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Original Article

Clock-dependent temporal regulation of IL-33/ST2-mediated mast cell response

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Abbreviation:

IL-33, interleukin-33; BMMCs, bone marrow-derived mast cells; BM basophils, bone marrow-derived basophils; ILC2s, group-2 innate lymphoid cells; SCN, suprachiasmatic nucleus; Per, Period; Cry, Cryptochrome; ZT, Zeitgeber time; ChIP, chromatin immunoprecipitation

ABSTRACT

Background: Interleukin-33 (IL-33) is an alarmin cytokine that binds to the interleukin 1 receptor-like 1 protein ST2. *Clock* is a key circadian gene that is essential for endogenous clockworks in mammals. This study investigated whether *Clock* temporally regulated IL-33-mediated responses in mast cells.

Methods: The kinetics of IL-33-mediated IL-6, IL-13, and TNF- α productions were compared between bone marrow-derived mast cells (BMMCs) from wild-type and *Clock*-mutated mice (*Clock* ^{Δ 19/ Δ 19} mice). The kinetics of the neutrophil influx into the peritoneal cavity or expression of IL-13 and Gob-5 in the lung in response to IL-33 were compared between wild-type and *Clock* ^{Δ 19/ Δ 19} mice. We also examined the kinetics of ST2 expression in mast cells and its association with *Clock* expression.

Results: There was a time-of-day-dependent variation in IL-33-mediated IL-6, IL-13, and TNF- α production in wild-type BMMCs, which was absent in *Clock*-mutated BMMCs. IL-33-induced neutrophil infiltration into the peritoneal cavity also showed a time-of-day-dependent variation in wild-type mice, which was absent in *Clock* ^{Δ 19/ Δ 19} mice. Furthermore, IL-33-induced IL-13 and Gob-5 expression in the lung exhibited a time-of-day-dependent variation in wild-type mice. These temporal variations in IL-33-mediated mast cell responses were associated with temporal variations of ST2 expression in mast cells. In addition, CLOCK bound to the promoter region of ST2 and *Clock* deletion resulted in down-regulation of ST2 expression in mast cells.

Conclusions: CLOCK temporally gates mast cell responses to IL-33 via regulation of ST2 expression. Our findings provide novel insights into IL-33/mast cell-associated physiology and pathologies.

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Introduction

Interleukin-33 (IL-33) is an alarmin cytokine that plays diverse roles in innate and acquired immune responses, including allergic reaction.^{1–3} IL-33 strongly stimulates innate immune cells such as mast cells, basophils, and group-2 innate lymphoid cells (ILC2s) to produce various cytokines/chemokines, including IL-6, IL-13, and TNF- α via the interleukin 1 receptor-like 1 protein ST2.² Although

the roles of IL-33/ST2 in innate immune responses have been extensively studied, regulation of the IL-33/ST2 pathway in innate immune cells remains incompletely understood.

The circadian clock is an essential timing system that drives daily oscillations of behavior and physiology, such as sleep–wake cycles and hormonal secretion.^{4,5} The mammalian circadian clock system consists of the central oscillator, located in the suprachiasmatic nucleus (SCN) of the hypothalamus, and peripheral oscillators in virtually all cell types including mast cells and basophils. The central SCN clock receives light input from the retina, which synchronizes internal clock timing to the external solar day, and passes this information on to peripheral clocks via neural and endocrine pathways. The molecular mechanisms of rhythm generation, which are highly similar in SCN and peripheral cells, are created and maintained by interlocked

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transcriptional–translational feedback loops consisting of several clock genes.^{4,5} Briefly, CLOCK and BMAL1 heterodimerize and activate transcription of *Period* (*Per*)1/2 (PER), *Cryptochrome* (*Cry*)1/2 (CRY), and other clock output genes via the E-box and E-box-like elements in their promoter regions. PER and CRY heterodimerize and suppress CLOCK/BMAL1 activity, thereby inhibiting their own transcription.

IL-33/mast cell-associated pathologies such as allergic reactions vary with time of day (e.g., asthma attacks).⁶ Therefore, in this study we investigated whether the circadian clock (CLOCK protein) regulates IL-33/ST2-mediated mast cell responses. For this purpose, we examined the kinetics of IL-33-mediated responses in mast cells from wild-type and *Clock*-mutated mice⁷ and investigated the mechanisms underlying *Clock*-dependent temporal regulation of IL-33-mediated mast cell responses.

Methods

Mice

C57BL/6 *Clock*^{Δ19/Δ19} mice,⁷ their littermates, and PER2^{LUCIFERASE} (PER2^{LUC}) knock-in mice⁸ (C57BL/6 background) were bred under specific pathogen-free conditions. *Clock*^{Δ19/Δ19} mice have an A-to-T point mutation in the 5' splice site of intron 19, which causes an in-frame deletion of the entire exon 19 (*Clock*^{Δ19/Δ19}), resulting in the loss of normal transcriptional activity.⁷ *Clock*^{Δ19/Δ19} mice were mated in our laboratory to generate *Clock*^{Δ19/Δ19} Per2^{Luc} knock-in mice. All mice were housed under 12-h light/12-h dark conditions (Light/Dark [L/D] 12:12; the light was turned on at 6:00 AM, Zeitgeber time [ZT] 0, and the light was turned off at 6:00 PM, ZT12), with *ad libitum* access to food and water, for at least 2 weeks. In some experiments, the mice were then housed under 10-h light/10-h dark conditions (L/D 10:10 cycle) for 8 weeks using special animal cages where light conditions can be freely controlled. This study was approved by the Animal Experiment Committee of University of Yamanashi Faculty of Medicine and followed by the Helsinki Protocol.

Recombinant mouse IL-33

Recombinant mouse IL-33 (the 18 kDa mature protein) was purchased from Enzo Biochem Inc. (Exeter, UK).

Preparation of bone marrow-derived mast cells (BMMCs) and basophils (BM basophils)

Bone marrow-derived mast cells (BMMCs) or basophils (BM basophils) were generated from femoral bone marrow cells of male mice as previously described.^{9,10}

Measurement of bioluminescence in BMMCs generated from Per2^{Luc} mice

BMMCs generated from Per2^{Luc} knock-in mice were placed in 35-mm Petri dishes following centrifugation at 400 g for 5 min, and then incubated at 37 °C. Bioluminescence was monitored at 10-min intervals for 120 h using a dish-type luminometer (Kronos DioAB-2550; ATTO Inc., Tokyo, Japan) as previously described.^{11,12}

FACS staining

BMMCs or peritoneal mast cells or BM basophils were incubated for 15 min with rat-anti-mouse Abs against CD16/32 (2.4G; BD Biosciences, San Diego, CA, USA) to block nonspecific binding, and then stained with FITC-conjugated anti-mouse FcεRIα (MAR-1;

eBioscience, San Diego, CA, USA) and PE-conjugated anti-mouse c-Kit Ab (2B8; BD PharMingen, San Diego, CA, USA) for 30 min on ice. After being washed with PBS, the stained cells (live-gated on the basis of forward and side scatter profiles) were analyzed on a BD Accuri™C6 flow cytometer (Becton Dickinson), and the data were processed using the CellQuest software (BD Biosciences).

ELISA

BMMCs or BM basophils were stimulated with 1 ng/ml of IL-33 for 6 h.

Levels of IL-6, IL-13, and TNF-α in the culture supernatants were measured using a mouse IL-6 (R&D Systems, Minneapolis, MN, USA) or IL-13 or TNF-α (eBioscience) ELISA kit.

Peritoneal neutrophil influx analysis

Mice were injected intraperitoneally with PBS or 0.5 μg rIL-33. After 3 h, the mice were killed, and peritoneal lavage was collected. Peritoneal cells were evaluated by flow cytometry. The following antibodies were used: anti-mouse CD16/CD32 (2.4G2; BD biosciences), FITC-conjugated anti-mouse Ly-6G (1A8; BD PharMingen), and PE-conjugated anti-mouse c-Kit Ab (2B8; BD PharMingen).

IL-13 and Gob-5 expression analysis in the lung

C57BL/6J mice were dosed intranasally with one or three repeated doses of rIL-33 (1 μg). IL-13 and Gob-5 mRNA levels in the lung were determined by qPCR after 3 days (for IL-13) or 1 day (for Gob-5).

Quantitative real-time PCR (qPCR)

Quantitative real-time PCR analysis using cDNA from BMMCs was performed using an AB Step One Plus Real-time PCR system (Applied Biosystems, Foster City, CA, USA), using primers and probes for mouse *Il1rl1* (ST2), *Period2* (Per2), *Bmal1* (Arntl), and *Gapdh* (Applied Biosystems) as previously described.¹² mRNA levels were normalized against the corresponding level of *Gapdh* mRNA, and relative expression levels are shown.

Detection of phosphorylated p38 and NF-κB in mast cells

Cells were collected and resuspended in 0.5 ml PBS, and formaldehyde was added to a final concentration of 4%. Cells were fixed for 10 min at 37 °C, and then chilled on ice for 1 min. For permeabilization, pre-chilled cells were centrifuged and resuspended in 90% ice-cold methanol, and then incubated for 30 min on ice. Permeabilized cells (1 × 10⁶) were mixed with 100 μl 0.5% bovine serum albumin (BSA)/PBS containing antibodies against phospho-p38 MAPK or phospho-NF-κB p65 (Cell Signaling Technology, Beverly, MA, USA) and incubated for 1 h at room temperature. Cells were washed with 0.1% BSA/PBS, followed by incubation with goat anti-rabbit immunoglobulin G (IgG) Alexa Fluor 647-conjugated antibodies (2 mg/ml) for 30 min at room temperature. Cells were washed again with 0.1% BSA/PBS, and then resuspended in 0.3 ml PBS and evaluated by FACS analysis.

Peritoneal mast cell assay

Peritoneal exudates were collected from wild-type mice at the indicated time points, and surface ST2 levels on mast cells gated by FcεRIα and c-kit were immediately assessed by flow cytometry.

Chromatin immunoprecipitation (ChIP) assay

ChIP assays were performed as previously described.¹² Antibodies used for the immunoprecipitation were as follows: anti-mouse/human KAT13D/CLOCK antibody ChIP grade (rabbit polyclonal IgG) (Abcam, Cambridge, MA, USA), control purified rabbit IgG (Invitrogen, Carlsbad, CA, USA), control purified mouse IgG (Life Technologies, Carlsbad, CA, USA). Equivalent masses of immunoprecipitated and input DNA were analyzed by real-time PCR using primers and a TaqMan probe for the promoter region of ST2, as described below.

ST2 primers;

Sense (5'-TGCAGAACTGTGTGGTGTC-3'),

Antisense (5'-GGAATTCGGTCTATCTACCTGCTC-3'),

TaqMan probe (5'-FAM-CCCTCAGAGCACAGCTG-MGB-3').

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

Retroviral construct and transfection of BMMCs

Murine *Period2* cDNA was subcloned into the retrovirus vector pMXs-puro, which was a gift from Dr. T. Kitamura (The University of Tokyo, Tokyo, Japan). Transduction of the vectors into BMMCs was performed as previously reported.¹³ In brief, a retrovirus-packaging cell line, PLAT-E, was transfected with each retrovirus vector using FuGENE 6 (Roche Diagnostics, Branford, CT, USA). The conditioned medium was incubated for 6 h at 37 °C in plates coated with RetroNectin (Takara Bio, Otsu, Japan) to allow attachment of virus particles. After removal of the supernatant, BMMCs that had been cultured for 2 weeks were plated onto virus-loaded plates and incubated 2 days. Infected cells were selected by additional culture in the presence of 1.6 µg/ml puromycin for 14 days.

Statistical analysis

Statistical analyses were performed using the unpaired Student's *t*-test for two-group comparisons or one-way ANOVA. The Statcel3 software (OMS Publishing, Saitama, Japan) was used for the analysis. A value of *P* < 0.05 was considered to be significant.

Results

IL-33-mediated mast cell responses vary with the time of day, dependent on functional CLOCK activity

The circadian clocks of cultured cells can be synchronized by several procedures that activate various signaling pathways, including a simple media change or serum shock. Thereafter, the cells exhibit circadian cycles of gene expression for several consecutive days.^{4,5} Hence, at least some cellular functions of circadian clocks can be investigated *in vitro*.

We showed previously that in *in vitro* culture, the mast cell clockwork is functional within a very narrow window of time (0–48 h after a media change for synchronization).^{11,12} This result was obtained by monitoring the bioluminescence of bone marrow-derived mast cells (BMMCs) derived from *Per2^{LUC}* knock-in mice,⁸ which express a PER2-luciferase fusion protein (PER2^{LUC} BMMCs) (Supplementary Fig. 1). This is likely due to a lack of oscillator coupling in dissociated cell cultures, leading to damping of the ensemble rhythm at the population level.^{4,5} A simple media change triggers synchronization of circadian clocks in peripheral cells, including mast cells, *in vitro*.^{11,12}

Therefore, we compared the extent of IL-33-mediated responses in mast cells between PER2^{LUC} BMMCs cultured for 12 and 24 h following a media change, representing the nadir and zenith of PER2^{LUC} protein level, respectively. IL-33-mediated IL-6, IL-13, and

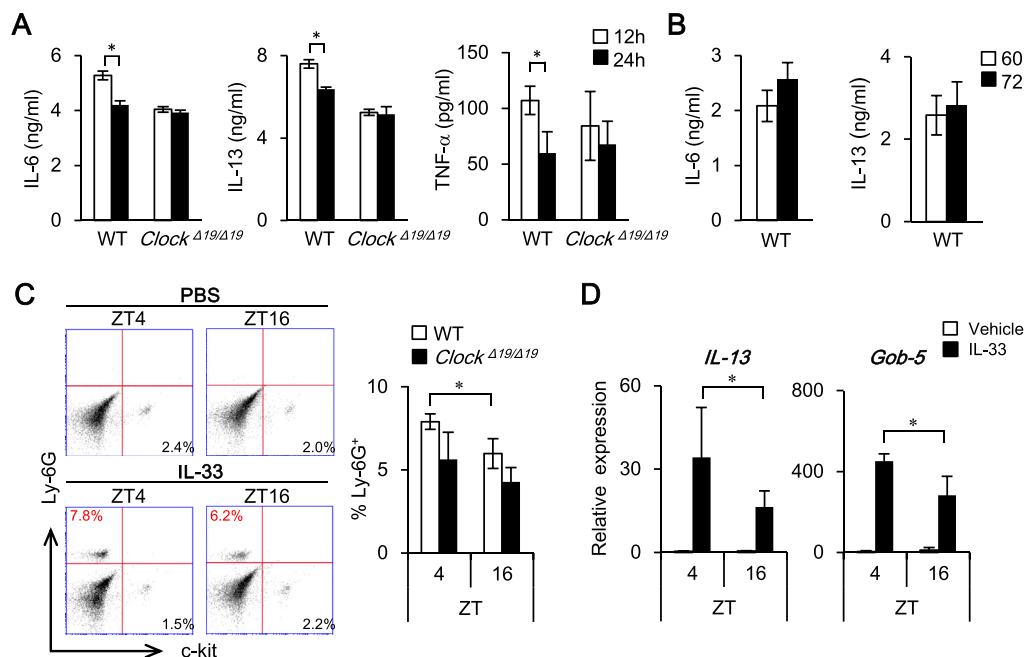


Fig. 1. IL-33-mediated mast cell responses vary with the time of day. (A) IL-33-mediated IL-6, IL-13, and TNF- α production in wild-type and *Clock*-mutated BMMCs, derived from PER2^{LUC} knock-in mice, cultured for 12 or 24 h after a media change (*n* = 4 per group). (B) IL-33-mediated IL-6 and IL-13 production from BMMCs, derived from PER2^{LUC} knock-in mice, cultured for 60 or 72 h after a media change (*n* = 4 per group). (C) IL-33-mediated neutrophil infiltration into the peritoneal cavity at ZT4 or ZT16 in wild-type or *Clock*-mutated mice. Neutrophils are represented by the Ly-6G⁺ population. (left) Representative plots in wild-type mice are shown. (right) Quantitative analysis (*n* = 4–6 per group). (D) Expression of IL-13 and Gob-5 mRNAs in the airway following intranasal administration of IL-33 to wild-type mice at ZT4 or ZT16 (*n* = 5–7 per group). Values represent means $\pm$ SD. **P* < 0.05. Similar results were obtained in at least three independent experiments (A–D).

TNF- α production was significantly higher at 12 h than at 24 h, but these variations were absent in *Clock*-mutated PER2^{LUC} BMMCs (Fig. 1A). By contrast, IL-33-mediated IL-6 and IL-13 production was comparable at 60 and 72 h (Fig. 1B), times at which the clockwork appeared to be no longer functional (Supplementary Fig. 1).

The intraperitoneal influx of neutrophils in response to IL-33 in wild-type mice is mast cell-dependent.¹⁴ Consistent with the *in vitro* findings, IL-33-induced neutrophil infiltration into the peritoneal cavity was more extensive in wild-type mice treated with IL-33 at Zeitgeber time [ZT] 4 than in mice treated at ZT16 (Fig. 1C). This variation was absent in *Clock*-mutated mice (Fig. 1C). In addition, intranasal administration of IL-33 to wild-type mice induces mast cell activation in the airway with increased expression of IL-13 and Gob-5.¹⁵ IL-33-induced IL-13 and Gob-5 mRNA expression in the lung was higher in mice treated with IL-33 at ZT4 than in mice treated at ZT16 (Fig. 1D). Collectively, these observations demonstrate that IL-33-mediated mast cell responses varied with the time of day, dependent on functional CLOCK activity.

CLOCK regulates ST2 expression in mast cells to temporally fine-tune IL-33/ST2-mediated signaling

To investigate how CLOCK temporally regulated IL-33-mediated mast cell responses, we examined the kinetics of surface ST2 expression in BMMCs. Surface ST2 levels were higher in BMMCs cultured for 12 h than in those cultured for 24 h, but these variations were absent in *Clock*-mutated BMMCs (Fig. 2A). Surface ST2 expression did not vary between 60 and 72 h (Fig. 2B), time points at which the clockwork appeared to be no longer functional. mRNA levels of ST2 and clock genes *Per2* and *Bmal1* (*Arntl*) exhibited temporal variations in wild-type BMMCs following a media change for synchronization, but these variations were absent in *Clock*-mutated BMMCs (Fig. 2C). Corresponding to the ST2 levels, IL-33-mediated phosphorylation of p38 and NF- κ B varied with the time of day in wild-type, but not *Clock*-mutated BMMCs (Fig. 2D). Furthermore, surface ST2 levels on peritoneal mast cells isolated from wild-type mice at ZT4 were higher than those isolated at ZT16, but these variations were absent in *Clock*-mutated mice (Fig. 2E).

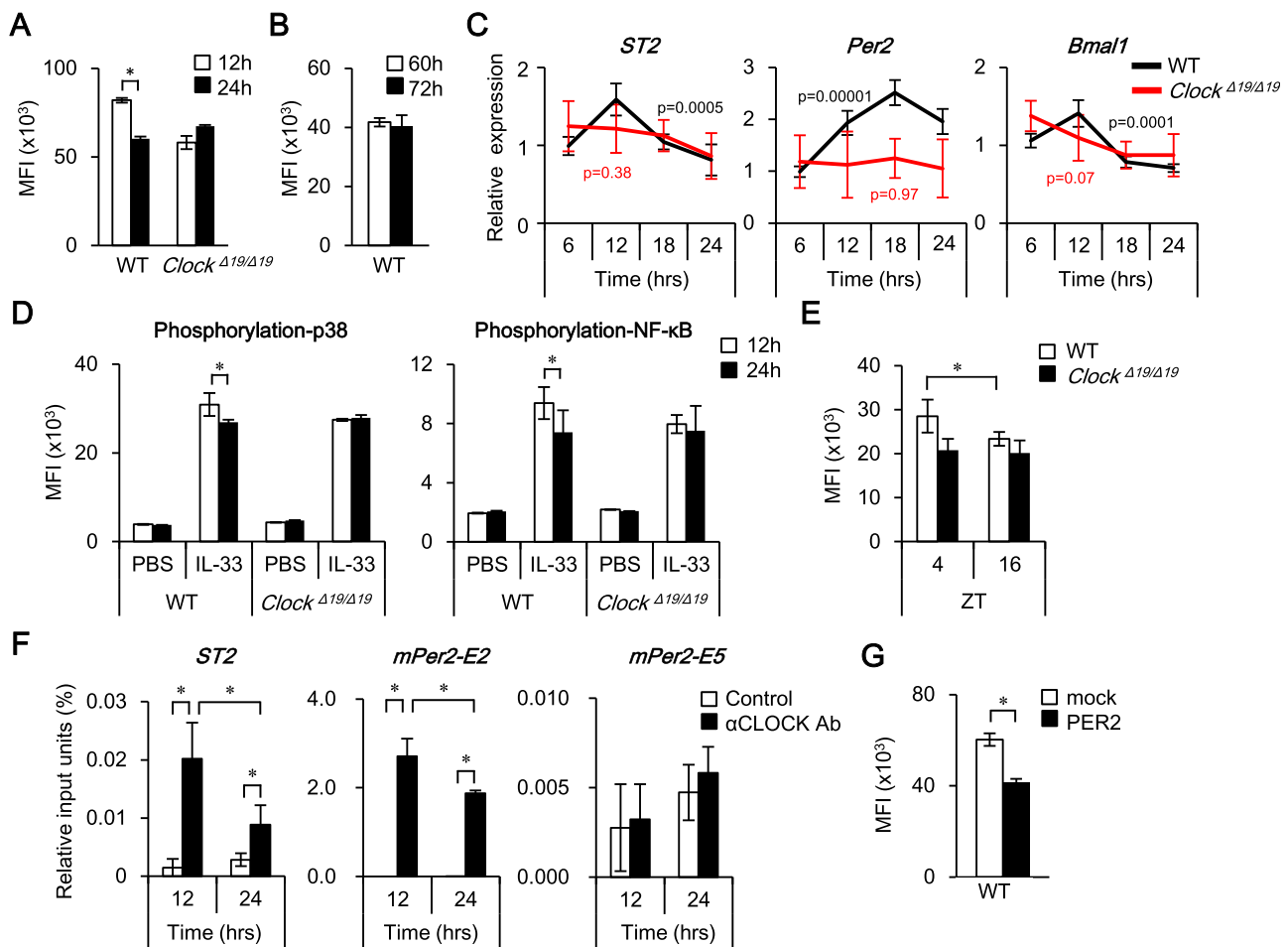


Fig. 2. CLOCK temporally regulates ST2 expression in mast cells. (A) Quantitative analysis of ST2 levels in wild-type and *Clock*-mutated BMMCs cultured for 12 or 24 h after a media change. MFI, mean fluorescence intensity ($n = 3$ per group). (B) Quantitative analysis of ST2 levels in wild-type BMMCs cultured for 60 or 72 h after a media change ($n = 3$ per group). (C) Wild-type or *Clock*-mutated BMMCs were cultured *in vitro* after a media change for synchronization. At the indicated time points, mRNA was extracted and qPCR analysis was performed on *ST2*, *Per2*, and *Bmal1* (*Arntl*) mRNAs. Values represent means $\pm$ SD. P values were calculated by one-way ANOVA ($n = 4$ per group). (D) Phosphorylation of p38 and NF- κ B following IL-33 stimulation in BMMCs. Wild-type or *Clock*-mutated BMMCs were stimulated with IL-33 for 15 min, and then IL-33-dependent p38 and NF- κ B phosphorylation levels were evaluated ($n = 6$ per group). (E) Quantitative analysis of ST2 levels on peritoneal mast cells from peritoneal lavages of wild-type or *Clock*-mutated mice, performed at different times of the day (ZT4 and ZT16) ($n = 6$ per group). (F) Detection of CLOCK binding to the promoter region of mouse *ST2* or the E2 or E5 enhancer of mouse *Period2* (*mPer2*) in wild-type BMMCs cultured for 12 or 24 h after a media change ($n = 3$ per group). (G) Quantitative analysis of ST2 levels on mock or PER2-overexpressing BMMCs 24 h after a media change ($n = 3$ per group). Values represent means $\pm$ SD. * $P < 0.05$. Similar results were obtained in at least three independent experiments (A–G).

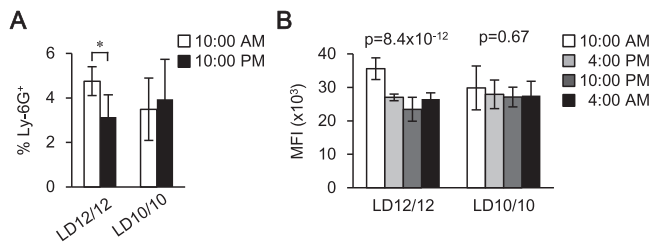


Fig. 3. Functional disruption of the circadian clock system results in the absence of temporal variations in IL-33-mediated responses in mice and surface ST2 expression on mast cells. **(A)** IL-33-mediated neutrophil infiltration into the peritoneal cavity at 10:00 AM or 10:00 PM in mice housed under a regular 12-h light/12-h dark (LD12/12) cycle or a 10-h light/10-h dark (LD10/10) cycle for 8 weeks. Neutrophils are represented by the Ly-6G⁺ population. Quantitative analysis ($n = 4-5$ per group). **(B)** Quantitative analysis of ST2 levels, determined by flow cytometry, on peritoneal mast cells isolated at 4:00 AM, 10:00 AM, 4:00 PM, or 10:00 PM from wild-type mice housed under LD12/12 or LD10/10 conditions. MFI, mean fluorescence intensity ($n = 9-10$ per group). Values represent means $\pm$ SD. * $P < 0.05$ (one-way ANOVA). Similar results were obtained in at least two independent experiments (**A**, **B**).

Thus, ST2 mRNA and protein levels also varied with the time of day in mast cells, dependent on functional CLOCK activity.

We next investigated whether CLOCK directly regulates the transcription of ST2. Several E-box or E-box-like elements to which CLOCK can bind¹⁶ are present in the promoter region of mouse ST2 (Supplementary Fig. 2). Consistent with this, ChIP assays revealed that CLOCK temporally bound to the promoter region of ST2 in wild-type BMMCs (Fig. 2F). The integrity of the ChIP samples was confirmed by detection of constitutive binding of CLOCK to the promoter of the *Per2* gene, which contains a non-canonical E-box enhancer 2 (E2) sequence but no E5 sequence, in wild-type BMMCs cultured for 12 or 24 h, as previously reported¹⁷ (Fig. 2F). Importantly, wild-type BMMCs transduced with retroviral vectors that overexpress PER2, an inhibitor of CLOCK activity, exhibited reduced cell surface ST2 expression relative to control BMMCs (Fig. 2G). These results suggested that CLOCK directly regulates ST2 expression in mast cells.

Aberrant light/dark conditions result in the absence of temporal variations in IL-33-mediated mast cell responses in vivo

If the IL-33/ST2 pathway in mast cells is under control of the circadian clock, functional disruption of the circadian clock system by housing mice under irregular circadian lighting environments^{4,5} should affect the IL-33/ST2-mediated mast cell responses. It was shown that mice housed under a 10-h light/10-h dark (LD10/10) cycle for 8 weeks, but not mice housed under a normal LD12/12

cycle, showed disruption of the circadian rhythms in locomotor activities.¹⁸

As expected, the temporal variations of IL-33-mediated neutrophil infiltration into the peritoneal cavity and ST2 expression in peritoneal mast cells were absent in mice housed under LD10/10 cycle for 8 weeks, but present in mice housed under a normal LD12/12 cycle (Fig. 3A, B). These results support the notion that the IL-33/ST2 pathway in mast cells is under the control of the circadian clock.

IL-33-mediated basophil responses vary with the time of day, dependent on functional CLOCK activity

We finally determined whether the findings in mast cells were relevant to basophils. For this purpose, we compared the extent of IL-33-mediated responses in basophils between bone marrow-derived basophils (BM basophils) cultured for 12 and 24 h following a media change. We have previously shown that the BM basophil clockwork is functional within this very narrow window of time (0–48 h after a media change for synchronization) by monitoring the bioluminescence of bone marrow-derived BM basophils derived from *Per2^{Luc}* knock-in mice.¹⁹ Similar to BMMCs, IL-33-mediated IL-6 production was significantly higher at 12 h than at 24 h, but these variations were absent in *Clock*-mutated BM basophils (Fig. 4A). Surface ST2 levels were also higher in BM basophils cultured for 12 h than in those cultured for 24 h, but these variations were absent in *Clock*-mutated BM basophils (Fig. 4B). mRNA levels of ST2 and clock genes *Per2* exhibited temporal variations with an opposite phase in wild-type BMMCs following a media change for synchronization, but these variations were absent in *Clock*-mutated BMMCs (Fig. 4C). These results suggest that IL-33-mediated basophil responses vary with the time of day, dependent on functional CLOCK activity.

Discussion

IL-33/ST2 signaling in innate immune cells is recently highlighted as a critical pathway for a variety of physiology and pathologies including allergic diseases.^{1–3} However, regulatory mechanisms of this important pathway remain poorly understood. We propose here-in that the circadian clock (CLOCK protein) is a novel regulator of the IL-33/ST2 pathway in mast cells. To the best of our knowledge, this is the first study showing that IL-33/ST2 signaling in innate immune cells is temporally regulated.

Enoksson *et al.* reported that the intraperitoneal influx of neutrophils in response to IL-33 in wild-type mice is partially dependent on mast cell-derived TNF- α .¹⁴ Because there is a time-of-day-dependent variation in IL-33-mediated TNF- α production as

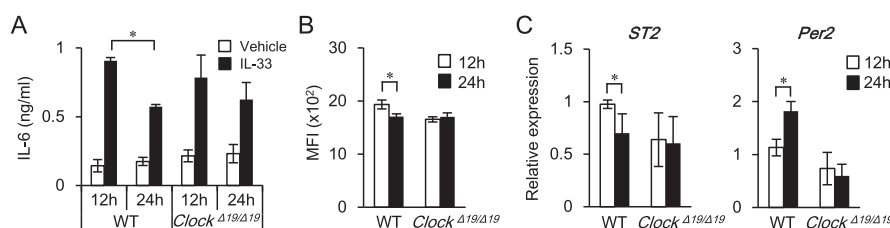


Fig. 4. IL-33-mediated mouse bone marrow-derived basophils responses vary with the time of day. **(A)** Bone marrow-derived basophils (BM basophils) generated from wild-type or *Clock*-mutated mice were cultured after a media change for synchronization. After 12 or 24 h, IL-33-mediated IL-6 production was measured by ELISA ($n = 4$ per group). **(B)** Quantitative analysis of ST2 levels, determined by flow cytometry, of the basophils described in **A**. MFI, mean fluorescence intensity ($n = 3$ per group). **(C)** qPCR analysis for ST2 or *Per2* mRNA using the basophils described in **A** ($n = 4$ per group). Values represent means $\pm$ SD. * $P < 0.05$. Similar results were obtained in at least three independent experiments (**A**–**C**).

well as IL-6 and IL-13 production in wild-type, but not *Clock*-mutated, BMMCs (Fig. 1A), the time-of-day-dependent variation of intraperitoneal influx of neutrophils in response to IL-33 in wild-type mice could be attributed to the temporal variations of IL-33-mediated TNF- α production. Unfortunately, we failed to detect TNF- α in the peritoneal lavage fluid samples of the IL-33-treated mice possibly because TNF- α concentrations were very low in the samples. Please note that Enoksson *et al.* also did not present the data regarding TNF- α levels in the peritoneal lavage fluid samples. They used mast cells derived from TNF- α -deficient mice and argue their conclusions.¹⁴

The current results suggest that CLOCK regulates ST2 expression via direct modulation of transcription, thereby temporally gates mast cell responses to IL-33. A time-of-day-dependent variation in IL-33-mediated phosphorylation of p38 and NF- κ B, downstream intracellular signaling molecules of ST2, in mast cells may support this notion. However it is possible that other mechanisms may operate underlying CLOCK-dependent regulation of IL-33/ST2 signaling in mast cells. For example, Keller *et al.* reported that components of the MAPK pathway (e.g., MEK1) or regulatory components of NF- κ B (e.g. I κ B α) exhibit circadian variations at mRNA levels in mouse macrophages.²⁰ These molecules may show temporal variation also in mast cells so that IL-33/ST2 signaling could be temporally modulated in mast cells. Alternatively, CLOCK might directly interact with NF- κ B, thereby affecting temporal IL-33/ST2 signaling in mast cells.^{21,22}

The kinetics pattern of *Per2* mRNA expression was quite different between BMMCs and BM-basophils, although the ST2 expression was similar (Fig. 2C, 4C). In general, the peak phases of a given core clock gene including *Per2* differ across organs, tissues, or cell types.²³ Thus, we speculate that the different kinetics of *Per2* mRNA between BMMCs and BM basophils might reflect the cell-type specific differences in the kinetic of core circadian gene mRNA expression. Given that the kinetic of ST2 mRNA expression were similar between BMMCs and BM basophils, *Per2* may only play a minor role in the regulation of *Clock*-mediated ST2 expression in mast cells or basophils. This issue should be investigated in future studies.

We have previously shown that IgE-mediated mast cell responses (e.g. degranulation) also exhibit a time-of-day-dependent variation, which relies on the functional circadian clock activity in mast cells.^{11,12} In this case, the β subunit of high-affinity IgE receptor (Fc ϵ RI β) is a CLOCK-target gene in mast cells.¹² The current findings thus expand the previous studies and reveal that IL-33/ST2-mediated mast cell responses are also under the control of the circadian clock and ST2 is another E-box-mediated target gene of CLOCK protein in mast cells.

Recent studies show that IL-33/mast cell-mediated responses play important roles in the development of allergic diseases,²⁴ of which pathophysiology exhibit prominent circadian aspects.^{6,25} Thus, *Clock*-dependent temporal regulation of the IL-33/ST2 pathway in mast cells and possibly in basophils may underlie the circadian pathophysiology of allergic diseases. For instance, since IL-33 potentiates IgE-mediated mast cell responses,^{26,27} *Clock*-dependent augmented IL-33-mediated mast cell responses in the resting phases (the night in humans) could contribute to the development of nocturnal symptoms in patients with asthma. Alternatively, since IL-33 plays a critical role in rhinovirus-induced asthma exacerbation,²⁸ circadian regulation of IL-33-mediated mast cell responses might lead to temporal variations in the severity of virus-induced exacerbation of asthma.

In summary, we suggest that IL-33-mediated mast cell responses exhibit a time-of-day-dependent variation, which relies on functional CLOCK activity. CLOCK likely does so by regulating ST2 expression in mast cells in a circadian manner. The current findings

revealing temporal regulation of IL-33/ST2-mediated signaling in mast cells will provide a novel insight into the IL-33-associated physiology or pathologies such as allergic diseases.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.alit.2017.02.004>.

Conflict of interest

The authors have no conflict of interest to declare.

Authors' contributions

TK, KI, and AN designed the study; TK, KI, YN, NN, and MH performed the experiments and analyzed the data; HO, KO, and SS provided the reagents; and TK, KI, YN, and AN wrote the manuscript.

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