Dissertation

The role of the molecular circadian clock in asthma

submitted by

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Abbreviation

AHR	Airway hyperresponsiveness
BMAL1	Basic Helix-Loop-Helix ARNT Like 1
BMDM	bone marrow–derived
BMI	body mass index
CCL	C-C motif chemokine ligand
CCR	C-C chemokine receptor type
Crs	compliance
Cry	Cryptochrome
CXCL	C-X-C motif chemokine ligand
ECP	eosinophil cationic protein
EGFR	epidermal growth factor receptor
EPX	eosinophil peroxidase
ERK	extracellular signal-regulated kinase
Ers	elastic stiffness
FeNO	fractional exhaled nitric oxide
G	tissue dampening
GM-CSF	granulocyte macrophage colony-stimulating factor
H	tissue elastance
H/E	hematoxylin and eosin
i.n.	intranasally
i.p.	intraperitoneal
IC	isotype control
ICAM	intercellular adhesion molecule
ICS	inhaled corticosteroids
IFN-γ	interferon gamma
IL	interleukin
LABA	long-acting beta-agonist
LPS	lipopolysaccharide
LuFu	lung function
MAPK	mitogen-activated protein kinase

MBP	major basic protein
MHC	major histocompatibility complex
Mo-AMs	monocyte-derived macrophages
n.a.	not analysed
Per	Period
PPR	Pattern recognition receptor
PSGL-1	P-selectin glycoprotein ligand-1
Rn	Newtonian resistance
ROR	receptor-related orphan receptor
Rrs	respiratory system
SABA	short-acting beta-agonist
SCN	superchiasmatic nucleus
TCR	T-cell receptor
TGF-β	transforming growth factor beta
Th2	T-helper 2
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor-α

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Zusammenfassung

Asthma ist eine chronisch-entzündliche Atemwegserkrankung mit ausgeprägten tageszeitlichen Schwankungen der Symptomatik, was auf eine regulatorische Rolle der molekularen zirkadianen Uhr hinweist. Diese Arbeit untersucht den Einfluss der molekularen zirkadianen Uhr auf die Immunantwort und ihre pathogenesebedingten Veränderungen bei allergischen Erkrankungen und Asthma. Dabei richtet sich der Fokus auf Veränderungen in eosinophilen und neutrophilen Granulozyten, T-Zellen, Monozyten und Makrophagen.

Mittels Flowzytometrie wurde die Expression zirkadianer Proteine (BMAL1, CLOCK, REV-ERBs, RORs) in peripheren Leukozyten im Tagesverlauf bestimmt. Bei Asthmapatient:innen zeigten Eosinophile, Monozyten und T-Zellen signifikante Unterschiede in der Proteinexpression, wobei eine krankheitsabhängige Abnahme der Expression mit der Schwere der Erkrankung und klinischen Parametern wie z. B. der Lungenfunktion korrelierte. *In vitro* Versuche bestätigten, dass ein entzündliches Milieu zu einer Reduktion der zirkadianen Proteine bei Eosinophilen und polarisierten Makrophagen führt.

Als pharmakotherapeutische Intervention, wurde der inverse ROR Agonist SR1001 zunächst *in vitro* eingesetzt. SR1001 normalisierte die Expression der zirkadianen Proteine, unterdrückte proinflammatorische Zytokine, verbesserte die Phagozytoseleistung von Makrophagen und reduzierte die Migration von Eosinophilen von allergischen Spender:innen. Die Effekte in Eosinophile kamen durch eine Hemmung des C-C-Chemokinrezeptors Typ 3 und des Epidermal Growth Factor Receptor Signalwegs zustande. In experimentellen Modellen der allergischer Atemwegsentzündung führte SR1001 nach systemischer Gabe zu einer verminderten Infiltration von Immunzellen und einer verbesserten Lungenfunktion – ohne die biologischen Rhythmen der Versuchstiere zu beeinflussen.

Insgesamt wies die Arbeit auf eine pathogenesebedingte Veränderung der molekularen zirkadianen Uhr in Immunzellen bei Allergiker:innen und Asthmatiker:innen hin und identifizierte SR1001 als einen vielversprechenden Therapieansatz für Asthma und andere eosinophile Erkrankungen.

Abstract

Asthma is a chronic inflammatory airway disease that exhibits pronounced diurnal variation in symptom severity, suggesting a regulatory role for the molecular circadian clock (MCC). This thesis investigates the impact of the MCC and its disruption on immune responses in allergic diseases such as asthma, focusing specifically on eosinophils, neutrophils, T cells, and monocyte-derived macrophages.

Using peripheral blood samples, oscillating clock protein expression of the stabilizing loop (BMAL1, CLOCK, REV-ERBs, and RORs) was profiled across a 24-hour cycle. In asthmatic subjects, eosinophils, monocytes, and T cells displayed significant alterations in the expression range of circadian clock protein levels. A disease-dependent decrease was observed, suggesting a negative correlation between clock protein expression and disease severity and clinical parameters e.g., lung function. In vitro studies demonstrated that the inflammatory environment of eosinophils and polarized macrophages is reflected in reduced clock protein expression.

To explore the therapeutic potential of the MCC, the inverse ROR agonist SR1001 was applied in vitro. SR1001 restored clock protein expression and showed anti-inflammatory properties such as suppressing pro-inflammatory cytokine secretion, enhancing macrophage phagocytic function, and reducing the migratory responsiveness of eosinophils from allergic donors. The anti-inflammatory effects on peripheral eosinophils are mediated via inhibition of C-C chemokine receptor type 3 and epidermal growth factor receptor signalling. In vivo, systemic administration of SR1001 in murine models of allergic airway inflammation reduced immune cell infiltration and improved pulmonary function without disrupting biological rhythms.

Collectively, these findings show that the MCC in immune cells is disrupted during allergy and asthma. Furthermore, we identified SR1001 as a promising clock-modulating treatment strategy for eosinophilic inflammation and improved asthma outcomes.

1. Introduction

1.1 Circadian system

Over the past decade, there has been substantial progress in our understanding of biological rhythms regulated by the body clock. In recognition of their ground-breaking work on the molecular mechanisms controlling circadian rhythms, Jeffrey C. Hall, Michael Rosbash, and Michael W. Young were awarded the Nobel Prize in Physiology or Medicine in 2017 (3). The molecular circadian clock plays a pivotal role in regulating daily physiological processes, and increasing evidence highlights the significance of timing in the activation of our immune system by infection (4) or vaccination (5). Notably, many inflammatory diseases, such as asthma, exhibit pronounced time-of-day variations in symptoms, further linking these conditions to circadian biology.

1.1.1 Circadian rhythm

Circadian rhythms are self-sustained, oscillating patterns of behaviour and physiology following a 24-hour cycle (6). The most apparent of these rhythms include sleep-wake cycles (7) and appetite (8). Our ability to establish and maintain circadian rhythms enables adaptation and anticipation to environmental changes, and hence optimizing the survival (9). Circadian rhythms originate from a central pacemaker in the human brain, the superchiasmatic nucleus (SCN). This central clock responds to daily environmental cues, known as Zeitgeber, such as light, eating rhythm or physical activity. Light is considered the strongest Zeitgeber, rapidly and directly affecting the SCN (6,10). From the central clock signals are projected into the periphery via hormonal cues such as cortisol or melatonin, systematic cues like feeding rhythm, or via neural pathways. One primary mechanism of communication is hormonal signalling: glucocorticoids, notably cortisol, peak in the early morning under SCN and HPA-axis control to prime metabolic activity and wakefulness, while melatonin, secreted by the pineal gland at night, promotes sleep and signals the onset of the biological night. Neural pathways also contribute, allowing the SCN to influence peripheral physiology via the autonomic nervous system, modulating functions such as lung activity, thermoregulation, and heart rate by adjusting sympathetic tone. Additionally, systemic cues such as feeding rhythms serve as powerful zeitgebers, particularly for peripheral clocks in metabolic organs like the liver and pancreas (11). In almost every cell

type including immune cells residing in the lung, a peripheral circadian clock is present that generates the circadian rhythmicity (6,10) (**Figure 1**).

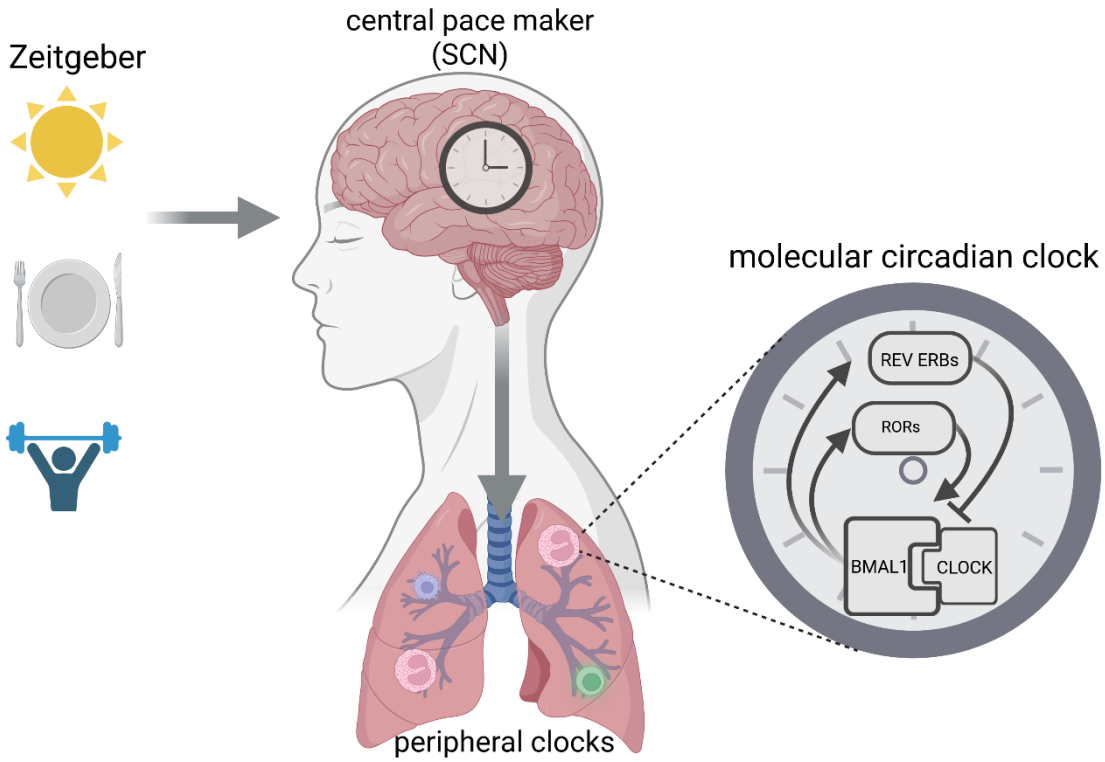

Figure 1: Circadian system. *The central clock (SCN) of the brain integrates light and dark information and relays the information downstream to the peripheral clocks by neural pathways, hormone release (glucocorticoids), and metabolic signals. The molecular circadian clock is found in almost every cell in the body consisting of interacting feedback loops including the stabilizing loop that is illustrated. This figure was created with BioRender.com.*

The peripheral clocks are autonomous; however, without the SCN as a central clock, rhythms in individual cells or tissues may become desynchronized. Disruptions within this circadian system significantly reduce our well-being and life quality and are implicated in the development of various disorders, including inflammatory diseases (12). For example, shift workers experience greater sleep disruption and have an increased risk of chronic metabolic diseases, cardiovascular diseases, and chronic inflammatory diseases such as asthma. As the cyclic occurrence of light and darkness entrains physiologic activity and is a Zeitgeber for circadian timing, artificial light enables the extension of the active period into the night. This mismatch between environment cues and the body clock can result in circadian misalignment. Thus, recent studies hypothesize a causal relationship between increased disease risk and the circadian disruption associated with shift work (13).

1.1.2 Molecular circadian clock

Circadian rhythms are generated by the molecular circadian clock, which consists of interacting transcriptional translational feedback loops. These loops are coordinated by two core transcription factors: clock circadian regulator (CLOCK) and Basic Helix-Loop-Helix ARNT Like 1 (BMAL1). BMAL1 is the main orchestrator of the molecular circadian clock as its deletion leads to a complete ablation of rhythmicity (14). By regulating numerous clock-controlled genes, BMAL1 drives the rhythmic expression of enzymes, receptors, and cytokines, thereby modulating various biological processes including the immune system.

The core loop, referred to as the activating loop, is driven by the transcription factors CLOCK and BMAL1, which promote the expression of the clock genes Period (Per1, 2) and Cryptochrome (Cry1, 2). As PER and CRY protein levels increase, they form complexes that translocate into the nucleus and repress CLOCK and BMAL1 activity, thereby inhibiting their own transcription. Enzymatic degradation of PER and CRY provides a delay mechanism prior to the onset of the next transcriptional cycle (15,16).

The so-called stabilizing loop, which is the focus of this thesis, includes the nuclear circadian receptor families REV ERB and ROR. The REV ERB family consists of two isoforms, REV-ERBα and REV-ERBβ, which function as transcriptional repressors of BMAL1 expression. In contrast, the ROR family comprises three isoforms, RORα**,** RORβ**,** and RORγ, which act as transcriptional activators of BMAL1 (10,16), as shown in Figure 1. The balance between these clock genes and nuclear circadian receptors is essential for a dynamic regulation of various biological processes due to their shared target genes (17). REV ERB and ROR form a stabilizing feedback loop that connects tightly with the core CLOCK–BMAL1–PER–CRY axis, reinforcing rhythmic stability and amplitude (15,16). By rhythmically repressing and activating Bmal1 transcription, REV-ERB and ROR respectively create a finely tuned oscillatory environment that synchronizes with the inhibitory actions of PER and CRY on CLOCK–BMAL1, ensuring coherence between the activating and repressing phases of the circadian cycle (16,17). In particular, the transcriptional output of REV-ERB and ROR indirectly regulates the timing of Per and Cry expression by modulating BMAL1 availability, thereby coupling the stabilizing loop to the

core feedback system (15,17). This multi-loop integration allows the clock to buffer against environmental fluctuations and maintain robust rhythmicity, highlighting the interdependence between the nuclear receptor–mediated loop and the PER/CRY negative feedback mechanism (15,16,18). Overall nearly 50% of human protein coding genes are clock controlled showing an oscillating gene expression over the day (18).

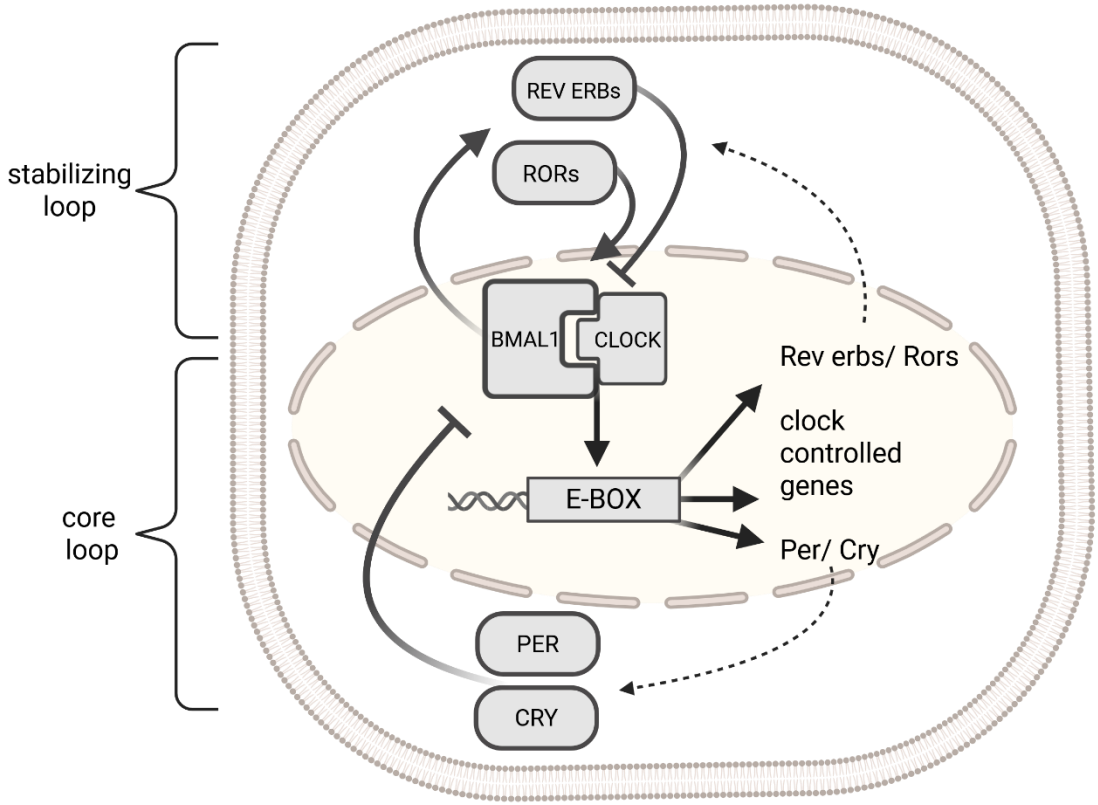

Figure 2: Transcriptional–translational feedback loops of the molecular circadian clock. *The core loop is driven by the CLOCK–BMAL1 complex, which activates* Per *and* Cry *transcription through E-box elements. PER and CRY proteins inhibit CLOCK–BMAL1 activity, forming a negative feedback loop. The stabilizing loop involves the nuclear receptors REV ERBs and RORs, which rhythmically repress or activate* Bmal1 *transcription, respectively. This loop reinforces circadian amplitude and phase stability by modulating BMAL1 levels and indirectly influencing* Per *and* Cry *expression. Integration of both loops ensures robust rhythmicity and temporal coherence of downstream clock-controlled genes.*

1.2 Interaction between the circadian clock and the immune system

The innate and adaptive immune systems exhibit circadian variation, influencing a broad range of processes including immune cell trafficking, pathogen recognition, cytokine secretion, and phagocytic activity (19). As noted, accumulating evidence indicates that the circadian regulation of immune responses contributes to time-of-day differences in infection susceptibility (4), inflammation severity, and even vaccine effectiveness (5). Increased immune surveillance and responsiveness are increased during the active phase (diurnal in human) including increased proinflammatory cytokine production, more antigen presentation and increased phagocytotic activity, enhanced blood circulation of immune cells and stronger responses to immune challenges (19–22).

Innate immune cells, such as monocytes, macrophages (1), neutrophils (23) and eosinophils (24), have intrinsic peripheral clocks. In myeloid cells, the core circadian gene BMAL1 has extensive immunomodulatory effects, largely through its transcriptional regulation of the nuclear receptor families REV ERBs and RORs, hence acting as the key circadian regulator of innate immunity (20). The peripheral molecular circadian clocks influence various functions of the immune cells including cytokine production, chemokine expression, pattern of recognition receptors or immune cell function.

Cytokine production:

The molecular circadian clock is regulating the timing and duration of proinflammatory cytokine expression in humans. Peak production of interferon gamma (IFN-γ), interleukin (IL)-1 and IL-12 and Tumor necrosis factor-α (TNF-α), occurs during the night and early morning at a time when plasma cortisol is lowest (19,25). Both IFN-γ and IL-10 display daily rhythms individually, with their ratio peaking in the early morning, influenced by cortisol and melatonin (26). The findings from Petrovsky et al. suggest a causal relationship between plasma cortisol and possibly melatonin regulate diurnal production of these pro-inflammatory cytokines (25,26). The increase in IL-2 production is

primarily sleep-dependent (27). Plasma IL-6 levels in healthy humans exhibit a circadian rhythm peaking in the late evening and early night (28).

Table 1: List of human circadian controlled pro-inflammatory cytokines.

cytokine	peak production	references
IFN-γ	night and early morning	26,27
IL-1	night and early morning	26
IL-2	during/after sleep	28
IL-6	late evening/ night	29
IL-10	early morning	27
IL-12	night and early morning	26
TNF- α	night and early morning	26

Chemokine expression:

Further, the molecular circadian clock influences the rhythmic homing of hematopoietic cells to the bone marrow and the recruitment of leukocytes into tissues such as the lungs. This regulation occurs partly through the oscillatory expression of adhesion molecules such as the intercellular adhesion molecule 1 gene (ICAM) and the chemokine gene C-C motif chemokine ligand 2 (CCL2/ MCP-1) and CCL5 (RANTES) (19,22). Human CCL2 (MCP-1) shows robust, endogenously controlled circadian oscillation, but exact peak times have not been quantified.

Pattern recognition receptors:

The expression and activity of pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs), which are crucial for sensing pathogens and triggering immune responses, is modulated by the circadian clock. In monocytes and macrophages, TLR expression fluctuates over the day, leading to time-of-day–dependent variations in cytokine production and inflammatory responses (29,30). For example, TLR9-mediated responses in macrophages peak during the active phase, enhancing pathogen recognition when the organism is most exposed to environmental challenges (30). Neutrophil functions like

oxidative burst and chemotaxis also display circadian oscillations linked to rhythmic TLR signalling (19). Even T cells, which express low levels of TLRs, show circadian variation in activation and cytokine secretion, possibly influenced by rhythmic TLR signalling pathways (21).

Immune cell function:

The molecular circadian clock exerts a profound influence on the fluctuation and effector functions of various immune cell types, including monocytes, macrophages, neutrophils, eosinophils, and T cells.

In monocytes and macrophages, core clock components such as BMAL1 and REV ERBα regulate the rhythmic expression of inflammatory genes, cytokines, and chemokines, thereby driving daily oscillations in immune responsiveness and trafficking (1,21,29). For instance, BMAL1-deficient macrophages exhibit exaggerated inflammatory responses**,** highlighting its role as a negative regulator of inflammation (21). In mice, neutrophil recruitment to tissues shows clear circadian patterns governed by clock-controlled C-X-C motif chemokine ligand 1 (CXCL1), CXCL2 and CXCL5, which optimize immune surveillance during the organism's active phase (19,22). While less studied, eosinophil counts and activation markers also display circadian variation, likely influenced by clock genes and diurnal release of cortisol (31), melatonin (10) and catecholamines (32), which may contribute to the daily oscillation of allergic inflammation such as asthma exacerbations. T cells demonstrate time-of-day differences in proliferation, cytokine production, and migration, with clock genes modulating their adaptive immune functions and balance between pro- and anti-inflammatory states (22). Together, these findings underscore the importance of the circadian clock in orchestrating immune cell behaviour to maintain homeostasis and effectively respond to environmental challenges.

On the one hand, various aspects of the immune system such as leukocyte trafficking or cytokine release show daily variation, which appears to be essential for a proper immune response. On the other hand, immune signals can feedback to modulate the circadian system. Inflammatory stimuli may dampen circadian oscillations, leading to a pro-inflammatory state and a manifestation of diseases. Inflammatory mediators such as tumor necrosis factor-

α **(**TNF-α) and IL-1β can suppress the expression or amplitude of core clock genes such as *Bmal1*, disrupting rhythmic gene expression and circadian physiology (33).

The previous sections highlighted the bidirectional relationship between the circadian clock and the immune system. To better understand this interplay, the following chapters focus on key immune cell types addressed in this PhD thesis.

1.2.1 Eosinophils

Historically, eosinophils have been recognized as prominent drivers of T-helper 2 (Th2)-driven immune responses, particularly in the context of parasitic infections and allergic reactions. Accordingly, eosinophils play a key role in primary immune defence and tissue homeostasis, but are also implicated in pathological conditions such as asthma (34,35). Under steady-state conditions, eosinophils constitute up to 6% of circulating white blood cells (22,36). They originate in the bone marrow, their differentiation and maturation from myeloid progenitors are primarily driven by IL-5, granulocyte macrophage colony-stimulating factor (GM-CSF) and IL-3. In healthy individuals, eosinophils reside in several tissues, including the thymus, lymphoid organs, uterus, and adipose tissue, while their occurrence in the mammary gland appears to be restricted to distinct developmental stages. (36,38).

Eosinophils are characterized by their prominent granularity and contribute to primary immune defence (36). Their main functions include host defence against helminth infections, maintenance of tissue homeostasis, and facilitation of tissue repair. Eosinophils internalize substances recognized as harmful by the immune system, such as components derived from parasites. Their function extends beyond the elimination of foreign material to the regulation of infections. When needed, eosinophils migrate from the circulation to sites of inflammation, a mechanism tightly regulated by various factors, including chemokines and cytokines (22,37). Thus, under inflammatory conditions, such as helminth infections or allergic responses, eosinophils are recruited to affected tissues such as the lung or oesophagus (38).

A crucial step in eosinophil recruitment is the extravasation from the bloodstream into tissues. Briefly, eosinophils initially bind to the endothelium via selectins such as L-selectin, which facilitates their rolling along the vascular wall. In addition to L-selectin, eosinophils also express P-selectin glycoprotein ligand-1 (PSGL-1), the binding partner of P-selectin (39). In allergic asthmatic patients, eosinophils exhibit elevated PSGL-1 levels, which enhances their adhesion to IL-4-stimulated endothelium (40). Interestingly, upon IL-13 stimulation, eosinophils adhere to the endothelium in a P-selectin-dependent manner, unlike neutrophils (39,41).

To recruit eosinophils successfully to peripheral organs such as the lung, adhesion to the epithelium is essential. Type 2 cytokines such as IL-4 and IL-13 lead to the expression of the adhesion molecule VCAM-1 on endothelial cells and to the production of CC-chemokines from epithelial cells, airway smooth muscle cells, and even airway fibroblasts. CC-chemokines and VCAM-1 effectively induce migration of eosinophils from the bloodstream into affected tissues (34,35).

Following migration into the tissue, other Th2 cytokines such as IL-5 and GM-CSF play important roles. IL-5, on the one hand, stimulates the development and maturation of eosinophils in the bone marrow. On the other hand, IL-5 also prolongs the survival and enhances the effector functions of transmigrated eosinophils (42). In addition, eosinophils can produce GM-CSF in an autocrine manner upon activation by cytokines or inflammatory signals, thereby prolonging their survival and enhancing their effector functions. (43). Consequently, many activated eosinophils accumulate at the affected site.

Upon stimulation, activated eosinophils release their granule contents into the environment, a process called degranulation, which involves the release of preformed cytotoxic proteins and other mediators leading to epithelial damage, increased vascular permeability, and activation of various other cell types such as basophils and neutrophils (44). Eosinophils secrete a range of inflammatory mediators, including cytokines, chemokines and interleukins such as GM-CSF, TNF-α or IFN-γ, and lipid mediators such as leukotrienes or prostaglandins. Furthermore, eosinophils can release growth factors including transforming growth factor beta (TGF-β), cytotoxic secretory products such as

eosinophil peroxidase (EPX) or major basic protein (MBP) and neuromediators like substance-P (**Figure 3**) (38,45). While these mechanisms support host defence against parasitic infections, they can also contribute to disease pathogenesis. For example, eosinophil granular proteins are present at elevated concentrations in respiratory secretions from patients with asthma, where their toxic effects on the respiratory epithelium lead to tissue damage (46,47). Eosinophil peroxidase and cationic protein exhibit oscillatory expression patterns in sputum, serum, and urine (45). Additionally, rhythmic expression of clock genes and eosinophil-specific genes has been observed in eosinophils from both healthy and allergic donors (48).

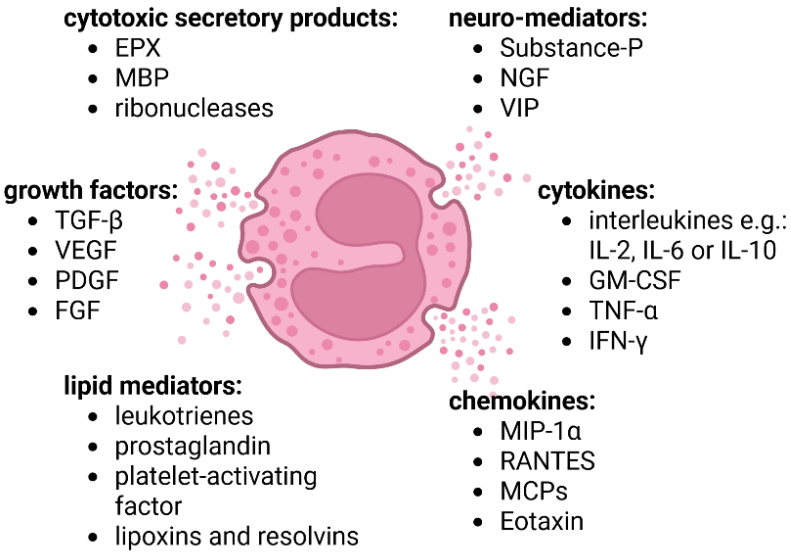

Figure 3: Eosinophils secrete a range of inflammatory mediators. *A large range of cytokines, chemokines, lipid mediators, growth factors, cytotoxic secretory products and neuro-mediators are released after activation as illustrated. Transforming Growth Factor-beta (TGF-β); Vascular Endothelial Growth Factor (VEGF); Platelet-Derived Growth Factor (PDGF); Nerve Growth Factor (NGF); Fibroblast Growth Factor (FGF); Vasoactive Intestinal Peptide (VIP). This illustration was created with BioRender.com.*

1.2.1.1 Circadian biology in eosinophils

The circadian clock is a master regulator of various physiological systems, including sleep, metabolism, and immune function. Immune responses exhibit temporal variations in both the abundance and sensitivity of immune cells, including eosinophils (22). The number of circulating eosinophils in the blood follows a circadian rhythm, a phenomenon first described in 1956 by Acland and Gould (49). Typically, eosinophil counts are lowest in the

morning and increase progressively from noon until after midnight (49). Similar to eosinophils, other leukocyte subsets including T-cells, B-cells, neutrophils, monocytes and natural killer (NK) cells also display circadian oscillations in the bloodstream during homeostasis and show time-of-day-dependent recruitment to organs (22). This rhythmic leukocyte trafficking highlights the interplay between the immune system and the circadian clock.

Eosinophil differentiation in the bone marrow, their release into the circulation, and their accumulation and survival in organs such as the lung are regulated by certain cytokines, including IL-5, IL-13, and GM-CSF. Notably, particularly IL-5, is rhythmically secreted by tissue-resident type 2 innate lymphoid cells (ILC2s) throughout the day (22,50,51). Th2 cells, NK cells, mast cells, eosinophils and basophils can also produce IL-5, especially during immune responses, however, their diurnal IL-5 secretion has not been demonstrated so far**.**

Recently, Baumann et al. demonstrated for the first time an oscillating pattern of clock gene expression in human eosinophils (24). During degranulation, eosinophils exert their inflammatory function by releasing granule-stored proteins, including major basic protein (MBP), EPX and ECP (52), all of which exhibit circadian variation in human serum samples (36,37,48). Lower levels of ECP and EPX have been reported in serum and urine during the daytime. Compared to healthy individuals, allergic patients show a peak in these eosinophil-specific markers during the early morning hours (53). In both resting and activated eosinophils, a functional core circadian loop was identified. Moreover, eosinophils display extracellular signal-regulated kinase (ERK) activation and circadian release of chemokines upon activation (35).

Overall, the mutual interplay between the molecular circadian clock and the immune system is well established. On the one hand, various immune processes, such as eosinophil migration and cytokine release, exhibit daily variation, which appears to be essential for an effective immune response. On the other hand, immune responses, for example to infections and chronic inflammation, can disrupt circadian rhythms, thereby disturbing the molecular clock and contributing to the development of inflammatory diseases such as asthma (10,22).

1.2.2 T cells

T cells are a central component of the adaptive immune system and play a pivotal role in orchestrating immune responses, including those involved in inflammation. Upon activation through recognition of peptide antigens presented by major histocompatibility complex (MHC) molecules on antigen-presenting cells, T cells differentiate into various subsets with distinct effector functions. Among these, $CD4^+$ T helper cells coordinate immune responses through cytokine secretion, while $CD8^+$ cytotoxic T cells are primarily involved in the direct elimination of infected or aberrant cells. In the context of inflammation, T cells contribute both to protective immunity and to pathological processes when their activation is dysregulated. The dysregulation of different T cell subsets is associated with chronic inflammatory diseases, including asthma, inflammatory bowel disease, and rheumatoid arthritis (54,55).

Understanding the roles of T cells in inflammation is therefore critical for the development of targeted therapies for a broad range of immune-mediated conditions. The pivotal role of T cells in the pathogenesis and regulation of asthma will be discussed in detail in a later chapter.

1.2.2.1 Circadian biology in T cells

Recent research has highlighted the significant influence of circadian biology on immune function, including the activity and behaviour of T cells. Both $CD4^+$ and $CD8^+$ T cells exhibit circadian oscillations in their trafficking, activation, and effector responses, regulated by intrinsic molecular clocks composed of core clock genes such as Bmal1 and Clock. These circadian mechanisms modulate T cell responses to antigens and control the timing of cytokine production and proliferation. For instance, $CD4^+$ T cells have been shown to display time-of-day-dependent variations in cytokine secretion such as IL-2, IL-4 or IFN-γ, influencing the balance between pro- and anti-inflammatory states. Similarly, $CD8^+$ T cells rely on both intrinsic circadian regulators—such as the core clock gene *Bmal1*—and extrinsic time-of-day–dependent cues, including hormonal and dendritic cell signals, for optimal priming, expansion, and cytotoxic function, which are critical during viral infections

and tumour surveillance. Disruption of circadian rhythms—through genetic ablation of clock genes or environmental factors such as shift work or jet lag—can impair T cell-mediated immunity and exacerbate inflammatory diseases (56–59). These findings underscore the importance of considering circadian timing in immunological studies and therapeutic strategies.

1.2.3 Monocytes and Macrophages

Macrophages are central components of the mononuclear phagocyte system, which includes bone marrow-derived cells such as blood monocytes and tissue-resident macrophages. They can arise from peripheral monocytes or develop from embryonic precursors, such as alveolar macrophages (60). Monocytes migrate from the bloodstream into various tissues, where they differentiate into macrophages. During this process, tissue resident cells such as endothelial cells or fibroblasts release chemokines like MCP-1 to attract monocytes by binding to their CCR2 receptor. Once monocytes reach the tissue, local cytokines including macrophage colony-stimulating factor (M-CSF) induce their differentiation into macrophages. During inflammation, macrophages perform essential functions including antigen presentation through MHC expression and T cell activation, phagocytosis, and immunomodulation via cytokines such as TNF-α or IL-1β, as well as growth factor production (61). They play a critical role in initiating, sustaining, and resolving inflammation, being activated and deactivated in response to inflammatory cues.

Macrophages play a central role in the initial immune response, particularly in the respiratory mucosa, where these responses are crucial for the development of type 2 immunity to inhaled allergens (62). Macrophage immune functions adapt to both internal physiological changes, such as nutritional challenges that reprogram macrophage activity, including shifts in polarization, cytokine secretion, and tissue infiltration (63,64), and external environmental changes, such as the well-characterized relationship between macrophages and aging, particularly in redox homeostasis (65,66). Daily regulation of immune activity also includes tissue infiltration and inflammatory function of macrophages (67).

Activation signals for macrophages include cytokines such as IFN-γ, GM-CSF, and TNF-α, as well as bacterial lipopolysaccharide, extracellular matrix proteins, and other chemical mediators (60,68). Inflammatory responses are tempered through the removal or deactivation of these mediators and inflammatory cells, allowing tissue repair. Anti-inflammatory cytokines like IL-10 and TGF-β, as well as cytokine antagonists primarily produced by macrophages, deactivate activated macrophages, thus participating in the autoregulatory loop of inflammation (69).

Due to their ability to produce a wide array of biologically active molecules that influence both beneficial and detrimental outcomes in inflammation, macrophages and their products present promising targets for therapeutic interventions aimed at controlling inflammatory diseases (60,68,69).

In many pulmonary diseases, particularly those involving type 1 or innate immune responses, macrophages are a major source of inflammatory cytokines such as TNF-α and IL-1β (70–72). In type 2 inflammation, such as in asthma, macrophages contribute to disease pathology by adopting an alternatively activated (M2) phenotype, supporting tissue remodelling, mucus production, and eosinophilic inflammation, although the primary type 2 cytokines (IL-4, IL-5, IL-13) are mainly produced by Th2 cells and ILC2s (72,73). Depending on environmental signals, macrophages polarize into different phenotypes, typically categorized as classical, pro-inflammatory M1, or non-classical, anti-inflammatory M2 subtypes (60). However, it is important to note that the M1/M2 classification is only a simplification of macrophage behaviour, as macrophage polarization exists along a broad and dynamic spectrum depending on the microenvironment.

In addition to their polarization status, macrophages can be classified by their location. Lung-resident alveolar macrophages are crucial for clearing airborne pathogens. They initiate essential pro-inflammatory responses to mobilize and activate peripheral phagocytes such as neutrophils and monocytes (71). During inflammation, lung tissue-resident macrophages, together with epithelial and other innate cells, release cytokines and chemokines to recruit classical monocytes to the lungs, where they differentiate into monocyte-derived macrophages (Mo-AMs). In the late stages of inflammation, Mo-AMs will either undergo apoptosis or contribute to the alveolar macrophage pool in some steady

states (71,74). Dysfunctions in macrophages, including changes in polarization or phagocytic behaviour, are closely linked to asthma pathology (73).

1.2.3.1 Circadian biology in macrophages

Macrophage immune responses are triggered by various stimuli, including lipopolysaccharide (LPS) and IFN-γ, a Th1 cytokines, as well as Th2 cytokines such as IL-4 and IL-13, which also influence macrophage polarization and reprogramming (72). Upon LPS exposure, macrophages activate intracellular signalling pathways leading to the production and secretion of a range of pro-inflammatory cytokines such as TNF-α, IL-1β or IL-6 as well as chemokines like MCP-1/CCL2 and macrophage inflammatory protein 1 alpha (MIP-1α/CCL3), which are essential for mediating inflammation and recruiting other immune cells to the site of infection. Concurrently, macrophages increase their phagocytic activity to clear pathogens. During resolution, macrophages contribute to tissue repair and remodelling by switching phenotypes and secreting anti-inflammatory cytokines such as IL-10 (60,75,76).

The response of macrophages to LPS exhibits a clear time-of-day variation, as reflected in the fluctuating secretion of inflammatory mediators including IL-6, IL-12, CCL2, CCL5, and CXCL1. Sato et al. highlighted the interplay between the molecular circadian clock and macrophage function, demonstrating that *Rev erbα*, the transcriptional repressor of *Bmal1*, suppresses inflammatory cytokine secretion (77). Peritoneal macrophages isolated from mice subjected to a light cycle shift mimicking chronic jet lag showed increased IL-6 expression upon LPS exposure (78). Moreover, peritoneal macrophages from *rev erbα* knockout mice exhibit elevated Ccl2 expression. REV ERBα directly binds to a ROR response element in the *Ccl2* promoter, repressing CCL2-mediated activation of mitogen-activated protein kinase (MAPK) pathways, including ERK phosphorylation. This repression impairs macrophage adhesion and migration (77). This suggests that while BMAL1 may inhibit CCL2 under normal rhythmic conditions, the anti-inflammatory role of REV ERB is mediated through additional mechanisms beyond BMAL1 repression. Thus, in REV ERB-deficient mice, the overall inflammatory response is enhanced, and CCL2 is elevated despite potentially increased BMAL1 activity.

TLR9 plays a critical role in both innate immunity—through recognition of bacterial or viral DNA—and adaptive immunity via antigen presentation by macrophages. The circadian clock regulates TLR9 expression and responsiveness, leading to daily variations in macrophage immunity (79). Key circadian factors, CLOCK and BMAL1, are essential for circadian regulation of *Tlr9* gene expression in peritoneal macrophages, with TLR9 ligand responses showing significant time-of-day dependence (67).

Various studies have demonstrated that macrophage phagocytic activity follows a circadian rhythm (1,80–82). Both tissue-resident and peripheral monocyte-derived macrophages exhibit similar oscillations in core clock gene expression, which autonomously regulate their phagocytic function. Specifically, phagocytic activity peaks during the resting period and oscillates in antiphase to Bmal1 expression. Deletion of BMAL1 in myeloid cells markedly enhances bacterial phagocytosis in macrophages, indicating that BMAL1 acts as a suppressor of bacterial phagocytosis, whereas effects in neutrophils are moderate and not statistically significant (83). Furthermore, in Ly6C^{high} monocytes, BMAL1 rhythmically inhibits chemokine gene expression of *Ccl2* and *Ccl8* which are important for monocyte recruitment and hence resulting in oscillatory trafficking of circulating monocytes to inflammatory sites (84).

1.3 Asthma

Asthma is a complex chronic inflammatory disease characterized by airway hyperresponsiveness, reversible airflow obstruction, and airway remodelling. It affects millions of individuals worldwide and is marked by an exaggerated immune response to environmental triggers, such as allergens, pollutants, and respiratory infections. Central to asthma pathogenesis is the interplay of multiple immune cells, including eosinophils, basophils and mast cells, T cells, monocytes, and macrophages as shown in **Figure 4**, which orchestrate the inflammation and structural changes in the airways (68,85–87).

The circadian regulation of these immune cells and the lungs plays a crucial role in the temporal pattern of asthma symptoms and exacerbations, which often worsen during the night and early morning. Understanding how circadian rhythms influence immune cell

function in asthma may clarify disease mechanisms and reveal novel therapeutic targets to improve disease management (10,88–90).

1.3.1 Allergic asthma

Persistent inhalation of allergens such as pollens, pet dander, or house dust mites can lead to chronic airway inflammation (91). In allergic asthma, sensitization induces an IgE-mediated immune response. Upon allergen exposure, binding of the allergen to IgE on mast cells triggers the release of granule contents—histamine, leukotrienes, prostaglandins, and other inflammatory mediators—which then recruit T helper cells, eosinophils, and macrophages to the site of allergen exposure (92).

1.3.2 Eosinophilic asthma

Eosinophilic asthma is the most prevalent and extensively studied asthma phenotype, accounting for over half of severe asthma cases. It is characterized by eosinophilia in sputum or peripheral blood, as well as eosinophilic infiltration within airway tissues (93,94). Eosinophils, key effector cells in asthma pathogenesis, drive airway hyperresponsiveness and Th2-type inflammation by releasing a variety of signalling molecules, including Th2 cytokines such as IL-4, IL-5, IL-10, TNF-α, and TGF-β, chemokines like MIP-1α, and cytotoxic granule proteins in response to allergens or infections (94,95).

Clinically, eosinophilic asthma is associated with airway tissue eosinophilia and basement membrane thickening and typically responds well to corticosteroid treatment (94). Although definitions vary, peripheral blood eosinophil counts—commonly using thresholds of ≥150, ≥300, or ≥400 cells/μL—along with elevated fractional exhaled nitric oxide (FeNO), serve as practical biomarkers to identify eosinophilic asthma in both research and routine clinical settings (96,97).

1.3.3 T cells in asthma

T cells play a central role in orchestrating the immune response in asthma, with distinct subsets—particularly CD4+ and CD8+ T cells—contributing to disease pathogenesis. CD4+ T cells, especially the Th2 subset, are primary drivers of airway inflammation and hyperresponsiveness. Upon activation, Th2 cells release cytokines such as IL-4, IL-5, IL-9, and IL-13, which promote key asthma features including airway eosinophilia, mucus hypersecretion, and activation of B cells (87,98,99).

Activation of Th2 cells occurs when their T-cell receptor (TCR) recognizes peptide antigens presented by MHC class II molecules on airway dendritic and epithelial cells. This antigen recognition initiates a cytokine cascade that drives the inflammatory response characteristic of asthma (99). Meanwhile, CD8+ T cells, traditionally associated with cytotoxic functions, are increasingly acknowledged for their modulatory roles in asthma. While their exact contribution remain under investigation, interactions between CD8+ and CD4+ T cells, along with the surrounding cytokine environment, collectively influence the chronic airway inflammation and remodelling that drive asthma progression (98,100).

Understanding the distinct and complementary roles of these T cell subsets is crucial for elucidating the immunopathology of asthma and for developing targeted therapies.

1.3.4 Macrophages: another key player in asthma

While type II immune responses in the airways are primarily driven by eosinophils, mast cells, and basophils, lung macrophages also play an important role in modulating type II inflammation and disease progression (101). Due to the lung’s unique microenvironment, resident macrophages predominantly exhibit an "M2"-like phenotype, responding to type 2 cytokines such as IL-4. This phenotype enables them to suppress asthmatic lung inflammation, as demonstrated in murine models of asthma (102,103). Tissue-resident macrophages promote the generation of regulatory T cells, further contributing to the suppression of allergic inflammation (103,104).

A protective mechanism of these macrophages involves the mannose receptor (MRC1/CD206), a surface marker associated with the M2 phenotype. Mice deficient in this receptor show exacerbated lung inflammation following allergen exposure, highlighting its role in controlling airway inflammation (105). Additionally, TGF-β1 produced by these macrophages is essential for their maturation and regulation of allergic responses. Deficiency of TGF-β1 results in increased production of monocyte-attractant chemokines and elevated type II inflammation, particularly in response to house dust mite allergens (69).

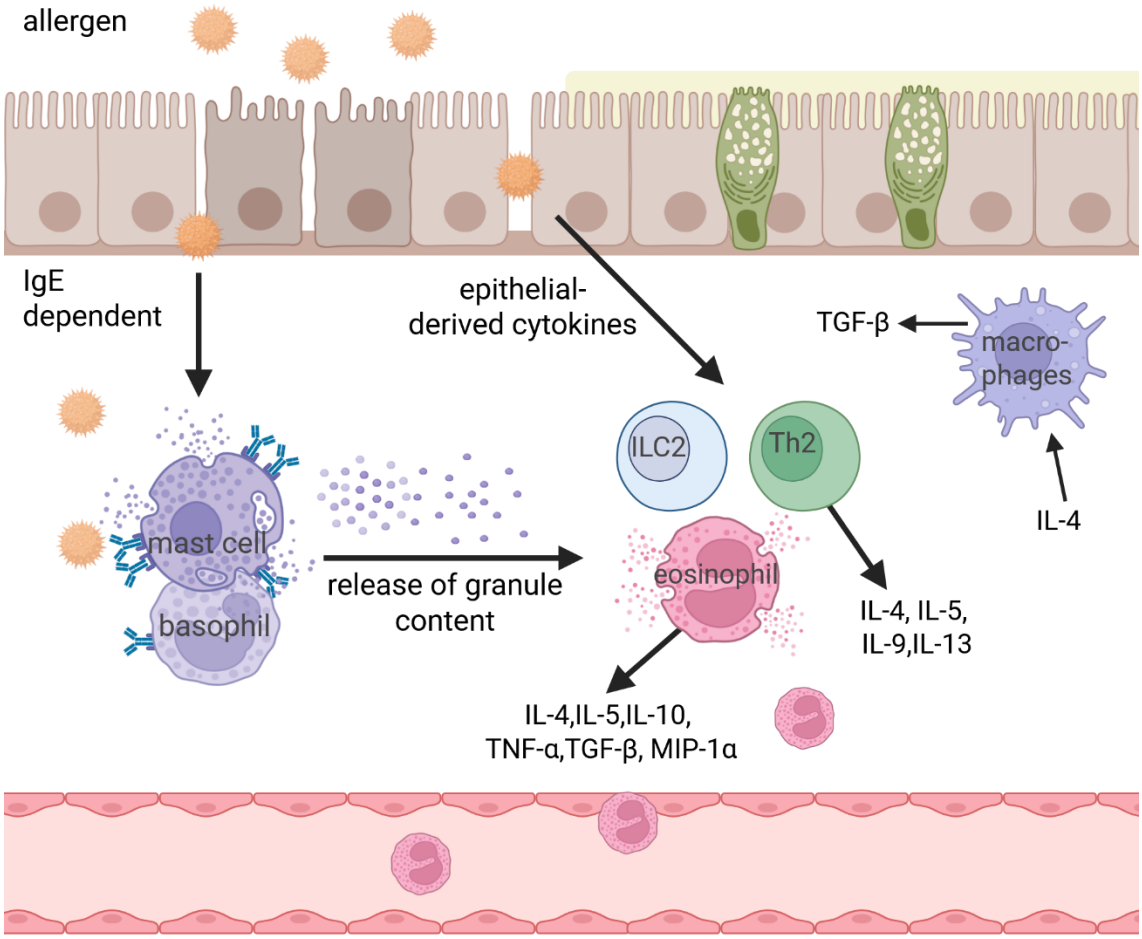

Figure 4: Simplified schema of the IgE-mediated and epithelial-driven immune response contributing to asthma. *Allergen specific IgE mediated degranulation of mast cells and basophils releases inflammatory mediators during an early asthmatic response. These mediators act on Th2 cells, ILC2s and eosinophils leading to bronchoconstriction and inflammation. In parallel, epithelial cells exposed to allergens release cytokines, which directly activate Th2 and ILC2 cells. Activated eosinophils, Th2 cells, and macrophages engage in reciprocal signalling, amplifying type 2 inflammation. In a late response the from mast cells and basophils released cytokines prepare the vessel wall for leukocyte migration. Schematic illustration was created with BioRender.com.*

1.3.5 Therapeutic approaches

1.3.5.1 Conventional therapy

Historically, asthma therapy focused on symptom relief and bronchodilation, with short-acting beta-agonists (SABAs) like salbutamol serving as the primary treatment. These betamimetics were effective in relaxing airway smooth muscles and offered immediate relief from bronchoconstriction (106). However, they did not address the underlying airway inflammation, and reliance on SABAs alone led to inadequate long-term control and increased risk of exacerbations. Moreover, the widespread use of hydrofluorocarbon (HFC)-propelled inhalers raised environmental concerns due to their significant contribution to greenhouse gas emissions.

Recognition of airway inflammation as a central driver of asthma pathogenesis marked a turning point in treatment strategy. This led to the introduction of inhaled corticosteroids (ICS), such as fluticasone furoate, which significantly improved control by targeting the inflammatory processes within the airways (107). Nevertheless, ICS therapy did not substantially alter the natural course of asthma and many patients continued to experience symptoms or frequent exacerbations despite treatment.

Additional challenges also complicated asthma management. The heterogeneity of asthma—encompassing a variety of phenotypes and endotypes—made a one-size-fits-all treatment approach inadequate. Traditional medications often failed to provide relief for patients with severe or non-type 2 asthma, and non-adherence to inhaled therapies further limited their effectiveness. Patients frequently cited concerns about side effects, complexity of dosing regimens, or a lack of perceived benefit as reasons for poor adherence.

1.3.5.2 Biological therapy

In the early 2000s, the treatment landscape shifted once more with the development of biological therapies. The first approved biologic, Omalizumab, a recombinant humanized monoclonal antibody targeting IgE, opened the door for precision medicine in asthma (108).

This was followed by therapies directed against key mediators of type 2 inflammation, including IL-5 (mepolizumab, reslizumab), IL-5R (benralizumab), IL-4/IL-13R (dupilumab), and TSLP (tezepelumab) (109). These biologics offer targeted treatment options for patients with severe asthma, many of whom previously had limited therapeutic choices.

Importantly, these innovations have led to a shift in perspective: asthma is no longer viewed solely as a controllable disease, but as one that may be modifiable or even remissible. Some researchers and clinicians now refer to these agents as "disease-modifying" therapies, reflecting their ability to alter the course and severity of asthma rather than just suppress symptoms (110).

Nonetheless, response to biologics is variable. In a cohort study of patients treated with anti-IL-5 or IL-5R therapies, only 14% met criteria for "super-responders" after two years, with responses associated with shorter disease duration and higher baseline lung function (111). Post hoc analyses of clinical trials also demonstrated that high blood eosinophil levels correlate with better treatment response to anti-IL-5 biologics (112,113). More refined biomarkers, such as induced sputum profiles—including eotaxin-1, IL-5, and EPX - may offer even greater predictive value (114).

Despite these advancements, several challenges remain, including variability in treatment response, high costs, limited accessibility, and the lack of validated biomarkers for personalized care. Moving forward, integrating clinical phenotyping, biomarker-guided therapy, and patient-centred care will be essential for achieving remission and improving long-term outcomes in asthma.

1.3.5.3 Implementation of circadian biology into asthma management

Chronotherapy:

Recent research on the role of circadian biology in asthma pathophysiology has opened new avenues for refining treatment strategies through chronotherapy - the timed administration of medications to align with the body’s biological rhythms. Circadian rhythms regulate airway function, inflammatory responses, and bronchial reactivity, with

symptoms commonly peaking at night. Notably, nocturnal asthma affects up to two-thirds of patients, with symptoms often worsening around 4 a.m., making the timing of drug delivery a critical factor in therapeutic efficacy (115). Moreover, circadian regulation extends to the immune system and inflammatory biomarkers of asthma, as daily oscillations in cytokine levels, immune cell function, and FeNO levels have been observed (110).

Beta2 agonists such as salbutamol, bambuterol, and formoterol act as bronchodilators by relaxing airway smooth muscles and, hence dilating the airways. Their effectiveness, however, fluctuates with circadian cycles, underscoring the importance of chronotherapy in asthma management. For example, Qureshi et al. developed a chrono-modulated drug delivery system for salbutamol designed to release the medication in alignment with nocturnal symptom peaks (116). Similarly, Barnes and colleagues formulated a prolonged-action inhaler for salmeterol, and clinical trials with bambuterol demonstrated that evening dosing produced a longer duration of bronchodilation compared to morning administration (115,117).

Clock-modulating ligands:

At the molecular level, nuclear receptors—ligand-activated transcription factors constituting a large family of druggable proteins—play key roles in circadian regulation and immune modulation (118). Targeting these receptors to modulate the circadian clock holds therapeutic promise but can produce complex effects, including both anti- and pro-inflammatory outcomes. Experimental studies have evaluated synthetic ligands for circadian nuclear receptors with encouraging results.

SR9009 and GSK4112 are synthetic small-molecule ligands classified as agonists of the nuclear receptors REV-ERBα and REV-ERBβ (17,119,120). Both ligands stabilize REV-ERBs' repressive conformation, enhancing their ability to regulate circadian rhythms and metabolism. GSK4112 was the first identified ligand but exhibits limited potency and bioavailability, whereas SR9009 is a more potent and bioavailable derivative suitable for in vivo studies (17,120). For example, the REV-ERBα agonist SR9009 significantly suppressed LPS-induced inflammation in bone marrow–derived macrophages (BMDMs), particularly in Bmal1 knockout models, and shifted macrophage polarization from the pro-inflammatory M1 phenotype toward the anti-inflammatory M2 state (119,120).

In contrast, SR1078 and SR1001 target the ROR family, with SR1078 acting as an agonist and SR1001 as a selective inverse agonist that stabilizes the receptor's inactive state, thereby reducing its baseline activity. Kojetin and Burris reviewed the molecular basis of REV ERB and ROR ligand interactions, highlighting their therapeutic potential (17,121). Despite efforts to improve selectivity, these ligands still exhibit off-target effects. SR9009 and GSK4112 can influence receptors beyond REV ERBs, complicating data interpretation (119,122). Although SR1078 and SR1001 were designed for selectivity, comprehensive pharmacological profiling remains essential to identify potential off-target effects and ensure safe therapeutic application. Thus, SR1078 and SR1001 show promising immunomodulatory effects, but require further pharmacological characterization before clinical use (1,2,121,123). The inverse agonist of RORs, SR1001, has demonstrated potent anti-inflammatory properties by modulating macrophage viability and function in human monocytic cell lines (70) and murine models (1,121–123).

Future perspectives:

Despite these advances, one major hurdle remains: the identification of reliable biomarkers to accurately predict asthma phenotypes, disease progression, and response to therapy. Current biomarkers such as blood eosinophil counts and FeNO provide useful but limited information, often insufficient to guide personalized therapy fully. Circadian biology offers a novel dimension in biomarker discovery by revealing temporal patterns in immune cell activity and inflammatory mediator levels, which may refine disease phenotyping and treatment timing (110,124).

Notably, a study by Gibbs et al. demonstrated that circadian clock genes modulate the inflammatory responses of airway epithelial cells, suggesting that time-of-day–dependent biomarker expression could inform optimal treatment windows (21). Another investigation by Durrington et al. showed that circadian oscillations in cytokines such as IL-6 and TNF-α correlated with nocturnal asthma symptom severity, supporting the potential for circadian-informed biomarker panels to improve clinical decision-making (125).

These findings highlight the therapeutic potential of targeting the molecular circadian machinery to both optimize timing of current asthma treatments and develop novel anti-inflammatory strategies.

Integrating circadian biology insights into asthma management represents a promising approach, with chronotherapy potentially enhancing drug efficacy, reducing side effects, and directly targeting the molecular mechanisms of airway inflammation.

1.4 Aims and hypothesis

There is compelling evidence that circadian rhythms, and particularly the molecular circadian clock, contribute to the pathogenesis of asthma.

Based on these observations, we hypothesize that the molecular circadian clock is disrupted in circulating leukocyte populations from asthmatic patients compared to healthy individuals.

Therefore, the aim of this thesis is to investigate the role of clock proteins, particularly those involved in the stabilizing loop, in the pathophysiology of asthma. While most existing studies focus on the RNA level of the molecular circadian clock, this thesis seeks to investigate the protein levels of the stabilizing loop. Furthermore, we aim to determine whether the nuclear circadian receptor families, REV ERB and ROR, could serve as potential targets for a novel treatment approach for asthma.

Hence, this thesis consists of three parts, each designed to investigate and describe the role of the molecular circadian clock in immune cells during inflammation, with a particular focus on asthma. The specific objectives are as follows:

1. **Expression of the molecular circadian clock at the protein level in human leukocyte populations:**
   Using whole blood staining, the expression of clock proteins of the stabilizing loop in various leukocyte subsets from healthy, allergic, mild and moderate asthmatic blood donors was examined over a 24-hour period. These comparisons reveal significant differences in period length, phase and expression levels of clock proteins among the groups.

2. **Clock protein expressions as a potential biomarker for asthma and respiratory inflammation**
   This aim assesses the responsiveness of the molecular circadian clock to inflammatory conditions and varying asthma severity, evaluating the potential of clock proteins as biomarkers for asthma and respiratory inflammation.

3. **Clock modulating ligands as a novel therapeutic target:**

   The third part of this thesis investigates whether synthetic ligands targeting the nuclear circadian receptor families REV ERB and ROR can modulate immune cell functions *in vitro* and *in vivo*. Additionally, we explore whether these ligands exhibit anti-inflammatory and broncho-protective properties, providing insight into their potential as novel therapeutic agents for asthma.

2. Materials and methods

Some parts of this section have been published in adapted form (1,2).

2.1 Materials

2.1.1 Antibodies

Table 2: Antibodies including primary and secondary antibodies that were used for staining experiments, western blots and phosphor flow

Antibodies	Company	Catalogue number	Species	Working dilution
Surface markers				
CD3 APC-Cy7	Biolegend	317342	mouse anti human	1:500
CD4 Alexa Fluor 488	Biolegend	317420	mouse anti human	1:500
CD8 BV510	Biolegend	344732	mouse anti human	1:500
CD14 BV421	Biolegend	301830	mouse anti human	1:250
CD16 PerCP-Cy5.5	Biolegend	301828	mouse anti human	1:500
Siglec-F PE	BD Pharmingen	552128	rat anti mouse	1:100
Ly6G APC	Biolegend	127614	rat anti mouse	1:500
CD11b PE-Cy7	BD Pharmingen	552850	rat anti mouse	1:200
CD11c BV421	BD Pharmingen	562782	hamster anti mouse	1:200
Zombie Green™ Fixable Viability Kit	Biolegend	423111		1:500
CD80 BV421	Biolegend	305221	mouse anti human	1:100
CD206 APC-Cy7	Biolegend	321119	mouse anti human	1:100
Primary antibodies				
BMAL1	Novusbio	NB100-2288	rabbit anti human	1:100-1:200
CLOCK	mybiosource	MBS4750976	rabbit anti human	1:200

REV ERB alpha	Abcam	ab174309	rabbit anti human	1:25
REV ERB beta	Novusbio	NBP2-19576	rabbit anti human	1:50
ROR alpha	ThermoFisher Scientific	PA1-812	rabbit anti human	1:50
ROR beta	Novusbio	NBP1-82532	rabbit anti human	1:20
ROR gamma -PE	R&D System	IC6006P	mouse anti human	2.5µg/mL
EPX	Dr. Elizabeth Jacobsen	MM25-82		2,5 µg/mL
CD68	Abcam	ab955	mouse anti human	1:100
Secondary antibodies				
Donkey anti-rabbit PE	Biolegend	406421	donkey anti rabbit	1:500
647	ThermoFisher Scientific	A32795	donkey anti rabbit	1:500
488	ThermoFisher Scientific	A32723	goat anti mouse	1:500

2.1.2 Buffers

+ Buffer:

- 25 mg BSA
- 45 mg Glucose
- 250 µL HEPES
- 25 mL PBS+/+ (with calcium and magnesium)

- Buffer:

- 25 mg BSA
- 45 mg Glucose
- 250 µL HEPES
- 25 mL PBS-/- (without calcium and magnesium)

BAL Buffer:

- 29 mg EDTA
- 250 µL HEPES
- 100 mL PBS-/- (without calcium and magnesium)

NH_4Cl_2 Buffer:

- 9g ammonium chloride
- 1g potassium bicarbonate
- 37 mg EDTA
- 100 mL aqua dest.

2.1.3 Clock-modulating ligands

Table 3: List of clock-modulating agonists:

Targeting REV ERB	Company	Catalog number	Working dilution [µM]
GSK4112	Sigma Aldrich	G0673	1-10
SR9009	Sigma Aldrich	1379686-30-2	1-10
SR8278	Sigma Aldrich	S9576	10
Targeting ROR			
SR1078	MedChemExpress	1334106-03-0	1-10
SR1001	MedChemExpress	1246525-60-9	10 (in vitro), 25mg/kg (in vivo)

2.2 Methods

2.2.1 Ethical Approval

All experiments involving primary cells from human subjects were approved by the Institutional Review Board of the Medical University of Graz (EK 17–291 ex 05/06 and 30-537 ex 17/18). All volunteers signed an informed consent. Experimental time frames were aligned with participant appointment schedules to ensure consistency across sample collections at both centres. Daily blood donors were routinely scheduled for their appointments at 8:00 am at the Division of Pharmacology, whereas asthma clinic sessions at Wythenshawe Hospital, Manchester, typically began at 9:00 am. Blood donors were divided into healthy, non-allergic, and allergic donors due to serologic tests. Staining experiments using blood samples of the moderate asthma cohort, respective controls, and sputum samples from severe asthmatics were approved by the Manchester University NHS Foundation Trust (M2023-133). Information on asthma diagnosis and medication was self-reported by the participants. The collection of human lung samples was approved by the Institutional Ethics Board (32–446 ex 19/20), following the patients' informed consent. All studies involving animal experiments were approved by the Animal Ethics Committee of the Austrian Federal Ministry of Science and Research and carried out in line with the European Community's Council Directive (BMWF-2022-0.626.093).

2.2.2 Whole blood staining

For whole blood samples leukocyte populations were stained with CD3-APC-Cy7 (Biolegend, California, USA, 317342), CD14-BV421 (Biolegend, 301830) and CD16-PerCP-Cy5.5 (Biolegend, 301828). Cells were fixed and permeabilized with FIX and PERM (Nordic-MUbio, Netherlands, GAS-002). Samples were then blocked with human TruStain FcX™ Fc Receptor Blocking Solution (Biolegend, 422302) and clock proteins were stained intracellularly using the following primary antibodies: NR1D1 (Abcam, United Kingdom, ab174309), REV ERB beta (Novusbio, Colorado, USA, NBP2-19576), BMAL1 (Novusbio, NB100-2288), CLOCK (Mybiosource, California, USA, MBS4750976), ROR alpha (ThermoFisher Scientific, Massachusetts, USA, PA1-812) ROR beta (Novusbio, NBP1-82532) and ROR gamma-PE (R&D Systems, Minnesota, USA, IC6006P). The secondary donkey anti-rabbit-PE antibody (Biolegend, 406421) was used for detection. Samples were measured on a BD FACSCanto II flow cytometer and analysed as FI-FMO using FlowJo 10.8.1.

2.2.3 Isolation of immune cells from whole blood

2.2.3.1 Isolation of peripheral blood mononuclear cells (PBMCs)

For the isolation of human mononuclear cells from peripheral blood, 70 mL of blood was drawn from healthy donors after they had given their consent in accordance with a protocol approved by the local ethics committee. Blood clotting was prevented by the addition of 3.8% sodium citrate. First the sodium citrate blood was centrifuged at 400 xg (low brake) for 20 minutes. Platelet-rich plasma was removed and followed by dextran sedimentation (6 % dextran spiked with 0.9 % saline). The samples were incubated for 30 minutes at room temperature to facilitate the aggregation of the erythrocytes. The upper phase, containing leucocytes, was transferred to 15 mL of PBMC Spin medium (pluriSpin, Leipzig, Germany) with a polysucrose solution (density of 1.077 g/mL) to separate the cells by centrifugation (400 xg, 20 min, low break) according to their density. The saline phase was discarded and the interphase with the PBMCs was transferred to a new Falcon. The cell pellet with the granulocytes was used for polymorphonuclear leukocyte isolation (see 2.2.3.2 Isolation of polymorphonuclear leukocytes). The interphase was filled up with wash buffer to achieve a final volume of 40 mL before centrifuging the cells at 400 xg for 7 minutes. The

supernatant was discarded, and the cells were washed once again in 20 mL wash buffer following a final centrifugation step for a final 7 minutes. For counting, the cells were stained with Kimura stain (1:10) and counted with a Neubauer chamber using a brightfield microscope.

2.2.3.2 Isolation of Polymorphonuclear Leukocytes (PMNLs)

The cell pellet derived from the immune cell separation using PBMC Spin medium was resuspended in 10 mL wash buffer following a centrifugation step at 400 xg for 7 minutes. The cell pellet was resuspended in 0.2 % saline, to remove remaining erythrocytes. Next the same volume of 1.6 % saline was added, before centrifuging the cells at 400 xg for 7 minutes. Again, cells were stained with Kimura stain (1:10) and counted with a Neubauer chamber using a brightfield microscope.

2.2.3.3 Purification of peripheral eosinophils

Isolation of eosinophil granulocytes was performed according to the manufacturer's protocol of the eosinophil Isolation Kit, human Miltenyi Biotec. In brief, the PMNL cell count was determined, and the cells were centrifuged at 400 xg for 7 minutes. The cell pellet was resuspended in 40 µl of buffer per 10^7 total cells. 10 µl of a cocktail of biotin-conjugated monoclonal antibodies (containing CD2, CD14, CD16, CD19, CD56, CD123 and CD235a) was added and the cells were incubated at 4 °C. After ten minutes, 30 µl of buffer and 20 µl of an anti-biotin antibody conjugated to MicroBeads were added. The cells were mixed and incubated for a further 15 minutes at 4 °C. Cells were washed, centrifuged at 400 xg for 7 minutes and resuspended in 1 mL of buffer. To isolate eosinophils, LS columns (developed for the gentle isolation of microbead-labelled cells) were washed with 3 mL of buffer, then the cell suspension was added and the eluent was collected by adding a further 3 mL for a total of three times. At a final volume of 12 mL, a final centrifugation step was performed and the cells were stained with Kimura stain (1:10) and counted using a Neubauer chamber.

2.2.4 Isolation of cells from sputum samples

The cell plug from the saliva of the sputum samples was divided and the selected sample weighed. A volume of 1:10 diluted DTT (D0632-5g, Sigma) was added 4 times in PBS (P4417, Sigma) and the sample vortexed for 15 seconds. The sample was placed on a tube roller for 15-30 Min at RT depending on the mucosity. The volume PBS was added 4 times and the sample vortexed for 15 seconds. The sputum sample was filtered with pre-wet mesh and the filtered sample centrifuged for 10 Min at 320 xg at 4°C. The cell pellet was resuspended in max. 1mL PBS and cytospins were made for diagnosis. Cells were spun for 10 Min at 320 xg at 4°C and it was continued with the same staining protocol used for whole blood samples.

2.2.5 Generation of Human Monocyte-Derived Macrophages

Human PBMCs were isolated from citrated blood by density gradient sedimentation. Peripheral blood monocytes in the PBMC fraction were resuspended in pre-warmed adhesion medium (RPMI supplemented with 5% human AB serum, non-essential amino acids, sodium pyruvate, HEPES and 1% penicillin/streptomycin) at a concentration of 10 Mio cells/mL and seeded onto Corning® CellBIND plates (Sigma-Aldrich, Missouri, USA) for 2 h at 37 °C in a humidified atmosphere with 5% CO_2. Non-adherent cells were washed off and enriched monocytes incubated with differentiation medium (10% FCS, 1% penicillin/streptomycin) and 20 ng/mL recombinant human (rh) M-CSF (Peprotech, New Jersey, USA, AF-300) for a week. Cultured human Monocyte-Derived Macrophages (MDMs) were polarized with 20 ng/mL rh IFN-γ (Immunotools, Germany, 11343534) and 100 ng/mL LPS (Sigma-Aldrich, L2880) into M1 cells or with 20 ng/mL rh IL-4 (Immunotools, 11340043) into M2 cells. The M1/M2 classification is a simplification of macrophage behaviour and is used in this thesis only to distinguish between these two mechanisms of polarization. To ensure a good viability a live dead staining was performed using zombie green (FITC-labelled, Biolegend, 42311, 1:500 dilution). Polarization was confirmed in accutase-detached macrophages by staining with monoclonal antibodies CD80-BV421 (Biolegend, 305222, 1:100 dilution) and CD206-APC-Cy7 (Biolegend, 321120, 1:100 dilution,) for 30 min.

2.2.6 Stimulations and treatments

2.2.6.1 Asthma/ allergy medication

Isolated PMNL were treated with 50 pM formoterol (Selleckchem, S2020), 500 pM fluticasone furoate (Selleckchem, S6476) or 400 nM levocetirizine (MCE, HY-B0814) to clarify the impact of commonly used allergy/asthma medication on clock protein expression in human PMNL.

2.2.6.2 Pro- or anti-inflammatory mediators

Isolated PMNL were stimulated with either 100 nM prostaglandin (PG) E_2 (Cayman Europe, 14010) or a pro-inflammatory interleukin cocktail. Due to the results of the human Th Cytokine 12 plex a mixture of IL-4 0,1 pg/mL (Immunotools, 1134004), I-L5 0,1 pg/mL (Peprotech, 200-05-10UG), IL-6 1,5 pg/mL (Peprotech, 200-06), IL-10 0,03 pg/mL (Immunotools, 11340103), IL-13 0,3 pg/mL (Biolegend, 571102) and IFN-γ 0,15 pg/mL (Immunotools, 1134353) was used. Mediators were added to RMPI medium supplemented with 1%FBS and 1% P/S to incubate the cells for 3 hours.

2.2.6.3 Clock-modulating ligands

Human PMNL or isolated eosinophils were pretreated with REV ERB or ROR agonists. For all experiments a concentration of 1-10 µM SR9009 (Sigma Aldrich, 1379686-30-2), 1-10 µM GSK4112 (Sigma Aldrich G0673), 10µM SR8278 (Sigma Aldrich, S9576), 10 µM SR1001 (MedChemExpress, 1334106-03-0) or 1-10 µM SR1078 (MedChemExpress, 1246525-60-9) was used.

2.2.7 Intracellular staining

Cells were fixed and permeabilized with FIX and PERM (Nordic-Mubio, GAS-002). Thereafter, the samples were blocked with human TruStain FcX™ Fc Receptor Blocking Solution (Biolegend, 422302) and clock proteins were stained intracellularly using primary antibodies NR1D1 (Abcam, ab174309), BMAL1 (Novusbio, NB100-2288) and ROR beta (Novusbio, NBP1-82532). The secondary donkey anti rabbit -PE (Biolegend, 406421)

antibody was used for detection. Samples were measured by flow cytometry and analysed as an increase over the isotype control.

2.2.8 Multiplex assays

2.2.8.1 Human Th Cytokine 12-plex

Serum samples from the monitoring experiment were used for simultaneous quantification of 12 human cytokines, including IL-2, 4, 5, 6, 9, 10, 13, 17A, 17F, 22, IFN-γ and TNF-α by the Human Th Cytokine Panel from Biolegend (741028) according to the manufacturer's instructions.

2.2.8.2 Human Chemokine 6-plex

Supernatant from macrophages was used to measure the concentrations of granulocyte colony stimulating factor (G-CSF), interleukin 8 (IL-8), monocyte chemoattractant protein 1 (MCP-1), monokine induced by interferon gamma (MIG), macrophage inflammatory protein (MIP) 1α and 1β using the human chemokine 6-plex FlowCytomix from eBioscience (California, USA) according to the manufacturer's instructions.

2.2.9 Analysis of Clock gene expression

Microarray data from Tsitsiou et.al. (126) were analysed to explore clock gene expression in circulating CD4+ and CD8+ T cells comparing 8 healthy controls and 8 severe asthmatic patients. The data discussed in this publication were accessed through GEO Series accession number GSE31773 (https://www.ncbi.nlm.nih.gov/sites/GDSbrowser).

Microarray data from Woodruff et.al. (127) were analysed to explore clock gene expression in alveolar macrophages comparing 15 non-smoking healthy controls and 15 non-smoking asthmatic patients. The data discussed in this publication were accessed through GEO Series accession number GSE2125 (https://www.ncbi.nlm.nih.gov/sites/GDSbrowser?acc=GDS1269#details).

2.2.10 Functional assays

2.2.10.1 Shape change

Following agonist treatment, PMNLs or isolated eosinophils were exposed to eotaxin-1 for 4 minutes at 37°C, and then rapidly fixed on ice. To differentiate eosinophils from neutrophils in the PMNL samples, the distinct autofluorescence characteristics of eosinophils were utilized. The cells were subsequently analysed for changes in forward scatter (FSC), which reflect alterations in cytoskeletal structure. Data were presented as the percentage increase in forward scatter relative to that observed in unstimulated control samples (128).

2.2.10.2 Chemotaxis

Prior to the experiment, a 48-well microBoyden chemotaxis chamber was incubated at 37°C for a couple of hours and PVP-free polycarbonate filters with a pore size of 5 µm were engulfed in +buffer for 30 minutes before the experiment. Isolated eosinophils were collected and resuspended in +buffer in concentration of 2 Mio/mL. Cells were transferred in FACS tubes and treated with clock modulating ligands. After an incubation period of 3 hours, cells were transferred onto the assembled Boyden chamber at a concentration of 100.000 cells per well. Cells were allowed to migrate for 60 minutes at 37°C. After that, upper wells were aspirated, and the content of the lower wells was fixed in 150 µL FIX and the migrated cells were enumerated by flow cytometry. Data were presented as the percentage increase in comparison to control samples (129).

2.2.10.3 Apoptosis

Purified eosinophils were cultured in RPMI 1640 (Fisher Scientific) supplemented with 1% FBS and 1% Penicillin/Streptomycin and 50 pM IL-5 (Peprotech, 200-05-10UG). After 0h, 3h and 22h cells were stained with APC-annexin-V (1:100, Biolegend, 640941) in the dark for 20 min at 4°C and Propidium iodide (1:50, Biolegend 421301) in the dark for 1 min at room temperature. Samples were immediately measured by flow cytometry for 1 min, and the total number of eosinophils gated on a forward scatter/side scatter plot and the percentage of alive cells (annexin-V negative/propidium iodide negative), early apoptotic cells (annexin-V positive/propidium iodide negative), late apoptotic cells (annexin-V

positive/propidium iodide positive) and necrotic cells (annexin-V negative/propidium iodide positive) were recorded. The data were presented as the percentage increase relative to control samples (130).

2.2.10.4 Respiratory burst

Purified human PMNL were pre-treated and stimulated with serial dilutions of the respective chemoattractant in the presence of 1 µM dihydrorhodamine-123 (Sigma Aldrich, D1054) for 20 min at 37 °C as described. Production of reactive oxygen species (ROS) was quantified by flow cytometry as the increase of fluorescence due to oxidation of nonfluorescent dihydrorhodamine-123 into fluorescent rhodamine-123 and analysed by FlowJo 10.7.1 software. Responses were expressed as geometric mean fluorescence intensity (MFI) (131).

2.2.10.5 Degranulation assay

PMNL were stained using CD16-PerCP-Cy5.5 (Biolegend, 301828) to divide them into CD16+ neutrophils and CD16- eosinophils. Further, cells were mixed with 5 µg/mL cytochalasin B (Sigma Aldrich, C6762), stained with FITC-CD63 (Biolegend, 353006) and samples measured by flow cytometry. The increase of geometric MFI during C5a stimulation was recorded.

2.2.10.6 Phagocytosis

The Vybrant Phagocytosis assay (ThermoFisher Scientific, V-6694) was performed and analysed according to the manufacturer's recommendation and measured by flow cytometry. In brief, fluorescein-labelled E. coli (K-12 strain) was internalized by the treated macrophages, followed by trypan blue staining to quench the fluorescence from the non-absorbed particles. Data were presented as the percentage increase in comparison to cell derived from healthy controls.

2.2.11 Signalling pathway analysis

2.2.11.1 Phosphokinase Array

The proteome profile phosphokinase array from Bio-Techne (ARY003C) was performed according to the manufacturer's instructions. In brief, isolated eosinophils from three asthmatic donors or three healthy donors were pooled together. Cells were divided again and treated with 10 µM SR1001 or DMSO as a control for 3 hours. After a washing step protein was extracted from the cells. The extracted protein was applied to a membrane containing capture antibodies spotted in duplicate. Subsequently, biotinylated detection antibodies were used to bind the captured proteins. Chemiluminescent detection reagents were applied to visualize the signal, which was captured using the Bio-Rad ChemiDoc Imaging System. Background signal was subtracted from the pixel density, and the mean value of the duplicates was calculated. The data from healthy donors were set as 100%, and comparisons were made with the values obtained from asthmatic donors.

2.2.11.2 Phospho-Flow

Eosinophils were treated with SR1001, SR1078 or DMSO as a control for 3 hours. Afterwards, cells were stimulated with eotaxin 1 nM -10 nM for 10 min. Eosinophils were fixed and stored in the fridge overnight. On the next day the cells were stained for total ERK (1:400 dilution, Cell signalling 4695S) and phospho-ERK (1:400 dilution, Cell signalling 90101S) for 30 Min at 4°C and PE-donkey anti-rabbit (1:500 dilution, Biolegend 406421) was used as a secondary Antibody to stain for 30 min. Samples were measured by flow cytometry.

2.2.11.3 Western Blot

Protein was extracted from isolated eosinophils using RIPA buffer supplemented with three-time protease inhibitors. Before loading the gel, protein content was determined by BCA according to the manufacturer's protocol. Next, 10 µg of protein were mixed with 6x Laemmli buffer containing 5 % β-mercaptoethanol and samples were cooked at 95°C for 5 minutes. Cells were loaded onto Novex 10 % Tris-Glycine gels and run 125 Volt for 1.5 hours before being washed and fast blotted onto a nitrocellulose membrane, using iBlot technology.

Membranes were blocked with 3% BSA-TBST and incubated with a primary pERK, tERK, and β-actin overnight. On the following day, the secondary antibodies were applied. HRP signal was measured using the iBright system.

2.2.12 Immunofluorescent staining

2.2.12.1 Human lung biopsies

Biopsies from asthmatic patients and non-tumorous human lung samples underwent deparaffinization followed by a heat-induced antigen retrieval in sodium citrate buffer (pH 6). Unspecific binding was blocked with 4% BSA and 10% goat serum in PBS for 2 h at room temperature. Primary antibodies BMAL1 (Novusbio, NB100-2288, 1:100 dilution) and EPX (clone MM25-82.2; 5 µg/mL; kindly donated by Dr. Elizabeth Jacobsen) were incubated overnight. Sections were incubated with secondary antibodies Cy3-labeled donkey anti-rabbit and goat anti-mouse conjugated with Alexa Fluor 488 (both from ThermoFisher Scientific, 1:500) for 2 h on the next day. Nuclear counterstaining was performed with DAPI-containing TrueVIEW® Autofluorescence Quenching Kit. All images were acquired using constant laser settings with a Nikon A1+ confocal microscope. Constant laser settings were applied for all image acquisitions. Control slides were stained with secondary antibodies only (132).

All images were acquired using constant laser settings with a Nikon A1+ confocal microscope. As a control, slides were stained with secondary antibodies only.

2.2.12.2 Human precision cut lung slices (PCLS)

Serial cut PCLS (4 µm) were deparaffinized, followed by a heat-induced antigen retrieval in sodium citrate buffer (pH 6). Unspecific binding was blocked with 4% BSA and 10% goat serum in PBS for 2 h at room temperature. Primary antibodies BMAL1 (Novusbio, NB100-2288, 1:100 dilution) and CD68 (Abcam, ab955, 1:100 dilution) were diluted in 1:10 diluted blocking solution and slides were incubated overnight at 4 °C. After washing steps, sections were incubated with secondary antibodies Cy3-labeld donkey anti-rabbit and goat anti-mouse and conjugated with Alexa Fluor 488 (both from ThermoFisher Scientific, 1:500) for 2 h. Nuclear counterstaining was performed with the DAPI containing mounting medium

TrueVIEW® Autofluorescence Quenching Kit (Vector Laboratories, California, USA). Images were taken with a fluorescence microscope (133).

All images were acquired using constant laser settings with an Olympus IX73 fluorescence microscope. Controls included unstained cells, slides stained with either BMAL1 only or CD68 only, and secondary antibodies only. Images were automatically analysed with the same threshold settings using Image J. In brief, FITC-channel CD68+ positive cells were considered as macrophages and the fluorescence intensity of BMAL1 was measured in Cy3 channel within these cells.

2.2.13 Animal experiment

2.2.13.1 In vivo migration model

7-10-week-old IL-5Tg mice were treated i.p. with SR1001 (25 mg/ kg/ twice a day) or vehicle for 5 times. *In vivo* chemotaxis of eosinophils was induced by intranasal instillation of 6 µg eotaxin. Bronchoalveolar lavage (BAL) fluid and blood was collected 4 hours afterwards, and cell populations were detected by flow cytometry using CD11b-PE-Cy7, CD11c-BV421, Ly6G-APC and Siglec-F-PE antibodies (134).

2.2.13.2 House dust mite (HDM) model

8-12-week-old BALB/c mice were challenged intranasally with 10 µg of house dust mite allergen (dissolved Acarizax SLIT-tablet) once a week. After 4 times the mice were treated i.p. with SR1001 (25 mg/ kg/ twice a day) or vehicle every 12 hours, in total for 5 times. Lung function/AHR of these mice was measured by a methacholine challenging test using the FlexiVent platform (Scireq). Additionally, BAL fluid and blood was collected and cell populations were detected by flow cytometry using CD11b-PE-Cy7, CD11c-BV421, Ly6G-APC and Siglec-F-PE antibodies. Lungs were either snap-frozen in liquid nitrogen or fixed with formalin for 1 h at room temperature and embedded in paraffin (1).

2.2.13.3 LabMaster system

The LabMaster system (TSE Systems, Bad Homburg, Germany) was employed to analyse the effect of SR1001 on the circadian pattern of locomotion, exploration, drinking and feeding in singly housed IL-5Tg mice and HDM challenged BALB/c mice. The locomotion, exploratory behaviour, and water and food intake of the test mice were continuously recorded in the home-like environment of the LabMaster system (TSE Systems, Bad Homburg, Germany) as previously described (135). For this purpose, transparent LabMaster experimental cages were surrounded by two frames emitting infrared beams to measure vertical exploratory behaviour as well as horizontal locomotor activity by counting infrared beam interruptions. In addition, two weight sensors attached to the cage lids were used to assess ingestive behaviour as a food container and a drinking bottle were attached to the sensors throughout the experiment. All recording devices were connected to a personal computer, which was used to record and analyse the data using LabMaster software. Food and water intake were recorded in grams of food (g) and millilitres of water (mL) respectively. Before starting the agonist treatment, the animals were habituated to the food containers and the drinking bottles used in the LabMaster system as well as to single housing for three days. In the LabMaster system, the mice need to be housed individually to allow accurate activity measurements. Animals were placed into LabMaster cages to habituate the mice to single housing and the used drinking bottles (135).

2.2.13.4 Lung function measurements

On the last day, HDM challenged mice were anaesthetized, and their lung function including parameters such as airway resistance and lung compliance were measured under methacholine challenge. Herein the lung function/AHR of these mice was detected in response to increasing concentrations of methacholine through the FlexiVent platform (Scireq) (136).

2.2.13.5 Haematoxylin and eosin staining

Lung sections of 5 μm were deparaffinized and stained with haematoxylin and eosin (H/E) staining Olympus IX73 fluorescence microscope. Six to eight photomicrographs at 100 × magnification were taken and automatically evaluated in a blind fashion by Image J.

2.2.13.6 Periodic-Acid-Schiff (PAS) staining

Mouse lung sections of 5 µm were deparaffinized followed by oxidation in a periodic acid solution for 5 min. Slides were washed in distilled water and placed in Schiff´s reagent for 15 min. Sections were washed for 5 min followed by a counterstain with Myer´s haematoxylin. Samples were dehydrated, put in xylene and covered in mounting media. All slides were scanned with the Aperio slide scanner (Leica) and analysed by Image J.

2.2.14 Statistical Analyses

All data are shown as mean ± SEM for n observations. Correlations were created and analysed with RStudio (PBC, Boston, MA, URL http://www.rstudio.com/). Other statistical analyses were performed using GraphPad Prism software 6.0 (La Jolla, CA; USA). Statistical outliers were identified using either the ROUT method (applicable exclusively to RNA data sets) or the Grubbs test. Groups were compared by t-test or Mann-Whitney, One-way or Two-way ANOVA followed by Bonferroni post hoc test. Probability values of $P < 0.05$ were considered statistically significant and are indicated as $*P < 0.05$; $**P < 0.01$; and $***P < 0.001$.

3. Results

Some parts of this section have been published in adapted form (1,2).

3.1 Expression of the molecular circadian clock at the protein level in human leukocyte populations

To validate the daily oscillation of clock proteins in human peripheral blood immune cell subsets, we conducted a 24-hour monitoring experiment utilizing a whole blood staining protocol as previously described (1,2). Blood samples were collected at 4 a.m., 12 p.m., and 8 p.m., with half of each sample stained immediately and the other half processed after four hours, resulting in six time points per day. Cells were gated based on their FSC/SSC properties and respective surface markers into autofluorescence+CD16- eosinophils, autofluorescence-CD16+ neutrophils, CD3+ T cells and CD14+ monocyte subsets. Due to the low abundance of intermediate and non-classical monocytes, it was not possible to distinguish between these two populations; however, analyses are represented as CD14+CD16- and CD14+CD16+ monocytes (**Figure 5**) (1,2).

All participants were categorized into two groups: healthy donors and mild asthmatics, based on clinical diagnoses by external respiratory physicians following GINA guidelines, total IgE (≥100 U/mL (128)), and specific IgE levels [U/mL] as summarized in the demographic table (**Table 4**) (2).

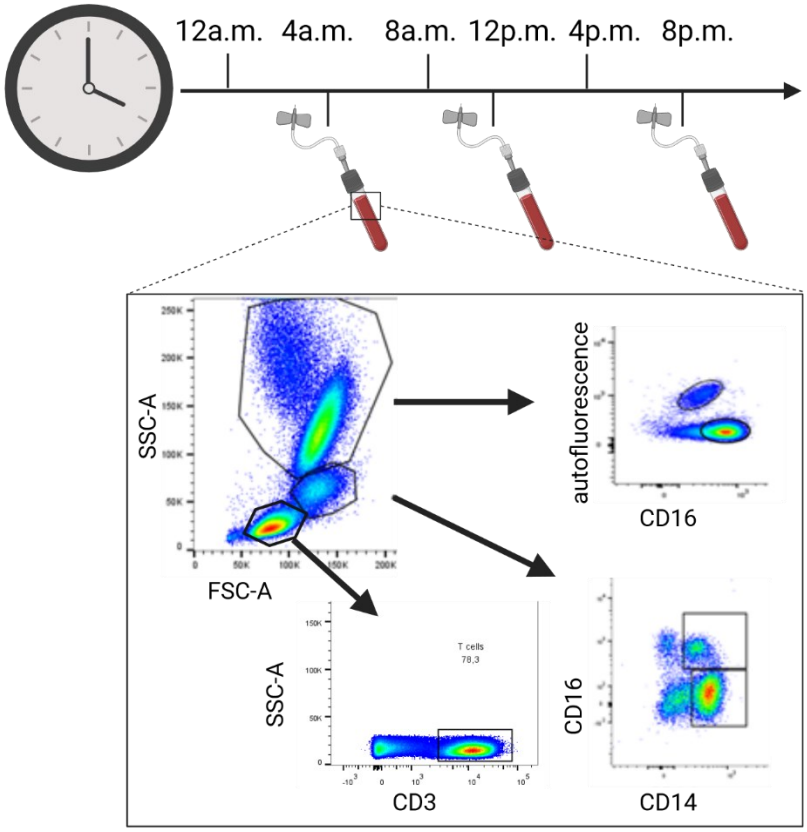

Figure 5: Experimental setup: *Blood samples were collected three times daily (at 4 a.m., 12 p.m., and 8 p.m.) and either processed immediately or incubated for an additional 4 hours, resulting in six evaluation time points per day. Eosinophils, neutrophils, T cells, CD14⁺CD16⁻ and CD14⁺CD16⁺ monocytes were identified based on forward/side scatter characteristics and surface markers. Schematic illustrations were created with BioRender.com. Figure adapted from (1)*

Table 4: Demographic table of healthy donors (n=8) and mild asthmatics (n=9). *Table adapted from (2)*

		healthy donors		mild asthmatics		
		average	SD	average	SD	p-value
participants		8		9		
age		34	5.92	34	10,8	0.2998
gender	female	3		5		
	male	5		4		
total IgE [U/mL]		31	24.44	278	0.53	**0.0152**
specific IgE [U/mL]	grass pollen	0.038	0.039	10.214	14.852	**0.0018**
	tree pollen	0.031	0.047	7.357	16.687	**0.0008**
	HDM	0.075	0.102	5.020	7.546	**0.0195**
	milk protein	0.036	0.037	0.088	0.132	0.4111
	peanut	0.044	0.085	1.387	3.092	**0.0169**
medication	anti-histamines	0		4		**0.0312**
	low daily ICS dose ± LABA	0		3		0.1685

Our results clearly demonstrate that the clock proteins of the stabilizing loop (BMAL1, CLOCK, REV ERBs, and RORs) are expressed in an oscillating manner in eosinophils (**Figure 6**), neutrophils (**Figure 12**) (2), T cells (**Figure 16**), classical CD14+CD16- monocytes (**Figure 24**), and CD14+CD16+ monocytes (**Figure 28**) (1) from healthy donors. A more detailed description of these oscillations is provided for each of the following immune cell types:

3.1.1 Eosinophils

In eosinophils, the core protein BMAL1 shows its lowest expression at 8 a.m. and peaks at 12 a.m. Its binding partner, CLOCK, has the highest protein expression in the first half of the day, with the lowest levels observed at 4 p.m. Consistent with expectations, the repressor of BMAL1, REV ERBα, exhibits an expression pattern opposite to BMAL1. REV ERBα peaks at 4 a.m. and shows the lowest level in the evening. The second member of this nuclear receptor family, REV ERBβ, also peaks at 4 a.m. and 8 p.m. Notably, higher expression levels of the BMAL1 activators RORα and RORβ are observed in the first half of the day, while elevated levels of RORγ persist until 4 p.m. (**Figure 6**) (2).

Peripheral eosinophils from blood donors with mild asthma show a reduced overall amplitude for CLOCK, REV ERBβ, and RORα, with a lesser reduction observed for REV ERBα, compared to healthy donors. Additionally, these eosinophils exhibit changes in period lengths and phase shifts in the oscillation of all clock proteins. Furthermore, a significant decrease in REV ERBα protein levels was observed at 4 a.m. when comparing expression of circulating eosinophils from mild asthmatics across specific time points. In the evening, REV ERBα expression was also notably increased. At 4 p.m., a marked reduction in CLOCK expression was detected in only healthy blood donors. Additionally, in peripheral eosinophils from mild asthmatic blood donors, lower expression levels within the activating receptor family ROR were evident at 8 a.m. in, with the most prominent decrease in RORβ expression occurring at 8 p.m., coinciding with a significant decline in REV ERBα levels. These results reflect an overall dampening of peripheral clock oscillation in blood eosinophils from asthmatic donors (**Figure 6**) (2).

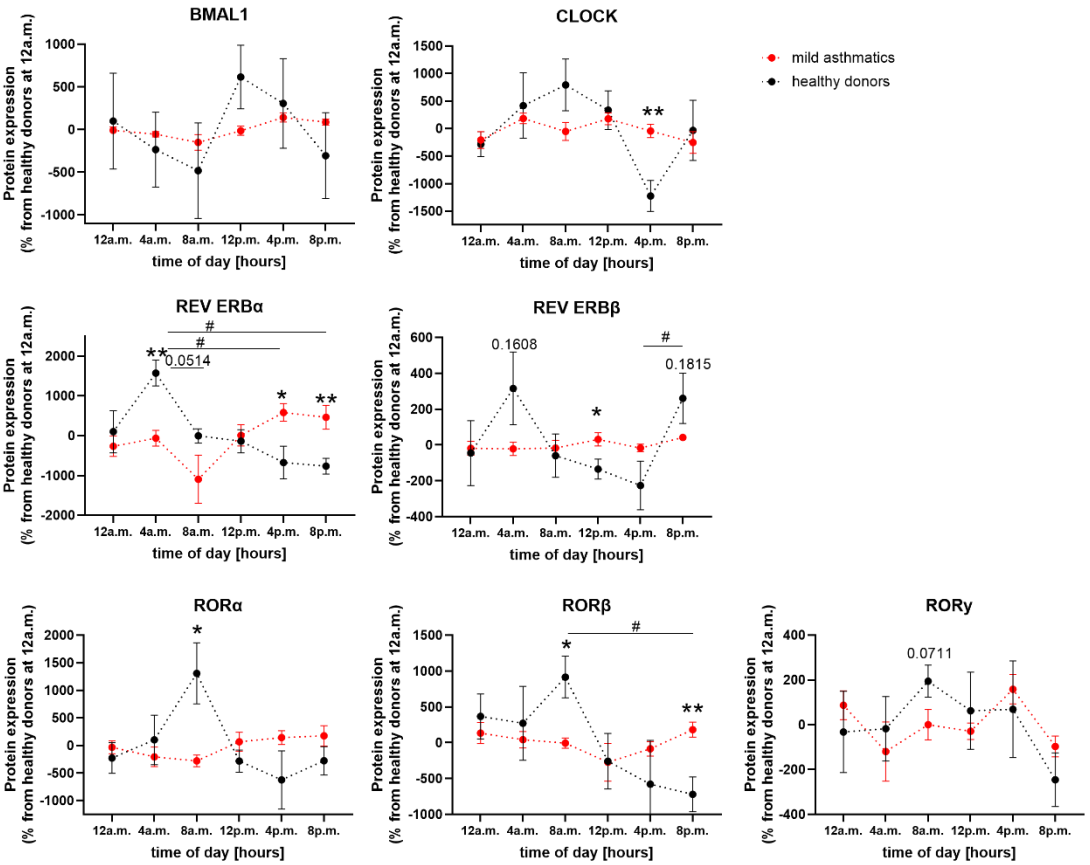

Figure 6: Peripheral blood eosinophils displayed oscillatory expression patterns of the clock proteins BMAL1, CLOCK, REV ERBs, and RORs, with significant differences observed between asthmatic patients and healthy control group. *For statistical evaluation, Z-scores were computed and normalized to the average value of the healthy control group at 12 a.m. Data are presented as mean ± SEM. Group-matched repeated measures were analysed using Two-Way ANOVA followed by Tukey's post hoc test. Intra-group comparisons are shown using coloured lines (red for mild asthmatics, black for healthy donors), while inter-group comparisons are marked with asterisks. * and # denote p < 0.05; ** indicates p < 0.01. Figure adapted from (2).*

When comparing clock protein expression in the forenoon (combining the morning time points 8 a.m. and 12 p.m.) and in the afternoon (4 p.m.), we observe reduced BMAL1 and RORβ levels in the forenoon in peripheral eosinophils from mild asthmatic donors. In the afternoon, significant lower expression of CLOCK, REV ERBs and RORγ is observed. Despite a small sample size, eosinophils from allergic but non-asthmatic donors display a similar trend, although these differences do not reach statistical significance (**Figure 7**).

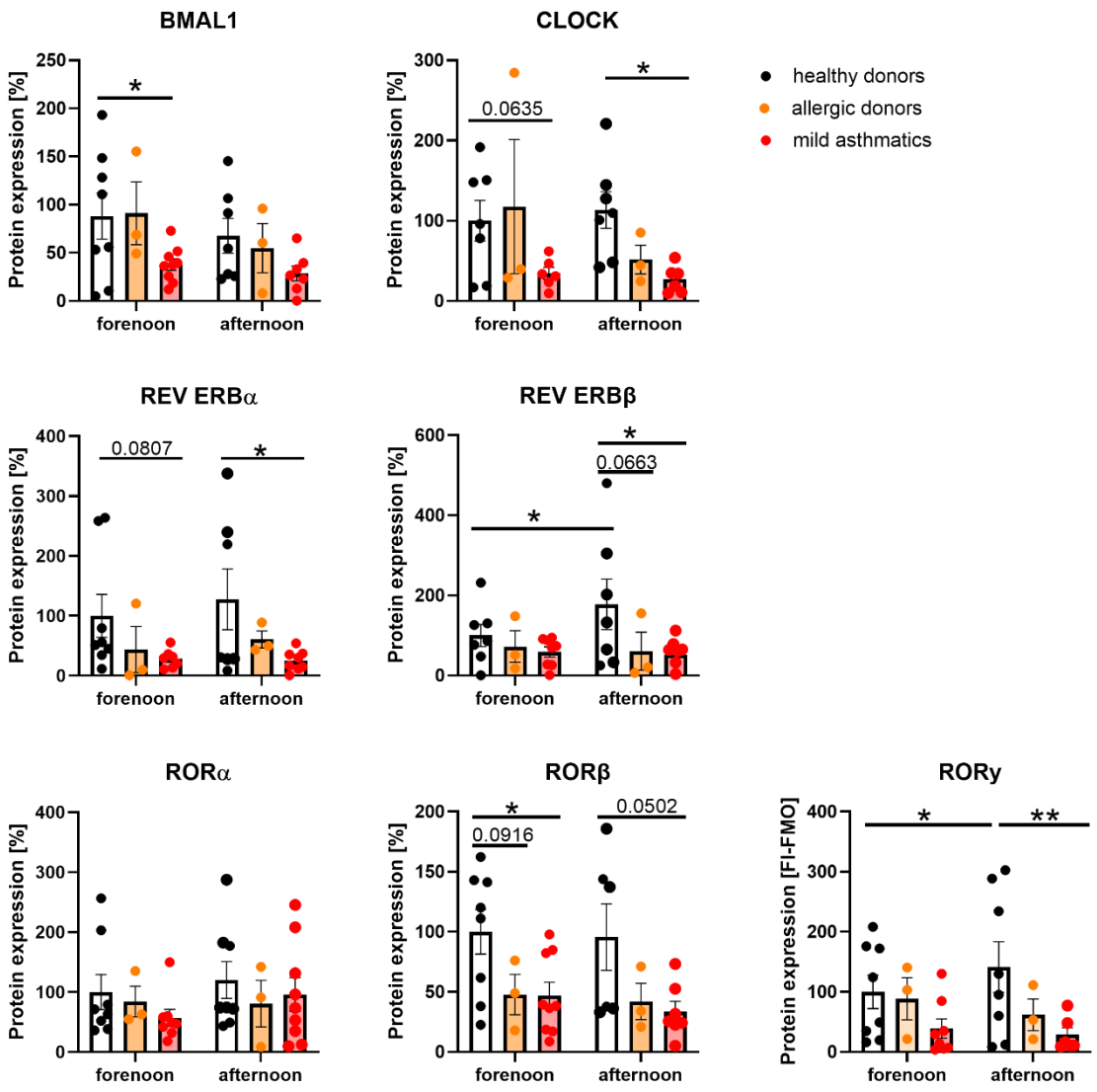

Figure 7: Clock protein expression in eosinophils was compared among healthy individuals, allergic subjects, and patients with mild asthma. *Measurements from 8 a.m. and 12 p.m. were averaged to represent the forenoon period, while data from 4 p.m. were used for the afternoon. Values were normalized to their corresponding control groups. Statistical analysis was performed using Two-Way ANOVA. Significance levels are indicated as follows: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.*

To further support these findings, we assessed clock protein expression in a well-characterized moderate asthma cohort, clustered by the time of blood collection as shown in **Table 5.** Compared to a healthy control group, significantly lower levels of all clock proteins from the stabilizing loop, except for REV ERBα, were detected in eosinophils in the forenoon. In the afternoon, peripheral eosinophils from individuals with moderate asthma showed a significant decrease in REV ERBα and RORβ expression, along with lower levels of BMAL1 and RORα, when compared to healthy blood donors (**Figure 8**). These findings are consistent with our previous experiments, which demonstrated that eosinophils from mild asthmatics exhibit significantly lower levels of BMAL1 and RORβ in the morning, as well as reduced levels of CLOCK and REV ERBα in the afternoon as shown in **Figure 7**.

Together, these data further support the hypothesis that the expression of clock proteins is diminished in the peripheral blood eosinophils of individuals with asthma (2).

Table 5: Demographic table of healthy donors and moderate asthmatics. *All participants were allocated to the forenoon or afternoon group depending on the time of evaluation. Table adapted from (2)*

		forenoon (9a.m. – 1p.m.)		afternoon (1p.m.–5 p.m.)	
		healthy donors	moderate asthmatics	healthy donors	moderate asthmatics
participants		5	12	5	7
age		54	48	36	45
gender	female	3	10	3	5
	male	2	2	2	2
BMI		29	30	27	33
high FeNO (>50ppb)		-	6	-	6
blood test	high eosinophils (>0.3*109/L)	0	8	0	3
	sensitized	1	9	0	3
	atopic	0	9	0	2
spirometry data [%]	FEV1 predicted	-	80	-	93
	FVC predicted	-	91	-	97
	obstructive	-	14	-	20
daily ICS dose ± LABA	medium	-	10	-	2
	high	-	3	-	2
SABA		-	9	-	2

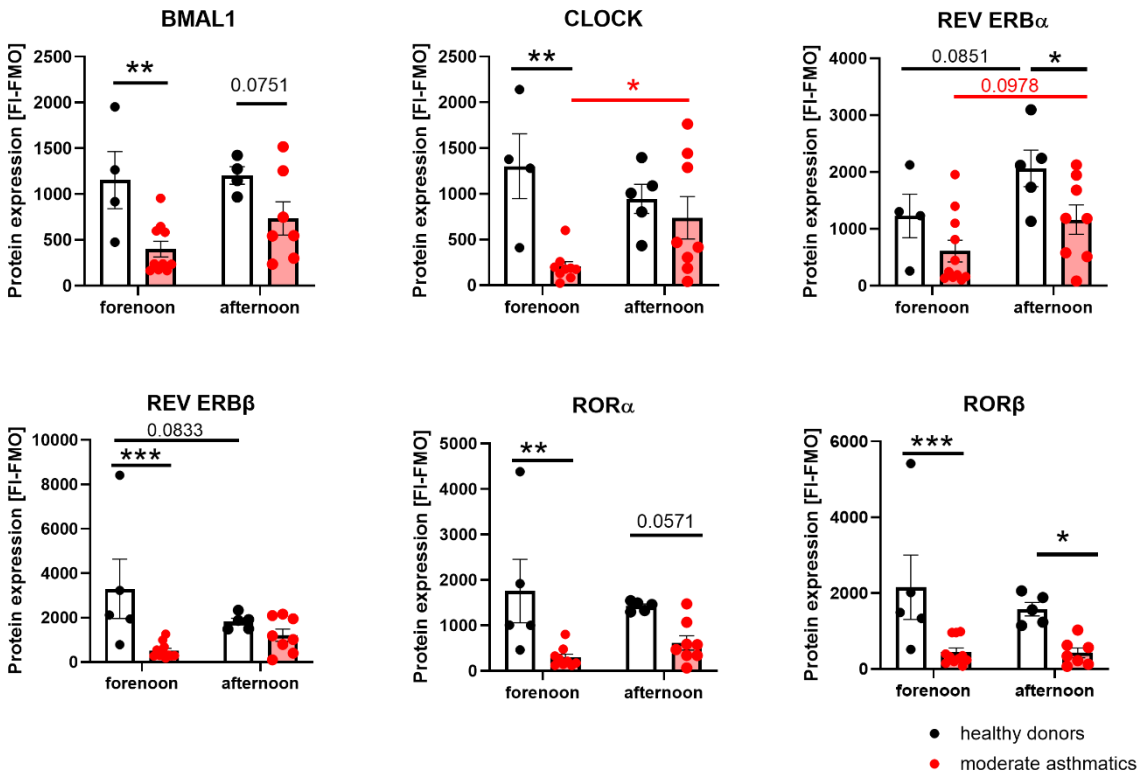

Figure 8: Eosinophils from individuals with moderate asthma exhibited reduced expression of clock proteins compared to healthy individuals. *Significantly lower protein levels were observed in the moderate asthma group (red) relative to healthy donors (black) across both groups. Statistical analysis was conducted using group-matched repeated measures Two-Way ANOVA followed by Tukey's post hoc test. Significance is indicated as follows: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Figure adapted from (2).*

Interestingly, in a small group of moderate asthmatics who worked shifts, we observed contrasting results. In eosinophils, an increased expression of CLOCK, REV ERBs, and RORα was measured compared to moderate asthmatics on a conventional work schedule (**Figure 9**) (2). However, it is important to emphasize that despite the very small sample size, these significant differences in clock protein levels were still evident. Further, shift work is known to disrupt the endogenous circadian system and its alignment with environmental cues, which may contribute to an increased risk of pulmonary diseases (129).

Table 6: Demographic table of moderate asthmatics with conventional work schedules and shift workers. *All participants were allocated to the morning (9 a.m.-1 p.m.). Table adapted from (2).*

moderate asthmatics		working schedule			
		conventional		shift worker	
		mean	SD	mean	SD
participants		12		3	
age		48	19,8	44	10,61
gender	female	8		2	
	male	4		1	
BMI		30	5,21	30	2,20
asthma	uncontrolled	1		1	
	high FeNO	6		1	
spirometry data	FVC predicted	90	19.09	88	4.55
	obstructive	3		2	
blood test	atopic	5		n.a.	
	high blood eosinophils (>0.3*10^9/L)	8		3	
environmental triggers	mould	2		2	
	pet owner	4		2	
smoking history	(ex-)smoker	5		2	
	pack years	2	3.52	6	9.66
shift worker		**0**		**3**	

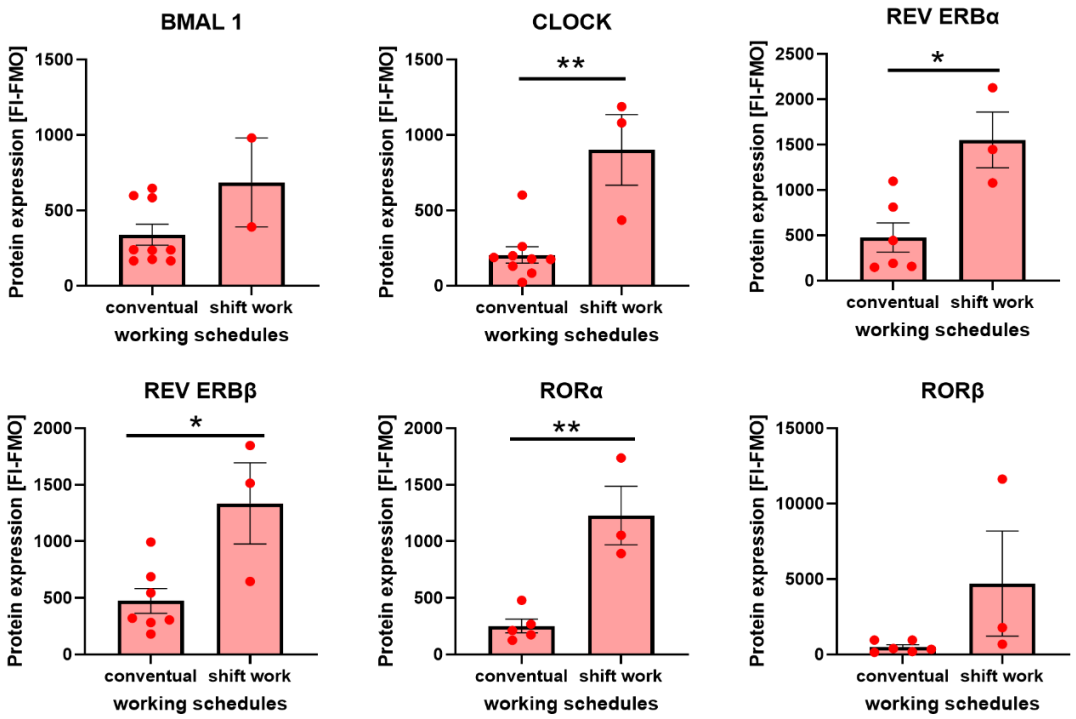

Figure 9: Forenoon expression of circadian proteins in peripheral eosinophils of shift-working moderate asthmatic patients is elevated. *(A) Demographic summary of moderate asthma patients in the morning group, categorized by conventional work schedule (n ≤ 7) or shift work (n = 3). (B) Significantly increased expression of REV ERB and ROR receptor families was detected in shift-working individuals compared to those with a standard daytime schedule. Statistical analysis was performed using a T-test. Significance is indicated as follows: * p < 0.05; ** p < 0.01; *** p < 0.001. Figure adapted from (2).*

3.1.1.1 Molecular circadian clock of lung-resident and sputum eosinophils

Under pathological conditions, peripheral blood eosinophils migrate from the circulation into the airways and lung tissue. In the context of airway inflammation, eosinophil levels increase in the sputum, indicating their recruitment and accumulation in the respiratory tract (130). Therefore, our aim was to detect the molecular circadian clock in sputum and lung resident eosinophils, in addition to peripheral eosinophils. Our study is the first to demonstrate that the circadian clock is also expressed and detectable in tissue and sputum eosinophils from asthmatics, as shown in **Figure 10** (2).

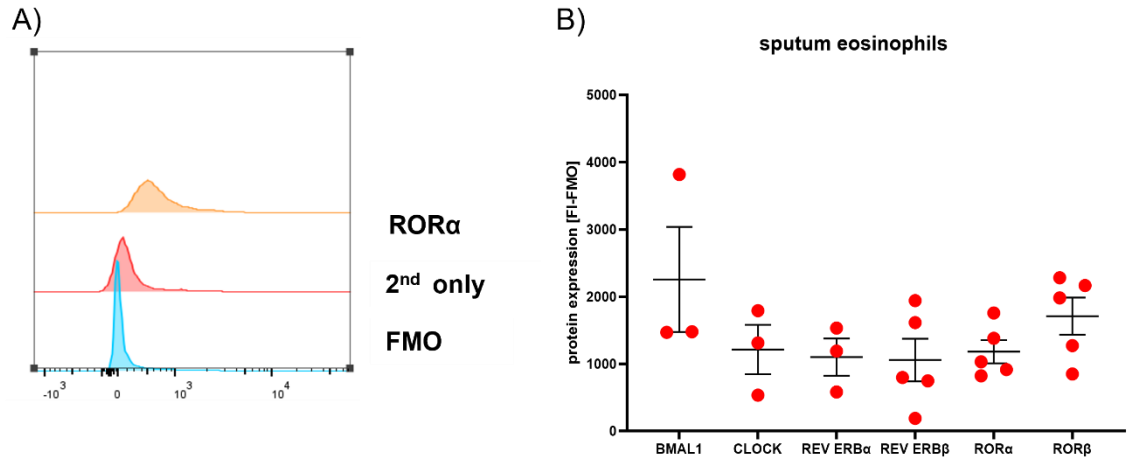

Figure 10: Clock protein expression was detected in sputum eosinophils from patients with severe asthma. *Cells were obtained from fresh spontaneous sputum samples using DTT treatment and analysed by flow cytometry following staining for surface markers and intracellular clock proteins (n ≥ 3; data presented as mean ± SEM). Figure adapted from (2)*

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Furthermore, we observed BMAL1 expression in lung-resident eosinophils from patients with asthma and in lung biopsies from non-asthmatic individuals (**Figure 11**) (2).

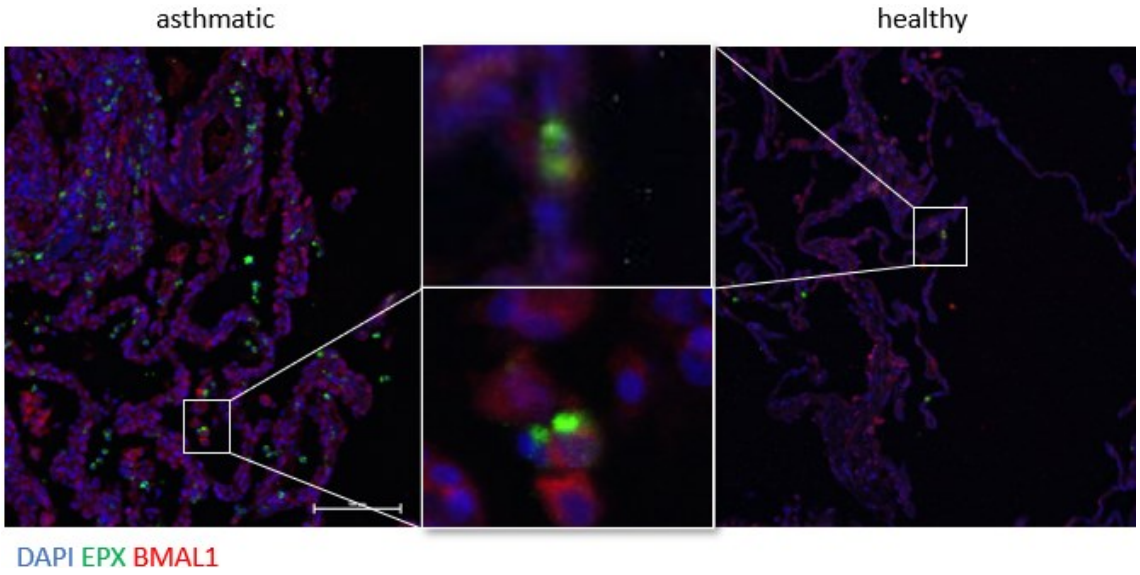

Figure 11: Clock proteins are expressed in tissue eosinophils of asthmatics. *(A) Cells were isolated with DTT from fresh spontaneous sputum samples from severe asthmatic patients. Samples were stained for surface markers and intracellular clock proteins by flow cytometry (n=4, mean ± SEM). (B) Biopsies from asthmatics and healthy controls were stained with anti-EPX for eosinophils and anti-BMAL1. Representative images are shown (scale bar 100 µm). EXP is shown in green and BMAL1 is labelled in red (n≥3). Images are published in (2).*

3.1.2 Neutrophils

In circulating neutrophils from non-allergic blood donors, the lowest levels of the main orchestrator BMAL1 were observed at 4 a.m. and 4 p.m. Interestingly, its binding partner CLOCK showed the highest expression peak at 4 p.m.. REV ERBα expression was lower during the day (8 a.m. to 4 p.m.) compared to night-time, while the second member of the REV ERB receptor family displayed a clear peak at midnight. Overall, ROR expression tended to be lower during the day, with peaks observed for RORα and RORγ during the night, and RORβ peaking at 8 p.m. (**Figure 12**) (2).

In contrast to the expression pattern of peripheral eosinophils (**Figure 6**), less differences are observed in the oscillating clock protein expression pattern of peripheral neutrophils from mild asthmatics. As shown in **Figure 12**, at 8 a.m., CLOCK expression reaches its lowest level in peripheral neutrophils from healthy blood donors, resulting in a significant difference compared to individuals with mild asthma. The BMAL1 activators, RORs, peaks at night, specifically at 12 a.m. and 8 p.m., when significantly higher levels of RORβ are observed in healthy blood donors compared to mild asthmatics (2).

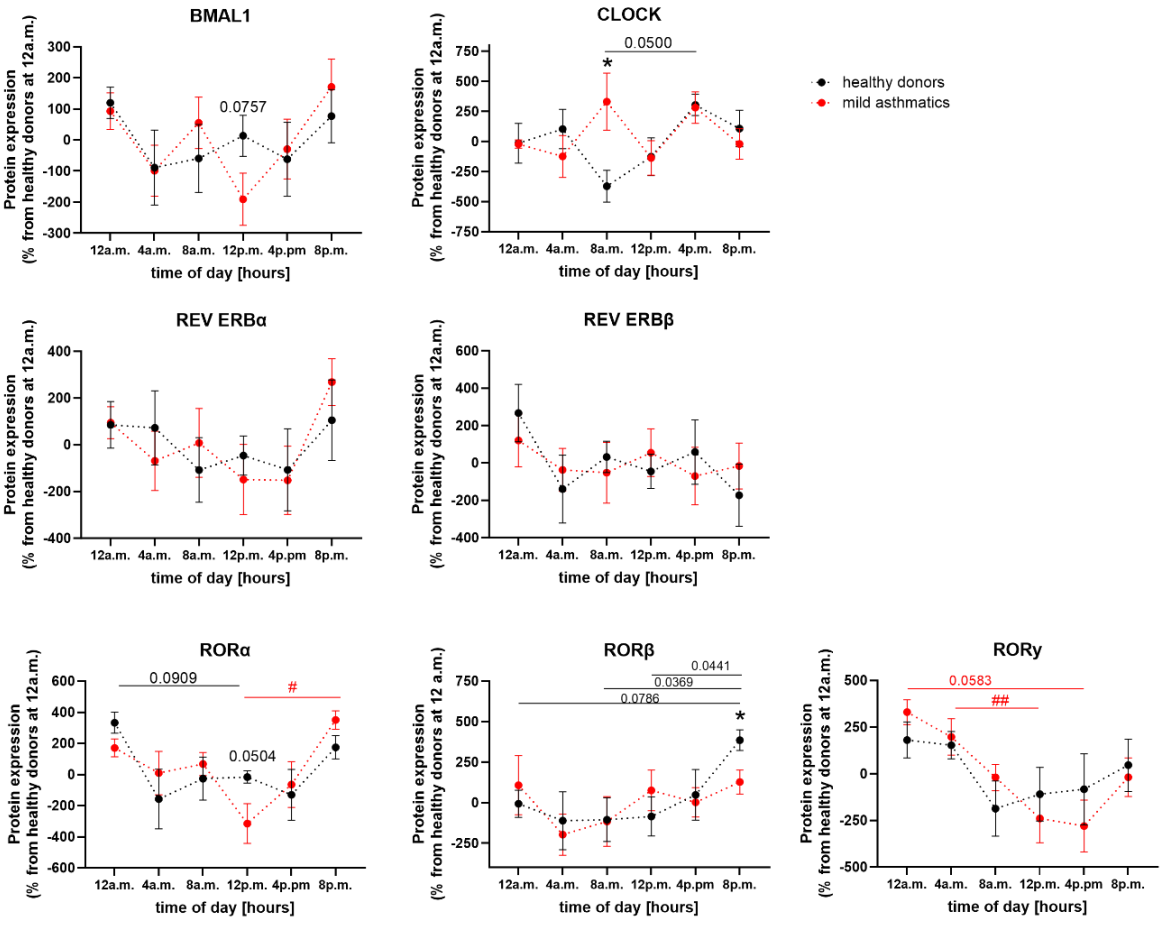

Figure 12: Human peripheral neutrophils display an oscillatory expression pattern of clock proteins. *Blood samples were collected every 4 hours from healthy individuals (black, n = 9) and mild asthma patients (red, n = 8), and expression of BMAL1, CLOCK, REV ERBs, and RORs was analysed by flow cytometry. Neutrophils were identified based on FSC/SSC characteristics and surface marker staining. Z-scores were calculated and normalized to the average of the healthy control group. Data are shown as mean ± SEM. Statistical analyses included the ROUT outlier test, paired Two-Way ANOVA, and Tukey's post hoc test. Intra-group comparisons are indicated with coloured lines and matching hashtags; inter-group comparisons are marked with asterisks. * and # denote p < 0.05; ** and ## indicate p < 0.01. Figure adapted from (2).*

Comparison of neutrophil clock protein levels from healthy, allergic, or asthmatic donors in the forenoon and afternoon, revealed no significant differences (**Figure 13**) together with fewer overall changes in neutrophils.

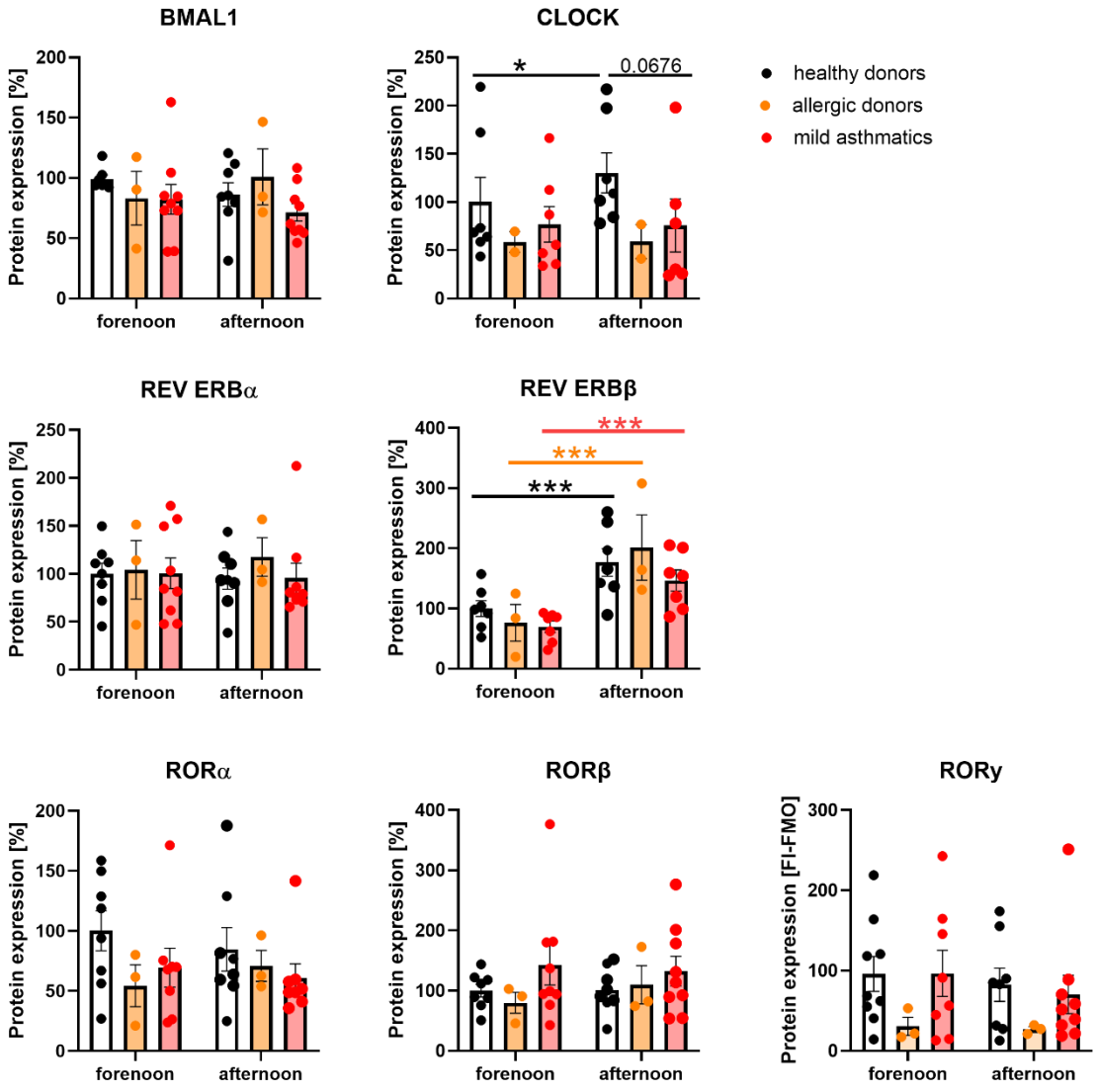

Figure 13: Clock protein levels in neutrophils were compared among healthy donors, allergic individuals, and patients with mild asthma. *Measurements taken at 8 a.m. and 12 p.m. were averaged to represent the morning time point, while 4 p.m. was used to represent the afternoon. Data were normalized to their respective control groups. Statistical analysis was performed using Two-Way ANOVA. Significance levels are indicated as follows: * p < 0.05; ** p < 0.01; *** p < 0.001; **** p < 0.0001.*

Similarly, to peripheral eosinophils (**Figure 8**), clock protein expression in blood neutrophils of moderate asthmatics was significantly reduced in the forenoon, except for REV ERBα. In the afternoon, lower levels of BMAL1, REV ERBα, and RORs were detected in comparison to healthy blood donors (**Figure 14**) (2).

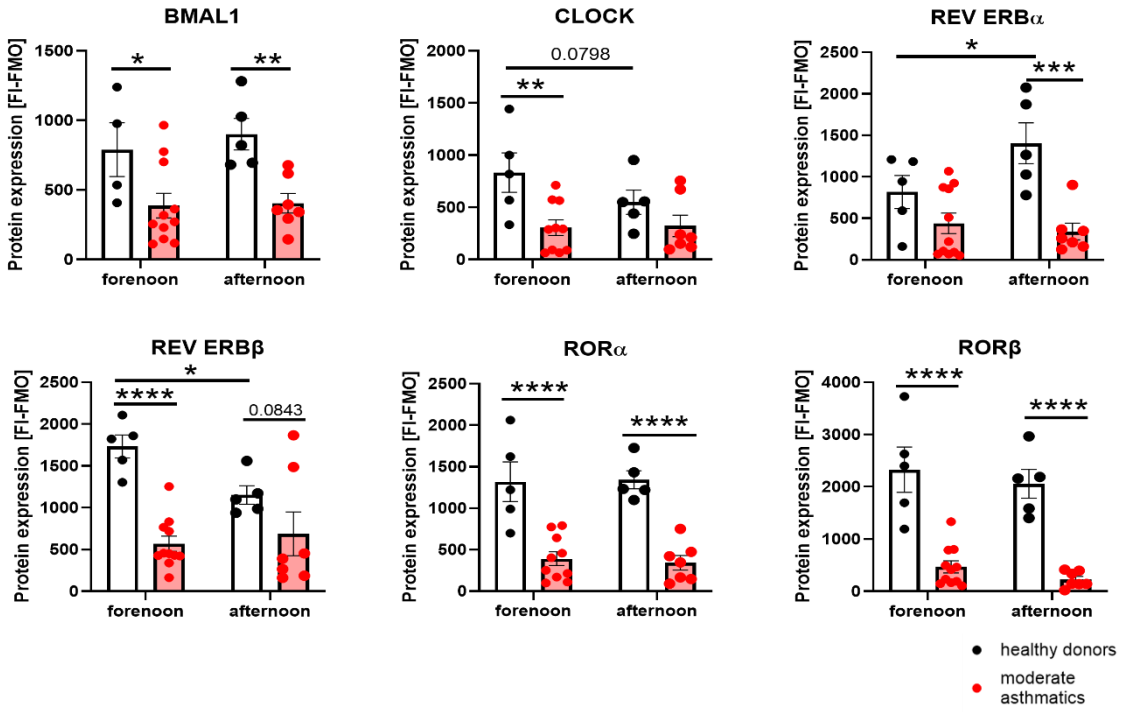

Figure 14: Reduced clock protein expression in neutrophils of patients with moderate asthma. *Clock protein levels were significantly lower in neutrophils from moderate asthmatic patients (red) compared to healthy controls (black) across both analysed groups. Statistical evaluation was performed using group-matched repeated measures Two-Way ANOVA followed by Tukey's post hoc test. Significance is indicated as follows: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Figure adapted from (2)*

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In contrast to eosinophils from moderate asthmatics working shifts, less significant differences were observed in neutrophils. A significant increase was observed only in RORβ (**Figure 15**) (2).

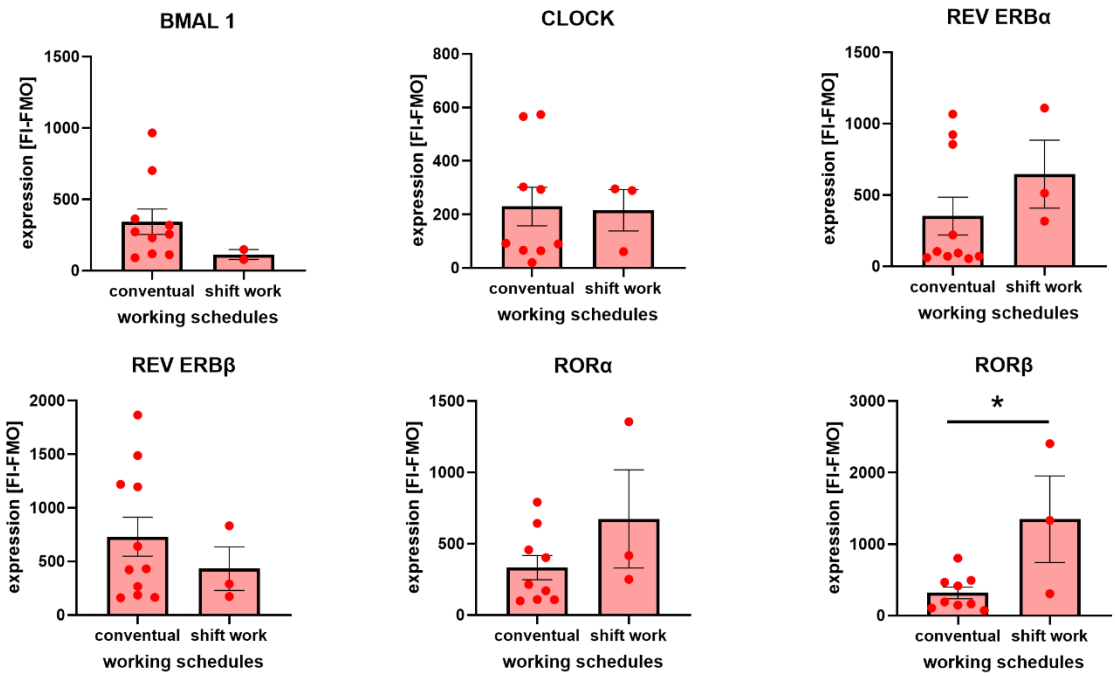

Figure 15: RORβ expression in neutrophils during the forenoon is elevated in moderate asthmatic patients engaged in shift work. *Significantly higher RORβ levels were detected in shift-working individuals (n = 3) compared to those following a conventional daytime schedule (n ≥ 7). Statistical analysis was performed using a T-test; * p < 0.05. Figure adapted from (2).*

3.1.3 T cells

In T cells from healthy individuals, the core protein BMAL1 exhibits its lowest expression in the morning, reaching its peak at midnight. Its binding partner, CLOCK, shows a shorter period length with highest protein expression around midnight and again between 8 a.m. and 12 p.m., while lowest levels are observed at 4 p.m. The BMAL1 repressor, REV ERBα, displays lower protein expression during the first half of the day, with a peak at 8 p.m. The second member of this nuclear receptor family, REV ERBβ, also demonstrates lower protein levels in the morning and at noon, peaking at 4 p.m. just before REV ERBα. As expected, the BMAL1 activator, RORα, follows a similar oscillatory pattern to BMAL1, with higher levels observed in the latter half of the day. The other nuclear receptor family members, RORβ and RORγ, show distinct expression patterns, with a longer period length observed for RORβ reaching its lowest expression towards the night, while RORγ exhibits its lowest protein levels at 8 a.m. (**Figure 16**).

In T cells from mild asthmatics different oscillation pattern are observed. For instance, the lowest expression of REV ERBs and RORs, with the exception of RORβ, is observed at 4 p.m. Compared to T cells from healthy blood donors, circulating T cells from mild asthmatics show an increase in REV

ERBα at noon and significant lower RORα levels at 4 p.m. (**Figure 16**). These findings indicate altered clock protein expression in blood T cells derived from asthmatic donors.

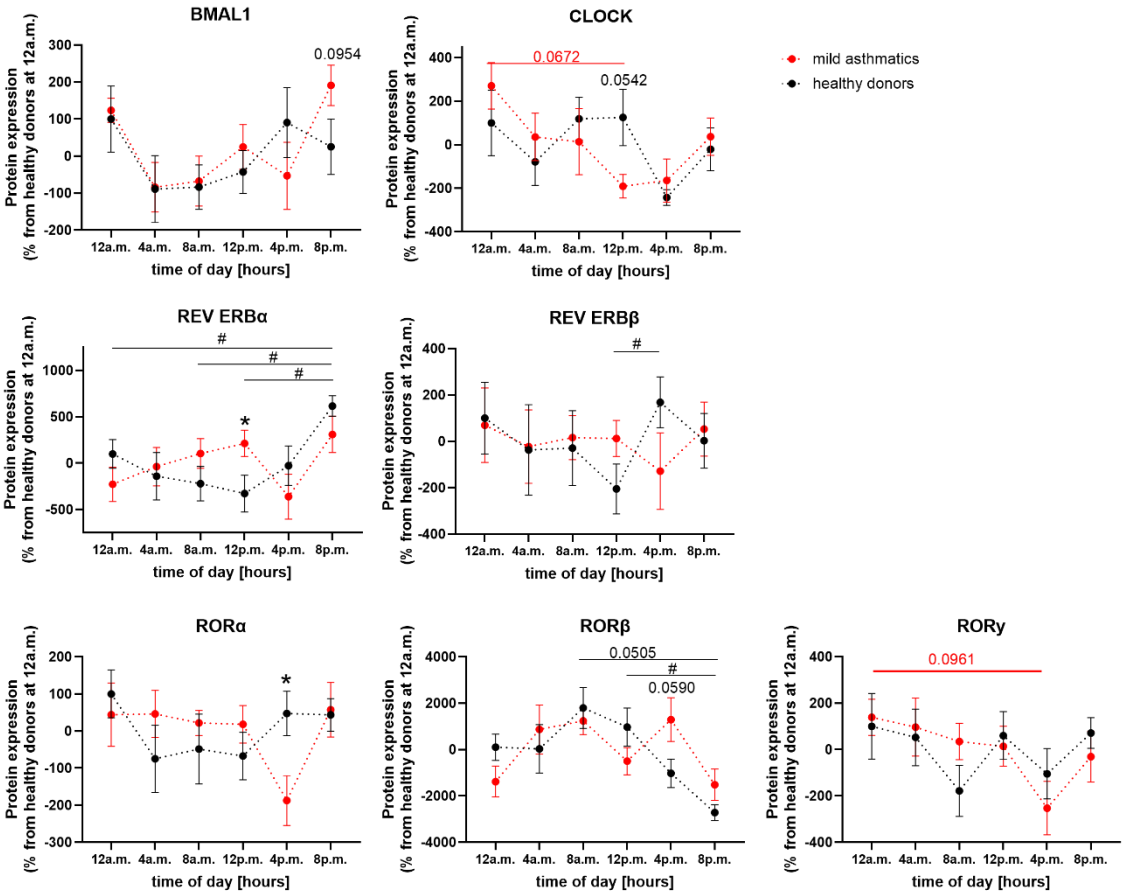

Figure 16: Distinct oscillation patterns of clock proteins in CD3⁺ T cells from mild asthmatics compared to healthy individuals. *Peripheral blood CD3⁺ T cells exhibited rhythmic expression of BMAL1, CLOCK, REV ERBs, and RORs. Z-scores were calculated and normalized to the mean of the healthy control group at 12 a.m. Data are presented as mean ± SEM. Statistical analysis was conducted using group-matched repeated measures Two-Way ANOVA followed by Tukey's post hoc test. Intra-group comparisons are shown with color-coded lines (red for mild asthmatics, black for healthy donors), while inter-group differences are indicated with asterisks. * and # denote $p < 0.05$; ** and ## indicate $p < 0.01$.*

Besides observed changes in period lengths and phase, we clearly show reduced CLOCK, REV ERBα and RORα levels in T cells from mild asthmatics in the forenoon as illustrated in **Figure 17**. T cells from allergic donors exhibited a similar trend, although the results were not statistically significant, possibly due to the small sample size.

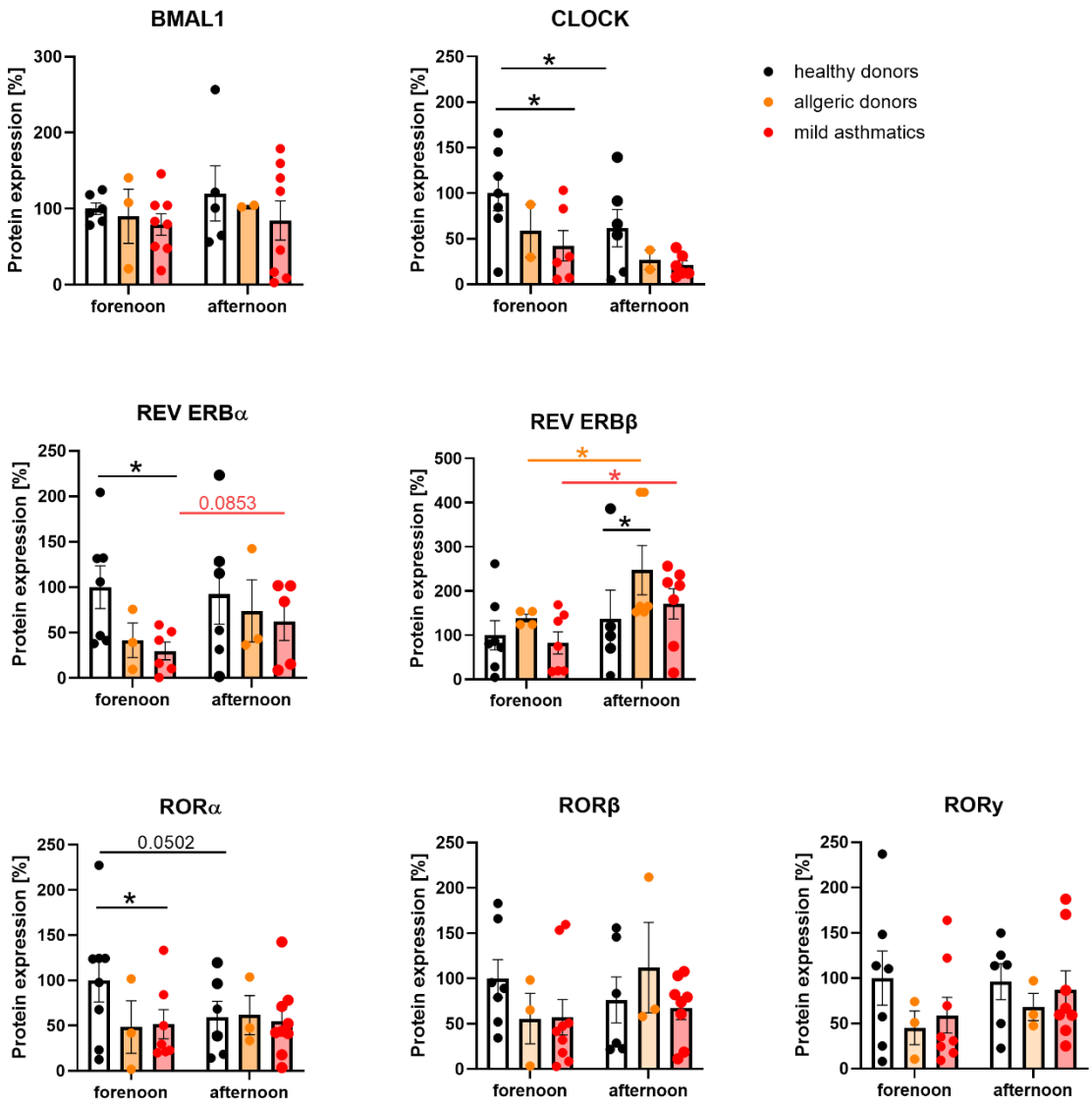

Figure 17: Clock protein levels in CD3⁺ T cells were compared among healthy donors, allergic individuals, and patients with mild asthma. *Values from the 8 a.m. and 12 p.m. time points were averaged to represent the morning group, while 4 p.m. data were used for the afternoon. All data were normalized to the corresponding control groups. Statistical analysis was performed using Two-Way ANOVA; * $p < 0.05$*

.

To further support our data, we assessed clock protein expression in T cells from the well-characterized moderate asthma cohort, which is clustered by the time of blood collection as shown in **Table 5**. As T cells are a very heterogenic population, we divided them roughly in CD4+ and CD8+ T cells for further experiments. CD4+ T cells, particularly Th2 cells, drive allergic asthma by secreting cytokines such as IL-4, IL-5, and IL-13, which promote class switch to IgE production, eosinophil recruitment, and mucus production, leading to airway inflammation and hyperresponsiveness (87).

In separated CD4+ and CD8+ T cells from healthy donors almost all clock proteins significantly increase in the afternoon (**Figure 18** and **Figure 19**), while this daytime variation is lost in CD4+ and CD8+ T cells derived from moderate asthmatics and a decrease in all clock proteins is observed (**Figure 18** and **Figure 19**). Notably, the dampening of clock protein expression in PMNLs derived from individuals with moderate asthma (eosinophils shown in **Figure 8** and neutrophils illustrated in **Figure 14**) was more pronounced in the forenoon. These data reinforce the hypothesis that clock protein expression is generally attenuated in peripheral blood leukocyte subset of individuals with asthma.

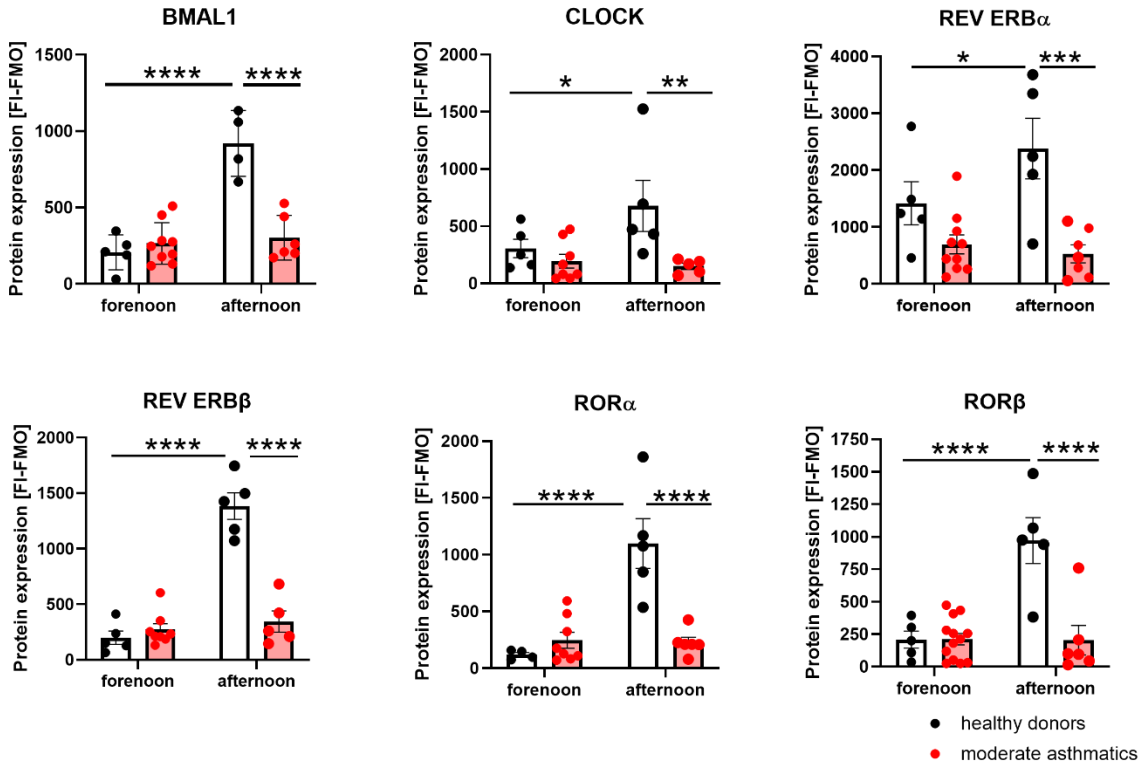

Figure 18: Clock protein expression is reduced in CD4⁺ T cells from patients with moderate asthma. *In the afternoon, significantly lower levels of clock proteins were observed in moderate asthmatic patients (red) compared to healthy controls (black). Statistical analysis was conducted using group-matched repeated measures Two-Way ANOVA followed by Tukey's post hoc test. Significance levels are indicated as follows: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.*

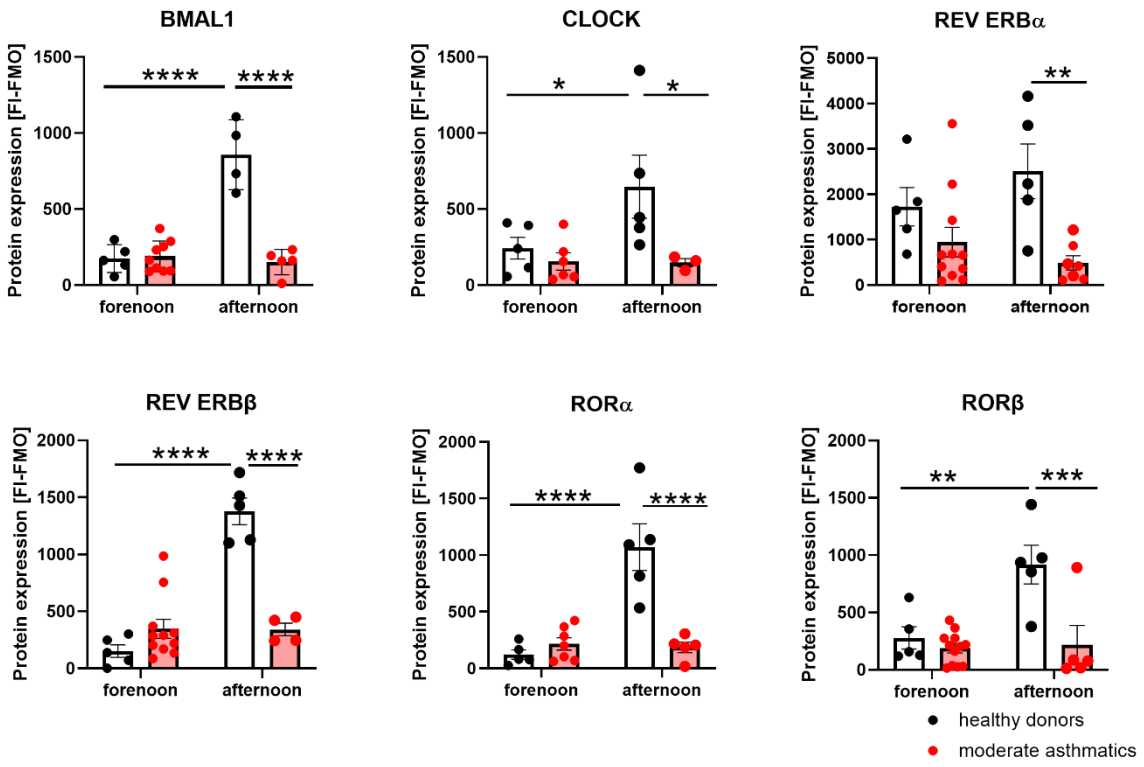

Figure 19: Clock protein expression is diminished in CD8⁺ T cells of individuals with moderate asthma. *In the afternoon, CD8⁺ T cells from moderate asthmatic patients (red) showed significantly lower clock protein levels compared to healthy controls (black). Statistical analysis was performed using group-matched repeated measures Two-Way ANOVA with Tukey's post hoc test. Significance is indicated as follows: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.*

In a comparison between a small group of moderate asthmatics working shift hours and those working conventional hours, a significant increase in REV ERBβ expression was observed in both T cell subsets (**Figure 20** and **Figure 21)**. Additionally, in CD8+ T cells RORα and RORβ levels were increased in shift workers, while only a trend was observed in CD4+ T cells (**Figure 21**).

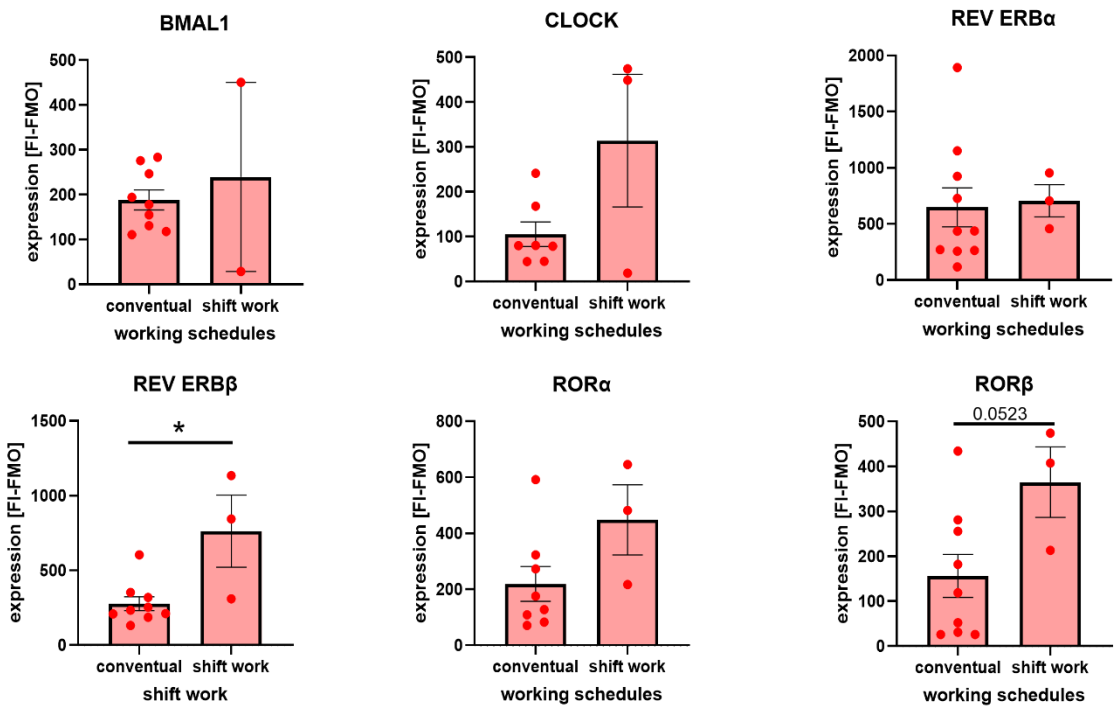

Figure 20: REV ERBβ expression in peripheral CD4⁺ T cells is elevated in the morning (9 a.m. – 1 p.m.) in moderate asthmatic patients engaged in shift work. *Significantly higher levels of RORα were observed in shift-working individuals (n = 3) compared to those following a conventional daytime schedule (n ≥ 7). Statistical analysis was conducted using a T-test; * p < 0.05.*

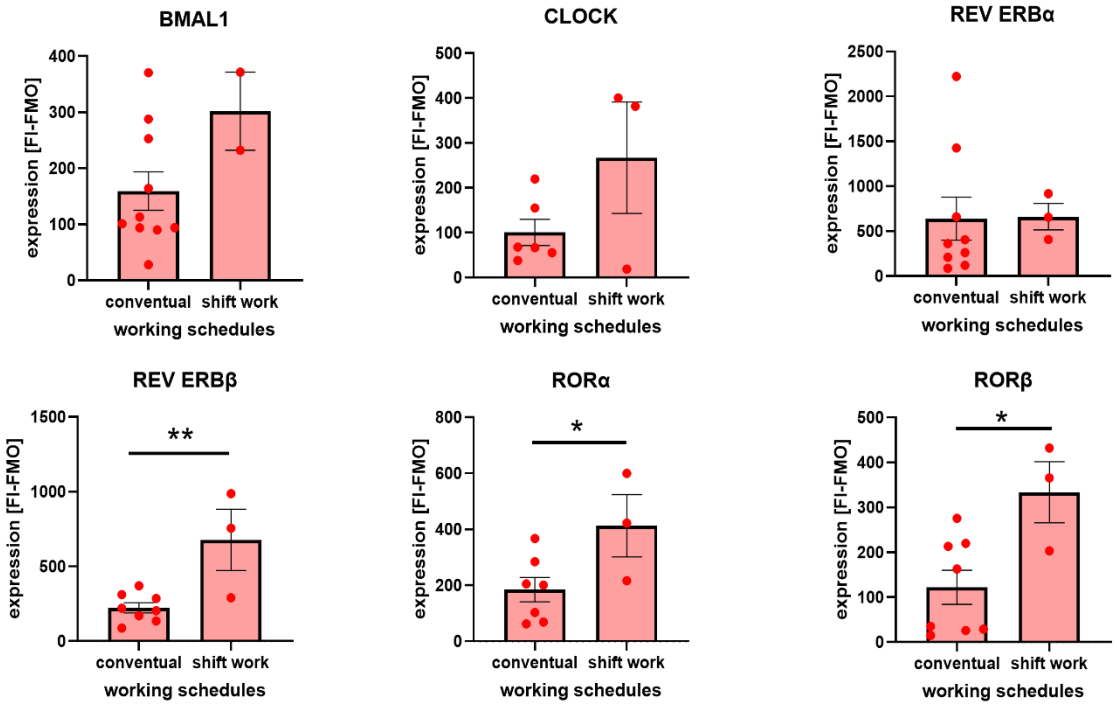

Figure 21: REV ERBβ and ROR protein expression in peripheral CD8⁺ T cells is elevated in the morning (9 a.m. – 1 p.m.) in moderate asthmatic patients engaged in shift work*. RORα levels were significantly higher in shift-working individuals (n = 3) compared to patients with a conventional work schedule (n ≥ 7). Statistical analysis was performed using a T-test; * p < 0.05.*

Having observed a damped molecular circadian clock in CD4+ and CD8+ T cells from moderate asthmatics, we utilized a publicly available microarray dataset published by Tsitsiou et.al. (126) to compare clock gene expression in both T cell subsets from healthy donors and severe asthmatic patients (**Table** 7**)**.

Table 7: Demographic table of severe asthmatic patients and healthy donors.

		healthy donors	severe asthmatics	p-value
participants		8.00	8.00	
age		36.13	44.75	0.1596
gender	female	4.00	7.00	
	male	4.00	1.00	
spirometry	FEV1 predicted [%]	96.00	69.13	**0.021**
	FVC predicted [%]	100.25	90.63	0.2972
	obstructive pattern	0.00	3.00	

Consistent with our findings from mild and moderate asthmatics, we observed significant differences in clock gene expression in CD4+ and CD8+ T cells. In both subsets, a significant decrease in *clock* gene expression was observed. Further, significant lower levels of *RORβ* were detected in CD4+ T cells (**Figure 22**), while in CD8+ T cells *Rev erbα* was significantly decreased. Notably, *Rorα* clock gene expression was significantly elevated in circulating CD8+ T cells from severe asthmatics (**Figure 23**).

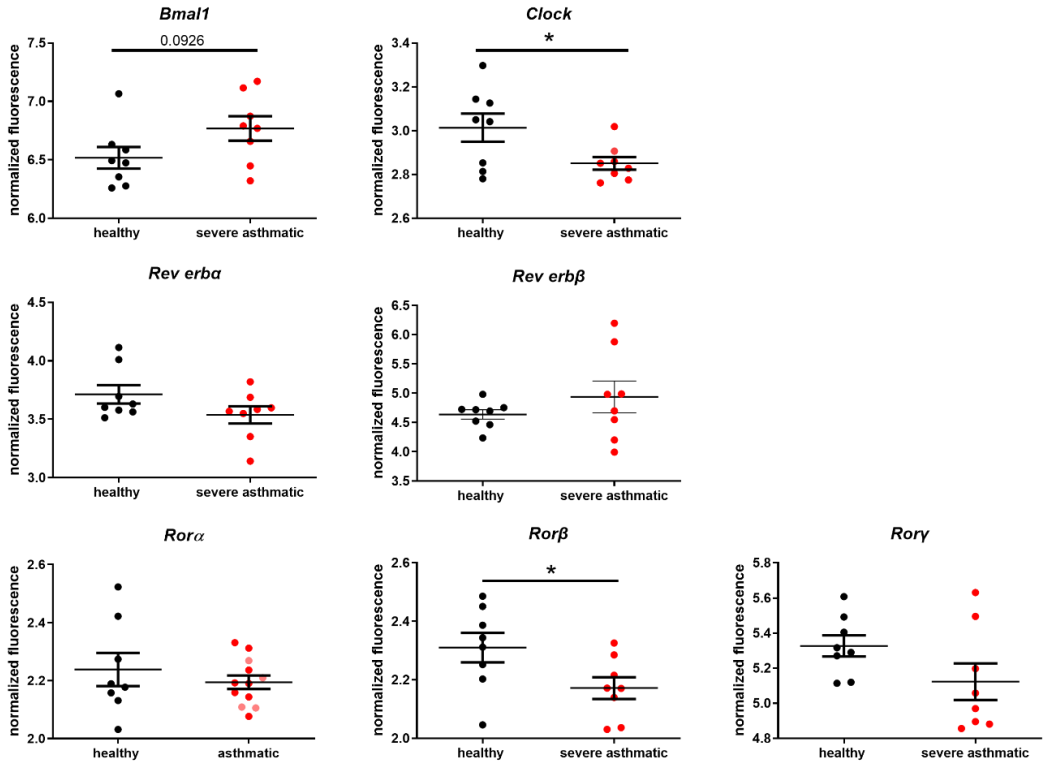

Figure 22: Clock and Rorβ gene expression is decreased in circulating CD4+ T cells from severe asthmatics compared to healthy controls. *Clock gene expression in CD4+ T cells from severe asthma patients was compared to healthy controls (n = 8). T-test, * p < 0.05.*

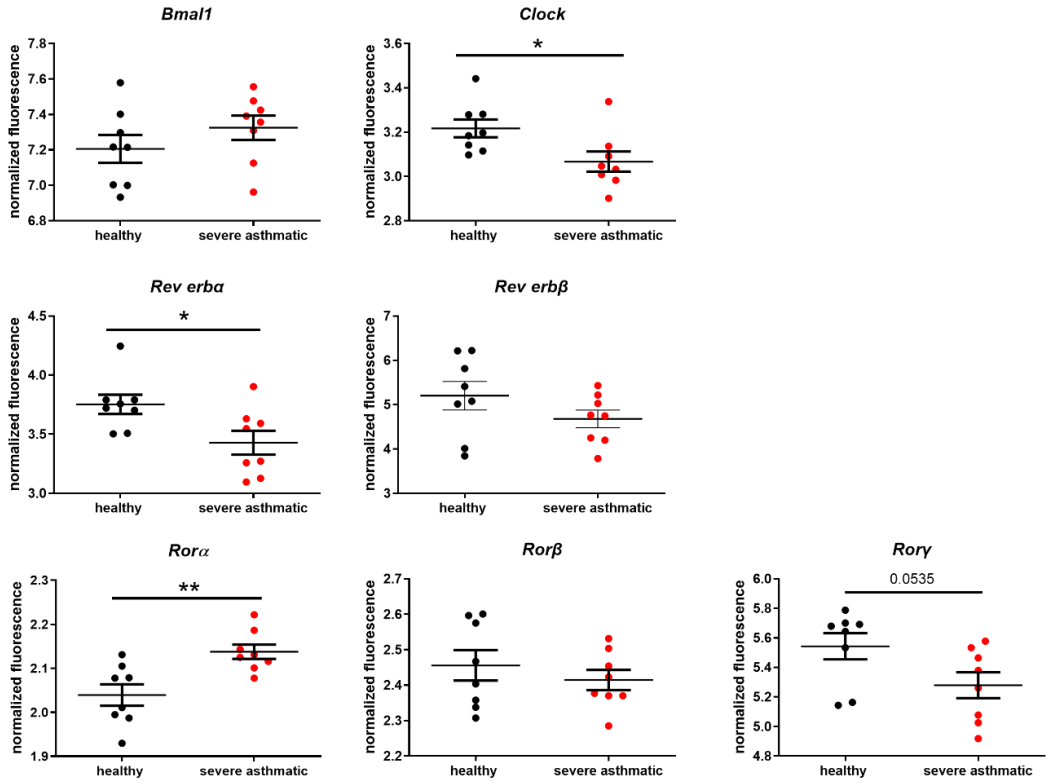

Figure 23: Clock, Rev erbα is decreased and Rorα gene expression is increased in circulating CD8+ T cells from severe asthmatics compared to healthy controls. *Significant lower Clock, Rev erbα and Rorα expression was observed in CD8+ T cells from severe asthma patients compared to healthy controls (n = 8). T-test, * p < 0.05, ** represent p < 0.01.*

3.1.4 Monocytes

Our results clearly demonstrate that the clock proteins of the stabilizing loop (BMAL1, CLOCK, REV ERBs, and RORs) are expressed in an oscillatory pattern in both peripheral CD14+CD16- monocytes (**Figure 24**) and CD14+CD16+ monocytes (**Figure 28**) from healthy and mild asthmatic donors (1).

In classical CD14+CD16- monocytes from healthy donors, BMAL1 exhibited low levels in the morning but showed increased expression towards the evening. CLOCK, which partners with BMAL1, reached peak expression at midnight, with the lowest levels observed at 4 p.m. REV ERBα, the repressor of BMAL1, had an expression pattern inversely correlated with BMAL1, peaking at 4 a.m. and showing minimal expression at 8 p.m.. REV ERBβ, displayed reduced overall oscillation amplitude, with nadirs at 4 p.m. and midnight. Higher expression of the BMAL1 activators, RORα and RORβ, were observed in the first half of the day, while RORγ showed a shorter phase but higher amplitude, peaking at 8 a.m. and 4 p.m. (**Figure 24**) (1).

Alterations in clock protein oscillation result in notable differences in expression when comparing classical CD14+CD16− monocytes from individuals with mild asthma to those from healthy blood donors. In classical monocytes from healthy blood donors, CLOCK protein expression is significantly reduced at 4 p.m., followed by a marked increase at 8 p.m. A similar pattern is observed in the expression of REV ERBβ. Conversely, RORβ expression tends to decrease at 8 p.m. in CD14+CD16− monocytes from mild asthmatics, indicating an opposing trend. (**Figure 24**) (1).

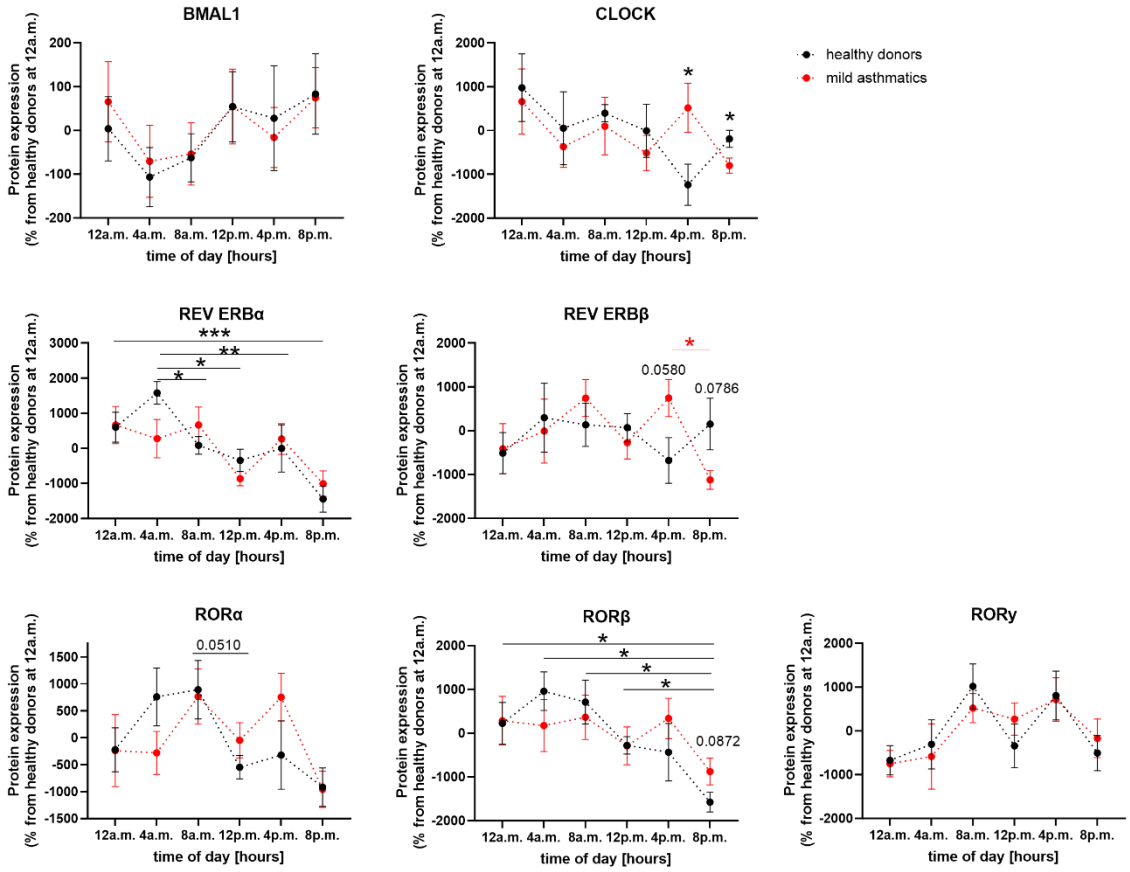

Figure 24: Distinct oscillatory patterns of clock protein expression were observed in CD14⁺CD16⁻ monocytes from mild asthmatic patients compared to healthy controls. *Peripheral blood CD14⁺CD16⁻ monocytes exhibited rhythmic expression of BMAL1, CLOCK, REV ERBs, and RORs. Z-scores were calculated and normalized to the mean of the healthy control group at 12 a.m. Data are presented as mean ± SEM. Statistical analysis was performed using group-matched repeated measures Two-Way ANOVA followed by Tukey's post hoc test. Within-group comparisons are shown with colour-matched lines (red for mild asthmatics, black for healthy donors); between-group comparisons are indicated by asterisks. * and # denote p < 0.05; ** and ## indicate p < 0.01. Figure adapted from (1).*

Comparing clock protein expression of classical monocytes from healthy, allergic and mild asthmatic donors, we observed a trend towards decreased CLOCK and RORγ expression in mild asthmatics in the forenoon. In the afternoon, significant lower BMAL1 and RORγ levels were found in classical monocytes from mild asthmatic donors (**Figure 25**).

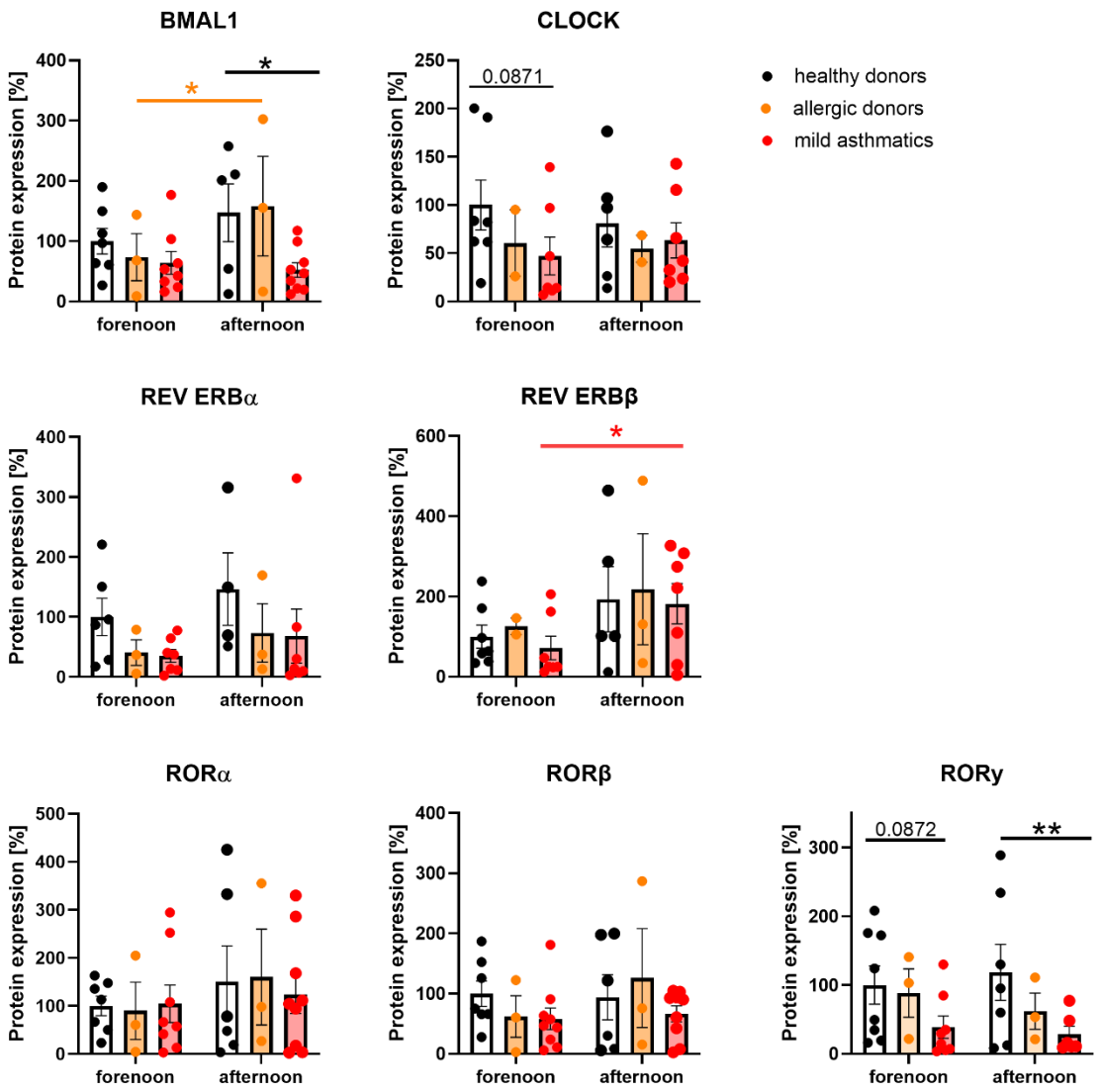

Figure 25: Comparison of clock protein expression in CD14⁺CD16⁻ monocytes among healthy donors, allergic individuals, and mild asthmatic patients. *Protein levels measured at 8 a.m. and 12 p.m. were averaged to represent the morning group, while 4 p.m. values were used for the afternoon. Data were normalized to the respective control groups. Statistical analysis was conducted using Two-Way ANOVA. Significance is indicated as follows: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.*

Compared to moderate asthmatics, healthy donors exhibited significantly higher levels of almost all clock proteins, with notable variations depending on the time of blood collection. In line with observations from the mild asthmatic cohort, CD14+CD16- monocytes from moderate asthmatics showed significantly lower expression of REV ERBα and RORα in the forenoon. Additionally, CD14+CD16- monocytes from moderate asthmatics showed reduced BMAL1 and RORβ expression in the forenoon, while REV ERBα expression was also decreased in the afternoon (**Figure 26**).

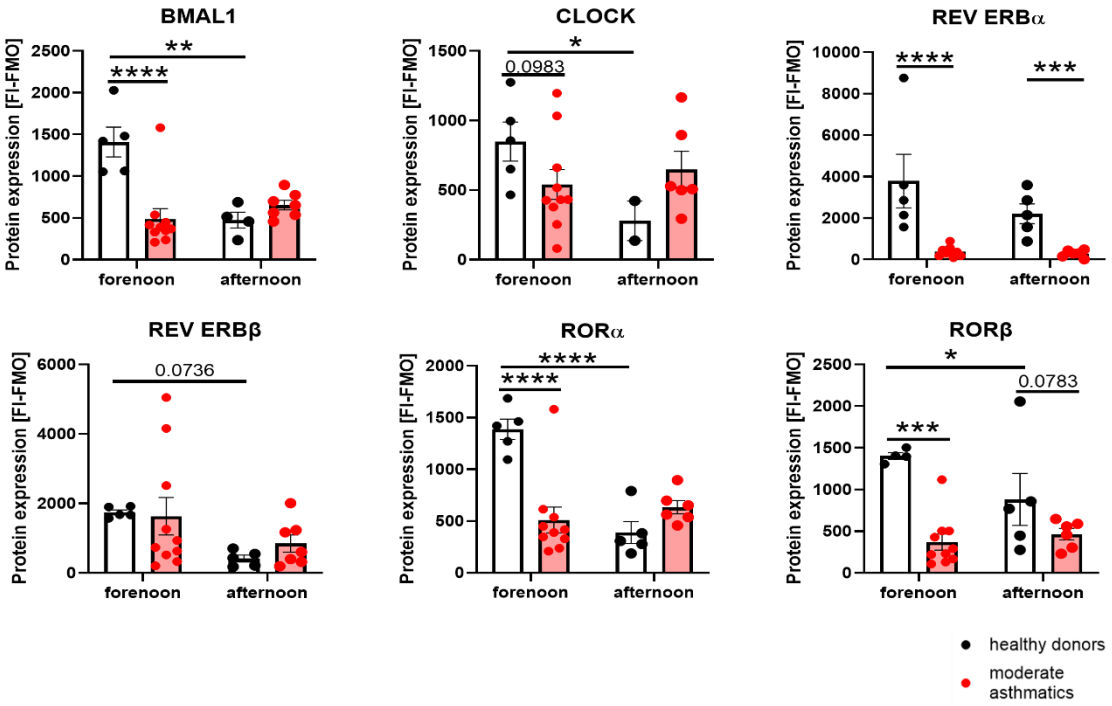

Figure 26: Reduced clock protein expression in CD14⁺CD16⁻ monocytes from patients with moderate asthma. *Clock protein levels were significantly lower in moderate asthmatics (red) compared to healthy controls (black) across both groups. Statistical analysis was performed using group-matched repeated measures Two-Way ANOVA followed by Tukey's post hoc test. Significance is denoted as follows: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.*

A comparison between a small cohort of moderate asthmatics working shift hours and those on conventional schedules revealed a significant upregulation of CLOCK protein expression in classical monocytes from shift workers (**Figure 27**).

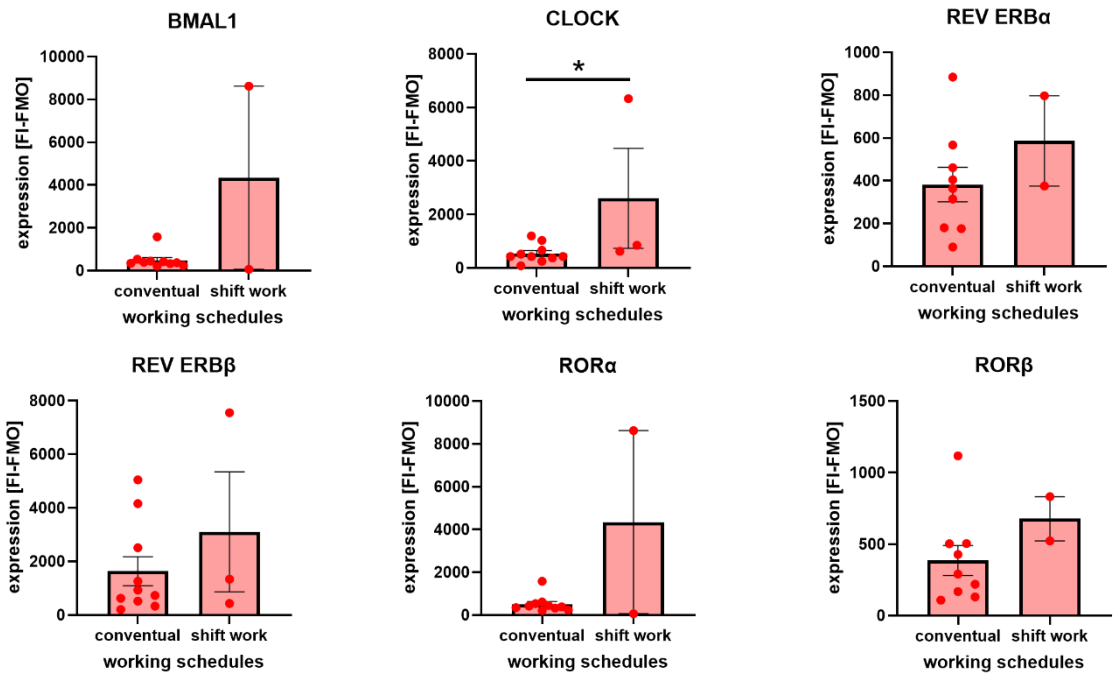

Figure 27: Forenoon CLOCK expression is elevated in peripheral CD14⁺CD16⁻ monocytes from moderate asthmatic patients engaged in shift work. *Significantly higher RORα levels were detected in shift-working patients (n = 3) compared to those with a conventional work schedule (n ≥ 7). Statistical analysis was performed using a t-test. * p < 0.05*

Analysis of protein expression at specific time points in CD14+CD16+ monocytes from blood donors with asthma revealed increased BMAL1 and CLOCK levels at noon. The nuclear receptors REV ERBα, RORα, and RORβ exhibited reduced expression at 4 a.m., a time point frequently linked to severe and nocturnal asthma episodes. Among the ROR family, RORγ showed its lowest expression at 4 p.m., with peak levels occurring during the night. Compared to other clock proteins, REV ERBβ displayed the shortest period length, with expression peaks detected at 8 a.m. and 4 p.m. (**Figure 28**).

A significant difference was observed between healthy blood donors and mild asthmatics, characterized by a pronounced decrease in REV ERBα and RORβ expression at 12 a.m. Most clock proteins of CD14+CD16+ monocytes from mild asthmatic donors peaked at noon, leading to significant differences in CLOCK, REV ERBβ and RORα expression compared to cells from healthy participants. Towards the night clock protein expression increases in CD14+CD16+ monocytes from healthy donors, leading to a significant difference in REV ERBβ and RORβ expression at 4p.m. (**Figure 28**). These findings strongly indicate that the molecular circadian clock in classical as well as CD14+CD16+ monocytes is altered in asthma (1).

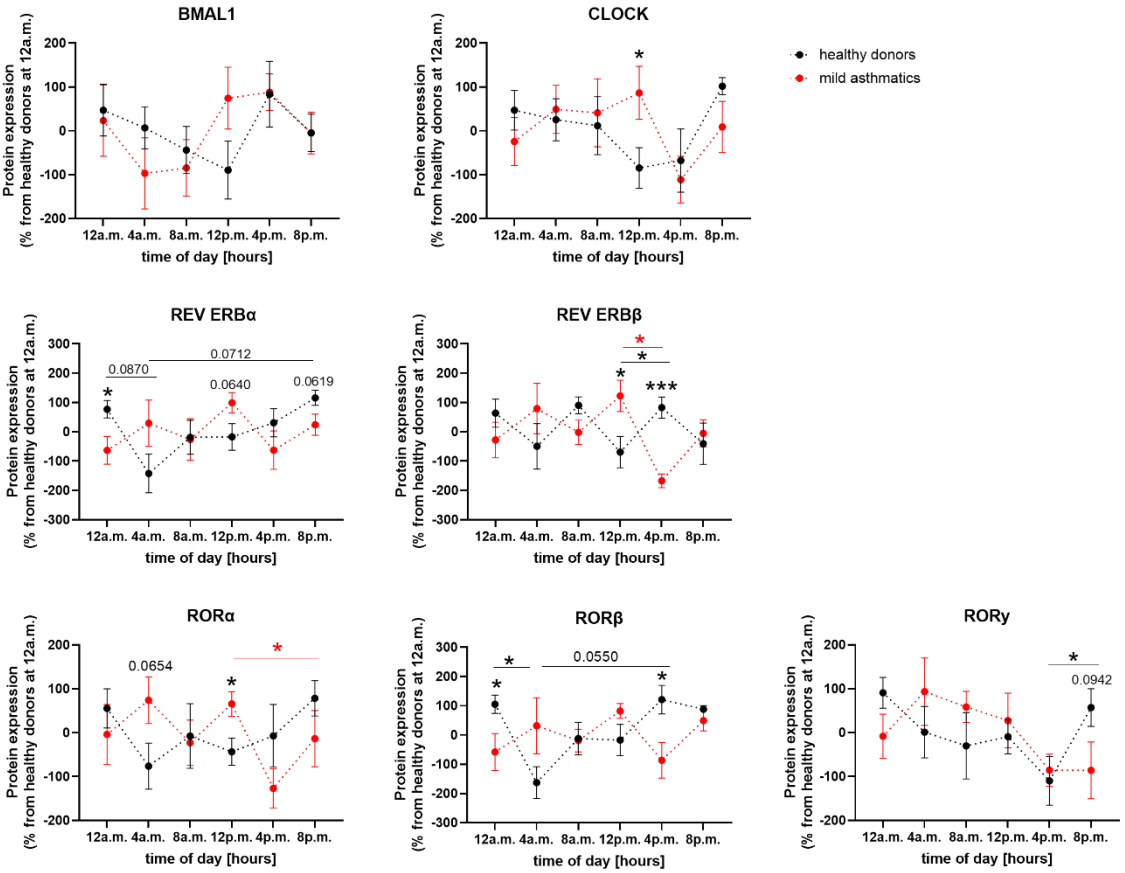

Figure 28: Significant differences in the oscillatory expression of clock proteins were observed between CD14⁺CD16⁺ monocytes from asthmatic patients and healthy controls. *Rhythmic patterns of BMAL1, CLOCK, REV ERB, and ROR proteins were detected in peripheral blood CD14⁺CD16⁺ monocytes. Z-scores were calculated and normalized to the mean value of the healthy control group at 12 a.m. Data are presented as mean ± SEM. Statistical analysis involved group-matched repeated measures Two-Way ANOVA followed by Tukey's post hoc test. Within-group comparisons are shown with color-coded lines (red for mild asthmatics, black for healthy donors), and between-group differences are indicated with asterisks. * $p < 0.05$; ** $p < 0.01$. Figure adapted from (1).*

When comparing forenoon and afternoon clock protein levels in peripheral CD14+CD16+ monocytes from healthy, allergic, and mild asthmatic blood donors, a pattern similar to that observed in classical monocytes was noted; however, only BMAL1 expression showed a significant reduction in mild asthmatics in the afternoon (**Figure 29**).

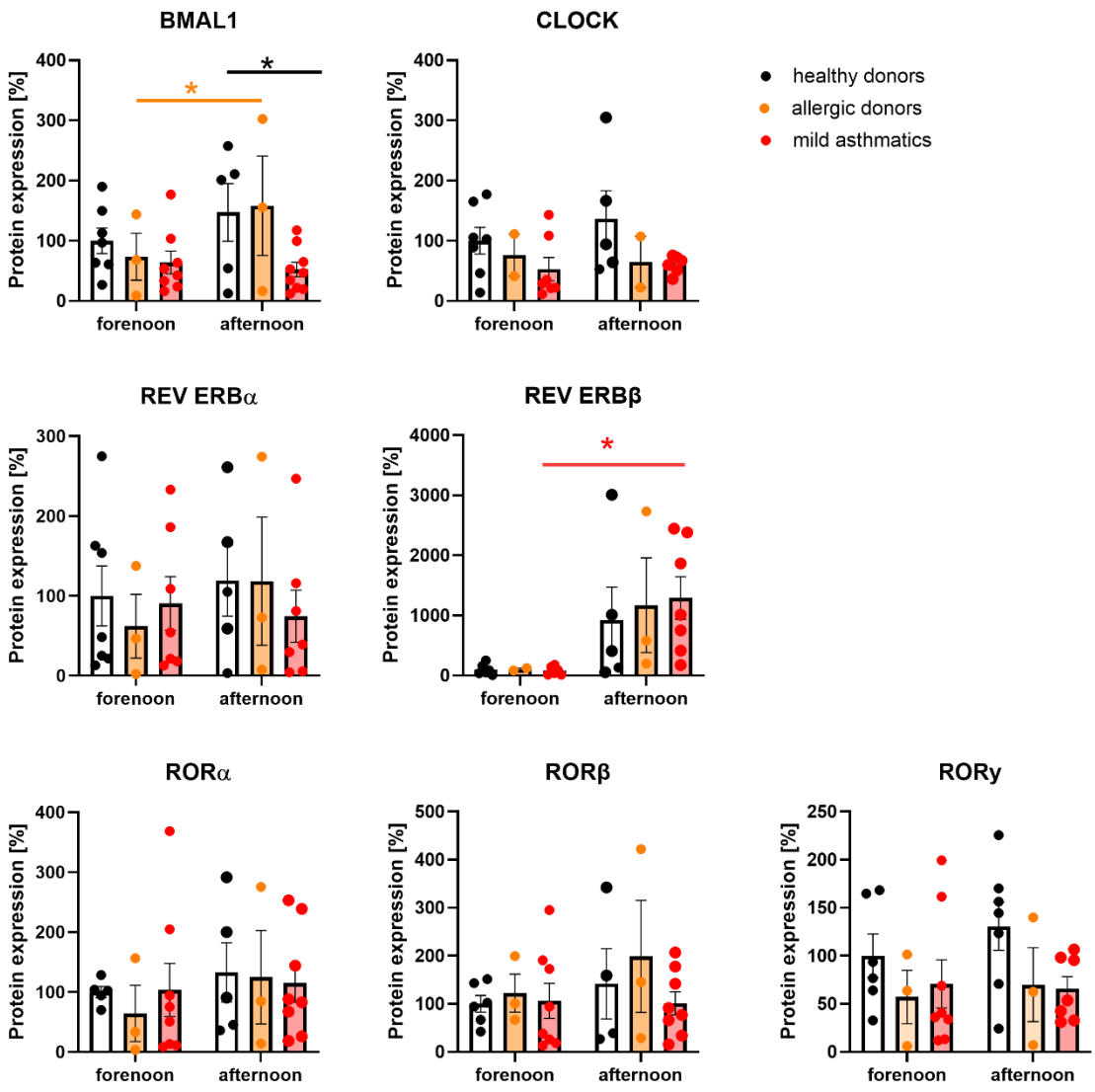

Figure 29: Comparison of clock protein levels in CD14⁺CD16⁺ monocytes from healthy donors, allergic individuals, and mild asthmatic patients. *Protein levels from the 8 a.m. and 12 p.m. time points were averaged to represent the morning group, while the 4 p.m. measurement was used for the afternoon. All data were normalized to their respective control groups. Statistical analysis was conducted using Two-Way ANOVA. Significance is indicated as follows: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.*

Consistent with our previous research indicating reduced clock protein levels in peripheral eosinophils, neutrophils, and T cell subsets, we observed decreased levels of clock proteins in circulating monocytes from individuals with moderate asthma, in both CD14+CD16- and CD14+CD16+ subsets. In both monocyte subsets, lower BMAL1, REV ERBα and ROR levels were observed in the forenoon (**Figure 26** and **Figure 30)**. In contrast to classical monocytes, significantly lower REV ERBβ, RORα and RORβ expression was measured in CD14+CD16+ monocytes from moderate asthmatics in the afternoon (**Figure 26** and **Figure 30)**.

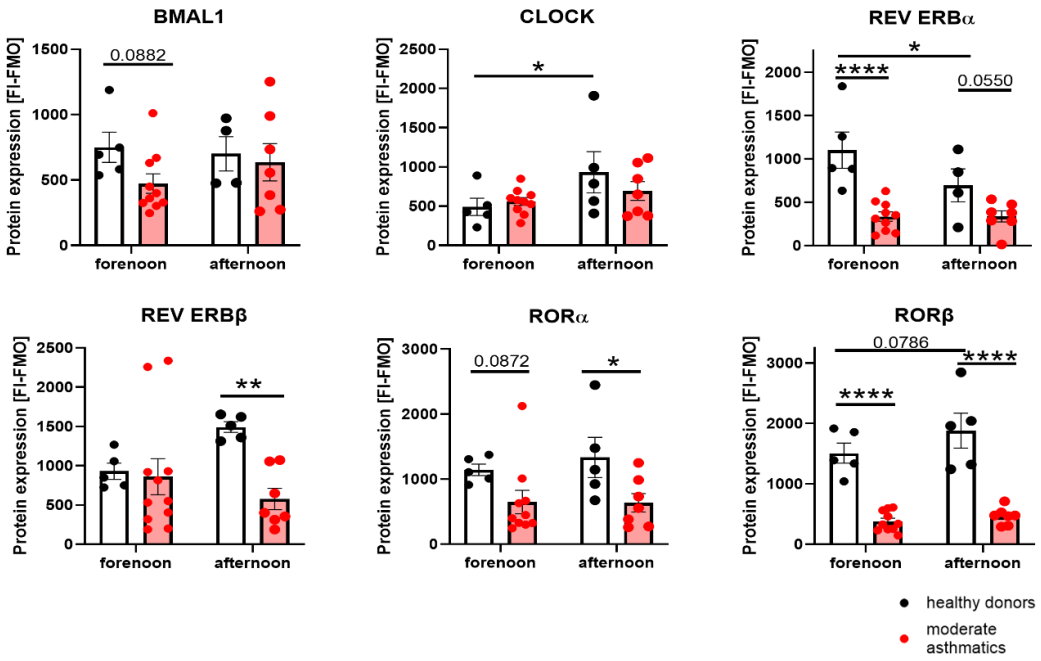

Figure 30: Clock protein expression is reduced in CD14⁺CD16⁺ monocytes from patients with moderate asthma. *Significantly lower clock protein levels were detected in moderate asthmatic patients (red) compared to healthy donors (black) across both groups. Statistical analysis was performed using group-matched repeated measures Two-Way ANOVA followed by Tukey's post hoc test. Significance is indicated as: * $p < 0.05$; ** $p < 0.01$; ***** $p < 0.0001$.*

In a comparison between a small group of moderate asthmatics working shift hours and those working conventional hours, a significant increase in CLOCK expression is observed in classical monocytes (**Figure 27**), whereas decreased BMAL1 and RORα levels are found in CD14+CD16+ monocytes from shift workers suffering from moderate asthma as illustrated in **Figure 31**.

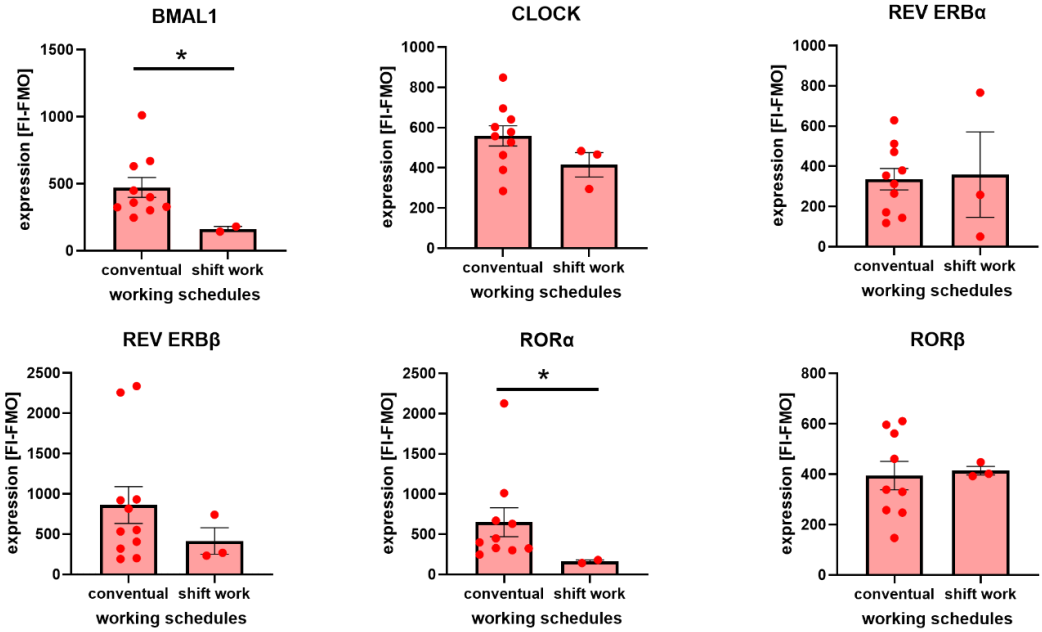

Figure 31: BMAL1 and RORα protein expression in peripheral CD14⁺CD16⁺ monocytes from moderate asthmatic patients engaged in shift work is reduced during the morning hours (9 a.m. – 1 p.m.). *Shift-working patients (n = 3) showed significantly higher RORα levels compared to those following a conventional work schedule (n ≥ 7). Statistical significance was determined using a T-test; * $p < 0.05$.*

In summary (**Table 8**), we found across various immune cell types, including eosinophils, neutrophils, T cells, and monocytes, a rhythmic expression pattern of clock proteins such as BMAL1, CLOCK, REV ERBs and RORs that vary throughout the day. In asthma, these oscillations are often blunted, with reduced overall amplitude leading to distinct expression differences.

Table 8: Summary of clock protein expression in peripheral leukocyte subsets comparing asthmatic to healthy blood donors. *Temporal expression patterns of clock components (BMAL1, CLOCK, REV-ERBs, and RORs) are shown for eosinophils, neutrophils, T cells, and monocyte subsets over a 24-hour cycle. In healthy individuals, oscillating protein expression is observed in all cell types, whereas clock protein expression is altered in asthmatics (indicated by arrows, X= no significant effect). Shift work further disrupts circadian protein expression. Further clock gene levels from CD4+ and CD8+ T cells are compared. n.a.= not analysed.*

molecular circadian clock		eosinophils	neutrophils	T-cells		monocytes	
				CD4	CD8	CD14+CD16-	CD14+CD16+
oscillating protein expression		√	√	√	√	√	√
differences in protein expression from asthmatics	hour of the day	reduced overall amplitude 4 a.m.: REV ERBα↓ 8 a.m.: RORα↓ RORβ↓ 12 p.m.: REV ERBβ↑ 4 p.m.: REV ERBα↑ CLOCK↑ 8p.m.: REV ERBα↑ RORβ↑	8 a.m.: CLOCK↑ 8 p.m.: RORβ↓	**in CD3**: 12 p.m.: REV ERBα↑ 4 p.m.: RORα ↓		4 p.m.: CLOCK↓ 8p.m.: CLOCK↑	12 a.m.: REV ERBα ↓ and RORβ ↓ 12 p.m.: CLOCK ↑ REV ERBβ ↑ RORα ↑ 4 p.m.: REV ERBβ↓ RORβ↓
	forenoon	BMAL1↓ CLOCK↓ REV ERBβ↓ RORs↓	BMAL1↓ CLOCK↓ REV ERBβ↓ RORs↓	-	-	BMAL1↓ REV ERBα↓ RORs↓	REV ERBα↓ RORβ↓
	afternoon	REV ERBα↓ RORβ↓	BMAL1 ↓ REV ERBα↓ RORs↓	↓	↓	REV ERBα↓	REV ERBβ↓ RORs↓
	working shifts	CLOCK↑ REV ERBS↑ RORα↑	RORβ↑	REV ERBβ↑	REV ERBβ↑	CLOCK↑	BMAL1↓ RORα↓
altered clock gene levels in asthma		n.a.	n.a.	*Clock*↓ *Rorβ*↓	*Clock*↓ *Rev erb*α↓ Rorα↑	n.a.	n.a.

3.2 Responsiveness of the molecular circadian clock to an inflammatory environment and asthma severity

3.2.1 Clock protein expression of peripheral eosinophils reflects asthma severity and inflammatory environment

Based on our observation of decreased peripheral clock protein levels in blood eosinophils from asthma patients, we further investigated whether these proteins could serve as suitable biomarkers for asthma management. Therefore, we correlated protein levels with clinical parameters such as FEV1 and FeNO, which are known to exhibit diurnal and seasonal variation (137–139). To this end, we assessed clock protein expression of the stabilizing loop in moderate asthmatic patients with both normal and obstructive spirometry patterns (FEV1:FVC ratio < 0.7, **Figure 32**). Further we compared those with $FeNO^{high}$ (>50 ppb, ATS guideline, **Figure 33**) to $FeNO^{low}$ patients. In addition, we analysed clock protein expression of moderate asthmatics based on their atopy status. Therefore, patients were again categorized based on the time of examination (2).

Among moderate asthmatic patients with obstructive spirometry patterns, we observed significant differences in clock protein expression of REV ERBs and RORα in the afternoon, including a loss of diurnal variation, compared to patients with better lung function. Overall, the expression differences of clock proteins, with the exception of RORβ, in peripheral eosinophils from moderate asthmatics were more pronounced in the afternoon than in the morning (**Figure 32)** (2).

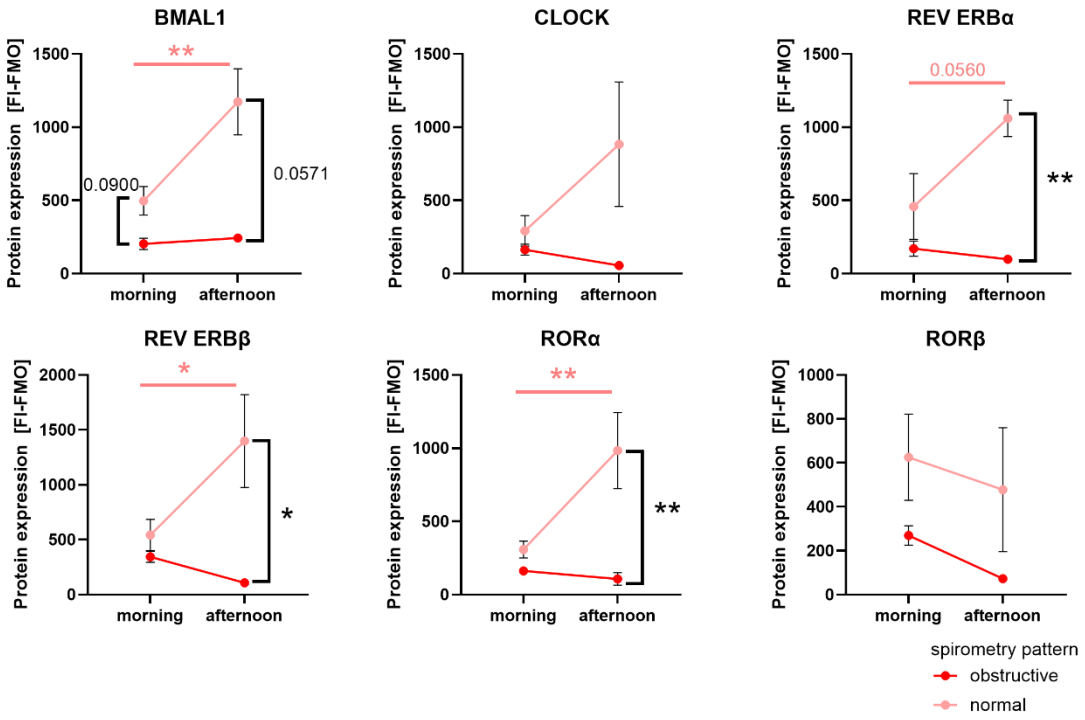

Figure 32: Reduced clock protein levels are associated with lung obstruction. *Clock protein expression was compared between moderate asthmatic patients exhibiting obstructive spirometry (FEV1:FVC < 0.7) and those with normal lung function (n ≥ 5). Statistical analysis was performed using Two-Way ANOVA. Significance is indicated as * p < 0.05 and ** p < 0.01. Figure adapted from (2).*

Similarly, patients with high FeNO values, an established biomarker for eosinophilic airway inflammation, showed significantly lower expression of BMAL and CLOCK in the afternoon. Further, loss of the typical time-of-day-dependent variation of clock proteins is observed in moderate asthmatics with higher FeNO levels. Similar to our correlation with airway obstruction, alterations in clock protein levels, with the exception of REV ERBα, were more pronounced in the afternoon than in the forenoon group (**Figure 33A**) (2).

Interestingly, moderate asthma patients allergic to at least one common allergen such as house dust mite, animal hair, or pollen exhibited significantly lower levels of CLOCK and RORs in circulating eosinophils, as measured in the morning as a single time point (**Figure 33B**) (2).

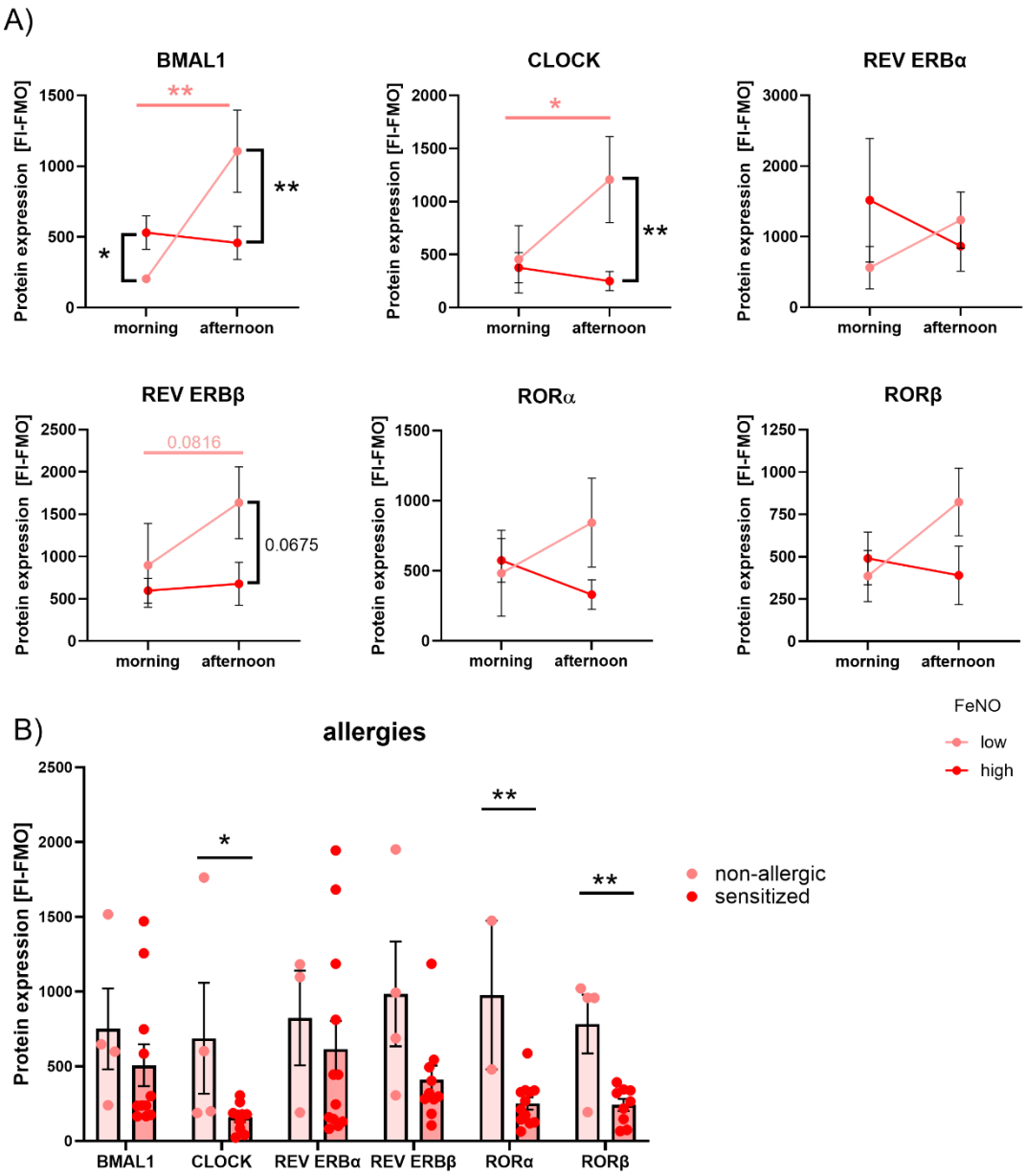

Figure 33: Low clock protein levels are associated with airway inflammation and allergic status in moderate asthmatic patients. *(A) Moderate asthmatics with elevated FeNO levels (threshold 50 ppb, n ≥ 4) show a loss of circadian variation in BMAL1 and CLOCK expression. (B) Allergic (sensitized) asthmatics display significantly reduced levels of CLOCK, RORα, and RORβ proteins compared to non-allergic asthmatics. Statistical significance was determined using multiple unpaired t-tests or Mann-Whitney U tests. * p < 0.05, ** p < 0.01. Figure adapted from (2).*

Given the observed link between the decreased peripheral clock of eosinophils and clinical asthma parameters including allergy status, we aimed to explore the bidirectional interplay between the immune system and the circadian clock in more detail. As anticipated, in mild asthmatics (**Table 4)**, we detected increased serum levels of IL-4 (140), IL-5 (141), IL-13 (142), IL-6 (143), IL-17-F, and TNF-α (144), cytokines which are all associated with inflammation and asthma. In healthy donors, cytokine secretion exhibited diurnal variation, with peak levels observed at 4 a.m., while asthmatic

patients showed elevated cytokine levels throughout the day, resulting in significant differences at noon and in the evening (**Figure 34**) (2).

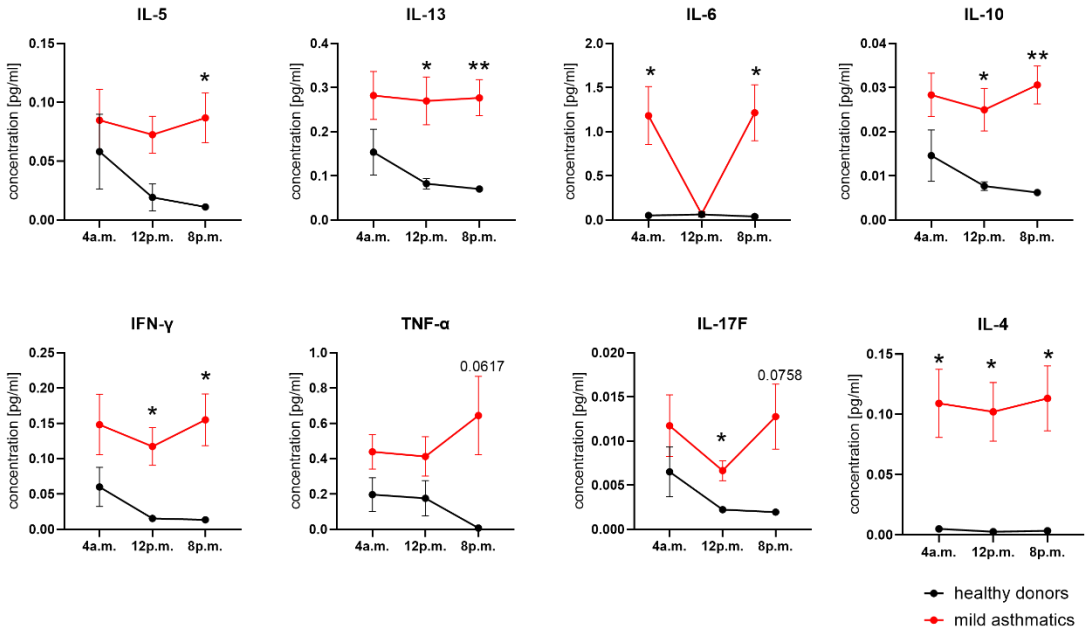

Figure 34: Circadian variations in circulating cytokine levels in mild asthmatic patients compared to healthy donors. *Serum cytokine concentrations were measured using a multiplex assay in mild asthmatic patients (red) and healthy controls (black) (n ≥ 8). Data are presented as mean ± SEM. Statistical analysis was performed using Two-Way ANOVA. Significance is indicated as * p < 0.05, ** p < 0.01. Figure adapted from (2).*

When analysed in the morning, we identified an association between reduced REV ERBα expression and allergies and/or asthma in blood eosinophils from our daily donors. Interestingly, we observed slightly reduced REV ERBα levels in asymptomatic blood donors, who self-reported as non-allergic but had positive serological results (**Figure 35A**). This finding further underscores the reciprocal influence between the immune system and the molecular circadian clock of blood eosinophils. To better understand this interaction, we exposed isolated PMNL from healthy donors to sera from asthmatic donors, which resulted in reduced BMAL1, CLOCK and REV ERBα protein levels in blood eosinophils. Sera from non-allergic individuals and autologous serum were used as control conditions (**Figure 35B**). Assuming that pro-inflammatory mediators present in the serum of asthma patients induced the observed reduction in clock protein levels, we next stimulated eosinophils from healthy donors with a cytokine cocktail, comprising IL-4, IL-5, IL-6, IL-10, IL-13 and IFN-γ, based on the results of the multiplex assay (**Figure 34**). As expected, incubation with the pro-inflammatory cytokine cocktail led to suppression of the peripheral clock proteins BMAL1 and REV ERBα in blood eosinophils. Conversely, PGE_2, a bronchoprotective prostaglandin with anti-

inflammatory properties relevant to asthma (145), upregulated REV ERBα expression (**Figure 35C**) (2).

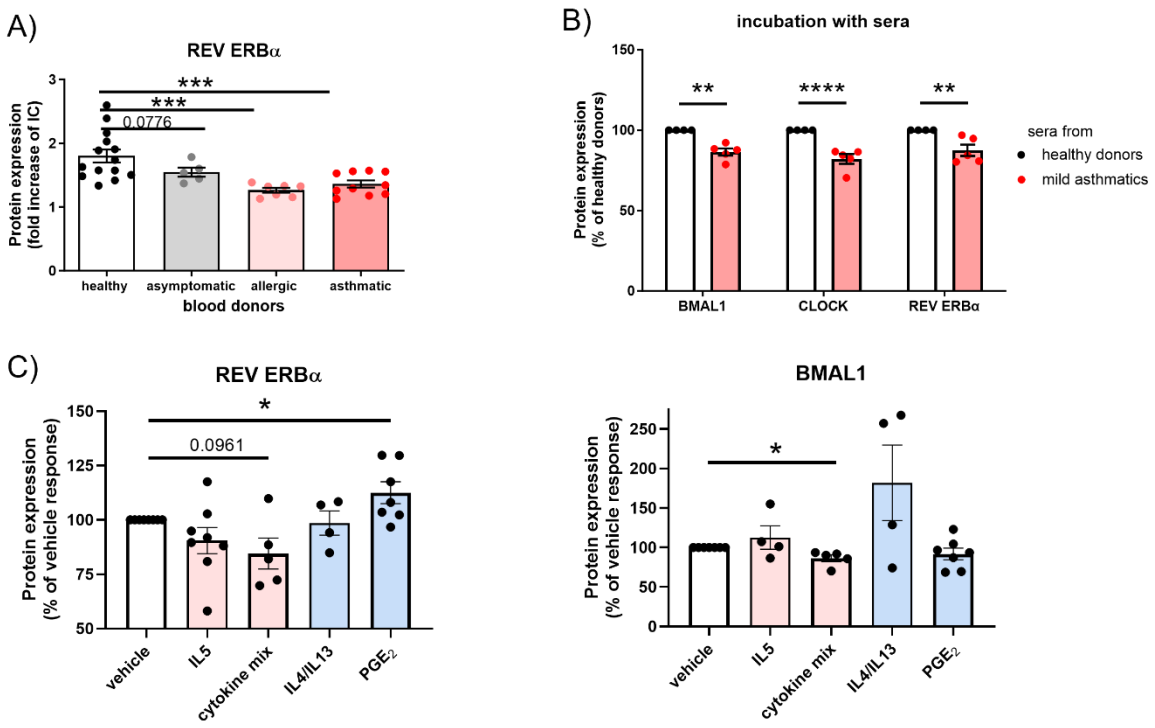

Figure 35: Clock protein levels in eosinophils are influenced by an inflammatory environment. *(A) REV ERBα expression is reduced in eosinophils from mild asthmatics and allergic donors but remains unchanged in asymptomatic sensitized individuals (n ≥ 5). (B) Exposure of eosinophils from healthy donors to serum from mild asthmatics results in a dampening of circadian protein expression (n ≥ 5). (C) Treatment of PMNLs from healthy donors with a pro-inflammatory cytokine mixture or the anti-inflammatory mediator PGE2 modifies REV ERBα levels (n ≥ 5). Statistical analyses included unpaired t-tests or Mann-Whitney U tests, One-Way ANOVA, and multiple t-tests. Significance is indicated as * p < 0.05, ** p < 0.01, *** p < 0.001. Figure adapted from (2).*

Notably, reduced REV ERBα expression in circulating neutrophils was observed in asthmatic patients, further supporting the bidirectional interaction between the immune system and the molecular circadian clock. However, no such difference was observed in peripheral neutrophils of allergic blood donors (**Figure 36A**). In contrast to eosinophils, no significant changes in REV ERBα expression were observed in healthy PMNL when exposed to sera from asthmatic donors **(Figure 36B)**, or when incubated with a cytokine cocktail or PGE_2 (**Figure 36C**). However, our previous monitoring experiments already showed less differences in clock protein expression between healthy, allergic and asthmatic blood donors in neutrophils (**Figure 12 and Figure 13**), but a more pronounced effect on eosinophils (**Figure 6** and **Figure 7**) (2).

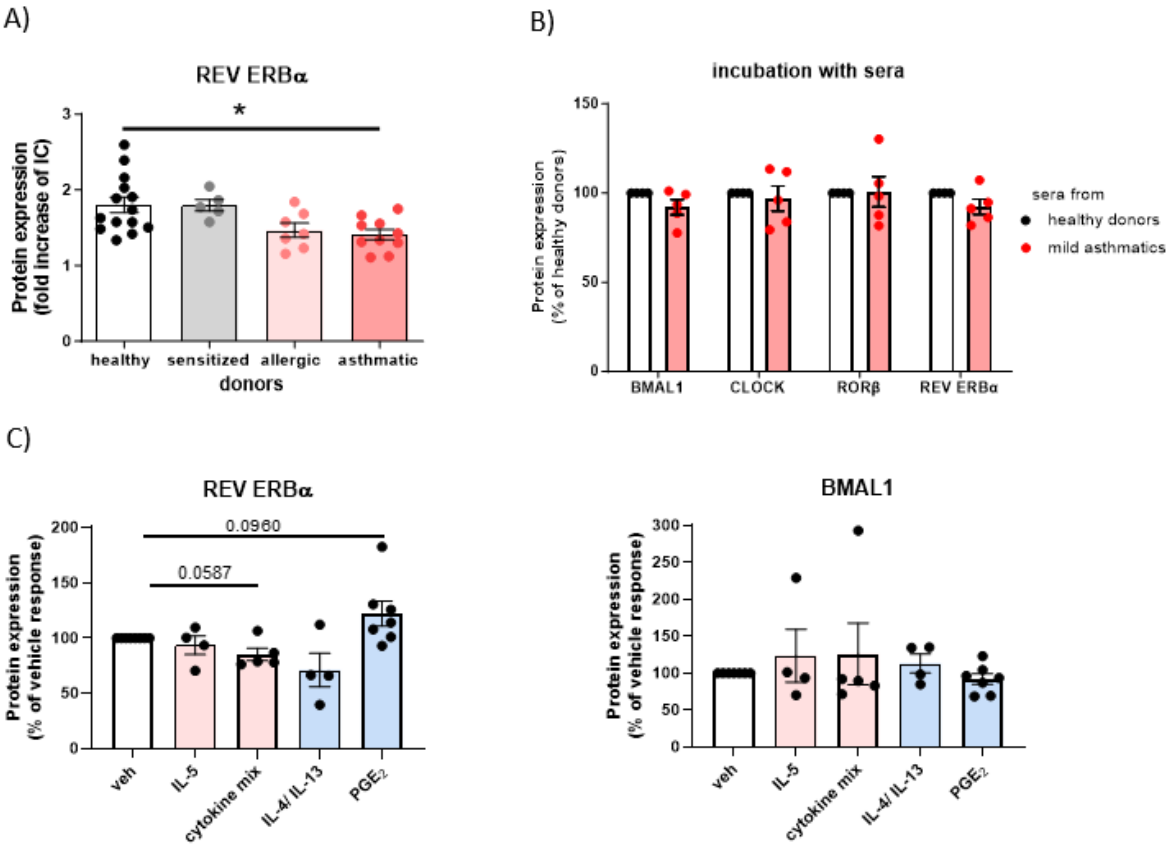

Figure 36: Clock protein expression in blood neutrophils shows only a weak response to inflammatory stimuli. *(A) REV ERBα levels are reduced in neutrophils from mild asthmatic donors compared to healthy controls (n ≥ 5). (B) Treatment of neutrophils from healthy donors with serum from mild asthmatics does not significantly alter circadian protein expression (n ≥ 5). (C) Exposure of PMNLs from healthy donors to a pro-inflammatory cytokine mixture results in a partial decrease of REV ERBα (n ≥ 5). Statistical analyses were performed using unpaired t-tests or Mann-Whitney tests, as well as One-Way or Two-Way ANOVA. Significance is indicated as * p < 0.05. Figure adapted from (2).*

Although clock protein levels of peripheral eosinophils respond to inflammatory conditions and reflect asthma severity, a four-hour incubation with asthma or allergy therapeutics, such as the beta-mimetic formoterol, the corticosteroid fluticasone, or the antihistamine levocetirizine, does not induce a direct short-term effect on the clock protein levels of the stabilizing loop in eosinophils and neutrophils. However, analyses were only performed after a single incubation at concentrations corresponding to systemic levels reached after inhalation (146–148) and hardly reflect the conditions in patients under long-term medication (**Figure 37**) (2).

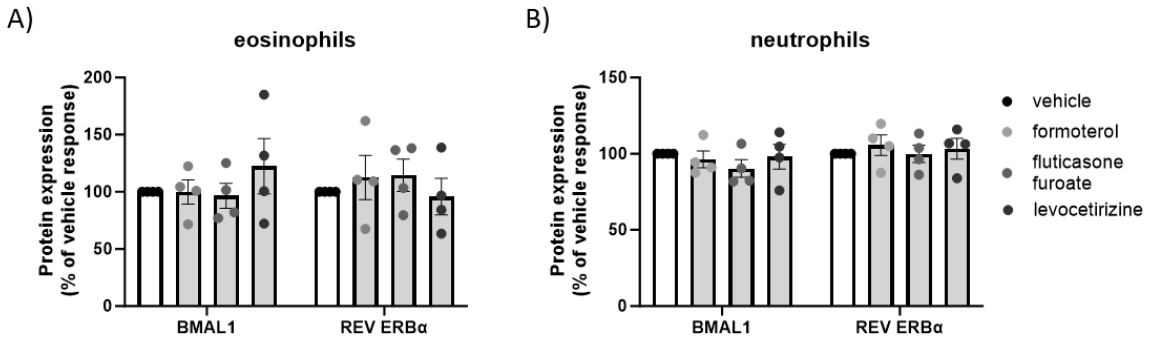

Figure 37: Allergy and asthma medications do not directly affect clock protein expression in the short term. *Four-hour incubation with asthma or allergy medications did not change clock protein levels in peripheral eosinophils (A) or neutrophils (B) (n = 4). Data are shown as mean ± SEM. Statistical analysis was performed using multiple t-tests. Figure adapted from (2).*

3.2.2 Clock protein levels of macrophages respond to inflammatory mediators

3.2.2.1 Monocyte-derived macrophages (MDMs)

In peripheral blood monocytes, we observed differences in clock protein expression between healthy donors and allergic asthmatic patients. To explore these variations further, we examined the expression of protein of the stabilizing loop in monocyte-derived macrophages (MDMs) from both healthy and allergic blood donors at a single time point. PBMCs were initially isolated, cultured, and differentiated into MDMs over a period of 7 days. The differentiated MDMs exhibited reduced expression of RORβ, as depicted in **Figure 38** (1).

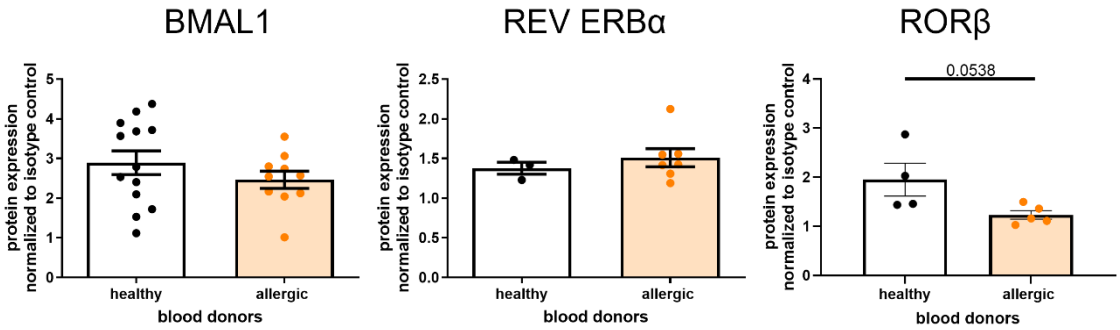

Figure 38: BMAL1, REV ERBα, and RORβ protein expression in monocyte-derived macrophages from healthy and allergic donors. *Peripheral blood monocytes were cultured in 12-well plates and differentiated into MDMs. Expression levels of circadian proteins were compared between MDMs from allergic and non-allergic donors (n ≥ 4). Data are presented as mean ± SEM. Statistical analysis was performed using a t-test. Figure adapted from (1).*

Following differentiation, MDMs were polarized with LPS/IFN-γ (to induce a classically activated M1 phenotype) or IL-4 (to induce an alternatively activated M2 phenotype) (**Figure 39A**). In the total cohort, polarization with IL-4 significantly increased BMAL1 compared to unpolarized MDMs. Additionally, REV ERBα, which represses BMAL1, was significantly lower in M2 macrophages compared to those polarized with LPS/IFN-γ (M1) (**Figure 39B**) (1).

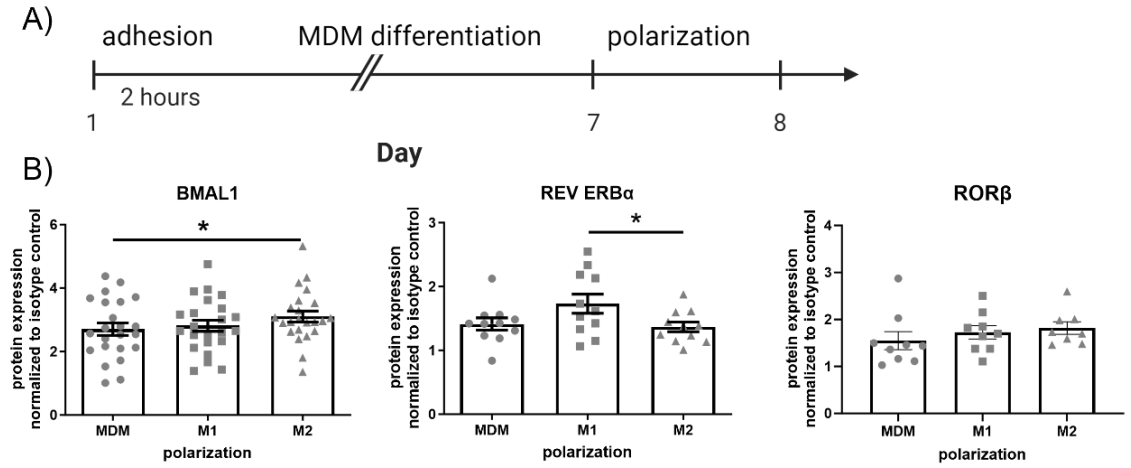

Figure 39: Clock protein expression in unpolarized and polarized macrophages. *(A) Human peripheral monocytes were cultured in 12-well plates and differentiated into monocyte-derived macrophages (MDMs). These MDMs were subsequently polarized toward M1 or M2 phenotypes using LPS/IFN-γ or IL-4, respectively. (B–C) Expression levels of BMAL1, REV ERBα, and RORβ were assessed by flow cytometry in MDM, M1, and M2 macrophages derived from (B) healthy donors (n ≥ 5) and (C) allergic donors (n ≥ 4). Protein levels were expressed as fold increase over isotype control (IC). Data are shown as mean ± SEM. Statistical analysis was performed using One-Way ANOVA. * p < 0.05. Figure adapted from (1).*

When analysing healthy and allergic donors separately, we found that altered clock protein expression in response to macrophage polarization was only observed in allergic donors, not in the healthy group (**Figure 40)**. Interestingly, in contrast to our previous findings from blood leukocytes, BMAL1 and its activator RORβ were significantly increased in IL-4 polarized cells compared to unpolarized macrophages, while REV ERBα was significantly elevated in the M1 phenotype (**Figure 40B**). These results suggest that the molecular circadian clock in macrophages from allergic patients is disrupted, leading to altered responses to polarization into both pro-inflammatory and resolution-associated phenotypes (1).

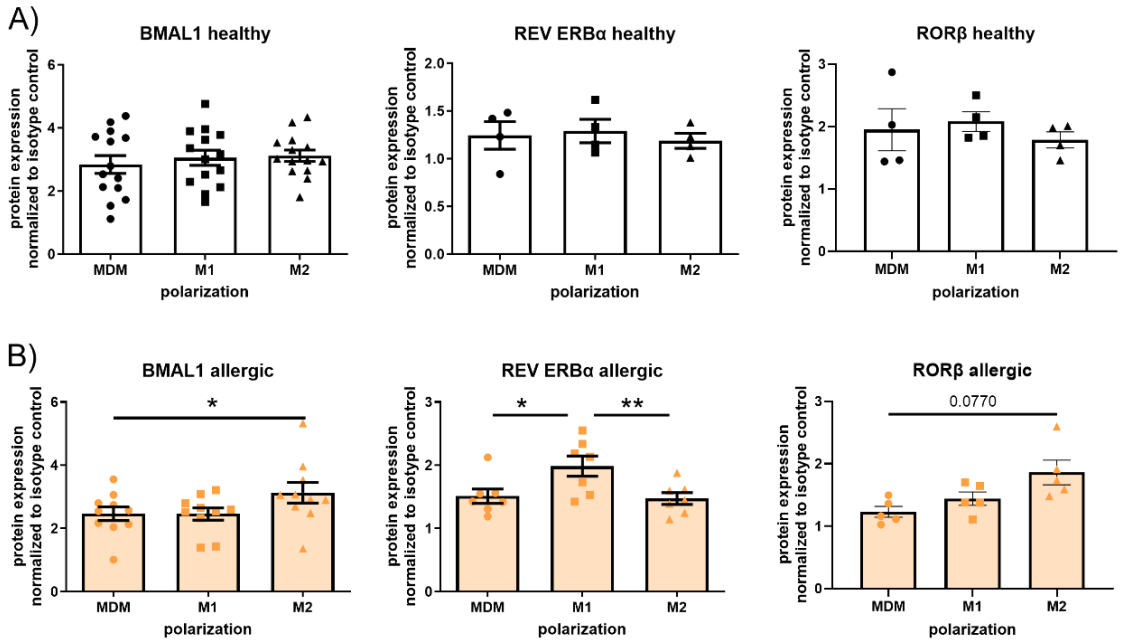

Figure 40: Clock protein expression in macrophage subtypes differs between allergic and healthy donors. *Levels of the circadian proteins BMAL1, REV ERBα, and RORβ were measured by flow cytometry in MDM, as well as in M1 and M2 polarized subtypes generated from (A) healthy (n ≥ 5) and (B) allergic blood donors (n ≥ 4). Results are expressed as fold increase over isotype control (IC). Data are presented as mean ± SEM. Statistical analysis was performed using One-Way ANOVA. * p < 0.05, ** p < 0.01. Figure adapted from (1).*

Given the pronounced differences in clock protein levels among macrophage subtypes derived from allergic blood donors, our subsequent aim was to directly compare unpolarized and polarized macrophages from both allergic and non-allergic donors. M1-polarized macrophages from both healthy and allergic donors exhibited significant changes in clock protein expression, including increased REV ERBα levels and correspondingly lower RORβ levels. These cells also showed reduced BMAL1 expression compared to M1 macrophages from non-allergic individuals (**Figure 41A**). Similarly, a comparable trend in RORβ was observed in unpolarized macrophages, while REV ERBα was only slightly elevated in M2 macrophages from allergic donors (**Figure 41B**) (1).

These results suggest that the molecular circadian clock in macrophages from allergic patients is disrupted, leading to altered responses to polarization into both pro-inflammatory and resolution-associated phenotypes.

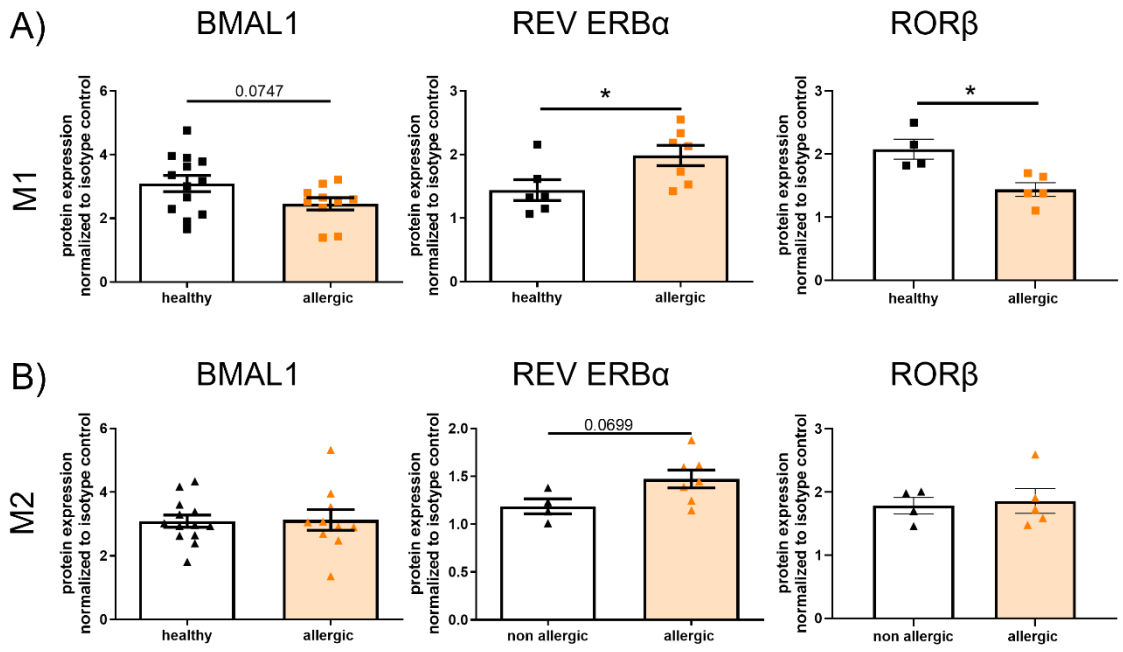

Figure 41: Clock protein expression in polarized monocyte-derived macrophages from healthy and allergic donors. Peripheral blood monocytes were cultured in 12-well plates and differentiated MDMs. *These MDMs were subsequently polarized into classically activated M1 macrophages using IFN-γ/LPS or into alternatively activated M2 macrophages using IL-4. Expression levels of clock proteins were analysed in (A) M1 and (B) M2 macrophages from allergic and non-allergic healthy donors (n ≥ 4). Data are shown as mean ± SEM. Statistical significance was determined using a t-test; * p < 0.05. Figure adapted from (1).*

3.2.2.2 Clock protein expression in human lung-resident macrophages depends on the inflammatory stimulus

To investigate macrophages in a more physiologic environment, we employed human PCLS as an *ex vivo* model. Therefore, PCLS obtained from non-tumor lung resections were stimulated for 6 or 24 hours with the same cytokines used for *in vitro* macrophage polarization: either LPS/IFN-γ or IL-4/IL-13. BMAL1 protein expression was assessed using immunofluorescence microscopy. Consistent with our previous *in vitro* findings from allergic donors, we observed higher BMAL1 levels in lung-resident macrophages from IL-4/IL-13-treated PCLS compared to those treated with LPS/IFN-γ, after both 6 and 24 hours. Notably, after 24 hours of stimulation, BMAL1 expression was significantly lower in LPS/IFN-γ-treated lung slices compared to vehicle-treated controls (**Figure 42**) (1).

These results suggest that BMAL1 expression in tissue-resident lung macrophages is influenced by the inflammatory environment and the phenotype of the cells, showing opposing responses depending on the type of inflammatory stimulus (1).

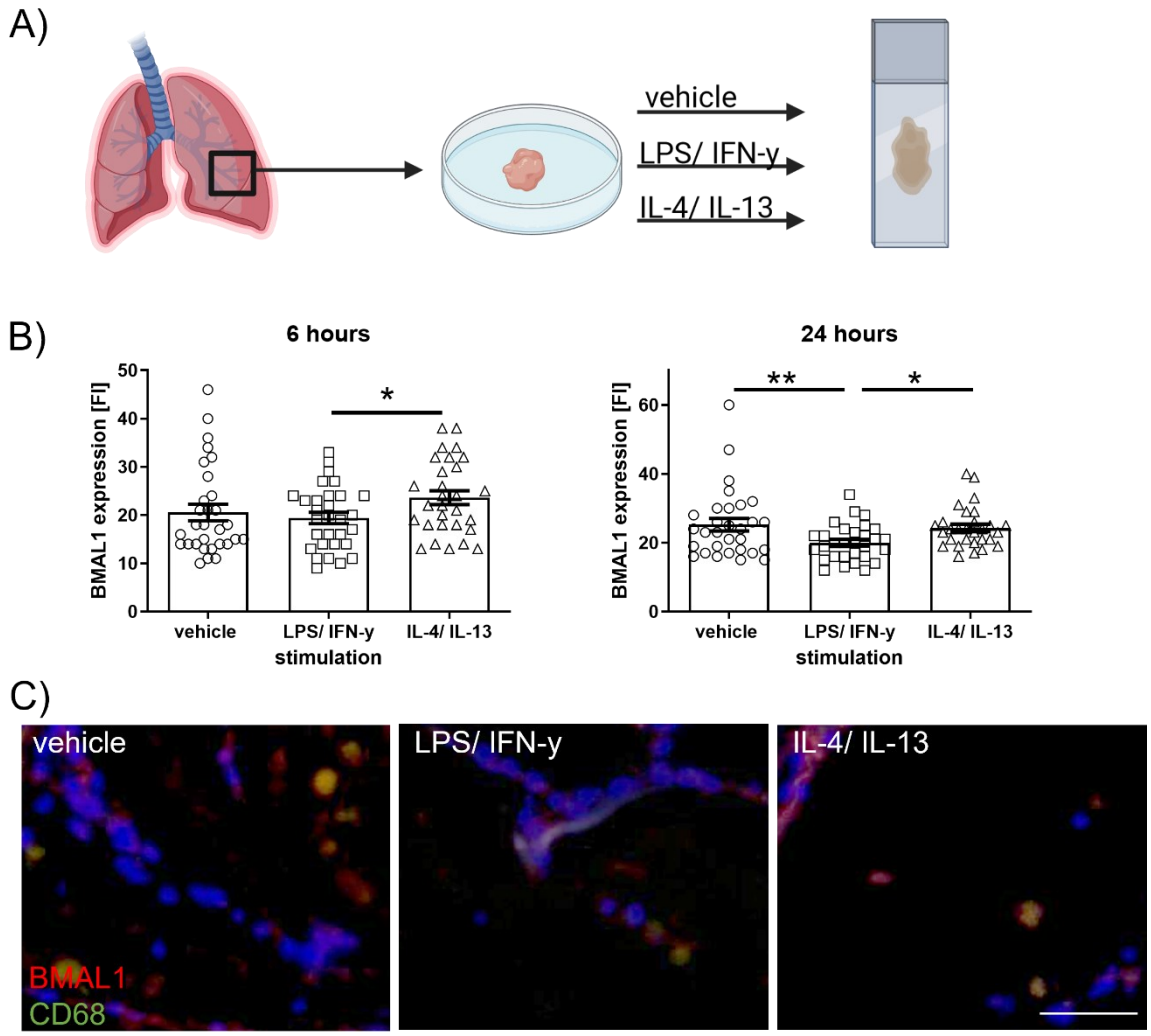

Figure 42: BMAL1 expression in human resident macrophages is differentially regulated by LPS/IFN-γ and IL-4/IL-13 stimulation. *(A) PCLS from non-tumorous human lung tissue were cultured and treated with either LPS/IFN-γ or IL-4/IL-13 for 6 or 24 hours. (B) Co-immunofluorescence staining for the macrophage marker CD68 and the clock protein BMAL1 confirmed BMAL1 expression in resident macrophages. IL-4/IL-13 stimulation for 6 hours led to increased BMAL1 expression compared to LPS/IFN-γ. This effect was more pronounced after 24 hours, while LPS/IFN-γ treatment for 24 hours significantly reduced BMAL1 levels. Fluorescence intensity (FI) was quantified using ImageJ. (C) Representative images of PCLS after 24-hour stimulation are shown (scale bar: 100 µm). CD68 is visualized in green and BMAL1 in red. Data are presented as mean ± SEM. Statistical significance: * $p < 0.05$, ** $p < 0.01$. Figure adapted from (1).*

3.2.2.3 Alveolar macrophages (AMs)

As previously mentioned, alveolar macrophages (AMs) are key players in asthma pathology. Therefore, following the detection of an altered molecular clock in peripheral monocytes, monocyte-derived macrophages, as well as tissue-resident macrophages, we utilized a publicly available microarray dataset published by Woodruff and colleagues (127) to compare clock gene expression in alveolar macrophages from healthy donors and asthmatic patients. Consistent with our findings from patient-derived monocytes, we observed significant differences in clock protein expression.

Specifically, *Bmal1* and its activator *Rorα* were significantly elevated, while mRNA levels of the repressor *Rev erbα* were reduced in alveolar macrophages from asthmatic patients (**Figure 43**) (1).

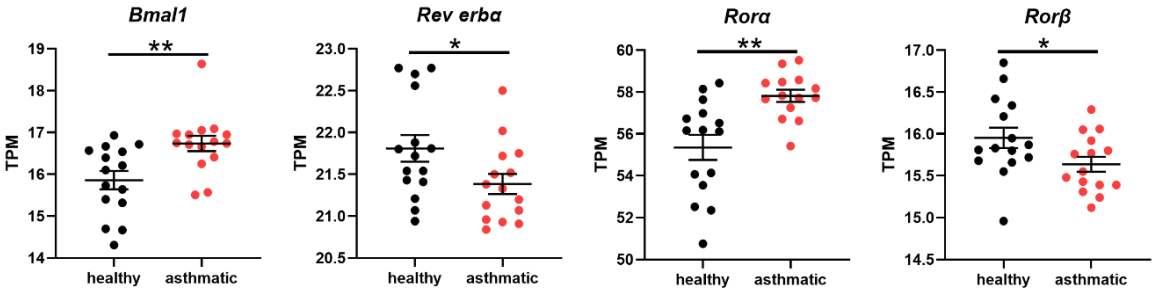

Figure 43: Clock gene dysregulation in alveolar macrophages of asthma patients. *mRNA expression levels of clock genes in alveolar macrophages from healthy individuals and asthma patients were analysed using microarray data (transcripts per kilobase million, TPM) available from GEO dataset GSE2125. Data are shown as mean ± SEM. Statistical analysis included the Kolmogorov-Smirnov test, unpaired t-test, and Mann-Whitney test. * $p < 0.05$; ** $p < 0.01$. Figure adapted from (1).*

Additionally, the typical positive correlation between *Bmal1* and *Rorα*, as well as the negative feedback loop where *Rev erbα* represses *Bmal1*, were disrupted in alveolar macrophages from asthmatics (**Figure 44**) (1).

These results underscore the circadian disruption and the associated imbalance of the peripheral clock in alveolar macrophages during chronic inflammatory conditions such as asthma.

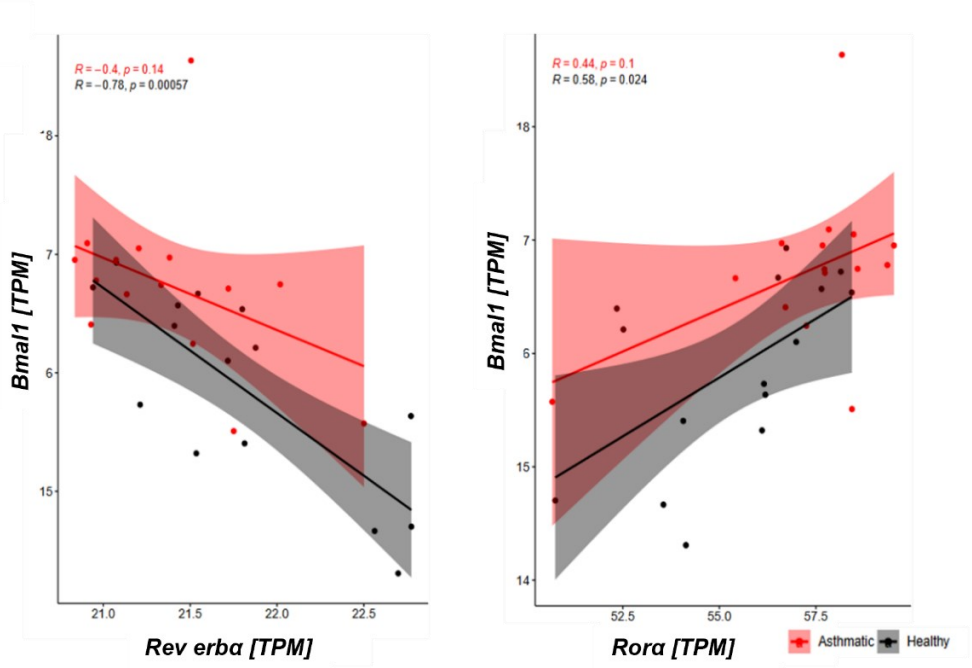

Figure 44: Clock gene correlations are lost in alveolar macrophages of asthma patients. *Scatter plots illustrate correlations between Bmal1 and Rev ERBα, as well as Bmal1 and RORα, in alveolar macrophages from healthy (black) and asthmatic (red) donors. Correlations were assessed using Pearson's test. Correlations published in (1).*

3.3 Clock-modulating ligands

Given the inflammation- and disease-related decline in clock protein expression, which is not reversed by short-term treatment with standard asthma therapeutics, we investigated whether synthetic clock-modulating ligands could restore the molecular circadian clock in the immune cell populations examined in this thesis.

3.3.1 REV ERB targeting ligands

Nuclear receptors are among the most effectively targeted proteins in pharmacology (118). Given the well-established bidirectional relationship between the circadian clock and the immune system and recognizing REV ERBα as a crucial link between these two systems (17,21), we ought to investigate the potential therapeutic effects of the REV ERB agonists GSK4112 and SR9009 (1,2).

3.3.1.1 REV ERB agonists impact eosinophil effector function

Both REV ERB agonists, GSK4112 and SR9009, were found to increase REV ERB expression while reducing BMAL1 levels in peripheral eosinophils and neutrophils (**Figure 45** and **Figure 46,** respectively). Therefore, we further investigated the potential therapeutic effects of these synthetic clock-modulating ligands. Targeting REV ERB with the agonist GSK4112 led to reduced eosinophil migration and increased apoptosis, effects that were partially reversed by the REV ERB antagonist SR8278. In contrast, SR9009 did not significantly affect eosinophil migration or apoptosis. Additionally, both agonists enhanced ROS production, an effect that was not blocked by the antagonist, suggesting that the ROS increase may be at least partially independent of REV ERB (**Figure 45**) (2).

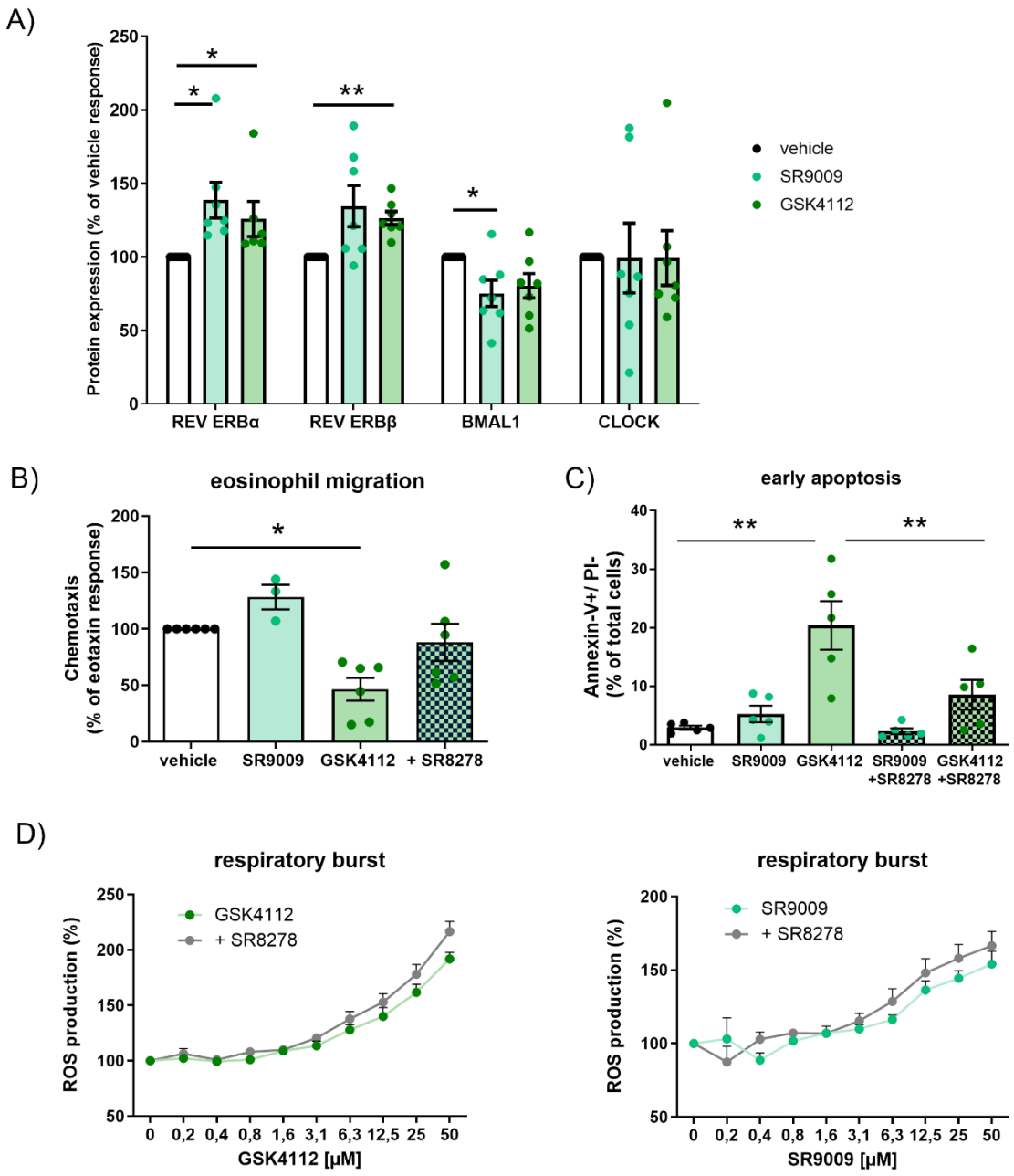

Figure 45: REV ERB agonists GSK4112 and SR9009 modulate eosinophil effector functions partially independently of REV ERBα. *(A) PMNL were incubated with 10 µM of the REV ERB agonists for 3 hours. Intracellular levels of REV ERBα, REV ERBβ, and BMAL1 were measured by flow cytometry, with eosinophils identified based on FSC/SSC characteristics and autofluorescence (n≥7). PMNL or purified eosinophils were pretreated with 10 µM SR8278 prior to a 3-hour incubation with either 10 µM SR9009 or GSK4112. (B) Chemotaxis assays were conducted to assess eosinophil migration toward 3 nM eotaxin-1 using a microBoyden chamber (n>3). (C) Apoptosis was evaluated using APC-Annexin V and Propidium Iodide staining followed by immediate flow cytometric acquisition (60 s at medium flow rate, n=5). (D) Reactive oxygen species (ROS) generation was determined via a dihydrorhodamine-123-based flow cytometric assay (n=5). Statistical comparisons were performed using One-Way or Two-Way ANOVA. *p < 0.05, **p < 0.01. Figure adapted from (2).*

Similarly, GSK4112 reduced neutrophil migration, an effect that could be blocked by the REV ERB antagonist SR8278. However, both agonists significantly induced ROS production, which was not reversed by SR8278, suggesting the involvement of REV ERB-independent off-target effects. (**Figure 46**) (2).

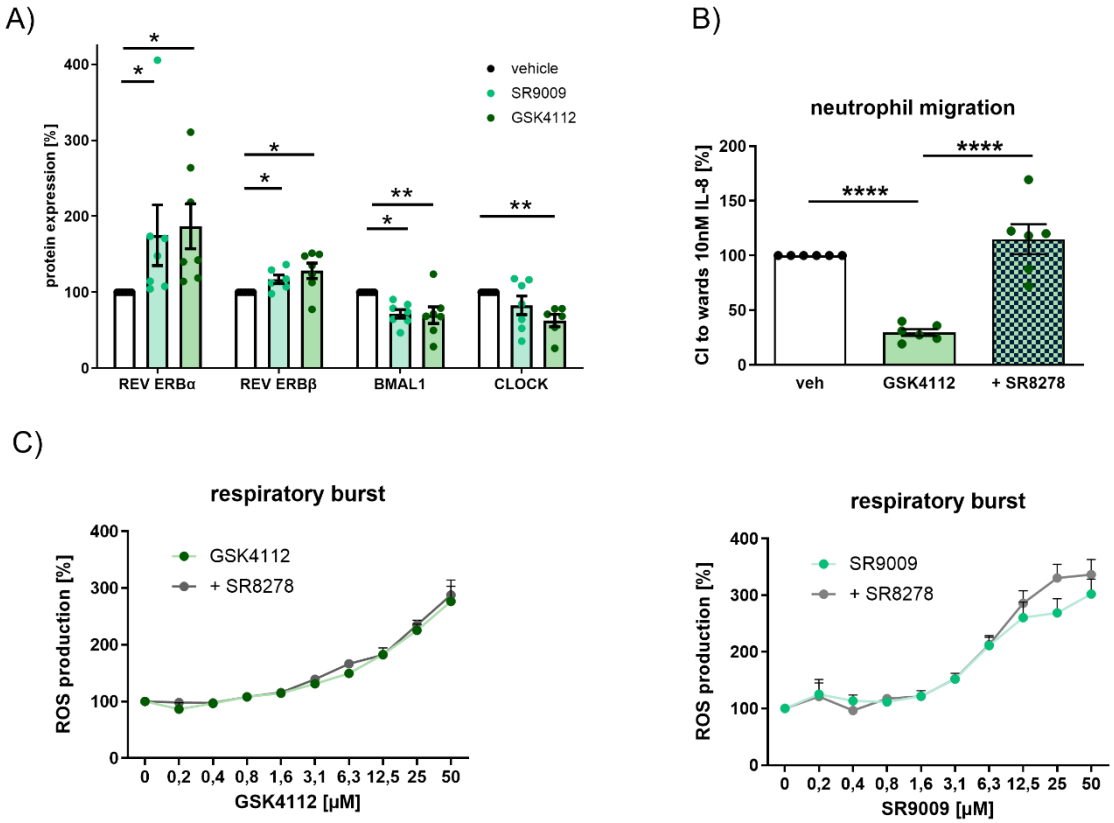

Figure 46: REV ERB agonists GSK4112 and SR9009 influence neutrophil functions through mechanisms partly independent of REV ERBα. *(A) PMNL were exposed to 10 µM of the indicated REV ERB agonist for 3 hours, followed by intracellular staining to assess REV ERBα, REV ERBβ, and BMAL1 protein expression via flow cytometry (n≥7). For inhibition experiments, PMNL were pretreated with 10 µM SR8278 before a 3-hour incubation with either SR9009 or GSK4112 at 10 µM. (B) Neutrophil chemotaxis was evaluated in response to 10 nM IL-8 using a microBoyden chamber assay (n=6). (C) Reactive oxygen species (ROS) levels were measured using a dihydrorhodamine-123-based flow cytometric assay (n=5). Statistical significance was determined by One-Way or Two-Way ANOVA. *p < 0.05, **p < 0.01, ***p < 0.001. Figure adapted from (2).*

3.3.1.2 REV ERB agonists alter the polarization state and effector function of macrophages

Given the inflammation-associated changes in BMAL1 and its regulatory receptor families, REV ERB and ROR in monocyte-derived macrophages, we aimed to investigate the potential therapeutic effects of synthetic clock-modulating ligands on macrophages.

To assess the proposed anti-inflammatory properties of the REV ERBα agonist SR9009 on macrophage polarization, MDMs were cultured and activated into M1 and M2 macrophages.

Subsequently, the expression of clock proteins and the macrophage surface markers CD80 and CD206, indicative of M1 and M2 activation, respectively, was evaluated. As shown in **Figure 47**, SR9009 decreased REV ERBα expression in M1 macrophages and slightly increased BMAL1 expression in M2 macrophages, highlighting its clock-modulating potential but also suggesting possible pro-inflammatory effects of this synthetic agonist. Notably, we also observed a reduction in the surface expression of CD206, indicating a shift towards a rather inflammatory or aged phenotype. However, consistent with previous findings indicating REV ERBα-independent effects in eosinophils and neutrophils, and as reported by Dierickx and colleagues (149), pre-treatment with the REV ERB antagonist SR8278 did not block or reverse the effects of SR9009 on M2 polarization (**Figure 47B**). Additionally, elevated concentrations of the pro-inflammatory macrophage inflammatory proteins MIP 1α and MIP 1β, which are strongly linked to allergic airway inflammation (150,151), were detected in the supernatants of SR9009-treated M1 macrophages (**Figure 47C**) (1).

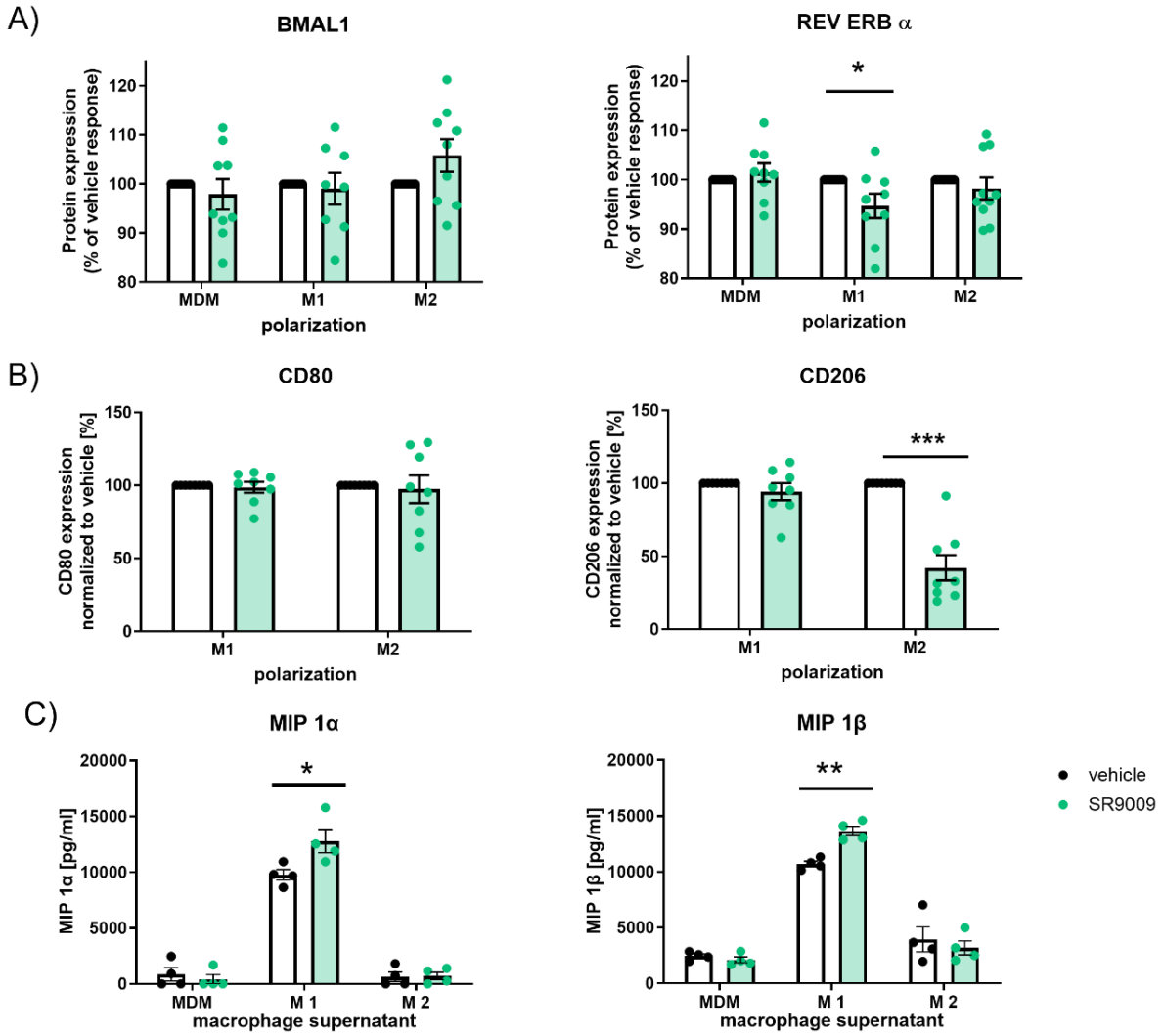

Figure 47: The REV ERBα agonist SR9009 promotes a pro-inflammatory phenotype in human macrophages. *Macrophages were generated from human peripheral blood mononuclear cells (PBMCs) and polarized using LPS/IFN-γ or IL-4, with or without SR9009 (10 μM). (A) Intracellular flow cytometry was used to assess the expression of REV ERBα and BMAL1 (n ≥ 8). (B) For reversal experiments, macrophages were pre-incubated with the REV ERB antagonist SR8278 for 5 hours prior to polarization in the presence of SR9009. Surface markers CD80 and CD206 were analysed via flow cytometry to confirm polarization (n ≥ 8). (C) Supernatants from SR9009-treated M1 macrophages showed elevated levels of macrophage inflammatory proteins (MIPs), measured using a human chemokine 6-plex assay (n ≥ 4). Data are presented as mean ± SEM; statistical analysis was performed using T-test or One-Way ANOVA. *p < 0.05, **p < 0.01, ***p < 0.001. Figure adapted from (1).*

These findings confirm the clock-modulating potential of SR9009, but also its pro-inflammatory properties. Consistent with previous studies (149,152), pre-treatment with a REV ERB antagonist did not prevent the observed effects, indicating that the pro-inflammatory response may not be mediated by REV ERB but other off-target pathways.

3.3.2 ROR targeting ligands

Given the unfavourable pro-inflammatory effects associated with REV ERB agonists, such as increased ROS production in eosinophils and neutrophils and elevated MIP release in macrophages, along with reported REV ERBα-independent effects of SR9009 in previous studies (149,152), we next focused on targeting the opposing ROR receptor family.

3.3.2.1 Inverse ROR agonist SR1001 exhibits anti-inflammatory properties *in vitro*

3.3.2.1.1 Eosinophils and neutrophils

Treatment with the inverse ROR agonist SR1001 led to increased expression of clock proteins, including BMAL1 and RORβ (**Figure 48A**). After confirming that SR1001 modulates clock protein expression, we investigated the effects of synthetic ROR ligands on eosinophil effector functions *in vitro*. Thereby, we found that the ROR agonist SR1078 induced pro-inflammatory responses such as cytoskeleton rearrangement measured by shape change assays (**Figure 48B**), a trend toward increased migration (**Figure 48C**) and elevated degranulation (**Figure 48D**), as well as increased production of ROS (**Figure 48E** and **F**). Indeed, pre-treatment with the inverse ROR agonist SR1001 attenuated pro-inflammatory responses by reducing shape change, degranulation, and oxidative stress in eosinophils (**Figure 48B,D-F**). Further, the inverse ROR agonist decreased the migratory responsiveness in eosinophils derived from allergic blood donors (**Figure 48C**) (2).

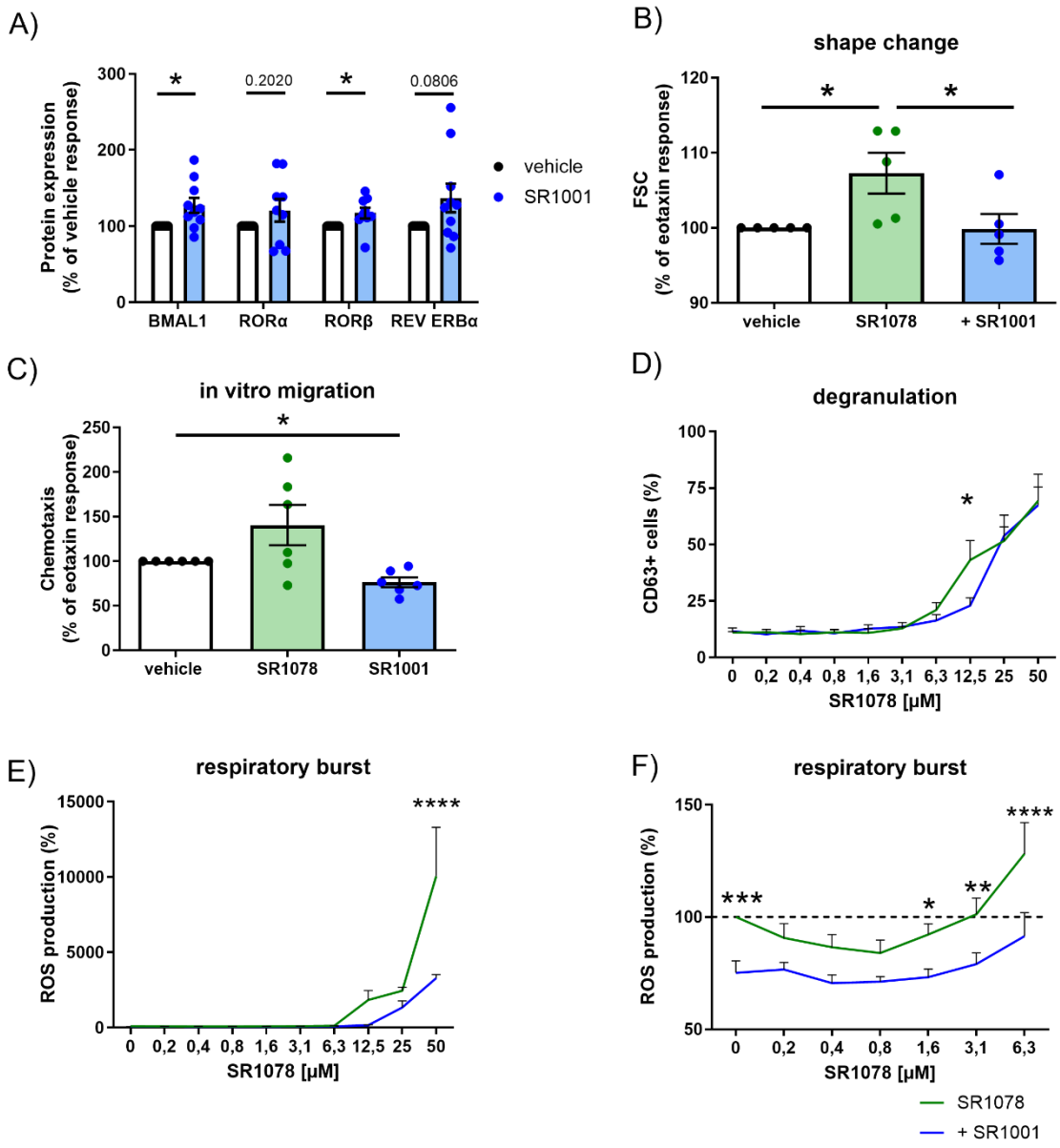

Figure 48: The ROR agonist SR1078 and inverse agonist SR1001 exert opposing effects on eosinophil effector functions. *PMNL (A, B, D) or purified eosinophils (C, E, F) from healthy and patient-derived blood samples were pre-treated with 10 µM SR1001 and/or exposed to 10 µM SR1078. (B) Following treatment, intracellular levels of the clock proteins REV ERBα, REV ERBβ, and BMAL1 were assessed by flow cytometry. Eosinophils were identified based on forward/side scatter characteristics and autofluorescence. (A) A shape change assay in response to eotaxin-1 was conducted (n = 5). (C) Chemotaxis towards eotaxin-1 was measured in eosinophils from allergic donors (n ≥ 4). (D) Degranulation was evaluated by staining with FITC-anti-CD63 after incubation with 5 µg/mL cytochalasin B and analysed via flow cytometry (n = 8). (E) ROS production was determined using a dihydrorhodamine-123-based respiratory burst assay (n = 10). Data are shown as mean ± SEM; statistical analysis was performed using unpaired t-tests, One-Way or Two-Way ANOVA. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. Figure adapted from (2).*

Given the promising anti-inflammatory effects of SR1001 observed in eosinophils *in vitro*, we next investigated the impact of SR1001 on neutrophil effector functions. Although treatment with SR1001 also increased RORα expression in neutrophils, the restoration of the circadian clock and the inhibitory effects on shape change and degranulation were observed exclusively in eosinophils. However, the inverse ROR agonist SR1001 reduced the respiratory burst in neutrophils induced by the ROR agonist SR1078 (**Figure 49**) (2).

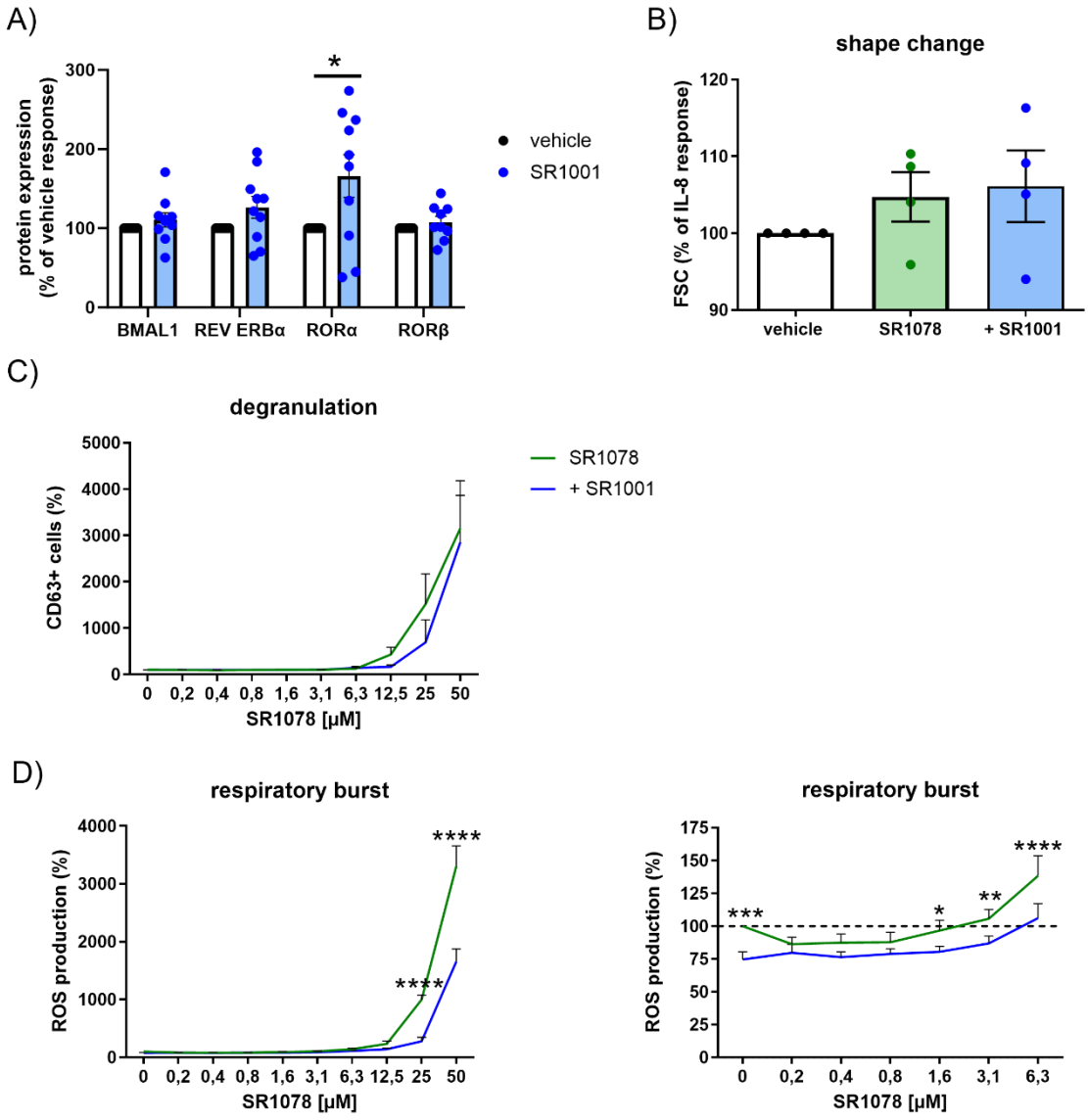

Figure 49: Limited impact of SR1001 on neutrophil functions. *(A) Following treatment with SR1001, intracellular levels of the clock proteins REV ERBα, REV ERBβ, and BMAL1 were measured by flow cytometry. (B–D) PMNL were pre-incubated with 10 µM SR1001 and/or exposed to 10 µM SR1078. (B) A shape change assay in response to IL-8 was conducted (n = 5). (C) Degranulation was assessed by staining with FITC-anti-CD63 after treatment with 5 µg/mL cytochalasin B, followed by flow cytometric analysis (n = 8). (D) ROS production was quantified using a dihydrorhodamine-123 respiratory burst assay (n = 10). Data are presented as mean ± SEM; statistical significance was determined by One-Way or Two-Way ANOVA. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. Figure adapted from (2).*

3.3.2.1.2 Macrophages

As anticipated, SR1001 treatment also affected circadian protein expression in MDMs and polarized macrophages. Specifically, the expression of the BMAL1 repressor REV ERBα was reduced in MDMs, but increased in M1 macrophages following SR1001 treatment. Accordingly, in M1 polarized macrophages BMAL1 expression was significantly reduced. Conversely, levels of the BMAL1 activator RORβ were significantly elevated in M2 macrophages treated with SR1001 compared to the vehicle-treated group (**Figure 50A**). As a result, SR1001-treated M1 macrophages exhibited significantly lower BMAL1 levels, which was associated with a tendency towards reduced cell viability (**Figure 50B**) (1).

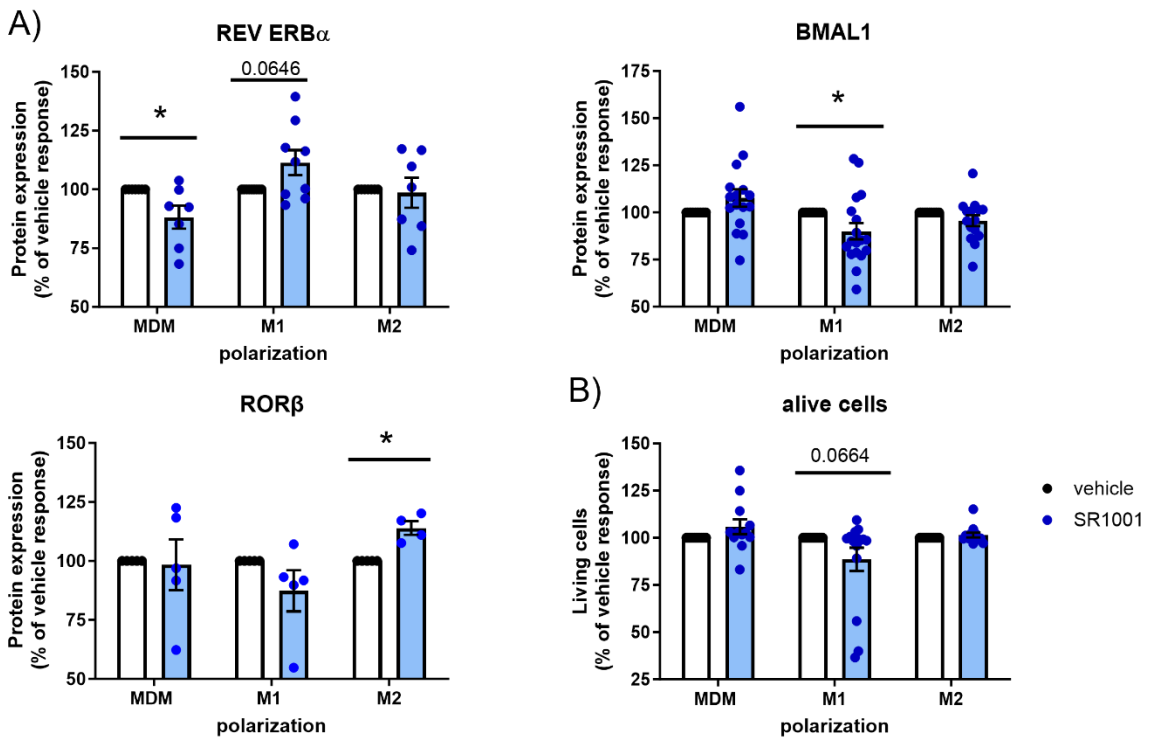

Figure 50: The inverse ROR agonist SR1001 modulates circadian protein expression in monocyte-derived macrophages and polarized human macrophages. *Macrophages were differentiated from human peripheral PBMCs and polarized with LPS/IFN-γ (M1) or IL-4 (M2) in the presence or absence of SR1001 (10 µM). (A) Intracellular expression levels of the clock proteins BMAL1, REV ERBα, and RORβ were measured by flow cytometry (n ≥ 5). (B) Cell viability was assessed using a zombie dye assay (n ≥ 10). Data are presented as mean ± SEM; statistical analysis was performed using T-tests or One-way ANOVA. *p < 0.05. Figure adapted from (1).*

Impaired phagocytosis has been observed in airway- and monocyte-derived macrophages from asthmatic patients (153–155), with evidence further indicating that macrophage phagocytic activity undergoes circadian oscillations in a BMAL1-dependent manner (83). Therefore, we investigated the phagocytic potential of SR1001-treated macrophages *in vitro*. We observed an increased phagocytic capacity in MDMs and M2 macrophages treated with SR1001 (**Figure 51A**). In contrast, treatment

with the ROR agonist SR1078 significantly decreased phagocytic potential in M1 polarized macrophages (**Figure 51B**) (1).

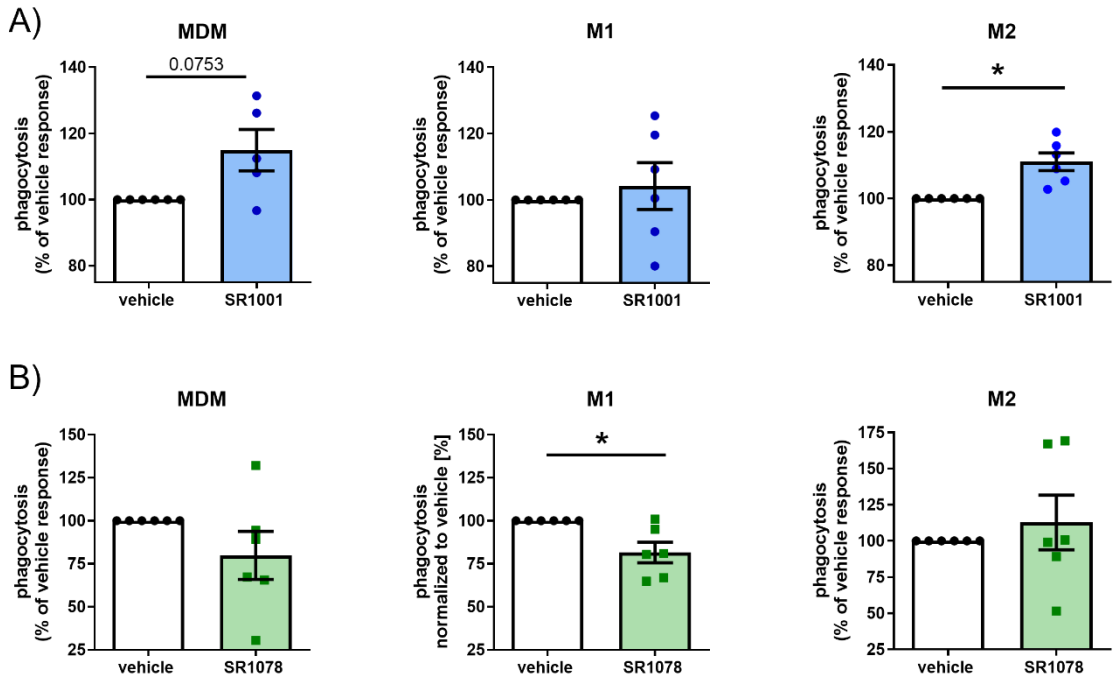

Figure 51: SR1001 increases the phagocytic capacity of monocyte-derived macrophages and polarized macrophages. *Human peripheral monocytes were differentiated into macrophages and polarized with LPS/IFN-γ (M1) or IL-4 (M2). Cells were treated with (A) SR1001 (10 µM) or (B) SR1078 (10 µM) during polarization. Phagocytosis was assessed using the Vybrant Phagocytosis Kit according to the manufacturer's instructions (n ≥ 5). Data are presented as mean ± SEM. Statistical analysis was performed using T-tests. *p < 0.05. Figure adapted from (1).*

In contrast to the REV ERB agonists SR9009 (**Figure** 47), treatment with the inverse ROR agonist SR1001 resulted in decreased secretion of chemokines by M1 macrophages (**Figure 52A**). Both MIP 1α and MIP 1β are chemokines that attract eosinophils to the asthmatic airways, hence contributing to airway inflammation (151,156). Consequently, we assessed the migratory responsiveness of purified human eosinophils to supernatants from M1 polarized macrophages treated with ROR agonists. We found that significantly fewer eosinophils migrated toward the supernatant from SR1001 treated macrophages compared to the vehicle group (**Figure 52B**) (1). In contrast to SR1001, treatment with the ROR agonist SR1078 did neither induce significant changes in MIP release nor eosinophil migration towards the supernatant of treated cells **(Figure 52C** and **D**). These results underscore the anti-inflammatory properties of the inverse ROR agonist SR1001, which not only inhibited pro-inflammatory chemokine release from M1 macrophages, but also enhanced the phagocytic activity of M2-like cells.

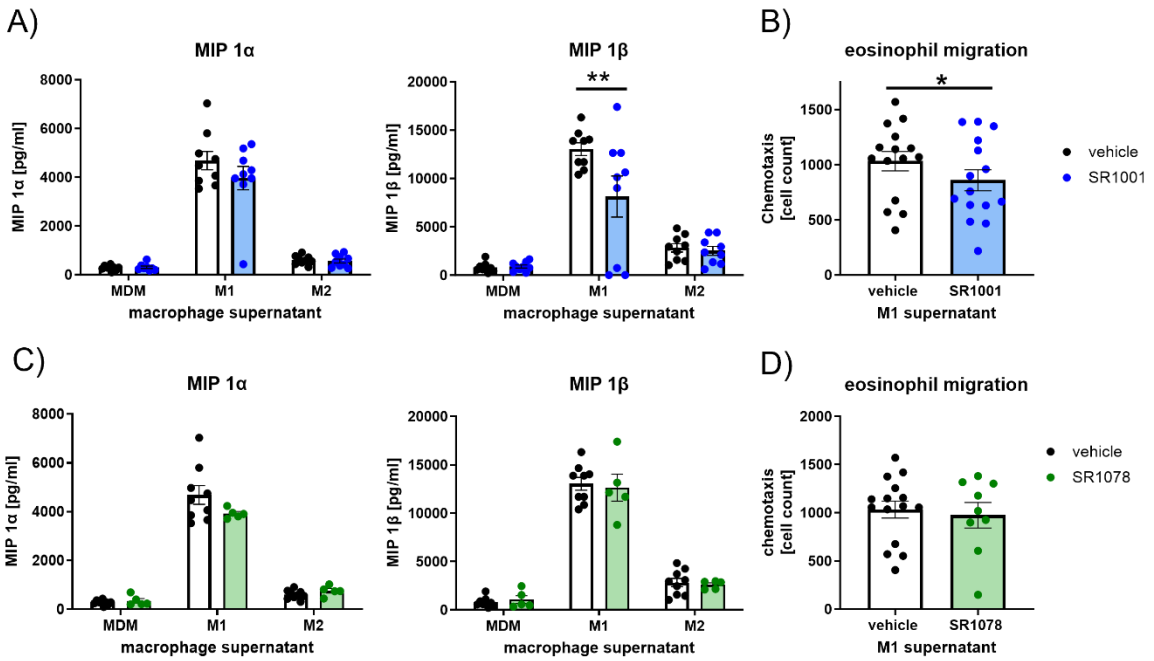

Figure 52: SR1001 exhibits anti-inflammatory effects in human monocyte-derived macrophages. *Macrophages were differentiated from human peripheral PBMCs and polarized with LPS/IFN-γ (M1) or IL-4 (M2). During polarization, cells were treated with (A) SR1001 (10 µM) or (B) SR1078 (10 µM). MIPs were quantified in culture supernatants using a human chemokine 6-plex assay (n ≥ 8). Eosinophil migration assays were performed using a microBoyden chemotaxis chamber (n=15). Data are shown as mean ± SEM. Statistical analysis was conducted using T-tests or One-way ANOVA. *p < 0.05, **p < 0.01. Figure adapted from (1).*

3.3.2.2 SR1001 promotes anti-inflammatory and bronchoprotective effects in vivo

Due to the promising anti-inflammatory effects of SR1001 observed *in vitro,* particularly the inhibition of the migratory responsiveness of peripheral eosinophils from allergic donors, we proceeded to further investigate its effects *in vivo*. In an *in vivo* migration model, IL-5 transgenic mice were pre-treated with SR1001 for three days before intranasal eotaxin-2/CCL24 instillation (**Figure 53A**). As shown in **Figure 53B**, significantly less eosinophils were recruited into the airways of SR1001-treated mice compared to control mice. Consistent with our *in vitro* findings, no significant changes were observed in the number of neutrophils in the BAL fluid. However, SR1001 treatment did reduce the proportion of pro-inflammatory CD11c^{high} Siglec-F^{low} monocyte-derived alveolar macrophages (Mo-AMs) in the BAL fluid of mice, although this reduction was not statistically significant. To ensure that targeting the stabilizing loop with SR1001 does not disrupt the animals' circadian rhythms, we monitored their behaviour using LabMaster cages. SR1001 treatment did not affect drinking behaviour, eating patterns, or physical activity rhythms during the experiment (**Figure 53C**) (2).

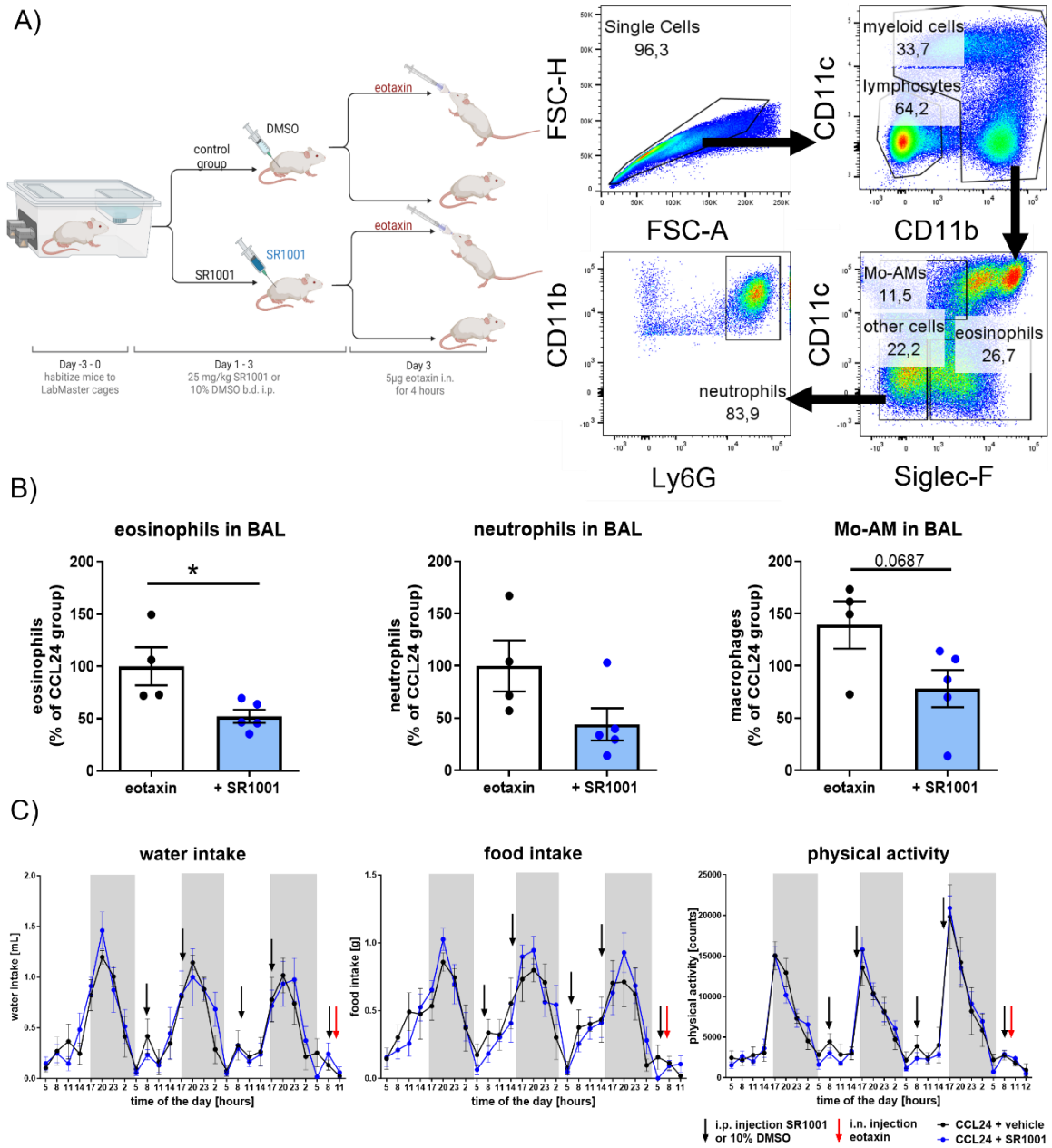

Figure 53: The inverse ROR agonist SR1001 prevents eosinophil recruitment in vivo. *(A) Mice were treated intranasally (i.n.) with house dust mite (HDM) extract (10 µg) or vehicle once weekly for four weeks. Starting on day 22, following a 3-day acclimatization in LabMaster cages, mice received five intraperitoneal (i.p.) injections of SR1001 (25 mg/kg). Bronchoalveolar lavage (BAL) fluid was collected after the final SR1001 injection on day 24. Schematic illustrations were created with BioRender.com. (B) Airway inflammation was assessed by flow cytometry analysis of BAL cells, quantifying CD11c^low Siglec-F^high eosinophils, CD11c^high Siglec-F^low monocyte-derived alveolar macrophages (Mo-AM), and CD11c^high Ly6-G^high neutrophils (n ≥ 4). (C) Circadian home-cage behaviour, including drinking, eating rhythms, and physical activity, was continuously monitored using the automated LabMaster system (n ≥ 5). Data are presented as mean ± SEM. Statistical analyses were performed using One-Way and Two-Way ANOVA. *p < 0.05. Figure adapted from (2).*

To further assess the therapeutic potential of targeting the ROR family, we applied the inverse ROR agonist SR1001 in a murine model of HDM-induced lung inflammation. Mice were subjected to HDM challenges to induce lung inflammation, followed by five injections of SR1001, this time as a

therapeutic intervention (**Figure 54A**). Consistent with our *in vitro* findings and the results from the *in vivo* migration model, SR1001 treatment significantly reduced the proportion of eosinophils in the BAL, while neutrophil numbers remained unchanged. Furthermore, the treatment led to a significant decrease in the proportion of pro-inflammatory Mo-AMs in the BAL (**Figure 54B**). The *in vivo* reduction of Mo-AMs is aligning with our *in vitro* data indicating a trend towards reduced M1 macrophage survival (**Figure 50B**). Consistent with previous results, no differences in biological rhythms were observed although the molecular circadian clock was targeted with the inverse ROR agonist SR1001 (**Figure 54C**) (1,2).

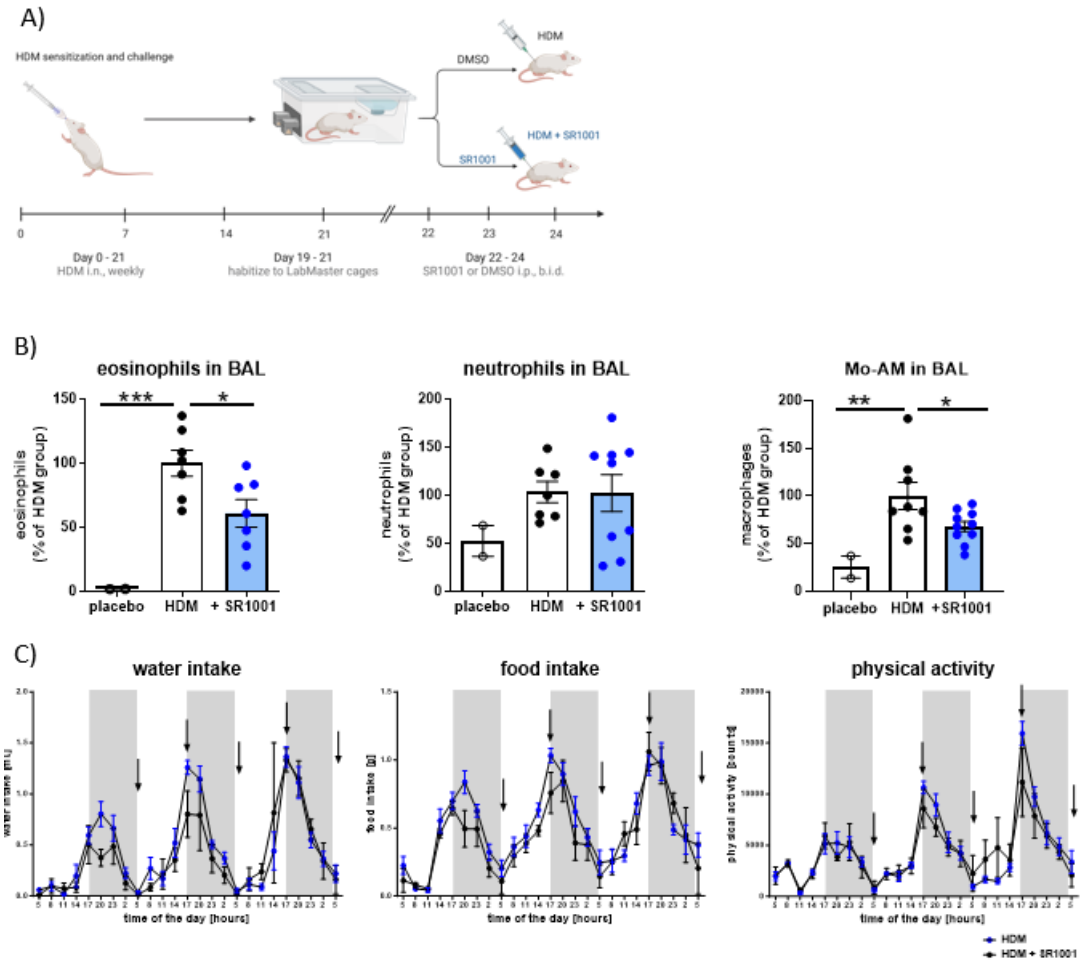

Figure 54: The inverse ROR agonist SR1001 promotes anti-inflammatory effects in HDM-induced airway inflammation. *(A) Mice were treated i.n. with HDM extract (10 µg) or vehicle once weekly for four weeks. Starting on day 22, following a 3-day acclimatization in LabMaster cages, mice received 5 intraperitoneal (i.p.) injections of SR1001 (25 mg/kg) twice daily (b.i.d.). Bronchoalveolar lavage (BAL) fluid was collected after the final injection on day 24. Schematic figure created with BioRender.com. (B) To assess airway inflammation, flow cytometry was used to quantify CD11c^low Siglec-F^high eosinophils, CD11c^high Siglec-F^low monocyte-derived alveolar macrophages (Mo-AM), and CD11c^high Ly6-G^high neutrophils in the BAL fluid (n ≥ 4). (C) Circadian home-cage behaviour—including drinking, eating rhythm, and physical activity—was recorded every minute using the automated LabMaster system (n ≥ 5). Data are shown as mean ± SEM. Statistical analyses: One-Way and Two-Way ANOVA; *p < 0.05, **p < 0.01, ***p < 0.001. Figure adapted from (1,2).*

In line with the reduction of immune cells in the BAL fluid, SR1001 treatment was associated with significantly decreased immune cell infiltration into the lung tissue and reduced goblet cell hyperplasia (**Figure 55A**). Additionally, compared to the HDM control group, lungs from SR1001-treated mice exhibited significantly reduced mucus plugging in PAS staining (**Figure 55B**). These findings clearly indicate an anti-inflammatory and bronchoprotective effect of SR1001, while preserving the overall circadian rhythm of the animals. Hence, clock-modulating ligands such as SR1001 might be novel treatment approaches for asthma in future (1,2).

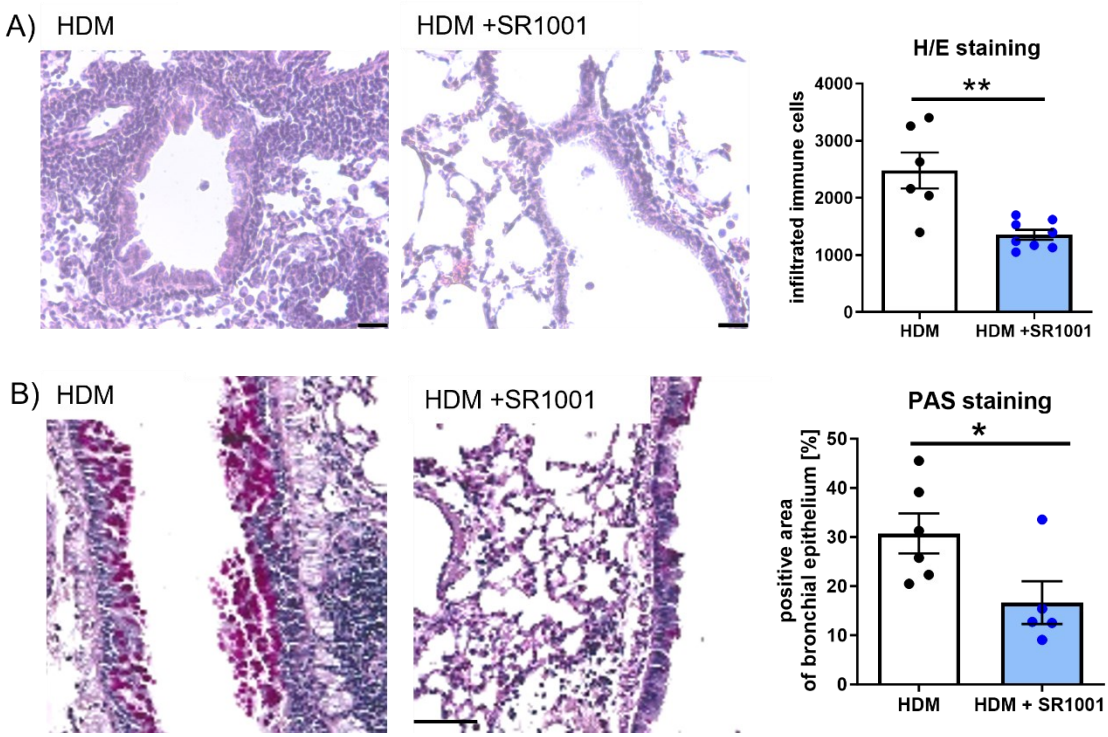

Figure 55: The inverse ROR agonist SR1001 reduces immune cell infiltration, hyperplasia, and mucus production. *(A) Hematoxylin and eosin (H/E) staining was performed on lung tissue sections and analysed automatically using ImageJ. Representative images are shown (scale bar, 10 µm; n ≥ 6). (B) Mucus production was evaluated histologically by periodic acid–Schiff (PAS) staining. Representative images are shown (scale bar, 100 µm; n ≥ 5). All images were analysed in a blinded manner using ImageJ. Data are presented as mean ± SEM. Statistical analysis: T-test, *p < 0.05, **p < 0.01. Figure adapted from (1,2).*

In addition, lung function was assessed by challenging both groups of mice with increasing concentrations of methacholine, a potent cholinergic bronchoconstrictor. Mice treated with SR1001 exhibited improved lung function, as measured by the FlexiVent system. As shown in **Figure 56**, SR1001 treatment led to decreased respiratory resistance of the respiratory system (Rrs) and lower resistance in the central airways (Rn). Furthermore, the treatment resulted in reduced stiffness (Ers and H), decreased dampening (G), and enhanced compliance (Crs) during methacholine challenge (2).

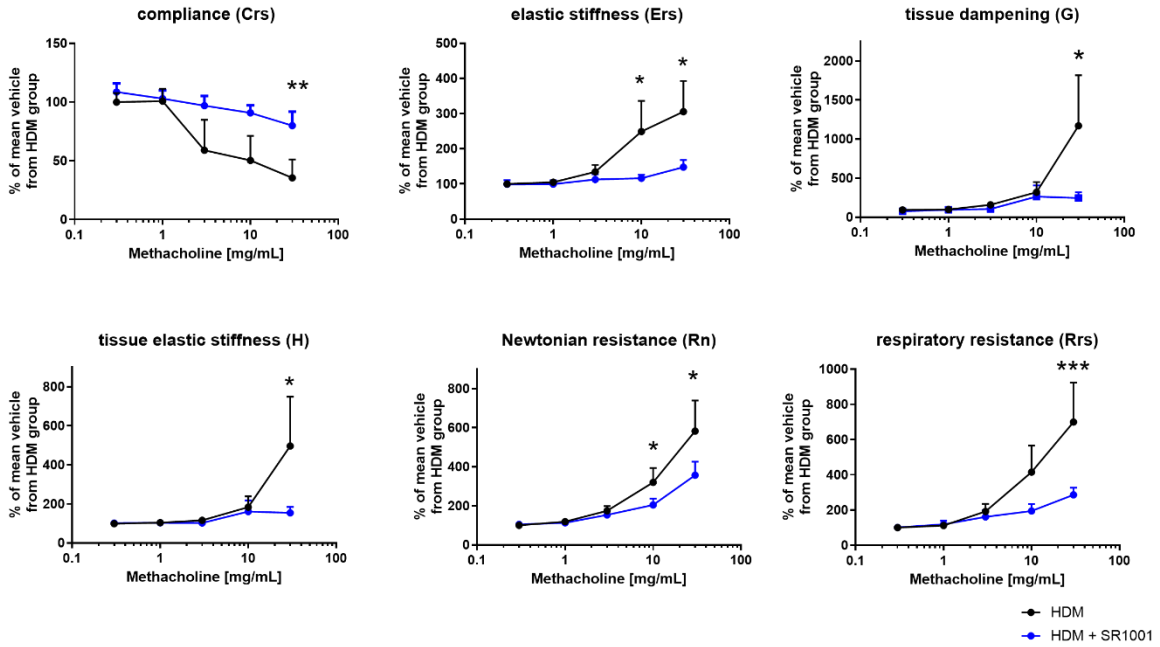

Figure 56: The inverse ROR agonist SR1001 promotes lung-protective effects in HDM-induced airway inflammation. *Airway hyperresponsiveness (AHR) in response to methacholine was assessed using the FlexiVent system (n ≥ 7). Data are presented as mean ± SEM. Statistical analysis: Two-Way ANOVA, *p < 0.05, **p < 0.01, ***p < 0.001. Figure adapted from (2).*

3.3.2.3 Inverse ROR agonist SR1001 mediates anti-inflammatory effects of eosinophils by blocking C-C type chemokine receptor (CCR3) and EGFR-signalling

Based on the observed anti-inflammatory effects in eosinophils, we aimed to investigate the underlying signalling mechanisms through which SR1001 reduces eosinophil effector cell function. Recent studies have suggested that upregulation of BMAL1 leads to decreased ERK phosphorylation (157). Given that ERK phosphorylation is a key signalling pathway for many pro-inflammatory mediators, such as eotaxin/CCL11, and is elevated in eosinophils from asthmatics (158), we investigated the impact of SR1001 on kinase phosphorylation in eosinophils (2).

In a Phospho-Flow assay, we observed that SR1001 significantly blocked eotaxin/CCL11-induced ERK phosphorylation in purified eosinophils (**Figure 57A**). Additionally, Western blot analysis revealed that SR1001 reduced the elevated levels of phospho-ERK in eosinophils from mild asthmatic patients compared to healthy controls (**Figure 57B**) (2).

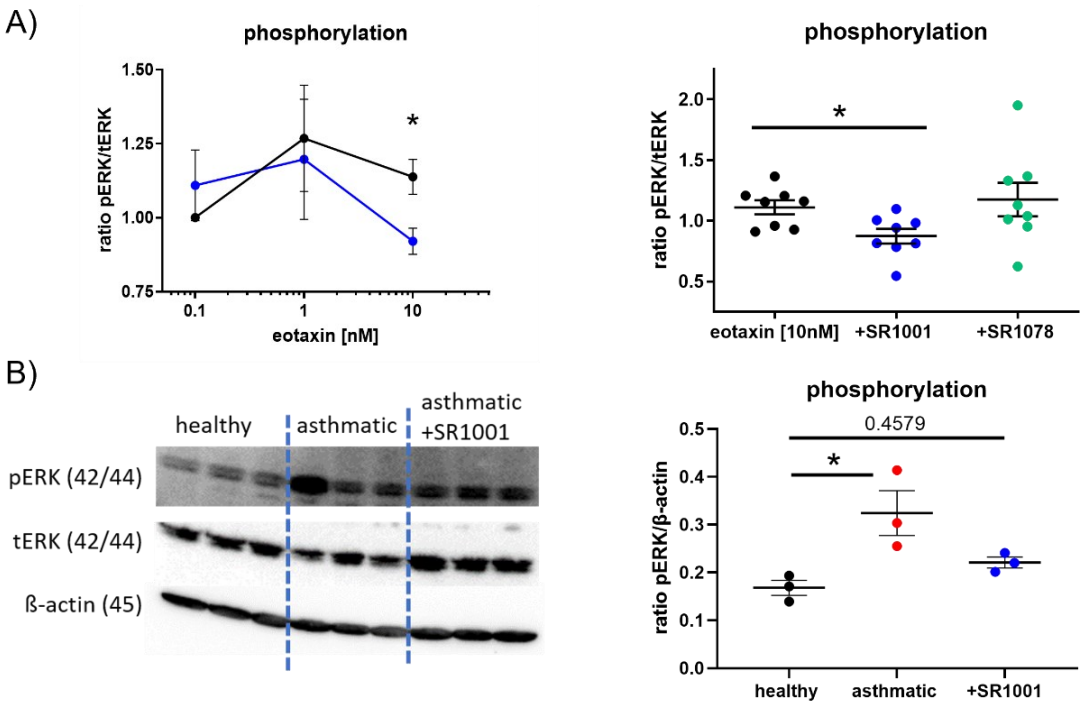

Figure 57: The inverse ROR agonist SR1001 inhibits ERK phosphorylation in circulating eosinophils. *(A) The impact on ERK phosphorylation was detected in a Phospho-Flow Assay in response to eotaxin-1. Data are presented as the ratio of phosphorylated ERK to total ERK (n=8). (B) Western blotting confirmed that SR1001 reduces the increased ERK phosphorylation in asthmatics (duplicates measured from n=3). Mean ± SEM; t-test, Two- or One-Way ANOVA, Tukey post hoc test * p < 0.01. Figure adapted from (2).*

A phosphokinase array further revealed that opposed to healthy controls, phosphorylation of epidermal growth factor receptor (EGFR), c-Jun-N-terminal kinase (JNK1/2/3), signal transducer and activator of transcription (STAT)1 and AKT1/2/3 is increased in eosinophils from asthmatics and again reduced after SR1001 treatment (**Figure 58**). These findings suggest that SR1001 reduces disease-induced eosinophil hyperactivation by inhibiting ERK- and EGFR-mediated signalling (2).

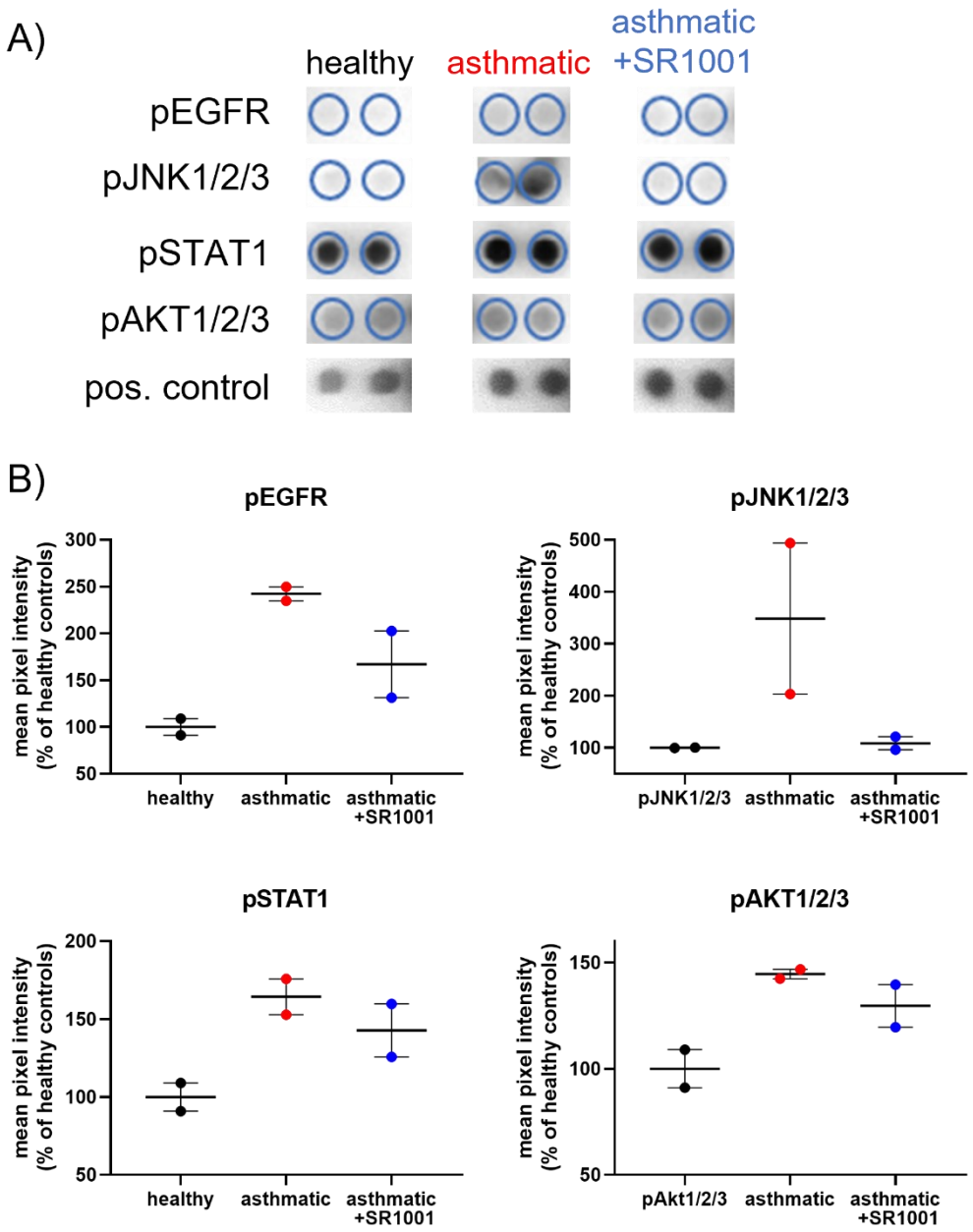

Figure 58: The inverse ROR agonist SR1001 blocks EGFR signalling in eosinophils. *Isolated eosinophils from three healthy or asthmatic blood donors were pooled to obtain 4.5 × 10⁶ cells per group. Cells were treated with either 10 µM SR1001 or vehicle control (DMSO) for 3 hours. A phosphokinase array was then performed.* ***(A)*** *Representative array membrane images from pooled eosinophils, highlighting SR1001-induced changes in site-specific phosphorylation.* ***(B)*** *Quantification of phosphorylation intensity values from (C), expressed as % of vehicle-treated control. Data are presented as mean ± SEM. Figure adapted from (2).*

As summarized in **Table 9**, pharmacological modulation of the molecular circadian clock targeting REV ERBs and RORs alters immune cell behaviour across eosinophils, neutrophils, and macrophages. Clock-modulating ligands lead to a change in clock protein expression and affecting functions such as migration, apoptosis, ROS production, and cytokine signalling. In vivo, SR1001 reduces cell infiltration and improves lung function in both murine models, highlighting therapeutic potential through circadian pathway regulation.

Table 9: Effects of clock-modulating ligands on immune cell circadian protein expression and function. *GSK4112, SR9009, and SR1001 influence clock protein expression and effector cell function (indicated with arrows, - = no significant effect) of eosinophils, neutrophils, and macrophage subsets. In vivo, SR1001 treatment diminishes eosinophil and macrophage accumulation in BAL and alleviates airway inflammation and mucus production, indicating the therapeutic relevance of targeting circadian regulators in pulmonary inflammation. n.a. = not analysed, LuFu = lung function*

clock-modulating ligands targeting		eosinophils	neutrophils	monocyte derived macrophages			lung
				MDM	M1	M2	
clock protein expression	GSK4112	REV ERBs↑	REV ERBs↑ BMAL1 ↓ CLOCK ↓	n.a.	n.a.	n.a.	
	SR9009	REV ERBα↑ BMAL1 ↓	REV ERBs↑ BMAL1↓	-	REV ERBα↓	-	
	SR1001	↑	RORα↑	REV ERBα↓	BMAL1↓	RORβ↑	
in vitro	GSK4112	migration↓ apoptosis ↑ ROS ↑	migration↓ ROS↑	n.a.	n.a.	n.a.	
	SR9009	migration - apoptosis - ROS ↑	ROS↑	MIP1 -	CD80 - CD206 - MIP1 ↑	CD80 - CD206 ↓ MIP1 -	
	SR1001	migration↓ degranulation↓ ROS ↓ EGFR-signalling ↓	Degranulation- ROS↓	MIP1 - phagocytosis -	MIP1β↓ phagocytosis - eosinophil migration ↓	MIP1 - phagocytosis ↑	
SR1001 *in vivo*	migration	↓ in BAL	-	↓ in BAL			
	HDM	↓ in BAL	-	↓ in BAL			H/E ↓ PAS↓ LuFu ↑

4. Discussion

This thesis aimed to explore the interaction between the molecular circadian clock and the immune system in the context of asthma. To clarify this relationship, we examined circadian protein expression in leukocyte subsets from both asthmatic and healthy, non-allergic donors. Further, we have investigated the responsiveness of the molecular circadian clock to inflammatory mediators. Finally, we examine whether clock-modulating ligands could serve as potential novel therapeutic approaches.

4.1 Molecular circadian clock is disrupted on a protein level in immune cells derived from asthmatics

First, we aimed to determine whether the molecular circadian clock is expressed at the protein level in a daily oscillating pattern. Recent studies have shown that clock genes oscillate in various peripheral tissues, including the lung (159–161). However, the characteristics of these oscillations within the human immune system remain largely unexplored. Baumann et al. recently showed oscillations of the circadian clock on mRNA level in eosinophils and mast cells (24). Similar, Bollinger and colleagues demonstrated that isolated human CD4+ T cells exhibit circadian oscillation of clock genes including *bmal1*, *clock* and *rev erbα* which persists over 48 hours in culture (57). Most studies on circadian regulation of monocytes and macrophages have yielded conflicting results, largely because they were conducted at the mRNA level or involved mouse macrophages. Boivin et al. were among the first to show oscillatory expression of the central clock loop in human PBMCs (162). Therefore, our initial aim was to explore the oscillatory patterns of the molecular circadian clock at the protein level in peripheral human immune cell subsets.

A significant challenge in circadian biology is accurately detecting human circadian rhythms in a manner that enables meaningful connections with the molecular clock. The expression of core clock genes involved in the transcription-translation feedback loop, can be directly assessed. Since the circadian state fluctuates throughout the day, it is essential to track these oscillations over a 24-hour period. This requires the collection of multiple samples at regular intervals. However, sampling frequency and duration are limited. To address this, we adopted a study design similar to that used by Baumann et al., who showed that the molecular clock in peripheral eosinophils and mast cells remains active for several hours after blood collection when incubated at 37 °C (163). Hence, we therefore collected blood samples every 8 hours, incubated a part of the blood for additional 4 hours, resulting

in six time points spaced 4 hours apart throughout the day. Given that asthma is a chronic inflammatory disease of the airways with circadian fluctuations in symptoms and biomarkers peaking around 4 a.m. (164), we ensured inclusion of this critical time point. Indeed, we are the first to detect oscillating clock protein expression patterns in peripheral eosinophils, neutrophils, T cells and both classical CD14+CD16- and CD14+CD16+ monocyte subsets (1,2). Notably, we identified significant variations in the amplitude of clock proteins between blood donors with asthma and healthy individuals, depending on the time of day. Consistent with previous reports on circadian gene expression of *bmal1* and *clock* in peripheral human eosinophils of non-allergic subjects (163), we observed increased BMAL1 protein expression at noon in circulating eosinophils from healthy blood donors. Furthermore, we observed that REV ERBα protein expression in $CD3^+$ T cells reached a nadir at noon, consistent with earlier findings of circadian *Rev erb*α gene expression in $CD4^+$ T cells. Taken together, these data support that immune cells, including eosinophils, T cells, and monocytes, maintain intrinsic circadian oscillations at the protein level.

Additionally, we are the first to observe a general attenuation of the molecular circadian clock at the protein level in asthmatics, with the effect being more pronounced in those with moderate asthma compared to blood donors with mild asthma, indicating a negative correlation of clock protein levels and disease severity. Although the consolidation of time frames was necessary due to differing participant schedules, it is important to consider that slight variations in sampling times could introduce variability in the data. However, given the close overlap of these time windows, any potential impact on the significance of the results on a protein level is expected to be minimal. Differences in phase and period length of clock proteins over a 24-hour period were observed even in leukocytes from patients with mild asthma. Consistent with this, *in vivo* studies have shown that ablation of clock genes (*bmal1* or *rev erb*) worsens inflammatory diseases such as pulmonary inflammation in mice (88,165–167). In line with these findings, Ehlers et al. reported reduced gene expression of *Rev erbs* and *Bmal* in the human lung, based on bronchial brushings from asthmatics, suggesting this may be a feature of the disease (88). Recently, Chen et.al. demonstrated that the expression of core loop genes, except for *Bmal1*, are downregulated in PBMCs derived from patients with bronchial asthma compared to healthy volunteers (168). Consistent with this study, we observed no significant reduction in BMAL1 expression, nor any alterations in phase or period in CD3+ T cells from mild asthmatic patients compared to healthy controls. However, we did find a significant reduction in CLOCK, REV ERBα, and RORα in CD3+ T cells from mild asthmatics during the forenoon. In contrast, in individuals with moderate asthma, a decrease in BMAL1 expression, along with other clock proteins, was evident in both CD4+ and CD8+ T cells, but exclusively during the afternoon. Additionally, analysis of publicly

available microarray data comparing circulating CD4+ and CD8+ T cells from severe asthmatics to healthy controls (126) revealed significantly lower expression of clock genes from the stabilizing loop, while *Bmal1* levels remained unaffected. Interestingly, *Rorα* gene expression was significantly elevated in human $CD8^+$ T cells from severe asthmatics. A non-significant trend towards increased RORα protein expression was observed in moderate asthmatics during the forenoon. Concurrently, CLOCK and REV ERBα expression levels appeared reduced, although these changes did not reach statistical significance. Comparison with the microarray data is limited, as disease severity differs between cohorts, and information regarding RNA sample collection time and participant medication status is unavailable. While $CD4^+$ T cells have traditionally dominated asthma research, $CD8^+$ T cells are now recognized as key contributors to severe and fatal asthma, particularly given their greater resistance to corticosteroids compared to $CD4^+$ T cells (169). Moreover, IL-23 has been implicated in steroid-resistant asthma, as it promotes enhanced IL-17 production by $CD8^+$ T cells and upregulates Rorα and Rorγt gene expression (170). This suggests that circadian disruption may be more pronounced in $CD8^+$ T cells in patients with severe asthma. Importantly, effector memory $CD8^+$ T cells—especially those expressing high IL-6Rα—are known to have increased GATA3 expression, which drives production of Th2 cytokines IL-5 and IL-13 (171). Because Rorα interacts with GATA3, facilitating the Th2 effector state, its upregulation in $CD8^+$ T cells likely contributes to the Th2-skewed inflammatory milieu characterizing severe asthma.

Differences in the incidence, prevalence, and severity of asthma between sexes are well documented. After puberty, women experience higher asthma rates, more severe symptoms, and greater mortality compared to men (172). Particularly in women, weight gain and body mass index (BMI) have been identified as risk factors for asthma (173–175). In our study we did not find significant sex- or BMI-related differences in clock protein levels, likely due to the limited sample size. Although our analyses yielded significant results, we acknowledge that these findings require validation in a larger, well-characterized patient cohort.

We highlight that, in a small group of shift workers with asthma, no clear expression pattern was observed. In contrast to asthmatics with regular working hour, CLOCK, REV ERBs, and RORα were all increased in eosinophils, whereas only RORβ levels were elevated in neutrophils—again, highlighting the more pronounced effects in eosinophils. In both T cell subsets, REV ERBβ expression was increased, with CD8+ T cells additionally showing elevated levels of RORs. In classical monocytes from moderate asthmatics working shifts, we observed increased CLOCK expression, while solely CD14+CD16+ monocytes exhibited lower levels of BMAL1 and RORα comparing

moderate asthmatics with a shift working schedule to conventional working hours. It is well-established that shift work disrupts the endogenous circadian system and causes misalignment with environmental cues, increasing the risk of various diseases including chronic pulmonary diseases (176). A recent study by Durrington et al. reported a 23% higher prevalence of asthma and significantly reduced lung function among night shift workers compared to day workers (13).

In asthma, eosinophils accumulate in the sputum, serving as an indicator of disease severity and a biomarker of airway inflammation (177,178). This prompted us to investigate the presence of the main orchestrator of the molecular circadian clock, BMAL1, in both sputum and lung-resident eosinophils, in addition to the peripheral eosinophils analysed previously. Again, our study is the first to demonstrate that the clock protein BMAL1 is not only expressed in peripheral immune cells but is also detectable in tissue and sputum eosinophils from individuals with asthma (2). Since spontaneous sputum samples were used in this study rather than induced sputum, inclusion of a healthy control group was not feasible for comparison.

Taken together we observed an oscillating expression pattern of the molecular clock in human peripheral leucocyte subsets including eosinophils, neutrophils, and monocytes that is lowered in asthma (1,2).

4.2 Responsiveness of clock protein expression to an inflammatory environment and asthma severity

Given our observation that the molecular circadian clock is expressed at the protein level and the disease-associated reduction in peripheral immune cells from asthmatic donors, our next step is to investigate whether there is a correlation between clock protein levels and the inflammatory environment in asthma, as well as disease severity.

We are the first to associate the disrupted molecular circadian clock in eosinophils with diagnostic parameters: decreased levels of BMAL1 and REV ERBα/β were linked to an obstructive respiratory pattern (FEV1/ FVC ratio < 0.7). Additionally, patients with high FeNO levels showed a lack of circadian variation in BMAL1 and CLOCK. These findings indicate that patients with active eosinophilic asthma have lower clock protein expression levels compared to those with better-controlled asthma. Furthermore, reduced CLOCK and RORα/β levels were associated with allergic asthma. To our knowledge we are the first to observe an overall reduction of clock proteins in eosinophils, dependent on disease severity. Notably, these effects were not specific to eosinophils but were also found, to a lesser extent, in neutrophils and monocytes. Consistent with this, Chen et al. found reduced *Bmal1* levels in PBMCs from well-controlled asthmatics with nocturnal symptoms (168). Additionally, murine studies indicate that disruptions in the molecular clock may influence circadian rhythms in IgE/mast cell-mediated allergic reactions (90).

Increasing evidence of a bidirectional interaction between the molecular circadian clock and the immune system is seen for instance in the daily fluctuations of circulating cytokines. Our study found that in healthy donors, circulating cytokine levels increase in the early morning hours, whereas in asthmatics, cytokine levels are generally elevated and tend to rise in the evening resulting in significant differences. This finding offers insight into the pathophysiology of nocturnal asthma attacks, which are thought to be driven by cytokines such as TNF-α. TNF-α plays a key role in the inflammatory response and can amplify the activity of other immune cells, including those responsible for bronchoconstriction and airway inflammation. Elevated levels of TNF-α during asthma attacks may thus contribute to nocturnal symptoms (179). In addition, IL-4 and IL-13 are important cytokines involved in asthma exacerbations. These cytokines contribute to airway inflammation by promoting mucus production, activating eosinophils, and enhancing the synthesis of IgE antibodies, which together drive airway hyperresponsiveness and inflammation during asthma attacks (180). IL-5**,** another key cytokine, is essential for the activation, survival, and recruitment of eosinophils, which

play a central role in the inflammatory process observed during asthma attacks (42). Further evidence supporting the regulatory role of the circadian clock in cytokine secretion comes from studies such as that by Tang et al., who reported elevated levels of IL-6 in patients with nocturnal asthma compared to those without nocturnal symptoms. This increase in IL-6 was specifically linked to the inhibition of the BMAL1/FOXA2 signalling pathway in airway epithelial cells (181). These inflammatory mediators tend to be more active during sleep, influenced by factors such as hormonal fluctuations (e.g., the nocturnal nadir of cortisol levels), cooler ambient temperatures, circadian rhythm changes, and environmental triggers such as allergen exposure (e.g., house dust mite) in the sleeping environment. Together, these factors contribute to the heightened risk of nocturnal asthma exacerbations (182). In our subsequent analysis, we confirmed that the disruption of the circadian clock in peripheral blood eosinophils in asthma is a result of the systemic increase in inflammatory mediators.

To further investigate the disease-associated disruption of the molecular circadian clock, we tested whether this disruption could result from a systemic increase in inflammatory mediators. We stimulated isolated immune cells from healthy donors with sera from asthmatics or with a cocktail of pro-inflammatory cytokines. Given our previous observation that circulating inflammatory mediators are significantly elevated in asthmatic individuals in the evening, a mixture of IL-4, IL-5, IL-6, IL-10, IL-13, and IFN-γ was employed for the experiment. After 3 hours of incubation a significant decrease in clock protein expression in eosinophils was observed, consistent with the previous findings in eosinophils from asthmatic patients. These results align with in vivo studies showing that Bmal1 ablation exacerbates acute lung inflammation in mice (88,166), and that increased inflammation is observed in Rev erb knockout mice (165,167). Interestingly, while pro-inflammatory stimuli reduced clock protein levels, the bronchoprotective mediator PGE_2 was found to increase the expression of the anti-inflammatory repressor REV ERBα. These results were particularly evident in eosinophils, whereas in neutrophils, a reduced REV ERBα expression was only observed in individuals with asthma. Our findings also align with data from Tsuchiya et al., demonstrating that PGE_2 functions as a clock-resetting agent both in vitro in cultured fibroblasts and in vivo in peripheral tissues (183).

Notably, these effects were specifically observed in eosinophils, with minimal or no discernible changes in other peripheral blood leukocytes, such as neutrophils. This selective response may be explained by the key role of eosinophils in the pathophysiology of T2-high asthma, and the predominate allergic and eosinophilic asthma phenotypes in our cohort as indicated by increased bronchial hyperresponsiveness, reduced lung function, and elevated IgE levels. Furthermore,

incubation with patient serum with increased levels of asthma-related cytokines or inflammatory mediators did not affect the expression of clock proteins in peripheral neutrophils, although some of these cytokines, particularly IL-6, IL-10, and IFN-γ, are known to activate both eosinophils and neutrophils (38,184). This lack of response may, in part, reflect the reduced active or less sustained activity of the molecular circadian clock in neutrophils. Due to their short lifespan (approximately 18–24 hours), neutrophils may exhibit relatively weak cell-intrinsic oscillations of clock gene and protein expression, relying instead on population-level rhythmicity driven by circadian regulation of bone marrow egress and tissue recruitment (23,185). On the other hand, IL-4 and IL-13 are crucial cytokines that drive eosinophil differentiation and activation, which, together with IL-5, plays a central role in the maturation, activation, and survival of eosinophils (38). These findings highlight the pivotal role of eosinophils in mediating the often overserved clinical features in asthma including elevated FeNO measurements, increased serum IgE or greater AHR along with poorer asthma control, reflecting their significant involvement in the inflammatory processes characteristic of this subtype of asthma (186).

Given that most asthma patients are treated with inhaled corticosteroids, β2-agonists, or oral antihistamines, we also evaluated the impact of these medications on the circadian clock. Concentrations corresponding to plasma levels following inhaled (fluticasone (146) and formoterol (147) or oral (levocetirizine (148) administration did not affect the stabilizing loop after four hours, suggesting that the observed alterations in asthmatics are not directly attributable to medication. Notably, resetting the molecular clock with REV ERB agonists or the inverse ROR agonist SR1001 led to an increased expression of REV ERBα and BMAL1 after four hours incubation. However, we did not assess the long-term effects of asthma or allergy medications on the circadian clock, which remains a limitation of this study.

As previously discussed, lung-resident immune cells are essential regulators of airway inflammation and immune responses to inhaled allergens. In the context of asthma, alveolar macrophages can exhibit functional dysregulation, contributing to sustained inflammation and disease progression. Hence, we observed that the connection between immune cell phenotypes and the molecular circadian clock also extended to alveolar macrophages. Analysis of lung-resident alveolar macrophages from asthmatic and healthy individuals revealed differences in the expression of clock genes, including *Bmal1, Rev erbα, RORα*, and *RORβ*. Notably, mRNA levels of *Bmal1* and *RORα* were significantly increased in asthmatics. Conversely, *Rev erbα* expression was significantly reduced in AMs from asthmatic patients compared to healthy controls. We hypothesize that these alterations disrupted the balance within the molecular circadian clock in macrophages from asthmatics, as

evidenced by the lack of correlation between the activator *RORα* and *Bmal1*, as well as between the repressor *Rev erbα* and *Bmal1* (187).

Macrophage polarization is a well-established process dependent on inflammatory environmental cues and is known to be altered in allergic and asthmatic patients (68). In this part of the study, we focused on clock protein expression in monocytes from allergic and non-allergic individuals, observing most pronounced differences in patients with allergic asthma. To explore this further, we differentiated human peripheral blood monocytes into macrophages and exposed them to LPS/IFN-γ or IL-4 for polarization into M1 or M2 macrophages, respectively. Multiple in vitro studies have shown that macrophages derived from peripheral monocytes display circadian rhythms in clock gene expression. However, the amplitude of these oscillations diminishes over time in cell culture, likely due to the absence of external cues (67). One limitation of this study is that, despite the known decline in oscillation amplitude over time in cell culture, we did not track the oscillations of clock proteins in macrophages throughout the 8-day culture period.

Polarization-induced changes in macrophages were observed exclusively in allergic donors. In particular, we observed significant increases in BMAL1 and its activator RORβ in IL-4-polarized macrophages, mirroring the clock gene pattern seen in tissue resident alveolar macrophages derived from asthmatic individuals. In contrast, the BMAL1 repressor, REV ERBα, was elevated in M1 macrophages. These observation aligns with recent findings from the Farkas lab, which reported differential effects of polarization on the amplitude and rhythmicity of the molecular circadian clock in macrophages (188). Specifically, M1 polarization via LPS and IFN-γ led to reduced BMAL1 expression, whereas M2 polarization with IL-4 resulted in increased BMAL1 levels. Notably, our study is the first to demonstrate that such strong circadian responsiveness occurs predominantly in macrophages derived from allergic donors, suggesting a unique feature of macrophage circadian regulation in allergic inflammation. Although LPS and IFN-γ are potent inducers of inflammation, the BMAL1 repressor REV ERBα was found to be upregulated in M1-polarized macrophages, in contrast to its expression in circulating PMNLs stimulated with an inflammatory cytokine cocktail, as well as in peripheral eosinophils and neutrophils from allergic and asthmatic donors. Several factors may explain this discrepancy. First, the duration of stimulation: Macrophages were differentiated for a week and polarized over 24 hours, whereas PMNLs were only exposed to inflammatory mediators for 4 hours. Secondly, cell-type specific differences: while macrophages show robust, high-amplitude circadian rhythms, the circadian machinery in neutrophils is comparatively limited (84). Neutrophils

exhibit a weaker circadian oscillation of core clock genes and rely more heavily on external synchronizing signals such as cortisol and CXCL12 (185).

To further validate these findings, we employed precision-cut lung slices (PCLS) as an ex vivo model to assess BMAL1 expression in tissue-resident macrophages within the human lung (189). PCLS retain the cellular complexity and tissue interactions of the lung, making them suitable for studying inflammation in the context of asthma. This model is well-established for investigating acute and allergic pulmonary inflammation, as evidenced by the successful induction of pro-inflammatory mediators upon stimulation with LPS (190–192). In line with our in vitro data, we observed that both LPS/IFN-γ and IL-4/IL-13 treatments, which promote M1 and M2 polarization respectively, induced similar changes in BMAL1 expression in PCLS. Specifically, IL-4/IL-13 treatment led to increased BMAL1 expression at both 6 and 24 hours, while LPS/IFN-γ treatment resulted in a decrease after 24 hours compared to vehicle-treated slices. These *ex vivo* results corroborate our in vitro observations and support the hypothesis that the molecular circadian clock in macrophages directly responds to tissue inflammation, thereby reflecting disrupted tissue homeostasis in allergic asthma.

Together, these findings underscore the dynamic and context-dependent regulation of the molecular circadian clock in immune cells, especially eosinophils and macrophages, in the setting of allergic inflammation. The differential expression of clock proteins in allergic donors and asthma patients highlights the potential of clock proteins as biomarkers or therapeutic targets in asthma and other inflammatory diseases. However, further studies are required to understand the mechanisms driving these changes and their implications for immune function and disease progression.

4.3 Clock-modulating ligands

The strong connection between the immune system and the circadian rhythm is known to be bidirectional. On the one hand, inflammatory stimuli can alter the circadian rhythm and disturb the balance of the molecular circadian clock leading to the manifestation and progression of inflammatory diseases such as asthma. On the other hand, the molecular circadian clock is known to time and regulate immune responses (193). Hence, clock-based therapeutic strategies, including chronotherapy, where dosing time is optimized for maximum therapeutic outcome, and pharmacological ligands that specifically modulate the molecular circadian clock might be of high potential for future treatment approaches of inflammatory disease.

4.3.1 REV ERB targeting ligands

As REV ERBα is known as the key link between the immune system and the molecular circadian clock (194) and given that a lack of REV ERBα enhances lung inflammation (195), we first tested the impact of the REV ERBα agonists GSK4112 and SR9009. Both agonists reset the molecular circadian clock by increasing clock protein levels and modulate neutrophil and eosinophil effector cell functions. The REV ERB agonist GSK4112 showed promising anti-inflammatory potential *in vitro*. However, due to its weak biological efficiency (196) it is not suitable for *in vivo* experiments. Although SR9009 is a REV ERB agonist with improved pharmacokinetic properties, we did not observe similar anti-inflammatory effects, such as reduced eosinophil migration and increased apoptosis. In fact, both agonists appear to induce receptor-independent or off-target effects such as pronounced ROS production which cannot be blocked by the REV ERB antagonist SR8278.

We also evaluated the impact of the REV ERB-modulating agonist on human MDMs. SR9009 induced coherent effects in macrophages, as the treatment promotes the classical, inflammatory M1 phenotype of the cells. This might be associated with counter-regulation of REV ERBα, along with increased MIP 1α and MIP 1β release. Furthermore, the REV ERB antagonist SR8278 could not reverse the SR9009-induced reduction in M2 polarization (152,197). This is consistent with previous findings from Dierickx et al., who reported REV ERB-independent effects of SR9009 on proliferation, metabolism and gene transcription (149). However these observations remain controversial, as other studies demonstrated anti-inflammatory properties of SR9009 in mouse bone marrow-derived macrophages (198,199).

4.3.2 ROR targeting ligands

Due to the controversial results with REV ERB ligands, we decided to use the inverse ROR agonist SR1001 for further experiments. Recent papers have shown that SR1001 reduces the mRNA level of Rev erbα in mice and indicate an anti-inflammatory potential of this compound (121–123). In addition to its role as an activator of BMAL1, RORα also contributes to immune functions. Similar to RORγ, RORα regulates the expression of Th17 signature genes, suggesting that targeting RORα could offer therapeutic benefits for treating autoimmune diseases (200,201). Correspondingly, we demonstrated the potential of SR1001 to reverse induced eosinophil effector functions in vitro such as

shape change, respiratory burst and degranulation. Notably, SR1001 treatment also reduces the migratory responsiveness of eosinophils isolated from allergic donors.

Furthermore, SR1001 treatment also affects macrophage viability and the expression of circadian proteins. Specifically, this agonist tends to reduce REV ERBα expression while increases BMAL1 expression. Macrophages play a significant role in asthma pathology by altering their function, particularly through increased cytokine production and impaired phagocytosis (73,85,202). Given this, we investigated the potential beneficial effects of SR1001 on the phagocytic capacity of human MDMs, as well as M1 and M2 macrophages. Our results demonstrated that SR1001 enhances macrophage-driven host defence by increasing phagocytic activity in unpolarized MDMs and M2 macrophages, highlighting an additional promising anti-inflammatory property of this inverse ROR agonist (73). This is in line with previous research by Kitchen et al., which showed a gain of phagocytic function due to the loss of BMAL1 in macrophages (203). Additionally, SR1001's ability to support macrophage effector functions, such as host defence through increased phagocytosis, is particularly relevant given that phagocytic activity is impaired in asthmatic patients (204). Recent studies suggest that macrophages contribute to asthma pathology not through changes in cell numbers, but rather through functional alterations in phagocytosis and cytokine production, as macrophage numbers in lung tissue remain unchanged in patients with asthma (205,206). Therefore, SR1001's influence on macrophage function, particularly phagocytosis and cytokine secretion, which are disrupted in asthma, presents a promising therapeutic avenue for mitigating inflammation in asthma.

Furthermore, SR1001 shows anti-inflammatory potential by reducing survival and chemokine release in M1 macrophages. MIPs are closely linked to airway inflammation, with elevated levels detected in the BAL fluid of patients with allergic asthma (150). Recently, MIP-1β has been recognized as a key driver of eosinophilic airway inflammation in an ovalbumin-induced model of allergen-induced airway inflammation (207). In line with this, we observed a reduction in eosinophil migration in response to the supernatant from SR1001-treated M1 macrophages. Furthermore, MIPs serve as potent chemoattractants for eosinophils, which are central to asthma pathology (151,156), as well as for monocytes and macrophages (208–210).

To confirm the *in vivo* relevance of the observed anti-inflammatory activities of SR1001, we employed both an established eosinophil migration model and an HDM model of allergen-induced airway inflammation. The accumulation and mobilization of eosinophils into the airways, driven by allergens, is mediated through the eotaxin-2/CCR3 pathway (38). In the eosinophil migration model, intranasal administration of human eotaxin-2/CCL24 induces directed movement of eosinophils into

the airways. In these IL-5 transgenic mice, eosinophils rapidly and selectively accumulate in the airways, which can be quantified in BAL fluid (134,211). Although mice do not naturally develop asthma, *in vivo* models effectively mimic several inflammatory alterations characteristic of the human condition, including eosinophil accumulation and Th2 cell activation (86). HDM mouse models elicit a strong inflammatory response and more accurately replicate human asthma due to the use of an allergen commonly associated with the disease in humans (212).

Previous studies have indicated the anti-inflammatory potential of SR1001 by reducing Th2 inflammation *in vivo* (121–123,213). As anticipated, in both models the SR1001-treated group showed reduced eosinophil influx and fewer pro-inflammatory CD11c^{high} Siglec-F^{low} Mo-AMs (74) in the BAL. These findings were supported by our in vitro results, where SR1001 reduced eosinophils migration, MIP release and M1 macrophage viability, while no prominent effect was observed in neutrophils. As inflammation progresses after repeated allergen exposure, monocytes are recruited to the lungs, where they differentiate into macrophages that drive pro-inflammatory responses (214). Additionally, tissue-resident alveolar macrophages can polarize to a pro-inflammatory phenotype, lose their suppressive function and further promote inflammation (60). In this context, reduced immune cells infiltration in the BAL fluid of treated mice corresponded with less immune cell infiltration in the lungs and diminished goblet cell hyperplasia. Further, in our model, SR1001 treatment after the HDM-challenge phase significantly suppressed airway hyperresponsiveness to methacholine, as measured by reduced lung and airway resistance and improved compliance (136,215). Importantly, monitoring the daily biological behaviour of the animals revealed no differences in movement, exploration, nor eating and drinking habits, suggesting that SR1001 did not disrupt the circadian rhythm despite targeting the molecular circadian clock. Hence, we demonstrated that SR1001 inhibits immune cell migration *in vitro* and *in vivo* without affecting the circadian rhythmicity of the animals (1,2). Overall, these findings highlight the potential of targeting alveolar macrophages under inflammatory conditions as a therapeutic strategy, with SR1001 demonstrating efficacy in inhibiting eosinophil migration, improving lung function, and reducing mucus production without affecting circadian rhythmicity.

In 2008, Chen et al. demonstrated that upregulation of BMAL1 can prevent ERK phosphorylation (157). Eotaxin-1/CCL11, a key chemotactic factor for eosinophils, plays a critical role in the pathogenesis of asthma. Durrington et al. observed a circadian variation in eotaxin-1 levels in sputum, peaking at 4 a.m., coinciding with increased eosinophil influx and a higher risk of severe asthma attacks (216). In this study, we show that SR1001 inhibits eotaxin/CCR3-induced ERK phosphorylation in eosinophils by increasing BMAL1 protein levels, thereby disrupting this important

pathogenic mechanism of asthma. Furthermore, SR1001 reduced ERK phosphorylation in eosinophils from asthmatic patients to near resting-state levels observed in healthy donors. Additionally, dysregulation of EGFR and its downstream signalling pathways are known to contribute to epithelial barrier dysfunction, mucus production, and airway inflammation (217). SR1001 effectively blocked excessive EGFR activation in eosinophils from asthmatic patients by inhibiting autophosphorylation and the subsequent activation of JNK/STAT/Akt pathways (218). This suggests a potential cross-talk between the circadian clock and EGFR signalling. To our knowledge, only one previous study has indicated a similar interaction between RORγ and EGFR/ERK signalling, and a CCR3-dependent activation of EGFR has been described in bronchial epithelial cells (219). However, further research is required to fully elucidate the role of the ROR/CCR3/EGFR/ERK axis in asthma pathophysiology.

4.4 Limitations

While the findings provide important insights, several limitations need to be considered. The reliance on peripheral blood sampling provides only discrete snapshots, making it difficult to capture the full dynamics of circadian rhythms, as sampling frequency and duration are inherently limited in studies of human circulating leukocytes. The heterogeneity of peripheral leukocyte subtypes can further obscure cell-type–specific rhythmicity when bulk populations are analysed. Environmental and behavioural factors, including light exposure, sleep–wake cycles, diet, and stress, complicate the interpretation of leukocyte rhythms, while interindividual variability in age, sex, chronotype, lifestyle, and genetics may mask or confound underlying patterns. Moreover, the timing of sample processing introduces uncertainty, as gene expression may reflect the time of fixation rather than the blood draw. Furthermore, a limitation of this study is the consolidation of differing time frames for sample collection, which may introduce minor variability, although this was necessary to accommodate participant scheduling. Comparisons with RNA datasets are also limited, as disease severity differs between cohorts and information on sample collection time and participant medication status is unavailable.

Sex- and BMI-related differences in asthma incidence, prevalence, and severity are well documented, particularly in women, where weight gain and higher BMI are risk factors. In our study, no significant sex- or BMI-related differences in clock protein levels were observed, likely due to the limited sample size. Similarly, in a small group of shift workers with asthma, no clear expression

pattern was detected, although shift work is known to disrupt the circadian system and increase disease risk. These findings, while significant, require validation in larger, well-characterized cohorts.

The use of spontaneous sputum samples precluded inclusion of a healthy control group. PCLS obtained from non-tumor lung resections may also be influenced by underlying conditions such as chronic inflammation, fibrosis, or systemic disease, potentially altering lung physiology. In vitro experiments assessing the effects of asthma/allergy medications were limited to a four-hour incubation, so longer-term effects on clock protein levels cannot be excluded. Clock-modulating ligands were tested for up to 24 hours in macrophages and 2.5 days in vitro, with additional injections; however, translation to humans may be limited, as mouse circadian biology differs from humans, including their nocturnal activity.

5. Conclusion

In the course of this PhD thesis, we describe for the first time that the molecular circadian clock is expressed at the protein level in human leukocyte subsets including eosinophils, neutrophils, T cells, monocytes, and macrophages. We further revealed significant differences in clock protein expression levels when comparing immune cells from asthmatic donors to healthy volunteers. Among all leukocyte subsets, the decrease in clock protein expression was most pronounced in eosinophils.

To further explore the disease-associated decrease, clinical parameters including lung function, FeNO measurements, and allergy status from a moderate asthma cohort were correlated with clock protein levels, revealing decreased clock protein levels in more severe asthmatics e.g., those with an obstructive spirometry pattern. Furthermore, we observed reduced clock protein expression levels when eosinophils derived from healthy donors were incubated with sera from asthmatics or stimulated with a mix of inflammatory mediators, found to be increased in serum samples from mild asthmatic patients. Similarly, clock protein expression in human monocyte-derived and lung resident macrophages responded to inflammatory mediators: the classical M1 activation using LPS and IFN-γ tended to decrease BMAL1 levels while the expression has risen after M2 activation with IL-4.

As nuclear receptors are among the most effectively targeted proteins in pharmacology, and both families of the stabilizing loop, REV ERB and ROR, can be targeted by synthetic ligands, we aimed to explore their potential to reverse the inflammation-associated decrease in clock protein expression. As REV ERBα is known as the key link between the immune system and the molecular circadian clock, and a lack of REV ERBα enhances lung inflammation, we first tested the impact of the REV ERBα agonists GSK4112 and SR9009. Both agonists increased clock protein levels and modulated neutrophil and eosinophil effector cell functions. Due to its poor pharmacokinetic profile after intraperitoneal administration (17), the REV ERB agonist GSK4112 is not suitable for *in vivo* experiments. Although SR9009 is a REV ERB agonist with improved pharmacokinetic properties, the anti-inflammatory effects such as reduced eosinophil migration and increased apoptosis were missing, likely due to REV ERB independent effects of SR9009. Further SR9009 induced the classical inflammatory M1 phenotype in MDMs and increased the secretion of proinflammatory mediators such as MIPs.

Therefore, we investigated the potential of clock-modulating ligands targeting the opposing nuclear receptor family ROR. The ROR agonist SR1078 increases eosinophil effector function such as shape change, degranulation or ROS production which can be blocked by the inverse ROR agonist

SR1001. Further, SR1001 increased the phagocytic capacity and reduced MIP-1 secretion in macrophages, resulting in decreased eosinophils migration towards the supernatant of exposed cells. Furthermore, SR1001 treatment reduced eosinophil migration *in vitro* and *in vivo*. In both mouse models, anti-inflammatory effects were observed mainly in eosinophils and macrophages, along with broncho-protective effects in lung function measurements. Finally, we demonstrated that the inverse ROR agonist SR1001 mediates the anti-inflammatory effects in eosinophils by blocking CCR3 and EGFR-signalling (**Figure 59**).

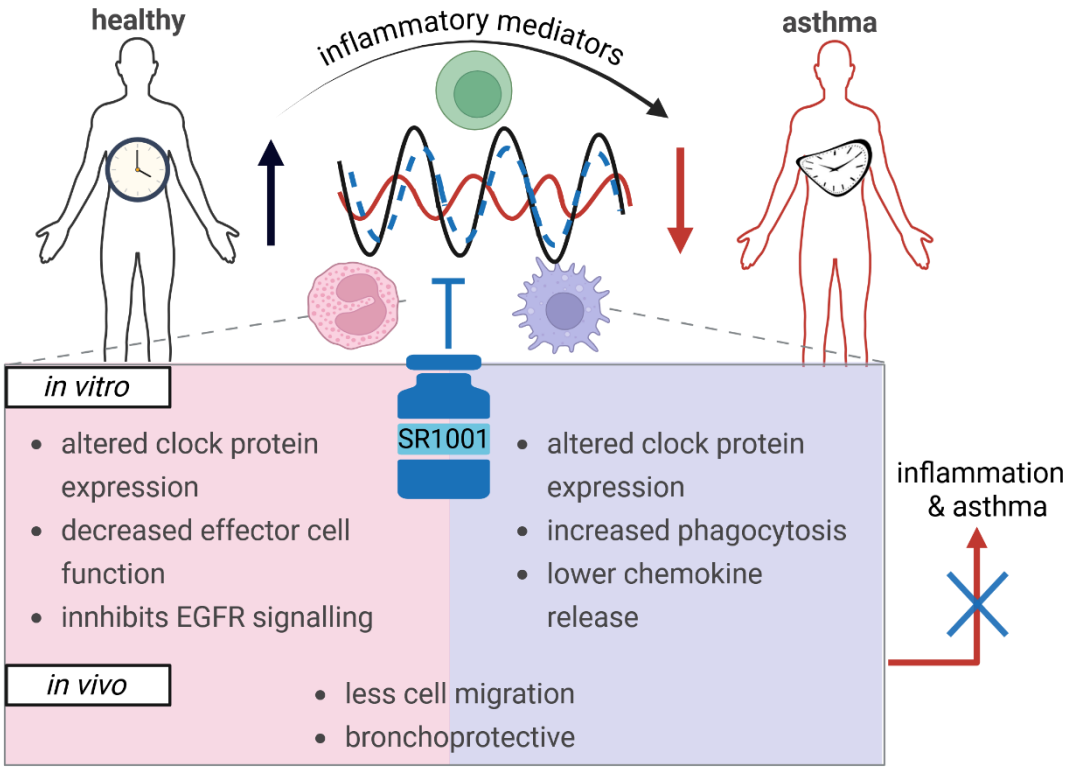

Figure 59: Schematic overview *showing decreased clock protein expression in immune cells from asthmatics compared to healthy individuals. Treatment with the ROR inverse agonist SR1001 restores clock protein expression, modulates effector functions in vitro, and reduced cell migration while having bronchoprotective effects in vivo.*

Hence, the molecular circadian clock in immune cells may serve as a potential biomarker for predicting asthma severity and phenotypes, and offers a new pathway for developing more effective treatments.

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