Heliyon 10 (2024) e27471

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon

Research article

Melatonin regulates circadian clock proteins expression in allergic airway inflammation

Si-Nuo Guo [a,b,1], Xu-Qin Jiang [b,c,1], Ning Chen [a,b], Si-Ming Song [a,b], Yu Fang [a,b], Qiu-Meng Xie [b,d], Guang-He Fei [b,d,**], Hui-Mei Wu [a,b,*]

[a] *Anhui Geriatric Institute, Department of Geriatric Respiratory and Critical Care Medicine, The First Affiliated Hospital of Anhui Medical University, Hefei, 230022, China*

[b] *Key Laboratory of Respiratory Disease research and Medical Transformation of Anhui Province, Hefei, 230022, China*

[c] *Department of Pulmonary and Critical Care Medicine, The First Affiliated Hospital of University of Science and Technology of China, Division of Life Sciences and Medicine, University of Science and Technology of China, Hefei, 230001, China*

[d] *Department of Respiratory and Critical Care Medicine, The First Affiliated Hospital of Anhui Medical University, Hefei, 230022, China*

ARTICLE INFO

Keywords:
Melatonin
Circadian clock
Per1
Allergic airway inflammation

ABSTRACT

Asthma demonstrates a strong circadian rhythm with disrupted molecular clock. Melatonin which can directly regulate circadian rhythm has been reported to alleviate asthma, but whether this effect is related to its regulation on circadian clock has not yet been known. Here, female C57BL/6 mice were challenged with ovalbumin (OVA) to establish allergic airway inflammation, and were treated with melatonin or Luzindole to investigate whether the expressions of circadian clock proteins were changed in response to OVA and were affected by exogenous/endogenous melatonin. Airway inflammation, mucus secretion, protein expressions of circadian proteins (Bmal1, Per1, Clock, Timeless, Cry1 and Cry2), melatonin biosynthetase (ASMT, AANAT) and melatonin receptor (Mel-1A/B–R) were analyzed accordingly. The results showed that in the successfully established allergic airway inflammation model, inflammatory cells infiltration, expressions of circadian clock proteins in the lung tissues of OVA-challenged mice were all notably up-regulated as compared to that of the vehicle mice. Meanwhile, the protein expression of ASMT and the level of melatonin in the lung tissues were reduced in allergic mice, while the expression of melatonin receptor Mel-1A/B–R was markedly increased. After addition of exogenous melatonin, the OVA-induced airway inflammation was pronouncedly ameliorated, while simultaneously the OVA-induced expressions of Per1 and Clock were further increased. However, a melatonin receptor antagonist Luzindole further augmented the OVA-induced airway inflammation, accompanied with remarkably decreased expressions of Per1, Bmal1, Cry1 and Cry2 but notably increased expression of Timeless. Collectively, our results demonstrated that the expression of circadian clock proteins was increased in the lungs during allergic airway inflammation, and Per1 was a clock protein that can be regulated by both exogenous and endogenous

* Corresponding author. Anhui Geriatric Institute, Department of Geriatric Respiratory and Critical Care Medicine, The First Affiliated Hospital of Anhui Medical University, Hefei, 230022, China.

** Corresponding author. Department of Respiratory and Critical Care Medicine, The First Affiliated Hospital of Anhui Medical University, Hefei, 230022, China

E-mail addresses: gh.fei@ahmu.edu.cn (G.-H. Fei), wuhm@ahmu.edu.cn (H.-M. Wu).

[1] These authors contribute equally.

https://doi.org/10.1016/j.heliyon.2024.e27471
Received 16 November 2023; Received in revised form 20 February 2024; Accepted 29 February 2024
Available online 8 March 2024

melatonin, suggesting Per1 may be an important potential circadian clock target for melatonin as a negative regulatory factor against Th2-type airway inflammation.

1. Introduction

Bronchial asthma is characterized by chronic airway inflammation, reversible airflow limitation and airway hyperreactivity [1]. Of important is that these symptoms display a circadian rhythm, about 75% of asthmatics are with symptoms frequently worsening at midnight or early morning [2].

Circadian rhythms are endogenous biological oscillations with about 24 h interval [3]. They are formed according to cyclical light: dark phases due to the earth's rotation, by this, live organisms partition their pathways especially those of immune responses to fit to the rhythmic environment [4]. Circadian rhythms are controlled by molecular circadian clock systems which consist of the "central

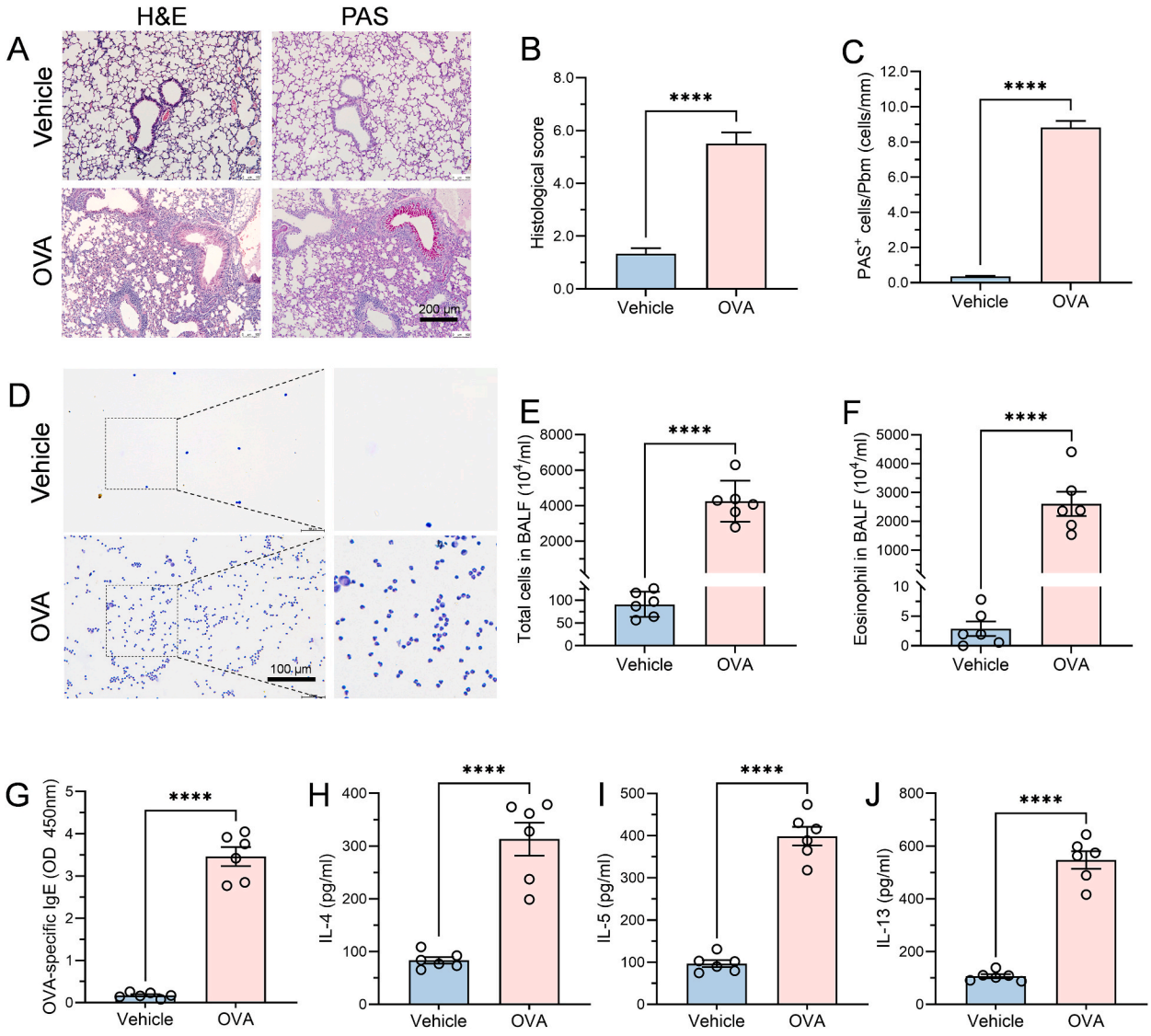

Fig. 1. Successful establishment of allergic airway inflammation induced by OVA. (A) Lung sections from vehicle and OVA groups were stained with H&E to analyze cell infiltration, or periodic acid-Schiff (PAS) to evaluate mucus secretion and goblet cell hyperplasia, scale bar: 200 μm. (B, C) Quantification of lung inflammation and goblet cell hyperplasia. (D) Representative stained BALF cell smears from vehicle and OVA groups, the cells were isolated and stained with Wright-Giemsa, scale bar: 100 μm. (E, F) Total cells and eosinophils count in BALF from vehicle and OVA-challenged mice. (G) ELISA was used to measure the level of OVA-specific IgE in serum. (H–J) The production of Th2 cytokines IL-4, IL-5 and IL-13 in BALF. Data were expressed as mean ± SEM (n = 6 per group). ****$p < 0.0001$.

clock" that resides in the suprachiasmatic nucleus (SCN) of the brain, it regulates the "peripheral clock" in almost all organs including the lung [5]. The core of the cellular circadian clock is a heterodimer composed of Bmal1 and Clock, which regulates the expression of Period (Per) and Cryptochrome (Cry). Per and Cry in turn form an autoregulatory transcriptional-translational feedback loop to regulate Clock/Bmal1 [6,7], interestingly, Timeless can also form heterodimer with Cry or Per and functions as a negative-feedback component of the mammalian clockwork [8]. It has been reported that acute or chronic house dust mite (HDM) exposure results in sex and time-of-day dependent circadian clock disruption [9,10], and dysfunction of circadian clock has been increasingly reported to exert a profound effect on lung inflammation, immunity and glucocorticoid response [11,12], indicating its importance in the pathogenesis of asthma [3,13].

Melatonin is an endogenous hormone produced by the pineal gland. Its level is tightly linked to the light/dark cycle and shows daily/seasonal rhythm [14]. Its production depends on two rate limiting enzymes, arylalkylamine N-acetyltransferase (AANAT) and acetylserotonin O-methyltransferase (ASMT) [15], and change of its level is associated with various diseases. For example, a significantly lower level of melatonin has been found in asthmatic patients [16,17] and asthmatic animal model [18,19]. As a key molecule to regulate the circadian rhythm, melatonin represents an important hormonal mechanism through which "central clock" sends signals to "peripheral clock" to synchronize it [20]. Besides, it can also modulate peripheral oscillators' clock genes and therefore exerts immunomodulatory role [21,22]. Administration of melatonin could significantly alleviate OVA-induced airway inflammation in experimental asthma [19,23,24]. However, whether this effect is related to its regulation on pulmonary circadian clock remains unclear.

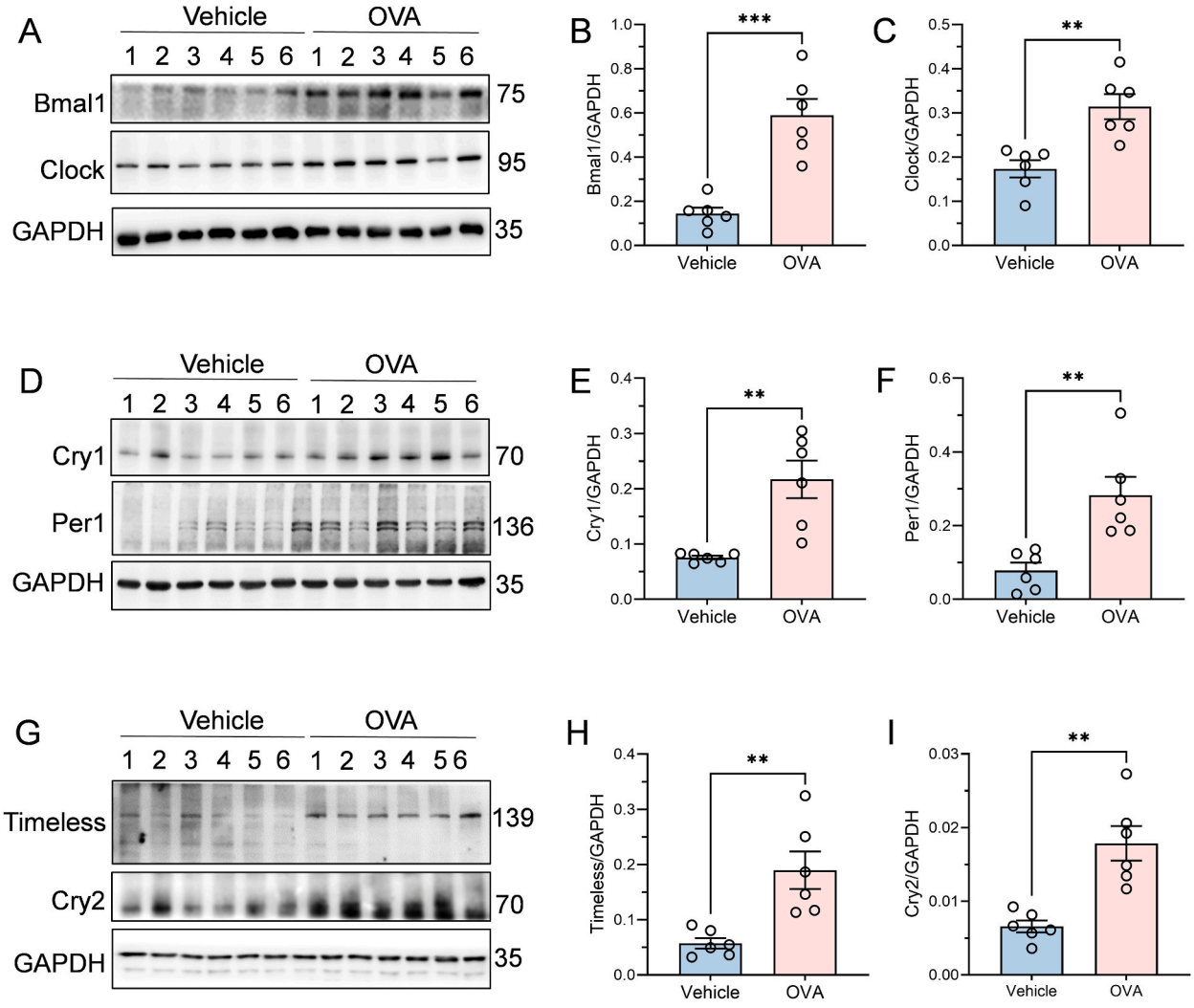

Fig. 2. Increased expression of circadian clock proteins in OVA-induced allergic airway inflammation model. (A, D, G) Representative blots showing the protein level of Bmal1, Clock, Cry1, Per1, Timeless and Cry2 in the lung tissues from vehicle and OVA groups. (B, C, E, F, H, I) Quantification of the expression of Bmal1, Clock, Cry1, Per1, Timeless and Cry2 as referencing to GAPDH in the lung. Cropped blots are shown, and Supplementary file 2: Fig. S1 presents the full-length blots. Data were expressed as mean ± SEM (n = 6 per group). **p < 0.01, ***p < 0.001.

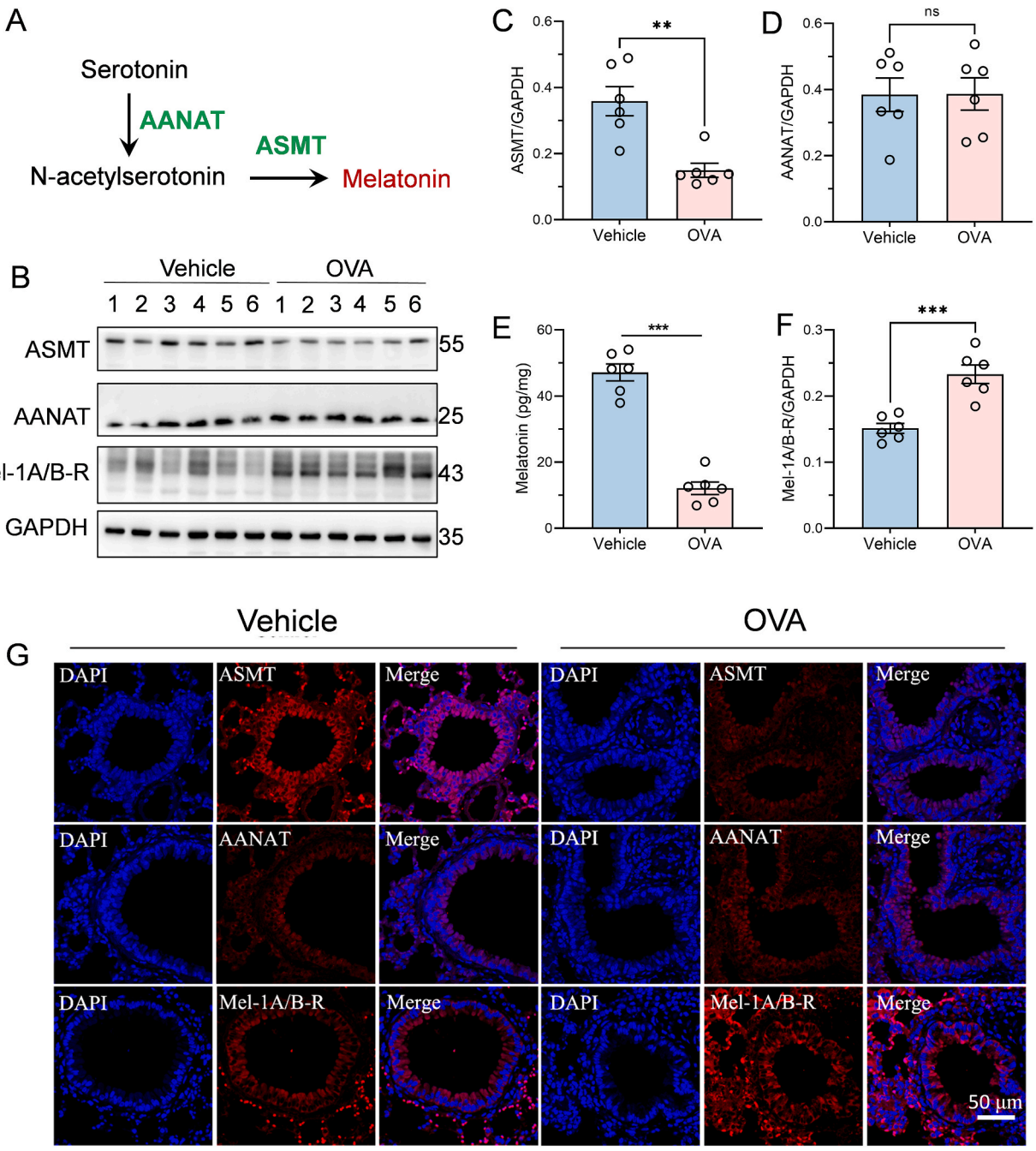

Fig. 3. The biosynthesis of melatonin was decreased in OVA-challenged mice. (A) Scheme showing the biosynthesis of melatonin. (B) The expression of ASMT, AANAT and Mel-1A/B–R in the lung tissues was analyzed by western blotting. Cropped blots are shown, and Supplementary file 2: Fig. S2 presents the full-length blots. (C, D) Quantification of the expression of ASMT and AANAT as referencing to GAPDH. (E) The level of melatonin in the lung tissues was analyzed by ELISA. (F) Quantification of the expression of Mel-1A/B–R as referencing to GAPDH. (G) The confocal microscopy images of ASMT, AANAT and Mel-1A/B–R in the lung tissues. Data were expressed as mean ± SEM (n = 6 per group). **p < 0.01, ***p < 0.001, ns represents no significant difference.

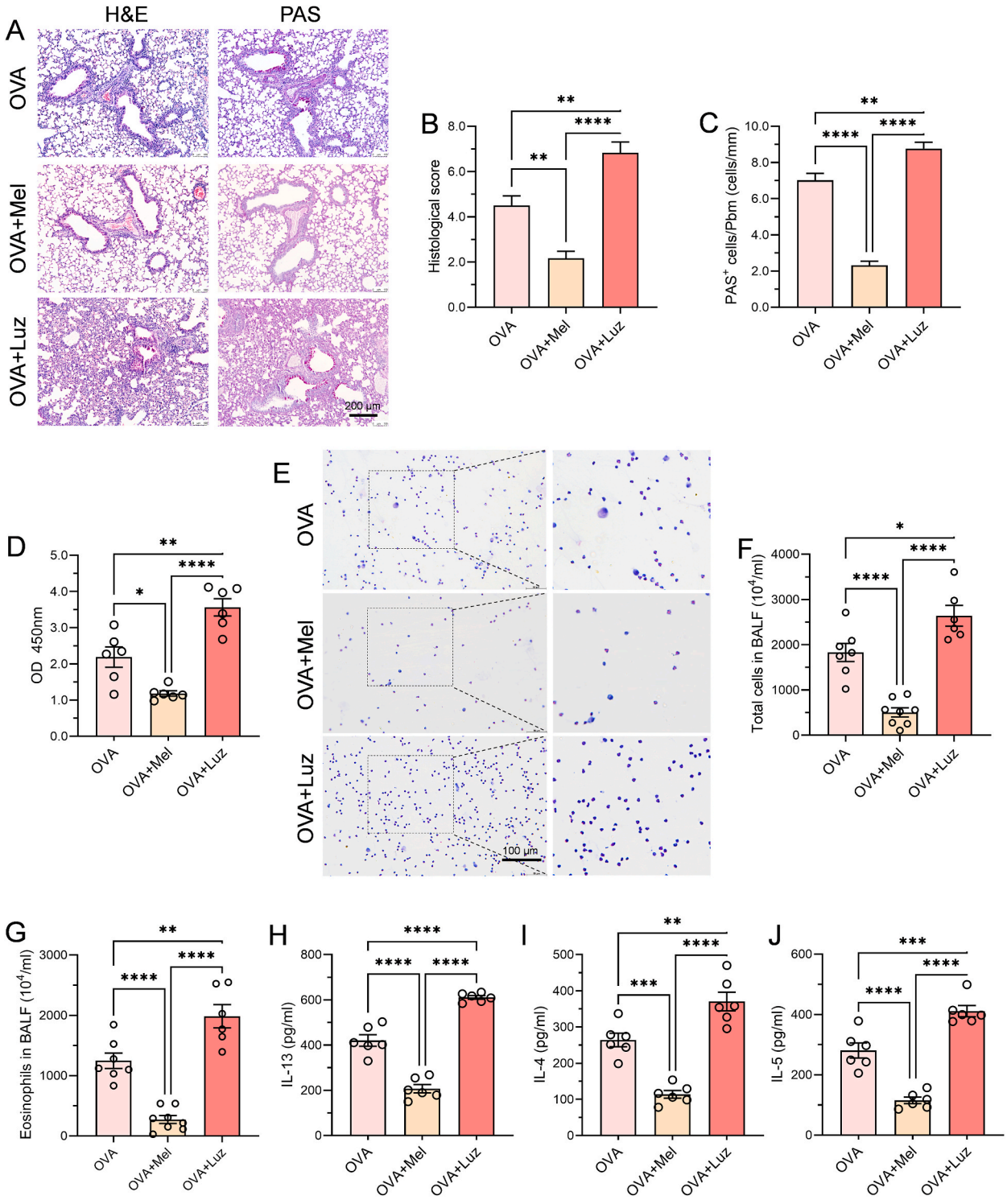

Fig. 4. The effect of melatonin or Luzindole on allergic airway inflammation. (A–C) Airway inflammation, goblet cell hyperplasia in OVA, OVA plus melatonin (Mel) and OVA plus Luzindole (Luz) groups were assessed by H&E, PAS, scale bar: 200 μm. (D) The level of OVA-specific IgE in serum was measured by ELISA. (E) Representative stained BALF cell smears from OVA, OVA plus melatonin and OVA plus Luzindole groups, scale bar: 100 μm. (F, G) Total cells and eosinophils count in BALF of OVA, OVA plus melatonin, and OVA plus Luzindole groups. (H–J) The production of Th2 cytokines IL-4, IL-5 and IL-13 in BALF. Data were expressed as mean ± SEM (n = 6 per group). *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

In the present study, we used ovalbumin (OVA) as an allergen to induce murine allergic airway inflammation and investigated whether the expressions of circadian clock proteins were changed in response to OVA, and were affected by exogenous/endogenous melatonin.

2. Results

2.1. Successful establishment of allergic airway inflammation induced by OVA

SPF C57BL/6 mice were used to establish the allergic airway inflammation model. As compared to that of vehicle group, challenged with 1% OVA for 7 consecutive days induced a large amount of airway inflammatory cells infiltration and goblet cells hyperplasia as analyzed by H&E and PAS staining (Fig. 1A–C). In addition, OVA challenge significantly increased the number of total cells, eosinophils, macrophages, lymphocytes and neutrophils, especially eosinophils in BALF (Fig. 1D–F, supplementary file 1: Fig. S1A). ELISA analysis indicated that the content of OVA-specific IgE (Fig. 1G) and the production of Th2-related cytokines (such as IL-4, IL-5, IL-13) were markedly elevated compared with vehicle group (Fig. 1H–J).

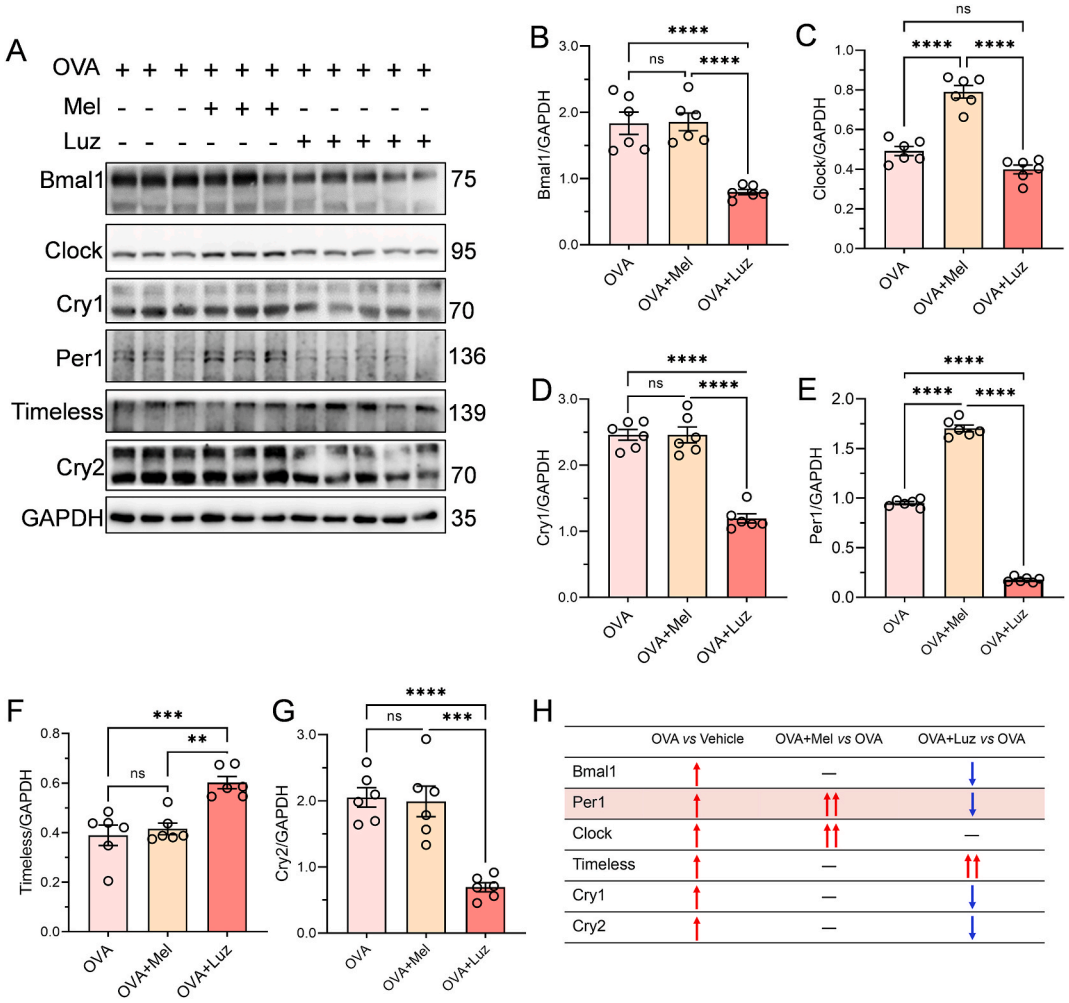

Fig. 5. Melatonin or Luzindole changed the expression of circadian clock proteins. (A) Representative blots showing the protein expressions of Bmal1, Clock, Cry1, Per1, Timeless and Cry2 in the lungs from OVA, OVA plus melatonin and OVA plus Luzindole groups. Cropped blots are shown, and Supplementary file 2: Fig. S3 presents the full-length blots. (B–G) Quantification of the expression of Bmal1, Clock, Cry1, Per1, Timeless and Cry2 as referencing to GAPDH. (H) Summary of the expression trend of circadian clock proteins in the comparison of OVA *vs* vehicle, OVA plus melatonin (Mel) *vs* OVA, OVA plus Luzindole (Luz) *vs* OVA. Data were expressed as mean ± SEM (n = 6 per group). $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

2.2. Increased expression of circadian clock proteins in OVA-induced murine allergic airway inflammation model

To study the changes of peripheral lung clock in OVA-induced allergic airway inflammation mice, western blotting was used for measuring the expression of circadian clock proteins in the lung tissue. In the present study, the expression of Bmal1 (Fig. 2A and B), Clock (Fig. 2A–C), Cry1 (Fig. 2D and E), Per1 (Fig. 2D–F), Timeless (Fig. 2G and H) and Cry2 (Fig. 2G–I) were all significantly increased in response to OVA challenge, suggesting the disruption of the lung circadian clock during allergic airway inflammation.

2.3. Decreased biosynthesis of melatonin and increased expression of melatonin receptor in OVA-challenged mice

Next, we detected the expression of melatonin biosynthesis enzymes (Fig. 3A) in the lung. We found that by western blotting (Fig. 3B–D) and immunofluorescence (Fig. 3G, supplementary file 1: Figs. S2A–B), the level of ASMT was significantly decreased while AANAT was not affected in OVA group as compared to that of vehicle mice. Consistently, the level of endogenous melatonin in lung tissue was markedly decreased in OVA-induced allergic airway inflammation (Fig. 3E). However, the expression of melatonin receptor protein Mel-1A/B–R was significantly increased in OVA group with comparison to that of vehicle mice (Fig. 3B–F, G, supplementary file 1: Fig. S2C).

2.4. Exogenous melatonin ameliorates while Luzindole augments allergic airway inflammation

To directly investigate the effect of melatonin on allergic airway inflammation, exogenous melatonin or endogenous melatonin receptor antagonist Luzindole was applied. Melatonin pretreatment markedly alleviated while Luzindole treatment further augmented OVA-induced lung inflammation (Fig. 4A and B), goblet cell hyperplasia (Fig. 4A–C), IgE level in serum (Fig. 4D), leukocytes recruitment (Fig. 4E–G, supplementary file 1: Fig. S1B) and Th2 cytokines (IL-4, IL-5, IL-13) production in BALF (Fig. 4H–J). Basing on these data, we confirmed that exogenous administration and endogenous melatonin have a profound inhibitory effect on allergic airway inflammation.

2.5. The effect of exogenous melatonin or Luzindole on the expression of circadian clock proteins

Finally, in order to further explore whether the protective effect of melatonin on allergic airway inflammation is related to its regulation of circadian clock, the expression of circadian clock proteins in the lungs was detected by western blotting. Interestingly, OVA-challenged mice treated with melatonin significantly up-regulated the levels of Per1 and Clock compared with the vehicle-treated OVA group (Fig. 5A–C, E), however, melatonin administration did not affect the expressions of Bmal1, Cry1, Cry2 and Timeless. On the other hand, after treatment with Luzindole, the expression of Per1 and other proteins (Bmal1, Cry1 and Cry2) decreased remarkably (Fig. 5A–F) accompanied by an increase of Timeless (Fig. 5A–G) in response to OVA. These data supported the contribution of circadian clock components to the beneficial role of melatonin in allergic airway inflammation, especially Per1 (Fig. 5H).

3. Discussion

In the present study, we demonstrated that OVA challenge induced typical Th2-type airway inflammation, accompanied by decreased biosynthesis of melatonin but increased expression of melatonin receptor and lung circadian clock proteins. Exogenous melatonin pronouncedly ameliorated while endogenous melatonin receptor antagonist Luzindole augmented OVA-induced airway inflammation. Simultaneously, melatonin treatment further increased the OVA-induced expressions of Per1 and Clock without affecting other circadian clock proteins expression, however, Luzindole remarkably decreased the OVA-induced expressions of Per1, Bmal1, Cry1 and Cry2 while further increased the OVA-induced expression of Timeless. These results demonstrated that the expression of circadian clock proteins was increased in allergic lungs and their levels can be regulated by exogenous or endogenous melatonin. Especially, Per1 was a clock protein that can be regulated by both exogenous and endogenous melatonin, that is the OVA-induced expression of Per1 was further up-regulated by exogenous melatonin but down-regulated by Luzindole, suggesting Per1 may be an important potential circadian clock target for melatonin as a negative regulatory factor against Th2-type airway inflammation.

Allergic airway inflammation is dominated by a Th2 response, characterized by eosinophil-rich inflammation, airway hyperresponsiveness, and mucus overproduction [25]. Using OVA sensitization and challenge, we demonstrated that OVA markedly induced lung inflammatory cells infiltrations, goblet cells proliferation and mucus secretion, increased OVA-specific IgE and Th2 cytokines production, indicating the successful establishment of the allergic airway inflammation model. Circadian clock dysfunction is associated with inflammatory responses, and their reciprocal relationship has been documented in the lungs that the circadian oscillations are related to airway hyperresponsiveness, airway smooth muscle tone and inflammation [26,27]. Studies have emphasized the importance of clock genes including Bmal1, Clock, Per1, Per2, Per3, Cry1, Cry2, Timeless and etc in the pathophysiological process of asthma. For example, Bmal1 has been reported to regulate asthma airway phenotypes [28,29], while REV-ERBα/Nr1d1 has been found to gate HDM-induced airway hyperresponsiveness [9,30] probably by regulating the polarization of T helper cells and differentiation [31]. Disruption of clock contributes to epithelial barrier dysfunction and deregulated immune responses to promote asthma [13]. In a clinical study contains 120 asthmatic patients and 60 healthy subjects, the researchers found altered mRNA expression of circadian clock genes in asthmatic patients as compared to healthy volunteers [3], in the not well-controlled asthmatics with night symptoms, the mRNA expression of Bmal1, Per1 and Per2 was significantly increased as compared with those without night symptoms. In the present study, we further confirmed the overall increase of molecular clock proteins including Bmal1, Per1, Clock, Timeless, Cry1 and

Cry2 in the lungs of murine allergic airway inflammation. It has been reported that allergen-induced Th2 cytokines influence the expression and function of clock proteins in lung epithelial cells, which subsequently increases the susceptibility of epithelial cells to allergens [32]. Moreover, knockdown of Bmal1 in macrophages significantly suppresses HDM-induced eosinophilic inflammation [29]. Therefore, the increases of clock proteins in allergic airway inflammation may be occurred in multiple cell types, including epithelial cells and immune cells, and the increase in Th2 cytokines during asthma may be one of the possible mechanisms by which clock targets are altered in the lungs in response to allergen.

As a key hormone secreted by the pineal gland, melatonin, can directly regulate circadian rhythm and rescue the endogenous period of internal biological clocks [33]. Of interest is that melatonin itself is regulated by daylight, and shows daily and seasonal rhythm [14]. Reports from our group and others have confirmed the disordered circadian rhythms of salivary melatonin in asthmatic patients [16,34]. Besides, the serum or salivary level of melatonin in patients with asthma is significantly lower than healthy controls, and its level is negatively correlated with disease symptoms [17]. This concept is confirmed in animal model that melatonin level in the serum, BALF or lung tissues of allergic mice is decreased [18,19]. The present study also confirmed that the expression of key melatonin bio-synthetase ASMT and the melatonin level in the allergic lung tissues was markedly decreased, suggesting that the locally secreted melatonin in the lung is involved in the pathogenesis of asthma. Contrarily, the expression of melatonin receptor (MT1/MT2) was significantly increased in allergic lungs. Melatonin possesses various biological activities such as anti-inflammatory and anti-oxidative stress [35,36], such effect is reported to be mediated by melatonin receptors especially MT2 [37]. Therefore, the elevated expression of melatonin receptor in allergic lungs may be a compensatory increase when melatonin level is low. Indeed, this hypothesis was confirmed by addition of exogenous melatonin, which markedly alleviated OVA-induced leukocytes recruitment, goblet cell hyperplasia, IgE and Th2 cytokines (IL-4, IL-5, IL-13) production in our present and previous studies [19]. Additionally, Luzindole is a non-selective MT1/MT2 receptor antagonist with much higher affinity for MT2 receptor, it reportedly blocks the effect of melatonin [38]. Current study revealed that blocking the effect of endogenous melatonin by Luzindole further augmented OVA-induced allergic airway inflammation. These results demonstrated that both exogenous and endogenous melatonin were capable to suppress allergic airway inflammation.

Next, we further investigate the influence of exogenous and endogenous melatonin on the expression of lung clock proteins. Several studies have shown that melatonin plays an immunomodulatory role by controlling clock genes. In breast cancer, melatonin-induced Bmal1 leads to suppression of immune response by upregulating oxidative phosphorylation (OXPHOS) and inhibiting glycolysis [21]. In patients with Parkinson's disease, melatonin increases Bmal1 level, but it does not affect the level of Per1 [22]. Several mechanisms could explain the regulation of circadian clock by melatonin, such as by binding to the melatonin receptors that causes the decrease of cAMP-PKA-CREB, and subsequently influences the expression of the gene Per, or by joining orphan RORα receptor that positively regulates BMAL1 level [39]. In our present study, exogenous melatonin administration further up-regulated the levels of Per1 and Clock as compared to that of OVA group, while the levels of Bmal1, Timeless, Cry1 and Cry2 were not affected. However, after treatment with Luzindole to block the effect of endogenous melatonin, the expression of Per1 and other proteins (Bmal1, Cry1 and Cry2) decreased remarkably, accompanied by Timeless increased significantly, which fully implicated the differential regulatory effect of exogenous melatonin and endogenous melatonergic pathway on clock proteins. Despite such differential regulation, Per1 was a clock protein that can be regulated by both exogenous and endogenous melatonin. As we know, Per1 mRNA expression can be strongly induced by a glucocorticoid hormone analogue, dexamethasone or β2-adrenoceptor agonist [40] which are both used clinically for asthma control, since Per1 contains both glucocorticoid response element (GRE) and cAMP response element (CRE). Therefore, our results support a contribution of the circadian clock proteins especially Per1 to the negative regulatory effect of melatonin against Th2-type airway inflammation. However, Per1 as an important target for melatonin against Th2-type airway inflammation needs further confirmation using Per1 knockout mice, and the specific molecular mechanisms how melatonin influences clock proteins also warrants further elucidation.

Taken together, the present study revealed that melatonin protected against allergic airway inflammation, and this may be related to its regulatory effect on circadian clock proteins expression especially Per1 in the lung. Pharmacologically targeting the clock components is a potent promising strategy for the treatment of asthma. To the best of our knowledge, this is the first study to investigate the effect of melatonin on pulmonary circadian clock proteins expression in allergic airway inflammation, and suggests that Per1 is an important potential circadian clock target for melatonin as a negative regulatory factor against Th2-type airway inflammation.

4. Materials and methods

4.1. Animals

SPF C57BL/6 mice (Female, 5-week-old, 16–18 g) were purchased from Anhui Laboratory Animal Center (Hefei, China). All animals were housed at a temperature of 20–26 °C, free to food and water, and on a 12 h light-dark alternating time. All experimental protocols were approved by the Animal Care and Use Committee of Anhui Medical University (Permit number: LLSC20221281).

4.2. OVA-induced acute allergic airway inflammation model and treatments

OVA-induced acute allergic airway inflammation model was established as reported in our previous study [41,42]. On days 0 and 7, mice were sensitized with 0.5 mL suspension consisting of 10 μg OVA (Sigma-Aldrich, St. Louis, MO, USA) and 1 mg KAl $(SO4)_2$ (Sangon Biotech, Shanghai, China) in saline intraperitoneally. Between day 14 and 20, mice were challenged by airway inhalation for

30 min with 1% OVA every day, the time of allergen challenge was among 5:00–7:00 p.m. due to mice showed a strong time-of-day response in features of airway inflammation [9,30]. Vehicle mice received inhalation of equivalent saline. Mice were randomly grouped, in OVA plus melatonin group and OVA plus Luzindole group, melatonin (10 mg/kg, Sigma) or Luzindole (30 mg/kg, Sigma) was intraperitoneally injected 1 h before OVA challenge for consecutive 7 days, respectively (supplementary file 1: Fig. S3).

4.3. Bronchoalveolar lavage fluid (BALF) collection and analysis

After 24 h of the last challenge, all the mice were sacrificed among 5:00–8:00 p.m. After exposure of trachea and lung, the right lung was ligated. Bronchoalveolar lavage was performed on the left lung for five times with 0.3 mL cold Phosphate Buffered Saline (PBS; Servicebio, Wuhan, China). The collected BALF was centrifuged at 4 °C, 1000 rpm for 10 min. Cell pellets were resuspended in 200 μL PBS. Total cell counts were performed using a hemocytometer (QIUJING, Shanghai, China) and Wright-Giemsa Stain (Baso, Zhuhai, China) was used for cellular differentiation including eosinophil, macrophage, lymphocyte and neutrophil in a blind manner. The supernatants were stored at −80 °C for Enzyme-linked immunosorbent assay (ELISA).

4.4. Hematoxylin-eosin (H&E) and periodic acid-Schiff (PAS) staining

After BALF was collected, in order to assess the degree of inflammation and mucus secretion around the bronchi, a lobe of the right lung was used for histopathology. Lung was fixed with 4% paraformaldehyde, dehydrated, then embedded in paraffin, and cut into 4 μm sections for HE and PAS staining. Quantification of lung inflammation and goblet cell hyperplasia were accomplished according to our previous study [18].

4.5. Western blotting

Lung tissue was homogenized on ice using Tissuelyser (Tiss-24, Shanghai Jingxin Industrial Development Co., Ltd, Shanghai, China)) with RIPA buffer lysate to extract the total protein. Equal amounts of protein were separated by a 12% SDS-PAGE and then transferred to PVDF membrane. The membranes were blocked in 5% non-fat milk for 1 h. The primary antibodies: Bmal1, Per1, Clock, Timeless, Cry1 and Cry2 (all from abcam, Boston, MA, USA), ASMT (abcam), AANAT (Sigma), Mel-1A/B–R (Santa Cruz Biotechnology, CA, USA), GAPDH (KANGCHEN Biotech, China) were incubated respectively at room temperature for 1 h and 4 °C overnight. The next day, after being washed with TBST thrice and incubated with appropriate HRP-conjugated secondary antibodies (Promega, madison, wi, USA) for 1 h, bands were washed with TBST for thrice again. Then, enhanced chemiluminescence (ECL, Biosharp, Beijing, China) and Chemiluminescence imaging system (Qing Xiang, Shanghai, China) were used to detect the protein immunostaining. The density of protein bands was obtained by using Image J 1.48v software (NIH, Bethesda, MD, USA).

5. Immunofluorescence

Lungs were processed by peroxidase removal and antigen retrieval, as well as 10% goat serum blocking. Then, sections were exposed to ASMT (1:100, abcam), AANAT (1:200, Sigma) and Mel-1A/B–R antibodies (1:100, Santa cruz) overnight at 4 °C. After washing with PBS, they were incubated with Alexa Fluor® 594 conjugated anti-mouse or anti-rabbit fluorescent secondary antibodies (Jackson ImmunoResearch Lab, Inc. West Grove, PA, USA) at 37 °C for 1 h in the dark. Finally, 4,6-diamino-2-phenyl indole (DAPI) staining was performed (Beyotime Biotechnology, China) for 8 min at room temperature. Result was observed under laser scanning confocal microscope (LSM880, Carl Zeiss AG, Oberkochen, GER).

5.1. Enzyme-linked immunosorbent assay (ELISA)

The level of OVA-specific IgE in serum was measured by ELISA kit (Cusabio, Wuhan, China). The levels of IL-4, IL-5 and IL-13 in BALF and the level of melatonin in the lung tissues were assessed by ELISA kits (Cloud-Clone Crop, Wuhan, China) according to the protocols from the manufacturer.

5.2. Statistics

Data was expressed as mean ± SEM (standard error of mean). Statistical comparisons among the groups were performed using a one-way analysis, while independent-sample *t*-test was used between two groups. Statistical significance was set at $p < 0.05$.

Ethical approval

All in vivo experimental protocols followed the guidelines of the Institutional Animal Care and Use Committee and were approved by the Animal Care and Use Committee of Anhui Medical University (Permit number: LLSC20221281).

Funding sources

The work was supported by National Natural Science Foundation of China (81770032), Natural Science Research Project of Anhui

Educational Committee (2022AH051168), Foundation of Anhui Medical University (2022xkj166), Provincial Foundation for Excellent Young Talents of Colleges and Universities of Anhui Province (gxyq2021175), Anhui Province Overseas Training Program for Outstanding Youth in Universities (gxfx2017012).

Data availability statement

Data will be made available on request.

CRediT authorship contribution statement

Si-Nuo Guo: Writing – original draft, Methodology, Investigation. **Xu-Qin Jiang:** Writing – original draft, Validation, Methodology. **Ning Chen:** Validation, Software, Methodology. **Si-Ming Song:** Visualization, Validation, Formal analysis. **Yu Fang:** Project administration, Methodology. **Qiu-Meng Xie:** Validation, Project administration. **Guang-He Fei:** Writing – review & editing, Supervision, Conceptualization. **Hui-Mei Wu:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e27471.

References

[1] S.T. Holgate, Innate and adaptive immune responses in asthma, Nat Med 18 (5) (2012) 673–683.
[2] S.E. Panzer, A.M. Dodge, E.A. Kelly, N.N. Jarjour, Circadian variation of sputum inflammatory cells in mild asthma, J. Allergy Clin. Immunol. 111 (2) (2003) 308–312.
[3] H.C. Chen, Y.C. Chen, T.N. Wang, W.F. Fang, Y.C. Chang, Y.M. Chen, et al., Disrupted expression of circadian clock genes in patients with bronchial asthma, J. Asthma Allergy 14 (2021) 371–380.
[4] F.W. Turek, Circadian clocks: not your grandfather's clock, Science 354 (6315) (2016) 992–993.
[5] A. Giri, Q. Wang, I. Rahman, I.K. Sundar, Circadian molecular clock disruption in chronic pulmonary diseases, Trends Mol. Med. 28 (6) (2022) 513–527.
[6] H. Jacob, A.M. Curtis, C.J. Kearney, Therapeutics on the clock: circadian medicine in the treatment of chronic inflammatory diseases, Biochem. Pharmacol. 182 (2020) 114254.
[7] I.K. Sundar, H. Yao, M.T. Sellix, I. Rahman, Circadian clock-coupled lung cellular and molecular functions in chronic airway diseases, Am. J. Respir. Cell Mol. Biol. 53 (3) (2015) 285–290.
[8] J.W. Barnes, S.A. Tischkau, J.A. Barnes, J.W. Mitchell, P.W. Burgoon, J.R. Hickok, et al., Requirement of mammalian Timeless for circadian rhythmicity, Science 302 (5644) (2003) 439–442.
[9] A. Srinivasan, A. Giri, S.K. Duraisamy, A. Alsup, M. Castro, I.K. Sundar, Acute HDM exposure shows time-of-day and sex-based differences in the severity of lung inflammation and circadian clock disruption, J Allergy Clin Immunol Glob 2 (4) (2023) 100155.
[10] A. Srinivasan, A. Giri, S.K. Duraisamy, A. Alsup, M. Castro, I.K. Sundar, Chronic HDM exposure shows time-of-day and sex-based differences in inflammatory response associated with lung circadian clock disruption, iScience 26 (9) (2023) 107580.
[11] J. Gibbs, L. Ince, L. Matthews, J. Mei, T. Bell, N. Yang, et al., An epithelial circadian clock controls pulmonary inflammation and glucocorticoid action, Nat Med 20 (8) (2014) 919–926.
[12] A.C. Silver, A. Arjona, W.E. Walker, E. Fikrig, The circadian clock controls toll-like receptor 9-mediated innate and adaptive immunity, Immunity 36 (2) (2012) 251–261.
[13] A. Nakao, Circadian regulation of the Biology of allergic disease: clock disruption can promote allergy, Front. Immunol. 11 (2020) 1237.
[14] M.F. Farez, I.D. Mascanfroni, S.P. Mendez-Huergo, A. Yeste, G. Murugaiyan, L.P. Garo, et al., Melatonin contributes to the Seasonality of multiple Sclerosis Relapses, Cell 162 (6) (2015) 1338–1352.
[15] G. Mazzoccoli, I. Kvetnoy, E. Mironova, P. Yablonskiy, E. Sokolovich, J. Krylova, et al., The melatonergic pathway and its interactions in modulating respiratory system disorders, Biomed. Pharmacother. 137 (2021) 111397.
[16] G-h Fei, Alterations in circadian rhythms of melatonin and cortisol in patients with bronchial asthma, Acta Pharmacol. Sin. 25 (5) (2003) 651–656.
[17] S. Habtemariam, M. Daglia, A. Sureda, Z. Selamoglu, M.F. Gulhan, S.M. Nabavi, Melatonin and respiratory diseases: a review, Curr. Top. Med. Chem. 17 (4) (2017) 467–488.
[18] H.M. Wu, Q.Y. Shen, L. Fang, S.H. Zhang, P.T. Shen, Y.J. Liu, et al., JNK-TLR9 signal pathway mediates allergic airway inflammation through suppressing melatonin biosynthesis, J. Pineal Res. 60 (4) (2016) 415–423.
[19] H.M. Wu, C.C. Zhao, Q.M. Xie, J. Xu, G.H. Fei, TLR2-Melatonin feedback loop regulates the activation of NLRP3 inflammasome in murine allergic airway inflammation, Front. Immunol. 11 (2020) 172.
[20] R. Hardeland, D.P. Cardinali, V. Srinivasan, D.W. Spence, G.M. Brown, S.R. Pandi-Perumal, Melatonin–a pleiotropic, orchestrating regulator molecule, Prog Neurobiol 93 (3) (2011) 350–384.
[21] G. Anderson, Daytime orexin and night-time melatonin regulation of mitochondria melatonin: roles in circadian oscillations systemically and centrally in breast cancer symptomatology, Melatonin Research 2 (4) (2019) 1–8.
[22] D.L. Delgado-Lara, G.V. Gonzalez-Enriquez, B.M. Torres-Mendoza, H. Gonzalez-Usigli, J. Cardenas-Bedoya, M.A. Macias-Islas, et al., Effect of melatonin administration on the PER1 and BMAL1 clock genes in patients with Parkinson's disease, Biomed. Pharmacother. 129 (2020) 110485.
[23] I.S. Shin, J.W. Park, N.R. Shin, C.M. Jeon, O.K. Kwon, J.S. Kim, et al., Melatonin reduces airway inflammation in ovalbumin-induced asthma, Immunobiology 219 (12) (2014) 901–908.

[24] I.S. Shin, J.W. Park, N.R. Shin, C.M. Jeon, O.K. Kwon, M.Y. Lee, et al., Melatonin inhibits MUC5AC production via suppression of MAPK signaling in human airway epithelial cells, J. Pineal Res. 56 (4) (2014) 398–407.
[25] M.Y. Lee, C.S. Seo, J.A. Lee, N.H. Lee, J.H. Kim, H. Ha, et al., Anti-asthmatic effects of Angelica dahurica against ovalbumin-induced airway inflammation via upregulation of heme oxygenase-1, Food Chem. Toxicol. 49 (4) (2011) 829–837.
[26] H.J. Durrington, S.N. Farrow, A.S. Loudon, D.W. Ray, The circadian clock and asthma, Thorax 69 (1) (2014) 90–92.
[27] M.H. Smolensky, B. Lemmer, A.E. Reinberg, Chronobiology and chronotherapy of allergic rhinitis and bronchial asthma, Adv. Drug Deliv. Rev. 59 (9–10) (2007) 852–882.
[28] A. Ehlers, W. Xie, E. Agapov, S. Brown, D. Steinberg, R. Tidwell, et al., BMAL1 links the circadian clock to viral airway pathology and asthma phenotypes, Mucosal Immunol. 11 (1) (2018) 97–111.
[29] H. Hong, J. Zhang, X. Cao, Y. Wu, T.F. Chan, X.Y. Tian, Myeloid Bmal1 deletion suppresses the house dust mite-induced chronic lung allergy, J. Leukoc. Biol. 115 (1) (2024) 164–176.
[30] H.J. Durrington, K. Krakowiak, P. Meijer, N. Begley, R. Maidstone, L. Goosey, et al., Circadian asthma airway responses are gated by REV-ERBalpha, Eur. Respir. J. 56 (6) (2020).
[31] D. Tiwari, N. Ahuja, S. Kumar, R. Kalra, R. Nanduri, S. Gupta, et al., Nuclear receptor Nr1d1 alleviates asthma by abating GATA3 gene expression and Th2 cell differentiation, Cell. Mol. Life Sci. 79 (6) (2022) 308.
[32] S.K. Duraisamy, A. Srinivasan, I.K. Sundar, House dust mite and Th2 cytokine-mediated epithelial barrier dysfunction attenuation by KL001 in 16-HBE cells, Tissue Barriers (2023) 2203841.
[33] J.B. Zawilska, D.J. Skene, J. Arendt, Physiology and pharmacology of melatonin in relation to biological rhythms, Pharmacol. Rep. 61 (3) (2009) 383–410.
[34] B. Kos-Kudla, Z. Ostrowska, B. Marek, N. Ciesielska-Kopacz, D. Kajdaniuk, M. Kudla, et al., Diurnal rhythm of serum melatonin, ACTH and cortisol in asthma patients with long term glucocorticoid treatment, Endocr. Regul. 31 (1) (1997) 47–54.
[35] J. Wang, H. Hao, L. Yao, X. Zhang, S. Zhao, E.A. Ling, et al., Melatonin suppresses migration and invasion via inhibition of oxidative stress pathway in glioma cells, J. Pineal Res. 53 (2) (2012) 180–187.
[36] J. Wang, C. Jiang, K. Zhang, X. Lan, X. Chen, W. Zang, et al., Melatonin receptor activation provides cerebral protection after traumatic brain injury by mitigating oxidative stress and inflammation via the Nrf2 signaling pathway, Free Radic. Biol. Med. 131 (2019) 345–355.
[37] M.L. Dubocovich, M. Markowska, Functional MT1 and MT2 melatonin receptors in mammals, Endocrine 27 (2) (2005) 101–110.
[38] M.L. Dubocovich, Luzindole (N-0774): a novel melatonin receptor antagonist, J Pharmacol Exp Ther 246 (3) (1988) 902–910.
[39] F.G.D. Amaral, J. Cipolla-Neto, A brief review about melatonin, a pineal hormone, Arch Endocrinol Metab 62 (4) (2018) 472–479.
[40] N. Burioka, Y. Fukuoka, M. Takata, M. Endo, M. Miyata, H. Chikumi, et al., Circadian rhythms in the CNS and peripheral clock disorders: function of clock genes: influence of medication for bronchial asthma on circadian gene, J. Pharmacol. Sci. 103 (2) (2007) 144–149.
[41] J.F. Sha, Q.M. Xie, N. Chen, S.M. Song, Y. Ruan, C.C. Zhao, et al., TLR2-hif1alpha-mediated glycolysis contributes to pyroptosis and oxidative stress in allergic airway inflammation, Free Radic. Biol. Med. 200 (2023) 102–116.
[42] H.M. Wu, L. Fang, Q.Y. Shen, R.Y. Liu, SP600125 promotes resolution of allergic airway inflammation via TLR9 in an OVA-induced murine acute asthma model, Mol. Immunol. 67 (2 Pt B) (2015) 311–316.