



House dust mite and Th2 cytokine-mediated epithelial barrier dysfunction attenuation by KL001 in 16-HBE cells

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ABSTRACT

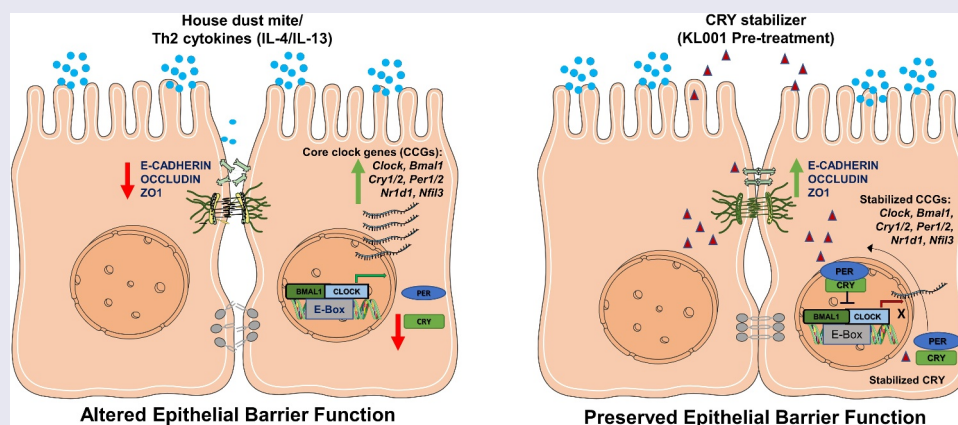
House dust mite (HDM) is a common aeroallergen that can disrupt the airway epithelial barrier leading to dysregulated immune response, resulting in allergic lung diseases such as asthma. Cryptochrome (CRY), a circadian clock gene, plays an important role in the regulation of metabolism, and immune response. It remains unclear whether stabilizing CRY using KL001 can attenuate HDM/Th2 cytokine-induced epithelial barrier dysfunction in 16-HBE cells. We evaluate the effect of KL001 (20 μ M) pre-treatment (4 hrs) in HDM/Th2 cytokine (IL-4 or IL-13)-mediated change in epithelial barrier function. HDM and Th2 cytokine-induced changes in transepithelial electrical resistance (TEER) were determined by an xCELLigence real-time cell analyzer and delocalization of adherens junction complex (AJC: E-cadherin and β -catenin) and tight junction proteins (TJP: Occludin and Zonula occludens-1) by immunostaining and confocal microscopy. Finally, quantitative real-time PCR (qRT-PCR) and Western blotting were used to measure altered gene expression and protein abundance of the epithelial barrier function and core clock genes, respectively. HDM and Th2 cytokine treatment significantly decreased TEER associated with altered gene expression and protein abundance of the selected epithelial barrier function and circadian clock genes. However, pre-treatment with KL001 attenuated HDM and Th2 cytokine-induced epithelial barrier dysfunction as early as 12–24 hrs. KL001 pre-treatment showed attenuation of HDM and Th2 cytokine-induced alteration in the localization and gene expression of AJP and TJP (*Cdh1*, *Ocln*, and *Zo1*) and core clock genes (*Clock*, *Arntl/Bmal1*, *Cry1/2*, *Per1/2*, *Nr1d1/Rev-erba*, and *Nfil3*). We demonstrate, for the first time, the protective role of KL001 in HDM and Th2 cytokine-mediated epithelial barrier dysfunction.

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Introduction

Epithelial barrier function is critical in the activation of allergic airway inflammatory response in asthma. We know that the circadian clock-dependent change in the heightened allergic

immune response is inversely proportional to epithelial barrier function.¹ Protease activity of allergens including house dust mites (HDM), harmful agents (lipopolysaccharide [LPS] and inhaled toxins), and Th2 cytokines (IL-4, IL-5, and IL-13) can affect the epithelial barrier

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function.^{2–4} The airway is constantly exposed to physical, chemical, and biological agents present in the inhaled air (e.g., airborne particles, allergens, infectious agents, etc.), and thus proper maintenance of effective epithelial barrier function is necessary for protection¹. The airway epithelial cells contain adherens junctions (AJs), tight junctions (TJs), and gap junctions along with ciliated cells and mucus-producing goblet cells forming the core components of the epithelial barrier, protecting the airway from any damage.^{5,6} Disruption or defects in the epithelial barrier leave the airway vulnerable to foreign particles resulting in various pathological conditions through the cytokines/chemokines released by the airway epithelial cells. Patients with allergic asthma show defective epithelial barrier function associated with patchy disruption of TJs.^{7,8} Asthmatics have rhythmic patterns in the symptoms and severity, characterized by significant day-night variations in pathophysiological phenotypes (altered lung function), biological markers, and therapeutic response.⁹ Evidence from the mouse model demonstrates circadian clock gene *Rev-erba* global knockout (KO) and *Bmal1* myeloid cell-specific KO augments HDM and ovalbumin (OVA)-induced lung inflammation and asthmatic phenotypes.^{10,11} These findings provide a strong connection between asthma and the circadian clock that is driven by a transcription-translation feedback loop, resulting in a robust 24-hour rhythm.^{12,13}

This biological clock oscillation depends on the synthesis of CLOCK, BMAL1, CRY1–2 (Cryptochrome), and PER1–3 (Period) proteins forming the core circadian clock machinery. Among these proteins, CLOCK:BMAL1 constitutes transcriptional activators, with PER and CRY being transcriptional repressors. During the daytime, transcription of CRYs and PERs is induced by CLOCK:BMAL1 heterodimers binding to E-box elements after nuclear translocation, resulting in transcriptional activation of CRY and PER.¹⁴ Cytosolic accumulation of CRY and PER leads to CRY:PER heterodimer, which then translocates to the nucleus and inhibits the binding of CLOCK:BMAL1 to the E-box promoter, repressing their own transcription.^{15,16} Degradation of CRY and PER proteins is controlled by phosphorylation post-translationally by casein kinase, controlling

the stability of clock proteins, thereby lengthening the circadian period.¹⁷ Additionally, CLOCK:BMAL1 complex regulates the transcription of REV-ERB α/β and ROR α/γ , which are responsible for maintaining the timing and expression levels of BMAL1, thus stabilizing the circadian oscillators.¹⁸ Current literature strongly supports that the clock in the airway epithelium plays an essential role in the circadian regulation of lung homeostasis during health and disease.^{13,19–21} Environmental agents such as HDM, pollens, and LPS can cause exaggerated inflammation in circadian clock gene-deficient mice, suggesting a potential role of clock genes in regulating inflammatory response associated with altered lung physiological functions, such as airway hyperresponsiveness (AHR), mucus hypersecretion, and epithelial barrier function.^{10,11,13,20,22}

The small-molecule KL001 (N-[3-(9 H-carbazol-9-yl)-2-hydroxypropyl]-N-2(2-furanylmethyl)-methanesulfonamide), a modulator of CRY protein, binds to its flavin adenine dinucleotide pocket, resulting in the blockage of ubiquitin-oriented breakdown of CRY1 and CRY2, thereby inducing lengthening of the circadian period.²³ Circadian clock protein CRY has been shown to regulate proinflammatory cytokines *in vitro* and *in vivo*.^{24,25} *Cry1/2* double KO (DKO) mice showed constitutively upregulated transcript levels of cytokines (*il6*, *Tnfa*, and *inos*) in the hypothalamus, fibroblasts, and bone marrow-derived macrophages via activation of NF- κ B-dependent signaling.²⁵ Another report suggests that CRY1/2 repressed inflammation in fibroblast-like synoviocytes (FLSs) in a mouse model of collagen-induced arthritis. TNF α stimulated *Cry1/2* DKO FLSs showed augmented transcript levels (*cxcl1*, *il6*, *cxcl5*, and *il1b*) and cytokine release of inflammatory mediators compared to WT cells. Activation of CRY using KL001 significantly reduced TNF α -induced cytokine (CXCL1, IL-6, and IL-1 β) responses in murine FLSs cells.²⁴ These studies indicate that CRY proteins regulate the cellular and physiological processes such as inflammation, immune response, and metabolic functions in a cell type-specific manner. As stated earlier, the epithelial barrier is under circadian control and KL001 may help maintain the epithelial barrier function by lengthening the circadian period. There is no direct evidence whether the circadian clock regulator, the cryptochromes

(CRYs: CRY1/2) lengthen the circadian period and thus regulate epithelial barrier function in the lung and epithelial permeability to allergens and/or Th2 cytokines (IL-4 and IL-13).

HDM and Th2 cytokines (IL-4/IL-13)-mediated change in membrane adherens junction complex (AJC: altered E-cadherin, α - and β -catenin) and TJ proteins (reduced Zonula occludens-1 [ZO-1] and Occludin) have been shown to affect epithelial barrier function confirmed by transepithelial electrical resistance (TEER), immunofluorescence, and Western blotting in 16HBE14o and Calu-3 cells.^{2,3,26} To date, whether CRY modulators can attenuate HDM or Th2 cytokine-induced epithelial barrier dysfunction has not been proven using cell culture models. To explore the functional significance of stabilizing CRY in epithelial barrier function, the small-molecule KL001 that stabilizes CRY1/2 was tested *in vitro* in 16-HBE cells. KL001 was used for all the experiments to determine the efficacy of CRY1/2 stabilization-mediated attenuation of epithelial barrier dysfunction.

Materials and methods

Cell culture and treatment

16HBE14o- human bronchial epithelial cells (16-HBE; Sigma Aldrich, St. Louis, MO) were grown in Dulbecco's Minimum Essential Medium (DMEM) supplemented with sodium pyruvate and L-glutamine containing high glucose (Life Technologies, Carlsbad, CA) along with 10% fetal bovine serum (FBS), Penicillin (100 U/ml), Streptomycin (100 μ g/ml), Amphotericin B (100 μ g/ml) and HEPES (10 mmol/L) (Life Technologies). Initially, the 16-HBE cells were grown in a humidified CO₂ incubator (5%) at 37°C using T-75 flasks, until they reach the desired confluence as described previously.²³ 16-HBE cells were seeded on 6-well plates at a density of 250,000 cells/well and grown to 100% confluence. The culture media was changed to a complete medium containing 1% FBS, 24 hrs before treatment. 16-HBE cells were treated with HDM (50 μ g/ml) or Th2 cytokines [IL-4 or IL-13 (50 ng/ml)] or KL001 alone (20 μ M/ml), or KL001+HDM or KL001+Th2 cytokines [pre-treated with KL001 for 4 hrs before HDM/Th2 cytokine treatment in the combination

groups] for 24 hrs to determine epithelial barrier function. Cells were harvested and stored at -80°C for mRNA and protein analysis.

Impedance-based assay by xCelligence Real-Time Cell Analysis (RTCA)

Cell impedance measurements were performed using the Agilent xCELLigence RTCA MP instrument connected to a laptop using the RTCA Software Pro (Agilent Technologies, Inc. Santa Clara, CA) as described previously.^{23,27} 16-HBE cells were seeded on E-plate 96 PET (Agilent Technologies, Inc.) at a density of 150,000 cells/well. Cells were grown to confluence (100%) during which they reach the steady-state (plateau) in cell impedance (This takes about 3–7 days depending on the seeding density of the cells in 96 well E-plates). When the cells were reaching maximal confluence, the culture medium was changed every alternate day, with real-time cell impedance measurement recordings every 15 min throughout the experiments. After the cells reached the maximal confluence, a complete medium containing 1% FBS was changed 24 hrs before treatment with HDM (50 μ g/ml) or Th2 cytokines [IL-4 or IL-13 (50 ng/ml)] to induce epithelial barrier dysfunction and monitored from 0–48 hrs. Regarding treatment with a small molecule (KL001; 20 μ M/ml) CRY activator, cells were pre-treated with KL001, 4 hrs before treatment with agents that cause barrier dysfunction (HDM or Th2 cytokines). The concentrations of the small-molecule KL001 and HDM or IL-4/IL-13 that induces epithelial barrier dysfunction were based on prior reports.^{3,23,26,28,29}

Cell impedance was recorded continuously every 15 min for the entire period of the experiment except during the addition of a small molecule (KL001) or agents (HDM or Th2 cytokines) that induce epithelial barrier dysfunction. The cell impedance of electron flow caused by adherent cells is stated without a specific unit referred to as Cell Index (CI), where $CI = (\text{impedance at time point } n - \text{impedance in the absence of cells}) / \text{nominal impedance value}$.^{30,31} The CI will be zero without adhesion of cells, and as cells adhere and grow to confluence on the surface of the gold electrode arrays printed in 96 well E-plates the CI increases. Data analysis of impedance values was

calculated as baseline normalized impedance as described previously.^{30,31} In brief, each time point average impedance value of untreated controls was subtracted and normalized as $CI_{(normalized)} = CI_{time\ x} / CI_{norm\ time}$ referred to as baseline normalized CI. The baseline normalized CI value was exported to Microsoft Excel. Graphical representation of the result was shown in figures and supplementary figures using GraphPad Prism 9 software or RTCA Software Pro. Results were shown as the average of eight independent wells treated with the indicated small molecule (KL001) or agents (HDM or Th2 cytokines [IL-4 or IL-13]) or their combinations (KL001+HDM/IL-4/IL-13).

Immunofluorescence staining of adherens junction complex (AJC) and tight junction (TJ) proteins

16-HBE cells were seeded on 4-well chamber slides at a density of 150,000 cells/well. Cells were grown to 100% confluence and then changed to a complete medium containing 1% FBS, 24 hrs before treatment. Treatment with HDM/Th2 cytokines and KL001 pre-treatment was performed as described earlier. After 24 hrs post-treatment, the cells were washed twice in 1× PBS and fixed in ice-cold 4% paraformaldehyde solution for 10 min followed by 3 washes (5 min each) with PBST (1× PBS with 0.05% Tween 20). Cells were permeabilized by adding 0.05% Triton X-100 for 5 min followed by 3 washes with PBST. The following AJC and TJ primary antibodies (E-cadherin [1:250], β -catenin [1:500], Occludin [1:250], Zona occludens-1 [1:500]) were diluted in 10% goat serum (blocking buffer) and incubated overnight at 4°C. The next day, cells were washed 4–5 times using PBST, then incubated with fluorescently conjugated secondary antibodies (1:800) for 1 hr at room temperature (RT) in dark. Finally, the cells were washed five times in PBST. The nuclei were stained using Hoechst dye (1:10,000) and incubated at RT for 5 min in dark, followed by four washes with PBST. The slides were removed and mounted with antifade mountant before confocal microscopy. Images were taken at 60× oil immersion using Nikon C2 confocal microscopy (Nikon Instruments Inc. Tokyo, Japan). After imaging

was done, the files were exported as tiff images and analyzed using Image J software (NIH, USA).

Total RNA isolation, cDNA synthesis, and quantitative real-time polymerase chain reaction (Qrt-PCR)

Cell pellets were thawed in ice, and total RNA was extracted using the RNeasy mini kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. During extraction, on-column DNase treatment was performed using DNase I (Zymo Research, USA). The concentration and purity of Total RNA were measured by an Epoch microplate spectrophotometer (BioTek, CA, USA).

cDNA was synthesized using the iScript cDNA Synthesis Kit (Bio-Rad, USA). In brief, total RNA (1,000 ng) was added to the master mix containing 8 μ l of 5× iScript reaction mix, 4 μ l of random primers, 2 μ l of iScript reverse transcriptase, and the final volume made up to 20 μ l using RNase-free water. PCR conditions used for cDNA synthesis were as follows: 25°C for 5 min, 46°C for 20 min, 95°C for 1 min, and hold at 4°C. After cDNA synthesis, 60 μ l of RNase-free water was added to dilute the cDNA to make the final volume 80 μ l.

All the qRT-PCRs for gene-specific targets (core circadian clock genes, AJC, TJ, and *Rn18S* reference gene) were performed in the CFX Opus 96 Real-time PCR System (Bio-Rad) using iTaq Universal SYBR Green Supermix (Bio-Rad, USA). Relative expression of the target genes was determined by the $2^{-\Delta\Delta C_t}$ method in comparison to the reference gene *Rn18S*. The human qRT-PCR primers used in the study are listed in Table 1.

Protein estimation and Western blot analysis

Cells were lysed using RIPA buffer containing protease inhibitor cocktail by vortexing the samples with multiple freeze-thaw cycles and finally centrifuged at 14,000 rpm for 10 min at 4°C. The clear lysate containing total protein was estimated using Pierce™ Micro BCA Protein Assay Kit (ThermoFisher Scientific, USA). Western blot was performed in 25 μ g of total protein using 4–15% gradient gels (Bio-Rad). Separated protein was transferred into nitrocellulose membrane using Bio-Rad Turbo Transblot system,

Table 1. Primer sequence used for Qrt-PCR analysis

Sl. No.	Target Gene	Forward (5'–3')	Reverse (5'–3')
1	<i>Arntl/Bmal1</i>	CATTGAAAAAGTTAAGCTTGGG	ACGGAGGTGCCTGTTTACC
2	<i>Clock</i>	GGACTGTAGAATTATCCAAAGGC	GAGAGCGCGAAGGAAATCT
3	<i>Cry1</i>	CCACGAATCACAAACAGACG	GTCTACATCCTGGACCCCTG
4	<i>Cry2</i>	TGCAGGTGTACTCTGCTGC	TGAAGAACTCAGCAACGCGG
5	<i>Per1</i>	TGGGGAGAACAGAACAGAGAA	AGGACACTCCTGCGACCA
6	<i>Per2</i>	ACATCTTCCTGCAGTGGGAC	GGAGCCTGAGGTCCGAA
7	<i>Nr1d1/Rev-erba</i>	GAAGCTGCCATTGGAGTTGT	GGTGAAGACATGACGACCCT
8	<i>Nr1d2/Rev-erbβ</i>	GCCTCCACAGAGTTGACGTT	GAGCAGGGGATCTGCTAAAC
9	<i>Rora</i>	AAAAGCCCTTGCAGCCTT	AGAGCTATTCCAGCACCAGC
10	<i>Dbp</i>	CTCGTTGTTCTGTACCGCC	ACCTTGACCCTCGAAGACA
11	<i>Cldn1</i>	CCTCCTGGGAGTGATAGCAAT	GGCAACTAAATAGCCAGACCT
12	<i>Cldn4</i>	TGGGGCTACAGGTAATGGG	GGTCTGCGAGGTGACAATGTT
13	<i>Cldn8</i>	CAACCCATGCCTTAGAAATCGC	TCACGCAATTCATCCACAGTC
14	<i>Cldn9</i>	CGGCTGCACTGCTTATGCT	GAGGGGATGGAGTAGCCCA
15	<i>Cldn16</i>	CCCCACTGTTGGTTACAGCC	GTGGCGGGTGTCACTCTTC
16	<i>Ocln</i>	ACAAGCGGTTTTATCCAGAGTC	GTCTATCCACAGGCGAAGTTAAT
17	<i>Zo1/Tjp1</i>	CAACATACAGTGACGCTTCACA	CACATTGACGTTTCCCACTC
18	<i>Cdh1</i>	CGAGAGCTACACGTTACGG	GGGTGTGAGGGGAAAAATAGG
19	<i>Ctnnb1</i>	AAAGCGGCTGTTAGTCACTGG	CGAGTCATTGCATCTGTCCAT
20	<i>Marvel2/Tricellulin</i>	ACAGGCGCTACGATGAGGT	GGTGGCTGTAATGGGAGAGG
21	<i>Nfil3</i>	CTGCTTTAACGGAAGTGTGAGA	TTCCTCCGACATGCAGAAGAT
22	<i>Rn18S</i>	GTAACCCGTTGAACCCCAT	CCATCCAATCGGTAGTAGCG

followed by ponceau S visualization, and blocking in 5% BSA in TBST (1× Tris-buffered saline [TBS] containing 0.05% Tween 20) at RT for 1 hr. The membrane was then washed twice in TBST at RT (5 min each) and incubated at 4°C overnight, with respective primary antibody (1:1000) diluted in blocking buffer. After overnight incubation the membrane was washed 3 times in TBST, followed by incubation with HRP-conjugated secondary antibody diluted in blocking buffer for 1 hr at RT. Post-incubation, the membranes were washed 3 times in TBST, and the image was acquired using Odyssey XF Dual-Mode Imaging System (LI-COR Biosciences, NE, USA) after the addition of chemiluminescence substrate (ThermoFisher Scientific, USA). Densitometry/quantification of Western blot images was performed using Image J software (NIH, USA).

Statistical analysis

Statistical analysis for significance was calculated between the untreated control and treatment groups (HDM/IL-4/IL-13, KL001, KL001+HDM/IL-4/IL-13) using one-way ANOVA and Tukey's multiple-comparison test with the GraphPad Prism 9 (La Jolla, CA). The results are shown as means ± SEM with a $P < 0.05$ is considered statistically significant. Statistical analysis was done

using 3–4 replicates/group. In some instances, outlier analysis was performed with Grubb's test, and outliers were removed before statistical analysis.

Results

HDM and Th2 cytokines (IL-4 and IL-13)-induced epithelial barrier dysfunction attenuation by KL001 in 16-HBE cells

We hypothesized that HDM and Th2 cytokines (IL-4/IL-13)-mediated epithelial barrier dysfunction may be attenuated by stabilizing CRY (CRY1/2: Cryptochromes) in the cytosol using KL001. We used the electrical impedance measurement by the xCELLigence Real-Time Cellular Analysis system to monitor alterations in transepithelial electrical resistance (TEER) induced by HDM or Th2 cytokines. Herein, we first utilized 16-HBE cells (in a 96-well E-plate) to demonstrate that HDM and Th2 cytokine treatment causes epithelial barrier dysfunction. Notably, HDM treatment (50 µg/ml) showed a significant change in cell impedance as early as 4 hrs with a maximal decrease in baseline normalized cell index at 24–48 hrs (Figure 1a and Supplementary Figure S1(a)). However, KL001 (20 µM/ml) treatment alone shows preserved epithelial barrier function as reflected by the increased baseline normalized cell index at 2-, 12-, and 24–48 hrs. Furthermore, we also observed that 4 hrs pre-

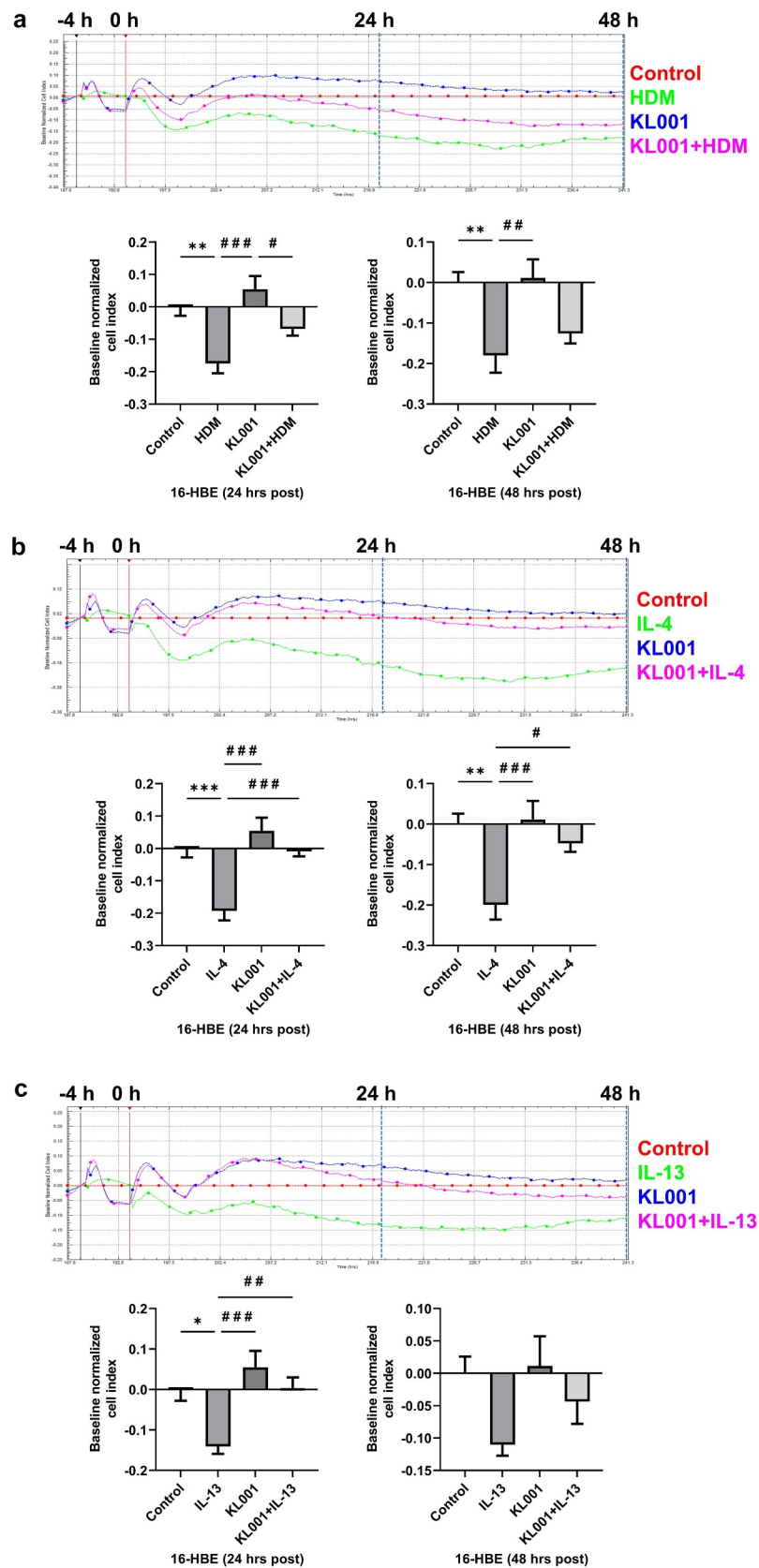


Figure 1. HDM and Th2 cytokine (IL-4 and IL-13)-induced epithelial barrier dysfunction attenuation by KL001 in 16-HBE cells. Note: (a) HDM, (b) IL-4, and (c) IL-13-induced change in maximal cell impedance (i.e., loss of epithelial barrier function) at 24–48 hrs post-treatment compared to untreated control. KL001 pre-treatment (for 4 hrs) along with HDM or IL-4/IL-13 showed attenuation of HDM or Th2 cytokine (IL-4/IL-13)-induced epithelial barrier function in 16-HBE cells at 24–48 hrs post-treatment. The line graphs shown in

treatment with KL001+HDM still showed attenuation of HDM-induced barrier dysfunction at 12- and 24 hrs, post-treatment (**Figure 1a** and Supplementary Figure S1(a)).

Additionally, we determined whether Th2 cytokine (IL-4/IL-13) treatment (50 ng/ml) also causes epithelial barrier dysfunction in 16-HBE cells, and KL001 pre-treatment for 4 hrs attenuates epithelial barrier dysfunction using the above-mentioned approach. Results revealed that IL-4 treatment reduced cell impedance as early as 4 hrs with a maximal reduction observed at 24 and 48 hrs. Similarly, KL001 alone showed preserved epithelial barrier function from 2–48 hrs. KL001 pre-treatment for 4 hrs along with IL-4 showed attenuation of IL-4-induced epithelial barrier dysfunction at 12-, 24-, and 48 hrs post-treatment (**Figure 1b** and Supplementary Figure S1(b)). Similarly, we found that IL-13 treatment caused a maximal change in epithelial barrier function at 24 hrs post-treatment. The KL001 alone treatment showed preserved epithelial barrier function from 12–24 hrs. Furthermore, 4 hrs pre-treatment with KL001+IL-13 showed attenuation of IL-13-induced epithelial barrier dysfunction at 12–24 hrs post-treatment (**Figure 1c** and Supplementary Figure S1(c)).

HDM and Th2 cytokine (IL-4 and IL-13)-induced epithelial barrier dysfunction attenuation by KL001 in 16-HBE cells confirmed by immunofluorescence and confocal microscopy

We used immunofluorescence staining and confocal microscopy to determine HDM and Th2 cytokine-induced epithelial barrier dysfunction and attenuation by evaluating the localization and expression of AJC (E-cadherin and β -catenin) and TJP (Occludin and Zonula occludens-1 [ZO1]) 24 hrs post-treatment in 16-HBE cells. 16-HBE cells were grown to 100% confluence in 4-well chamber slides and then treated with HDM or Th2 cytokines

(IL-4 or IL-13) or KL001 alone or KL001+HDM or KL001+Th2 cytokines to cause delocalization of AJC and TJ proteins. Treatment with HDM/IL-4/IL-13 differentially reduced the protein expression of AJC (E-cadherin and β -catenin) and TJP (Occludin and ZO1) compared to untreated control at 24 hrs (**Figures 2a-b and 3a-b**). KL001 alone treatment showed slightly increased E-cadherin (AJC) and ZO1 (TJP) compared to the control. Pre-treatment with KL001 for 4 hrs showed slight attenuation in the protein expression of E-cadherin (AJC), Occludin, and ZO1 (TJP) among KL001+HDM/KL001+IL-4/KL001+IL-13 groups (**Figures 2a-b and 3a-b**). Overall, the AJC proteins showed the highest disruption/delocalization in β -catenin followed by E-cadherin in HDM and Th2 cytokine-treated groups, whereas in TJP, Occludin was significantly reduced followed by ZO1 in both HDM and Th2 cytokine-treated groups. IL-13 treatment showed a significant delocalization in AJC and TJP followed by IL-4 and HDM in 16-HBE cells confirmed by immunostaining and confocal microscopy. KL001 pre-treatment in the HDM and Th2 cytokine combination group showed attenuation in the epithelial barrier as demonstrated by relatively normal localization of AJC and TJ proteins in 16-HBE cells (**Figures 2a-b and 3a-b**). These results altogether indicate that pre-treatment with KL001 attenuated HDM and Th2 cytokine-induced epithelial barrier dysfunction by regulating the protein abundance and stabilizing localization of AJC and TJ proteins in human bronchial epithelial cells.

HDM and Th2 cytokine-induced epithelial barrier dysfunction attenuation by KL001 in 16-HBE cells affects the mRNA expression and protein levels of AJC and TJ proteins

We have shown that HDM and Th2 cytokines-induced epithelial barrier dysfunction as measured

Figure 1a-c represent the average time-dependent change in cell impedance for 48 hrs post-treatment (HDM or IL-4 or IL-13, KL001, and KL001+HDM or KL001+IL-4 or KL001+IL-13), graphs were exported from RTCA Software Pro. -4 h to 0 h marked in the line graph refers to 4 hrs KL001 pre-treatment before HDM or IL-4 or IL-13 alone or combination treatment. Bar graphs represent baseline normalized cell index for 24- and 48-hrs post-treatment. Data are shown as mean $\pm$ SEM (n = 8/group; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, significant compared to untreated control; # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$, significant compared to KL001 or KL001+HDM/KL001+IL-4/KL001+IL-13 groups; One-way ANOVA with Tukey's multiple comparison test).

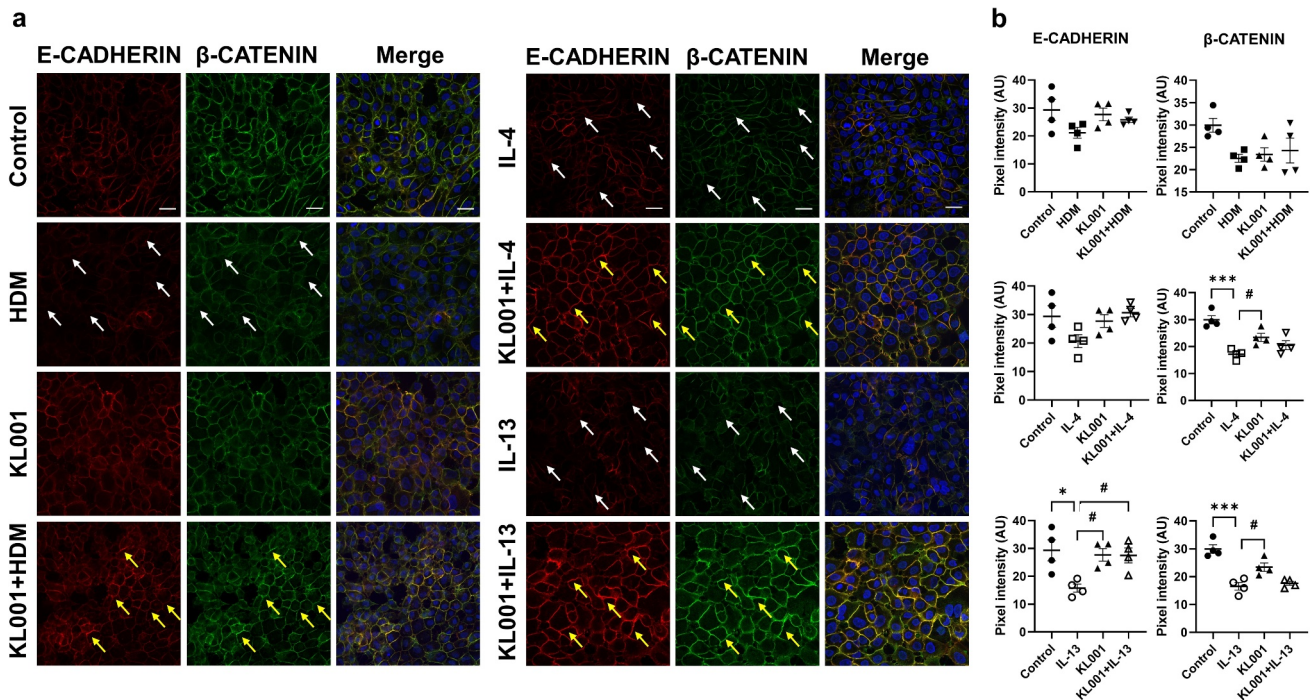


Figure 2. KL001 treatment restored HDM and Th2 cytokine (IL-4 and IL-13)-induced disruption of adherens junction complex proteins in 16-HBE cells. Note: (a) Representative immunostaining images for adherens junction complex (AJC) proteins E-cadherin and β -catenin (red and green color, respectively), 24 hrs post-treatment (HDM or IL-4 or IL-13) compared to untreated control. KL001 pre-treatment (for 4 hrs) along with HDM or IL-4/IL-13 restored HDM or IL-4/IL-13-induced epithelial barrier disruption in 16-HBE cells at 24 hrs post-treatment. (Images were captured using 60 $\times$ magnification; Scale bar: 25 μ m). Merge shows double or single immunostaining using target-specific antibodies counterstained with Hoechst nuclear stain (blue). (b) The scatter plot shows the quantification of immunostaining AJC proteins analyzed using ImageJ with mean pixel intensity (AU). Arrows (white) indicate disrupted AJCs in 16-HBE cells treated with HDM or IL-4/IL-13; Arrows (yellow) indicate restored AJC proteins in 16-HBE cells pre-treated with KL001 (for 4 hrs) along with HDM or IL-4/IL-13 combination 24 hrs post-treatment. Data are shown as mean $\pm$ SEM ($n = 4$ /group; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, significant compared to untreated control; # $P < 0.05$, significant compared to KL001+IL-13; # $P < 0.05$, significant compared to KL001 or KL001+IL-4/KL001+IL-13 groups; One-way ANOVA with Tukey's multiple comparison test).

by TEER and immunostaining followed by confocal microscopy. Here, we show how pre-treatment with KL001 before treatment with HDM or Th2 cytokines affects the AJC and TJP at the mRNA and protein levels in 16-HBE cells. We found HDM/Th2 cytokine (IL-4/IL-13)-treated cells showed significantly increased mRNA expression of AJC genes *Cdh1* and *Cttnb1* (E-cadherin and β -catenin) and TJP genes (*Ocln*, *Zo1/Tjp1*, *Cldn1*, and *Marveld2/Tricellulin*) compared to control (Figure 4a-c and Supplementary Figure S2(A-C)). KL001 alone treatment showed a slight change in the mRNA expression of both AJC (*Cdh1* and *Cttnb1*) and TJP genes (*Ocln*, *Zo1/Tjp1*, *Cldn1*, *Cldn4*, and *Marveld2*) compared to control or HDM/Th2 cytokine-treated 16-HBE cells. In addition, we found a slight increase in *Cldn4* and *Cldn16*, and a reduced trend in the expression of

Cldn8 and *Cldn18* in HDM and Th2 cytokine-treated cells compared to the control (Supplementary Figure S2A-C). Pre-treatment with KL001 for 4 hrs before HDM/IL-4/IL-13 treatment significantly reduced mRNA expression of AJC (*Cdh1* and *Cttnb1* [except in HDM]) and TJP (*Ocln*, *Zo1/Tjp1*, *Cldn1*, and *Marveld2*) compared to HDM/IL-4/IL-13-treated group (relative to the control group) (Figure 4a-c and Supplementary Figure S2 A-C). In addition, we noted a slight increase in the *Cldn8* and *Cldn18* in KL001+IL-4/KL001+IL-13 compared to the group treated with IL-4/IL-13 alone.

Furthermore, we evaluated the protein abundance of AJC (E-CADHERIN and β -CATENIN) and TJP (OCCLUDIN, CLAUDIN 4, and ZO1) in HDM or Th2 cytokine-treated 16-HBE cells using Western blot analysis. HDM treatment significantly

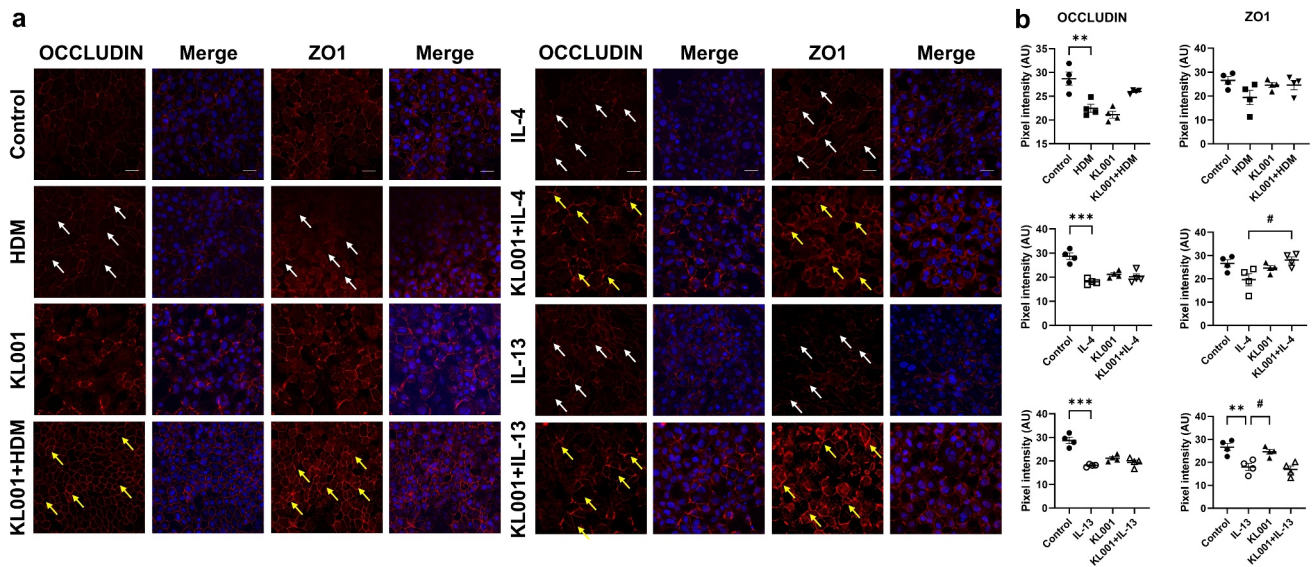


Figure 3. KL001 treatment restored HDM and Th2 cytokine (IL-4 and IL-13)-induced disruption of tight junction proteins in 16-HBE cells. Note: (a) Representative immunostaining images for tight junction proteins Occludin and ZO-1 (red) 24 hrs post-treatment (HDM or IL-4 or IL-13) compared to untreated control. KL001 pre-treatment (for 4 hrs) along with HDM or IL-4/IL-13 restored HDM or IL-4/IL-13-induced tight junction protein disruption in 16-HBE cells at 24 hrs post-treatment. (Images were captured using 60 $\times$ magnification; Scale bar: 25 μ m). Merge shows single immunostaining using target-specific antibodies counterstained with Hoechst nuclear stain (blue). (b) The scatter plot shows the quantification of immunostaining TJ proteins analyzed using ImageJ with mean pixel intensity (AU). Arrows (white) indicate disrupted TJ proteins in 16-HBE cells treated with HDM or IL-4/IL-13; Arrows (yellow) indicate restored TJ proteins in 16-HBE cells pre-treated with KL001 (for 4 hrs) along with HDM or IL-4/IL-13 combination 24 hrs post-treatment. Data are shown as mean $\pm$ SEM ($n = 4$ /group; ** $P < 0.01$, *** $P < 0.001$, significant compared to untreated control; # $P < 0.05$, significant compared to KL001 or KL001+IL-4; One-way ANOVA with Tukey's multiple comparison test).

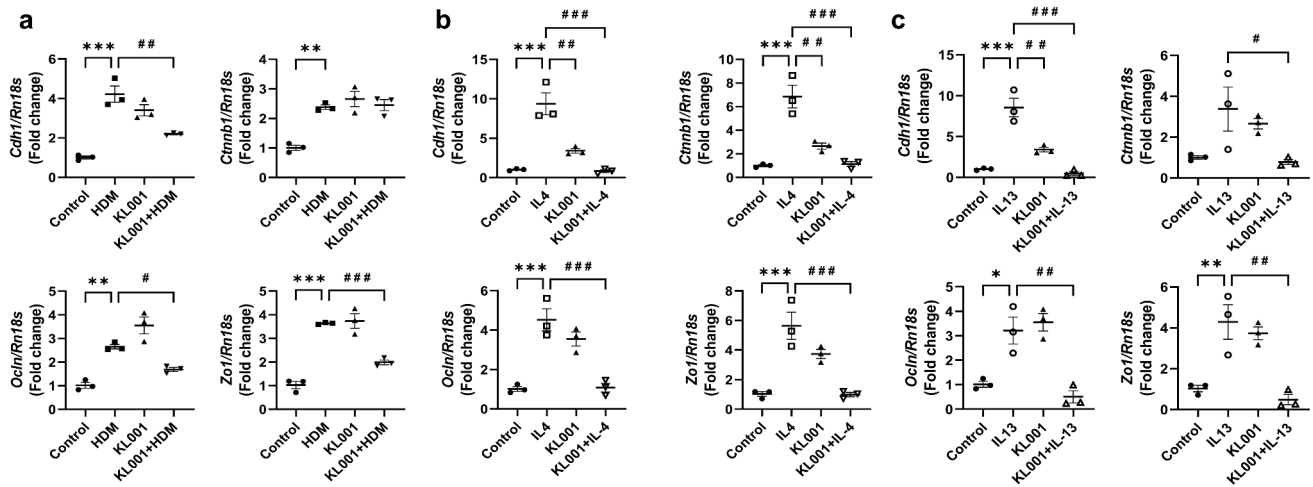


Figure 4. KL001 treatment attenuates HDM/Th2 cytokine (IL-4 and IL-13)-induced change in mRNA expression of adherens junction complex and tight junction protein genes in 16-HBE cells. Note: (a) HDM, (b) IL-4, and (c) IL-13 treatment altered mRNA expression of AJC protein genes (*Cdh1/E-cadherin* and *Cttnb1/ β -catenin*) and TJ protein genes (*Ocln*, *Zo1/Tjp1*), post-24 hrs compared to untreated control. KL001 pre-treatment (for 4 hrs) restored HDM/Th2 cytokine-induced change in transcript levels of selected AJC and TJ protein genes in 16-HBE cells. Relative gene expression of target genes was determined by the $2^{-\Delta\Delta C_t}$ method with *Rn18s* as the housekeeping gene. Data are shown as mean $\pm$ SEM ($n = 3$ /group; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, significant compared to untreated control; # $P < 0.05$, # $P < 0.01$, # $P < 0.001$, significant compared to KL001 or KL001+HDM/KL001+IL-4/KL001+IL-13 groups; One-way ANOVA with Tukey's multiple comparison test).

increased the protein abundance of AJC (E-CADHERIN) but reduced TJP (OCCLUDIN and ZO1) compared to the control (Supplementary Figure S3A-B). KL001 alone treatment showed slightly reduced protein abundance in AJC β -CATENIN (but not E-CADHERIN), TJP OCCLUDIN, and CLAUDIN 4 (but not ZO1) compared to the control. KL001+HDM treatment showed comparable levels of AJC and TJP protein abundance relative to the control. Th2 cytokine IL-4 and IL-13 treatment reduced the protein abundance of both AJC (E-CADHERIN and β -CATENIN) and TJP (OCCLUDIN, CLAUDIN 4, and ZO1) differentially compared to the control (Supplementary Figure S3A-B). However, KL001+IL-4 and KL001+IL-13 treatment showed relatively reduced protein abundance of E-CADHERIN (AJC) and CLAUDIN 4 (TJP) compared to IL-4 alone group. Similar trends were observed in the protein abundance of β -CATENIN and ZO1 in KL001+IL-13 compared to IL-13 alone group (Supplementary Figure S2A-B). Overall, we found E-CADHERIN and CLAUDIN 4 were significantly increased at the protein level in HDM-treated group, and E-CADHERIN, β -CATENIN, and CLAUDIN 4 protein abundance was significantly decreased in IL-13-treated group compared to the control.

HDM and Th2 cytokine-induced epithelial barrier dysfunction attenuation by KL001 in 16-HBE cells affects the mRNA expression and protein levels of core circadian clock targets

It is unclear whether HDM and Th2 cytokine treatment for 24 hrs affects the mRNA and protein levels of core circadian clock targets in 16-HBE cells, thereby contributing to epithelial barrier dysfunction. We evaluated whether HDM and Th2 cytokine affects circadian clock targets and pre-treatment with KL001 before HDM or Th2 cytokine treatment attenuates the altered core clock genes (CCGs) at the mRNA and protein level by qRT-PCR and Western blot analysis. HDM/Th2 cytokine (IL-4/IL-13) treatment significantly increased mRNA expression of several CCGs such as *Clock*, *Bmal1*, *Cry1*, *Cry2*, *Per1*, *Per2*, *Nr1d1*, *Nr1d2*, and *Nfil3* compared to the control (Figure 5a-c and Supplementary Figure S4(A-C)).

Rora is the only gene not significantly affected by both HDM and Th2 cytokine treatment compared to the control. Interestingly, KL001 alone treatment showed attenuation in mRNA expression of selected genes (reduced expression of *Cry1*, *Cry2*, and *Per2*) compared to HDM/Th2 cytokine-treated group. Pre-treatment with KL001 for 4 hrs before HDM/IL-4/IL-13 treatment significantly reduced mRNA expression of most CCGs (*Clock*, *Bmal1*, *Cry1*, *Cry2*, *Per1*, *Per2*, *Nr1d1*, *Nr1d2* (except KL001+HDM), and *Nfil3*) compared to HDM/IL-4/IL-13 alone treated group (relative to the mRNA levels observed in untreated control) (Figure 5a-c and Supplementary Figure S3(A-C)).

Additionally, HDM/Th2 cytokine treatment for 24 hrs in 16-HBE cells analyzed by Western blotting revealed altered protein abundance of circadian clock targets compared to control (Supplementary Figure S5A-B). We found that HDM treatment significantly reduced CLOCK protein abundance and slightly reduced both BMAL1 and REV-ERBa compared to the control. However, the protein abundance of CRY1/2 was not affected in HDM-treated group. IL-4/IL-13 treatment showed reduced protein abundance in CLOCK, BMAL1, and CRY2 but increased protein levels of REV-ERBa (except in IL-13) and CRY1 compared to the control (Supplementary Figure S5A-B). KL001 alone treatment showed reduced protein abundance of CLOCK, BMAL1, and REV-ERBa compared to the control. Interestingly, CRY1 was slightly increased and CRY2 remained relative to control in KL001-treated group (Supplementary Figure S5A-B). KL001+HDM treatment showed increased protein abundance of CLOCK, BMAL1, and CRY1 but reduced CRY2 compared to HDM-treated group. KL001+IL-4 treatment increased the protein abundance of CLOCK, BMAL1, and CRY1 compared to IL-4-treated group. Furthermore, KL001+IL-13 treatment showed increased protein abundance in REV-ERBa, and CRY1 compared to IL-13-treated group (Supplementary Figure S5A-B). Overall, small-molecule KL001 pre-treatment along with HDM or Th2 cytokines showed a differential response in stabilizing core clock targets at the transcriptional and translational level that may have a potential role in maintaining their homeostasis, thereby attenuating epithelial barrier function in 16-HBE cells.

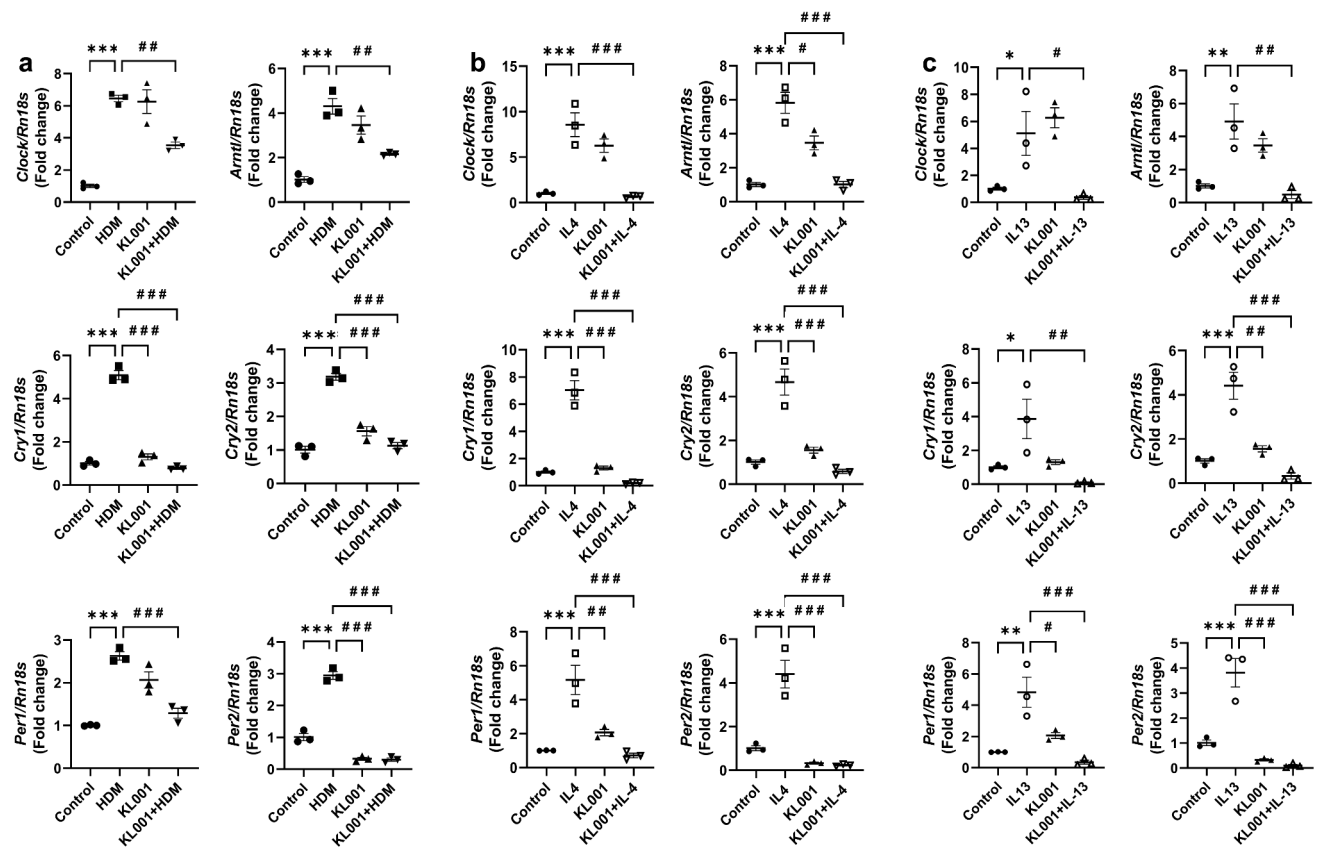


Figure 5. KL001 treatment attenuates HDM/Th2 cytokine (IL-4 and IL-13)-induced change in mRNA expression of circadian clock genes in 16-HBE cells. Note: (a) HDM, (b) IL-4, and (c) IL-13 treatment altered mRNA expression of core clock genes (*Clock*, *Bmal1*, *Cry1*, *Cry2*, *Per1*, and *Per2*) post-24 hrs compared to untreated control. KL001 pre-treatment (for 4 hrs) attenuated HDM/Th2 cytokine-induced change in transcript levels of selected clock genes in 16-HBE cells. Relative gene expression of target genes was determined by the $2^{-\Delta\Delta C_t}$ method with *Rn18S* as the housekeeping gene. Data are shown as mean $\pm$ SEM ($n = 3/\text{group}$; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, significant compared to untreated control; # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$, significant compared to KL001 or KL001+HDM/KL001+IL-4/KL001+IL-13 groups; One-way ANOVA with Tukey's multiple comparison test).

Discussion

Airway epithelial cells through AJC and TJP maintain barrier integrity of the lung epithelium by protecting against environmental particles/dust/infectious agents entering the respiratory tract. Disruption of the epithelial barrier makes the airway vulnerable to foreign particles resulting in various pathological conditions mediated by the release of cytokines/chemokines from epithelial cells. Patients suffering from chronic lung diseases such as asthma and allergic rhinitis are extremely sensitive to allergens, air pollution (PM_{2.5} and PM₁₀), and commercial dust due to impaired barrier function.^{5,6,32,33}

The 16-HBE cells derived from the human airway have been widely used to study various airway-related pathophysiological processes and

mechanisms. Through the formation of adherens junctions (AJs), and tight junctions (TJs), epithelial cells maintain the barrier integrity of the respiratory tract. Localization of AJC and TJP is important in maintaining the airway epithelial resistance, thus controlling the paracellular permeability. Changes in TJP localization can lead to altered epithelial resistance and loss of paracellular permeability. *Staphylococcus aureus* exposure showed a direct association between delocalized ZO1 and reduced TEER in nasal epithelial cells.³⁴ Similarly, pollens have been shown to reduce E-cadherin, Occludin, ZO1, and Claudin 1 resulting in compromised epithelial barrier function.^{35–37} Low molecular weight components of pollen differentially affect ionic permeability and innate cytokine production in bronchial epithelial cells.³⁸

Studies have demonstrated that airborne allergens such as HDM induce leaks in the epithelial barrier, leading to an increased inflammatory response that drives allergic diseases such as asthma and allergic rhinitis.^{5,6,32} Similarly, Th2 cytokines IL-4 and IL-13 have been reported to disrupt the epithelial barrier integrity in 16-HBE cells, leading to a progressive reduction in TEER values between 24–72 hrs.^{3,39,40} In this study, we noticed that treatment with HDM/Th2 cytokines significantly reduced the cell impedance as early as 4 hrs, with maximal reduction noted between 24–48 hrs. HDM or Th2 cytokine-induced reduction in cell impedance is due to changes in the mRNA and protein abundance of AJC/TJP and altered expression of core clock genes (CCGs) that may be essential to regulate homeostatic function in epithelial cells. Interestingly, KL001 pre-treatment attenuated the HDM/Th2 cytokine-induced barrier dysfunction at 12- and 24 hrs post-treatment, possibly via stabilizing CRY-dependent regulation of AJC/TJP in 16-HBE cells as evidenced by immunostaining and confocal microscopy.

Bronchial brushings from asthmatics revealed reduced expression of clock genes (*Bmal1*, *Npas2*, *Per2*, *Dbp*, *Nr1d1*, and *Nr1d2*) compared to healthy controls.⁴¹ Patients with nocturnal asthma even with controlled asthma showed relatively lower levels of *Bmal1*, *Clock*, *Cry1*, *Cry2*, and *Per1* compared to asthmatics without nocturnal symptoms.²² These studies indirectly suggest that there may be an underlying clock-controlled mechanism that contributes to this observed phenotype that relates to compromised epithelial barrier function in asthmatics. A prior *in vivo* study has shown that LPS-induced inflammatory response was higher at CT0 compared to CT12 with increased infiltration of immune cells in the BAL fluid.⁴² The observed time-of-day response was completely abolished in LPS-treated *Bmal1* KO mice, suggesting a potential role of BMAL1 in the regulation of lung inflammatory response.⁴² Using immunohistochemistry and immunofluorescence staining from both mouse and human lungs revealed abundant protein expression of CLOCK and PER2 in the nuclei surrounding the bronchioles (airway) and relatively less in the alveolar epithelium.²¹ Enhanced response to LPS in the lung, irrespective of the time of day, was

observed in lung epithelial cell-specific *Bmal1* KO compared to WT.²⁰ In an ovalbumin-induced allergic inflammation model myeloid cell-specific *Bmal1* KO mice showed increased lung eosinophilia along with augmented Th2 cytokines in the lungs.¹¹ In this study, we noted that HDM and Th2 cytokine treatment significantly increased several core clock genes including *Bmal1* expression compared to untreated control, possibly contributing to the compromised inflammation-induced epithelial barrier dysfunction. Pre-treatment with KL001 (CRY activator/stabilizer) not only reduced the *Bmal1* expression but also stabilized the expression of other core clock genes, selected AJC, and TJP genes in 16-HBE cells. These results indicate that stabilizing CRY may have potentially reduced the HDM/Th2 cytokine-induced epithelial barrier dysfunction via regulating core clocks (CLOCK:BMAL1) and CCGs (*Cry1/2*, *Per1/2/3*, and *Nr1d1/2*) that directly influence inflammatory signaling pathways in the airway epithelium. Maintaining the expression of core clocks at an optimal level might be crucial in regulating inflammation-dependent cellular and physiological processes in bronchial epithelium, which needs further investigation using cell type-specific KO models.

Cry1/2 knockout (KO) mice develop severe arthritis with increased CD3⁺ and CD69⁺ T lymphocyte activation and increased TNF α secretion by splenocytes. In addition, *Cry1/2* KO mice had increased TNF α , IL-1 β , IL-6, and matrix metalloproteinase-3 (MMP3) in the serum.⁴³ They also demonstrated that CRY regulates TNF α , by upregulating promoter activity of TNF α in *Cry1/2* KO mouse embryonic fibroblasts, leading to a more proinflammatory state. Similarly, another report showed that decreased NF- κ B activation and CRY protein deficiency promotes the production of proinflammatory cytokines and increases sensitivity to TNF α -induced apoptosis.⁴⁴ Altogether, these results indicate that CRY protein stabilization can potentially reduce proinflammatory response. The KL001-mediated protection against HDM and Th2 cytokine-induced epithelial barrier dysfunction observed in this study may be due to a similar mechanism via regulating the inflammatory response in 16-HBE cells, which remains unexplored in the present study.

Allergen exposure induces Th2 cytokine response, subsequently promoting TGF β -mediated epithelial-to-mesenchymal transition (EMT) in the airway⁴. Similarly, the Wnt/ β -catenin pathway has been shown to induce EMT via TGF β activation, leading to increased airway inflammation and remodeling in asthma.⁴⁵ Additionally, β -catenin has been shown as a master regulator of epithelial barrier function that affects both circadian clock genes and MMPs (MMP2 and MMP9), thereby reducing tight junction proteins and resulting in increased intestinal permeability in the gut.⁴⁶ In this study, HDM and Th2 cytokine treatment did not affect the protein levels of β -catenin compared to the control. However, HDM/Th2 cytokine significantly increased the transcript levels of β -catenin, and pre-treatment with KL001 attenuated the Th2 cytokine-induced alterations, relative to untreated control. We are not sure if similar signal transduction pathways are affected concordantly during HDM and Th2 cytokine-induced altered epithelial barrier function in the airway epithelium. Future studies will investigate the potential role of KL001 pre-treatment on other transcription factors and regulators that directly affect EMT signaling targets in the presence of HDM and Th2 cytokine treatment in bronchial epithelial cells.

Rora is a transcription factor that competitively binds to the RORE to regulate *Bmal1* expression. REV-ERBa binding to the RORE inhibits the expression of *Bmal1*. A prior report showed that *Rora* plays an essential role in OVA-induced lung inflammation in mice.⁴⁷ In this study, we found gene expression of *Rora* was slightly decreased in the KL001+HDM group but increased in the KL001+IL-4 group. Additionally, we observed KL001 pre-treatment significantly increased *Rora* expression in IL-13-treated group. However, the exact mechanism by which CRY stabilization during IL-13 treatment augments *Rora* expression remains unclear. We speculate that KL001-mediated CRY stabilization might have influenced core clock CLOCK:BMAL1 expression, thereby affecting output genes including *Rora* as a result of the regulatory feedback mechanism in bronchial epithelial cells. The exact mechanism by which *Rora* is affected by CRY stabilization

using KL001 requires further investigation. CRY stabilization-mediated protection in HDM and Th2 cytokine-induced epithelial barrier function may also be due to direct or indirect regulation of inflammatory mediators (TNF α and CXCL5 or other cytokines/chemokines) in epithelial cells, as reported previously.^{24,25}

Asthma is a chronic inflammatory lung disease that is under the regulation of the circadian clock.⁹ Efforts are underway to find novel clock-based chronotherapeutics for the treatment/management of asthma and other chronic inflammatory lung diseases.⁴⁸ CRY is one of the core clock gene that remains unexplored, with the potential to attenuate HDM-induced epithelial barrier dysfunction. Consistently throughout the experiments using TEER, immunofluorescence, and protein expression, we found that stabilizing CRY protein abundance in 16-HBE cells reversed the HDM and Th2 cytokine-mediated change in epithelial barrier integrity.

KL001, a carbazole scaffold, inhibits ubiquitin-mediated CRY degradation by prolonging the repression of BMAL1. KL001 treatment in models, *in vitro* and *in vivo*, demonstrates anti-glycemic, anti-inflammatory, and anti-cancer properties.^{24,27,49} However, the effects of CRY stabilizer on other clock genes (other than CRY1/2) and whether it can attenuate HDM and Th2 cytokine-induced epithelial barrier dysfunction has not been investigated to date. Our study for the first time shows KL001-mediated protection against HDM and Th2 cytokine-induced epithelial barrier dysfunction via modulation of both AJC and TJ proteins/genes and core circadian clock genes. These results indicate that KL001 through CRY stabilization may be used as a therapeutic candidate to alleviate airway inflammation by regulating the circadian clock and has the potential to treat chronic conditions associated with circadian disruption (e.g., asthma and allergic rhinitis).

In summary, we showed that small-molecule CRY stabilizer KL001 may be a novel modulator that can attenuate epithelial barrier dysfunction, which has implications for the treatment of allergen/Th2 cytokine-induced allergic immune response. It may be possible that the small-molecule KL001-mediated attenuation of HDM and/or Th2 cytokine-induced epithelial barrier

dysfunction is due to its ability to repress proinflammatory cytokines/chemokines by regulating intracellular kinase signaling and/or stabilizing the AJC and TJ protein expression in 16-HBE cells needs further investigation. Overall, we showed, for the first time, that circadian cryptochrome modulator KL001 can attenuate HDM- and Th2 cytokine-induced epithelial barrier dysfunction, which may have broad implications for the development of new therapeutics to treat chronic lung diseases such as asthma that needs to be further explored.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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Author contribution

SKD, AS, IKS: conception and design of the *in vitro* study and performed experiments.

SKD, IKS: Data analysis, interpretation, and manuscript drafting.

SKD, AS: acquisition of data, and partly conducted the experiments within the study.

All authors have checked the revised manuscript and approved this for publication.

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