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Circadian regulation of allergic reaction by the mast cell clock in mice
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Circadian regulation of allergic reactions by the mast cell clock in mice

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Background: It remains elusive how allergic symptoms exhibit prominent 24-hour variations. In mammals the circadian clocks present in nearly all cells, including mast cells, drive the daily rhythms of physiology. Recently, we have shown that the circadian clocks drive the daily rhythms in IgE/mast cell–mediated allergic reactions. However, the precise mechanisms, particularly the specific roles of the mast cell–intrinsic clockwork in temporal regulation, remain unclear.

Objective: We determined whether the mast cell clockwork contributes to the temporal regulation of IgE/mast cell–mediated allergic reaction.

Methods: The kinetics of a time of day–dependent variation in passive cutaneous anaphylactic reactions were compared between mast cell–deficient mice reconstituted with bone marrow–derived cultured mast cells generated from mice with a wild-type allele and a dominant negative type mutation of the key clock gene *Clock*. We also examined the temporal responses of wild-type and *Clock*-mutated bone marrow–derived cultured mast cells to IgE stimulation *in vitro*. Furthermore, factors influencing the mast cell clockwork were determined by using *in vivo* imaging.

Results: The *Clock* mutation in mast cells resulted in the absence of temporal variations in IgE-mediated degranulation in mast cells both *in vivo* and *in vitro* associated with the loss of temporal regulation of FcεRI expression and signaling. Additionally, adrenalectomy abolished the mast cell clockwork *in vivo*.

Conclusion: The mast cell–intrinsic clockwork, entrained by humoral factors from the adrenal gland, primarily contributes to the temporal regulation of IgE/mast cell–mediated allergic reactions. Our results reveal a novel regulatory mechanism for IgE-mediated mast cell responses that might underlie the circadian pathophysiology in patients with allergic diseases.

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Key words: *The circadian clock, mast cells, IgE, allergy, mouse*

Abbreviations used

BMCMC:	Bone marrow–derived cultured mast cell
ChIP:	Chromatin immunoprecipitation
mMCP:	Mouse mast cell protease
PCA:	Passive cutaneous anaphylaxis
PER2::LUC:	Period2::Luciferase
siRNA:	Small interfering RNA
ZT:	Zeitgeber time

Allergic diseases are characterized by symptoms that exhibit prominent 24-hour variations.[1,2] For instance, in patients with allergic rhinitis, the symptoms are worse overnight or early in the morning ("morning attack") and often compromise nighttime sleep, resulting in a poor daytime quality of life.[3] Although these phenomena have been recognized for decades,[4,5] the precise mechanisms remain unclear.

The circadian clocks drive daily rhythms in physiology that enable organisms to keep track of the time of day. In mammals the light-entrained central oscillator located in the suprachiasmatic nucleus of the hypothalamus synchronizes the peripheral oscillators present in nearly all cell types, including mast cells, through neural and endocrine pathways.[6-11] The mechanisms of rhythm generation are based on transcriptional-translational feedback loops, wherein 2 transcription factors, CLOCK and BMAL1, activate the transcription of the Period *(Per)* and Cryptochrome *(Cry)* genes. The PER and CRY proteins in turn inhibit their own expression by repressing CLOCK/BMAL1 activity.[6-9]

In a classical mouse model of IgE/mast cell–mediated allergic reaction (passive cutaneous anaphylactic [PCA] reaction), we have recently shown that there are time of day–dependent variations that rely on the normal activity of a key clock gene, *Period2* (*Per2*),[10] suggesting that the circadian clocks drive the daily rhythms in IgE/mast cell–mediated allergic reactions. However, the precise mechanisms, particularly the specific roles of the mast cell–intrinsic clockwork in the temporal regulation, have been unclear. This study aimed to determine whether the mast cell clockwork contributes to the temporal regulation of IgE/mast cell–mediated allergic reactions.

METHODS

For more information, see the Methods section in this article's Online Repository at www.jacionline.org.

Mice

Male 5- to 6-week-old C57BL/6 mice, mast cell–deficient WBB6F1-W/Wv mice (Japan SLC, Tokyo, Japan), PER2::Luciferase (PER2::LUC) knock-in mice (C57BL/6 background),[12] and C57BL/6 $Clock^{\Delta 19/\Delta 19}$ mice[13] were bred under specific pathogen-free conditions. $Clock^{\Delta 19/\Delta 19}$ mice have an

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A to T point mutation in the 5′ splice site of intron 19 and, as a consequence, an in-frame deletion of the entire exon 19 ($Clock^{\Delta 19/\Delta 19}$), which results in loss of normal transcriptional activity.[13] This autosomal dominant mutation eventually provokes arrhythmicity in mice. All mice were housed under 12-hour light/12-hour dark conditions (the light was turned on at 6 AM, which is Zeitgeber time [ZT] 0, and the light was turned off at 6 PM, which is ZT12) with *ad libitum* access to food and water for at least 2 weeks.

Preparation of bone marrow–derived cultured mast cells

Bone marrow–derived cultured mast cells (BMCMCs) were generated from the femoral bone marrow cells of male mice.[14]

PCA reaction

Mast cell–deficient WBB6F1-W/Wv mice were reconstituted with subcutaneous injections (dorsal skin) of BMCMCs (1.5×10^6 per mouse) derived from wild-type C57BL/6 mice or C57BL/6 $Clock^{\Delta 19/\Delta 19}$ mice. Six weeks after reconstitution, the mice were sensitized subcutaneously in the BMCMC-injected dorsal skin region with mouse anti-TNP IgE (0.5 μg/ 20 μL; BD Biosciences, San Jose, Calif) to induce a PCA reaction. Saline alone was used as a negative control. The mice were then challenged intravenously 24 hours later with 50 μg of DNP-BSA (Cosmo Bio, Tokyo, Japan) with 0.5% Evans blue dye. Quantitative analysis of the PCA reaction was performed, as previously described.[10]

Statistical analysis

The statistical analyses were performed by using the unpaired Student *t* test for 2-group comparisons and ANOVA for comparison of more than 2 groups. Statcel3 software (OMS Publishing, Saitama, Japan) was used for the analysis. A P value of less than .05 was considered significant, unless otherwise indicated.

RESULTS

The mast cell clock times PCA reactions

To determine whether the mast cell–intrinsic clockwork contributes to the temporal regulation of IgE/mast cell–mediated allergic reactions, we compared the kinetics of a time of day–dependent variation in the PCA reaction between mast cell–deficient W/Wv mice subcutaneously reconstituted with BMCMCs generated from wild-type mice and mice with a dominant negative–type mutation of *Clock* ($Clock^{\Delta 19/\Delta 19}$ mice).[13] There were comparable levels of cell-surface FcεRIα and c-kit expression (without synchronization, please see below), mouse mast cell protease (mMCP) 5 and mMCP-6 mRNA expression, and Syk and Lyn protein expression and similar morphology between wild-type and *Clock*-mutated BMCMCs (see Fig E1 in this article's Online Repository at www.jacionline.org), suggesting that the *Clock* mutation did not affect the differentiation of mast cells. We also confirmed that the numbers of mast cells in the skin were comparable between mice reconstituted with wild-type BMCMCs and those reconstituted with *Clock*-mutated BMCMCs (see Fig E2 in this article's Online Repository at www.jacionline.org).

The extent of PCA reactions showed a time of day–dependent variation in control mice, with a clear nadir around the onset of night (10 PM, which was ZT16; Fig 1, *A* and *B*), as described previously in conventional wild-type mice.[10] This variation was absent in mast cell–specific *Clock*-mutated mice (Fig 1, *A* and *B*). In contrast, the daily profiles and levels of serum corticosterone and IgE were comparable between the mice (Fig 1, *C* and *D*). These results suggest that the mast cell–intrinsic clockwork is critical to the daily rhythm generation in the PCA reaction.

IgE-mediated mast cell responses show temporal variations *in vitro*

To support the *in vivo* findings, we examined the temporal responses of mast cells to IgE stimulation by using BMCMCs generated from knock-in mice expressing a PER2::LUC fusion protein (PER2::LUC BMCMCs),[12] wild-type mice, and $Clock^{\Delta 19/\Delta 19}$ mice[13] *in vitro*. We noted that the time window when the mast cell clockwork was functional appeared to be very limited (approximately 0-36 hours after medium change) in *in vitro* culture conditions based on the monitoring of bioluminescent emission of PER2::LUC BMCMCs (see Fig E3 in this article's Online Repository at www.jacionline.org).[10] This might be due to a lack of oscillator coupling in the dissociated cell cultures, leading to damping of the ensemble rhythm at the population level.[15] Therefore we compared the extent of IgE-mediated degranulation in mast cells between 12-hour cultured PER2::LUC BMCMCs (after a medium change) with the nadir of the PER2::LUC protein level and 24-hour cultured PER2::LUC BMCMCs with the peak PER2::LUC protein level. A simple medium change is a trigger to synchronize the circadian clocks in peripheral cells *in vitro*.[6-9] We avoided using potent reagents to synchronize peripheral clocks (eg, cyclic AMP activators and dexamethasone) for the *in vitro* experiments because such reagents affect IgE-mediated signaling in mast cells independent of "clock" function.[16,17]

The extent of IgE-mediated β-hexosaminidase release was significantly higher in the 12-hour cultured PER2::LUC BMCMCs than in the 24-hour cultured PER2::LUC BMCMCs (Fig 2, *A*). More detailed kinetic studies using the 6-, 12-, 18-, and 24-hour cultured BMCMCs after the medium change showed similar findings in wild-type BMCMCs but not in *Clock*-mutated BMCMCs (Fig 2, *B*). There were little differences in spontaneous β-hexosaminidase release (ie, without IgE stimulation) between wild-type and *Clock*-mutated BMCMCs at the representative time points (see Fig E4 in this article's Online Repository at www.jacionline.org). In contrast, the extent of IgE-mediated β-hexosaminidase release was comparable between the 48- and 60-hour cultured wild-type BMCMCs (Fig 2, *C*), both of which appeared to no longer have functional clockwork (see Fig E3). The extent of IgE-mediated IL-13 production also showed similar temporal variations in 12- and 24-hour cultured wild-type BMCMCs (Fig 2, *D*). Consistently, the extent of IgE-dependent intracellular Ca^{2+} mobilization and the total tyrosine phosphorylation levels of cell lysates were higher in the 12-hour cultured wild-type BMCMCs than those in the 24-hour cultured wild-type BMCMCs, which were absent in *Clock*-mutated BMCMCs (Fig 2, *E* and *F*). Interestingly, FcεRI-independent stimulation of the 12- and 24-hour cultured wild-type BMCMCs (and also *Clock*-mutated BMCMCs) with calcium ionophore A23187 showed comparable β-hexosaminidase release (Fig. 2, *G*). Collectively, the IgE-mediated degranulation and signaling in mast cells showed temporal variations *in vitro*, and disruption of the mast cell clockwork by either a *Clock* mutation or long-term culture without a medium change (synchronization) abolished the variations.

FcεRIβ transcription is under circadian control by the mast cell clock

The high-affinity IgE receptor (FcεRI) on mast cells consists of 3 subunits (FcεRI α, β, and γ chains), and IgE binds to FcεRIα with a high affinity, whereas the β and γ chains transduce the extracellular signals into the intracellular signaling pathways,

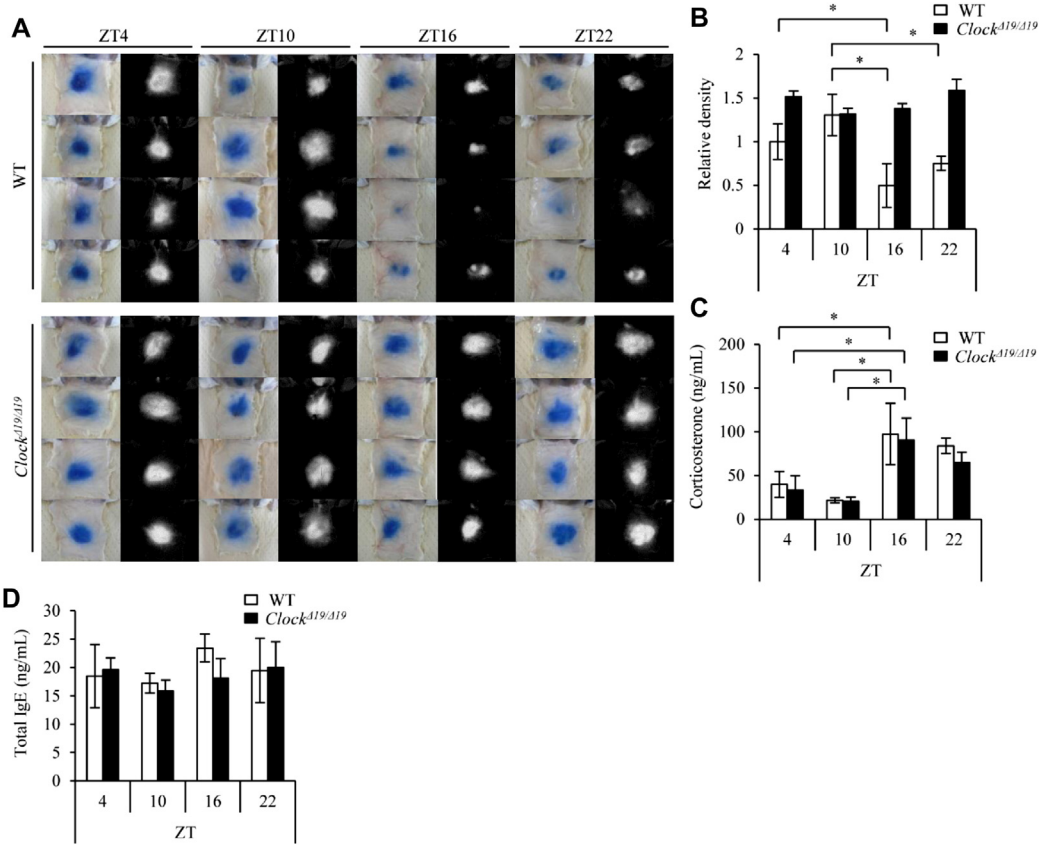

FIG 1. The mast cell–intrinsic clockwork is critical for time of day–dependent variations in PCA reactions. **A,** Representative pictures of skin color reactions in W/Wv mice reconstituted with wild-type BMCMCs *(WT)* or *Clock*-mutated BMCMCs (*Clock*$^{\Delta 19/\Delta 19}$, *left panels*) and digitalized images of density value evaluations *(right panels)* at the indicated time points. Data shown are results from the same group of wild-type or *Clock*$^{\Delta 19/\Delta 19}$ mice challenged at the indicated time points. **B,** Quantitative analysis of the data in Fig 1, *A*. **C** and **D,** Serum corticosterone (Fig 1, *C*) and total IgE (Fig 1, *D*) levels at the indicated time points. Values represent means ± SDs (n = 4 per group). A representative result of 2 independent experiments is shown. **P* < .05.

including Ca^{2+} signaling.[18-20] Given the findings that IgE-mediated intracellular Ca^{2+} mobilization showed temporal variations and that direct stimulation of Ca^{2+} signaling with A23187 did not show temporal variations in degranulation in mast cells (Fig 2, *E* and *G*), we thought that the mast cell–intrinsic clockwork likely targets FcεRI signaling–associated molecules upstream of Ca^{2+} signaling in mast cells, thereby temporally fine tuning FcεRI signaling.

A quantitative PCR analysis revealed that *FcεRIβ*, but not other FcεRI signaling–associated molecules upstream of Ca^{2+} signaling, such as *FcεRIα* and *FcεRIγ*, *Syk*, and *STIM1*,[18-20] showed circadian mRNA expression with a peak at approximately 8 hours and a nadir at approximately 20 hours after a medium change in wild-type BMCMCs (Fig 3). The circadian oscillation of FcεRIβ mRNA was not observed in *Clock*-mutated BMCMCs. We confirmed that *Per2* mRNA showed circadian oscillations in wild-type BMCMCs but not in *Clock*-mutated BMCMCs. These data suggest that FcεRIβ transcription is under circadian control by the mast cell–intrinsic clock.

CLOCK binds to the promoter of FcεRIβ and modulates its transcription

Several E-box–like elements to which the CLOCK/BMAL1 complex theoretically can bind[21] are present in the promoter regions of the mouse *FcεRIβ* (see Fig E5 in this article's Online Repository at www.jacionline.org).[22] To directly show that *FcεRIβ* is a target of the mast cell clockwork, we performed chromatin immunoprecipitation (ChIP) assays using anti-CLOCK antibody.

CLOCK bound to the promoter of *FcεRIβ* in the 6- and 24-hour, but not 12-hour, cultured wild-type BMCMCs (Fig 4, *A*), which was largely consistent with the mRNA kinetics of *FcεRIβ* (Fig 3). The integrity of the ChIP samples was confirmed by means of constitutive binding of CLOCK to the promoter of the *Per2* gene containing the noncanonical E-box enhancer 2 (E2) sequence, but not the E5 sequence, in wild-type BMCMCs, as previously reported (Fig 4, *B*).[23] CLOCK levels were comparable in the 12- and 24-hour cultured wild-type BMCMCs (Fig 4, *C*).

We then determined whether CLOCK affected the transcription of FcεRIβ in mast cells. Clock small interfering RNA (siRNA), but not control siRNA, suppressed Clock mRNA and protein levels in wild-type BMCMCs at 48 hours after siRNA transfection (Fig 4, *D* and *E*). At this time point, *FcεRIβ*, but not *Syk* and *Lyn*, mRNA levels decreased in association with the reduction of IgE-mediated β-hexosaminidase release in Clock, but not control, siRNA-treated wild-type BMCMCs (Fig 4, *F* and *G*). Consistently, the extent of the IgE-dependent increase in total tyrosine phosphorylation levels of cell lysates was reduced in Clock

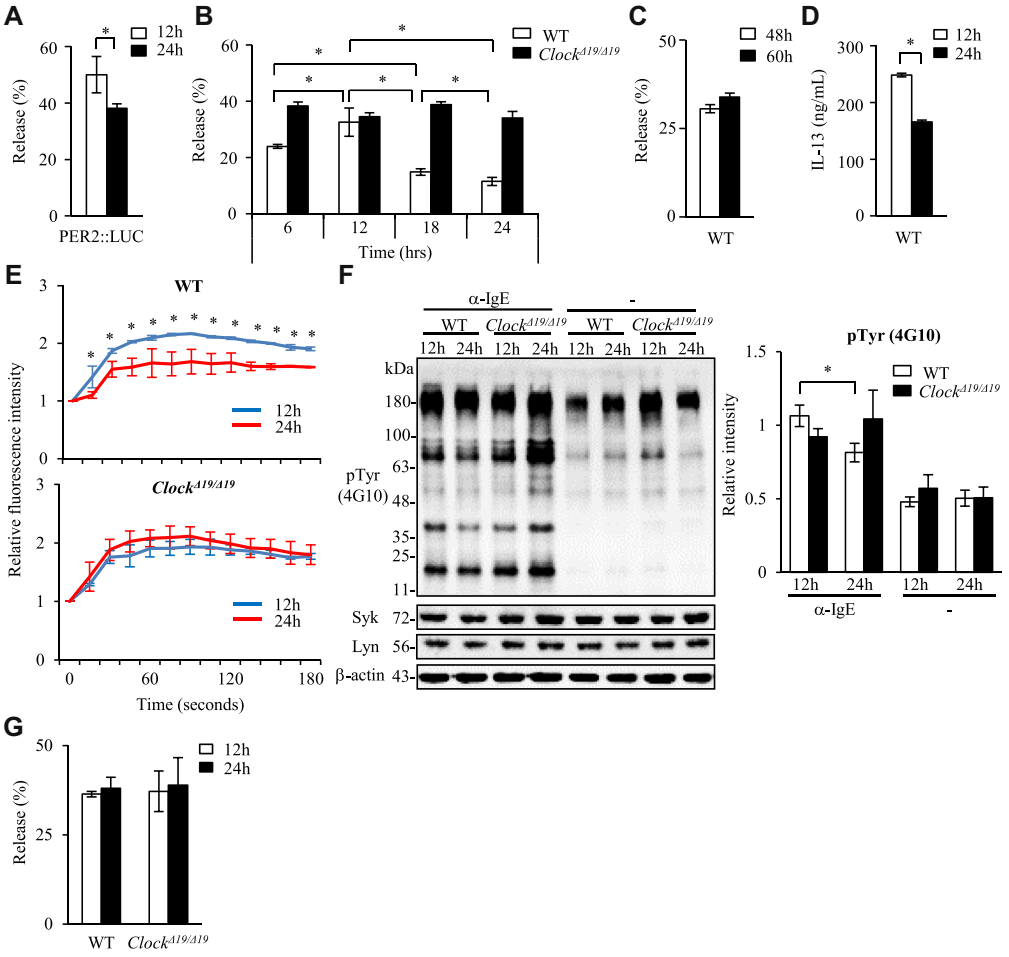

FIG 2. The mast cell–intrinsic clockwork temporally regulates IgE-dependent degranulation and signaling in mast cells *in vitro*. **A,** Release of β-hexosaminidase from the 12- or 24-hour cultured (after the medium change) BMCMCs derived from PER2::LUC knock-in mice (PER2::LUC). **B,** Release of β-hexosaminidase from the 6-, 12-, 18-, or 24-hour cultured (after the medium change) BMCMCs derived from wild-type *(WT)* and *Clock*$^{\Delta 19/\Delta 19}$ mice. **C,** Release of β-hexosaminidase from the 48- or 60-hour cultured (after the medium change) wild-type BMCMCs. **D,** IgE-dependent IL-13 production from the 12- or 24-hour cultured (after the medium change) wild-type BMCMCs. **E,** IgE-dependent intracellular Ca^{2+} mobilization in the 12- or 24-hour cultured (after the medium change) BMCMCs derived from WT and *Clock*$^{\Delta 19/\Delta 19}$ mice. **F,** *Left panel,* IgE-dependent total tyrosine phosphorylation levels in the 12- or 24-hour cultured (after the medium change) BMCMCs derived from WT and *Clock*$^{\Delta 19/\Delta 19}$ mice. *Right panel,* Quantitative analysis. **G,** Release of β-hexosaminidase from the 12- or 24-hour cultured (after the medium change) BMCMCs stimulated with A23187 derived from WT and *Clock*$^{\Delta 19/\Delta 19}$ mice. Values represent means ± SDs (n = 3 per group). Similar results were obtained in at least 3 independent experiments. The statistical analysis in Fig 2, *E*, was done with the unpaired Student *t* test. *$P < .05$.

siRNA–treated wild-type BMCMCs compared with that seen in control siRNA–treated wild-type BMCMCs (see Fig E6 in this article's Online Repository at www.jacionline.org). Furthermore, CLOCK overexpression in wild-type BMCMCs enhanced the promoter activity of FcεRIβ in a reporter assay (see Fig E7 in this article's Online Repository at www.jacionline.org).[22] These results suggest that CLOCK temporally binds to the promoter of *FcεRIβ* and modulates its transcription.

Unfortunately, we were unable to evaluate the FcεRIβ protein levels directly because of the lack of a good antibody. Therefore to confirm that temporal regulation of *FcεRIβ* transcription by the mast cell clock was indeed translated into protein levels, we examined cell-surface FcεRIα expression levels in the 12- and 24-hour cultured wild-type BMCMCs because the cell-surface FcεRIα levels are regulated by FcεRIβ protein levels in mast cells.[24-26] As expected, the cell-surface FcεRIα expression levels increased in the 12-hour cultured wild-type BMCMCs compared with those in the 24-hour cultured wild-type BMCMCs, which was absent in *Clock*-mutated BMCMCs (Fig 4, *H*). We also found that the cell-surface FcεRIα expression levels decreased in Clock, but not control, siRNA–treated wild-type BMCMCs (see Fig E8 in this article's Online Repository at www.jacionline.org). Notably, the expression levels of FcεRI at the cell surfaces of peritoneal mast cells from peritoneal lavage fluid performed at different times of day (ZT4 and ZT16) showed a time of day–dependent variation in wild-type mice, whereas this variation was absent in *Clock*-mutated mice (Fig 4, *I*). Collectively, it is likely that temporal modulation of *FcεRIβ*

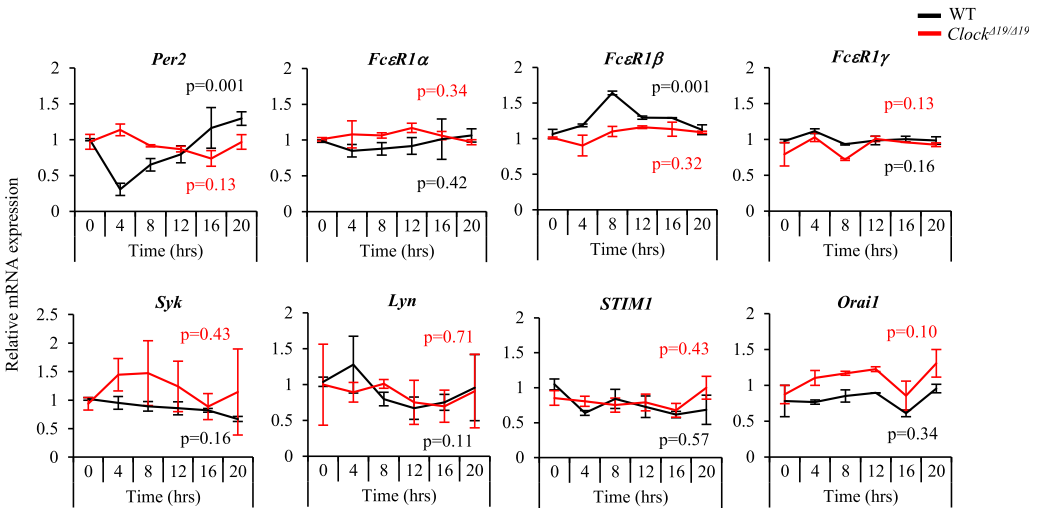

FIG 3. The mRNA expression kinetics of FcεRI-related signaling molecules in wild-type *(WT)* and *Clock*-mutated BMMCs. Wild-type or *Clock*-mutated *(Clock$^{\Delta 19/\Delta 19}$)* BMCMCs were consistently cultured *in vitro* after the medium change, and then, at the indicated time points, mRNA was extracted, and a quantitative PCR analysis was performed for *Per2*, *FcεRIα*, *FcεRIβ*, *FcεRIγ*, *Syk*, *Lyn*, *STIM1*, and *Orai1* mRNA. The values represent means ± SDs (n = 3 per group). *P* values are shown (1-way ANOVA).

transcription by CLOCK is indeed translated into protein levels in mast cells in a circadian manner, thereby timing FcεRI expression and signaling.

The adrenal gland is important to synchronize the mast cell clockwork

The data (Figs 1-4) suggest that if the individual mast cell clockwork does not synchronize with internal zeitgebers ("time givers" in German), this would lead to damping of the ensemble rhythm at population levels of mast cells and might result in uncoupled IgE-dependent responses. However, there is no information available about how mast cells tell time in the body.

We screened several bioactive substances for this effect by monitoring the bioluminescence emission in consistent cultures of PER2::LUC BMCMCs (see Fig E9 in this article's Online Repository at www.jacionline.org). The addition of corticosterone to PER2::LUC BMCMCs before the medium change or at 12 or 24 hours after the medium change either enhanced the amplitude of or phase shifted the mast cell clockwork, respectively (see Fig E9, *A*, and Fig 5, *A*). Consistently, corticosterone induced *Per1* and *Per2* mRNA expression in wild-type BMCMCs (Fig 5, *B*). Interestingly, corticosterone induced *Per1*, but not *Per2*, mRNA in *Clock*-mutated BMCMCs (Fig 5, *B*). The bioluminescence emission from mast cell–deficient W/Wv mice subcutaneously reconstituted with PER2::LUC BMCMCs showed a time of day–dependent variation in the PER2::LUC protein in sham-operated mice, with a peak at 10 PM (ZT16), as previously described,[10] whereas adrenalectomy resulted in the absence of variation (Fig 5, *C* and *D*). Conversely, dexamethasone treatment of W/Wv mice subcutaneously reconstituted with PER2::LUC BMCMCs at ZT4 enhanced the PER2::LUC protein levels at ZT10 compared with those seen in PBS-treated mice (Fig 5, *E* and *F*). These results suggest that humoral factors derived from the adrenal gland (one candidate is corticosterone) might play a critical role in synchronizing (or resetting) the mast cell clockwork *in vivo*.

DISCUSSION

This study demonstrated that the mast cell–intrinsic clock, entrained by internal zeitgebers originated from the adrenal gland, temporally gated IgE-mediated mast cell responses, at least in part, by timing FcεRI expression and signaling. The results identify a novel regulatory mechanism for IgE-mediated mast cell responses and provide a new insight into the allergic diseases that are regulated by many environmental and internal causes and triggers.[27]

The extent of the PCA reactions showed a time of day–dependent variation in W/Wv mice reconstituted with wild-type BMCMCs similar to that seen in conventional wild-type mice (Fig 1, *A* and *B*),[10] suggesting that this model of mast cell–specific gene inactivation reliably reflected at least the temporal phenotype in normal mice. In contrast, this variation was absent in W/Wv mice reconstituted with *Clock*-mutated BMCMCs (Fig 1, *A* and *B*). Additionally, corticosterone did not induce *Per2* mRNA in *Clock*-mutated, but not wild-type, BMCMCs (Fig 5, *B*), suggesting that the circadian clock in *Clock*-mutated mast cells cannot reset its clockwork according to a systemic timing cue. Because the daily profiles and levels of serum corticosterone were comparable between the mice (Fig 1, *C*), these findings strongly suggest that the mast cell–intrinsic clockwork is critical to the circadian regulation of PCA reactions.

Wang et al[11] reported that IgE-mediated degranulation in BMCMCs did not show temporal variations, although they did not show the actual data. We also reported that *Per2* mutation did not affect IgE-mediated degranulation in BMCMCs when we used wild-type and *Per2*-mutated BMCMCs cultured for more than 48 hours after a medium change (synchronization) and evaluated IgE-mediated degranulation at one time point alone.[10] This study suggests that it is important to use BMCMCs with functional clockwork to evaluate their circadian outputs and that timing of the analysis is also critical. Thus different experimental conditions of BMCMCs are likely to explain some different (contradictory) results between the previous[10,11] and current studies.

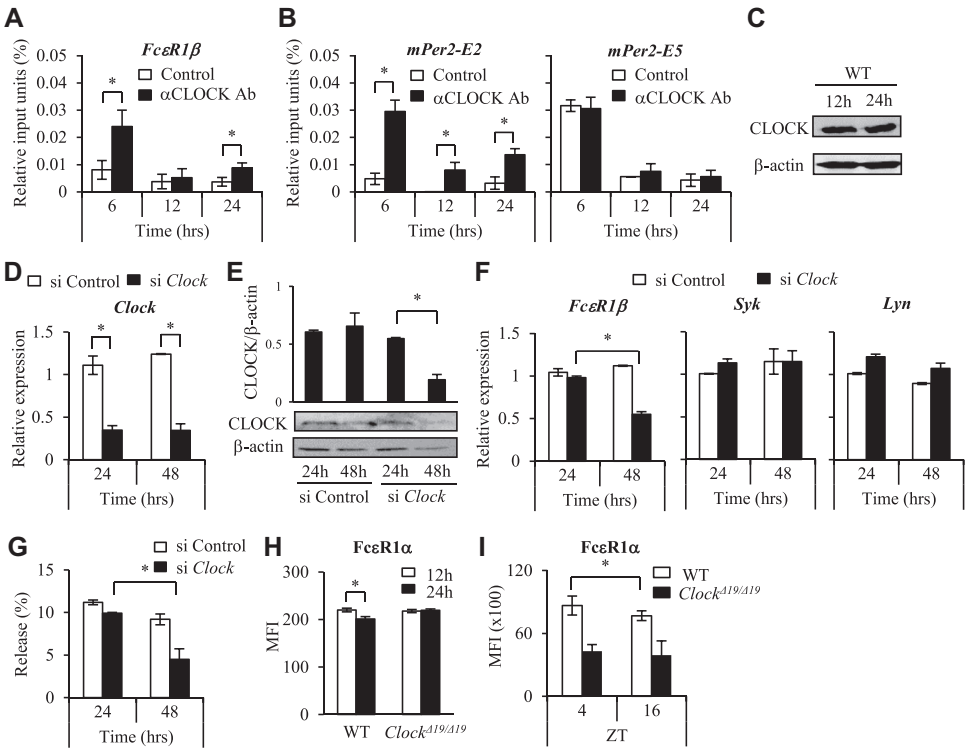

FIG 4. CLOCK binds to the promoter of *FcεRIβ* and modulates its transcription in mast cells. **A** and **B,** Detection of CLOCK binding to the promoter region of mouse *FcεRIβ* (Fig 4, *A*) or E2 or E5 enhancer of *mPer2* (Fig 4, *B*) in the 6-, 12-, and 24-hour cultured (after the medium change) wild-type BMCMCs. **C,** CLOCK and β-actin protein expression in the 12- and 24-hour cultured wild-type *(WT)* BMCMCs (from the medium change). **D,** Clock mRNA expression in wild-type BMCMCs at 24 and 48 hours after Clock siRNA transfection. **E,** CLOCK protein expression in wild-type BMCMCs at 24 and 48 hours after Clock siRNA transfection and quantitative analysis. **F,** *FcεRIβ*, *Syk*, and *Lyn* mRNA expression in wild-type BMCMCs at 24 and 48 hours after Clock siRNA transfection. **G,** Release of β-hexosaminidase from wild-type BMCMCs at 24 and 48 hours after Clock siRNA transfection. **H,** A quantitative analysis of FcεRIα levels on the 12- and 24-hour cultured wild-type and *Clock*-mutated BMCMCs (after the medium change) by using fluorescence-activated cell sorting staining. *MFI*, Mean fluorescence intensity. **I,** Quantitative analysis of FcεRIα levels on peritoneal mast cells from peritoneal lavage fluid of wild-type and $Clock^{\Delta 19/\Delta 19}$ mice performed at different times of the day (ZT4 and ZT16) by using fluorescence-activated cell sorting staining. *MFI*, Mean fluorescence intensity. Values represent means ± SDs (n = 3 per group). Similar results were obtained in at least 3 independent experiments. *$P < .05$.

We suggest that FcεRIβ is a key molecule that links the mast cell clockwork and IgE-mediated mast cell responses. Because FcεRIβ is a critical modulator of FcεRI expression and signaling,[24-26] CLOCK targeting FcεRIβ might be a reasonable strategy for temporally fine tuning FcεRI signaling in mast cells. However, it remains to be determined precisely how the mast cell clockwork modulates the transcription of *FcεRIβ* in mast cells. Because rhythmic histone modifications (acetylation and methylation) are involved in circadian regulation of transcription and the CLOCK/BMAL1 complex is associated with histone acetyltransferase and methyltransferases,[28] we speculate that the CLOCK/BMAL1 complex might induce histone modifications that encompassed the *FcεRIβ* promoter in a circadian manner, thereby temporally controlling FcεRIβ transcription.

Indeed, we found that the acetylation status of histone H4 and the methylation status of histone H3K4 (typical markers for active chromatin) showed temporal alterations in the mouse FcεRIβ promoter (see Fig E10 in this article's Online Repository at www.jacionline.org), which was largely associated with temporal CLOCK binding to the promoter (Fig 4, *A*). How these epigenetic changes occur in a temporal manner in the promoter region of FcεRIβ remains to be determined.

Wang et al[11] reported that *FcεRIα* mRNA displayed a circadian oscillation in BMCMCs, which was not observed herein (Fig 3). To synchronize BMCMCs, they used serum shock (50% horse serum) that might contain factors strongly influencing the mast cell clockwork (eg, glucocorticoid).[16,17] Therefore the discrepancy might be due to different synchronization procedures. In addition, the genetic backgrounds of mice might affect the circadian outputs[29] because BMCMCs from ICR mice did not show circadian expression of FcεRIβ mRNA[10] in contrast to this study. Consistently, a time of day–dependent variation in the expression levels of FcεRI at the cell surfaces of peritoneal mast cells observed in wild-type C57BL/6 mice (Fig 4, *I*) was not observed in wild-type ICR mice (data not shown).

Opposite results appeared to be obtained in IgE-mediated degranulation between *Clock*-mutated $(Clock^{\Delta 19/\Delta 19})$ and *Clock*-deleted (Clock siRNA–treated) BMCMCs: unlike *Clock*-deleted BMCMCs (Fig 4), $Clock^{\Delta 19/\Delta 19}$ BMCMCs did not show reduction of FcεRIβ transcription and IgE-mediated degranulation at any

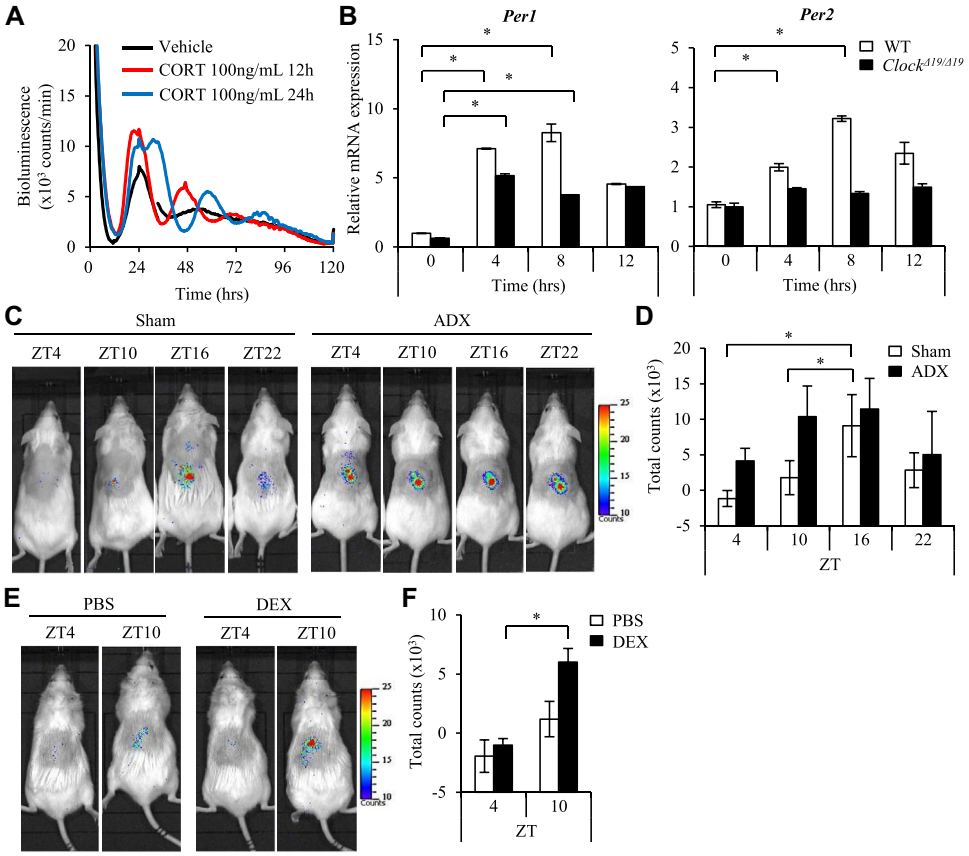

FIG 5. Factors derived from the adrenal glands synchronize or reset the mast cell–intrinsic clockwork. **A,** Monitoring of PER2::LUC bioluminescence of BMCMCs from PER2::LUC knock-in mice from the medium change for 120 hours. Corticosterone (*CORT*; 100 ng/mL) was added to the culture at 12 or 24 hours after the start of monitoring. **B,** Wild-type *(WT)* or *Clock*-mutated *(Clock*$^{\Delta 19/\Delta 19}$*)* BMCMCs were stimulated with or without 100 ng/mL corticosterone for the indicated times, and then quantitative PCR was performed for *Per1* and *Per2* mRNA detection. **C,** Representative pictures of *in vivo* imaging of W/Wv mice reconstituted with subcutaneous injections of BMCMCs derived from PER2::LUC knock-in mice at the indicated time points. Before the *in vivo* imaging, the mice were subjected to a sham operation *(Sham)* or adrenalectomy *(ADX)*. **D,** Quantitative analysis of the data in Fig 5, *C*. **E,** Representative pictures of *in vivo* imaging of W/Wv mice reconstituted with subcutaneous injections of BMCMCs derived from PER2::LUC knock-in mice. The mice were treated with dexamethasone (*DEX*; 1 mg/kg) or PBS at ZT4. The bioluminescence emission from the mice was recorded at ZT4 (before dexamethasone or PBS administration) and ZT10. **F,** Quantitative analysis of the data in Fig 5, *E* (n = 5 per group). Values represent means ± SDs (n = 3 per group unless otherwise indicated). Similar results were obtained in at least 2 independent experiments. **P* < .05.

time of day either *in vitro* or *in vivo* (Figs 1-3). *Clock*$^{\Delta 19/\Delta 19}$ mice have more severe phenotypes in circadian behavior and physiology relative to *Clock*-deficient mice,[30] which is probably due to a dominant negative–type capability of the mutant *Clock*$^{\Delta 19/\Delta 19}$ protein that can inhibit both CLOCK and CLOCK homolog NPAS2 functions.[29] Therefore *Clock*$^{\Delta 19/\Delta 19}$ BMCMCs likely have severe attenuation of the circadian clock machinery compared with *Clock*-deleted BMCMCs, which might result in aberrant temporal regulation of FcεRIβ expression through undefined mechanisms.

This study suggests that corticosterone might function as a timing signal to synchronize (or reset) the mast cell clockwork *in vivo*, as with other peripheral clocks, such as those in the liver.[31] However, we cannot exclude that the net endocrine and metabolic changes after the adrenalectomy (eg, induction of low body temperature) might lead to desynchronization of the mast cell clockwork *in vivo* because peripheral clocks are, in many cases, entrained by several different pathways *in vivo*.[6-9]

On the basis of the current findings that the disruption of mast cell–intrinsic clockwork alone results in the absence of daily rhythms in PCA reactions regardless of the intact daily profiles and levels of serum corticosterone (Fig 1), we think that aberrant corticosterone (or glucocorticoid) profiles and levels might indirectly affect the daily rhythms in PCA reactions by influencing the mast cell clockwork. The precise roles of corticosterone (or steroids treatment) in the circadian regulation of PCA reactions remain to be determined.

In summary, we suggest that the peripheral, but not systemic, circadian clock in mast cells is a primary driver for the daily rhythm generation in IgE/mast cell–mediated allergic reaction. It remains to be determined whether the current findings in mice are relevant to the mechanisms underlying the prominent 24-hour variations in symptoms characteristic of patients with allergic diseases.

We thank Suguru Fukumoto, Ms Kazuko Nakamura, Tomoko Tohno, and Yuko Ohnuma for their assistance and Professor Hitoshi Okamura (Kyoto University, Japan) for providing us with mouse Clock expression plasmid.

Clinical implications: The mast cell–intrinsic clock, entrained by internal zeitgebers originated from the adrenal gland, temporally gates IgE/mast cell–mediated allergic reactions, which might underlie the prominent approximately 24-hour variations in allergic symptoms.

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METHODS

Mice

All animal experiments were approved by the Institutional Review Board of the University of Yamanashi and the Committee for Animal Experimentation of the School of Science and Engineering at Waseda University.

Preparation of BMCMCs

BMCMCs were generated from the femoral bone marrow cells of male mice and cultured as described previously.[E1] Diff-Quik stain (a modified May-Giemsa stain; Sysmex Ltd, Hyogo, Japan) was used to identify mast cells morphologically.

Fluorescence-activated cell sorting staining of BMCMCs

BMCMCs or peritoneal exudates collected from mice were incubated for 15 minutes with rat anti-mouse antibodies to CD16/32 (2.4G, BD Biosciences) to block nonspecific binding and then were stained with fluorescein isothiocyanate–conjugated anti-mouse FcεRIα (MAR-1; eBioscience, San Diego, Calif) and a phycoerythrin-conjugated anti-mouse c-Kit antibody (2B8; BD PharMingen, San Diego, Calif) in PBS for 30 minutes on ice. The stained cells (live gated on the basis of forward and side scatter profiles) were analyzed on a FACSCalibur instrument (BD Biosciences), and data were processed with the CellQuest software program (BD Biosciences).

Staining and quantification of mast cells

Mouse tissue samples of back skin were fixed and embedded in paraffin, ensuring a cross-sectional orientation, and 2-μm sections were cut. Slides of paraffinized sections were dewaxed, rehydrated, and stained metachromatically with 0.05% toluidine blue (pH 4.1). Mast cells were expressed in numbers per millimeter of horizontal field length.

Evaluation of PCA reactions

Vascular permeability was visualized 30 minutes later by means of blue staining of the injection sites on the reverse side of the skin. These staining sites were digitized with a high-resolution color camera (digital camera LUMIX DE-991; Panasonic, Tokyo, Japan), and the images were saved in Windows photo viewer as 8-bit color-scale JPEG files. The open source ImageJ 1.43 software package (National Institutes of Health, Bethesda, Md) was used for the image analysis, as described previously.[E2] Briefly, color-scale images exported from Windows photo viewer were converted to "hue/saturation/brightness" stack-type images by using the Image tool. Thereafter, the hue/saturation/brightness stack images were split into hue, saturation, and brightness images, respectively. Only blue color–stained areas were selected from the hue image by using the threshold tool. These images were then combined with the saturation image and the density values for the blue color–stained areas and measured with the analytic tool.

Corticosterone and IgE ELISA

The serum corticosterone and IgE levels at the indicated time points were determined by using the AssayMax Corticosterone ELISA kit (AssayPro, Charles, Mo) or mouse total IgE ELISA kit (Morinaga Institute of Biological Science, Kanagawa, Japan).

Measurement of bioluminescence in BMCMCs

BMCMCs generated from PER2::LUC knock-in mice were placed in a 35-mm Petri dish after centrifugation at 1500 rpm for 5 minutes and incubated at 37°C. The bioluminescence was monitored at 10-minute intervals for 120 hours with a dish-type luminometer (Kronos DioAB-2550; ATTO, Tokyo, Japan).

The reagents used were cell membrane–permeable dibutyl cyclic AMP, corticosterone, adrenaline, noradrenaline, and melatonin (Sigma-Aldrich, St Louis, Mo) and human TGF-β1, mouse thymic stromal lymphopoietin, and mouse TNF-α (R&D Systems, Minneapolis, Minn).

Quantitative real-time PCR

Quantitative real-time RT-PCR with specific primers and probes for mouse *FcεRIα*, *FcεRIβ*, *FcεRIγ*, *Syk*, *Lyn*, *STIM1*, *Orai1*, *Per2*, *mMCP-5*, *mMCP-6*, and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*; Applied Biosystems, Foster City, Calif) was performed with the AB7300 real-time PCR system (Applied Biosystems).

β-Hexosaminidase release assay

The β-hexosaminidase release assay was performed and analyzed, as described previously.[E1] Briefly, BMCMCs (5×10^6 cells/mL) were incubated for 1 hour at 4°C with 1 μg/mL anti-DNP mouse IgE mAb (BD Biosciences) and then stimulated for 40 minutes at 37°C with 1 μg/mL anti-mouse IgE antibody (BD Biosciences). In some experiments BMCMCs were stimulated with calcium ionophore A23187 (3 μmol/L, Sigma-Aldrich).

IL-13 ELISA

The 12- or 24-hour cultured BMCMCs were stimulated with anti-mouse IgE antibody, as described above, in a β-hexosaminidase release assay and then cultured for 24 hours, and the culture supernatants were collected. The concentrations of IL-13 in the culture supernatants were measured by using the mouse IL-13 ELISA kit (R&D Systems).

Measurement of $[Ca^{2+}]_i$ mobilization

Measurement of $[Ca^{2+}]_i$ mobilization was performed, as previously described.[E3] Briefly, the IgE-sensitized BMCMCs (1×10^6 cells/mL) were loaded with 4 μmol/L Fluo-3 AM (Dojindo, Kumamoto, Japan) for 30 minutes at 37°C. The cells were resuspended in 1× Tyrode buffer, and then changes in dye fluorescence on addition of 1 μg/mL anti-mouse IgE were monitored by using flow cytometry. $[Ca^{2+}]_i$ mobilization was expressed as the relative fluorescence intensity.

Western blot

Western blotting was performed, as previously described.[E4] Briefly, IgE-sensitized BMCMCs (5×10^6 cell/mL) were stimulated with anti-IgE (1 μg/mL) for 5 minutes at 37°C. After stimulation and centrifugation, the cells were lysed by means of direct addition of sampling buffer (Bio-Rad Laboratories, Hercules, Calif). The cell lysates were electrophoretically removed in 10% SDS polyacrylamide gel and transferred onto Bio Trace PVDF membrane (Pall Corporation, Port Washington, NY). The membrane was incubated with a primary antibody and an appropriated secondary horseradish peroxidase–conjugated antibody. The signals were detected by using ECL (GE Healthcare Bioscience, Bucks, United Kingdom). The immunoreactive bands were visualized with the ChemiDoc XRS-J imaging system (Bio-Rad Laboratories) and analyzed with Quantity One (Bio-Rad Laboratories) to determine their relative intensity. Primary antibodies used are anti–phospho-tyrosine antibody 4G10 (Millipore, Billerica, Mass), anti-mouse Syk antibody and anti-mouse Lyn antibody (Cell Signaling Technology, Beverly, Mass), ChIP grade anti-KAT13D/CLOCK antibody (rabbit polyclonal IgG; Abcam, Cambridge, Mass), and anti–β-actin antibody (Santa Cruz Biotechnology, Santa Cruz, Calif).

ChIP assay

The ChIP assay was performed, as previously described.[E5] Antibodies used for the immunoprecipitation are ChIP grade anti-mouse/human KAT13D/CLOCK antibody (rabbit polyclonal IgG, Abcam), control purified rabbit IgG (Invitrogen, Carlsbad, Calif), control purified mouse IgG (Life Technologies, Carlsbad, Calif), anti-dimethyl histone H3 (Lys4) antibody (clone, MABI0303; Cell Signaling Technology, Beverly, Mass), anti–trimethyl-histone H3 (Lys9) antibody (clone, MABI0308; Cell Signaling Technology), anti-acetyl histone H3 antibody (Millipore, Billerica, Mass), and anti-acetyl histone H4 antibody (Millipore). Equivalent masses of immunoprecipitated and input DNA were analyzed by using real-time PCR with primers and a TaqMan probe for the promoter region of the *FcεRIβ* chain.[E6]

mPer2-E2 and *mPer2-E5*[E7] primers were as follows: *FcεRIβ* sense, 5′-ACAGCAAGAGAAAGGAGTCACTGAT-3′;

FcεRIβ antisense, 5′-CATGCGGAACCTACTTGTCAGA-3′; and *FcεRIβ* TaqMan probe, 5′-FAM-CAATCAGCCTGGAGACT-MGB-3′.

mPer2-E2 primers were as follows: sense, 5′-CCACCAATTGATGAGCG TAGC-3′; antisense, 5′-CGTCGCCCTCCGCTG-3′; and TaqMan probe, 5′-FAM-TCACGTTTTCCACTATGTG-MGB-3′.

mPer2-E5 primers were as follows: sense, 5′-TCCTGCCACATTGAG ATTTGG-3′; antisense, 5′-GTGATTGCCCCACACTCACA-3′; and TaqMan probe, 5′-AAGAGATGGCACGTTAGT-MGB-3′.

Data are presented as the ratio of the cycling threshold value of immunoprecipitated DNA to that of input DNA.

Transfection by means of electroporation

Wild-type BMCMCs were plated at 1×10^6 cells/mL in 10-cm Petri dish 24 hours before transfection. Transfection of BMCMCs with siRNAs or plasmids was performed with the Mouse Macrophage Nucleofector kit (VPA-1009; Amaxa Biosystems, now Lonza, Basel, Switzerland), according to the manufacturer's protocol.

siRNA reagents

Specific siRNAs against Clock (Stealth RNAi, ClockMS203030 3_RNAI) and the negative control (Stealth RNAi; Negative control Low GC Duplex #2) were purchased from Invitrogen.

Luciferase assay

The reporter plasmid (100 ng) −2.4k/pGL3-Basic, containing 2.4 kb 5′-upstream from the translation initiation codon of the mouse β-chain gene[E6] or pGL3-Basic was transfected into wild-type BMCMCs (2×10^6) with the internal control plasmid pRL-CMV (25 ng; Promega, Madison, Wis) in the presence or absence of mouse Clock expression plasmid (provided by Professor Hitoshi Okamura, Kyoto University) or pcDNA3.1 (Invitrogen; 100 ng). The relative luciferase activity of −2.4k/pGL3-Basic is represented as the ratio to the activity driven by pGL3-Basic after adjustment of the transfection efficiency. After 20 to 24 hours of cultivation, the cells were harvested and treated with a Dual-luciferase assay kit (Promega) for the measurement of luciferase activity. The luminescence was measured with Gene Light 55 (Microtec, Chiba, Japan).

Analysis of peritoneal mast cells

Peritoneal exudates were collected from wild-type or *Clock*-mutated mice at the indicated time points (ZT4 and ZT16), and the surface FcεRIα levels on mast cells gated by FcεRIα and c-kit were assessed by means of fluorescence-activated cell sorting analysis (histogram).

In vivo imaging

Mast cell–deficient WBB6F1-W/Wv mice were reconstituted with subcutaneous injections of BMCMCs (1.5×10^6 per mouse) derived from PER2::LUC mice or with PBS alone. Six weeks after reconstitution, 20 μL of luciferin (50 mg/mL) was subcutaneously administered at the indicated time points, and then the bioluminescence emission from the mice was recorded, as described previously.[E2] In our preliminary study we did not find any bioluminescence emission from the W/Wv mice reconstituted with BMCMCs (1.5×10^6 per mouse) derived from wild-type C57BL/6 mice after the luciferin injection (data not shown). For some experiments, the mice were treated intraperitoneally with water-soluble dexamethasone (1 mg/kg, Sigma-Aldrich) or PBS at ZT4, and the mice were then analyzed by means of *in vivo* imaging at ZT10.

Adrenalectomy

Bilateral adrenalectomy was carried out, and the adrenalectomized mice were maintained as reported previously.[E8]

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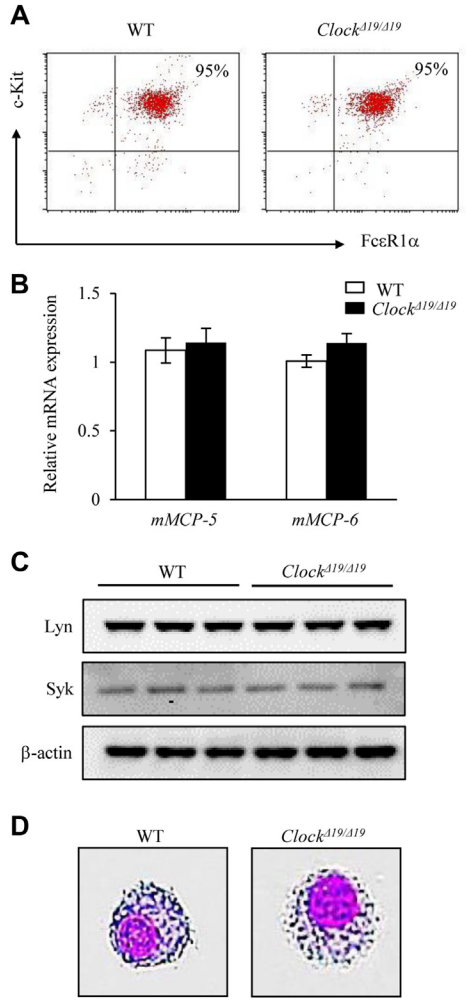

FIG E1. *Clock* mutation does not affect the differentiation of mast cells. BMCMCs were generated from wild-type *(WT)* or *Clock*-mutated *(Clock$^{\Delta 19/\Delta 19}$)* mice. Five weeks after the start of bone marrow cell culture, flow cytometric analysis with anti-FcεRIα and an anti–c-Kit antibody **(A)**, real-time PCR analysis for mMCP-5 and mMCP-6 mRNA expression **(B)**, Western blot analysis for Lyn and Syk protein expression **(C)**, and Giemsa staining **(D)** were performed. Representative data from 3 independent experiments with similar results are shown.

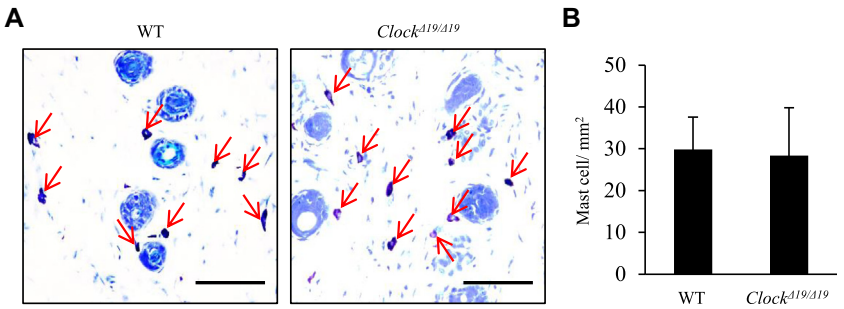

FIG E2. Toluidine blue staining. **A,** Toluidine blue staining of mast cells (indicated by *arrows*) on the back skin of mast cell–deficient W/Wv mice reconstituted with subcutaneous injections (back skin) of BMCMCs (1.5×10^6 per mouse) derived from wild-type *(WT)* or $Clock^{\Delta 19/\Delta 19}$ mice. *Scale bar* = 100 μm. **B,** Quantitative analysis of Fig E2, *A* (n = 4).

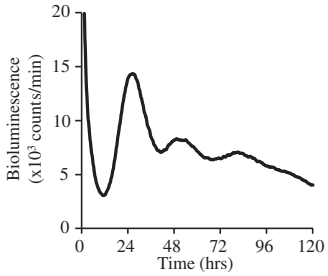

FIG E3. Monitoring of PER2:LUC protein expression in mast cells. Monitoring of PER2::LUC bioluminescence of BMCMCs derived from PER2::LUC knock-in mice after a medium change (synchronization) for 120 hours is shown.[E2]

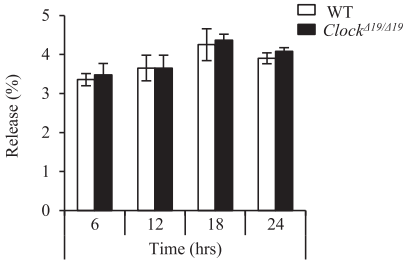

FIG E4. Spontaneous β-hexosaminidase release from wild-type *(WT)* and *Clock*-mutated BMCMCs. Release of spontaneous β-hexosaminidase (without IgE stimulation) from the 6-, 12-, 18-, or 24-hour cultured (after the medium change) BMCMCs derived from wild-type and *Clock*-mutated mice ($Clock^{\Delta 19/\Delta 19}$, n = 3).

tcccaggaaa ataactgagg tgatttaggg tactatggat agtatcttgc agttgtgaaa

aaccaatcat agaactgaac tgaaatataa gcaaggcaga cctgttctct cttgtgcatt

gtgttctggg gtagttactt gtgataaagt cgagtgaaca ctgtgtctgg gcagacatgt

tgctcagttt ccactattca gacttgctca caccatttct atgacattca catccctcca

tattttcctt gtcttgttct taccattaca aaaagtgcag cagttgccac acacatgttc

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tgcagaagac aggaagagaa aatgagaaag gggtgaggag agatgggtga gagggagagg

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catacagca agagaaagga gtcactgata tcaatcagcc tggagactta tctgacaagt
Sense primer
TaqMan probe
aggttccgca tgaagataat cattggtatt cagagccaac ccatcttaac tgcccatcca
Antisense primer
gagcacaccg catttctgtg taacagtatc tttcattcct ggatagccca attaatgaaa

aaATGgacac agaaaatagg agcagagcag atcttgctct cccaaatcca caagaatcct

FIG E5. E-box–like elements in the promoter region of the mouse *FcεRIβ*. There are several noncanonical E-box–like elements ("CANNTG" or "CANNTT") present in the promoter region of the mouse *FcεRIβ*.[E6] CLOCK/BMAL1-mediated circadian control of a gene transcription can be driven by noncanonical E-box sequences in the promoter region of the gene.[E7] "CANNTG" or "CANNTT" or their reverse sequences are indicated in green. The translation start site (ATGgacaac….) is indicated in *blue*. Primers and probe sequence sites used for the ChIP assay (*FcεRIβ*) are also shown in *red*.

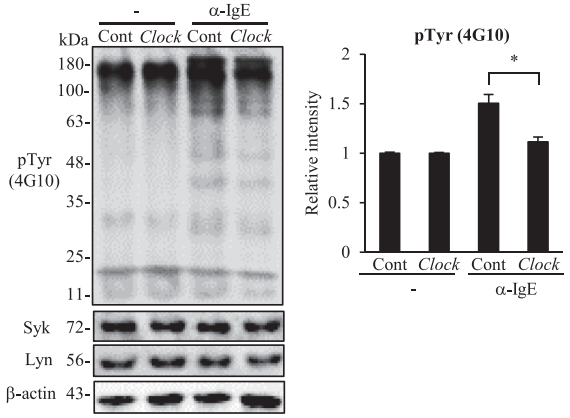

FIG E6. Knockdown of *Clock* reduces IgE-dependent increases in the total tyrosine phosphorylation in mast cells. IgE-dependent increases in the total tyrosine phosphorylation levels of the cell lysates were evaluated by means of Western blot analysis in Clock or control siRNA–treated wild-type BMCMCs at 48 hours after siRNA transfection. *Left panel*, Representative pictures of Western blot analysis with anti–phospho-tyrosine antibody 4G10. *Right panel*, Quantitative analysis (n = 3). $*P < .05$.

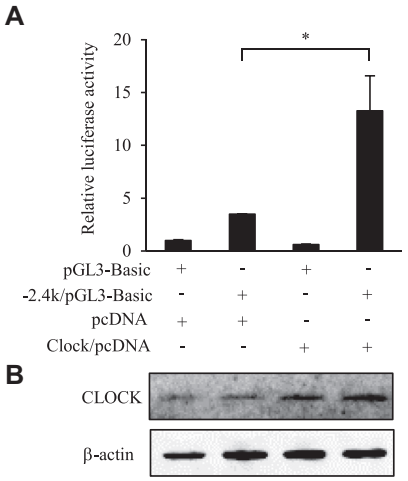

FIG E7. CLOCK overexpression enhances promoter activity of FcεRIβ in mast cells. **A,** The reporter plasmid −2.4k/pGL3-Basic containing 2.4 kb 5′-upstream from the translation initiation codon of the mouse β-chain gene or pGL3-Basic was transfected into wild-type BMCMCs with the internal control plasmid pRL-CMV. The relative luciferase activity of −2.4k/pGL3-Basic is represented as the ratio to the activity driven by pGL3-Basic (n = 3). **B,** Western blot analysis of the cell lysates described in Fig E7, *A*, with anti-CLOCK or anti–β-actin antibody. **P* < .05.

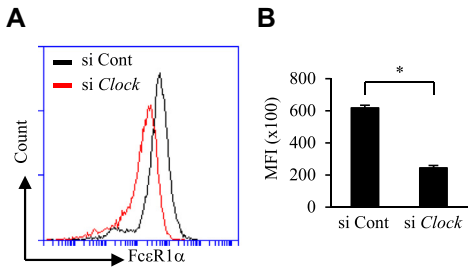

FIG E8. Knockdown of *Clock* reduces surface FcεRIα levels on mast cells. Cell-surface FcεRIα expression levels were evaluated by means of fluorescence-activated cell sorting *(FACS)* analysis in Clock or control siRNA–treated wild-type BMCMCs at 48 hours after siRNA transfection. **A,** Representative histogram. **B,** Quantitative analysis (n = 3). **P* < .05. *MFI,* Mean fluorescence intensity.

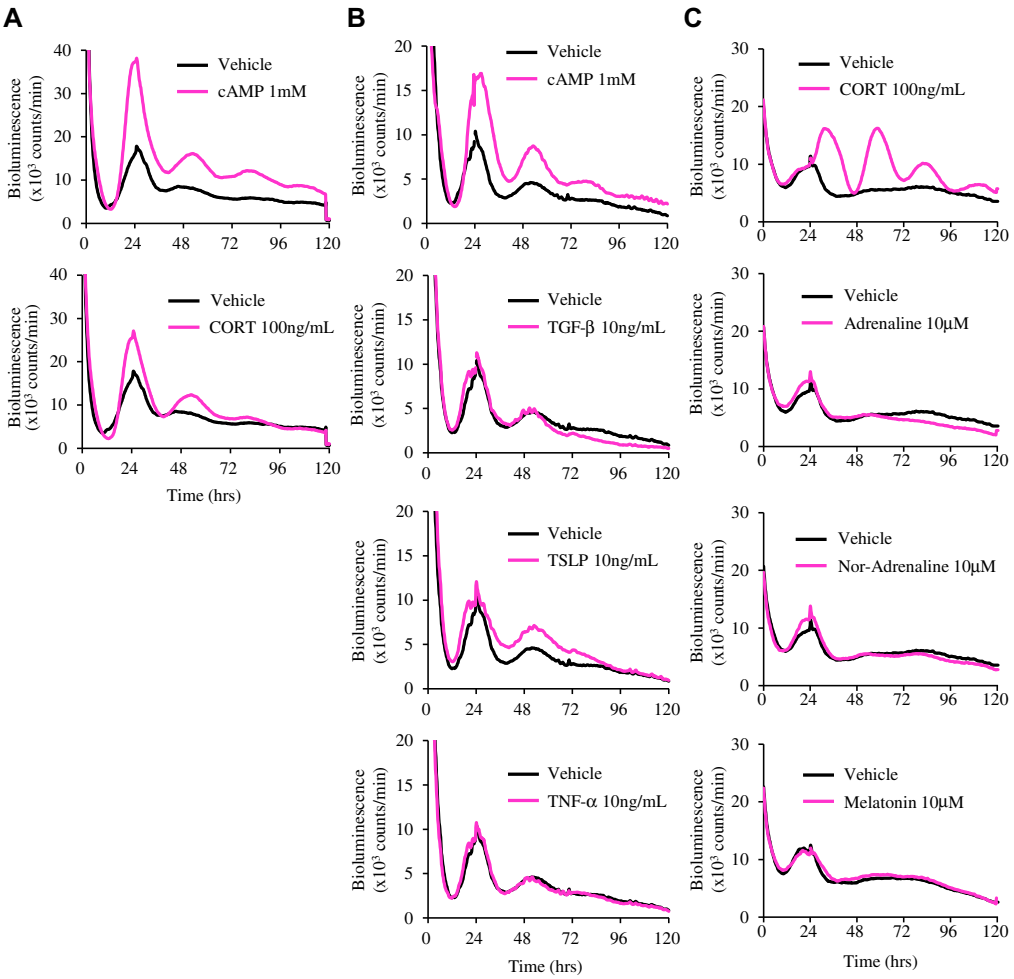

FIG E9. Screening several bioactive molecules for mast cell clockwork modulatory activity. **A** and **B,** Indicated concentrations of bioactive molecules or vehicles were added to consistent cultures of BMCMCs derived from PER2::LUC knock-in mice (PER2::LUC BMCMCs) for 2 hours, and then the PER2::LUC bioluminescence was monitored for 120 hours with a dish-type luminometer. **A** and **B,** The experiments were performed independently. Dibutyl cyclic AMP was used as a positive control, and it enhanced the amplitude of the mast cell clockwork (PER2::LUC expression). *CORT*, Corticosterone. **C,** Indicated concentrations of bioactive molecules or vehicles were added to consistent cultures of BMCMCs derived from PER2::LUC knock-in mice (PER2::LUC BMCMCs) at 24 hours after the medium change. PER2::LUC bioluminescence was monitored for 96 hours after stimulation (total of 120 hours), as described above. Note that there were little changes in the kinetics of bioluminescence after the additions of adrenaline, noradrenaline, and melatonin but not corticosterone *(CORT)*.

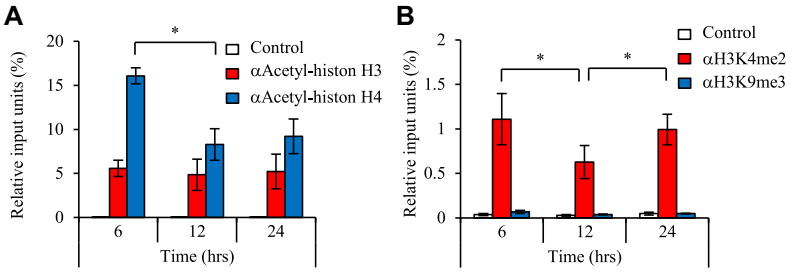

FIG E10. Epigenetic changes in the promoter region of FcεRIβ. **A,** The acetylation status of histone H3 and H4 in the promoter region of mouse *FcεRIβ* in the 6-, 12-, and 24-hour cultured (after the medium change) wild-type BMCMCs was evaluated by means of ChIP analysis. **B,** The methylation status of histone H3K4 and H4K9 in the promoter region of mouse *FcεRIβ* in the 6-, 12-, and 24-hour cultured (after the medium change) wild-type BMCMCs was evaluated by means of ChIP analysis (n = 3). **P* < .05.