

Allergy 2022 年掲載予定 巻 頁 未定

Circadian rhythm affects the magnitude of contact hypersensitivity response in mice

Short title: Circadian rhythm and severity of CHS response

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Abbreviations

ACD: allergic contact dermatitis, β 2AR: β 2 adrenergic receptor, cDC: conventional dendritic cell, CHS: contact hypersensitivity, DC: dendritic cell, DNBS: dinitrobenzene sulfonic acid, DNFB: dinitrofluorobenzene, FITC: fluorescein isothiocyanate, HSV: herpes simplex virus, IFN- γ : interferon- γ , LABA: long-acting beta agonist, LN: lymph node, XCR1: X-C Motif Chemokine Receptor 1, ZT: zeitgeber time

ABSTRACT

The circadian rhythm controls multiple biological processes, including immune responses; however, its impact on cutaneous adaptive immune response remains unclear. To address this issue, we used a well-established cutaneous type IV allergy model, contact hypersensitivity (CHS). We induced CHS using dinitrofluorobenzene (DNFB). Mice were sensitized and elicited with DNFB in the daytime or at night. In mice, a nocturnally active animal, we found that ear swelling increased when mice were sensitized at night compared with in the daytime. In addition, cell proliferation and cytokine production in the draining lymph nodes (LNs) were promoted when sensitized at night. We hypothesized that these differences were due to the oscillation of leukocyte distribution in the body through the circadian production of adrenergic hormones. Administration of a β 2-adrenergic receptor (β 2AR) agonist salbutamol in the daytime decreased the number of immune cells in blood and increased the number of immune cells in LNs. In contrast, a β 2AR antagonist ICI18551 administration at night increased the number of immune cells in blood and decreased the number of immune cells in LNs. Accordingly, the severity of CHS response was exacerbated by salbutamol administration in the daytime and attenuated by ICI18551 administration at night. Taken together, our study demonstrated that the magnitude of adaptive CHS response depends on the circadian rhythm and this knowledge may improve the management of allergic contact dermatitis (ACD) in humans.

Key words

Adrenal hormone, Circadian rhythm, Contact hypersensitivity

INTRODUCTION

The circadian rhythm is a 24-hour cyclic internal body clock that dictates numerous biological processes. It affects not only sleeping and feeding patterns¹, but also body temperature, hormone production, and cell regeneration²⁻⁴. Of note, recent studies highlighted a specific role of the circadian clock to coordinate functions of the immune system. Time-of-day-dependent variables are found in inflammatory responses and host-parasite interactions⁵. For example, the severity of pulmonary response to endotoxin challenge is exacerbated during the daytime in mice depending on the circadian oscillation of the potent neutrophil chemoattractant Cxcl5 produced by bronchial epithelial cells⁶. Herpes simplex virus (HSV) 2 skin infection becomes more severe in mice infected in the nighttime than in the daytime depending on the circadian expression of the HSV entry receptor Nectin-1⁷. In addition, the circadian rhythm controls the distribution of immune cells in blood and lymphoid tissues. In mice, the number of blood-circulating B cells and T cells peaks in the daytime, whereas that in lymphoid tissues peaks in the evening depending on the circadian expression levels of chemokine receptors (CXCR4 and CCR7) and adhesion molecules (ICAM-1, ICAM-2, VCAM-1 and CD44) on immune cells and blood endothelial cells⁸⁻¹⁰. This suggests a fundamental impact of circadian rhythm on the immune system. Furthermore, previous reports demonstrated that lymphocytes predominantly express $\beta 2$ adrenergic receptor ($\beta 2AR$) among adrenergic receptors, and $\beta 2AR$ stimulation inhibits lymphocyte egress from LNs¹¹. It was also reported that the amount of noradrenaline, an agonist of $\beta 2AR$, increases in the murine skin draining LNs at night compared with daytime¹².

As for the skin, previous studies reported that disturbance of the circadian rhythm exacerbated skin inflammation in contact hypersensitivity (CHS), which is a prototypic

murine model of cutaneous type IV allergy that mimics allergic contact dermatitis (ACD) in humans¹³. Constant light exposure and deficiency of *Bmal1* (one of the key circadian genes) exacerbate ear swelling, increase skin infiltrating immune cells, and decrease skin infiltrating regulatory T cells¹⁴. In addition, deficiency of another circadian-related gene, *Clock*, exacerbates ear swelling and increases dermal mast cells¹⁵. However, it remains unclear whether and how time-of-day-dependent variables are observed in the CHS response under physiological conditions. Currently, topical corticosteroids, oral corticosteroids, cyclosporine, and ultraviolet light therapy are used to treat ACD in human, and moisturizing is recommended to prevent the relapse. Nevertheless, the effects of circadian rhythm have not been considered in the treatments. In this study, we demonstrated that the timing of sensitization affects the magnitude of CHS response, and that an adrenaline receptor agonist/antagonist disturbs the circadian distribution of immune cells and affects the CHS response.

RESULTS

The timing of sensitization affects the magnitude of the CHS response

To investigate whether the timing of elicitation or sensitization, or both dictates the magnitude of CHS response, we induced CHS using dinitrofluorobenzene (DNFB). Mice were maintained on a 12/12-h light-dark cycle (lights on at zeitgeber time (ZT)0 [=8 a.m.], lights off at ZT12 [=8 p.m.]) and divided in the following four groups (**Fig. 1A**): Group [S5-E5]; sensitized and elicited at daytime (1 p.m.=ZT5), Group [S5-E13]; sensitized at daytime (ZT5) and elicited at nighttime (9 p.m.= ZT13). Group [S13-E5]; sensitized at nighttime (ZT13) and elicited at daytime (ZT5). Group [S13-E13]; sensitized and elicited at nighttime (ZT13). These timings of sensitization/elicitation were determined based on previous studies demonstrating that the number of blood-circulating lymphocytes peaks at ZT5, whereas that in lymphoid tissues peaks at ZT13¹². Twenty-four hours after elicitation, ear swelling was measured (**Fig. 1B-E**) and ears were harvested for histological analysis (**Fig. 2A-D**).

Each group developed dermatitis and showed general time course of CHS. In each group, the ear swelling reached a peak at 24 to 48 h after elicitation and began to decrease at 72 h after elicitation (**Supplementary Fig.1**). No significant difference in ear swelling was observed between [S5-E5] and [S5-E13] (**Fig. 1B**) or between [S13-E5] and [S13-E13] (**Fig. 1C**), suggesting that the timing of the elicitation does not affect the severity of CHS response. In contrast, there was a significant difference in ear swelling when we compared groups with different sensitization timing. Ear swelling was higher in [S13-E5] than in [S5-E5] (**Fig. 1D**), and higher in [S13-E13] than in [S5-E13] (**Fig. 1E**). Consistent with this, histological analysis revealed that ears in ZT13-sensitized group exhibited a larger accumulation of mononuclear cells (**Fig. 2C, D**) than

those in ZT5-sensitized group (**Fig. 2A, B**), and immunohistochemistry showed infiltration of CD8⁺ T cells (main effector immune cells in CHS) into dermis and epidermis (**Supplementary Fig. 2A, B**). Furthermore, flowcytometric analysis revealed that the number of ly6G⁺ neutrophils and CD8⁺ T cells in ear twenty-four hours after elicitation increased significantly in [S13-E5] and [S13-E13] compared to [S5-E5] and [S5-E13]. On the other hand, F4/80⁺ macrophages, dendritic cells (DCs), CD4⁺ T cells and B cells tended to increase in [S13-E5] and [S13-E13] (**Fig. 2E-J**). These results support histological findings.

Taken together, our findings suggest the magnitude of CHS response is affected by circadian rhythm, especially by the timing of sensitization. Mice sensitized at night (ZT13) had a more marked CHS response than those sensitized in the daytime (ZT5).

The migration kinetics and activation status of cutaneous DCs are not affected by the timing of sensitization

As the sensitization phase of CHS is initiated by the migration of antigen-bearing dendritic cells (DCs) from skin to draining lymph nodes (LNs), we examined whether the timing of sensitization affects migration kinetics and/or activation status of cutaneous DCs. Twenty-four hours after sensitization on the abdomen with fluorescein isothiocyanate (FITC), we harvested skin-draining inguinal LNs and FITC⁺ skin-derived DCs were identified as migratory DCs from skin to draining LNs by flow cytometry (**Fig. 3A**). The numbers of FITC⁺ DCs in LNs were comparable between ZT5-sensitized and ZT13-sensitized groups (**Fig. 3B**). In addition, the mean fluorescence intensity (MFI) of activation/costimulatory markers of DCs, such as CD80 and CD86, on FITC⁺ DCs were comparable between ZT5-sensitized and ZT13-sensitized groups (**Fig. 3C**).

Similarly, there was no difference in the proportion of CCR7 positive DCs and costimulatory factor CD40 and OX40L positive DCs (Fig. 3D). Following this, we examined migration kinetics and activation status in each conventional DC (cDC) subset. Each cDC subset has different functions and surface phenotypes. In mice, cDC1 activates mainly CD8⁺ T cells and Th1 CD4⁺ T cells, while cDC2 activates Th2 and Th17 CD4⁺ T cells. We identified cDC1 as X-C Motif Chemokine Receptor 1 (XCR1)-positive DCs, and cDC2 as CD11b-positive DCs. The numbers of FITC⁺ cDC1s and cDC2s in LNs were comparable between ZT5-sensitized and ZT13-sensitized groups (Fig. 3E), and the ratio of activated CD80⁺CD86⁺ cDCs in FITC⁺ cDCs in LNs were comparable between ZT5-sensitized and ZT13-sensitized groups (Fig. 3F).

This suggests that migration kinetics and activation status of cutaneous DCs are not affected by the timing of sensitization during the CHS response.

Circadian rhythm affects the leukocyte distribution in blood and LNs, but not in skin

To investigate the mechanism of how the timing of sensitization, but not elicitation, influences the magnitude of CHS response, we examined leukocyte counts in blood, LNs and skin at different time points (ZT5 and ZT13) in C57BL/6 mice. Based on flow cytometric analysis (Fig. 4A), the numbers of blood-circulating B cells, CD4⁺ and CD8⁺ T cells, neutrophils and monocytes increased at ZT5 compared with ZT13, but the number of DCs did not change significantly (Fig. 4B). As for neutrophils and monocytes, it is known that intrinsic circadian clock genes control their trafficking and affect the numbers in blood^{10,16}. This result is consistent with previous reports. In contrast, the numbers of B cells and CD4⁺ and CD8⁺ T cells in LNs at ZT13 were higher

than those at ZT5, but the number of DCs, neutrophils, and macrophages did not change significantly (**Fig. 4C**), as reported previously^{12,17}. As for skin, no significant difference was observed in the number of B cells, CD4⁺ and CD8⁺ T cells, DCs, neutrophils or macrophages at these time points (**Fig. 4D**). This suggests that a higher number of B cells and CD4⁺ and CD8⁺ T cells circulates in blood in daytime and shifts to LNs at night in mice, which is a high-activity period over the course of a day, and that skin is sequestered from this circadian oscillation.

Lymphocyte proliferation and IFN- γ production in the skin draining LNs vary by the timing of sensitization

To investigate whether the timing of sensitization affects lymphocyte proliferation and cytokine production in skin draining LNs, regional inguinal LNs were harvested 5 days after DNFB or vehicle sensitization on abdomen, and LN cell suspensions were incubated *in vitro* with ³H-thymidine with or without dinitrobenzene sulfonic acid (DNBS).

LN cells from ZT13-sensitized mice incorporated higher amounts of ³H-thymidine than those from ZT5-sensitized mice in the presence of DNBS (**Fig. 5A**). In addition, supernatant of LN cells incubated in the presence of DNBS from ZT13-sensitized mice contained higher interferon (IFN)- γ levels than that from ZT5-sensitized mice (**Fig. 5B**). On the other hand, other cytokines such as IL-4, IL-10, IL-12 IL-13, and IL-17A did not show significant difference (Data not shown). Furthermore, we analyzed the numbers of naive (CD44⁻CD62L⁺), central memory (CD44⁺CD62L⁺) and effector cells (CD44⁺CD62L⁻) in CD8⁺ population by flowcytometry (**Fig. 5C**). The number of total and effector CD8⁺ T cells increased in LNs of ZT13-sensitized mice. These results

suggest that effector CD8⁺ T cells increase in skin draining LNs of ZT13-sensitized mice, and cell proliferation and IFN- γ production are higher than those in ZT5-sensitized mice, which is consistent with the exacerbated CHS response *in vivo*.

Circadian oscillation of adrenal hormone affects the CHS response

Previous reports demonstrated that lymphocyte distribution is controlled by noradrenaline through β 2AR signaling¹². Therefore, we hypothesized that CHS response is affected by the noradrenaline- β 2AR pathway. Firstly, we checked β 2AR expression in immune cells by quantitative reverse transcription polymerase chain reaction (RT-PCR). CD4⁺ T cells, CD8⁺ T cells B cells, DCs, and neutrophils expressed *Adrb2* gene (**Supplementary Fig. 3**).

To verify this hypothesis, we treated mice with salbutamol (a specific β 2AR agonist), ICI118551 (a specific β 2AR antagonist), or vehicle 30 min before sensitization with DNFB. Five days later, these mice were challenged with DNFB on their ears. After β 2AR agonist administration, ZT5-sensitized mice exhibited more marked ear swelling than vehicle-treated mice, and the swelling was comparable with that observed in vehicle-treated, ZT13-sensitized mice (**Fig. 6A**). On the other hand, β 2AR agonist treatment had no effect on ZT13-sensitized mice. After β 2AR antagonist administration, the ear swelling was attenuated in ZT13-sensitized mice compared with vehicle-treated mice, and the swelling was comparable with that observed in vehicle-treated, ZT5-sensitized mice (**Fig. 6B**), whereas β 2AR antagonist did not affect ZT5-sensitized mice. In summary, β 2AR agonist administration increased CHS response to the same degree as in ZT13-sensitized mice, and β 2AR antagonist administration attenuated CHS response down to the level in ZT5-sensitized mice.

We further investigated the effects of β 2AR agonist, β 2AR antagonist, or vehicle treatment on lymphocyte counts in blood, LNs and skin at ZT5 and ZT13 (**Fig. 6C-E, Supplementary Fig. 4**). In blood, the number of B cells and CD4⁺ and CD8⁺ T cells decreased after β 2AR agonist administration in ZT5-sensitized mice, but not in ZT13-sensitized mice, and increased after β 2AR antagonist administration in ZT13-sensitized mice, but not in ZT5-sensitized mice (**Fig. 6C**). In contrast, the numbers of B cells and CD4⁺ and CD8⁺ T cells in the skin-draining LNs increased after β 2AR agonist administration in ZT5-sensitized mice, but not in ZT13-sensitized mice, and decreased after β 2AR antagonist administration at ZT13-sensitized mice, but not in ZT5-sensitized mice (**Fig. 6D**). However, β 2AR agonist and antagonist administration did not affect the numbers of B cells, CD4⁺ and CD8⁺ T cells in skin significantly (**Fig. 6E**). On the other hand, the number of neutrophils, monocytes, and macrophages were not affected by β 2AR agonist and antagonist administration (**Supplementary Fig.4**). Taken together, intrinsic β 2AR stimulation at night increases the number of lymphocytes in skin-draining LNs and potentiates the magnitude of CHS response.

DISCUSSION

In this study, we demonstrated that the severity of the CHS response depends on the timing of sensitization. A stronger CHS response was observed if mice were sensitized at night (ZT13), when more lymphocytes were distributed in LNs. β 2AR agonist increased the number of lymphocytes in LNs and exacerbated the CHS response when sensitized in the daytime. On the other hand, β 2AR antagonist reduced the number of lymphocytes in LNs and ameliorated CHS response when sensitized at night. β 2AR agonist only exerted an effect on CHS when the mice were treated during the day while

β 2AR antagonist only exerted an effect when treated at night. We hypothesize that intrinsic noradrenaline sufficiently stimulates T cells in LNs and β 2AR agonists only influenced CHS when the mice were treated during the day while β 2AR antagonists only influenced CHS when the mice were treated at night.

For mice, a nocturnal animal, the nighttime is a high-activity period over the course of a day. Thus, at night, mice have a higher risk of injury or exposure to harmful antigens and microorganisms. Taking this into account, it is reasonable that the immune system becomes more sensitive during the active time. In humans, a diurnal animal, the adrenergic nerve activity, and lymphocyte distribution exhibit an opposing circadian pattern to those in mice. Thus, sensitization to haptens may be more easily established during daytime in humans and is suppressed at nighttime, contrary to the murine CHS model. Indeed, in humans, it was demonstrated that immunization with antituberculosis vaccine¹⁸, or influenza vaccination¹⁹ more efficiently induced trained immunity when they were administered in the morning compared with that in the evening.

In this study, we demonstrated that a β 2AR antagonist exacerbates the CHS response and shifts lymphocytes to LNs. Consistent with this, long-acting beta agonist (LABA) monotherapy is related to excess asthma-related mortality^{20,21} and LABAs increase the allergic airway response in mice²². In addition, a previous study demonstrated that a polymorphism in the β 2AR gene is associated with the development of intrinsic atopic dermatitis in humans²³. This suggests that alteration of the β 2AR signal affects the magnitude of allergic immune responses in the airway and skin. Whether β 2AR antagonists promote percutaneous sensitization in humans is of great interest and should be addressed in future studies. Our study may be applied to the management of human ACD. Antigen sensitization plays an important role in the pathogenesis of some

inflammatory skin diseases, including ACD and atopic dermatitis. Considering our study, the skin barrier function should be well maintained during the daytime when the antigen sensitization may be more intense. Recommendations for frequent moisturizing during the daytime for patients with atopic dermatitis and recurrent ACD may be effective in controlling disease activity. Furthermore, to prevent the relapse in atopic dermatitis, proactive therapy with topical corticosteroids and tacrolimus is recommended to maintain remission. The daytime-topical application of proactive therapy may also enhance its preventive effect.

A limitation of this study is that we did not account for a “central clock” and “peripheral clock” in keratinocytes. The central clock is located in the suprachiasmatic nucleus of the hypothalamus, which is innervated by the retina and entrained by solar light/dark cycles. The peripheral clock is present in almost all cell types, including keratinocytes, and individual cells are autonomously controlled by clock genes. Indeed, keratinocytes express circadian clock genes, such as *Clock* and *Period2*, and diurnal oscillation has been observed in sebum production, transepidermal water loss, keratinocyte proliferation, skin blood flow, and skin temperature²⁴. Previous studies found that constant light exposure exacerbated the CHS response¹⁴, suggesting that derangement of the central clock affects the skin immune response. In addition, *Clock* gene mutation promoted T-helper type 1 immune response and exacerbated the CHS response in mice^{15,25}. Therefore, the cutaneous immune reaction is affected by the circadian rhythm in multiple aspects, and biological processes in immune cells, keratinocytes, and other cell types should be under the influence of circadian regulation.

Another remaining question is which cells respond to β 2 adrenergic signals during the elicitation of CHS. Previous reports showed that circadian oscillation of leukocyte

distribution disappears in *Adrb2*^{-/-} mice^{12,17}, and not only immune cells but also keratinocytes and fibroblasts express this receptor^{26,27}. This issue should be addressed using cell-specific targeting of *Adrb2* in the future.

In summary, our study demonstrated that the magnitude of the CHS response is time dependent. A better understanding of the circadian oscillation in immune responses may improve time-dependent preventive medicine, examinations, and treatment.

Materials and Methods

Mice

C57BL/6N mice were purchased from Oriental Bioservice, Inc. (Kyoto, Japan). Eight- to twelve-week-old mice were used for experiments. All mice were acclimated for two weeks before being used in the experiments. Mice were housed in a 12/12-h light-dark cycle (light period from 8 a.m. to 8 p.m.) with *ad libitum* feeding in a specific pathogen-free facility maintained at 22°C. All experiments were performed in accordance with protocols approved by the Kyoto University Animal Experimentation Committee.

Cell isolation from blood, LNs, and skin

Blood was drawn from the superficial temporal vein. Erythrocytes were lysed with ammonium chloride sodium buffer. Bilateral inguinal LNs were collected and disrupted by passing through a 40- μ m cell strainer (Falcon/Corning Incorporated, Corning, NY). The split ears were incubated for 1 h at 37°C in digestion buffer (RPMI medium supplemented with 2% FCS, 1000 U/ml of collagenase type 2 (Worthington Biochemical corporation, Lakewood, NJ), and 0.1% DNase I (Sigma-Aldrich Japan, Tokyo, Japan). After incubation, the ears were disrupted by passage through a 40- μ m cell strainer.

Contact hypersensitivity

Mice were sensitized by a single application of 50 μ l of 0.5% (w/v) DNFB (Nacalai Tesque, Kyoto, Japan) dissolved in acetone and olive oil at a volume ratio of 4:1 to the shaved abdominal skin 5 days before elicitation, and elicited by a single application of 10 μ l of 0.2% (w/v) DNFB to both sides of the right and left ears. Ear swelling was

evaluated by measuring ear thickness with a caliper before elicitation and 24 h after elicitation.

DC migration assay

Mice were sensitized by the application of 150 μ l of 0.5% (w/v) FITC isomer I (Sigma-Aldrich Japan) dissolved in acetone and dibutyl phthalate (1:1) to the shaved abdominal skin. Twenty-four hours after sensitization, we harvested bilateral inguinal LNs. The isolated cells were analyzed by flow cytometry to identify FITC⁺ skin-derived DCs.

Flow cytometry

Isolated cells in blood, LNs, and skin were stained with the following: antibodies against mouse CD45 (30-F11; BD Biosciences, San Jose, CA), CD4 (RM4-5; BD Biosciences), CD8b (H35-17.2; Invitrogen, Waltham, MA), CD8a (53-6.7; BioLegend, San Diego, CA), TCRb (H57-597; BioLegend), CD11c (N418; BioLegend), MHC class II (M5/114.15.2; eBioscience, Waltham, MA), CD11b (M1/70; BD Biosciences), XCR1 (ZET; BioLegend), Ly6G (1A8; BD Biosciences), CD115 (AFS98; BioLegend), F4/80 (BM8; BioLegend), CD3e (145-2C11; eBioscience), B220 (RA3-6B2; eBioscience), MHC class II (M5/114.15.2; BioLegend), CD80 (16-10A1; eBioscience), CD86 (GL1; eBioscience), CCR7 (4B12; BioLegend), CD40 (1C10; eBioscience), OX40L (RM134L; eBioscience), CD62L (MEL-14; BioLegend), and CD44 (IM7; eBioscience). Flow cytometry was performed using LSR Fortessa (BD Biosciences) and analyzed by FlowJo software (BD Biosciences).

Histopathology

Ear skin specimens were obtained and fixed in 4% paraformaldehyde and dehydrated in 20% sucrose. The samples were embedded in OCT compound. The specimens were cut into 5- μ m-thick sections and stained with hematoxylin and eosin (H&E) for histological examination.

ELISA

For cytokine production, cells (5×10^5 /well) were incubated in 96-well round bottom microplates (Falcon/Corning Incorporated) at 37 °C in 5% CO₂ in cRPMI medium (RPMI medium supplemented with 1% L-glutamine, 1% HEPES, 0.1% 2-mercaptoethanol, and 10% fetal bovine serum) for 72 h with or without 10 mg/ml of DNBS (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan). Cytokine production was measured in medium conditioned by LN cells using commercially available ELISA sets for murine IFN- γ (BD Biosciences). Cytokine titers were calculated with reference to a standard curve constructed using the known amounts of respective kit-provided recombinant cytokines. The sensitivity of the IFN- γ kits was 15 pg/ml.

³H-thymidine incorporation assay

For proliferation measurements, isolated dLN cells (0.5×10^6 /well) 5 days after sensitization were cultured in 96-well microplates (Falcon/Corning Incorporated) for 16 h with 0.5 mCi [³H]-thymidine/well added at onset. [³H]-Thymidine incorporation was measured using a scintillation counter and proliferation was expressed as counts per minute (cpm).

β 2AR agonist and antagonist treatment

Salbutamol (2 mg/kg) (Sigma-Aldrich Japan) or ICI118551 (12 mg/kg) (Abcam PLC, Cambridge, UK) was injected intraperitoneally 30 minutes before sensitization or elicitation.

Statistical analysis

GraphPad Prism (GraphPad Software, San Diego, CA) was used for all statistical analyses. For comparison of two nonparametric datasets, the unpaired Student's t test was used. For multigroup comparisons, we applied Tukey's multiple comparison test.

Acknowledgement

This study was supported by the Japan Society for the Promotion of Science KAKENHI (JP15H05790, 20H05697), Takeda Science Foundation, and the Japan Agency for Medical Research and Development (AMED) (16ek0410011h0003, 16he0902003h0002, and JP19gm1210006). We thank H. Doi for her technical assistance.

Conflict of Interests

The authors declare no competing financial interests.

Author Contributions

TMi and GE designed the experiments. ZC, RA, MO, SNakaj, TN, YI, TMu, and AK supported the planning and validated the strategy. TMi, RS, SNakam, and GE performed experiments. TMi, GE, and KK wrote the manuscript. TMi and GE performed data analysis. All authors participated in editing the manuscript.

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Figure legends

Figure 1: Circadian rhythm affects the CHS response severity

A: Mice were divided into four groups and sensitized and elicited using dinitrofluorobenzene (DNFB) at different timing in the following four groups: sensitized at 1 p.m. (zeitgeber time (ZT)5 as daytime) and five days later, elicited at ZT5 (group [S5 (sensitized at ZT5)- E5 (elicited at ZT5)]) or at 9 p.m. (ZT13 as nighttime) (group [S5-E13 (elicited at ZT13)]), or sensitized at ZT13 and elicited at ZT5 (group [S13 (sensitized at ZT13)-E5]) or at ZT13 (group [S13-E13]). Zeitgeber time is the time of the body clock during a 12/12-hour light/dark cycle, with ZT0 as the start of the light cycle and ZT12 as the start of the dark cycle. **B-E:** Ear swelling of the mice 24 h after elicitation. Data are presented as the mean $\pm$ SD from three independent experiments, with six mice per treatment. *P*-values were obtained by the unpaired Student's *t* test. *, *P* < 0.05.

Figure 2: Circadian rhythm affects the number of immune cells that infiltrate into skin in CHS response

A-D: Histology of the ear skin stained by hematoxylin and eosin. **E-J:** Numbers of neutrophils (**E**), macrophages (**F**), dendritic cells (DCs) (**G**), B cells (**H**) and CD4⁺ T cells (**I**) and CD8⁺ T cells (**J**) of C57BL/6 mice. Data are representative of three experiments and shown as the mean $\pm$ SD of six mice. *, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001. *P*-values were obtained by the unpaired Student's *t* test (**E-J**).

Figure 3: Migration kinetics and activation level of DCs are invariable throughout the day

A: Representative flow cytometry plots showing the identification of MHC Class II^{high} FITC⁺ migratory DCs. Cells were pre-gated as single live CD45⁺ cells. **B:** Numbers and percentage of FITC⁺ migratory DCs in skin-draining lymph nodes (LNs) of C57BL/6 mice sensitized at the indicated zeitgeber time (ZT). **C:** Mean fluorescent intensity (MFI) of CD80 and CD86 on FITC⁺ migratory DCs in skin-draining LNs of C57BL/6 mice sensitized at the indicated ZT. **D:** Percentage of CCR7⁺, CD40⁺, or OX40L⁺ migratory conventional DCs (cDCs) in skin-draining LNs of C57BL/6 mice sensitized at the indicated ZT. **E:** Numbers of FITC⁺ migratory cDCs in skin-draining LNs of C57BL/6 mice sensitized at the indicated ZT. **F:** Percentage of CD80⁺ CD86⁺ migratory cDCs in skin-draining LNs of C57BL/6 mice sensitized at the indicated ZT. Data are representative of three experiments and shown as the mean $\pm$ SD of six mice (**B-D**) or twelve mice (**E, F**). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. P -values were obtained by the unpaired Student's t test (**B-F**).

Figure 4: The circadian oscillation of leukocyte counts in blood, LNs, and skin

A: Representative flow cytometry plots showing the identification of DCs, B cells, CD4⁺T cells, CD8⁺ T cells, neutrophils, and monocytes in blood and macrophages and neutrophils in LN. Cells were pre-gated as single live CD45⁺ cells. **B-D:** Numbers of B cells, CD4⁺ and CD8⁺ T cells, neutrophils and monocytes or macrophages in blood (**B**), LNs (**C**), and skin (**D**) of C57BL/6 mice at the indicated ZT. Data are representative of three experiments and shown as the mean $\pm$ SD of seven mice. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. P -values were obtained by the unpaired Student's t test (**B-D**).

Figure 5: Cell proliferation and IFN- γ production in LNs vary by the timing of sensitization

A: ^3H -thymidine incorporation assay. Mice were sensitized on ZT5/13, and 5 days later, the skin draining LN cells were incubated with or without dinitrobenzene sulfonic acid (DNBS). Data are presented as the mean $\pm$ SD with five mice per treatment. **B:** Interferon- γ (IFN- γ) production in each supernatant of LN cells from ZT5/13-sensitized mice. **C:** Numbers of total CD8 $^+$ T cells and naïve, central memory and effector CD8 $^+$ T cells in the LNs. Data are representative of two experiments and shown as the mean $\pm$ SD of ten or six mice per time point. *, $P < 0.05$; P -values were obtained by the unpaired Student's t test.

Figure 6: Circadian oscillation of adrenal hormone affects the CHS response

A, B: Ear swelling 24 h after the sensitization in salbutamol/vehicle-treated (**A**) or ICI118551/vehicle-treated mice (**B**). Data are presented as the mean $\pm$ SD from three independent experiments, with six mice per treatment. **C-E:** Cell counts in blood (**C**), LNs (**D**) and skin (**E**) from salbutamol, ICI118551, or vehicle-treated mice at the indicated ZT. Data are representative of three experiments and shown as the mean $\pm$ SD of four or six mice. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. P -values were obtained by the unpaired Student's t test (**A, B**) or Tukey's multiple comparison test (**C-E**).