Supplementary Materials and Methods

Mice

Female 9-11 week-old mice were used for this study. *Clock*$^{\Delta 19/\Delta 19}$ mice have an 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 the loss of normal transcriptional activity (Vitaterna et al. 1994). *mPer2*$^{m/m}$ mice have an in-frame deletion mutation in the PAS domain (a dimerization domain found in Per, Arnt and Sim) of *mPer2*, which results in the loss of normal transcriptional activity (Zheng et al. 1999). Mast cell–deficient WBB6F1-W/Wv mice and control wild-type WBB6F1-$^{+/+}$ mice were purchased from Japan SLC. All animal experiments were approved by the Institutional Review Board of the University of Yamanashi and performed under the Declaration of Helsinki protocols.

IMQ-induced psoriasis-like skin inflammation

Mice were treated daily for 5 consecutive days on both ears and shaved dorsal skin with 31.2 mg of commercially available imiquimod (IMQ) cream (5% Beselna cream, Mochida Pharmaceutical, Tokyo, Japan) or Vaseline at ZT2. Imiquimod cream mixed

Vaseline in one to one and used. All animal experiments were approved by the Institutional Review Board of the University of Yamanashi.

Intradermal IL-23 Injection

To induce IL-23-mediated psoriasis-like skin inflammation, 20 μl PBS, either alone or containing 500 ng recombinant mouse IL-23 (Biolegend, San Diego, CA) was intradermally injected into the ears of anesthetized mice using a 30-gauge needle under the microscope (Wraymer Inc., Ohsaka, Japan) at ZT2. *Clock*$^{\Delta 19/\Delta 19}$ mice (C57BL/6 genetic background) and their control mice were injected every other day for 5 days. *mPer2*$^{m/m}$ mice (ICR genetic background) and their control mice were injected every other day for 10 days. Ear thickness was measured every day. On the injected days, ear thickness was measured immediately before injections.

Scoring severity of skin inflammation

Ear thickness was measured using an engineer's micrometer at indicated time points. The Δ ear thickness is calculated as the changes in ear thickness ([ear thickness at the indicated time points] - [ear thickness before treatment on day 0]). A scoring system based on the clinical Psoriasis Area and Severity Index, except for the affected skin area,

was used (van der Fits et al, 2009). Erythema, scaling, and thickening of ears were scored at ZT2 independently from 0 to 4: 0, none; 1, slight; 2, moderate; 3, marked; and 4, very marked. The scoring was performed in a blinded fashion: investigators were not informed which mice were treated or untreated with IMQ or IL-23. The cumulative score served as a measure of the severity of inflammation (scale 0-12).

Isolation of leukocytes from the ear

The ear was cut into small pieces and incubated in RPMI 1640 medium containing Liberase TL (0.5 mg/ml, Roche, Basel, Switzerland) for 1 hour at 37°C. A suspension of single cells was separated from undigested tissue by passing through a 40-μm nylon cell strainer.

FACS staining

Monoclonal fluorescent antibodies against mouse CD3ε, $\gamma\delta^+$ TCR, B220, IL-22, IL-23p40 were purchased from eBioscience (San Diego, CA). Monoclonal fluorescent antibodies against mouse CD4 and IL-23R were purchased from Beckman Coulters (Brea, CA) and R&D (Minneapolis, MN). CD8a, I-A/I-E, IL-17A were purchased from BD Pharmingen (San Diego, CA), respectively. The spleen, lymph nodes or thymus

from mice were incubated for 15 minutes with Fc receptor blocking mAb (BD Biosciences, San Diego, CA) to block nonspecific bindings, and then were stained with FITC-conjugated anti-mouse CD4 Ab or B220 Ab, PE-conjugated anti-mouse γδTCR Ab or I-A/I-E Ab, PerCP-conjugated anti-mouse IL-23R Ab and APC-conjugated anti-mouse CD3εAb or CD8a Ab in PBS for 30 minutes on ice. The stained cells (live-gated on the basis of forward and side scatter profiles) were analyzed. Isolated cells from ear were incubated for 15 minutes with Fc receptor blocking mAb (BD Bioscience), and then were stained with FITC-conjugated Annexin V (BD Pharmingen), PE-conjugated anti-mouse $\gamma\delta^{+}$TCR Ab and APC-conjugated anti-mouse CD3ε in PBS for 30 minutes on ice. Viable cells were identified by Annexin V uptake. For intracellular cytokine staining, cells were incubated in the presence of 2 μg/ml brefedrinA for 4 hours. Viable cells were identified by APC-conjugated Fixable viability Dye cell staining (eBioscience). Successively, cells were incubated with Fc receptor blocking mAb (BD Bioscience), and stained surface antigens. Cells were then fixed and permeabilized using eBioscience IC Fixation and Permeabilization Kit. And then were stained with FITC-conjugated anti-mouse IL-23p40 Ab, Alexa Fluor488-conjugated

anti-mouse IL-17A or PerCP-conjugated anti-mouse IL-22 Ab for 30 minutes. All samples were analyzed with BD Accuri™C6 flow cytometry (Becton Dickinson, Franklin Lakes, NJ).

Splenic γ/δ$^+$ T cell preparation and culture

γ/δ$^+$ T cells were purified from spleens by mouse γ/δ$^+$ TCR$^+$ T cell Isolation Kit (Milteny Biotec, Bergisch Gladbach, Germany) at ZT2. Cells were stimulated for 72 hours with IL-23 (10 ng/ml, R&D) or medium alone in the presence of anti-CD3 (2 μg/ml, eBioscience). After 72 hours, IL-17A or IL-22 concentrations in the supernatants were determined by ELISA (eBioscience).

ELISA

A Ready-Set-Go kit was used according to the manufacturer's protocol (eBioscience) for ELISA of IL-17A and IL-22. Mouse IgG ELISA Quantitation set (Bethyl Laboratories Inc, TX), Mouse IgA ELISA kit (Immunology consultants Laboratories Inc, Portland) and Mouse IgE ELISA kit (Morinaga, Yokohama, Japan) was used according to the manufacturer's protocol for ELISA of serum and fecal immunoglobulins.

Quantitative real-time PCR (qPCR)

RNA was harvested from splenic γ/δ^+ T cells or skin with RNeasy® Plus Micro Kit or RNeasy® Protect Mini Kit (QIAGEN, Venlo, Netherland) according to the manufacture's protocol. RT was performed using ReverTra Ace® qPCR RT Kit (TOYOBO, Ohsaka, Japan). Analysis of *IL-17A, IL-22, IL-23R, IL-23p19, S100A8, Keratin16, Reg3g*, *Period2* (*Per2*), *Bmal1* (*Arntl*), and *Clock* mRNA levels was performed using commercially available primer/probe sets (TaqMan ® Gene Expression Assay, Applied Biosystems, Foster City, CA). The accession numbers of the primer/probe sets are as follows; *Bmal1 (Arntl*): Mm00500226-m1, *Clock*: Mm00455950-m1, *Gapdh*: Mm99999915-g1, *IL-17A*: Mm00439618-m1, *IL-22*: Mm01226722-g1, *IL-23p19*: Mm01160011-g1, *IL-23R*: Mm00519943-m1, *Keratin16*: Mm01306670-g1, *Per2*: Mm00478113-m1, *Reg3g*: Mm00441127-m1, *S100A8*: Mm00496696-g1. The *Clock* primers/probe (Mm00455950-m1) are designed to amplify the specific sequences in the exon 15-16 of the gene and the deletion of the exon 19 ($Clock^{\Delta 19/\Delta 19}$) does not affect the mRNA stability of mutated *Clock* (Vitaterna et al. 1994). Relative levels of expression were determined by normalization to *GAPDH*. Samples were assayed on Step One Plus™ Real Time PCR Systems (Applied

Biosystems).

Chromatin immunoprecipitation (ChIP) assay

ChIP assay was performed as previously described (Nakamura et al. 2014). Antibodies used for the immunoprecipitation are anti-mouse/human KAT13D/CLOCK antibody ChIP grade (rabbit polyclonal IgG) (Abcam, Cambridge, MA), control purified rabbit IgG (Invitrogen, Inc., Carlsbad, CA), control purified mouse IgG (Life Technologies Cooperation, Carlsbad, CA). Equivalent masses of immunoprecipitated and input DNA were analyzed by real-time PCR using primers and a TaqMan probe for the promoter region of IL-23R as described below.

IL-23R primers;

Sense (5’-CTCCAAATCCAGGCCTGTCTT-3’),

Antisense (5’-CGGCCCTGTAAATCCTGAAGAT-3’),

TaqMan probe (5’-FAM-CTCCTCTTGGCATTTGT-MGB-3’).

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

Histology and immunohistochemistry

Sections from 4% paraformaldehyde, paraffin-embedded ears and back skins were stained with H&E. For immunohistochemistry, sections were deparaffinized and hydrated by washing sections in Xylene followed by a graded alcohol series. Sections were incubated in target retrieval solution (DAKO #S2367) 95°C for 30 min, and endogenous peroxidase activity was quenched by treating sections with 0.3% hydrogen peroxide methanol for 10 min at room temperature. Sections were blocked for 60 min (DS pharma #UKB40) at room temperature followed by incubation with primary Abs (goat anti-CD3ε, Santa Cruz #SC-1127, ×2000) over night at 4°C. Samples were washed and incubated for 60 min with secondary Abs (rabbit anti-goat immunoglobulins/biotinylated, DAKO #E-0466, ×500) and developed using DAKO LSAB+System-HRP (#K0679). All stained sections were counterstained with hematoxylin. The staining intensity was measured in five randomly selected fields (660 × 875 μm) at a magnification of ×200. The mean intensity was calculated for each specimen.

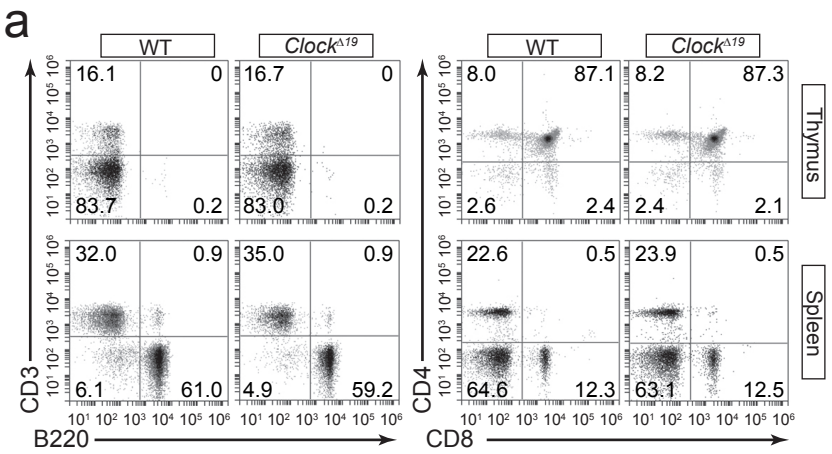
Statistical analysis

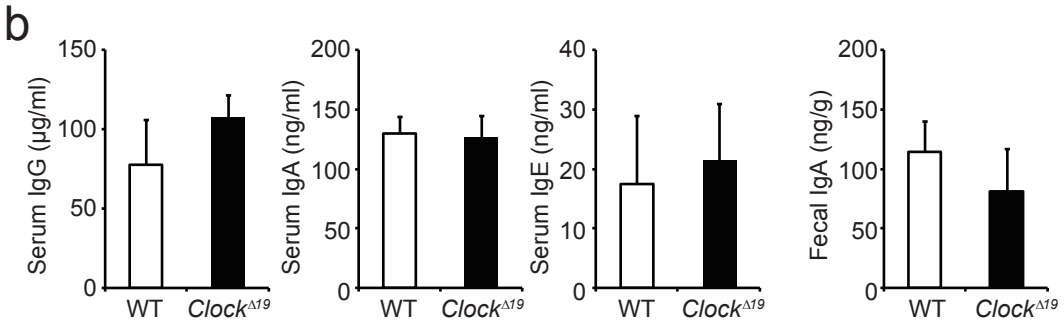
The statistical analyses were performed using the unpaired Student's *t*-test for two

group-comparisons, and ANOVA for comparison of more than two groups.

Supplementary Reference

Nakamura Y, Nakano N, Ishimaru K, *et al*. (2014) Circadian regulation of allergic reactions by the mast cell clock in mice. *J Allergy Clin Immunol* 133:568-575.





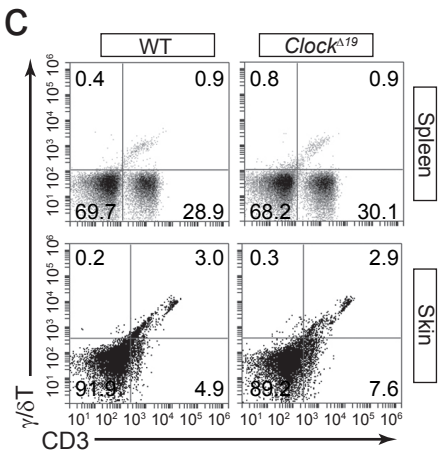


Figure S1

Clock$^{\Delta 19/\Delta 19}$ mice do not have obvious developmental defects in the immune system.

a. Frequency of T cells and B cells in the thymus and spleen obtained from wild-type and *Clock*$^{\Delta 19/\Delta 19}$ mice.

b. Serum IgG, IgA, IgE, and fecal IgA levels in wild-type and *Clock*$^{\Delta 19/\Delta 19}$ mice

c. Frequency of γ/δ T cells in the spleen and skin in wild-type and *Clock*$^{\Delta 19/\Delta 19}$ mice at ZT2.

Representative data (a-c) out of 3 independent experiments are shown.

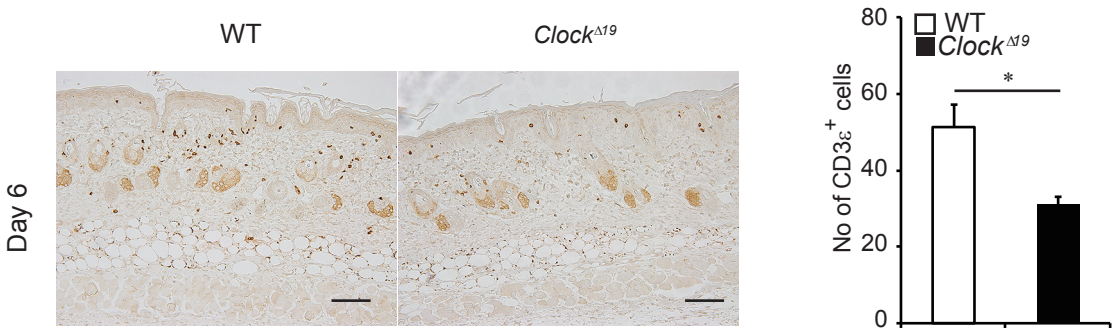


Figure S2

The number of infiltrating CD3+ T cells into the mouse skin.

The number of infiltrating CD3+ T cells into the skin in IMQ-treated wild-type mice or Clock$^{\Delta 19/\Delta 19}$ mice on Day 6 (n=3 per group). Scale bar=100 μm.

The values represent the means ± SD. *p < 0.05

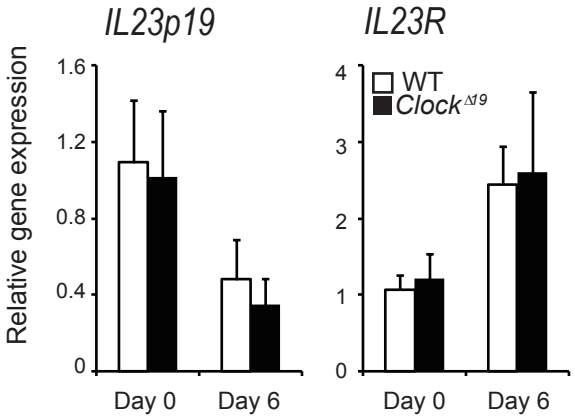


Figure S3

IL-23 and IL-23R mRNA expression in the mouse skin on Day 0 and Day 6

IL-23 or IL-23R mRNA expression in the skin of IMQ-treated wild-type mice or $Clock^{\Delta 19/\Delta 19}$ mice at ZT2 on Day 0 and Day 6 (n=3-5 per group).

The values represent the means ± SD.

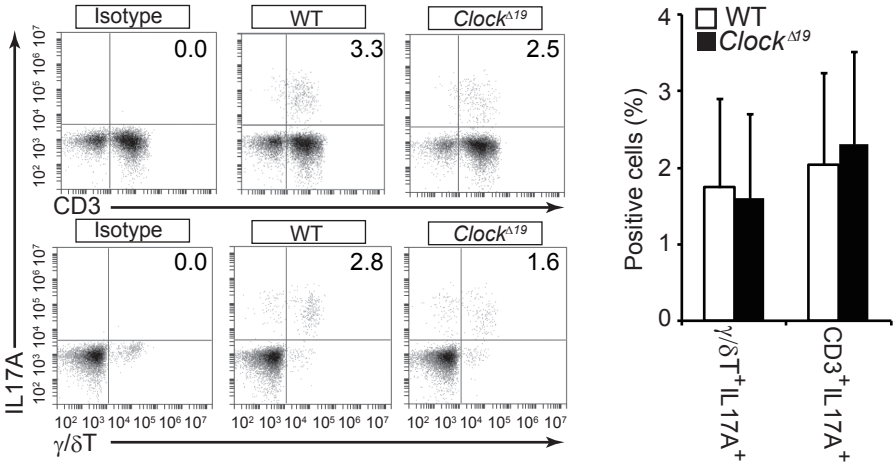


Figure S4

Frequency of IL-17$^+$ T cells in the cervical lymph nodes of IMQ-treated mice

Frequency of IL-17$^+$ T cells (γ/δ^+IL-17$^+$ cells, CD3$^+$IL-17$^+$ cells) in the cervical lymph nodes evaluated by FACS analysis in IMQ-treated wild-type or Clock$^{\Delta 19/\Delta 19}$ mice on day 6 (n=3-5 per group). The values represent the means ± SD.

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Sense primer TaqMan probe Antisense primer

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ACAGCCAGGAGGGCAGCGGCTTGATTCTGAAGAGAGAAAGTAATGCAAACAAATGAAAAGAG

Figure S5

E-box-like elements in the promoter region of the mouse *IL-23R*

There are several E-box-like elements (“CANNTG” or “CANNTT”) present in the promoter region of the mouse *IL-23R*. CLOCK/BMAL1-mediated circadian control of a gene transcription can be driven by non-canonical E-box sequences in the promoter region of the gene. “CANNTG” or “CANNTT” indicate green.

The 1st exon sequence indicates blue.

Primers and probe sequence sites used for the ChIP assay (*IL-23R*) are also shown in red.

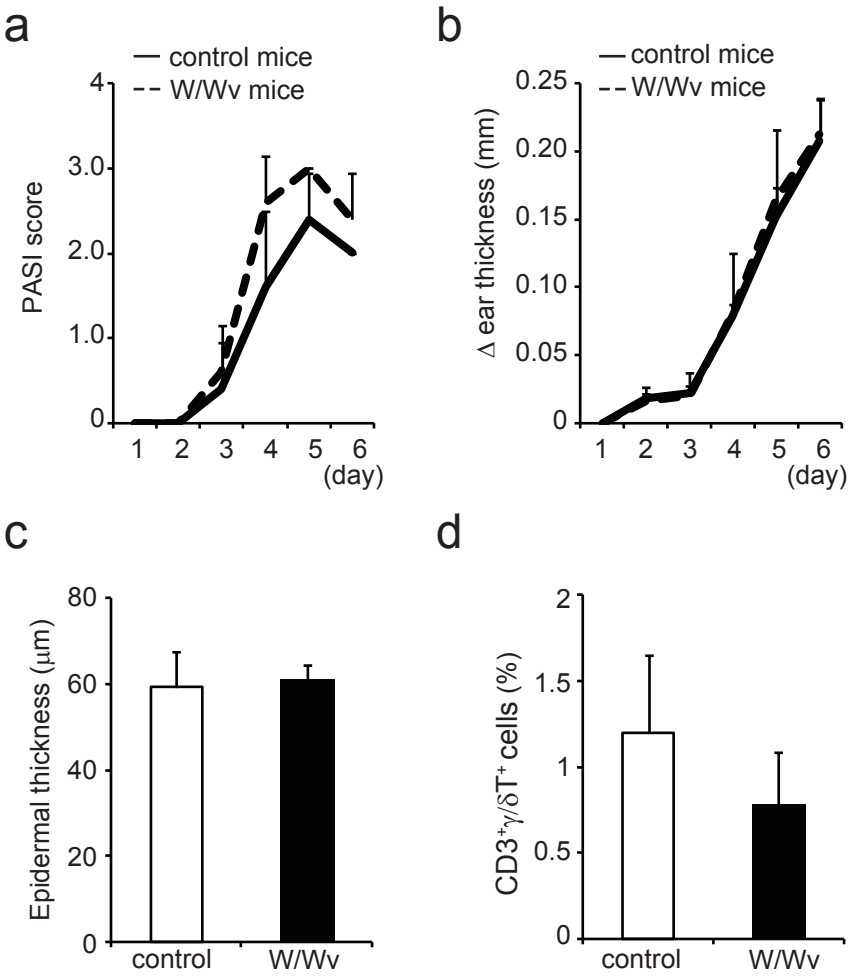

Figure S6

Mast cells play a marginal role in IMQ-induced dermatitis in the current experimental conditions.

a-d. Mast cell-deficient W/Wv mice and their control mice were treated daily with topical IMQ for 5 days and PASI score in the dorsal skin (a), ear thickness (b), epidermal thickness (c),

and frequency of $CD3^{+}\gamma/\delta^{+}$T cells in the cervical lymph nodes (d) are shown (n=5 per group).

The values represent the means ± SD.