a circadian oscillation in pineal gland (Ganguly et al., 2007), however, it remains unknown if FcεRIα expression is also under circadian control in mast cells. FcεR1α mRNA levels were determined by quantitative real-time PCR in BMMC every 4 h up to 72 h following synchronization. FcεR1α mRNA expression displayed a circadian oscillation ($A = 41.8$, $p = 0.028$), with a robust peak at 12 h and a depression around 22 h following synchronization (Fig. 5A). Further, this circadian oscillation of FcεR1α mRNA levels was maintained up to 72 h following synchronization.

To investigate whether FcεRIα protein is expressed in a circadian pattern, we measured surface expression of FcεRI by flow cytometry every 4 h following synchronization. Fig. 5B shows the surface expression profiles of FcεRI at representative time points following synchronization of BMMCs. FcεRIα expression at 32 h displayed a left shift as compared to 8 or 64 h indicating that receptor expression at 32 h is higher than the other 2 time points. Mean fluorescence intensity (MFI) values of FcεRIα expression showed a circadian oscillation, with a period around 28.6 h and a robust peak around 24 h following synchronization (Fig. 5C and Table 2). The rhythm in FcεRIα surface expression levels was out of phase with mRNA levels (Fig. 5). Without synchronization, MFI values of FcεRIα expression on BMMCs did not show any oscillation over 72 h (MFI: mean ± SEM = 118.91 ± 1.54, oscillation data not shown).

4. Discussion

To our knowledge, this is the first report on expression and function of circadian clock genes in mast cells. The most important finding in this study is that circadian clock genes are rhythmically expressed in serum synchronized mouse bone marrow-derived mast cells. Cytokine mRNA expression following IgE-dependent activation of BMMCs and serum synchronization exhibited a trend towards a circadian pattern. However, IgE/Ag stimulation of BMMCs did not shift existing circadian expression of a key circadian gene, *mPer2*. Following serum synchronization, the expression of FcεRIα displayed a circadian pattern that was associated with changes in cytokine mRNA expression in activated BMMCs.

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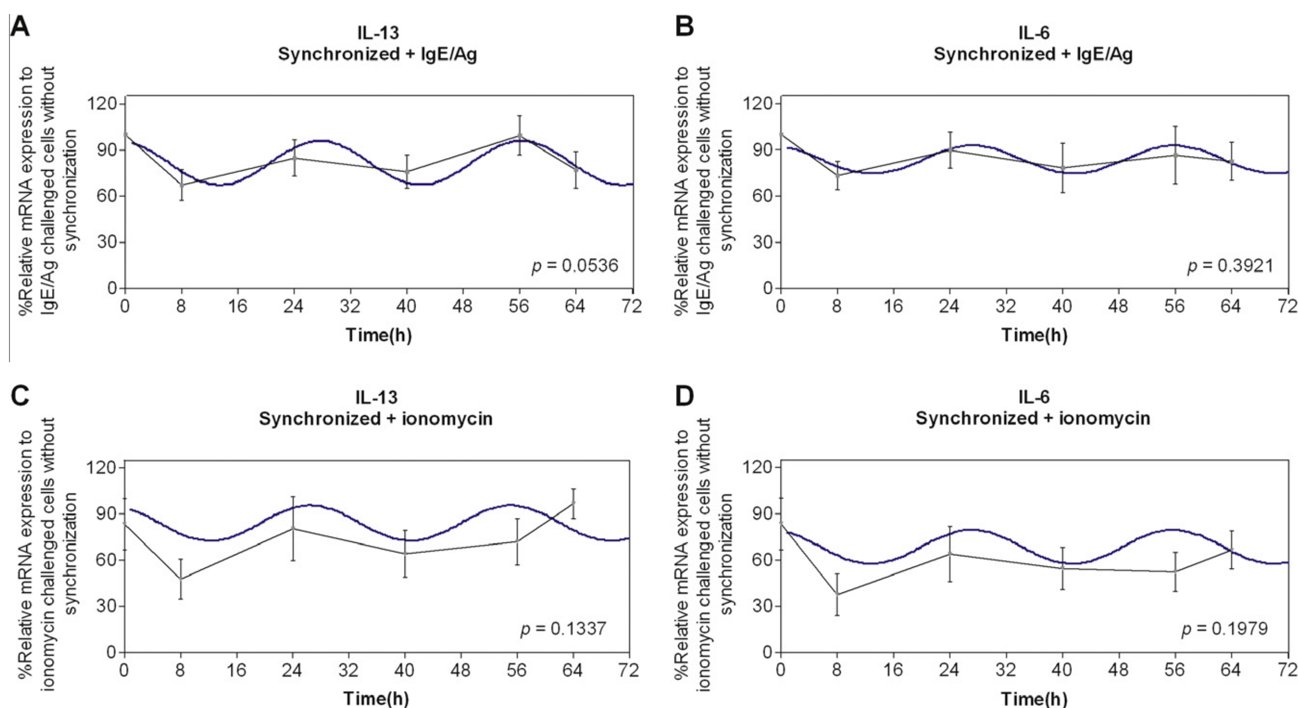

Fig. 3. Cytokine expression in synchronized BMMCs following IgE-dependent and -independent activation. BMMCs, following serum shock, were sensitized with IgE anti-DNP and stimulated for 30 min with (A and C) DNP or (B and D) ionomycin, and cells were collected. (A and B) IL-13 and (C and D) IL-6 mRNA expression following activation were normalized to non-oscillating Gapdh expression levels and expressed as a percentage of the non-serum shocked controls. Gray lines are presented as means ± SEM. The blue line displays cosine analysis fitting curve predicted by Time Series Analysis-Single Cosinor v. 6.3 software. $n = 6$ independent experiments. p-values (H0: Amplitude = 0) have been calculated by Time Series Analysis-Single Cosinor v. 6.3 software. (For interpretation of the references in colour in this figure legend, the reader is referred to the web version of this article.)

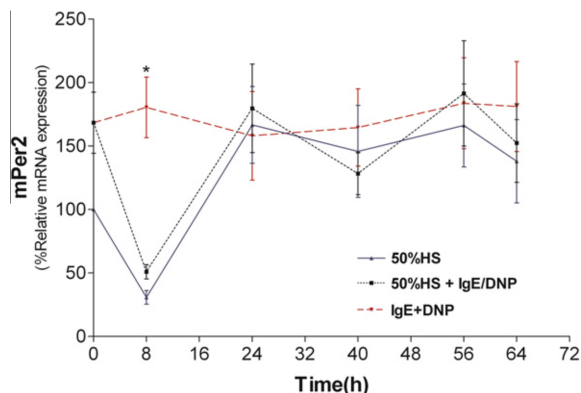

Fig. 4. Effect of FcεRIα-dependent activation on circadian gene expression. BMMCs were treated with serum, IgE + DNP, or both. Serum shock alone (blue), IgE + DNP alone (red), serum shock in combination with IgE + DNP (black). mPer2 expression were determined by quantitative real-time PCR, normalized to non-oscillating Gapdh, and expressed as percentage of 0 h without serum shock. Data is presented as means ± SEM. $n = 6$ independent experiments. *Significantly different ($p < 0.05$) from other two groups. (For interpretation of the references in colour in this figure legend, the reader is referred to the web version of this article.)

Circadian clock genes are expressed in a number of immune cells including natural killer (NK) cells (Arjona and Sarkar, 2008, 2006, 2005), macrophages (Keller et al., 2009), and T-cell subsets (Dimitrov et al., 2009). In this study, we provide the first evidence that circadian clock genes are expressed in BMMCs in a circadian pattern for up to 72 h following serum shock. Serum shock is a common method utilized to synchronize the circadian clock in cell cultures and is equivalent to the ability of light cycles to entrain the circadian clock *in vivo* (Hastings et al., 2003). It does not jumpstart damped circadian oscillators but instead synchronizes dephased circadian oscillators. This has been demonstrated in fibroblasts, where individual cells have been found capable of generating self-sustained circadian rhythms of gene expression (Nagoshi et al., 2004; Balsalobre et al., 1998). Here we report that serum shock synchronizes individual rhythms in BMMCs to a common phase as does it in fibroblasts (Nagoshi et al., 2004; Balsalobre et al., 1998), adipose-derived stem cells (Wu et al., 2007), natural killer cells (Murphy et al., 2007), and cardiomyocytes (Durgan et al., 2005). The mechanism(s) underlying serum shock synchronization of circadian gene expression *in vitro* is still unknown. Neither the activation of cAMP-dependent kinases nor the glucocorticoid receptor appear to be required for the synchronization of circadian gene expression by serum shock (Balsalobre et al., 2000b).

The phase relationship between rhythms of circadian genes in BMMCs is consistent with that in other cells. As reported in other cells, the heterodimer Bmal1 and Clock bind to E-box motifs in the promoter region of circadian genes to initiate their expression (Ripperger and Schibler, 2006; Gekakis et al., 1998). In BMMCs, the expression of *Bmal1* occurs earlier than *Rev-erbα*, *mPer2*, and *Dbp* suggesting that the heterodimer Bmal1 and Clock bind to the promoter region of *Rev-erbα*, *mPer2*, and *Dbp*. Further, the increased expression of *Rev-erbα* correlates with decreased expression of *Bmal1*. Previous studies indicate that *mPer2* protein interacts with BMAL1 and CLOCK to negatively regulate of the circadian clock (Langmesser et al., 2008). Dbp, a PAR leucine zipper transcription factor, is one of the clock-controlled genes and is transcriptionally regulated by BMAL1–CLOCK heterodimer (Ripperger and Schibler, 2006). The expression of Dbp has little effect on circadian clock gene expression, but rather is an output from the circadian system that regulates transcription of downstream genes (Wang et al., 2010). Taken together, these results indicate that mast cells contain a local intrinsic clock.

The function of a local intrinsic clock in BMMCs was investigated by measuring the activation of cells at specific time points following serum shock synchronization. Several studies have shown a circadian variation of cytokine levels in NK cells and

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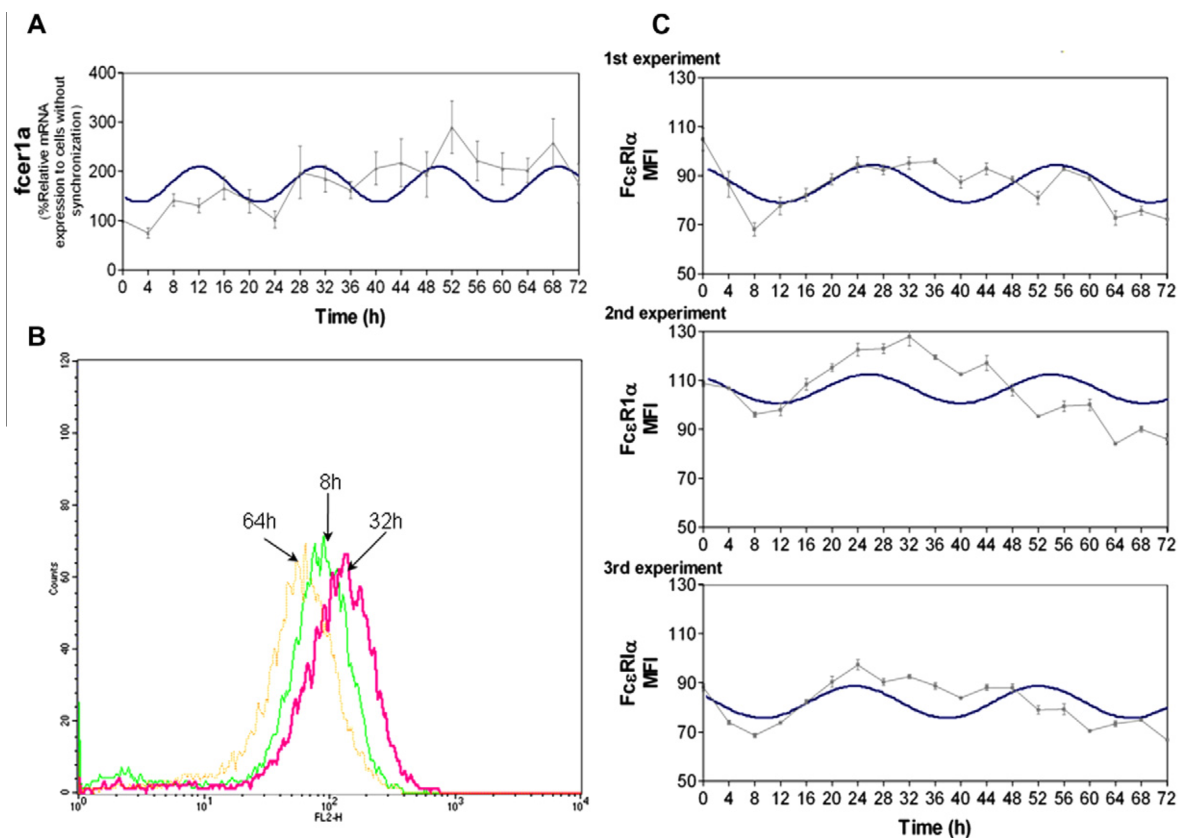

Fig. 5. FcεRIα expression in BMMCs following synchronization. (A) mRNA levels of fcer1a in BMMCs following serum shock (mean ± SEM, $n = 6$, best-fitting cosine blue line). (B) Surface protein expression of FcεRIα in BMMCs following serum shock. Histogram on the left shows 3 representative time points (8, 32, and 64 h). (C) Mean fluorescence intensity (MFI) values are shown as means ± SEM (gray line) at each time point with best-fitting cosine blue line ($n = 3$). (For interpretation of the references in colour in this figure legend, the reader is referred to the web version of this article.)

Table 2
Amplitudes of MFI for FcεRIα in BMMCs.

Experiment no.	Amplitude[a]	*p* Value[b]
1	7.71	0.0001
2	5.93	0.0477
3	6.57	0.0004

$n = 6$ independent experiments.
[a] Amplitudes (H0: Amplitude = 0) have been calculated by Time Series Analysis-Single Cosinor v. 6.3 software.
[b] *p* Values (H0: Amplitude = 0) have been calculated by Time Series Analysis-Single Cosinor v. 6.3 software.

macrophages (Keller et al., 2009; Hayashi et al., 2007; Arjona and Sarkar, 2005). TNF-α and IL-6 secretion patterns exhibit a circadian oscillation in *ex vivo* LPS-stimulated macrophages (Keller et al., 2009). Here, we report rhythms in mRNA levels of IL-13 and IL-6 in IgE-mediated activation of synchronized BMMCs. Mast cells, activated through antigen- and IgE-dependent aggregation of the high-affinity IgE receptor, FcεRI, produce a broad range of mediators, including preformed mediators and newly synthesized cytokines/chemokines (Galli et al., 2008; Kalesnikoff and Galli, 2008; Brown et al., 2008). Newly synthesized cytokines produced by mast cells include, but are not limited to, IL-6, IL-13, and TNF-α. We tested IL-13 production over time due to the important role of IL-13 in allergic asthma through initiation of a late phase response (Toru et al., 1998), induction of IgE synthesis by B cells (Punnonen and de Vries, 1994), proliferation of mast cells, and increasing FcεRI expression (Kaur et al., 2006). In human asthma, IL-13 mRNA in BAL cells and IL-13 in BAL fluid are significantly increased (Huang et al., 1995). The increase in IL-13 is associated predominantly with the late asthmatic response and is correlated with eosinophil numbers. Furthermore, mast cells are a source of IL-13. In the present study, we showed that IL-13 mRNA oscillated in mast cells. Studies have shown that the number of BAL cells expressing IL-13 mRNA and IL-13 levels in BAL fluid are increased at 4:00 AM as compared with 4:00 PM (Kraft et al., 2001). Taken together, these observations indicate that oscillation of IL-13 mRNA in mast cells can directly influence nocturnal expression of asthma symptoms in human.

Compared to IL-13, IL-6 is synthesized earlier in FcεRI-stimulated BMMCs and promotes the proliferation of mucosal T helper T(h)2 cells, which are critical effector cells in asthma (Scheller et al., 2006). The phases of IL-13 mRNA and IL-6 mRNA were identical, with nadirs at 8 and 40 h and peaks at 24 and 56 h. These observations suggest mast cells stimulated by IgE/Ag along the circadian cycle could possibly have different outcomes. The oscillation of cytokine mRNA produced by IgE-dependent activation of mast cells may be associated with circadian pathological responses that have been observed in patients with mastocytosis (Catini et al., 1994) and nocturnal responses in allergic asthma (Smolensky et al., 2007).

Other than activation through FcεRI, mast cells can also participate in physiological and pathological processes as a result of their activation by certain chemicals. To verify whether circadian oscillation of mast cell activation is dependent upon FcεRI expression or represents an intrinsic change in mast cell state, we used ionomycin to activate synchronized BMMCs independent of FcεRI and measured cytokine production over 72 h. Interestingly, IL-13 mRNA and IL-6 mRNA did not display an oscillation pattern

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following ionomycin challenge as observed following IgE/Ag stimulation of BMMCs. This result provides evidence that circadian regulation of mast cell activation may be FcεRI-dependent.

To further investigate IgE-dependent activation of BMMCs, FcεRI expression was measured up to 72 h. The diurnal variations in FcεRIα expression first observed in rat pineal glands suggests a biological significance of the timing of receptor function (Ganguly et al., 2007). Although we cannot formally exclude other influences, we speculate that oscillation of cytokine production in IgE/Ag stimulated BMMCs is due to circadian variation in FcεRIα expression. In fact, the IL-13 and IL-6 mRNA levels observed in this study from IgE/Ag stimulated BMMCs peak at 24 and 56 h, when the surface expression of FcεRIα peaks. Further experiments are required to unravel the underlying mechanism and determine which circadian factors that may regulate the oscillation of FcεRIα in BMMCs.

Cytokine proteins produced by IgE-dependent activation of synchronized BMMCs did not show circadian oscillation. The lack of observable oscillation in cytokine protein may have been due to the 4 h delay between stimulation of the cells and collection of medium bathing the cells. During the 4 h period, FcεRI expression on the surface of the cells was probably changing. Exposing mast cells to IgE *in vitro* leads to recruitments of FcεR1 to the cell surface (Kitaura et al., 2004). In addition to IgE receptor recruitment, cytokines released during the 4 h period accumulate in the medium, decreasing the ability to detect circadian oscillations in cytokine release. Cytokine mRNA levels were measured in a much shorter period than that of protein levels. Therefore, without time disturbance, the circadian oscillation of cytokine mRNA was observed in synchronized BMMC through an IgE-dependent pathway. The non-oscillation of mast cell degranulation begins to suggest that circadian clock genes only influence de novo synthesis of mediators within BMMCs.

Even though it appears that the circadian system can influence IgE/Ag activated BMMCs, the influence of IgE/Ag stimulation on the phases, phase relations, and amplitudes of circadian genes is still unknown in mast cells. Evidence suggests that the relationship of the immune system and the circadian system is bidirectional (Coogan and Wyse, 2008). In this study, we show circadian expression of *mPer2* can be triggered by serum shock but not IgE/Ag stimulation alone. Meanwhile, the initiated circadian phase of *mPer2* by serum shock cannot be shifted via IgE/Ag stimulation. We cannot, however, exclude the prolonged effects of IgE/Ag on BMMCs, including the production of proinflammatory cytokines, such as TNF-α, which could phase shift the clock in BMMCs.

Here, we report that a circadian clock located in mast cells regulates surface expression of IgE receptors. It is possible that IgE receptor expression might be higher during the early night than other time points influenced by low levels of cortisol. If asthmatics are exposed to Ag during periods of higher expression of the IgE receptor, activation of mast cells may produce more IL-13 which can recruit eosinophils into the airway and contribute to the nocturnal asthmatic response.

In conclusion, BMMCs rhythmically express circadian clock genes and BMMC activation through IgE/Ag exhibits a trend towards a circadian pattern which is linked to circadian expression of FcεRIα. The local conserved circadian clock in mast cells begins to provide further understanding of the dynamics in allergic disease and in particular diseases such as allergic asthma and mastocytosis where diurnal patterns have been observed. Meanwhile, this research should be considered when planning future *in vivo* studies involving mast cells. The phenotype of mast cells will vary significantly throughout the day due to synchronization with the SCN. The same experiment done with different time of the day could yield different data. By increasing our understanding of the circadian regulation of mast cells, we may begin to explore novel therapeutic strategies in the treatment of allergic disease.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbi.2010.09.007.

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