The circadian protein BMAL1 in myeloid cells is a negative regulator of allergic asthma


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Running head: BMAL1 inhibits asthma
Abstract

Our body clock drives rhythms in the expression of genes that have a 24-hour periodicity. BMAL1 is a transcription factor, which is a crucial component in the molecular clock. A number of physiological processes, including immune function are modulated by the circadian clock. Asthma is of particular relevance to the area of circadian control of immunity, since it is a disease with very strong clinical evidence demonstrating regulation by circadian variation. Airway hypersensitivity and asthma attacks are more common at night in humans. The molecular basis for this is unknown and no model of asthma in animals with genetic distortion of the molecular clock exists. In our study we have used mice lacking BMAL1 in myeloid cells (BMAL1-LysM⁻⁻) to determine the role of BMAL1 in allergic asthma. Using the Ovalbumin model of allergic asthma we demonstrated that BMAL1-LysM⁻⁻ mice have markedly increased asthma features such as increased lung inflammation demonstrated by drastically higher numbers of eosinophils and increased IL-5 levels in the lung and serum. *In vitro* studies demonstrated that IL-4 as well as LPS treated macrophages from BMAL1-LysM⁻⁻ mice have increased pro-inflammatory chemokine and mannose receptor expression compared to wild type controls. This suggests that Bmal1 is a potent negative regulator, in myeloid cells in the context of allergic asthma. Our findings might explain the increase in asthma incidents during the night when BMAL1 expression is low.
Introduction

Life on earth follows a daily 24-hour rhythm that impacts on a range of behaviors such as activity and feeding. Alterations in physiology that peak and trough in a 24 h timescale are classified as circadian and are caused by oscillations in the proteins of the molecular circadian clock (9, 18). Circadian oscillations of immune mediators coincide with the activity and trafficking of the immune system (12), helping to promote tissue recovery and handle microbial threats more efficiently (35). Disruption of circadian rhythms has been linked to inflammatory pathologies and recent studies have identified the critical importance of the 24 h cycle on immune function (19).

BMAL1 is the main orchestrator of the molecular clock, as it is the only single clock gene deletion that leads to complete ablation of the molecular clock, resulting in a loss of all rhythmic behavioral activity (5). The molecular clock exists in immune cells (36) including macrophages, where it plays key functions involving control of inflammatory responses (14, 24). Use of a genetic approach to disturb the molecular clock made it possible to study a plethora of inflammatory disorders regulated by circadian rhythms, such as sepsis, allergies, rheumatoid arthritis or atherosclerosis (7).

Although strong clinical evidence exists linking asthma with the circadian clock (15, 22, 28, 33, 41), no animal model of asthma has been shown to have circadian features. The importance of BMAL1 in lung inflammation has been previously shown and attributed to structural cells, namely epithelial cells (16).
However no study has examined BMAL1 deficient immune cells in the context of asthma. Since alveolar macrophages (AMs) are key orchestrators of pulmonary immune responses (21) and have crucial role in a mouse model of asthma (46), we have decided to use myeloid cell-specific deletion of Bmal1. Macrophages from mice lacking BMAL1 in myeloid cells exhibit augmented responses to LPS (10). Moreover these mice have lost circadian rhythm of inflammatory monocytes, exhibit increased CCL2 production, and decreased survival in response to bacterial infection (31). The significance of BMAL1 in structural or immune cells has never been tested in a Th2 type disease such as asthma.

Asthma symptoms worsen at night, particularly in the early hours of the morning (13). Lung function fluctuates in healthy individuals over 24 h period and these fluctuations are even more pronounced in asthmatics (26). These changes are reflected in lung immune cell composition. Lung macrophages and eosinophils from alveolar space have circadian variation in numbers which correlate with lung function (26). In fact, even during homeostasis, blood eosinophils demonstrate circadian cycling (17), which correlate with serum IL-5 levels (32). The role of BMAL1 in the regulation of eosinophil numbers and function in asthma has never been addressed.

We demonstrated here for the first time that BMAL1 expression in myeloid cells inhibits allergic asthma. We have observed a significant increase in lung eosinophila in mice lacking BMAL1 in myeloid cells, which correlates with asthma severity. Overall our study shows an inhibitory role of BMAL1 expressed by myeloid cells in asthma and suggests that the low expression of BMAL1 at
night might explain the well-known nocturnal increase in asthma symptoms in humans.
Materials and Methods

Animals

BMAL1-LysM−/− mice and corresponding wild type control mice were bred under specific pathogen–free conditions in Trinity Biomedical Science Institute animal facility. Mice with the gene Bmal1 containing loxP sites at either side of exon 8 were originally obtained from Jackson Labs (stock no. 007668) and are on C57BL/6J background. For experiments we have used male mice between 6 to 12 weeks of age. All experiments were carried out with prior ethical approval from the Trinity College Dublin Animal Research Ethics Committee.

Cell culture

Bone marrow–derived macrophages (BMDMs) were cultured in 20% L929 media until day 6, after which adherent fraction was collected. BMDMs were counted and re-plated on 12 well plates at concentration of 1x10^6 cells/mL in a volume of 0.5 mL.

Asthma protocol

OVA-induced acute allergic inflammation was elicited as previously described by i.p. sensitization with 20 μg OVA (Sigma-Aldrich, St. Louis, MO) mixed with 2 mg alum (Thermo Fisher Scientific, Waltham, MA) (14) on day 0, followed by two nebulizer-delivered airway challenges with 1% OVA on days 7 and 8 (15). Sensitization, as well as airway challenge phase was performed in the morning when BMAL1 expression was highest in wild type mice and therefore the difference in BMAL1 expression between the two genotypes was greatest. Mice were sacrificed and samples were collected at day 9. Similarly to sensitization
and airway challenge, sample collection was performed in the morning to ensure high BMAL1 expression. These acute allergic inflammation protocols resulted in eosinophilic accumulation measured in bronchoalveolar lavage fluid (BALF).

**Isolation of cells from BALF**

Lung lavage was performed according to a previously described protocol (20) employing 800-μl aliquots of sterile PBS/2 mM EDTA (pH 7.2) until a BALF volume of 3 ml was recovered. Total cells from BALF were suspended in PBS and counted by light microscopy. Aliquots were taken to prepare cytopsins for H&E staining and differential counting, which was performed on 100 cells from each mouse.

**Isolation of cells from lung digests and cell sorting**

Single-cell suspensions from lung digests were prepared as described in detail previously (19). Briefly, lungs were perfused with PBS, then removed and transferred into petri dishes containing 0.7 mg/ml collagenase A (Roche Diagnostic, Indianapolis, IN) and 50 μg/ml DNase I (Roche) in RPMI 1640 medium. Lungs were minced and cut into small pieces, agitated on a shaker (30 min, room temperature), and then incubated at 37°C for 30 min in a humidified atmosphere containing 5% CO2. Cell aggregates were dispersed by repeated passage through a syringe and filtered through a 70-μm cell strainer (BD Biosciences, San Jose, CA) to obtain a single-cell suspension. Erythrolysis was performed with 10 ml 0.8% ammonium chloride lysis buffer, and cells were subsequently washed, suspended in PBS/2 mM EDTA/0.5% FCS, counted, and subjected to flow cytometric staining. Fc receptor–mediated and nonspecific Ab
binding was blocked by addition of excess CD16/CD32 (BD Pharamingen, San Jose, CA). Staining for cell surface receptors was performed at 4°C in the dark for 15 min with previously established dilutions. Cell sorting was performed with a BD FACSAria Fusion High Performance Cell Sorter. Purity of sorted cells was ≥ 98% as determined by flow cytometry. Following murine antibodies were used in the study: CD11c-PerCP/Cy5.5 (N418; BioLegend, San Diego, CA); CD11b-FITC (M1/70; BD Pharminogen); Siglec F-PE (E50-2440; BD Pharminogen), CD45-APC (30-F11, BioLegend).

**RNA Isolation and Quantitative Real-Time PCR**

RNA extraction was performed using the RNeasy Mini Kit (QIAGEN), and cDNA was generated using the Applied Biosystems High-Capacity cDNA Archive Kit. The qRT-PCR analysis was performed with a 7500 Fast Real Time PCR System (Applied Biosystems). Reactions were set up with the SYBR™Green PCR Core Reagents (Invitrogen). Data were normalized to beta-actin and mRNA expression fold-change relative to controls was calculated. Following intron-spanning murine primer sequences used were: beta-actin, 5'-ACC CTA AGG CCA ACC GTG A-3' (f), 5'-CAG AGG CATA CAG GGA CAG CA-3' (r); ccl2, 5'-AGC ATC CA C GTG TTG GCT C-3' (f), 5'-CCA GCC TAC TCA TTG GGA TCA T-3' (r); muc5ac, 5'-AAA GAC ACC AGT AGT CAC TCA GCA A-3' (f), 5'- CTG GGA AGT CAG T GT CAA ACC A-3' (r); mrc1, 5'-GGC GAG CAT CAA GAG TAA AGA-3' (f), 5'-CAT AGG TCA GTC CCA ACC A-3' (r); cxcl10, 5'-TTT CTG CCT CAT CCT GCT G-3' (f), 5'- TCC CTA TGG CCC TCA TTC T-3' (r); bmal1, 5'-TGC AAT GTC CAG GAA GTT AGA T-3' (f), 5'-GTT TGC TTC TGT GTA TGG GTT G-3' (r).
The quantification of murine IL-5 protein was performed by commercially available ELISA kit following the instructions of the manufacturer (R&D Systems, Minneapolis, MN).

**Statistical analysis**

Data are presented as mean ± SEM. Statistical significance was analyzed using the GraphPad Prism 5.0 statistical program (GraphPad Software, La Jolla, CA). Comparisons between two experimental groups were performed using the Student *t* test. A *p* value < 0.05 was considered statistically significant.
Results

BMAL1-LysM-/- mice display increased eosinophilic inflammation and IL-5 levels in a model of allergic asthma

We first examined allergic lung inflammation in mice where BMAL1 is removed from the myeloid lineage (BMAL1-LysM-/-) (10), using the previously described (46) Ovalbumin (OVA) induced model of acute lung inflammation (Fig. 1A) resulting in predominantly eosinophilic infiltration. Mice were subjected to the OVA asthma model and lungs were examined for leukocyte infiltration at day 9 of the protocol. BMAL1-LysM-/- mice compared to wild types had significantly higher amount of leukocytes in lavage fluid (Fig. 1B). Upon histological examination we have determined that eosinophils were significantly higher in BALF from BMAL1-LysM-/- lungs, while AM numbers remained unchanged (Fig. 1C and D). Neutrophils and lymphocytes accounted for <3% of total cells in OVA-challenged lungs and did not change between genotypes (data not shown). IL-5 is a critical effector in asthma and key activator of eosinophils. We went on and demonstrated that asthmatic BMAL1-LysM-/- mice had increased levels of IL-5 in the lung (Fig. 2A), as well as systemically as evidenced by elevated serum IL-5 levels (Fig. 2B). Splenocytes were isolated at day 9 of the protocol and subsequently cultured in vitro for 3 days with varying concentrations of OVA. Splenocytes from asthmatic BMAL1-LysM-/- mice had increased IL-5 production (Fig. 2C) suggesting enhanced allergen sensitization. Taken together we
conclude that BMAL1 expression in myeloid cells inhibits eosinophil accumulation and IL-5 expression in allergic lung inflammation.

**Characterization of eosinophils from asthmatic BMAL1-LysM$^{-/-}$ mice**

We wanted to determine if BMAL1 was deleted in eosinophils, which are of myeloid lineage, in our BMAL1-LysM$^{-/-}$ mice. Since eosinophils under steady state conditions are in very low numbers, we examined their BMAL1 expression in asthmatic mice, where eosinophil numbers are significantly elevated. We have collected samples in the morning when BMAL1 expression is highest. Eosinophils and AMs were flow sorted from lung digests of asthmatic BMAL1-LysM$^{-/-}$ and corresponding controls mice to high purity (Fig. 3A). WT AMs expressed BMAL1 while BMAL1-LysM$^{-/-}$ AMs had significantly diminished bmal1 expression (Fig. 3B). Expression of BMAL1 in eosinophils from both genotypes was very low and not significantly different between the two genotypes (Fig. 3B). Eosinophils from BMAL1-LysM$^{-/-}$ lungs had higher expression of major airway mucin-muc5ac compared to WT controls, while AMs from both genotypes lacked muc5ac expression (Fig. 3B). High muc5ac expression correlated with low BMAL1 (Fig. 3B). These data indicates that eosinophils from asthmatic BMAL1-LysM$^{-/-}$ and WT mice are genotypically different with eosinophils from BMAL1-LysM$^{-/-}$ mice having high muc5ac expression. Alternatively, the difference in eosinophil expression of muc5ac between two genotypes could be due to the inflammatory signals such as IL-5, which are enhanced in asthmatic BMAL1-LysM$^{-/-}$ mice. Increased severity of asthma in BMAL1-LysM$^{-/-}$ mice might therefore not only be attributed to higher eosinophil numbers, but also to their
more inflammatory phenotype. Since expression of BMAL1 in eosinophils is very
low and similar between BMAL1-LysM−/− and WT, augmented eosinophil numbers
and their inflammatory phenotype in BMAL1-LysM−/− asthmatic mice might be
regulated indirectly by macrophages.

BMAL1-LysM−/− macrophages have augmented expression of proinflammatory
chemokines

We next characterized further macrophages from BMAL1-LysM−/− mice. Bone
marrow derived macrophages were isolated from BMAL1-LysM−/− mice and
stimulated with LPS to assess chemokines relevant to asthma, namely CCL2 and
CXCL10 (Fig. 4). Expression levels of both CCL2, responsible for recruitment of
monocytes in allergic inflammation (1), and CXCL10, which is known to activate
of eosinophils (42) and mast cells in asthma (4), were significantly higher in
macrophages from BMAL1-LysM−/− mice. Moreover expression of mannose
receptor, which is associated with asthma (20), was increased in BMAL1-LysM−/−
macrophages when stimulated with IL-4 (Fig. 4). These experiments suggest that
BMAL1-LysM−/− macrophages are not only more responsive to typical M1 (LPS)
stimulation, but also M2 (IL-4). This observation is relevant in diseases such as
asthma, where a mixture of AMs with M1 and M2 polarization states exists (29).
Discussion

In our study we have demonstrated that BMAL1 expression specifically in myeloid cells is a negative regulator of allergic lung inflammation. These findings are especially relevant clinically since BMAL1 is a major component of the molecular clock, and asthma is a disease where chronotherapy has practical applications (13). We provide evidence for the BMAL1 pathway as a novel target for pharmacological management of asthma.

BMAL1 expression in myeloid cells inhibits eosinophil accumulation and IL-5 expression in allergic lung inflammation. Since eosinophils themselves are of myeloid origin we examined whether dramatically augmented numbers as well as inflammatory phenotype of eosinophils in BMAL1-LysM<sup>-/-</sup> asthmatic mice are directly regulated by expression of their BMAL1 or indirectly by macrophages. Additionally blood eosinophils from healthy and allergic subjects were demonstrated to have circadian variations, which include rhythmic expression of BMAL1 (2). We wanted to examine the characteristics of functional eosinophils which left circulation and entered lung tissue. Although lung eosinophilia is a hallmark of allergic asthma, eosinophils are extremely rare in the lung at baseline (34). Therefore we were able to assess BMAL1 expression only in asthmatic mice. Since BMAL1 levels in eosinophils were very low and not different between BMAL1-LysM<sup>-/-</sup> and wild type mice we concluded that eosinophil numbers and phenotype is controlled indirectly by macrophages or possibly other myeloid cells. Neutrophils being another relevant myeloid cell type can also be involved,
however since they were found in BALF in low numbers we were not able to examine their phenotype, and therefore focused on alveolar macrophages. We therefore speculate that a major immune mediator candidate for crosstalk between macrophages and eosinophils responsible for regulating eosinophil numbers and function is IL-33. *In vivo* as well as *in vitro* IL-33 induces IL-5 production and eosinophilia (37) and recently was shown to be critical for eosinophil development and homeostasis (23). Future studies should also assess numbers and phenotype of Type 2 innate lymphoid cells in asthmatic BMAL1-LysM−/− mice, which were demonstrated to control eosinophil homeostasis (32) and are key component of allergic asthma (11).

Most molecular clock knockout studies performed in mice have focused on Th1 type of inflammation (7), however the need to study allergic diseases is evident by symptoms that exhibit prominent 24-hour variations (38). Although no asthma model was examined in mice with genetic distortion of molecular clock, some allergic diseases have been assessed in animal models. Mast cells intrinsic clock has been found to regulate IgE production (30), and CLOCK mutant mice had exacerbated contact hypersensitivity (44). Importantly BMAL1-LysM−/− mice used in our study did not have altered expression of CLOCK gene (data not shown). The OVA model of food allergy was a first study demonstrating that antigen exposure in the late light period induced more severe symptoms of disease (45). Our OVA model examined allergic asthma, which is another Th2 type of disease, and similar to food allergy is affected significantly by environmental stimuli, which
alter molecular clock gene expression (40). Our study therefore identifies BMAL1
as potential target for therapeutic intervention.

Data presented in this study highlight the relevance of myeloid cells in allergic
asthma and provide a good rationale to examine circulating myeloid cells from
asthmatic patients. Studying peripheral blood cells provides a readily accessible
way to evaluate expression of peripheral clock genes in humans (6). Oscillations
in molecular clock genes in peripheral blood mononuclear cells have been
reported previously in human populations (3, 25, 43), and can serve as an
alternative to future animal asthma models in dissecting chronobiology of
asthma. As depicted in Fig. 4 BMAL1 has the capacity to inhibit both LPS and IL-
4 driven macrophage responses, therefore we expect that a broad range of
inflammatory disorders will be affected by BMAL1 expression. This data identifies
BMAL1 as a very potent immune regulator and future studies will likely discover
further functions for BMAL1 outside the clock.

BMAL1 controls fundamental aspects of the innate immune response (8). In fact,
8% of the macrophage transcriptome undergoes a circadian variation (24).
Importantly, the TLR4 pathway, including genes involved in all aspects of the
TLR4 response, is under circadian control leading to clock-controlled enhanced
sensitivity and immunosurveillance (8). This includes chemokines and leukocyte
trafficking, which were exacerbated in our model. LPS is known to exacerbate
asthma features (27, 39), therefore BMAL1 may negatively regulate asthma by
inhibition of LPS responses. Although asthma features in humans are
exacerbated at night, in contrast to humans mice are nocturnal and active at
night. As evidenced by LPS study, murine and human immune responses are more severe at night indicating that perhaps light and not activity is more relevant for BMAL1 expression and function. Using the Ovalbumin model of allergic asthma we have demonstrated for the first time that mice with Bmal1-deficient myeloid cells have markedly increased asthma-associated inflammation, demonstrated by significantly greater recruitment of eosinophils and increased IL-5 levels in the lung and serum. Our results suggest that Bmal1 limits myeloid cell responses, which leads to inhibition of allergic asthma. The low expression of BMAL1 at night might explain the increase in asthma during the night.
References


the mannose receptor gene (MRC1) are associated with asthma in two independent populations. *Immunogenetics* 61: 731-738, 2009.


**Figure Description**

1. **Enhanced OVA-induced eosinophilic lung inflammation in BMAL1-LysM⁻/⁻ mice.** (A) Schematic representation of experimental protocol employing OVA sensitization and airway challenge phase (B) BALF numbers of total cells in mice subjected to OVA-induced asthma. Mice were sacrificed at day 9 of the protocol. Total cells from BALF were counted by light microscopy (C,D) differential cell count after Wright–Giemsa staining was performed on 100 cells. Data presented in all panels are expressed as the mean ± SEM from three experiments with two to three mice per group per experiment.*p < 0.05. WT-wild type, KO- BMAL1-LysM⁻/⁻

2. **Increased IL-5 levels in BMAL1-LysM⁻/⁻ asthmatic mice.** (A) BALF and (B) serum levels of IL-5 were measured by ELISA in mice from both genotypes subjected to OVA-induced asthma. (C) Splenocytes were isolated after second airway challenge and cultured for 3 days in the presence of OVA at 10 and 100 μg/ml concentration. Data presented in all panels are expressed as the mean ± SEM from three experiments with two to three mice per group per experiment.*p < 0.05, **p < 0.01. WT-wild type, KO- BMAL1-LysM⁻/⁻

3. **Expression of Bmal1 is very low in lung eosinophils and does not differ between wild type and BMAL1-LysM⁻/⁻ mice.** At day 9 of the protocol, mice were sacrificed, and lung digests were collected. After obtaining single cell suspension lung cells were stained for flow sorting. AMs were defined based on size and autofluorescence and as CD45⁺, CD11b⁻, SiglecF⁺, and CD11c⁺, eosinophils were defined as CD45⁺, CD11b⁺, SiglecF⁺, and CD11c⁻ cells.
AMs and eosinophils were sorted, assessed for purity and subjected to (B) RNA and (C) protein isolation protocols. Data in panel B is from two independent experiments with three mice per group. *p < 0.05, **p < 0.01. WT-wild type, KO-BMAL1-LysM−/−

4. BMAL1-LysM+/− BMDMs have augmented expression of inflammatory chemokines and M2 markers. BMDMs from both genotypes were stimulated with 100 ng/ml of LPS or 20 ng/ml of IL-4 for 24 h and mRNA was subjected to qPCR analysis. Data shown from 3-4 independent experiments **p < .01, ***p < .001 multiple comparison (Anova) WT-wild type, KO- BMAL1-LysM+/−
Fig. 1

A. 20μg OVA/2mg Alum (i.p.) 1% OVA in PBS (aerosol)

Sample collection

0 7 8 9

B. BALF

Total cells x million

WT KO

B. BALF

Total cells x million

WT KO

C. WT KO

D. BALF

Total cells x million

AM AM EOS EOS

Fig. 1
Fig. 2

A. IL-5 BALF

B. Serum IL-5

C. IL-5 splenocytes
Fig. 3

A) Purity sorting

B) muc5ac

C) Bmal