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Lentinan Inhibited the Activation of Th2 Cells in Allergic Mice by Reducing the Amplitude of Changes in Biological Rhythm

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Keywords

Lentinan · Biological rhythm · Allergy · Th2 cells · Murine model

Abstract

Introduction: Biological rhythm is inextricably linked to the physiological mechanisms of allergic diseases, but the exact mechanisms are still poorly understood. Clinical studies have reported rhythmic fluctuations in allergic diseases. The search for natural and harmless active ingredients based on biological rhythm with which to regulate allergic diseases is essential for the control of food allergy. ***Methods:*** In this study, mice were treated at different time points to determine the link between the severity of allergic reactions and the circadian clock genes. The mice were treated with lentinan, either continuously or discontinuously, to assess their clinical symptoms, vascular permeability, immune cells, cytokines, and clock genes. Specifically, rat basophilic leukemia (RBL-2H3) cells were treated with lentinan and the rhythmic changes of cell degranulation were measured. ***Results:*** The results in different models showed that the allergic reactions in mice treated at different time points were significantly different and thus related to fluctuations in biological rhythm. Treatment with lentinan was found to reduce the amplitude of changes in the clock genes, such as the activation of Per and Cry proteins in allergic mice, as well as to regulate biological rhythm in cells, inhibit the activation of Th2 cells, and alleviate allergic reactions. Furthermore, lentinan changed the rhythm of degranulation in RBL-2H3 cells. ***Conclusion:*** Lentinan was, therefore, determined to successfully alleviate allergic reactions by reducing the amplitude of changes in the body's biological rhythm, inhibiting the activation of Th2 cells, and affecting the immune microenvironment.

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Introduction

The biological rhythm plays a key role in many physiological processes and behaviors, which is an endogenous rhythmic life activity that reflects and adapts to the cyclical changes in the natural environment, such as mammalian and human sleep-wake activities, hormone secretion, blood pressure in the body, and other rhythmic changes

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[1]. Epidemiological studies have found long-term internal biological rhythm disorders, brought on by factors such as the irregular meal times of night shift workers, can increase the risk of metabolic diseases [2]. The mammalian biological rhythm is formed by the periodic expression of genes. Circadian genes can play a direct or indirect role in tumorigenesis by regulating the expression of proto-oncogenes, tumor suppressor genes, and genes encoding caspases and transcription factors [3]. The core clock genes, Bmal1, Clock, Per, Cry, Rev-erbα, Rora, and E4bp4, have been studied extensively [4, 5]. Among them, Per2 is considered the main circadian clock gene, as it can act on the clock mechanism mediated by Clock/Bmal1, become an important part of the negative feedback regulation loop, and also regulate key factors involved in the inflammatory response [6].

Food allergies have become a serious problem in the world. Allergens entering the human body can cause a series of adverse reactions in the digestive tract, respiratory tract, and skin, most of which manifest as dermatitis, asthma, diarrhea, and other symptoms [7, 8]. In the past decade or more, the incidence of food allergy has increased annually. According to statistics, 5% of adults and 8% of children in western countries suffer from food allergies [9]. Clinically, many symptoms and test indicators in allergic patients indicate obvious diurnal changes [10, 11]. Asthma patients, for example, experience peak allergic activity at midnight or early morning [12, 13], which affects nighttime sleep and reduces quality of life. However, the nature of the relationship between biological rhythm and food allergy remains unclear. At present, the most effective way to treat allergic diseases is simply to avoid contact with allergens since the current treatment drugs, such as antihistamines and steroids, can cause undesired side effects in some people. Therefore, it is important to find a more effective way to treat allergic diseases.

Shiitake mushroom is a large fungus which has good medical and health care effects. Lentinan, a β–1, 3-glucan with 2 β–1, 6-glucose branches every 5 glucose residues in the backbone from shiitake mushroom, is well known as a type of biological response modifier [14]. It exhibits many biological activities in life activities through different mechanisms, such as immunomodulation [15], antitumor [16], antivirus, hypolipidemic, and antioxidant [17]. Lentinan binds to lymphocyte surfaces or serum-specific proteins, which activates macrophage, T-helper cells, natural killer cells, and other effector cells [14]. Furthermore, there is evidence that lentinan not only upregulated expressions of the tumor suppressor p53, cell cycle arrest in p21, and proapoptotic proteins of Bax and caspase 3/9 but also downregulated PARP1 and antiapoptotic protein Bcl-2 expressions in tumor tissues. Lentinan sharply promoted immune cells accumulation into tumors accompanied by cells apoptosis and inhibition of cells proliferation during tumor development [18]. At present, there is no evidence to show the effects of lentinan on biological rhythm, but the related literature [18, 19] shows that lentinan can inhibit the development of tumors by regulating the expression of apoptosis-related factors, which is similar to the regulation of circadian clock genes on tumors. Therefore, this study considers whether lentinan can regulate biological rhythm, and whether it can alleviate food allergy symptoms by adjusting the biological rhythm.

In this study, a passive cutaneous anaphylaxis (PCA) and active allergic murine model were established, and mice were treated at different time points to explore the correlation between biological rhythm and food allergy. The effects of lentinan on biological rhythm and allergic reactions induced by ovalbumin (OVA) in sensitized mice were studied by treating them with lentinan. Rat basophilic leukemia (RBL-2H3) cells were used to verify the regulation of lentinan on cell degranulation and biological rhythm, so as to determine whether lentinan can alleviate food allergy by regulating biological rhythm.

Materials and Methods

Materials and Reagents

A detailed description of materials and regents is provided in see online suppl. material (for all online suppl. material, see www.karger.com/doi/10.1159/000509437).

Experimental Animals and Management

Female mice (5–6 weeks old, BALB/c) were purchased from Weitong Lihua Experimental Animal Technology Co., Ltd. (Beijing, China; No: SCXK [Jing] 2012-0036) and acclimatized to their new housing for a week before beginning experimental protocols. Mice were bred and housed under specific pathogen-free conditions, in the animal laboratory of the College of Food Science and Nutritional Engineering, China Agricultural University (Beijing, China). Experimental mice rooms were maintained with temperature of 25 ± 1°C, light/dark cycle of 12 h (the light was turned on at 6:00 a.m., zeitgeber time [ZT] 0, and the light was turned off at 6:00 p.m., ZT12). All experiments were performed under the China Agricultural University Animal Experimental Welfare and Ethical Inspection Committee approved protocols and in accordance with its guidelines. All efforts were made to minimize the suffering of experimental animals.

Passive Cutaneous Anaphylaxis

As previously described with some modifications [20], to assess the relationship between allergic reactions and biological rhythm,

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mice were randomly divided (n = 6) into 4 groups, labeled ZT2, ZT8, ZT14, and ZT20. Mice were injected intradermally in the right ear with 500 ng of anti-DNP IgE at their group's indicated ZT, respectively, to induce a passive cutaneous anaphylactic reaction. After 24 h, each mouse received an injection of 200 μL DNP-HSA (50 μg/mL) and 0.5% Evans blue mixture via the tail vein. Vascular permeability was visualized 30 min later by the blue staining of the injection sites. The ears were photographed, and mice were euthanized after blood collection. Samples of 1 cm^2 ear tissues were cut out and dissolved in 1 mL formamide solution overnight. The supernatant was measured for absorbance (Abs) at 620 nm.

Establishment of OVA-Induced Allergy Model in BALB/C Mice

Mice were randomly divided into 3 groups (n = 24), namely, control (CK), ZT2, and ZT14 groups. In ZT2 and ZT14 groups, mice were intraperitoneally injected with 10 and 20 μg OVA on the 4th and 20th days, respectively, and gavaged with 10 and 30 mg on the 27th and 30th days, respectively. On the 20th and 27th days, blood samples were collected from the mice eyes orbital venous plexus 24 h after OVA challenge. On day 30, blood samples were collected and 1 batch was euthanized 30 min after OVA challenge. Blood samples were centrifuged for 15 min at 4,000 *g*, 4°C, and serum were collected and stored at −80°C. Plasma was collected in centrifugal tubes which contained heparin. Mice were injected intraperitoneally with 1 mL saline, kneaded for >1 min, and the abdominal fluid was collected. The supernatant was collected by centrifugation at 4°C for 15 min and stored at −80°C. The Abs of the supernatant was measured at 562 nm to calculate the protein concentration in the sample using BCA protein assay kit according to the manufacturer's instructions. Subsequently, a batch was euthanized every 6 h. The injection/gavage times for ZT2 and ZT14 groups were 8 a.m. and 8 p.m., respectively, and the CK group was given PBS at ZT2 (8 a.m.).

Treatment with Lentinan in Allergic Mice Induced by OVA

In order to study the effects of lentinan on food allergy, we established 2 murine models, in which mice were treated with different doses of lentinan continuously or discontinuously (Fig. 4a, b). Overall, mice were randomly divided into 8 groups (n = 24), namely, CK group, allergy group (OVA), lentinan group continuously (100, 200, and 400 mg/kg), and lentinan group discontinuously (100, 200, and 400 mg/kg). On the 4th and 20th days, mice were intraperitoneally injected with 10 and 20 μg OVA, respectively. In the continuous model, mice were gavaged with lentinan every 2 days from 0 to 28 days. Mice in the discontinuous model were gavaged with lentinan every 2 days from 0 to 8 days and from 16 to 24 days. Mice in the OVA group were gavaged with PBS instead of lentinan every 2 days from 0 to 28 days. Mice in the CK group were gavaged with PBS instead of OVA and gavaged with saline instead of lentinan every 2 days from 0 to 28 days. On the 22nd and 28th days, blood samples were collected from the mice eyes orbital venous plexus. On the 30th day, mice were gavaged with 50 mg OVA. The OVA injection/gavage time was ZT2 (8 a.m.), mice were gavaged with lentinan at 7 a.m.

Thirty minutes after OVA challenge, the body temperature was observed using a rectal probe. The allergic symptoms were observed by using a scoring system as formerly described [21], with scoring-specific criteria as follows: 0 = no symptoms; 1 = nose, lip, and eye puffiness; 2 = decreased activities; 3 = dyspnea, cyanosis; 4 = no response after challenge or convulsion; and 5 = death. On day 30, blood samples were collected and 1 batch was euthanized 30 min after OVA challenge. After centrifugation, the supernatant of abdominal fluid was measured at 562 nm to calculate the protein concentration using BCA protein assay kit according to the manufacturer's instructions. Subsequently, a batch was euthanized every 6 h.

Serum IgE, IgG1 Levels and Routine Blood Test

Serum levels of IgE and IgG1 were determined using the mouse ELISA kit according to the manufacturer's instructions (eBioscience, Inc., San Diego, CA, USA). According to the routine blood test method, detect the levels of eosinophils, basophils, neutrophils, and lymphocytes in plasma.

Detection of Histamine and Cytokines

Serum levels of IL-4, IFN-γ, IL-10, IL-17A and level of plasma histamine were measured using ELISA kit according to the manufacturer's instructions (eBioscience, Inc.,USA).

Cell Line

RBL-2H3 cells were derived from the National Experimental Cell Resource Platform (Beijing, China). Cells were cultured in Eagle's minimum essential medium (MEM) containing 15% fetal bovine serum and 1% penicillin/streptomycin/amphotericin B and incubated at 37°C in a humidified atmosphere with 5% CO_2.

Cell Proliferation Assay

The effects of lentinan on cell proliferation were determined by CCK8 assay. RBL-2H3 cells at an initial density of 5×10^3 cells/well were seeded into the 96-well plates. After 12 h, cells were treated with 10 μL lentinan (10, 10^2, and 10^3 μg/mL, respectively) and CCK8 (10 μL), then incubated for 1 h. The Abs of each well was measured at 450 nm with a microplate reader. Control group was added with 10 μL of MEM basic medium and 10 μL CCK-8, which was determined as 100%, and the other groups were converted.

β-Hexosaminidase Release Assay

β-hexosaminidase release assay was carried out following procedure previously described with some modification [22]. A detailed description of β-hexosaminidase release assay is provided in online Suppl. material. The formula of the release of β-hexosaminidase calculation is as follows:

$$\beta - \text{Hexosaminidase release}\,(100\%) = \frac{\text{Absorbance}_{\text{supernatant}} - \text{Absorbance}_{\text{blank of supernatant}}}{\text{Absorbance}_{\text{Trix}} - \text{Absorbance}_{\text{blank of Trix}}} \times 100\%.$$

In order to detect the effects of biological rhythm on cell degranulation, RBL-2H3 cells were cultured at 37°C for 12 h, and the basic MEM medium was added to start timing. Every 6 h, C48/80 was added for 1 h and the supernatant was collected. In addition, the incubation time of lentinan was 6 h, that is, 100 μL lentinan (10^3 μg/mL) was added 6 h before C48/80 stimulation. After C48/80 stimulation, the supernatant was collected and stored at −20°C, and the total RNA of cells was extracted. The release of β-hexosaminidase in the supernatant was detected (the release of Trix group as 100%).

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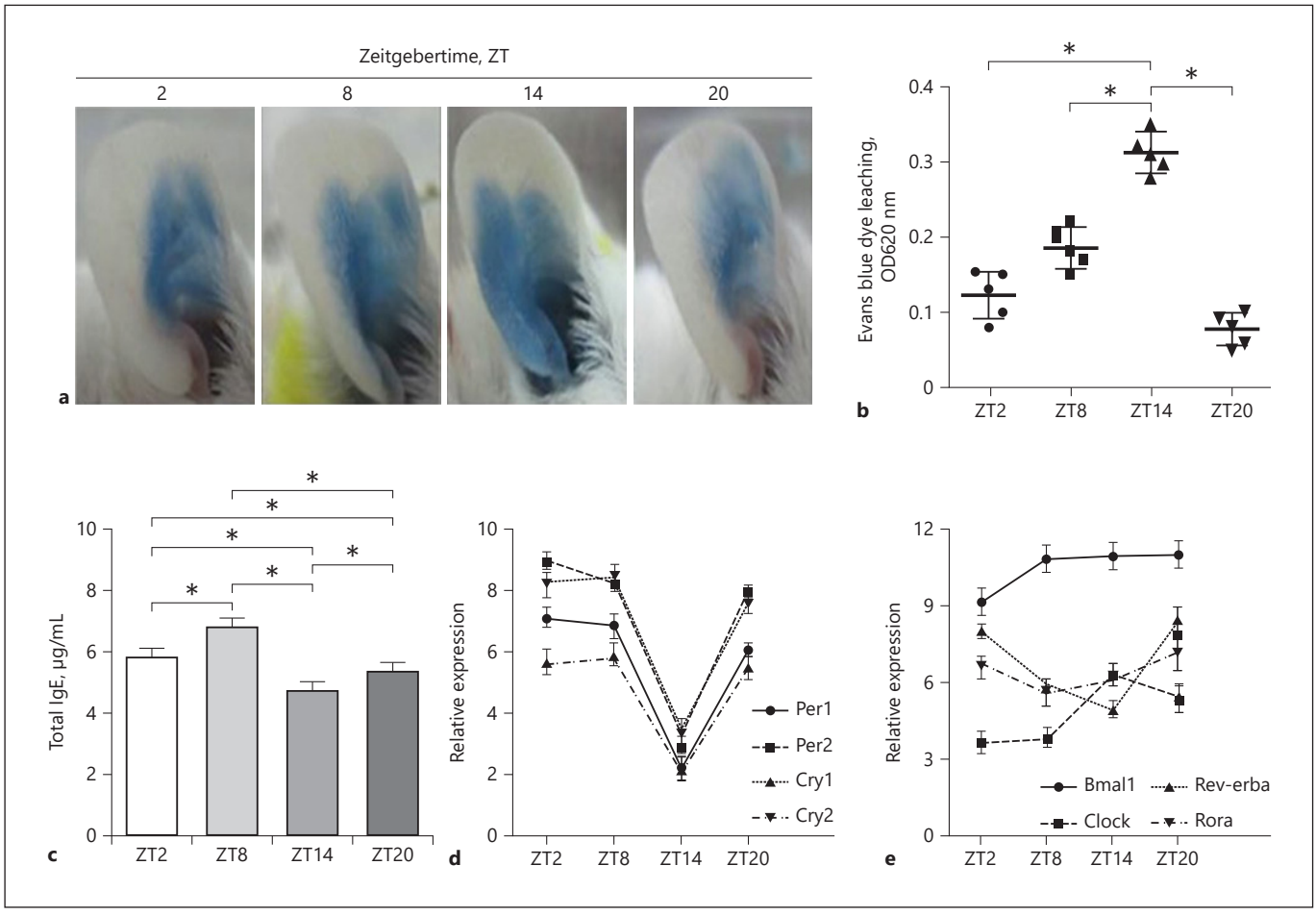

Fig. 1. PCA in mice. Representative photographs (**a**) and quantified maps of Evans blue dye leaching amount in ears at ZT2, ZT8, ZT14, and ZT20 (**b**); serum total IgE (**c**) and rhythmic expression of spleen circadian clock genes, over time, with Gapdh as an internal reference (**d**, **e**). Values are represented as mean ± SDs ($n = 6$). A pairwise comparison, * indicates a significant difference ($p < 0.05$). PCA, passive cutaneous anaphylaxis; SD, standard deviation.

Real-Time PCR

RNA extraction and assay were carried out following the procedure previously described [23]. Total RNA was extracted from BALB/c mice spleen/RBL-2H3 cells using TranZol RNA Extraction Kit (TransGen Biotech, China). A detailed description of real-time PCR is provided in online suppl. material. The real-time PCR primer sequences of the genes are in online suppl. Tables 1 and 2.

Statistical Analysis

One-way ANOVA using GraphPad Prism 5.01 was used to determine statistical significance. All data were expressed as mean ± standard deviation of 3 replicates with a significance set to $p < 0.05$.

Results

Allergic Reactions Had Biological Rhythm

Mice were treated at ZT2, ZT8, ZT14, and ZT20. It was found that there were differences in ear color among mice treated at different time points. Ear color was deepest in the ZT14 group (Fig. 1a) and quantitative analysis showed the Abs of ear extracts in this group to be the highest, indicating that the degree of allergy reached the highest peak at ZT14 (Fig. 1b). There were significant differences observed between each group. In addition, mice in the ZT14 group had the lowest total IgE levels (Fig. 1c), possibly because more IgE bound to the cell surface when allergic reactions were most severe, resulting in a decrease

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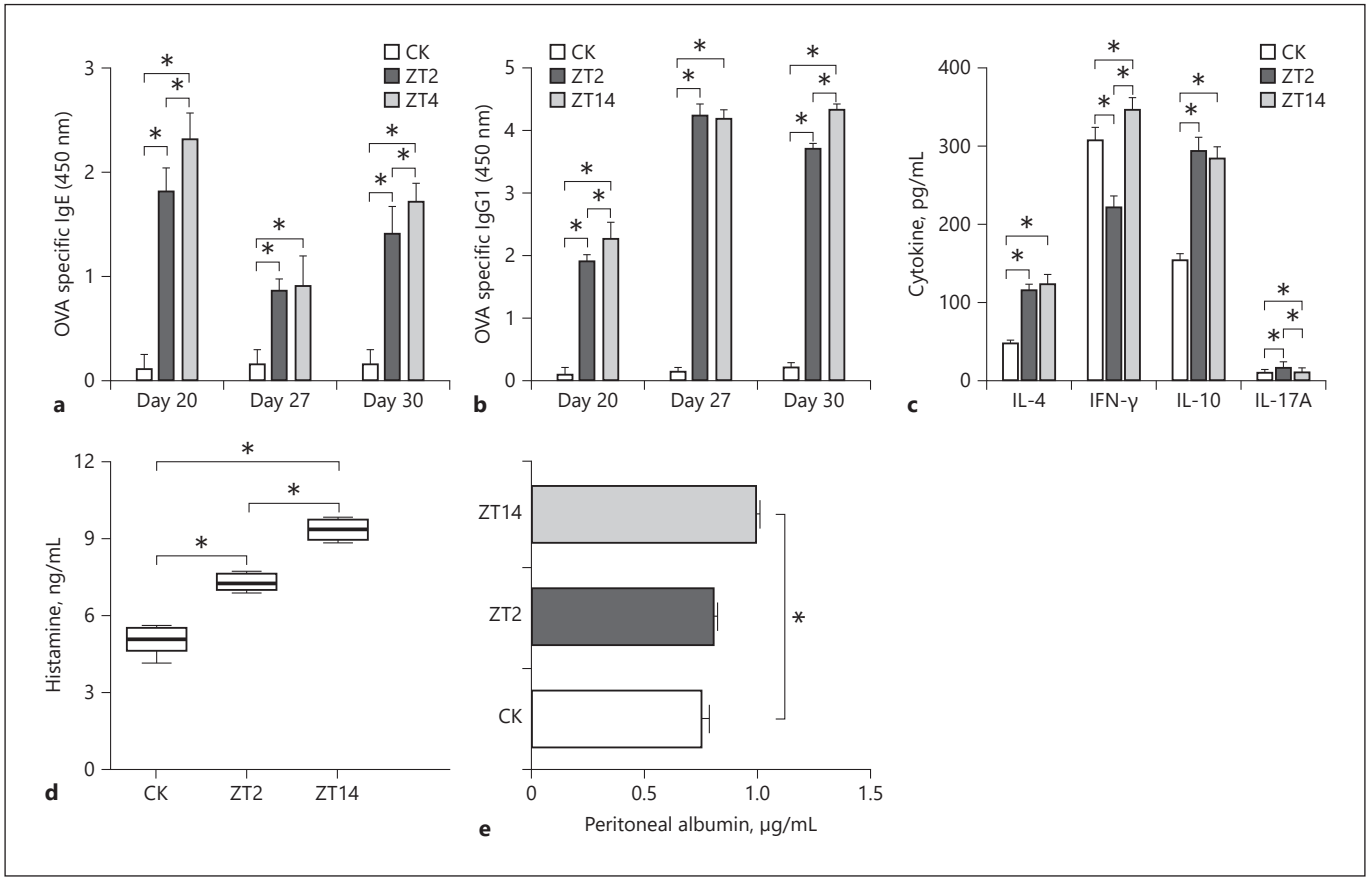

Fig. 2. Specific antibodies and cytokine levels. Serum-specific IgE (**a**) and IgG1 (**b**) levels. On days 20, 27, and 30, blood was collected 24 h after challenge with OVA. OVA-specific IgE and IgG1 were detected by ELISA microplate reader (450 nm). The levels of IL-4, IFN-γ, IL-10, and IL-17A in serum (**c**) and plasma histamine (**d**), and albumin content in abdominal fluid (**e**). Values are represented as mean ± SDs (n = 8). A pairwise comparison, *indicates a significant difference ($p < 0.05$). CK, control; OVA, ovalbumin; SD, standard deviation.

in free IgE levels in serum. It is worth noting that at ZT14, mice were most allergic, and the levels of Per1, Per2, Cry1, Cry2, and Rev-erbα in spleen reached their lowest points during the day, while the expressions of Clock and Bmal1 reached their highest points (Fig. 1d, e). These results indicate that Per, Cry and Clock, and Bmal1 had opposite trends, suggesting that negative feedback regulation among circadian clock genes had a certain correlation with the occurrence of allergy.

Furthermore, ZT2 and ZT14 time points were selected to establish an OVA-induced allergic murine model. The levels of specific IgE (Fig. 2a) and IgG1 (Fig. 2b) in the ZT2 and ZT14 groups on days 20, 27, and 30 were significantly different from those in the CK group. After challenge on the 30th day, there was a significant increase in serum specific IgE and IgG1 levels in the ZT14 group compared to the ZT2 group. Moreover, the levels of IL-4, IFN-γ, and IL-10 in serum and plasma histamine were significantly increased in the ZT14 group compared to the CK group (Fig. 2c, d), while the serum IL-17A level in the ZT2 group was significantly increased. In addition, the albumin content in abdominal fluid in mice of ZT14 group was significantly higher than that in the CK group (Fig. 2e), indicating that the changes in vascular permeability were more pronounced in ZT14-treated mice. The above data indicated that treatment at ZT14 will produce stronger allergic reactions, which is consistent with the result in the PCA model, suggesting that allergy is related to biological rhythm.

On day 30, 6 h after challenge at ZT2, the circadian clock genes in spleen changed in comparison to the CK group. Specifically, Per1/2 and Cry1/2 were downregu-

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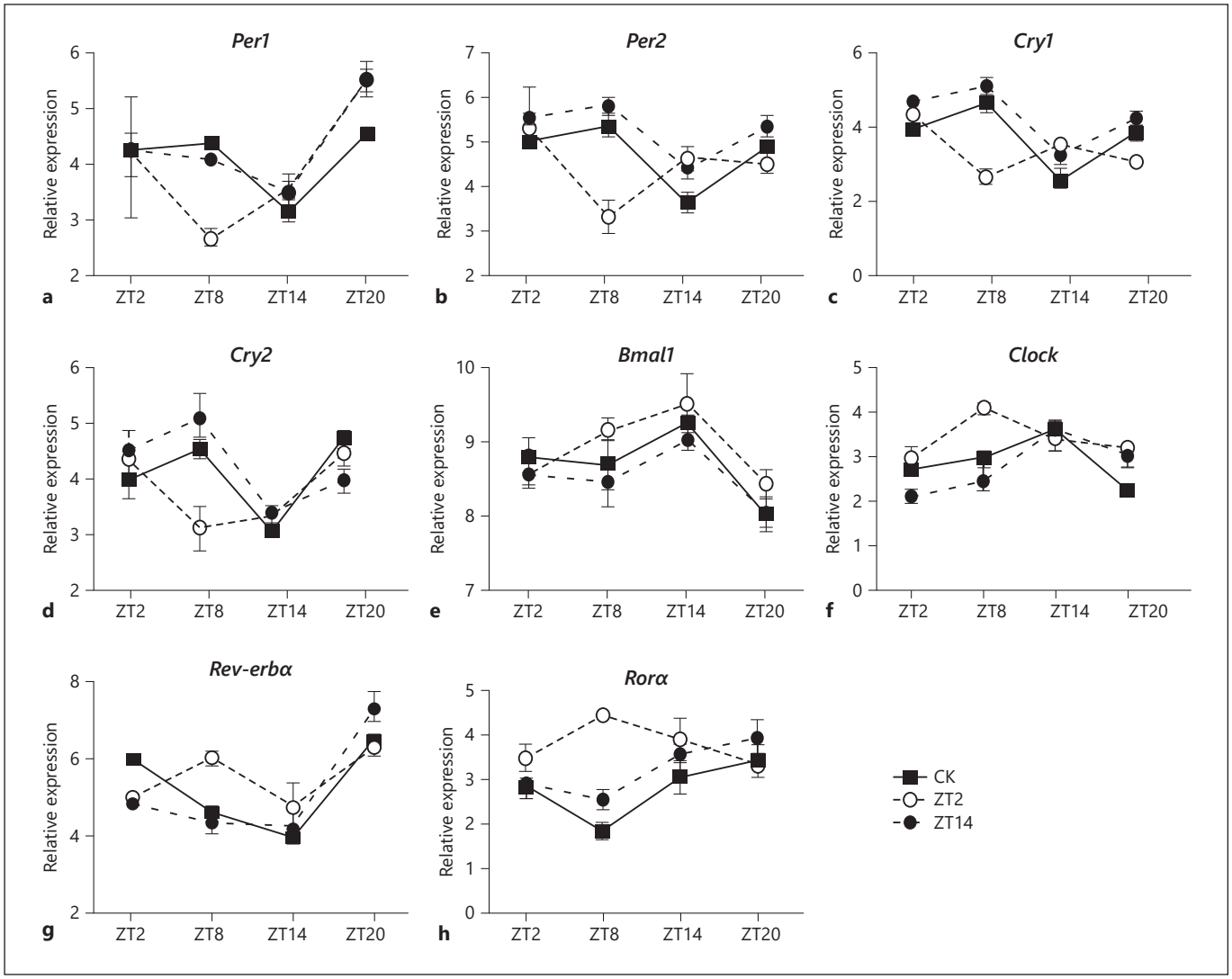

Fig. 3. Levels of spleen circadian clock genes. Mice were challenged with OVA at ZT2 and ZT14, respectively, and then were sacrificed every 6 h to quantitatively detect circadian clock genes Per1 (**a**), Per2 (**b**), Cry1 (**c**), Cry2 (**d**), Bmal1 (**e**), Clock (**f**), Rev-erbα (**g**), and Rorα (**h**). Values indicate the relative content of this gene to Gapdh ± SDs (n = 6/6 h). CK, control; OVA, ovalbumin; SD, standard deviation.

lated (Fig. 3a–d), while Rev-erbα, Rorα, Bmal1, and Clock were upregulated (Fig. 3e–h), then gradually recovered to equal the rhythm in the CK group. These changes indicated allergic reactions and thus further reflect the correlation between allergy and biological rhythm. However, when the mice were challenged at ZT14, no rhythmic changes were detected in these genes after 6 h (ZT20), presumably because ZT14 was 8 p.m. and additional stimulation and light during treatment were found to have certain effects on the sleep-wake cycle of mice. Therefore, in order to reduce the impact of light stimulation, ZT2 was selected for subsequent experiments. In addition, the upregulation of Rev-erbα and Rorα expression explained the higher level of IL-17A after ZT2 challenge. These results suggest that regardless of the type of allergy, changes in biological rhythm are associated with its occurrence and severity. It was, thus, speculated that fluctuations of biological rhythm in the body may affect the occurrence of allergic reactions; however, the potential to alleviate allergic reactions by regulating biological rhythm required further research.

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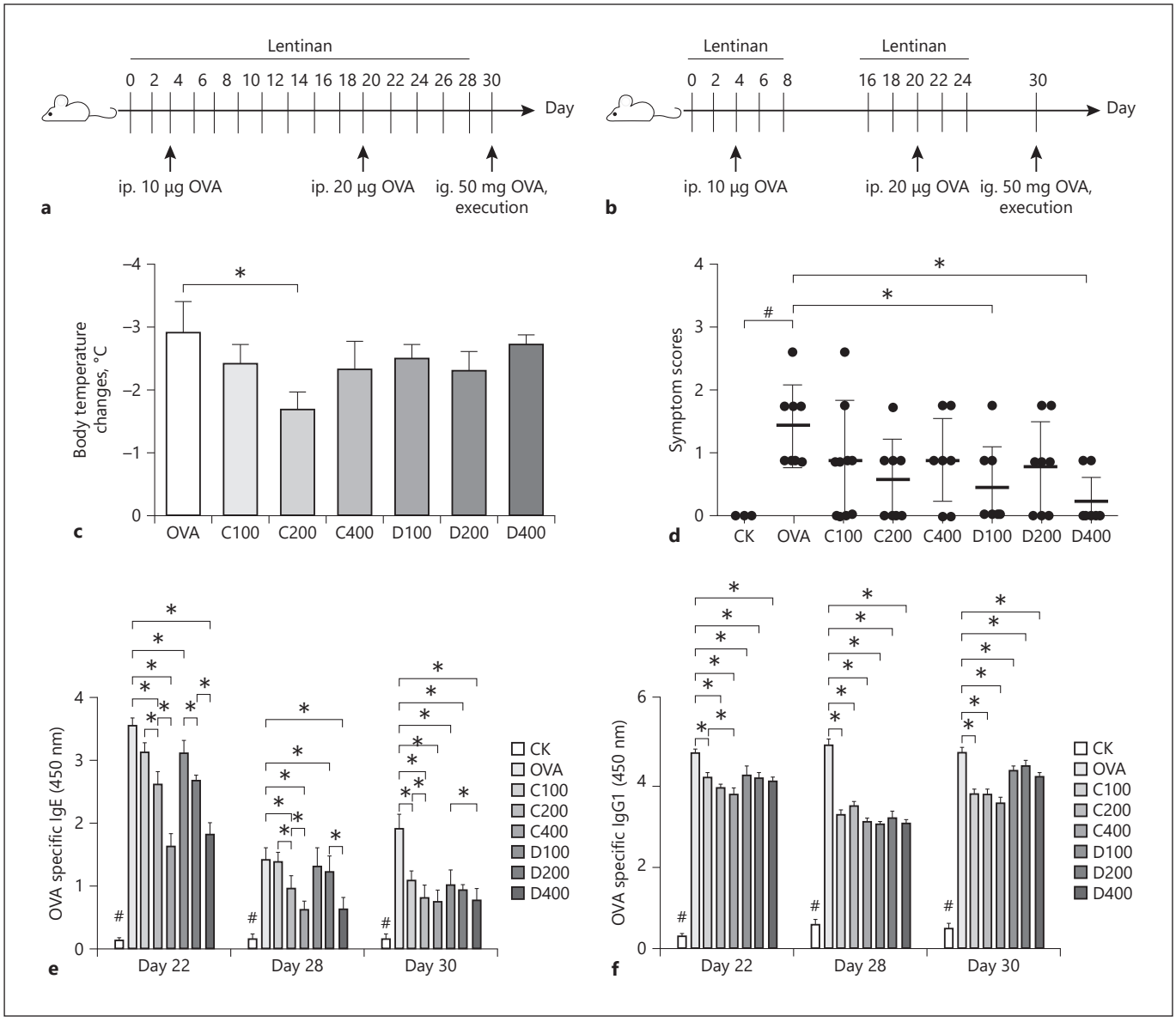

Fig. 4. Clinical symptoms and antibody levels in mice. Two models with (**a**) continuous and (**b**) discontinuous lentinan treatment. Body temperature changes (with that in the CK group as baseline) (**c**) and symptom evaluation 30 min after challenge on day 30 (**d**). Specific IgE and IgG1 (**e**, **f**) on days 22, 28, and 30. Values indicate Abs values (mean ± SDs) at a wavelength of 450 nm ($n = 8$).* indicates a significant difference in lentinan versus OVA or lentinan versus lentinan ($p < 0.05$), # indicated a significant difference in CK versus OVA ($p < 0.05$). CK, control; OVA, ovalbumin; Abs, absorbance.

Lentinan Reduced Allergic Symptoms in OVA-Induced Mice

To study whether lentinan can exert mitigating effects on allergic reactions, and whether its mechanism is influenced by the regulation of biological rhythm, we established 2 murine models, which were then treated with lentinan either continuously (Fig. 4a) or discontinuously (Fig. 4b), respectively. Body temperature and symptoms were observed 30 min after challenge on the 30th day. Taking body temperature in the CK group as baseline, it was found that the body temperature decreased by about 2–3°C after challenge, most seriously in the OVA group

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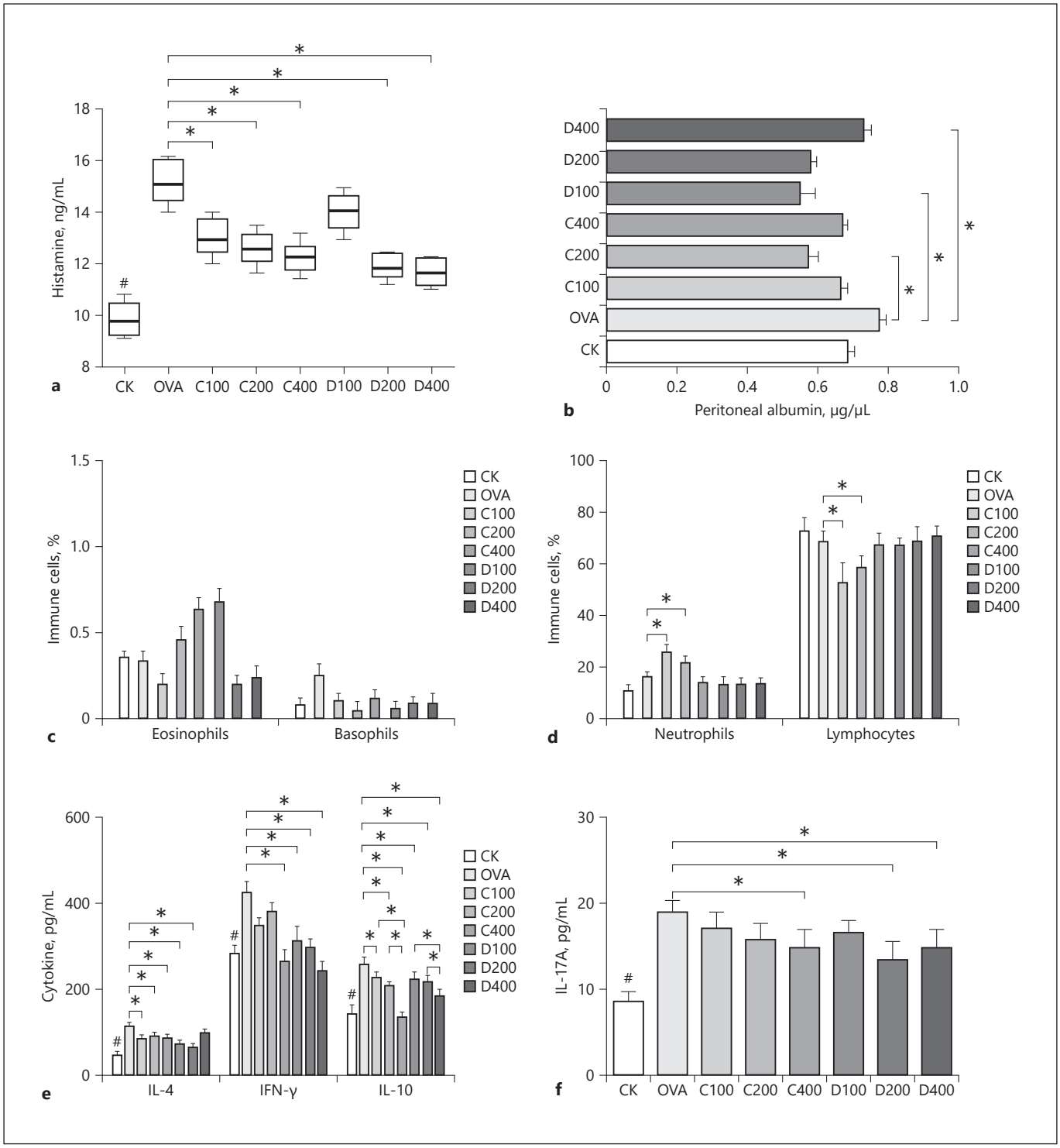

Fig. 5. Effects of lentinan on leukocyte and cytokine levels in mice. The content of (**a**) histamine and (**b**) albumin in abdominal fluid. The percentage of eosinophils, basophils (**c**), neutrophils, lymphocytes (**d**), and IL-4, IFN-γ, IL-10 (**e**), and IL-17A (**f**) levels in serum. Values are represented as mean ± SDs ($n = 8$). Pairwise comparison of lentinan versus OVA or lentinan versus lentinan, * indicates a significant difference ($p < 0.05$). CK versus OVA, #indicates a significant difference. CK, control; OVA, ovalbumin; SD, standard deviation.

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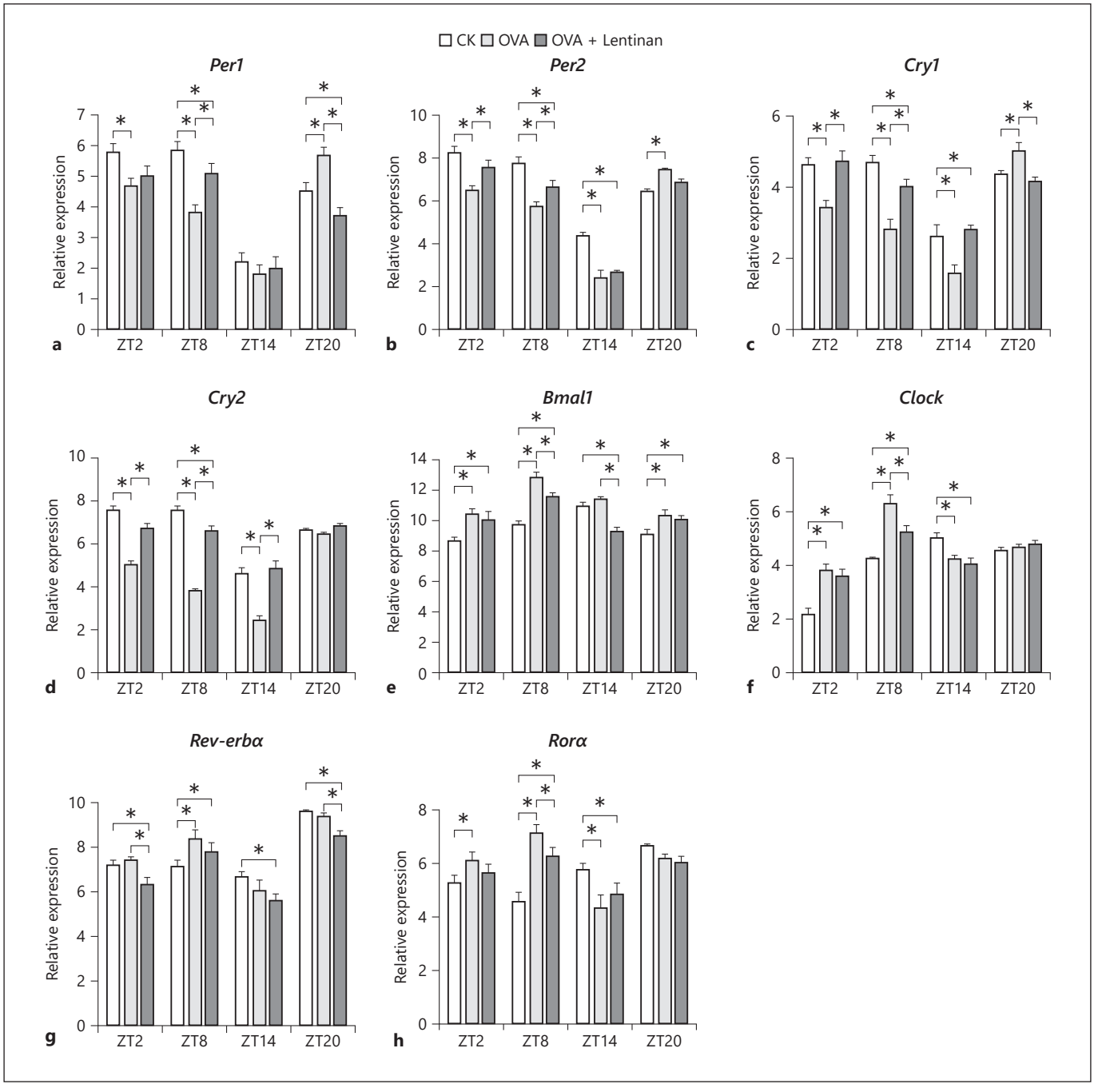

Fig. 6. Lentinan reduced the fluctuation of biological rhythm. After challenge on day 30, the expression of Per1 (**a**), Per2 (**b**), Cry1 (**c**), Cry2 (**d**), Bmal1 (**e**), Clock (**f**), Rev-erbα (**g**), and Rorα gene (**h**). Values are represented as mean ± SDs (n = 6/6 h). A pairwise comparison of CK versus OVA or lentinan versus OVA, * indicates a significant difference ($p < 0.05$). CK, control; OVA, ovalbumin; SD, standard deviation.

(Fig. 4c). The decrease of body temperature in the continuous lentinan-treated mice (200 mg/kg) was significantly less than that in the OVA group, while body temperature in the other lentinan-treated mice was only slightly reduced. In terms of clinical symptoms, the scores in the OVA group were significantly increased compared to those of the CK group. In the discontinuous treatment model, the symptom scores in both the low- and high-

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dose lentinan groups were significantly lower than those in the OVA group (Fig. 4d). Although the other lentinan treatment groups showed no significant difference in scores compared to the OVA group, they all showed a decreasing trend. These results suggest that both continuous and discontinuous lentinan treatments can alleviate allergic symptoms and allergic inflammatory responses in mice.

Lentinan Reduced OVA-Specific Antibody Levels

To further determine the effects of lentinan treatment on allergy, we measured serum OVA-specific IgE (Fig. 4e) and IgG1 (Fig. 4f) levels on days 22, 28, and 30. In both models, specific IgE and IgGl were significantly increased compared to the levels in the CK group. Specific IgE levels were similar in the 2 models on day 22, indicating that both medium and high doses of lentinan significantly reduced IgE levels in a dose-dependent manner. Moreover, every dose of lentinan treatment in both models significantly reduced IgG1, indicating that treatment with lentinan on days 10, 12, and 14 did not significantly affect the effects of lentinan on specific antibodies. On day 28, compared to the OVA group, the IgG1 levels in both models in the lentinan group were significantly reduced but not dose-dependent, while medium and high doses of lentinan treatment were found to significantly reduce the IgE levels. The above results suggest that both lentinan treatments can inhibit the production of OVA-specific IgE and IgG1, and the effects on the 2 models were similar. Consequently, we hypothesized that lentinan treatment may affect the activation of either T cells or B cells and thus inhibit the production of specific antibodies.

Lentinan Reduced Vascular Permeability in Allergic Mice and Inhibited the Release of Histamine from Mast Cells

The occurrence of allergic reactions is often accompanied by an increase in vascular permeability. By measuring albumin content of abdominal fluid, it was found that every dose of lentinan treatment reduced the content of albumin to a certain extent. Significant differences were observed between the continuous lentinan treatment (200 mg/kg) (Fig. 5b) and discontinuous treatment (100 and 200 mg/kg) groups, indicating that lentinan had certain effects on vascular permeability in allergic mice. The level of plasma histamine in the OVA group showed a significant increase compared to that in the CK group (Fig. 5a), while in comparison to the OVA group, the lentinan treatment was ascertained to significantly reduce histamine levels. The data indicated that lentinan treatment could inhibit the activation of mast cells, hinder the release of histamine, and reduce vascular permeability in allergic mice, thus suggesting that lentinan may inhibit the activation of Th2 cells and affect the production of Th2 cytokines.

Lentinan Inhibited the Release of Cytokines in Allergic Mice

In order to further explore the effects of lentinan on allergic reactions, we investigated whether lentinan treatment had regulatory effects on the Th2 cells environment. Discontinuous lentinan treatment showed no significant changes in eosinophils, basophils, neutrophils, and lymphocytes in plasma (Fig. 5c, d). However, in the continuous lentinan groups (100 and 200 mg/kg), the proportion of lymphocytes was significantly decreased (Fig. 5d). The results suggest that the effects of lentinan on allergic reactions may not directly affect the number of leukocytes in the body but may inhibit cells activation and the release of inflammatory mediators. Further analysis of cytokine levels in the serum showed a significant increase in IL-4, IFN-γ, IL-10, and IL-17A levels in the OVA group compared to the CK group (Fig. 5e, f). In comparison to the OVA group, lentinan significantly reduced the levels of IL-4, IFN-γ, and IL-10 in the 2 models, with significant dose-dependent differences. Continuous (400 mg/kg) and discontinuous (200, 400 mg/kg) treatments significantly reduced the level of IL-17A compared to that in the OVA group. IL-17A belongs to Th17-type cytokine, which promotes the production of IL-6 and IL-8 by T cells and promotes the production of inflammation. IL-4 promotes B-cell proliferation, activates B cells and T cells, and this antibody class turns to IgE to enhance allergic reactions. Lentinan regulated the levels of cytokines in the allergic mice, inhibited the activation of Th2 and Th17 cells, and alleviated allergic reactions. However, the regulation of cytokines by lentinan was found to be different depending on the dose.

Lentinan Reduced the Fluctuation of Biological Rhythm

After determining that lentinan can alleviate allergic reactions, we considered whether this mechanism is exerted by regulation of biological rhythm and consequently compared the expression of Per1, Per2, and other circadian clock genes in spleen in the CK group, OVA group,and continuous lentinan group (200 mg/kg). Per1, Per2, Cry1, Cry2, Bmal1, Clock, Rorα, and Rev-erbα genes in the CK group maintained the biological rhythm (Fig. 6a–h). In comparison, however, in the OVA group,

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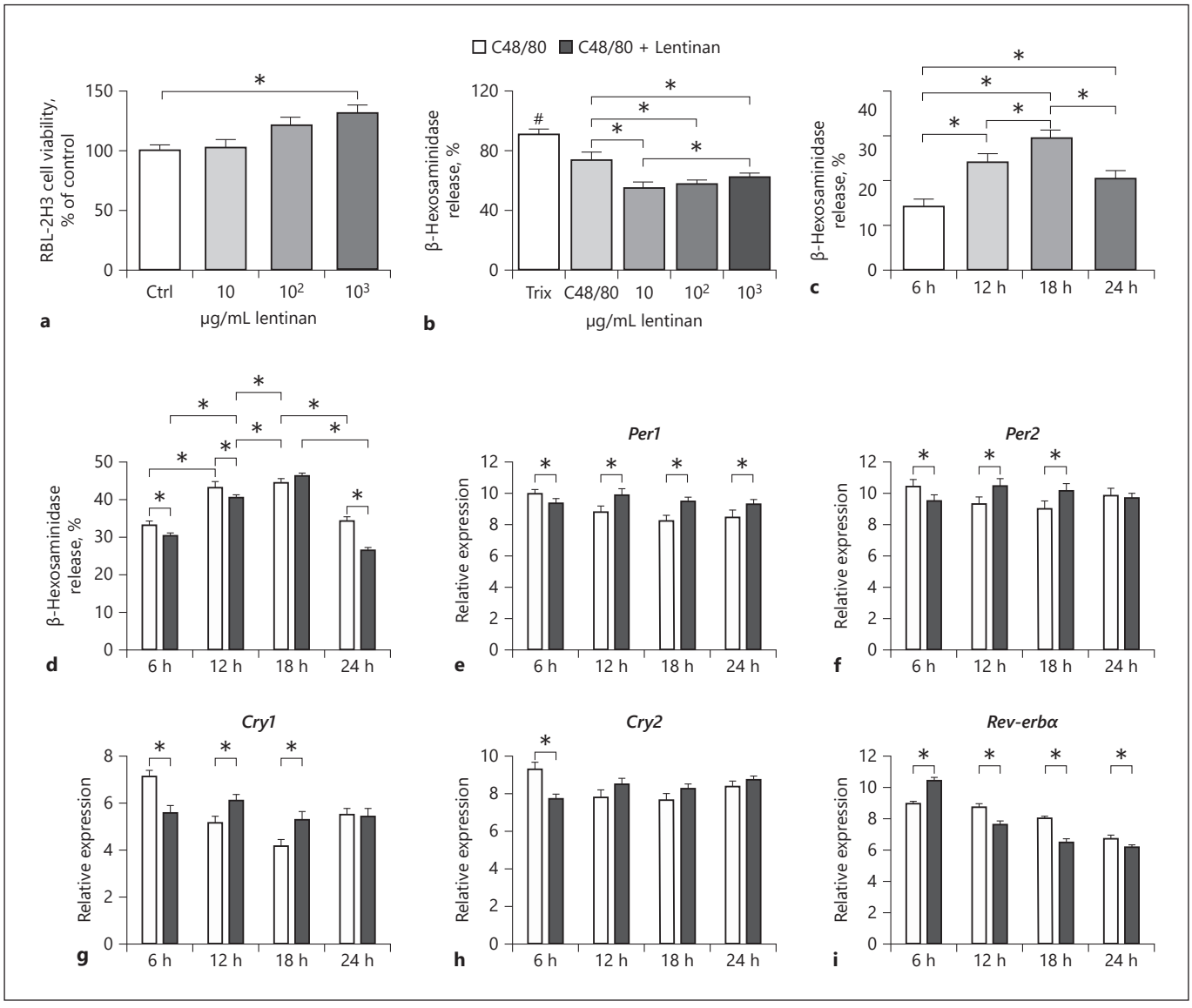

Fig. 7. Lentinan regulated circadian clock genes to reduce the level of degranulation of RBL-2H3 cells. **a** The concentration of lentinan was 10, 10^2, 10^3 μg/mL, and the larger the value, the higher the cells survival rate. **b** Different doses of lentinan can inhibit the release of β-hexosaminidase from RBL-2H3 cells induced by C48/80. **c** After liquid exchange, C48/80 was added every 6 h to induce degranulation of RBL-2H3 cells. **d** Preincubation of 10^3 μg/mL lentinan was added 6 h before the addition of C48/80, and the Abs was measured at 450 nm. The expression of Per1 (**e**), Per2 (**f**), Cry1 (**g**), Cry2 (**h**), Rev-erbα (**i**) 6–24 h after liquid exchange . Values are represented as mean ± SDs. Comparison between the 2, *indicates a significant difference ($p < 0.05$). RBL-2H3, rat basophilic leukemia; Abs, absorbance; SD, standard deviation.

Per1, Per2, Cry1, and Cry2 genes were significantly decreased, while Bmal1, Clock, Rev-erbα, and Rorα genes were upregulated after challenge at ZT2. After treatment with lentinan, the levels of Per1, Per2, Cry1, and Cry2 were increased; Bmal1, Clock, Rorα, and Rev-erbα were inhibited; and the fluctuation of circadian clock genes in allergic mice was reduced. To some extent, the biorhythm trend in allergic mice was restored to that of the CK group. Moreover, compared to the CK group, no significant differences were observed in Per1, Per2, Cry1, Cry2, and Rorα genes in the lentinan group after ZT2 challenge. Treatment with lentinan also reduced variations in the

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circadian clock genes and promoted the synchronization of biological rhythm with that of CK group. On the whole, allergic reactions can cause disorder in the body's biological rhythm and change the levels of circadian clock genes. Treatment with lentinan can reduce the variation of circadian clock genes Per1/2, Cry1/2, Bmal1, Clock, Rorα, Rev-erbα in allergic mice and can regulate biological rhythm to alleviate allergic reactions.

Lentinan Regulated the Rhythm of RBL-2H3 Cell Degranulation

Different doses of lentinan were tested for effects on cells proliferation. It was found that lentinan promoted the proliferation of RBL-2H3 cells and was significant at 10^3 μg/mL (Fig. 7a). C48/80 promoted the degranulation of RBL-2H3 cells, while treatment with lentinan significantly inhibited cells degranulation (Fig. 7b). Considering whether RBL-2H3 cells degranulation had biological rhythm, the time range was set at 24 h. The release of β-hexosaminidase was significantly increased every 6 h after liquid exchange and significantly decreased 24 h after exchange (Fig. 7c), thus indicating that cells degranulation was rhythmic. After preincubation of RBL-2H3 cells with 10^3 μg/mL lentinan, the effects of lentinan on the release of β-hexosaminidase was measured every 6 h after liquid exchange. Preincubation of lentinan at 0, 6, and 18 h after simultaneous liquid exchange significantly reduced cells degranulation levels (Fig. 7d), suggesting that the regulation of lentinan on cells may be related to biological rhythm.

To investigate the relationship between the rhythm of cells degranulation and circadian clock genes, the changes in circadian clock genes during degranulation were examined. The level of degranulation induced by C48/80 was detected every 6 h after liquid exchange, and Per1, Per2, Cry1, Cry2, and Rev-erbα showed rhythmic changes (Fig. 7e–i), reaching their lowest levels 18 h after liquid exchange. At this time, the level of β-hexosaminidase was the highest, which was consistent with the results in vivo that Per1/2 and Cry1/2 were negatively correlated with allergic severity. Lentinan was preincubated for 6 h to determine whether it could affect cells degranulation by regulating circadian clock genes. The levels of Per and Cry in the preincubation lentinan group were significantly higher than those in the control group 12–24 h after liquid exchange, while Rev-erbα was lower. It is worth noting that when lentinan inhibited cell degranulation, the expression of Per and Cry was enhanced. It was, thus, verified that in cellular level, lentinan can regulate the biological rhythm of RBL-2H3 cells, thereby affecting cells degranulation. These results are basically consistent with those of the in vivo experiments. However, it is necessary to further investigate the signal pathway through which the changes in biological rhythm occur in cells affected by degranulation.

Discussion

Biological rhythm is involved in numerous physiological processes, such as cells growth and regulation of the immune system. Abnormal biological rhythm can cause circadian rhythm disorders in the body, thereby increasing the chances of metabolic and immune system diseases. Previous studies suggest that IgE-mediated immediate-type reactions in the skin show diurnal variations [24]. Seery et al. [25] reported that the magnitude of cutaneous hypersensitivity reactions to allergens in patients with nocturnal asthma exhibits a circadian rhythm. This study established a PCA and a systemic food allergy model induced by OVA, showing that there is a correlation between food allergy and biological rhythm. Per and Cry genes were negatively correlated with Bmal1 and Clock genes. When allergic reactions were severe, the levels of Per1/2 and Cry1/2 were downregulated. In 1976, Miller and Church [26] found anaphylactic sensitivity in the pinna of a mouse to be subject to diurnal variation. In this study, mice were challenged at different time points (ZT), the vascular permeability of ear was the highest at ZT14, and the levels of Per1/2 and Cry1/2 genes reached the lowest within 1 day, consistent with previous reports. After treatment with lentinan, the levels of Per1/2, Cry1/2 were increased, Bmal1, Clock, Rorα, and Rev-erbα were inhibited, and the fluctuation of circadian clock genes in allergic mice was reduced. In addition, Nakamura et al. [24] found that Per2 mutant mice were less sensitive to the inhibitory effects of dexamethasone on the passive cutaneous anaphylactic reactions. Per2 mutation decreased sensitivity to the inhibitory effects of dexamethasone on IgE-mediated degranulation in BMMCs.

In OVA-induced allergy model, the allergic symptoms of mice in ZT2 and ZT14 groups were different, and the levels of circadian clock genes changed, compared with the CK group, indicating that food allergy are related to biological rhythm. In the circadian clock system, the central clock receives innervation from the retina, allowing it to be entrained by solar light/dark cycles. Actually, light signaling increases Per expression in the SCN neurons and induces phase shifts of the circadian rhythms [27]. Injecting or gavaging at ZT14 (8 p.m.) may affect biolog-

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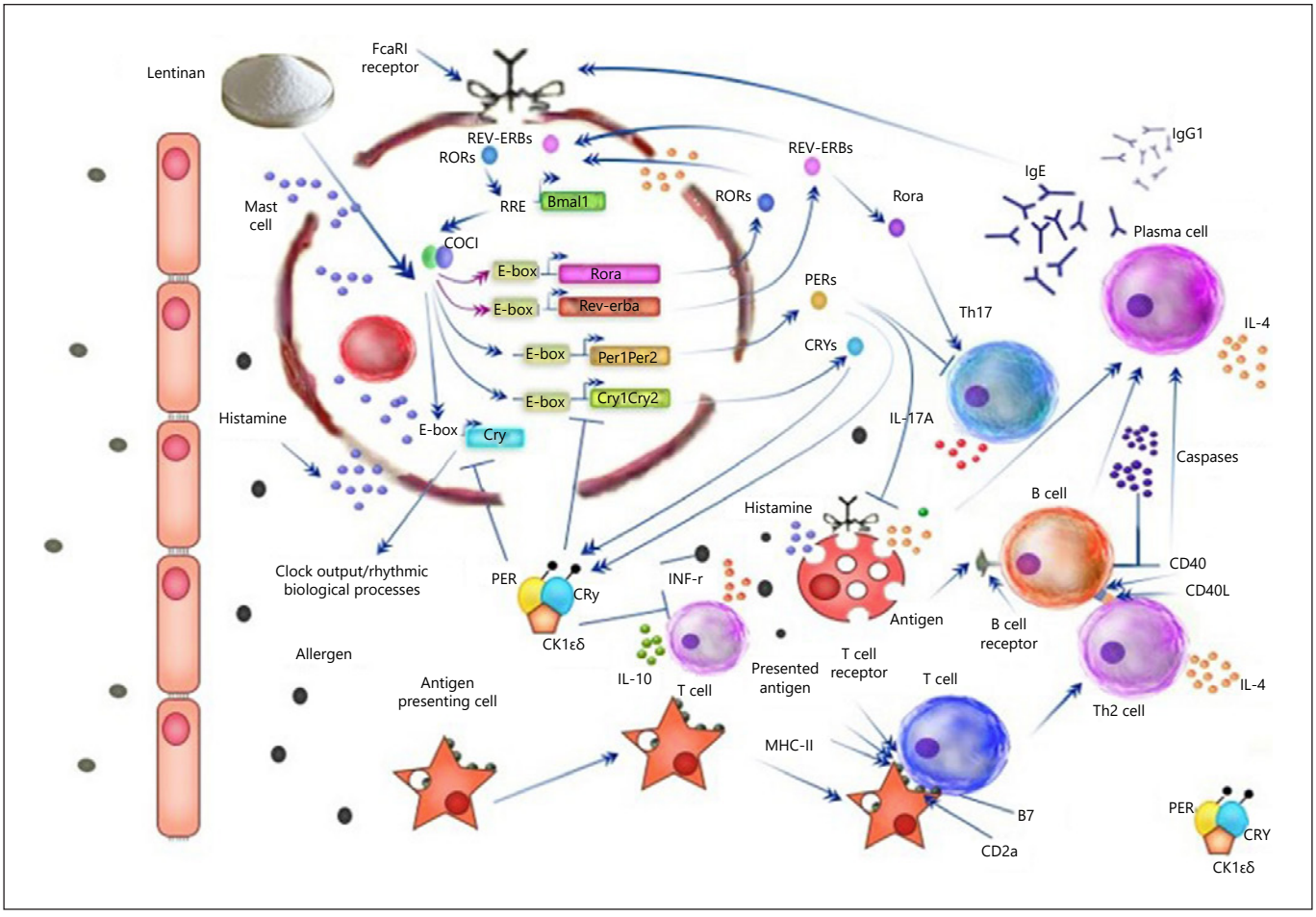

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Fig. 8. Molecular mechanism of lentinan regulating biological rhythm.

ical rhythm of mice due to light. Therefore, mice were treated with lentinan at the ZT2 time point. Lentinan can inhibit the increase of vascular permeability induced by OVA in allergic mice. Previous studies have shown lentinan not only inhibited angiogenesis by suppressing VEGF expression, leading to slow progression of tumors, but also had an intestinal anti-inflammatory through alleviating the degree of epithelial damage. Lin et al. [28] found that lentinan can reduce asthma-like eosinophilic esophagitis-like inflammation and inhibit asthma airway remodeling. However, in this study, lentinan did not significantly change the number of eosinophils. It may be that lentinan does not play a role in eosinophils in food allergy that is mainly gastrointestinal.

In addition, after challenge at ZT2, changes in the levels of circadian clock genes in spleen at ZT20 indicate that the role of lentinan may be time-sensitive or affected by other conditions in body. The specific reasons need to be further studied. In the cellular level, the levels of Per1/2 and Cry1/2 in lentinan the preincubation group were lower than those in the control group, while the Rev-erbα gene was higher. Unlike the regulation of the large environment in vivo, the regulation effects of lentinan on the body were relatively rapid and they took time to adapt to environmental stimulation in the cellular level. When lentinan inhibited cell degranulation, the expression of Per and Cry was enhanced, which is similar with that, Per2 mutation decreased sensitivity to the inhibitory effects of dexamethasone on IgE-mediated degranulation in BMMCs.

This study determined whether lentinan can regulate the body's biological rhythm and alleviate food allergies. In the regulation of biological rhythm, Per and Cry proteins form an inhibitory complex, which enters the nucle-

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us with the help of CKI ε protein (phosphorylation) to inhibit the Clock/Bmal1-mediated activation of Per and Cry. Secretion of Pers into the extracellular region can inhibit the expression of IgE receptor (FcεRI) on the surface of mast cells and inhibit the binding of IgE to its receptor. In addition, Rev-erb and Ror proteins act as co-transcription factors and compete to bind to the Rore site near the Bmal1 gene promoter. Rors can promote the transcription of Bmal1, play an important role in the differentiation of Th17, promote the secretion of IL-17A, enhance the activation of Th2 cells, and stimulate the occurrence of inflammatory reactions, including allergy, while Rev-erb binding inhibits Bmal1 transcription [29, 30]. Treatment with lentinan reduced the fluctuation of clock genes levels in allergic mice and affected the biological rhythm (Fig. 8). Among them, Pers, Crys, and Rev-erbs, as negative regulators of Clock/Bmal1, can regulate the transcription factors and genes related to cell growth cycle, affect the cycle of Th2 cells, inhibit the activation of Th2 cells, and regulate the production of IgE and the secretion of cytokines to alleviate allergic reactions.

The results have shown that food allergies are related to biological rhythm. If lentinan is treated at 2 different time points (ZT2 and ZT14), the effects on allergic reactions in mice are analyzed, demonstrating that lentinan affects allergic reactions through biological rhythm, which can be further improved in subsequent work. The deletion of Cry1 and Cry2 causes the activation of protein kinase A, mediated p65 phosphorylation, induced NF-κB activation and the expression of IL-6 and TNF-α [31]. Rev-erbα inhibits MAPK/ERK and p38 MAPK signaling-mediated inflammatory response downstream of CCL2, regulates the inflammatory infiltration of macrophages [32]. Research has shown lentinan exhibits intestinal anti-inflammatory activity through inhibition of IL-8 mRNA expression associated with the inhibition of NF-κB activation which is triggered by TNFR1 endocytosis and lowering of their expression in IECs [33]. Lentinan effectively attenuated ROS production, iNOS expression, nitric oxide release, and the activation of JNK and p38 MAPK. Moreover, lentinan dose-dependently prevented inhibition of insulin synthesis by blocking the activation of nuclear factor kappa beta in INS-1 cells [17]. Our previous results have shown that quail eggs inhibit PAR2-mediated MAPK and NF-κB activation, inhibit mast cell degranulation, and alleviate symptoms of food allergic EoE-like diseases [22, 23]. Whether lentinan can reduce food allergic symptoms through MAPK and NF-κB signaling pathways will be our future research direction.

In summary, this study suggests that food allergy is related to circadian rhythm. Lentinan can reduce the variation of circadian clock genes, for example, it can regulate the activation of Per and Cry proteins in allergic mice, as well as inhibit the activation of Th2 cells, thereby reducing allergies. Finally, elucidating that lentinan regulates biological rhythm, inhibits Th2 cells activation, and alleviates allergic reactions will provide valuable information toward the establishment of new food allergy therapies and also determine the new functional properties of lentinan.

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Statement of Ethics

All animal experiments were conducted in accordance with the Animal Experimental Welfare and Ethical Guidelines and approved by the Animal Housing Laboratory Experimental Welfare and Ethics Committee of the College of Food Science and Nutritional Engineering, China Agricultural University.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

Shuai Yang, Huifang Chew, and Yuchi Jiang are mainly responsible for animal experiments; Lei Cheng and Xiaoya Guo are mainly responsible for cells experiments; Na Sun is responsible for drawing mechanism diagram; and Huilian Che is responsible for guiding experiments. All were involved in data processing, and Shuai Yang mainly participated in the composing and modification of the manuscript.

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Materials and reagents

Lentinan was purchased from Future Industrial Co., Ltd. (Shanghai, China), with purity >98% (CAS 37339-90-5). Mouse anti-DNP-IgE monoclonal antibody, DNP-HSA, aluminum adjuvant (Alum), RNase AWAY, ovalbumin (OVA), and compound 48/80 (C48/80) were purchased from Sigma (St. Louis, MO, USA). TranZol RNA Extraction Kit (ET101), TransScript One-Step gDNA Removal and cDNA Synthesis SuperMix (AT311), TransStart Top Green qPCR SuperMix (AQ131) were purchased from TransGen Biotech(Beijing, China). A bicinchoninic acid (BCA) protein assay kit was purchased from Beyotime Biotechnology (Shanghai, China). Commercial mouse ELISA kits were obtained from eBioscience (USA). Other reagents and chemicals were of analytical grade.

β-hexosaminidase release assay

RBL-2H3 cells at an initial density of 1× 105 cells/well were seeded into the 96-well plates. After 12 h, cells were pre-incubated with 100 μL lentinan (0, 50, 70, 100 μg/mL, respectively, prepared from MEM basic medium) for 6 h. Afterward, cells were stimulated with 100 μg/mL C48/80 for 1-1.5 h at 37 °C. cells solution was centrifuged at 1,500 rpm for 5 minutes, 30 μL of supernatant was collected and transferred to a 96-well plate and incubated with 50 μL of p-Nitrophenyl- N-Acetyl-β-DGlucosaminide (1.3 mg/mL in 0.1 M citric acid buffer, pH 4.5) for 1 h at 37 °C. The reaction was terminated by adding 200 μL stop solution (0.1 M Na2CO3/NaHCO3, pH 10.0). Each well absorbance was measured at 405 nm using a microplate reader. In addition, add 100 μL C48/80 or 1% Triton X-100（Trix) to the cells not treated with lentinan to determine the release of β-hexosaminidase, and set the release rate of Trix group as 100%.

Real Time PCR

Total RNA was extracted from BALB/c mice spleen/RBL-2H3 cells using TranZol RNA Extraction Kit (TransGen Biotech, China), reverse transcribed to generate cDNA synthesis (TransScript One-Step gDNA Removal and cDNA Synthesis SuperMix, TransGen Biotech, China), and SYBR Green Master Mix kit (TransStart Top Green qPCR SuperMix, TransGen Biotech, China) was used for real time PCR. Quantitative real time PCR was performed using 96 well plates on the Bio-Rad iCycler iQ system (Bio-Rad, USA).

Quantitative real-time PCR was performed under the thermal cycling conditions included denaturation step at 95 °C for 1 minute, followed by 40 cycles at 94 °C for 30 s, 60 °C for 30 s, 72 °C for 30 s, and the final melting curve program with raping rate 0.5 °C/0.05 sec from 55 °C to 95 °C. Using Gapdh as the internal control gene, the relative quantitative level of mRNA was calculated by 2−△△Ct method. Ct was the threshold cycle and △Ct was calculated from test Ct-Gapdh Ct.

Table S1 Primer sequences of genes in spleen tissue of BALB/c mice

genes	Sequences(5'→3')	amplification products(bp)
Clock	Forward: TTCGACAGGACTGGAAACCC	201
	Reverse: GTTCTCCCTCTGGGATAAAA	
Bmal1	Forward: GCAGTGCCACTGACTACCAGTT	207
	Reverse: TGTGCTGAACAGCCATCCTTAG	
Per1	Forward: GGGAGCTCAAACTTCGACTG	437
	Reverse: GAGACCTGAACCTGCAGAGG	
Per2	Forward: GTGAAGCAGGTGAAGGCTAAT	312
	Reverse: AAGCTTGTAAGGGGTGGTGTAG	
Cry1	Forward: TTCGGGGACAGCCAGCTGAT	415
	Reverse: TCTCCTCCTGGCCACACTGCAGA	
Cry2	Forward: GGCAGAAACCACCCCTTA	159
	Reverse: CCAGGGAAGGCACGCCAT	
Rev-erba	Forward: CTTCCGTGACCTTTCTCAAC	235
	Reverse: CAGCTCCTCCTCGGTAAGTG	
Rora	Forward: ACGCCCACCTACAACATCTC	191
	Reverse: TCACATATGGGTTCGGGTTT	
Gapdh	Forward: GTATGACGTGGAGTCTACTG	923
	Reverse: TACTCCTTGGAGGCCATGTA	

Table S2 Primer sequences of genes in RBL-2H3 cells

genes	Sequences(5'→3')	amplification products(bp)
Per1	Forward: TGGTAAAGCACCAGGGACAAC	102
	Reverse: GAAGGACTTTGGCCTTGAATGTAC	
Per2	Forward: CTGGAAAGAACAGGAAACTGAA	205
	Reverse: GGGAACACAGGTAGTGGGTAAG	
Cry1	Forward: CGACGACCATGATGAGAAGT	229
	Reverse: ACAACCGAAGCGGAGATAAG	
Cry2	Forward: ATTGAGCGGATGAAGCAGAT	83
	Reverse: TCTACACAGGAAGGGACAGATG	
Rev-erba	Forward: ACAGCTGACACCACCCAGATC	101
	Reverse: CATGGGCATAGGTGAAGATTTCT	
Gapdh	Forward: GACAACTTTGGCATCGTGGA	133
	Reverse: ATGCAGGGATGATGTTCTGG	