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Circadian clock modulates epithelial cell autophagy to alleviate airway inflammation in asthma

Lei Zhang[a,b], **Zhou Jiang**[a,*], **Hanmin Liu**[a,c,d,*], **ShuTing Cheng**[a], **Xing Zuo**[a]

[a] NHC Key Laboratory of Chronobiology (Sichuan University), Chengdu 610041 China
[b] Department of Pediatric Chengdu Women's and Children's Central Hospital, School of Medicine, University of Electronic Science and Technology of China, Chengdu, Sichuan 610017China
[c] Department of Pediatric Pulmonology and Immunology, West China Second University Hospital, Sichuan University, Chengdu 610041 China
[d] Key Laboratory of Birth Defects and Related Diseases of Women and Children (Sichuan University), West China Second University Hospital, Ministry of Education, Sichuan 610041 China

*Corresponding authors, e-mail: Jiang-yuqian@126.com, liuhm@scu.edu.cn

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ABSTRACT: The circadian regulation of autophagy in airway epithelial inflammation remains poorly understood, particularly in the context of asthma. This study investigated how nuclear receptor subfamily 1 group D member 1 (NR1D1)-mediated downregulation of myosin-like BCL2-interacting protein (BECLIN1) influences autophagy in airway epithelial cells to alleviate inflammation. An ovalbumin-induced asthma mouse model and a Beas-2b cell line with type 2 inflammation were established. Rhythmic fluctuations in circadian clock genes (*Circadian locomotor output cycles Kaput* [*Clock*], *Brain and muscle Arnt-like protein 1* [*Bmal1*], and *Nr1d1*) and autophagy-related genes (*Autophagy Related 5* [*Atg5*], *Beclin1*, and *microtubule-associated proteins 1A/1B light chain 3B* [*Lc3*]) were monitored. *In vitro*, NR1D1 expression was modulated using SR9009 or siRNA, and effects on BECLIN1 and LC3 were assessed. BECLIN1 expression was altered via plasmid transfection to evaluate effects on NR1D1. NR1D1 bonded with and repressed the BECLIN1 promoter. In asthmatic mice and Beas-2b cells, NR1D1 expression was reduced, whereas ATG5, BECLIN1, and LC3 were elevated. CLOCK, BMAL1, NR1D1, ATG5, BECLIN1, and LC3 levels in the lung tissue showed cyclic fluctuations, with NR1D1 and BECLIN1 negatively correlated. SR9009 reduced BECLIN1 and LC3 levels, while NR1D1 knockdown increased them *in vitro*. BECLIN1 overexpression elevated NR1D1, whereas its inhibition reduced NR1D1. Circadian and autophagy-related genes showed pronounced periodic oscillation in asthmatic mice, coinciding with airway inflammation. NR1D1 negatively regulates BECLIN1 to suppress autophagy and mitigate airway inflammation in asthma, indicating a possible therapeutic target.

KEYWORDS: circadian clock, autophagy, asthma, airway epithelial cells, NR1D1, BECLIN1

INTRODUCTION

Asthma is one of the most common chronic respiratory diseases worldwide, the prevalence rates range from 3.9% to 10.5% in Asian countries, including China [1]. However, the asthma control rate remains inadequate [2]. Approximately 420,000 fatalities are attributed to the disease [3]. Airway inflammation pervades the entire trajectory of acute and chronic asthma, and focused strategies have been devised to tackle the inflammatory response pertinent to this disorder. However, the clinical therapeutic efficacy of these strategies remains unsatisfactory for some patients [4]. Airway structural cells, including epithelial cells, are important. Recent studies have demonstrated the critical immunoregulatory function of passive responders to asthma [5]. Empirical studies have demonstrated that interleukin (IL)-13 can induce inflammatory responses in airway epithelial cells and promote mucus production in the airways [6]. Impaired airway epithelium discharges a greater amount of pro-inflammatory mediators such as IL-25 and IL-33, thereby intensifying the development of asthma [7]. Elucidating these mechanisms may facilitate the mitigation of airway inflammation through targeted therapeutic interventions aimed at the airway epithelium [8].

In mammals, circadian rhythms synchronize diverse behaviors and physiological functions with the 24-h light-dark cycle to support environmental adaptation and maintain homeostasis [9, 10]. Patients with chronic inflammatory pulmonary diseases, such as asthma and chronic obstructive pulmonary disease, experience increased airway resistance at night or in the early morning [11]. However, the temporal dynamics of airway inflammation in patients with asthma have garnered limited interest. Therefore, a detailed investigation of this aspect may be crucial for addressing the bottlenecks in asthma treatment. The components of the circadian clock interact with key elements of the epithelial barrier function and immune response, thereby consistently regulating these biological processes over a 24-h cycle [12]. Current scholarly endeavors suggest that a multiplicity of circadian rhythm genes, such as *Clock*, *Bmal1*, and *Nr1d1*, may be implicated in the governance of immune cell circadian rhythms in the realm of respiratory allergic disorders

[13]. Nuclear receptor subfamily 1 group D member 1 (NR1D1) modulates the circadian rhythm associated with asthma and influences muscarinic receptors [14]. These findings emphasize the importance of circadian rhythm regulation in time-based therapies for asthma.

Autophagy plays an important role in the development of allergies and asthma [15]. Autophagy-related microtubule-associated protein 1 light chain 3 (Map1lc3b, LC3), autophagy-related protein 5 (ATG5), and BECLIN1 are involved in autophagosome formation and are associated with allergic inflammation [16]. Moreover, they regulate airway inflammation in asthma through the mediation of epithelial autophagy [17]. Autophagy is influenced by circadian rhythms, and autophagy genes (*Lc3* and *Atg4*) show rhythmic expression in the liver of zebrafish after fasting [18]. The circadian clock modulates the rhythmic expression of ATG5 through NR1D1-mediated mechanisms and inhibits autophagy in granulosa cells [19]. Although the intricate relationship between the circadian system and autophagy is being increasingly acknowledged, the rhythmic role of autophagy in mediating airway inflammation within the airway epithelium remains unclear. Furthermore, the potential mutual regulation between the circadian system and autophagy in the asthma-related airway epithelium has yet to be thoroughly investigated. This study aimed to elucidate the biological mechanisms by which circadian genes relieve airway inflammation in asthma through autophagy, thereby providing a theoretical foundation for the development of chronobiological therapy of asthma and new therapeutic targets.

MATERIALS AND METHODS

Induction of asthma in mice via ovalbumin (OVA)

The Sichuan University Ethics Committee (Approved Number: KS2023570, China) gave its approval to all experimental methods.

The mice were randomly allocated into two groups, the OVA group and the control group, with each group comprising six mice. A solution of OVA was prepared at a concentration of 0.8 mg/ml by dilution in physiological saline, and aluminum hydroxide gel was used as an immune adjuvant to concentrate the OVA sensitizer. Each mouse was administered an intraperitoneal injection of 80 µg (0.2 ml) OVA for sensitization on days 0, 7, and 14. Subsequently, a 5% OVA aerosol treatment was dispensed over four consecutive days, commencing on days 18, 20, 22, and 24. The control group received an equivalent volume of physiological saline instead of OVA at the same time points using the same methods. Both groups of mice were euthanized within 24 h of the final aerosol treatment, and blood, bronchoalveolar lavage fluid (BALF), and lung tissue samples were collected.

Monitoring of circadian rhythms in mice with OVA-induced asthma and wild-type (WT) mice

Artificial lighting was programmed to follow a 12-h light/12-h dark cycle, with light from 8 am to 8 pm and darkness from 8 pm to 8 am. The timing factor, known as zeitgeber time (ZT), was defined such that ZT0 corresponds to the activation of light in the control box, and ZT12 indicates its deactivation. At each time point, six mice were allocated to each group, and lung tissue samples were collected at 4-h intervals.

Establishment of a Beas-2b cell-based type 2 (Th2) inflammatory model

A type 2 (Th2) inflammatory response triggered by IL-13 was elicited as per the process outlined in a previous study [20]. After 24 h of culturing airway human epithelial cells (Beas-2b) in six-well plates with serum-containing medium, the old medium was removed, and 2 µl of IL-13 diluted to 10 ng/µl was added to each well to obtain a final concentration of 10 ng/ml. The cells were then incubated in a constant-temperature incubator for another 48 h and collected for subsequent tests.

Establishment of a 24-h circadian rhythm model using Beas-2b cells

The pre-prepared horse serum culture medium was introduced into Beas-2b cells, and this time point was designated as ZT0. Subsequently, cells were collected every 4 h starting from ZT4 (4 h after the addition of the culture medium), and RNA and proteins were extracted for further analysis.

Enhancement of the expression of NR1D1 by SR9009 in Beas-2b cells

In a six-well plate containing Beas-2b cells, three adjacent wells were designated in the same column as the control group (NC, Negative Control), whereas the remaining three wells served as the SR9009 treatment group. Following confirmation of cell adhesion to the plate, an appropriate volume of SR9009 was added to achieve a final concentration of 20 µmol/l in the SR9009 treatment group. The control group received an equivalent volume of PBS buffer.

NR1D1 siRNA transfection

In a six-well plate of Beas-2b cells, three wells were set as the NC group and the other three as the siRNA group. The transfection complex formulated by combining 10 µl of mM siRNA stock solution, 120 µl of riboFECT™ CP Buffer, and 12 µl of riboFECT™ CP Reagent was synthesized by RIBOBIO (Guangzhou, China). Finally, the complex was added to the complete medium without double antibodies and mixed. The medium in the six-well plate was aspirated and discarded, new medium with the complex was added,

and the plate was incubated for 72 h before collecting the cell samples.

sh+*BECLIN1* and OE+*BECLIN1* plasmid transfection

In a six-well plate of Beas-2b cells, three wells were set as the NC group and the other three as the plasmid group. Cell density was allowed to reach 30%–50% during transfection. The transfection complex was formulated by combining 4.8 µl of the plasmid storage solution synthesized by Shanghai GeneChen Co., Ltd. (Shanghai, China), 200 µl of jetPRIME Buffer, and 4 µl of jetPRIME Reagent (Polyplus-Transfection, France). Finally, the complex was added to the complete medium without double antibodies and mixed. The medium in the six-well plate was aspirated and discarded, new medium with the complex was added, and the plate was incubated for 72 h before collecting the cell samples.

RNA Extraction and qPCR

Mouse lung tissue samples and human bronchial epithelial Beas-2b cells were collected at the indicated time points. Total RNA was extracted and purified from tissues and cells using the Fast Pure Cell/Tissue Total RNA Isolation KitV2 (Vazyme) (Nanjing, China), according to the manufacturer's protocol. cDNA was generated using a HiScript III RT Reagent Kit (Vazyme). The qPCR reactions were carried out in a 20 µl reaction solution consisting of 10 ng cDNA with specific primers (200 nM) using the AceQ qPCR SYBR Green Master Mixa (Vazyme) and a CFX96 qRT-PCR system (LightCycler 96, USA). All primers are shown in Table S1.

Protein extraction and Western blot (WB)

Mouse lung tissue samples and Beas-2b cells were collected at the indicated time points. Following the manufacturer's protocol, RIPA Lysis Buffer (Beyotime, Shanghai, China) was added to lyse mouse lung tissue and Beas-2b cells. Lysates were centrifuged at 12,000 × *g* for 10 min at 4 °C, and the supernatant was aspirated. Subsequently, total proteins were extracted after mixing with 5 loading buffer and boiling for 10 min at 100 °C. The BCA method (Kagan Biotechnology, Nanjing, China) was used to detect the total protein concentrations. WB analysis was performed to assess the expression levels of relevant proteins.

ELISA

ELISA was conducted to analyze BALF and serum samples from mice following the guidelines provided by the manufacturer (quantification of IL-4, IL-13, and LC3 [MEIMIAN, Jiangsu, China]).

Dual-luciferase reporter assay system

Through bioinformatics analysis, the promoter regions of Beclin1 and Atg5, as well as their binding sites for transcription factors, were identified. HEK293T cells plated in 12-well plates in different groups separately were co-transfected with the relevant plasmids, including pGL4.20-basic and pRL-CMV, using 9 µl of FuGENE 6 Transfection Reagent (FuGENE, USA) as indicated. Cells were collected at 30 h post-transfection, luciferase activity was measured, and the Firefly-Luc/Renilla-Luc ratio was recorded. The experimental groups are provided in Table S2.

Statistical analyses

Data are expressed as the mean ± standard error of at least three independent experiments, each conducted in triplicate. Statistical analyses were performed using one-way ANOVA followed by appropriate post-hoc tests, or two-way ANOVA with subsequent post hoc analyses, utilizing t-tests with GraphPad Prism v. 7.0 (GraphPad, San Diego, CA, USA). The circadian rhythmicity of gene expression was determined using the single Cosinor method with the Time Series Single 6.3 (Expert Soft Tech., Richelieu, France). Statistical significance was set at $p < 0.05$.

RESULTS

Differential expression of circadian clock and autophagy genes in the lung tissues of WT versus asthmatic mice

In the HE staining of mouse lung tissue, compared to WT mice, asthmatic mice exhibited thicker airway walls, increased inflammatory cell infiltration around the airways, and a significantly higher airway inflammation score ($p < 0.001$). Immunohistochemical analysis showed no significant difference in the expression of the epithelial cell marker uteroglobin (UG) ($p > 0.05$) but a significant increase in the expression of the autophagy marker LC3 in airway epithelial cells ($p = 0.042$) (Fig. 1A). Electron microscopy revealed a significantly greater number of autophagosomes in the airways of asthmatic mice than in those of WT mice ($p < 0.001$) (Fig. 1B). Compared with WT mice, asthmatic mice showed significantly elevated levels of IL-4 ($p < 0.001$), IL-13 ($p = 0.005$), and LC3 ($p = 0.003$) in bronchoalveolar lavage fluid (BALF), whereas no significant differences were observed in the serum levels of these markers between asthmatic and WT mice ($p > 0.05$) (Fig. 1C).

Compared to WT mice, the mRNA expression levels of *Bmal1* ($p = 0.006$) and *Nr1d1* ($p < 0.001$) were significantly reduced in asthmatic mice, whereas no significant difference was observed in the mRNA expression level of *Clock* ($p > 0.05$). Conversely, the mRNA expression levels of *Atg5*, *Beclin1*, and *Lc3* were significantly elevated in asthmatic mice ($p = 0.002$, $p = 0.016$, and $p < 0.001$, respectively). Compared to WT mice, no significant difference was noted in the protein expression levels of BMAL1 and CLOCK in asthmatic mice ($p > 0.05$). However, the protein expression level of NR1D1 was significantly reduced

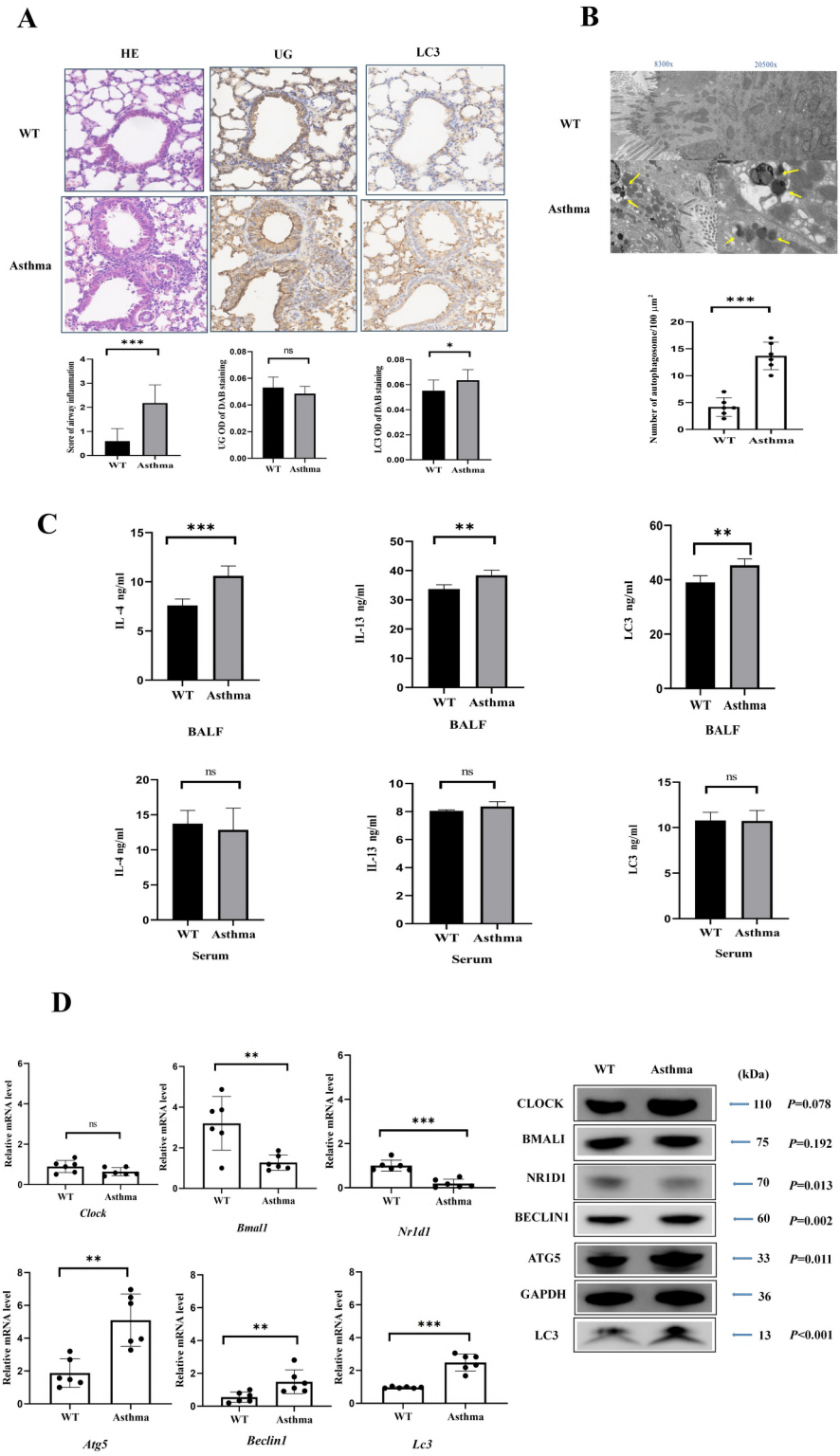

Fig. 1 Expression of circadian rhythm and autophagy genes in the lung tissues of mice. (A) In the lung tissue of asthmatic mice stained with HE (× 20), a marked increase in inflammatory cell infiltration in the airways was noted. UG immunohistochemistry confirmed the presence of airway epithelial cells, whereas LC3 immunohistochemistry demonstrated elevated expression of LC3 in the airway epithelial cells of asthmatic mice. (B) Electron microscopy at various magnifications revealed the presence of autophagosomes in the airway epithelial cells of both WT mice and asthmatic mice. (C) The expression of IL-4, IL-13 and LC3 in BALF and serum of mice. (D) The expression of circadian clock and autophagy genes in the lung tissues of mice. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

($p < 0.05$). Additionally, the protein expression levels of ATG5, BECLIN1, and LC3 were significantly elevated ($p < 0.05$) (Fig. 1D).

Differential expression of circadian rhythm and autophagy genes in Beas-2b and Beas-2b (Th2) cells

Following IL-13 stimulation, no significant differences were observed in the mRNA or protein expression levels of *CLOCK* in Beas-2b cells ($p > 0.05$). In contrast, both mRNA and protein levels of NR1D1 exhibited a statistically significant decrease in Beas-2b (Th2) cells with p values of 0.029 and 0.001, respectively. The *BMAL1* mRNA level was significantly elevated in Beas-2b (Th2) cells ($p = 0.042$), whereas BMAL1 protein levels were not significantly different ($p > 0.05$). The mRNA levels of *ATG5*, *BECLIN1*, and *LC3* were all found to be increased in Beas-2b (Th2) cells. However, changes in *ATG5* mRNA levels did not reach statistical significance ($p > 0.05$), whereas *BECLIN1* and *LC3* mRNA levels demonstrated statistically significant increases in Beas-2b (Th2) cells, with p-values of 0.014 and 0.034, respectively. Similarly, the protein expression levels of ATG5, BECLIN1, and LC3 also showed an upward trend in Beas-2b (Th2) cells; nonetheless, no statistically significant difference was noted in LC3 protein expression ($p > 0.05$), whereas those of ATG5 and BECLIN1 showed statistically significant differences ($p = 0.032$ and $p = 0.012$, respectively (Fig. 2).

Analysis of rhythmic fluctuations in circadian rhythm and autophagy genes in WT and asthmatic mice over 24 h

The expression levels of CLOCK, BMAL1, and NR1D1 proteins in the lung tissues of WT and asthmatic mice (asthma) showed rhythmic fluctuations with statistically significant differences ($p < 0.05$). In the lung tissue of WT mice, a trend of gradually increasing CLOCK protein expression was observed, with the lowest expression at ZT4 and the highest at ZT16. BMAL1 expression initially decreased and subsequently increased, with the lowest expression at ZT8. The expression levels of NR1D1 protein likewise exhibited a trend of an initial decrease and then an increase (Fig. 3A). In comparison with WT mice, the fluctuation range of CLOCK, BMAL1, and NR1D1 in the lung tissues of asthmatic mice was larger. Peak CLOCK protein expression was noted at ZT8. The expression levels of BMAL1 protein reached a maximum at ZT8 and ZT12. The expression levels of NR1D1 protein were highest at ZT4 and lowest at ZT16 (Fig. 3B).

The expression levels of ATG5, BECLIN1, and LC3 proteins in the lung tissue of WT and asthmatic (asthma) mice showed rhythmic fluctuations with statistically significant differences ($p < 0.05$). The peak expression level of the ATG5 protein in the lung tissue of WT mice occurred at ZT8 and ZT12. The lowest expression level of ATG5 protein in the lung tissue of asthmatic mice was noted at ZT8, and the peak expression level was recorded at ZT16. The expression of LC3 protein in the lung tissues of both groups of mice was consistent, manifesting a gradual increase over time, and was more pronounced in asthmatic mice. In the lung tissues of both groups of mice, the trends in the expression of the BECLIN1 protein were consistent, with the lowest expression occurring at ZT4 and the highest at ZT12. Nevertheless, the fluctuation amplitude of BECLIN1 protein was greater in asthmatic mice. The expressions of NR1D1 and BECLIN1 proteins exhibited a tendency toward negative fluctuation.

Analysis of the rhythmic fluctuations of circadian rhythm genes and autophagy genes in Beas-2b over 24 h

Apart from CLOCK protein, the levels of BMAL1, NR1D1, ATG5, LC3, and BECLIN1 showed rhythmic fluctuations in Beas-2b cells, with statistically significant differences ($p < 0.05$). The expressions of NR1D1 and BECLIN1 proteins exhibited a tendency toward negative fluctuation (Fig. 4).

Analysis of the expression of IL-13, IL-4, and LC3 in mouse airway lavage fluid over 24 h

The expression levels of LC3 in the BALF of WT and asthmatic mice varied at different time points ($p < 0.05$). The expression of LC3 in the BALF of asthmatic mice gradually increased from ZT0 to ZT20. Differences were observed in the expression of IL-4 and IL-13 in the BALF at different time points in both groups ($p < 0.05$). At ZT8 and ZT12, the expression levels of IL-4 and IL-13 in the BALF of asthmatic mice were significantly higher than those in the BALF of WT mice (Fig. 5).

Variations in BECLIN1 and LC3 in Beas-2b cells following upregulation of *NR1D1* by SR9009 treatment and the downregulation of *NR1D1* by siRNA transfection

The treatment of Beas-2b cells with the agonist SR9009 (20 μm/l) for 2 h led to an increase in *NR1D1* mRNA expression, which subsequently decreased gradually over time ($p < 0.001$). The expression of *BECLIN1* and *LC3* mRNA reached their lowest levels 8 h after SR9009 administration and increased after 12 h ($p < 0.001$) (Fig. 6A). A total of 2 h after the addition of SR9009, the expression of NR1D1 protein increased and subsequently decreased gradually over time ($p < 0.001$). The expression of BECLIN1 protein showed a gradual decrease and reached its lowest point 12 h after the addition of SR9009 ($p < 0.001$). Following the administration of SR9009 for 9 h, the expression of the LC3 protein initially declined and subsequently increased ($p < 0.001$) (Fig. 6B). Following siRNA transfection aimed at the reduction of NR1D1 in Beas-2bB cells, the expression of BECLIN1 and LC3 proteins decreased ($p < 0.001$) (Fig. 6D). Nonetheless, the

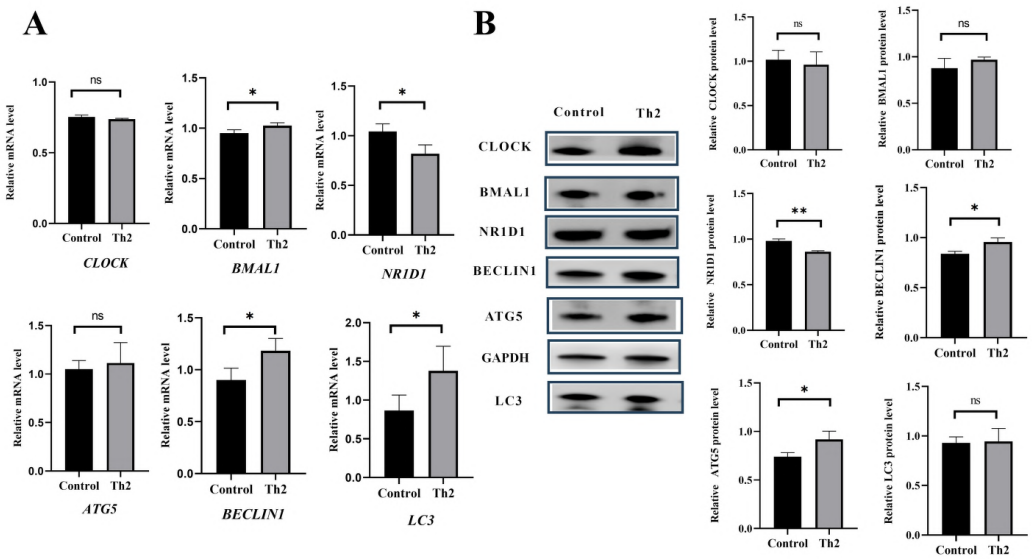

Fig. 2 Expression of circadian rhythm and autophagy genes in Beas-2b cells. (A) The expression of mRNA of circadian rhythm and autophagy genes in Beas-2b and Beas-2b (Th2) cells. (B) The expression of protein of circadian rhythm and autophagy genes in Beas-2b and Beas-2b (Th2) cells. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

trends in the *BECLIN1* and *LC3* mRNA levels were not significant ($p > 0.05$). After IL-4 (10 ng/ml) was added for 2 h, the group with decreased NR1D1 manifested a remarkable increase in *BECLIN1* ($p = 0.038$) and LC3 *mRNA* levels ($p < 0.001$) (Fig. 6C).

Variations in NR1D1 subsequent to overexpression (OE-BECLIN1) and suppression (sh-BECLIN1) of BECLIN1 via plasmid transfection in Beas-2b cells

In Beas-2b cells, overexpression of BECLIN1 was accomplished by plasmid transfection, leading to an augmented expression of *NR1D1* and *LC3* mRNA ($p < 0.05$). The expression levels of NR1D1 and LC3 proteins were also elevated ($p < 0.05$) (Fig. 7A). Following transfection with a plasmid to inhibit the expression of BECLIN1, the expression of *NR1D1* and *LC3* mRNA conspicuously decreased ($p < 0.05$). The expression of NR1D1 and LC3 proteins significantly decreased ($p < 0.05$) (Fig. 7B).

NR1D1 inhibits BECLIN1 and ATG5 transcription within its promoter

Dual-luciferase reporter assays revealed that NR1D1 binds to the promoters of *BECLIN1* and *ATG5*, thereby mediating negative regulation. Binding to the *BECLIN1* promoter was more pronounced. Both interactions showed statistically significant differences, with p values of 0.004 and 0.006, respectively. (Fig. 8).

DISCUSSION

Approximately half of the patients with asthma exhibit type 2 inflammation, primarily characterized by airway inflammation mediated by Th2 cytokines such as IL-4 and IL-13. Consequently, in the present study, we developed an OVA-sensitized asthma murine model and a Beas-2b cell line model of type 2 inflammation. The elevated levels of IL-4 and IL-13 in the BALF of asthmatic mice, in the absence of corresponding changes in blood, indicate the organ-specific nature of the inflammatory response.

Injury to the bronchial epithelium represents a critical component and initial phase in the pathogenesis of asthma, and is intricately associated with the severity of airway inflammatory conditions [21]. Therefore, in this study, we investigated airway epithelial cells. In *in vivo* experiments, we examined the expression levels of circadian rhythm genes (*Clock*, *Bmal1*, and *Nr1d1*) in the lung tissues of asthmatic mice. We found that the expression of CLOCK was not significantly altered, whereas the expression of BMAL1 and NR1D1 decreased, with a more pronounced reduction observed in NR1D1. In the *in vitro* experiments, no significant difference was observed in CLOCK expression, whereas NR1D1 expression was markedly reduced and BMAL1 expression was elevated in the type 2 inflammation model of Beas-2b cells. Both *in vivo* and *in vitro* experiments suggest that BMAL1 and NR1D1 may have positive and negative regulatory relationships, respectively. It has been hypothesized that NR1D1 levels are reduced in asthma and that the extent of this reduction may influence BMAL1 expression, resulting in both increased and decreased BMAL1 levels. BMAL1 regulates the lung tissue response to common viruses and plays a significant role in developing asthma-associated airway diseases [22].

When *Nr1d1*$^{-/-}$ mice were exposed to acute house dust mites (HDM), they exhibited exacerbated lung inflammation associated with elevated levels of pro-

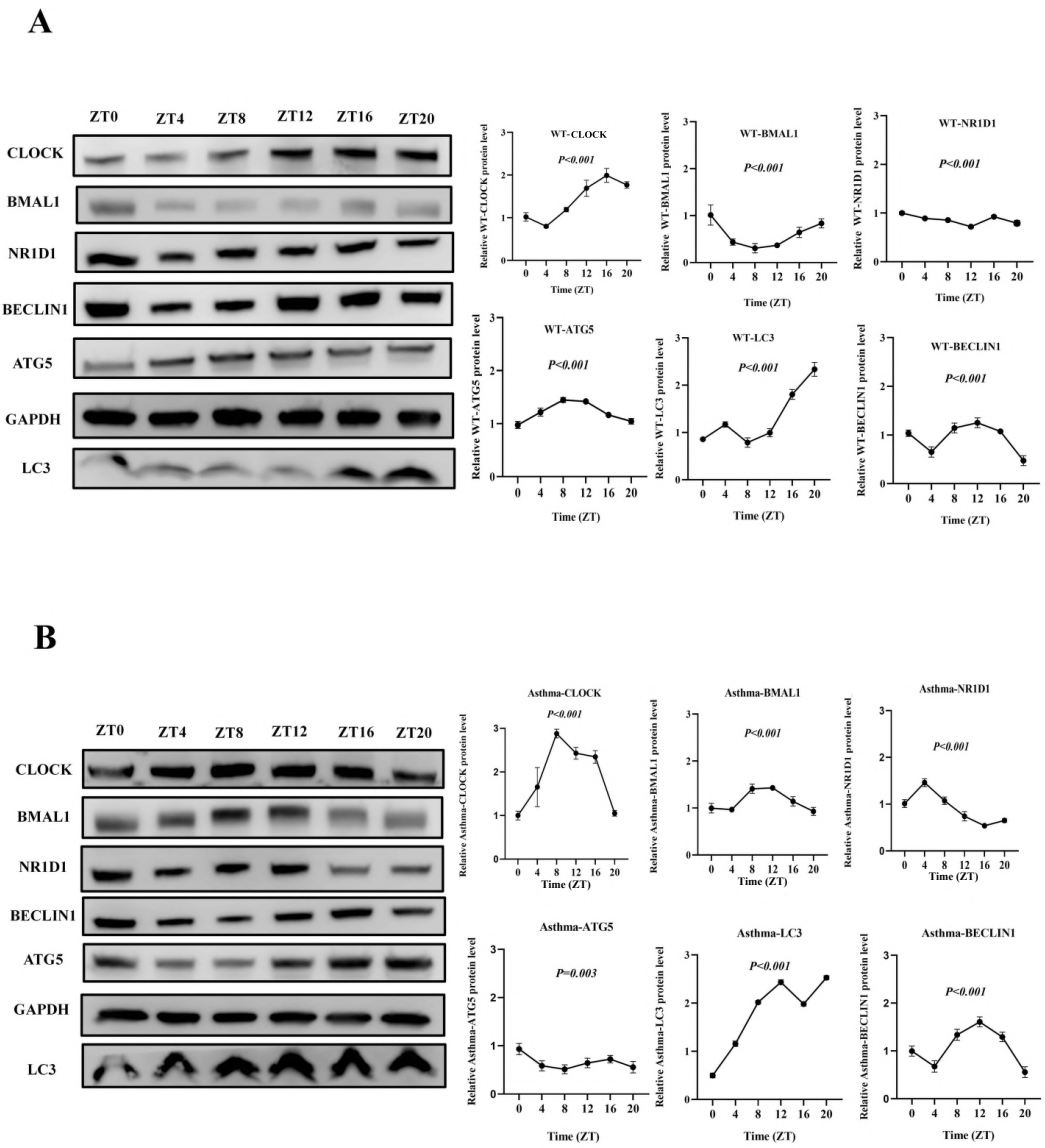

Fig. 3 Expression of circadian rhythm and autophagy proteins in the lungs of mice over 24 h. Protein expression levels of circadian rhythm-associated genes and autophagy-related genes across multiple time points, along with comparative trend plots depicting the relative abundance of the target gene protein normalized to the corresponding internal reference protein at each time point in WT (A) and asthmatic mice (B). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

inflammatory cytokines in the BALF [23]. Compared with the healthy control group, the BALF of patients with asthma exhibited significantly decreased expression of the circadian rhythm genes *BMAL1*, *PEF3*, and *NR1D11*, whereas *CLOCK* showed no significant difference [22]. The circadian rhythm genes *BMAL1* and *NR1D1* may be negatively correlated with asthma [14]. The results of these basic and clinical studies are consistent with our findings, indicating that the circadian rhythm genes BMAL11 and NR1D11 are associated with asthma onset both *in vitro* and *in vivo*.

HDM, dust, and air pollution particles can influence the severity of asthma by modulating airway epithelial autophagy [24]. In this study, the levels of IL-4 and IL-13, which are associated with type 2 airway inflammation in asthmatic mice, were elevated in the BALF, and the expression of LC3, an autophagy marker, also increased. These results are consistent with previous reports demonstrating that IL-13 activates autophagy to regulate airway epithelial cell secretion [25].

A substantial body of research, based on genomic sequencing and genomics, has identified susceptibility genes associated with an increased risk of asthma. Sev-

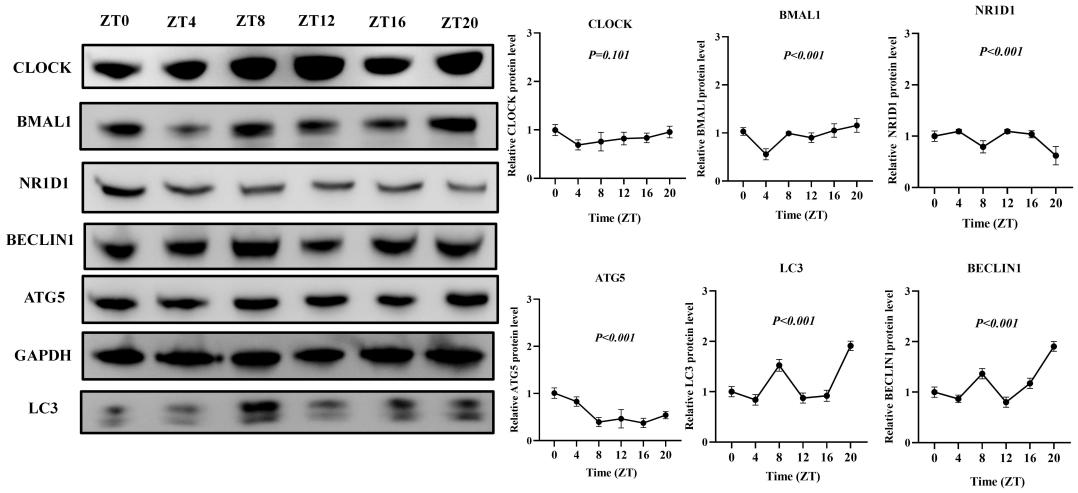

Fig. 4 Expression of circadian rhythm and autophagy proteins in Beas-2b cells over 24 h. Protein expression levels of circadian rhythm–associated genes and autophagy–related genes across multiple time points, along with comparative trend plots depicting the relative abundance of the target gene protein normalized to the corresponding internal reference protein at each time point in Beas-2b. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

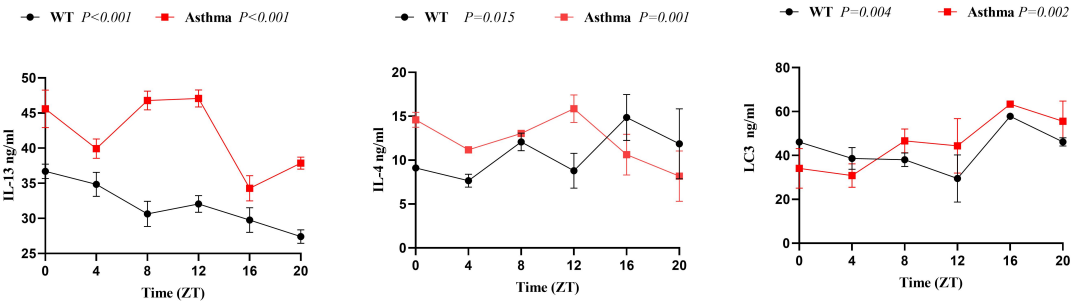

Fig. 5 Expression of IL13, IL4, and LC3 in BALF over 24 h.

eral of these genes play critical roles in the pathogenesis of asthma, including multiple autophagy-related genes [26]. The expression of BECLIN1, ATG5, and LC3 is increased in the lung tissue and type 2 inflammatory airway epithelial cells of asthmatic mice. Additionally, our findings confirm that genes related to autophagy are overexpressed in OVA-sensitized asthmatic mice [27]. The inhibition of airway inflammation via the autophagy pathway provides a novel therapeutic approach for the treatment of asthma.

Autophagy plays a crucial role in the biological clock-dependent rhythmic activation of the body to monitor health or the presence of specific diseases. Consequently, various studies have investigated the mechanisms underlying clock-regulated autophagy to identify potential clinical treatment options [28]. In this study of asthmatic mouse lung tissue and airway epithelial cells in a type 2 inflammation model, we found that the expression of circadian rhythm genes (*Nr1d1* and *Bmal1*) was altered, whereas the expression of autophagy-related genes (*Atg5*, *Beclin1*, and *Lc3*) was increased. These findings suggest that circadian rhythm genes play a role in the regulation of autophagy in asthma. We investigated the temporal expression patterns of rhythmic and autophagy-related genes in asthma. *In vitro* experiments indicated that *Clock* expression did not exhibit rhythmic fluctuations, which is consistent with previous findings in the liver and intestine [29]. Meanwhile, the expression of *Bmal1* and *Nr1d1* exhibited significant temporal fluctuations, with greater amplitude of these fluctuations in asthmatic mice, which is consistent with previous reports [12]. Our study demonstrated that the lowest BMAL1 secretion in asthmatic mice occurred during sleep, which was significantly different from that observed in WT mice. This finding is consistent with clinical studies showing that the lowest levels of BMAL1 secretion at night and during sleep contribute

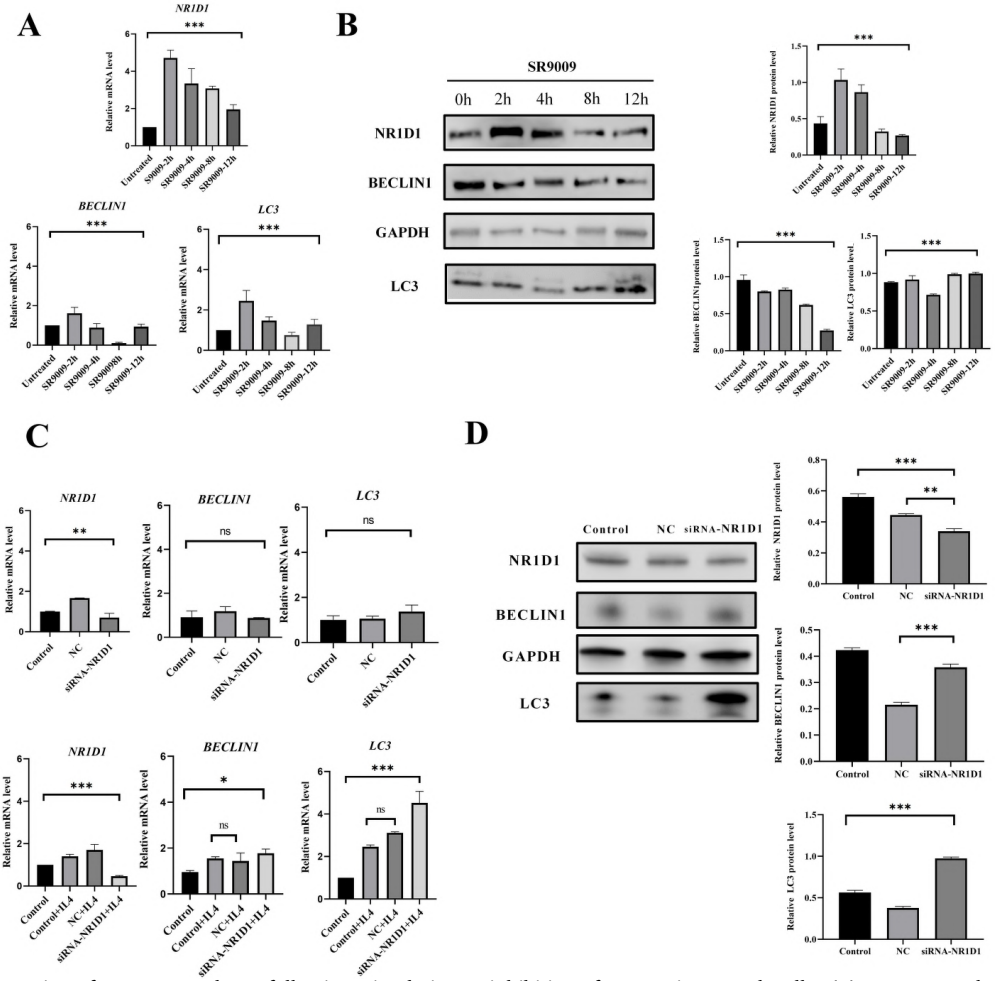

Fig. 6 Expression of BECLIN1 and LC3 following stimulation or inhibition of NR1D1 in Beas-2b cells. (A) *BECLIN1* and *LC3* mRNA following stimulation of NR1D1; (B) BECLIN1 and LC3 protein following stimulation of NR1D1; (C) *BECLIN1* and *LC3* mRNA following inhibition of NR1D1; (D) BECLIN1 and LC3 protein expression following inhibition of NR1D1. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

to the exacerbation of night-time asthma in humans [30]. Relatively limited research has been conducted on the rhythmic changes in NR1D1 in asthma. The trend of NR1D1 expression observed in airway epithelial cells and WT mouse lung tissue in this study was consistent with the findings reported by Weston et al [31]. However, the trend of NR1D1 fluctuation in the lung tissues of asthmatic mice exhibited a significant change. This alteration may also be influenced by changes in the expression of BMAL1.

Our *in vivo* and *in vitro* studies demonstrated that the expression levels of *Atg5*, *Beclin1*, and *Lc3* exhibited rhythmic oscillations. In asthmatic mice, the amplitude of these autophagy gene rhythms was more pronounced than in WT mice, resembling fluctuations in circadian rhythm genes. These findings suggest that autophagy genes are regulated by circadian rhythm genes and exhibit temporal variability. Inhibiting ATG5, BECLIN1, and LC3 can significantly reduce the airway inflammation associated with asthma [27].

Therefore, selecting an appropriate time point to regulate autophagy may enhance its effectiveness in reducing airway inflammation. In the granulosa cells of the ovary, the expression levels of NR1D1 and ATG5 are negatively correlated. The regulation of NR1D1 can inhibit ATG5 expression, thereby alleviating autophagy in granulosa cells [18]. This study showed that NR1D1 and BECLIN exhibited a negative fluctuation trend in both the lung tissue and airway epithelial cells, with no clear consistency with the fluctuation pattern of ATG5. These results indicate that the regulatory mechanisms of circadian rhythmic genes in autophagy may differ among various cell types.

NR1D1 belongs to the nuclear receptor family and functions as a heme-binding component of the circadian clock. It plays a coordinating role in circadian oscillators. In addition to suppressing the transcription of multiple circadian-related rhythm genes, NR1D1 also

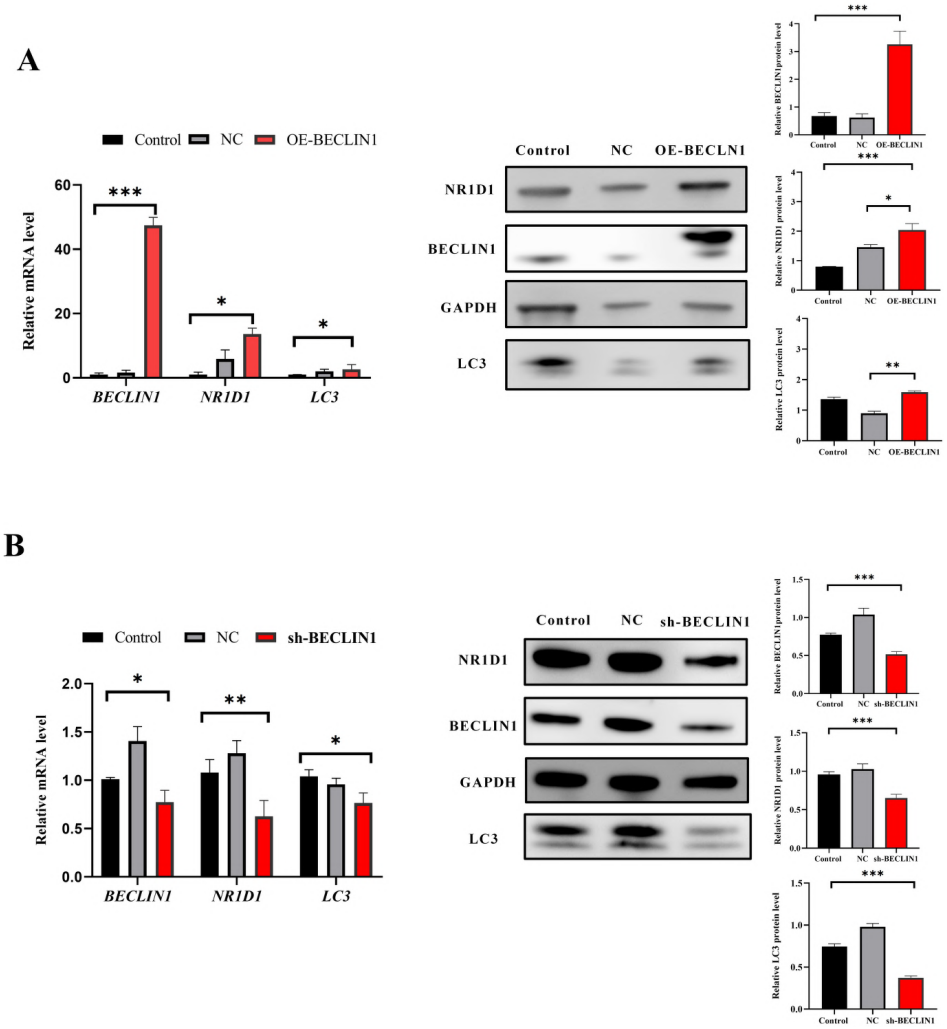

Fig. 7 Variations in NR1D1 following the stimulation or inhibition of BECLIN1 in Beas-2b cells. (A) Variations in NR1D1 and LC3 following the stimulation of BECLIN1. (B) Variations in NR1D1 and LC3 following the inhibition of BECLIN1. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

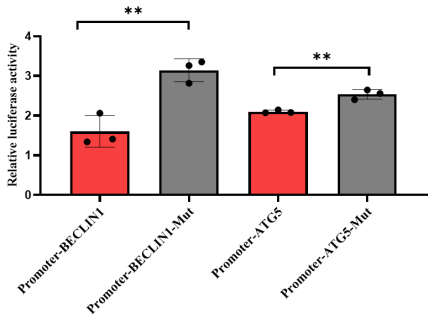

Fig. 8 NR1D1 regulates BECLIN1 and ATG5 transcription within its promoter. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

regulates numerous downstream target genes that are closely linked to autophagy, immunity, inflammation, metabolism, and organ aging [32].

Pharmacological regulation of circadian rhythm mechanisms may be an effective treatment for diseases. Although some studies have suggested that the effects of SR9009 on cellular metabolism are not entirely caused by NR1D1. The NR1D1 agonist SR9009 has been investigated for the treatment of various diseases, including tumors, cardiovascular diseases, and reproductive health [32, 33]. However, research on its effects on airways remains limited. Based on a preliminary CCK8 experiment and previous reports [33], we selected an optimal concentration of SR9009 for Beas-2b cells. The expression of NR1D1 peaked 2 h after the addition of SR9009, and the stimulatory effect subsequently diminished over time. A total of 8 h after the addition of SR9009, the expression of *BECLIN1* mRNA and *LC3* mRNA reached their lowest levels, and the reductions in BECLIN1 and LC3 protein levels were

comparable. These results support the conclusion that NR1D1 inhibits autophagy. This study also suggests that targeting NR1D1 in the airway tissue with SR9009 may be beneficial for asthma studies.

Secondly, in Beas-2b cells, we used siRNA to inhibit the expression of *NR1D1*. We observed that the protein levels of BECLIN1 showed an increasing trend, whereas those of LC3 exhibited a significant increase. However, there were no significant changes in the levels of *BECLIN1* mRNA or *LC3* mRNA. The *BECLIN1* pathway is a proactive target of autophagy independent of mTOR regulation. LC3 plays a crucial role in the selective recruitment of autophagosomes by acting as an autophagic carrier. It is involved in vesicle transport and autophagosome maturation, promoting the formation of the autophagic membrane and the fusion of autophagosomes with lysosomes. LC3 is translocated to the autophagosomal membrane via a fusion protein, ultimately facilitating formation of autophagosomes [34]. Therefore, LC3 is regulated by multiple upstream genes, and the cumulative changes may be more pronounced than those in BECLIN1. However, BECLIN1 and LC3 may contain multiple gene transcripts, which may account for the inconsistencies between their mRNA and protein expression in this context.

Because IL-4 is one of the primary inflammatory factors in type 2 inflammation, when Beas-2b cells were stimulated with IL-4 after suppressing NR1D1 expression, the mRNA levels of *BECLIN1* and *LC3* increased significantly. This suggests that autophagy sensitivity may increase following a reduction in NR1D1 expression. Furthermore, we observed changes in the expression of NR1D1 in Beas-2b cells upon stimulation and inhibition of BECLIN1. We found that NR1D1 expression increased when BECLIN1 was upregulated and decreased when BECLIN1 was downregulated. Autophagy plays a dual role in the body, and BECLIN1 is a key regulatory factor in the autophagy pathway. An increase or decrease in BECLIN1 expression may indicate that cells modulate their autophagic activity to enhance their survival under stressful conditions. The positive correlation between BECLIN1 and NR1D1 expression suggests that circadian clock regulation becomes more active during stress or disease, potentially reversing the effects of autophagy to maintain its beneficial effects on organisms.

In granulosa cells of the ovary, NR1D1 binds to the *ATG5* promoter to regulate autophagy [18]. Therefore, we subsequently verified the binding of NR1D1 to both *ATG5* and *BECLIN1* promoters, and the results showed that NR1D1 also binds to the *BECLIN1* promoter to regulate autophagy. Treatment of tilapia with NR1D1 agonists reduced the expression of most autophagy-related genes, including *Atg4*, *Ulk1*, and *Beclin1*, whereas the expression of *Atg4* and *Lc3* exhibited rhythmic fluctuating [35]. In our study, NR1D1 was found to bind to the promoters of *ATG5* and *BECLIN1*; however, only BECLIN1 exhibited fluctuations related to circadian genes. We speculated that NR1D1 binds to the promoters of multiple autophagy genes to regulate autophagy. Circadian genes, acting as upstream regulators, control the expression of autophagy genes that display varying expression levels and rhythmic fluctuations across different cell types. These variations are maintained by the autophagy pathway to ensure the optimal function of different organs. We also demonstrated for the first time that NR1D1 regulates autophagy by binding to the *BECLIN1* promoter, thereby alleviating airway inflammation. The use of NR1D1 agonists has provided new targets for asthma treatment.

CONCLUSION

A limitations of the study subsection should be included. First, the regulatory effects of NR1D1 on core ATGs beyond BECN1 remain uncharacterized. Second, an asthma mouse model with elevated NR1D1 expression has not been successfully established. Third, time-point-specific extraction of primary cells from asthmatic mice for precise validation was not performed. Notwithstanding these limitations, our research also indicates that Circadian and autophagy-related genes showed pronounced periodic oscillation in asthmatic mice, coinciding with airway inflammation. NR1D1 negatively regulates BECLIN1 to suppress autophagy and mitigate airway inflammation in asthma, indicating a possible therapeutic target. Future work will extend mechanistic validation to additional ATGs and advance toward clinical trials.

Appendix A. Supplementary data

Supplementary data associated with this article can be found at https://dx.doi.org/10.2306/scienceasia1513-1874.2026.039.

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Appendix A. Supplementary data

Table S1 Primers for amplification of fragments.

Mus musculus		*Homo sapiens*	
Clock	F:AGTACATCAACTCAGAGTCAACA R:TACAGTGGGGCTGTGAGAGT	*CLOCK*	F:GGCGCTCGGTTTCTCTTCTT R:GCCAGAGCCAACTCCAGAAA
Bmal1	F:ACTGACTACCAGTTAGAATATGCAG R:ATTTTGTCCCGACGCCTCTT	*BMAL1*	F:CCTGGGCCTTCATTGGTTCC R:TTTTCAGGCGGTCAGCTTCT
Nr1d1	F:ACAAGCAACATTACCAAGCTGA R:CACTCCATAGTGGAAGCCTGA	*NR1D1*	F:GGTCCAGTTTGAATGACCGC R:TGATGACGCCACCTGTGTTG
Atg5	F:ATCCAAGGATGCGGTTGAGG R:GTCATTCTGCAGTCCCATCCA	*ATG5*	F:CGGGTGAAGGTGGTTCCT R:TTTCAACCAAAGCCAAACTTAGTA
Lc3	F:CGCTACAAGGGTGAGAAGCA R:GCGGCGCCGGATGAT	*LC3*	F:AGACCGGCCTTTCAAGCAG R:GCTCGATGATCACCGGGATTT
Beclin1	F:GCTGTAGCCAGCCTCTGAAA R:AATGGCTCCTGTGAGTTCCTG	*BECLIN1*	F:CAAGATCCTGGACCGTGTCAC R:CACCATCCTGGCGAGGAG
Gapdh	F:TGCGACTTCAACAGCAACTC R:ATGTAGGCAATGAGGTCCAC	*GAPDH*	F:GAAAGCCTGCCGGTGACTAA R:GCCCAATACGACCAAATCAGAG

Table S2 The experimental groups in Dual-Luciferase Reporter Assay System.

OE-NC + Promoter-NC + RL
OE-NC + Promoter-NC + RL
OE-NR1D1 + Promoter-NC + RL
OE-NC + Promoter-*BECLIN1* + RL
OE-NR1D1 + Promoter-*BECLIN1* + RL
OE-NC + Promoter-*BECLIN1*-Mut + RL
OE-NR1D1 + Promoter-*BECLIN1*-Mut + RL
OE-NC + Promoter-*ATG5* + RL
OE-NR1D1 + Promoter-*ATG5* + RL
OE-NC + Promoter-*ATG5*-Mut + RL
OE-NR1D1 + Promoter-*ATG5*-Mut + RL