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Biological clock dysfunction exacerbates contact hypersensitivity in mice

Running head: Contact hypersensitivity and circadian clock

E. Takita*¹, S. Yokota*², Y. Tahara¹, A. Hirao¹, N. Aoki¹, Y. Nakamura³, A. Nakao³, S. Shibata¹

¹Department of Physiology and Pharmacology, School of Advanced Science and Engineering,
Waseda University, Tokyo, Japan

²Department of Veterinary Internal medicine, Faculty of Agriculture, Tottori University, Tottori,
Japan

³Department of Immunology, Faculty of Medicine, University of Yamanashi, Yamanashi, Japan

Correspondence: Shigenobu Shibata

E-mail: shibatas@waseda.jp

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Although circadian rhythms play important roles in immediate-type skin allergic reactions, such as passive cutaneous anaphylactic reaction, there are no reports on the roles of circadian rhythm in contact hypersensitivity.

We report that a deficit of the circadian clock using *Clock* mutant mice exacerbated 2,4,6-trinitro-1-chlorobenzene–induced contact hypersensitivity via a deficit in corticosterone secretion. We conclude that circadian rhythm modulates not only immediate-type but also delayed-type skin allergic reactions.

ABSTRACTS

Background Immediate-type skin allergic reactions, such as passive cutaneous anaphylactic reaction, are associated with circadian rhythm, but the role of circadian mechanisms on delayed-type skin allergic reactions, such as contact hypersensitivity (CHS), remains uncertain. In mice, CHS, a T cell–mediated immune response, is a classic model of human allergic contact dermatitis.

Objectives We investigated whether biological clock dysfunction affects CHS pathogenesis in *Clock* mutant mice compared with wild-type (WT) mice.

Methods Mice were treated with 2,4,6-trinitro-1-chlorobenzene (TNCB) on the abdominal skin on day 0 (sensitisation) and then treated with TNCB on the ears on day 5 (challenge).

Results We found that biological clock dysfunction resulted in severe inflammation. Ear swelling, serum IgE level, and mast cell number were significantly increased in *Clock* mutant mice compared with WT mice. These results provide evidence that *Clock* mutation promotes the T helper type

(Th)2 immune response and exacerbates CHS. Corticosterone has a protective effect on CHS. The serum corticosterone level lost rhythmicity and showed a decreased daily level in *Clock* mutant mice compared with WT mice, supporting the exacerbating effect of *Clock* mutation on CHS. Adrenalectomy markedly worsened TNCB-induced CHS in WT mice but not in *Clock* mutant mice. In addition, dramatic dexamethasone-induced protection of CHS was observed in *Clock* mutant mice compared with WT mice.

Conclusions The present results suggest that circadian rhythm might be an important factor in the regulation of CHS via corticosterone rhythmicity and/or level.

INTRODUCTION

Some allergic diseases are associated with circadian rhythm. For example, asthma is a common disease with a large circadian variation ¹. Circadian rhythm also plays an important role in the pathogenesis of arthritis ². In addition, we have previously demonstrated that immediate-type skin allergic responses, such as passive cutaneous anaphylactic reaction, are influenced by the circadian clock ³. In mice, contact hypersensitivity (CHS), a T cell-mediated immune response, is one of the most common models for studying human allergic contact dermatitis, and CHS is known to be a representative example of delayed-type skin allergic response ⁴. CHS is induced experimentally in mice by exposure to simple reactive chemicals ⁵. Details of circadian rhythm mechanisms underlying CHS remain unclear though. A T helper type 1 (Th1-type) immune response associated with activation of Th1 cells and cytokines such as interferon gamma (IFN- γ) and interleukin 12 (IL-12) was originally thought to be responsible ⁶. However, it was recently suggested that Th2 cytokines, IgE, and mast cells play crucial roles in the induction of CHS ⁷. There is also evidence that signal transducer and activator of transcription 6 (STAT6), the signalling pathway which is activated by Th2-type cytokines such as IL-4 and IL-13, is essential for the induction of CHS ⁸. Tumour necrosis factor alpha (TNF- α) and IL-1 β are also involved in the pathogenesis of CHS;

their signalling induces potent mature antigen-presenting cells.

In mammals, various physiologic phenomena such as body temperature, food intake, and the sleep-wake cycle are under the control of an endogenous circadian clock located in the suprachiasmatic nucleus of the hypothalamus⁹. Many clock genes, such as *Per1*, *Per2*, *Clock*, *Bmal1*, *Cry1*, *Cry2*, and *Rev-erb α* have been discovered, and molecular mechanisms of circadian oscillation have been established^{10,11}. Clock genes are expressed not only in the central autonomic clock (i.e., suprachiasmatic nucleus) but also in other brain regions and various peripheral tissues¹⁰,¹¹ including the heart, liver, and kidney. Among mutant mice carrying clock gene mutations, *Clock* mutant mice were the first described and are widely used as a model of circadian deficiency¹²; they are unable to generate circadian rhythms in behavior or hepatic clock gene expression^{12–15}.

Corticosterone is the principal glucocorticoid in rodents and is known to suppress the pathogenesis of human allergic contact dermatitis¹⁶. The secretion of corticosterone is under the control of the suprachiasmatic nucleus^{17, 18}. Interestingly, *Clock* mutant mice exhibit a decrease in serum corticosterone rhythmicity and also a suppression of corticosterone secretion throughout the day¹⁴,¹⁹. These results suggest that *Clock* mutant mice might show an exacerbation effect on 2,4,6-trinitro-1-chlorobenzene (TNCB)-induced CHS.

In the present study, we examined whether *Clock* mutation affects the expression of CHS. Possible involvement of adrenal cortex activity was also examined by adrenalectomy (ADx) and dexamethasone administration. In a recently published study, we demonstrated that both mast cell response and passive cutaneous anaphylactic reaction were less sensitive to dexamethasone in *Per2* mutant mice compared to wild-type (WT) mice, and ADx increased the passive cutaneous anaphylactic reaction.³ In the present study, ADx *Clock* mutant mice were used to investigate the effect of clock impairment on the pathogenesis of CHS under the condition of suppressed corticosterone secretion. Dexamethasone was used to investigate the difference in sensitivity to glucocorticoid between WT mice and *Clock* mutant mice.

MATERIALS AND METHODS

Mice

A breeding colony of *Clock* mutant mice with a Balb/c background was developed with mice originally supplied by Dr. J. S. Takahashi ¹¹. Genotypes were determined by polymerase chain reaction (PCR), as described previously ¹². Mice (7–15 weeks old) were housed at a temperature of 22 °C ± 2 °C, a humidity of 60% ± 5%, and a 12-h light/12-h dark cycle (lights on from 08:00 to 20:00). The mice were fed a normal commercial diet (Catalog #MF; Oriental Yeast Co. Ltd., Tokyo, Japan) and provided water *ad libitum*. In this study, zeitgeber time 0 (ZT0) was designated as lights-on time and ZT12 as lights-off time under the light-dark cycle. Experimental animal care was conducted with permission from the Committee for Animal Experimentation of the School of Science and Engineering at Waseda University (permission #09A11, 10A11) and in accordance with the law (No. 105) and notification (No. 6) of the Japanese Government.

Induction of CHS

Mice were treated with 100 µL of 5% (w/v) TNCB (Nacalai Tesque, Kyoto, Japan) dissolved in acetone (Wako Pure Chemical Industries, Ltd., Osaka, Japan) on the shaved abdominal skin on day 0 (sensitisation) and 20 µL of 1% (w/v) TNCB on both sides of the right and left ears on day 5 (challenge). Acetone alone was used as a vehicle control. Mice were killed at 0 h (before challenge), or 2, 4, 6, 18, or 24 h after challenge. Ear thickness (µm) was measured at 0 h and at each time point with a gauge (Ozaki Seisakusho, Tokyo, Japan), and the difference in thickness between 0 h and each time point (swelling) was recorded.

Adrenalectomy

Bilateral adrenalectomy (ADx) was carried out via a dorsal approach under midazolam/xylazine anesthesia, as described previously²⁰. In brief, the skin on the back was shaved and disinfected, and an incision of approximately 1 cm was made above and parallel to the spinal cord. The adrenal glands were removed from the surrounding fat tissue via small openings made in the muscle layer to the left and right of the spinal cord. The mice were subsequently provided with 0.9% saline solution instead of water to maintain appropriate mineral balance. ADx was performed at least 1 week before sensitisation, to allow for recovery. Some mice (5/18) with recovery deficits were excluded from further experiments.

Dexamethasone treatment

Dexamethasone treatment was performed as described previously.²¹ Treatment was initiated with an intraperitoneal injection of or 20 µg dexamethasone (Sigma-Aldrich, Tokyo, Japan) and was continued with 20 mg/L dexamethasone in the drinking water to maintain hormone levels for 24 h. The injection was performed 1 h before challenge. Injected saline and drinking water were used as vehicle control.

RNA isolation and real-time reverse-transcription PCR

Total RNA was extracted with phenol (Omega Bio-Tek Inc., Lilburn, GA). A 50-ng aliquot of total RNA was reverse-transcribed and amplified with a One-Step SYBR RT-PCR kit (Takara, Otsu, Japan) in a Step-One-Plus system (ABI, Foster, CA). Specific primer pairs (Table 1) were designed with Primer3 Input software (version 0.4.0). The conditions of real-time reverse-transcription PCR were the same as those in our previous publication²². The specificity of gene amplification was confirmed by measuring the size and purity of the PCR products by gel electrophoresis. The relative

light unit of the PCR product of the target gene was normalized to that of *18s rRNA*. Melt curve analysis was then performed.

Enzyme-linked immunosorbent assay

Specific antibodies, hormones and cytokines were measured in serum samples by enzyme-linked immunosorbent assay (ELISA). We used the following kits: total IgE ELISA (Yamasa, Chiba, Japan), corticosterone ELISA (AssayPro, Charles, MO), TNF- α and IFN- γ ELISA (R&D Systems, Minneapolis, MN), and IL-13 ELISA (Bender MedSystems, San Diego, CA). Assays were performed according to the manufacturers' instructions.

Histologic examination

Ear skin specimens were obtained and fixed in 4% paraformaldehyde (Sigma-Aldrich). The specimens were then cut into 10- μ m-thick sections and stained with toluidine blue (Waldeck GmbH & Co., Munster, Germany) for histologic examination. Numbers of mast cells infiltrating the dermis were counted and are expressed as the number of cells/mm².

Statistical analysis

All values are expressed as mean $\pm$ SE. For statistical analyses, one-way or two-way analysis of variance (ANOVA) with StatView software (SAS Institute, Cary, NC) was applied, and a post-hoc analysis was conducted with the Tukey-Kramer test. We also assessed statistical significance with the Student *t* test. A P value of <0.05 was considered statistically significant.

RESULTS

***Clock* mutation promotes the Th2-type immune response and exacerbates the pathogenesis of CHS**

Mice were sensitised and challenged at ZT6 and killed at 2, 4, 6, 18, 24, 48 or 72 h after challenge. *Clock* mutant mice showed increased ear swelling compared to WT mice at all time points for 24 h (Fig. 1). Ear swelling reached a plateau at 18 h in both WT and *Clock* mutant mice. In both WT and *Clock* mutant mice, ear swelling reduced to approximately 200 μm (211 ± 26 for WT, 238 ± 20 for *Clock* mutant) and 100 μm (90 ± 31 for WT, 117 ± 18 for *Clock* mutant) thickness at 48 h and 72 h after challenge, respectively. mRNA expression levels in ear samples of mice exposed to TNCB were determined at 2 h after challenge. The mRNA expression levels of *Ifn- γ* and *Il-12* (Th1-type cytokine) in *Clock* mutant mice were less than those in WT mice. In contrast, the mRNA expression levels of *Il-4*, *Il-13* (Th2-type cytokine), *Tnf- α* and *Il-1 β* in *Clock* mutant mice were greater than those in WT mice (Fig. 2A).

Serum IgE level in mice exposed to TNCB was determined at 2 h and 24 h after challenge. No significant difference in serum IgE level at 2 h was observed between WT mice and *Clock* mutant mice, but that at 24 h was significantly increased in *Clock* mutant mice compared to WT mice (Fig. 2B). Serum cytokine levels in mice exposed to TNCB were determined at 2, 18, and 24 h after challenge, because the peak production time varied according to cytokine type. The serum IFN- γ level at 18 h (peak) was decreased in *Clock* mutant mice compared to WT mice (Fig. 2C). In contrast, the serum level of IL-13 at 2 h (peak) was increased in *Clock* mutant mice compared to WT mice. Unexpectedly, the serum level of TNF- α , which showed a significant difference in mRNA expression between WT mice and *Clock* mutant mice, was almost equal (Fig 2C). Serum levels of IL-13 and TNF- α at 18 h and 24 h were undetectable.

Numbers of mast cells were determined at 24 h after challenge. There were many mast cells in *Clock* mutant CHS mice (Fig. 3A (d,e)) compared with WT CHS mice (Fig. 3A (c)). Mast cells were undetectable in WT and *Clock* mutant mice not exposed to TNCB (Fig. 3A(a,b)). The number of mast cells in *Clock* mutant mice was significantly increased compared to those in WT mice ($p<0.01$, Student's *t*-test) (Fig. 3B).

The pathogenesis of CHS is exacerbated in *Clock* mutant mice as a result of decreased corticosterone secretion

To investigate circadian variation in serum corticosterone level, intact mice (no TNCB exposure) were killed at ZT0, ZT6, ZT12, or ZT18. The serum corticosterone level in WT mice showed a circadian rhythm, with a peak at ZT12 (Fig 4A) ($F_{3,13}=14.4$, $p<0.001$ for one-way ANOVA). In contrast, the circadian rhythm was dampened in *Clock* mutant mice ($F_{3,11}=0.3$, $p=0.81$ for one-way ANOVA). There were significant differences in time $\times$ mutation as evaluated by two-way ANOVA ($F_{3,24}=6.2$, $p<0.01$). In addition, *Clock* mutant mice showed weak secretion of corticosterone compared to WT mice throughout the day. The serum corticosterone level in *Clock* mutant mice was also lower than that in WT mice, both early (2–6 h) ($p<0.01$, Student's *t*-test) and late (18–24 h) ($p<0.01$, Student's *t*-test) after challenge (Fig. 4B). In both WT and *Clock* mutant mice, the corticosterone level (Fig. 4B) was approximately three times higher than that before TNCB injection (Fig. 4A).

In the next experiment, we investigated whether *Clock* mutation affects CHS pathogenesis under the condition of suppressed corticosterone secretion in ADx mice. ADx or sham-operated mice were sensitised and challenged at ZT6 and killed at 24 h after challenge. Similar to the results of Figs. 1 and 2, *Clock* mutant sham-operated mice exhibited high sensitivity to TNCB-induced CHS. In WT mice, ADx resulted in significantly increased ear swelling compared with sham-operated mice (Fig.

5A). ADx did not result in increased ear swelling in *Clock* mutant mice. Serum IgE level showed similar results to ear swelling (Fig. 5B). The lack of ADx effect in *Clock* mutant mice might be due to the low secretion of corticosterone, suggesting that the sensitivity of glucocorticoid to the pathogenesis of CHS might differ between WT and *Clock* mutant mice.

To confirm this possibility, mice were treated with dexamethasone during the challenge phase. Mice were sensitised and challenged at ZT6 and killed at 24 h after challenge. The administration of dexamethasone decreased ear swelling in both WT mice and *Clock* mutant mice (Fig. 6A). The serum IgE level showed similar results to ear swelling (Fig. 6B), and dexamethasone dramatically decreased IgE level in *Clock* mutant mice (76% reduction) compared with WT mice (37% reduction). The total amount of dexamethasone intake was similar between WT and *Clock* mutant mice because the amount of drinking water was similar between WT mice and *Clock* mutant mice (WT: 3.60 ± 0.31 g/mouse; *Clock* mutant: 3.60 ± 0.14 g/mouse).

DISCUSSION

In this study, we investigated whether biological clock dysfunction affects the pathogenesis of CHS. The increase in ear swelling directly shows the severity of CHS; it occurs as a result of vascular hyperpermeability and migration of immune cells. *Clock* mutant mice showed increased ear swelling compared to WT mice. In parallel with symptom severity, the numbers of mast cells and serum IgE concentration were also significantly increased in *Clock* mutant mice. Mast cells are key promoters of contact allergy²³ and mediate many aspects of CHS. We recently reported that a passive systematic anaphylactic reaction is augmented throughout the day in *Per2* mutant mice³. Thus, we believe that the symptom of CHS is more severe in *Clock* mutant mice, and impairment of circadian rhythm exacerbated not only immediate-type skin allergic reactions, such as a passive systematic anaphylactic reaction,³ but also delayed-type allergic reactions, such as a CHS, as in the present results. It is well known that passive systematic anaphylactic reaction occurs via an

IgE/mast cell-mediated anaphylactic reaction. In the present results, TNCB-induced CHS might also be involved in IgE/mast cell-mediated events via a Th2-type immune response. It has already been suggested that Th2 cytokines, IgE, and mast cells play crucial roles in the induction of CHS⁷. The reason why the *Tnf- α* mRNA expression level (Fig. 2A), but not the serum TNF- α level (Fig. 2C), showed a significant difference between WT mice and *Clock* mutant mice might be that the mRNA expression level reflected local (ear) events and that the serum protein level reflected systemic events.

We found that *Clock* mutation promoted the Th2-type immune response and exacerbated the pathogenesis of CHS. We hypothesized that differences in serum corticosterone level between WT and *Clock* mutant mice affect the pathogenesis of CHS because corticosterone plays important roles in both immune and circadian systems²⁴⁻²⁶. In the present study, *Clock* mutant mice showed a low level of corticosterone secretion compared with WT mice before and after CHS. Consequently, the present results suggest that *Clock* mutation impaired both circadian- and stress-induced secretion of corticosterone and resulted in exacerbation of the CHS response.

Alternatively, we should consider that *Clock* is a transcription factor that presumably alters gene expression throughout the mouse. These changes are not all due to the loss of rhythmicity. Gene expression change not related to clock function by *Clock* mutation might affect the pathogenesis of CHS under the present experimental conditions. Such non-circadian functions of clock genes are currently being discussed with respect to several physiological functions such as sleep, addiction, and psychiatric disorders²⁷.

We next investigated the effect of *Clock* mutation on the pathogenesis of CHS under the condition of suppressed corticosterone secretion. Corticosterone is released predominantly by the adrenal cortex; therefore, mice were subjected to ADx. It is well known that ADx increases passive cutaneous anaphylactic reaction³ and other allergic diseases²⁸. In the present study, ADx affected the pathogenesis of CHS in WT mice but not in *Clock* mutant mice. We believe that the serum

corticosterone level after ADx is greatly decreased in WT mice but weakly decreased in *Clock* mutant mice. In other words, *Clock* mutant mice might adapt to the low corticosterone level. This result also implied that the sensitivity to glucocorticoid is important in the regulation of CHS pathogenesis. The difference in sensitivity to glucocorticoid between WT and *Clock* mutant mice might also affect the pathogenesis of CHS.

To investigate sensitivity to glucocorticoid, we administered dexamethasone to mice and performed the CHS protocol. Although the administration of dexamethasone significantly suppressed the pathogenesis of CHS in both WT and *Clock* mutant mice, the sensitivity to glucocorticoid was high in *Clock* mutant mice. In the present results, *Clock* mutant mice exhibited a low serum corticosterone level compared with WT mice. These data suggest that glucocorticoid receptors might be up-regulated in *Clock* mutant mice.

Functional analysis of mast cells or dendritic cells in *Clock* mutant mice should be performed to confirm that the exacerbated CHS is caused only by corticosterone. In our recent paper³, we found an expression rhythm of *Per2*, but no expression rhythm of *FcεRIα* or *FcεRIβ* genes of IgE receptor in mast cells³. The number of mast cells expressing *FcεRIα* and the release of β-hexaminidase from mast cells was similar between WT and *Per2* mutant mice³. In addition, differentiation, maturation, and function of bone marrow-derived dendritic cells (BMDCs) were comparable between WT mice and *Clock* mutant mice (Supplemental Fig 1). Taken together, we believe that there are no significant differences in mast cells and dendritic cells functions between WT mice and *Clock* mutant mice.

In summary, the present results showed that *Clock* mutant mice exhibited exacerbation of TNCB-induced CHS via modulation of glucocorticoid level.

AUTHORSHIP

ET, SY, and SS designed the experiments, collected data and wrote the manuscript. YT, AH, YN, and NA participated in data collection and data analysis. AN participated in discussion of the results. SS managed the laboratory and participated in the analysis and discussion of the results. All authors read and approved the manuscript. ET and SY contributed equally to this manuscript (*).

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FIGURE LEGENDS

Fig. 1. Time course for ear swelling in response to 2,4,6-trinitro-1-chlorobenzene (TNCB) in wild-type (WT) mice and *Clock* mutant mice

Mice were sensitised and challenged at ZT6 and sacrificed at 2, 4, 6, 18, or 24 h after challenge. Ear thickness (μm) was measured at 0 h and at each time point. The difference in ear thickness between 0 h and each end point (swelling) was recorded. White column: WT mice; black column: *Clock* mutant mice. Data are presented as mean $\pm$ SEM ($n = 4$ for each time point). ** $p < 0.01$ and *** $p < 0.001$ between WT mice and *Clock* mutant mice, as determined by Student's *t*-test.

Fig. 2. Effect of *Clock* mutation on the pathogenesis of contact hypersensitivity (CHS)

Mice were sensitised and challenged at ZT6 and killed at 2, 4, 6, 18, or 24 h after challenge. (A) mRNA expression for *Ifn- γ* , *Il-12*, *Il-4*, *Il-13*, *Tnf- α* and *Il-1 β* in ear samples from mice exposed to TNCB. mRNA expression was determined at 2 h after challenge. White column: WT mice; black column: *Clock* mutant mice. Data are presented as mean $\pm$ SEM ($n = 4$ for each column). ** $p < 0.01$ between WT mice and *Clock* mutant mice, as determined by Student's *t*-test. (B) Serum IgE level in mice exposed to TNCB. Serum IgE level was determined at 2 h and 24 h after challenge. ** $p < 0.01$ between WT mice and *Clock* mutant mice; # $p < 0.05$ between 2 h and 24 h, as determined by Tukey-Kramer test. (C) Serum levels of interferon (IFN)- γ , interleukin (IL)-13, and tumour necrosis factor (TNF)- α were determined at 2, 18, and 24 h after challenge because the peak time of production varied according to cytokine type. (Serum levels of IL-13 and TNF- α at 18 and 24 h were undetectable.) Data are presented as mean $\pm$ SEM ($n = 4$ for each column).

Fig. 3. Histologic analysis of CHS in WT mice and *Clock* mutant mice

Mice were sensitised and challenged at ZT6 and sacrificed at 24 h after challenge. (A) Sections were stained with toluidine blue. (a–d): A representative section from each experimental condition. Bar = 50 μ m. (e): Enlargement of the square area in (d). Bar = 250 μ m. (B) Numbers of mast cells in ear specimens of mice exposed to TNCB. White column: WT mice; black column: *Clock* mutant mice. Data are presented as mean $\pm$ SEM ($n = 5$ for each column). $**p < 0.01$ between WT mice and *Clock* mutant mice, as determined by Student *t* test.

Fig 4. Serum corticosterone level in WT mice and *Clock* mutant mice before and after TNCB treatment

(A) Circadian variations in serum corticosterone level in WT mice and *Clock* mutant mice. Intact mice (not exposed to TNCB) were sacrificed at ZT0, ZT6, ZT12, or ZT18. Data are presented as mean $\pm$ SEM ($n = 3–5$ for each time point). White column: WT mice; black column: *Clock* mutant mice. $*p < 0.05$ and $**p < 0.01$ between WT mice and *Clock* mutant mice, as determined by Student's *t*-test. (B) Time course of serum corticosterone level in mice exposed to TNCB. Mice were sensitised and challenged at ZT6 and sacrificed at 2, 4, or 6 h for early phase, or 18 or 24 h for late phase after challenge. We averaged the serum corticosterone levels for 2–6 h and for 18–24 h. Data are presented as mean $\pm$ SEM ($n = 12$ for 2–6 h, $n = 8$ for 18–24 h). $**p < 0.01$ between WT mice and *Clock* mutant mice, as determined by Tukey-Kramer test.

Fig. 5. Effect of adrenalectomy (ADx) on the pathogenesis of CHS

ADx or sham operation was performed at least 1 week before sensitisation, to allow for recovery. Mice were then sensitised and challenged at ZT6 and sacrificed at 24 h after challenge. (A) Ear swelling in mice exposed to TNCB. The difference in ear thickness (μm) between 0 h and endpoint (swelling) was recorded. (B) Serum IgE level in the mice exposed to TNCB. White column: WT mice; black column: *Clock* mutant mice. Data are presented as mean $\pm$ SEM ($n = 6-7$ for each column). ** $p < 0.01$ between WT mice and *Clock* mutant mice; # $p < 0.05$ between sham-operated mice and ADx mice, as determined by Tukey-Kramer test.

Fig. 6. Effect of dexamethasone (Dex) treatment on the pathogenesis of CHS

Mice were treated with Dex during the challenge phase, with an initial intraperitoneal injection of 20 μg Dex and continued with 20 mg/L Dex in the drinking water to maintain hormone levels for 24 h. The injection was performed 1 h before challenge. Mice were sensitised and challenged at ZT6 and sacrificed at 24 h after challenge. (A) Ear swelling in mice exposed to TNCB. The difference in ear thickness (μm) between 0 h and endpoint (swelling) was recorded. (B) Serum IgE level in mice exposed to TNCB. White column: WT mice; black column: *Clock* mutant mice. Data are presented as mean $\pm$ SEM ($n = 5-6$ for each column). ** $p < 0.01$ between WT mice and *Clock* mutant mice; ## $p < 0.01$ between vehicle and Dex treatment, as determined by Tukey-Kramer test.

Table 1. Specific Primer Pairs

gene name	Forward	Reverse
18s rRNA	GGGGAGTATGGTTGCAAAGC	TGTCAATCCTGTCCGTGTCC
Ifn- γ	GGCCATCAGCAACAACATAAG	CATCCTTTTTTCGCCTTGCTG
Il-12	CACATCTGCTGCTCCACAAG	AACCGTCCGGAGTAATTTGG
Il-4	CGAAGAACACCACAGAGAGTG AGCT	GACTCATTCATGGTGCAGCTT ATCG
Il-13	ATGGCCTCTGTAACCGCAAG	GGCGAAACAGTTGCTTTGTG
Tnf- α	ATCCGCGACGTGGAAGT	ACCGCCTGGAGTTCTGGAA
IL-1b	TCCAGGATGAGGACATGAGC	ATGGGAACGTCACACACCAG

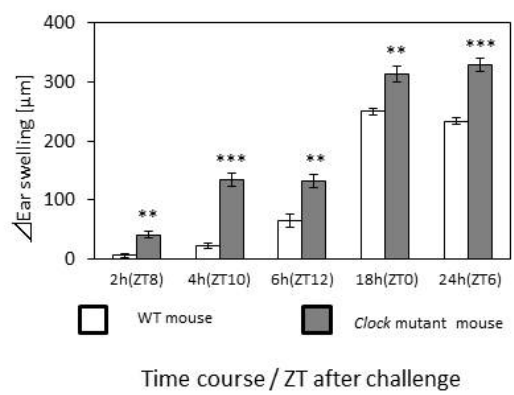


Fig. 1.

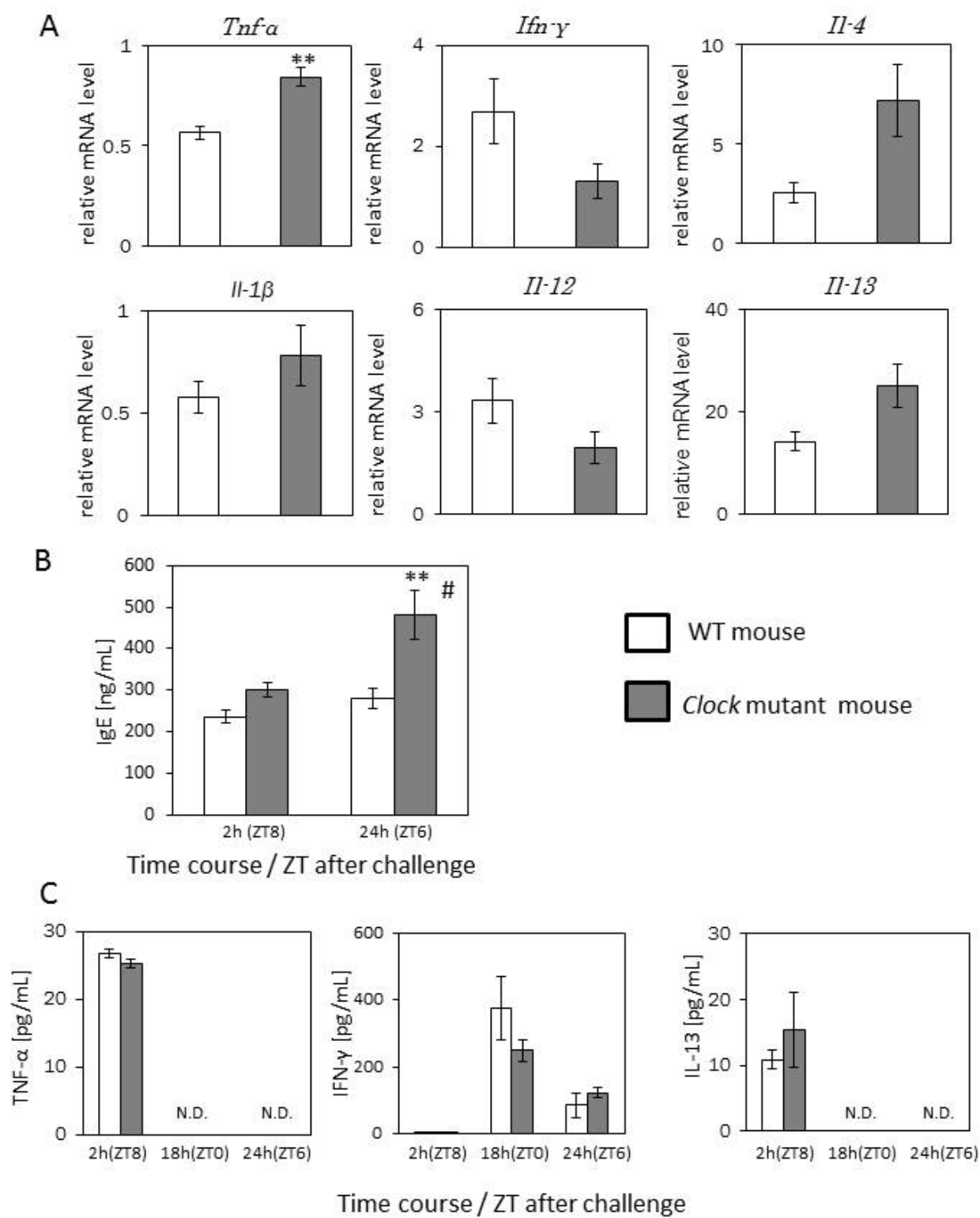


Fig. 2

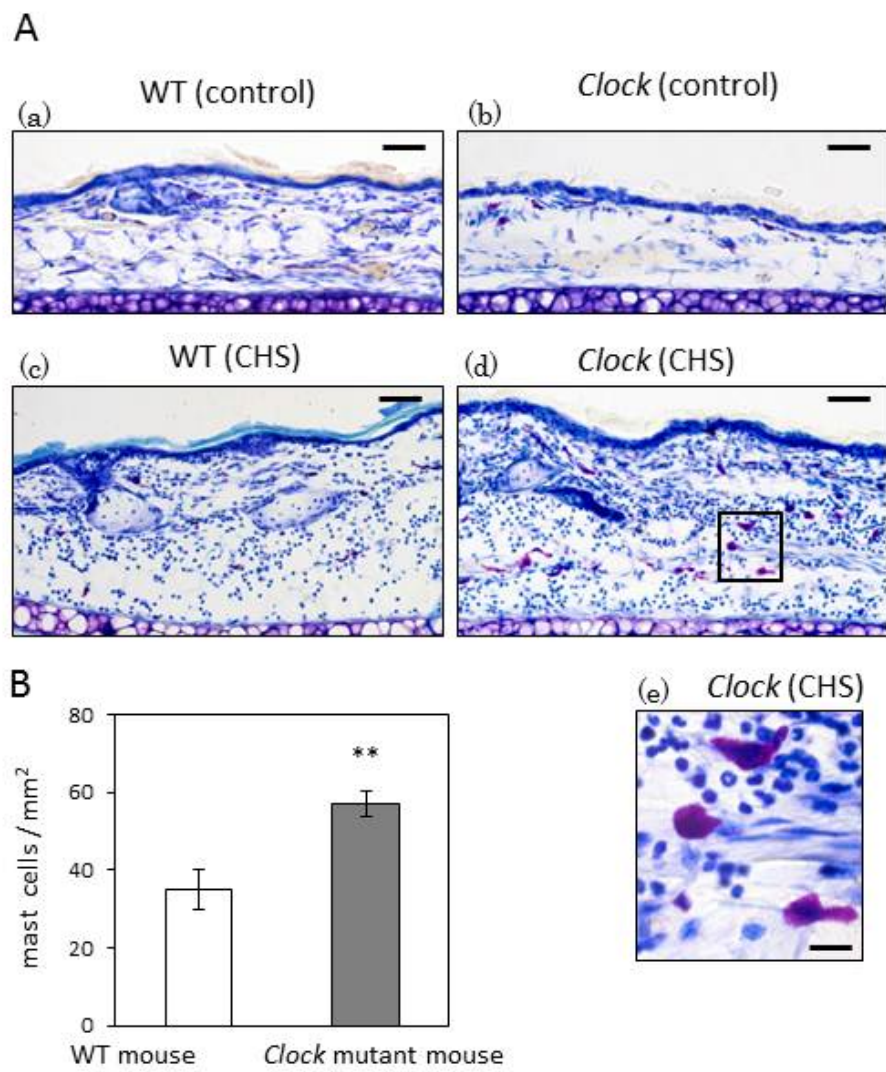
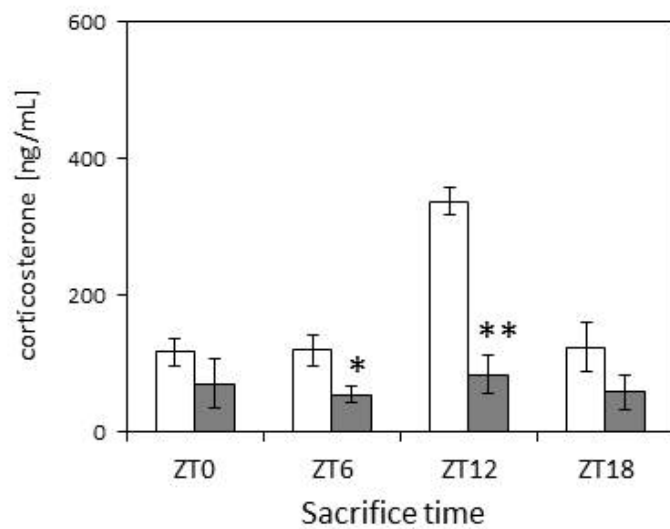


Fig. 3.

A



B

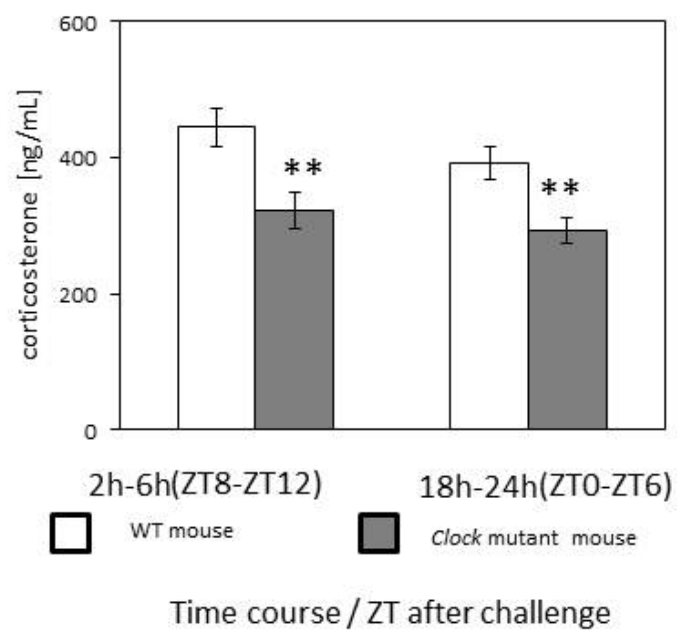
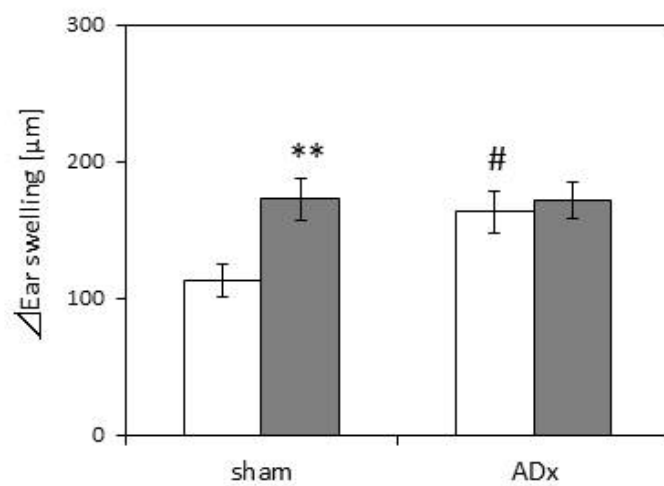


Fig. 4.

A



B

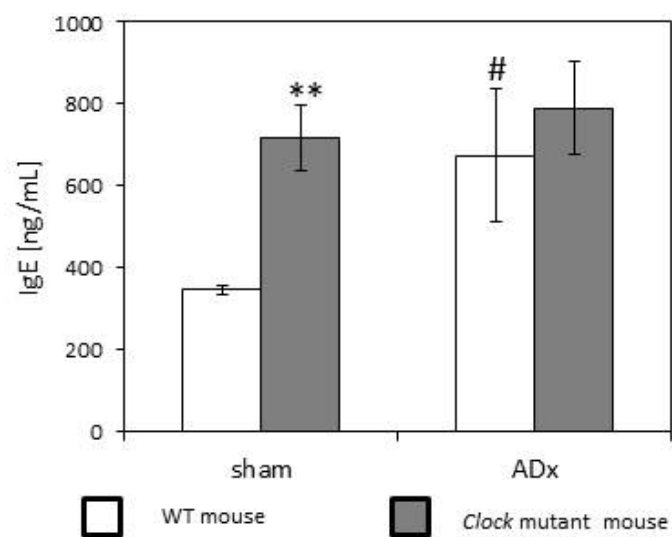
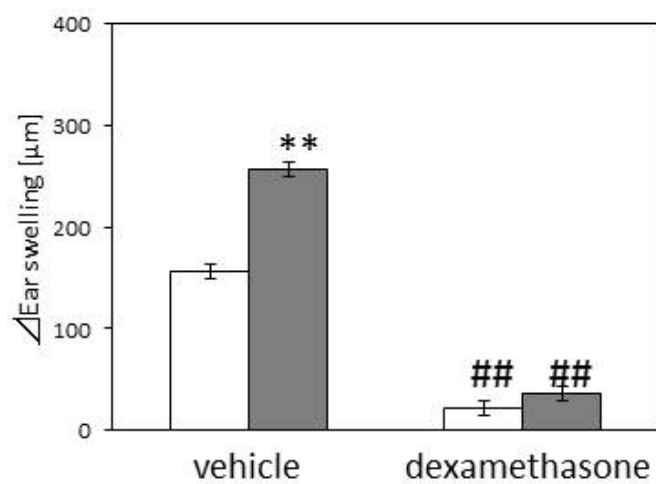


Fig. 5.

A



B

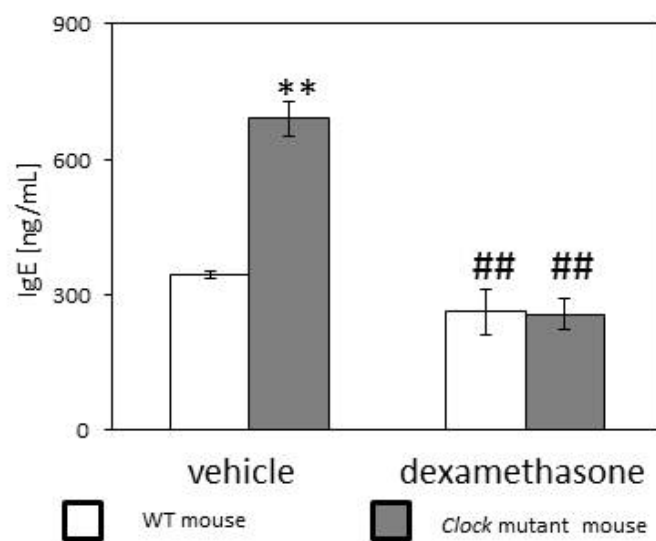


Fig. 6.