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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 ($Clock^{\Delta 19/\Delta 19}$ 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 $Clock^{\Delta 19/\Delta 19}$ 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 $Clock^{\Delta 19/\Delta 19}$ 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.

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 mice[7] and investigated the mechanisms underlying *Clock*-dependent temporal regulation of IL-33-mediated mast cell responses.

Methods

Mice

C57BL/6 $Clock^{\Delta 19/\Delta 19}$ mice,[7] their littermates, and $PER2^{LUCIFERASE}$ ($PER2^{LUC}$) knock-in mice[8] (C57BL/6 background) were bred under specific pathogen-free conditions. $Clock^{\Delta 19/\Delta 19}$ 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 ($Clock^{\Delta 19/\Delta 19}$), resulting in the loss of normal transcriptional activity.[7] $Clock^{\Delta 19/\Delta 19}$ mice were mated in our laboratory to generate $Clock^{\Delta 19/\Delta 19}$ $Per2^{Luc}$ 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 (1×10^6) 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.